Title: Unveiling the intricacies: Investigating the impact of paternal aging on murine sperm miRNA profiles and their potential link to autism spectrum disorder

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Abstract

Background

Paternal aging has been consistently linked to an increased risk of neurodevelopmental disorders, including autism spectrum disorder (ASD), in offspring. While *de novo* mutations arising from paternal aging have been extensively studied, recent evidence has highlighted the role of epigenetic factors. We have previously revealed age-related DNA hypomethylation in mouse sperm and alterations in histone modifications during spermatogenesis.

Objectives

This study aimed to investigate age-related alterations in the microRNA (miRNA) profile of mouse sperm and analyze the target genes regulated by differentially expressed miRNAs.

Materials and Methods

Microarray analyses were conducted to identify differentially expressed miRNAs in sperm samples from mice at different ages: 3 months (3M), over 12M, and beyond 20M. The expression levels of genes potentially regulated by these miRNAs were also accessed. Comprehensive bioinformatic analyses were performed to unravel the intricate relationship between differentially expressed miRNAs and their target genes.

Results

We identified 26 miRNAs exhibiting differential expression between the 3M and 20M, 34 miRNAs between the 12M and 20M, and 2 miRNAs between the 3M and 12M mice. Notably, the target genes regulated by these differentially expressed miRNAs were significantly associated with apoptosis and ferroptosis pathways. Some of these target genes were relevant to the nervous system and potentially involvement in ASD.

Discussion

The findings suggest that aging induces alterations in the sperm miRNA profiles . Importantly, the target genes regulated by these differentially expressed miRNAs are associated with cell death and ASD, implying a potential link between paternal aging and an increased risk of neurodevelopmental disorders.

Conclusion

The observed age-related changes in sperm miRNA profiles have the potential to impact sperm quality and subsequently affect offspring development. Further validation and through investigations are necessary to gain a comprehensive understanding of the precise effects of paternal aging on subsequent generations.

1 INTRODUCTION

With the increasing trend of delayed parenthood, there has been a burgeoning focus on understanding the intricate relationship between aging and germ-line cells. While the effect of maternal aging on offspring, such as an elevated risk of miscarriage and Down syndrome, have garnered significant attention, the impact of male aging has received comparatively less recognition. However, recent research has revealed the advancing paternal age not only influences sperm quality, but also has implications for DNA integrity and epigenetic factors[1-4]. These alterations in sperm composition due to paternal aging have been implicated in perturbations during the developmental process of offspring, potentially contributing to spectrum of health issues[5].

In recent decades, investigations into the origins of health and disease in future generations have gained paramount significance. The concept of "Developmental Origin of Health and Disease (DOHaD)" has conventionally emphasized the impact of environmental factors on fetal development within the maternal uterus. However, there is now an emerging recognition that paternal factors also play a pivotal role in the risk of diseases manifestation in children, giving rise to the concept of the "Paternal Origin of Health and Disease (POHaD)" [6,7]. Paternal aging, in particular, has been linked to not only to adverse incidents during pregnancy, such as miscarriage[8] and stillbirth[9], but also postnatal health issues, including low birth weight[10], congenital heart defects[11], and malignant tumors[12]. Although the concept of POHaD is a relatively nascent, delving into this perspective holds the potential to provide insights into diseases that remain incompletely understood.

The concept of POHaD has emerged as a compelling framework, offering substantial evidence linking paternal factors to a range of neuropsychiatric disorders in offspring. Notably, advanced paternal age has been consistently demonstrated an increased risk of schizophrenia[13], bipolar disorder[14], attention-deficit/hyperactivity disorder (ADHD) [15], neurocognitive dysfunction[16], autism spectrum disorder (ASD) [17] in subsequent generations. The global surge in ASD[18] has been influenced by a complex interplay of social and biological factors, encompassing updated diagnostic criteria, hightened social awareness, maternal drug exposure, and parental aging[17,19-21]. Remarkably, a meta-analysis of six million subjects across five countries has revealed that paternal aging exerts a more pronounced impact on ASD risk compared to maternal aging[17]. Consequently, further exploration on the effects of POHaD may hold significant potential in providing invaluable insights into understanding pathophysiology of ASD.

The precise mechanism underlying the impact of paternal aging on the health of the next generation remains incompletely understood. *De novo* mutations, which are mutations that are not present in eighter parent but are observed in the child, have been associated with paternal aging and are believed to contribute to the increased disease risk in offspring[22-24]. Specifically, *de novo* mutations of paternal origin have been found to accumulate with advancing paternal age, particularly in individuals diagnosed with ASD and schizophrenia[23,25]. Nevertheless, it is important to note that *de novo* mutations derived from aged fathers account for only a fraction, approximately 10-20%, of the overall risk of psychiatric disorders in children[26,27]. This suggests the involvement of additional factors that necessitate careful consideration in understanding the complete picture.

Epigenetic modifications in sperm have emerged as another plausible mechanism underlying the effects of paternal aging effects on offspring[28]. We have previously revealed age-related modifications in DNA methylation in mouse sperm[4] and changes in histone modifications within the testis[29], suggesting their potential influence on offspring development. Micro RNAs (miRNAs) are another crucial epigenetic factor involved in gene expression regulation by inhibiting translation. These short non-coding RNA molecules (21-25 nucleotides) form complexes known as RNA-induced silencing complexes with their mRNA target sequences, ultimately suppressing translation[30,31]. The biomedical research field actively investigates miRNAs due to their potential as biomarkers, including applications in oncology[32,33]. Notably, aberrant miRNA profiles have been identified in individuals with ASD, with altered miRNA expression observed in various samples such as the brain, blood, serum, and saliva[34-37]. Similar findings have been reported in animal models of ASD[38]. However, the the precise mechanisms by which these changes in miRNA expression contribute to the pathogenesis of ASD remain unclear.

In a previous study on human sperm samples, age-related alterations in miRNA expression have been reported, with certain miRNAs exhibiting increased levels while others show decreased levels[2]. Additionally, alterations in mouse sperm miRNA expression, induced by factors such as diet and exercise, can impact the insulin sensitivity of female offspring[39]. Nevertheless, the comprehensive changes in overall miRNA profiles and the precise role of the target genes regulated by these altered miRNAs remain elusive. Therefore, the primary objective of this study is to meticulously examine the expression profiles of miRNAs in sperm obtained from aged mice in comparison to young counterparts. Furthermore, we aim to unravel the potential

functions of differentially expressed miRNA's target genes, shedding light on their roles in the context of paternal aging and potential implications for offspring mental health.

2 MATERIALS ANS METHODS

2.1 Animals

C57BL/6J mice at 3 months old were purchased from a breeder (Charles River Laboratories, Japan) and were raised to 12 or 20 months old at the Institute for Animal Experimentation at the Tohoku University Graduate School of Medicine; they were then used for miRNA and mRNA analysis. The mice were then sampled for microarray analyses at 3 months old (3M), >12M or >20M (**Figure 1**). All animals were housed in standard cages in a temperature- and humidity-controlled room with a 12-hour light/dark cycle (light on at 8 am), and they had free access to standard food and water. All experimental procedures were approved by the Ethics Committee for Animal Experiments of the Tohoku University Graduate School of Medicine (#2017- MED210, #2019- MED280-03, 2020BeLMO-005-01 and 2020BeA-015-01), and the animals were treated according to the National Institutes of Health guidance for the care and use of laboratory animals.

2.2 Sperm collection for miRNA and mRNA analyses

For microarray analyses, three mice for each age were used. Mice were anesthetized with isoflurane (MSD Animal Health) and perfused with PBS (pH 7.4) to remove blood. After perfusion, the cauda epididymis was removed from fat and other connective tissues to collect sperm by retrograde perfusion[40-42]. Sperm were centrifuged on a 27% Percoll solution (2005-001 or 2001-017, NK system). The pellet was resuspended in PBS and recentrifuged (400×g for 2 min) to remove excess Percoll. The purity of the pellet was confirmed by microscopy. Samples were then subjected to extracting miRNA and mRNA.

2.3 RNA extraction and microarray analysis

Sperm samples were subjected to extract total RNA including miRNA or mRNA by using miRNeasy Mini Kit (217004, QIAGEN). We confirmed whether total RNA including miRNA was sufficiently collected by Agilent Small RNA Reagent kit (5067-1549) with 2100 Bioanalyzer Instrument (G2939BA). RNA samples were subjected to microarray analyses. Clariom™ D Assay for the mouse (902513, ThermoFisher Scientific) and GeneChip™ miRNA 4.0 Array (902412, ThermoFisher Scientific) were used for mRNA and miRNA analyses, respectively. Transcript expression values were normalized using Signal Space Transformation (SST-RMA) method. Raw intensity data (i.e., CEL files) was analyzed by using Transcriptome Analysis Console (TAC) Software version 4.0.2 (ThermoFisher Scientific). We identified differentially expressed miRNAs based on >2-fold up- or down-regulation

along with a *p*-value <0.05. Microarray data were deposited into the Gene Expression Omnibus database under accession number GSE232620 and are available at the following URL: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE232620. Pathway analyses were conducted using the Reactome pathway analysis package in R[43]. Networks were constructed using Cytoscape[44], which visualized relationships between enriched pathways and genes. Pearson`s correlation test was utilized to analyze the correlation between miRNAs (i.e., *mmu-let-7b-5p*, *mmu-miR-10a-5p* and *mmu-miR-24-3p*) and their target genes related with apoptosis (i.e., *Ppp3r1* and *Bcl2l11*). We also compared among groups the expression levels of the five miRNAs that have previously been reported as candidate miRNAs (i.e., miR-10a-5p, miR-146a-5p, miR-34c-5p, miR-143-3p, and miR-146b-5p) transmitted from sperm to fertilized egg[45]. In these analyses, $p<0.05$ was considered significant. We used Bonferroni's multiple comparison test to verify the significance of the microarray results.

2.4 Analyses exploring possible function of target genes of altered miRNAs

To investigate possible functions of the target genes of differentially expressed miRNAs in relation to ASD, we utilized the autism-related gene database SFARI[46]. We constructed a network of miRNA-target gene interactions by using Cytoscape. Additionally, we also conducted Pearson`s correlation test between the expression levels of miRNAs (*mmu-miR-466j*, *mmu-miR-24-3p*, *mmu-miR-690*, and *mmu-let-7b-5p*) and their target SFARI genes (*Oxtr*, *Gabrb2*, *Ctnnb1*, and *Grik2*).

3 RESULTS

3.1 Age-related changes in expression of miRNAs in the mouse sperm

We first analyzed age-related changes in sperm miRNA expression patterns. 298, 273, and 364 miRNAs were expressed in sperm samples of 3 M, 12 M, and 20 M, respectively (**Figure 2A**). There were 221 miRNAs commonly expressed in 3 M and 12 M, and 248 miRNAs commonly expressed in 3 M and 20 M. In 12 M and 20 M, 229 miRNAs were commonly expressed, and 210 miRNAs were expressed in all three groups (**Figure 2B**).

The miRNAs were then compared between the two groups, respectively. We considered the miRNAs that showed >2-fold up- or down-regulation along with $p<0.05$ in expression as significantly different. Two miRNAs were upregulated in the sperm sample collected from 12M compared with the sperm of 3M, 21 miRNAs were upregulated, and 5 miRNAs were downregulated in 3M vs 20M samples, and 26 and 8 miRNAs were up- or down-regulated, respectively, in 12M vs 20M samples (**Figures 2B-E**). The differentially expressed miRNAs at each comparison are summarized in **Table 1**.

No overlap was observed between the miRNAs that exhibited alterations in the 3M vs 12M comparison and those in the 3M vs 20M comparison (**Figure 2F)**. In the 3M vs 12M comparison, two miRNAs that displayed changes in the 3M vs 12M comparison were also found to be altered (4.5% of the total altered miRNAs). Furthermore, out of the 26 miRNAs that showed alteration in the 3M vs 20M comparison, 16 of them were similarly altered in the 12M vs 20M comparison (36% of the total altered miRNAs). Notably, this subset of miRNAs included *let-7b-5p*, *miR-10a-5p*, and *miR-24-3p*, which target apoptosis-related genes (**Figure 2F; Supplementary Table S1)**. These results indicate a distinct age-associated shift in the sperm miRNA profiles, particularly when comparing 3M vs 20M and 12M vs 20M age groups.

3.2 Targets of age-related miRNAs in the mouse sperm

Next, miRNAs significantly altered in the comparison between groups were listed and their target genes (**Supplementary Table S1**) were used for enrichment analyses by using an R package for Reactome Pathway Analysis[43]. Although, the 3M vs 12M comparison did not have any pathways that were significantly different (**Figure 3A**), comparison between 3M and 20M samples yielded differential pathways including “Dimerization of procaspase-8”, “CASP8 activity is inhibited”, “Regulation by c-FLIP”, “Apoptosis”, “Caspase activation via Death Receptors in the presence of ligand”, and

"Caspase activation via extrinsic apoptotic signaling pathway" (**Figure 3B**). Most of these pathways are thus related to the regulation of apoptosis.

In our comparison between 12M and 20M, we also found several pathways that showed significant differences. These included the same apoptosis-related pathways such as "Caspase activation via Death Receptors", "Dimerization of procaspase-8" and "Apoptosis", as well as other pathways such as "Signaling by Receptor Tyrosine Kinases", "Transmission across Chemical Synapses", and "NCAM signaling for neurite out-growth" and "Neuronal System" (**Figure 3C**). Those pathways related to the nervus system were not significantly enriched in comparison between 3M vs 20M (**Figure 3B**). Furthermore, four genes related to ferroptosis, another type of cell death, including *Acsl4*, *Hmox1*, *Slc11a2*, and *Slc3a2*, were identified as target genes of altered miRNAs between 3M vs 20M. Among them, *Acsl4* and *Hmox1* were also target genes in comparison between 12M vs 20M. Therefore, our findings suggest that aging may be linked with cell death during spermatogenesis.

In summary, we found more differentially expressed miRNAs in comparison of 12M vs 20M sperm samples, which yielded more target genes in unique pathways. The significant pathways in comparison between 3M vs 20M samples overlapped with those that were significant between 12M vs. 20M (**Figure 3D**). These results indicate that the target genes of miRNAs that change with aging were significantly enriched in pathways associated with cell death and the nervous system.

3.3 Changes in mRNA expression of age-associated miRNA target genes

We measured expression levels of target genes using a commercial microarray kit and the top 10 enriched pathways and genes were visualized using Cytoscape[44] (**Figure 4**). Among these pathways, we focused on the apoptosis-related pathways (such as "Caspase activation via Death Receptors", "Dimerization of procaspase-8" and "Apoptosis") and investigated whether the expression levels of the target genes regulated by altered miRNAs were affected by aging (**Supplementary Table S2**). The network representations in **Figure 4** revealed that the expression levels of most genes, including *Casp9*, *Tnfsf10*, and *Fadd*, exhibited minimal changes. However, the expression levels of *Ppp3r1*, regulated by *mmu-let-7b-5p*, and *Bcl2l11*, regulated by *mmu-miR-10a-5p* and *mmu-miR-24-3p*, increased with sperm aging. Notably, *Ppp3r1* showed a significant increase in 20M mice compared to 3M mice (fold change=1.5, p=0.029). To validate the association between changes in gene expression, we conducted Pearson's correlation tests. The results demonstrated a significant positive correlation between the expression levels of *Ppp3r1* and *mmu-let-7b-5p* (**Figure 5A**:

r=0.840, *p*= 0.005). Although *Bcl2l11* also exhibited a positive trend with *mmu-miR-10a-5p* and *mmu-miR-24-3p*, the correlation did not reach statistical significance (**Figure 5B** and **4C**; *mmu-miR-10a-5p*: *r*=0.646, *p*=0.060; *mmu-miR-24-3p*; *r*=0.613, *p*=0.079).

3.4 Target genes of altered miRNAs related with ASD

Since paternal aging is a risk for ASD[17,21], we analyzed whether the target genes of the age-related miRNAs might have some relation with ASD. We searched the autism-related gene database SFARI[46], and identified an overlap of 28 and 38 SFARI genes with the target genes of differentially-expressed miRNAs between 3M vs 20M and 12M vs 20M, respectively (**Supplementary Table S3**). No SFARI genes were identified as miRNA targets in comparisons with 3M and 12M. The relationship between miRNAs and SFARI genes is visualized in **Figure 6** as a network. We focused on four SFARI genes (i.e., *Oxtr*, *Gabrb2*, *Ctnnb1*, and *Grik2*), whose expression levels differed significantly between the groups, and confirmed correlations of the expression levels and their regulatory miRNAs. These included significant negative correlations between *mmu-miR-466j* vs *Oxtr* (**Figure 7A**; *r*=-0.701, *p*= 0.035) and *mmu-miR-24-3p* vs *Gabrb2* (**Figure 7B**; *r*=-0.707, *p*=0.033), while there was a tendency of positive correlation between *mmu-miR-690* vs *Ctnnb1* (**Figure 7C**; *r*=0.615, *p*=0.078). A negative correlation between *mmu-let-7b-5p* vs *Grik2* was not significant (**Figure 7D**; *r*=-0.246, *p*= 0.523).

3.5 Changes in expression of miRNAs that may possibly be transmitted from sperm to fertilized eggs

Finally, we focused on candidate miRNAs that can be carried from sperm to the fertilized egg, based on a previous mouse research comparing miRNA profiles of sperm, oocytes and fertilized eggs[45]. We examined, using the microarray, expression of the miRNAs, *miR-10a-5p*, *miR-146a-5p*, *miR-34c-5p*, *miR-143-3p*, *miR-146b-5p*. Our results revealed significant upregulation of *miR-10a-5p* and *miR-146a-5p* at 20M in the mouse (**Figure 7E-I**). This finding highlights the potential significance of *miR-10a-5p* and *miR-146a-5p* as candidate sperm miRNAs transmitted to the zygote and their possible implications for subsequent offspring development.

4 DISCUSSION

The present study aimed to investigate the effect of paternal aging on miRNA profiles in mouse sperm. We identified differentially expressed miRNAs with aging, which were significantly enriched in pathways related to apoptosis and the nervous system. We observed that the expression levels of *Ppp3r1* and *Bcl2l11*, two target genes of these miRNAs, showed trends of changes with aging and correlations with their respective regulatory miRNAs. Moreover, we found upregulation of *miR-10a-5p* and *miR-146a-5p* at 20M, suggesting the possibility of these miRNAs being transmitted to the zygote and having impact on offspring's development. Our study provided evidence that age-related changes in miRNAs might have an impact on the regulation of miRNAs in sperm, which may subsequently affect sperm quality and health of offspring.

4.1 Changes in expression of miRNAs with aging: implications for cell death or the nervous system

Our analyses of miRNAs in sperm samples obtained from mice at different ages (3M, 12 M, and 20M) revealed distinct patterns of miRNA expression. Notably, when comparing the miRNA profiles between the youngest (3M) and the oldest (20M) groups, we observed significant associations with pathways related to apoptosis (**Figure 3B**). This finding aligns with previous studies demonstrating an increase in apoptosis and DNA damage in in human and mouse sperm with advanced age[47,48].

Through our analyses, we identified miRNA target genes that both promote (e.g., *Bcl2l11*, *Casp9*, and *Ppp3r1*) and inhibit (e.g., *Tnfsf10*, *Fadd*, and *Fas*) apoptosis. The expression levels of these genes play critical role in regulating apoptosis. For instance, decreased expression of *Bcl2l11* and *Casp9* has been associated with suppression of sperm apoptosis[49]. Furthermore, exposure of recombinant tumor necrosis factor-related apoptosis-inducing ligand (coded by *Tnfsf10*) can modulate sperm apoptosis[50]. Our findings revealed variations in the expression levels of certain genes, including *Bcl2l11* and *Ppp3r1*, across different age groups, suggesting that changes in miRNA profile may contribute to the aberrant expression of these target genes.

Our findings suggest that paternal aging may not only promote apoptosis but also influence other forms of cell death in sperm. Previous research has reported age-related variations in the ratio of apoptosis and necrosis in sperm[47]. Moreover, our analyses revealed potential implications of paternal aging on a distinct type of cell death known as ferroptosis, which characterized by iron accumulation and lipid peroxidation, plays a vital role in spermatogenesis[51,52].

Through our investigations, we identified four genes (*Acsl4*, *Slc11a2* , *Slc3a2* and *Hmox1*) that are associated with ferroptosis as target genes of the altered miRNAs resulting from paternal aging. *Acsl4*, known as a driver of ferroptosis, modulates the content of polyunsaturated fatty acids in phospholipids, which are preferentially oxidized during ferroptosis[53]. This process is facilitated by the cytosolic iron released by Slc11a2. Slc3a2 is a subunit of system Xc-, plays a crucial role in antioxidant defense and the inhibition of ferroptosis. Hmox1, on the other hand, exhibits dual roles as it can either induce or prevent ferroptosis depending on the context[54].

Given that 20M represents a highly advanced age, it is important to consider that the aging process is accompanied by multitude of factors, including increased oxidative stress, lipid peroxidation, iron accumulation, and chronic inflammation. These factors may collectively contribute to heightened aging-related stress, which may not only promote apoptosis and ferroptosis but also increase the occurrence of necrosis. Our analysis revealed the presence of pathways related to apoptosis and ferroptosis in the comparison between 12M and 20M groups (**Figure 3C**), further supporting the notion that changes in miRNA profile of sperm resulting from paternal aging can influence different forms of cell death.

Our investigation uncovered several intriguing aging-related pathways, such as "Signaling by Receptor Tyrosine Kinases", "Transmission across Chemical Synapses", "Neuronal System" and "NCAM signaling for neurite out-growth". The consistency of these findings is particularly noteworthy, considering the shared expression of many genes in both the brain and testis[55]. One example is genes related with calcium signaling, a crucial process in both neuron and sperm. Calcium channels play a central role in neurotransmission in the brain[56] and are essential for sperm motility and capacitation[57]. In our study, we identified target genes involved in calcium signaling, including *Camkk1* and *Cacna2d2*. *Cacna2d2* codes for the alpha-2/delta subunit of the voltage-dependent calcium channel complex, while *Camkk1* codes for the Ca^{2+}/calmodulin-dependent protein kinase α, both of which are essential for proper calcium signaling in sperm[58,59]. Consequently, aberrant expression of these genes due to miRNAs may disrupt sperm motility and capacitation[57], both of which are also associated with paternal aging[1,60]. Thus, our findings suggest that changes in signaling pathways related to calcium signaling and other pathways identified in sperm from aged mice could contribute to the observed decline of sperm quality in aged males.

4.2 Changes in expression levels of genes caused by altered miRNAs with aging

Our study provides compelling evidence that altered miRNAs have an impact on the expression levels of specific genes in the mouse sperm. Particularly noteworthy were the age-related changes in the expression levels of the two apoptosis-related genes, *Ppp3r1* and *Bcl2l11*. These changes demonstrated a proportional association with the expression levels of their regulatory miRNAs, namely *mmu-let-7b-5p*, *mmu-miR-10a-5p*, and *mmu-miR-24-3p*, as quantitatively determined by microarray analyses. Previous research has indicated that the upregulation of these three miRNAs is associated with apoptosis[61-63]. For instance, Huang, Y. et al. has demonstrated that *let-7b-5p* promotes apoptosis, and that knockdown of *let-7b-5p* suppresses apoptosis in cultured human cells[61]. These findings lend further support to our hypothesis that these three miRNAs may indeed contribute to sperm apoptosis.

The positive correlation observed between the regulatory miRNAs and their target genes (**Figure 4A-C**) suggests the possibility of upregulation of *Ppp3r1* and *Bcl2l11* by these miRNAs. While miRNAs are commonly known for their role in repressing gene expression, it is important to note that they can also have the capacity to promote gene expression in cells at G0 phase, such as oocytes[64]. Although specific studies investigating the effects of these miRNAs on mRNA upregulation in sperm are currently lacking, it is plausible to consider that the target genes influenced by these miRNAs could undergo upregulation in sperm obtained from aged mice, potentially leading to induction of apoptosis.

4.3 Paternal aging and ASD risk of offspring

We further investigated the impact of altered miRNAs on autism-related SFARI genes through microarray analyses. Although changes in the expression levels of SFARI genes were generally limited, intriguing associations were found between the expression levels of *Oxtr* and *Gabrb2* and their regulatory miRNAs. *Oxtr* encodes an oxytocin receptor, a crucial component in regulation of social behavior[65]. A meta-analysis has previously identified four polymorphisms in the human *OXTR* gene (rs7632287, rs237887, rs2268491, and rs2254298) that are linked to ASD[66]. Similarly, *Gabrb22* encodes the gamma-aminobutyric acid (GABA) type A receptor subunit beta2, an important component of the inhibitory system in the brain[67]. Disruptions of GABA system have been implicated in ASD risk[68,69]. Polymorphisms on *GABRB2*, such as rs2617503 and rs12187676, have been implicated in ASD risk[70]. Although the roles of *Oxtr* and *Gabrb2* in germ-line cells are not well understood, their crucial functions in brain development are known unknown. Aberrant expressions of these genes due to

miRNA changes in sperm may provide partial insights into the relationship between paternal aging and the increased risk of ASD in offspring.

4.4 Limitation

While our study provides valuable insights into the role of miRNAs in paternal aging and their potential association with ASD risk, several limitations should be acknowledged. First, to fully understand the physiological function of these miRNAs in fertilized eggs and their relationship to ASD, additional investigations involving techniques such as miRNA injection or knockdown in fertilized mouse eggs are necessary. Despite the relative low abundance of miRNAs in sperm compared to the fertilized egg, they still hold promise as potential biomarkers for predicting the physical conditions of offspring.

Second, it is important to note that our analyses were conducted using a single mouse strain, C57BL/6J. It has been reported that miRNAs can vary among different mouse strains[71,72]. Therefore, it is essential to validate our findings in other mouse strains to ensure the generalizability of the results.

Moreover, extending our analyses to include human samples is crucial for a comprehensive understanding of the role of miRNAs in paternal aging and ASD. Although there is a shared commonality between humans and mice[73,74], species-specific differences and complexities cannot be overlooked. Investigating miRNAs and their impact on post-transcriptional gene regulation in human samples will provide valuable insights and potentially uncover novel causes of ASD.

Lastly, it is important to acknowledge that our study focused on miRNAs and their target genes, but other regulatory mechanisms and factors may also contribute to the development of ASD. Exploring additional epigenetic modifications and factors that influence gene expression could provide a more comprehensive understanding of the paternal contribution to ASD.

5 CONCLUSION

In this study, we aimed to investigate epigenetic changes in male germline cells induced by aging and their potential impact on the development of the next generation. Our findings shed light on the alterations in the miRNA profile of sperm as a result of aging, which can subsequently lead to changes in gene expression levels. These changes have the potentials to contribute to a wide range of symptoms associated with ASD and other transgenerational epigenetic changes. Future research is needed to elucidate the precise mechanism by which the sperm epigenome influences the disease risk in the offspring. Such investigations will not only deepen our understanding of the function of the sperm epigenome functions but also contribute to the development of novel therapeutic and preventive strategies for the diseases in the next generation. In addition to the well-established importance of the maternal environment in the DOHaD concept, our study highlights the growing relevance of investigating the effects of POHaD. By exploring both maternal and paternal contributions, we can gain comprehensive insights into the complex interplay between genetics, epigenetics, and environmental factors in shaping the health outcome of future generations.

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AUTHORS` CONTRIBUTIONS

MT, and NO designed the study. MT, KM, and TK performed the experiments. MT and KM undertook the bioinformatic and statistical analyses. KM, MT and NO wrote the manuscript. All authors contributed and have approved the final manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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FIGURE LEGENDS

FIGURE 1: Experimental diagram for the strategy of analyzing miRNAs. Sperm samples were collected from the cauda epididymis of three C57BL/6J mice at 3M, >12M, and >20M via retrograde perfusion. Total RNA, including miRNAs, was extracted from the collected samples and subjected to microarray analyses. Further details can be found in the text.

FIGURE 2: Analyses of differentially expressed miRNAs with aging. (A) Venn diagram showing the number of miRNAs expressed in sperm samples from mice aged 3M, 12M and 20M, respectively. A total of 447 miRNAs were expressed. White letters indicate the numbers of common miRNAs and their percentages against the total numbers. (B) Differentially expressed miRNAs were defined as those showing >2-fold up- or down-regulation and $p<0.05$. Red bars indicate upregulated miRNAs and green bars indicate downregulated miRNAs. (C-E) Volcano plots showing differentially expressed miRNAs in sperm samples from mice aged 3M vs 12M (C), 3M vs 20M (D), and 12M vs 20M (E). Differentially expressed miRNAs were defined as those showing >2-fold up- or down-regulation and $p<0.05$. Red dots indicate upregulated miRNAs and green dots indicate downregulated miRNAs. (F) Venn diagram showing differentially expressed miRNAs in sperm samples comparing 3M vs 12M, 3M vs 20M, and 12M vs 20M. A total of 46 differentially expressed miRNAs were identified. White letters indicate the number of common miRNAs and their percentage against the total number.

FIGURE 3: Pathway analyses of miRNA target genes. Pathway analyses of miRNA target genes were performed on miRNA target genes significantly altered the sperm samples of 3M vs 12M, 3M vs 20M, and 12M vs 20M, respectively using the Reactome pathway analysis package in R. The number of genes associated with each pathway is shown on the horizontal axis in (A-C). The gene ratio in (D) indicates the percentage of genes associated with each pathway among the genes listed as targets of the miRNAs.

FIGURE 4: Network of biological pathways and genes. Network of significantly enriched pathways and target genes belonged to those pathways when comparing 3M vs 20M (A) and 12M vs 20M (B). Node color indicates the fold change of the gene expression. Node size indicates the significance of the differential expression; larger sizes indicate smaller *p* values.

FIGURE 5: Correlation analysis between apoptosis-related genes, *Ppp3r1* and *Bcl2l11*, and their regulator miRNAs. Scatter plot shows the correlation between *Ppp3r1* and *mmu-let-7b-5p* (A), *Bcl2l11* and *mmu-miR-10a-5p* (B), and *Bcl2l11* and *mmu-miR-24-3p* (C).

FIGURE 6: Network of miRNAs and SFARI genes. Network of significantly altered miRNAs and their target autism-related SFARI genes when comparing 3M vs 20M (A) and 12M vs 20M (B). Node color indicates the fold change of the miRNA and target gene expression. Node size indicates the significance of the differential expression; larger sizes indicate smaller *p* values.

FIGURE 7: Expression levels of specific miRNAs. (A-D) Correlation analyses between SFARI genes whose expression levels were varied between groups and their regulatory miRNAs. (A) *mmu-miR-466j* vs *Oxtr*, (B) *mmu-miR-24-3p* vs *Gabrb2*, (C) *mmu-miR-690* vs *Ctnnb1*, (D) *mmu-let-7b-5p* vs *Grik2*. (E-I) Expression scores in this microarray analysis of miRNAs that are previously reported to be inherited from sperm to fertilized egg [45]. (E) *miR-10a-5p*, (F) *miR-146a-5p*, (G) *miR-34c-5p*, (H) *miR-143-3p*, and (I) *miR-146b-5p*. Bars show the mean of the signals of the three samples in each group. Bonferroni's multiple comparison test was used to test for significant differences. *$p<0.05$, ** $p<0.01$.

TABLE 1: The differentially expressed miRNAs with aging

Group	Transcript ID (Array Design)	Fold change	*p*_value	Accession	Chromosome
3M vs 12M	*mmu-miR-1941-5p*	3.51	0.0003	MIMAT0009405	chr15
3M vs 12M	*mmu-miR-877-5p*	2.52	0.0084	MIMAT0004861	chr17
3M vs 20M	*mmu-miR-24-3p*	4.16	0.0001	MIMAT0000219	chr13
3M vs 20M	*mmu-miR-10a-5p*	2.27	0.0011	MIMAT0000648	chr11
3M vs 20M	*mmu-miR-677-3p*	2.87	0.0013	MIMAT0017246	chr10
3M vs 20M	*mmu-miR-195a-3p*	18.41	0.0016	MIMAT0017000	chr11
3M vs 20M	*mmu-miR-1249-5p*	2.73	0.0042	MIMAT0014804	chr15
3M vs 20M	*mmu-let-7e-5p*	3.52	0.0043	MIMAT0000524	chr17
3M vs 20M	*mmu-miR-8095*	2.56	0.0046	MIMAT0031396	chr16
3M vs 20M	*mmu-miR-6906-5p*	-3.26	0.0056	MIMAT0027712	chr10
3M vs 20M	*mmu-miR-7054-5p*	-2.22	0.0074	MIMAT0028012	chr7
3M vs 20M	*mmu-miR-709*	2.69	0.0113	MIMAT0003499	chr8
3M vs 20M	*mmu-miR-690*	3.96	0.0132	MIMAT0003469	chr16
3M vs 20M	*mmu-miR-146a-5p*	2.38	0.0167	MIMAT0000158	chr11
3M vs 20M	*mmu-miR-3072-5p*	2.04	0.0176	MIMAT0014852	chr12
3M vs 20M	*mmu-let-7b-5p*	2.56	0.0203	MIMAT0000522	chr15
3M vs 20M	*mmu-mir-7017*	2.58	0.0254	MI0022866	chr4
3M vs 20M	*mmu-miR-6923-5p*	2.11	0.0255	MIMAT0027746	chr11
3M vs 20M	*mmu-miR-669f-5p*	2.45	0.0263	MIMAT0017327	chr2
3M vs 20M	*mmu-miR-668-5p*	-2.20	0.0276	MIMAT0017237	chr12
3M vs 20M	*mmu-miR-7033-5p*	4.19	0.0310	MIMAT0027970	chr5
3M vs 20M	*mmu-miR-16-1-3p*	9.76	0.0369	MIMAT0004625	chr14
3M vs 20M	*mmu-miR-455-3p*	-2.02	0.0389	MIMAT0003742	chr4
3M vs 20M	*mmu-miR-5107-5p*	3.14	0.0394	MIMAT0020615	chr18
3M vs 20M	*mmu-miR-7025-5p*	-2.50	0.0423	MIMAT0027954	chr5
3M vs 20M	*mmu-miR-3535*	6.25	0.0431	MIMAT0031410	chr1
3M vs 20M	*mmu-miR-1895*	4.89	0.0459	MIMAT0007867	chr3
3M vs 20M	*mmu-miR-3084-3p*	2.07	0.0500	MIMAT0014877	chr19
12M vs 20M	*mmu-miR-24-3p*	3.45	0.0003	MIMAT0000219	chr13
12M vs 20M	*mmu-miR-10a-5p*	2.39	0.0004	MIMAT0000648	chr11
12M vs 20M	*mmu-miR-7001-5p*	-2.37	0.0005	MIMAT0027904	chr2
12M vs 20M	*mmu-miR-1941-5p*	-3.04	0.0006	MIMAT0009405	chr15
12M vs 20M	*mmu-miR-6906-5p*	-2.83	0.0015	MIMAT0027712	chr10

12M vs 20M	*mmu-miR-677-3p*	2.38	0.0017	MIMAT0017246	chr10
12M vs 20M	*mmu-miR-10b-5p*	2.08	0.0023	MIMAT0000208	chr2
12M vs 20M	*mmu-miR-23a-3p*	2.75	0.0026	MIMAT0000532	chr8
12M vs 20M	*mmu-miR-877-5p*	-4.73	0.0026	MIMAT0004861	chr17
12M vs 20M	*mmu-miR-195a-3p*	21.29	0.0026	MIMAT0017000	chr11
12M vs 20M	*mmu-miR-690*	5.30	0.0039	MIMAT0003469	chr16
12M vs 20M	*mmu-miR-8095*	2.56	0.0046	MIMAT0031396	chr16
12M vs 20M	*mmu-miR-3535*	4.47	0.0089	MIMAT0031410	chr1
12M vs 20M	*mmu-miR-146a-5p*	2.45	0.0092	MIMAT0000158	chr11
12M vs 20M	*mmu-miR-7222-3p*	2.08	0.0165	MIMAT0028413	chr2
12M vs 20M	*mmu-miR-30a-5p*	3.54	0.0169	MIMAT0000128	chr1
12M vs 20M	*mmu-miR-3072-5p*	2.05	0.0184	MIMAT0014852	chr12
12M vs 20M	*mmu-let-7e-5p*	3.31	0.0188	MIMAT0000524	chr17
12M vs 20M	*mmu-let-7b-5p*	2.51	0.0198	MIMAT0000522	chr15
12M vs 20M	*mmu-miR-7025-5p*	-2.4	0.0245	MIMAT0027954	chr5
12M vs 20M	*mmu-miR-29a-3p*	2.72	0.0245	MIMAT0000535	chr6
12M vs 20M	*mmu-miR-16-1-3p*	12.14	0.0250	MIMAT0004625	chr14
12M vs 20M	*mmu-miR-8101*	2.11	0.0284	MIMAT0031405	chr11
12M vs 20M	*mmu-miR-7118-5p*	2.06	0.0300	MIMAT0028133	chr15
12M vs 20M	*mmu-miR-297a-5p*	2.02	0.0327	MIMAT0000375	chr2
12M vs 20M	*mmu-miR-145a-5p*	2.96	0.0342	MIMAT0000157	chr18
12M vs 20M	*mmu-miR-5135*	-2.26	0.0359	MIMAT0020646	chr12
12M vs 20M	*mmu-miR-3084-3p*	2.52	0.0390	MIMAT0014877	chr19
12M vs 20M	*mmu-miR-125b-5p*	3.79	0.0412	MIMAT0000136	chr16
12M vs 20M	*mmu-miR-6347*	-2.78	0.0421	MIMAT0025090	chr1
12M vs 20M	*mmu-miR-667-5p*	-2.03	0.0429	MIMAT0017239	chr12
12M vs 20M	*mmu-miR-466h-5p*	2.01	0.0454	MIMAT0004884	chr2
12M vs 20M	*mmu-miR-466j*	3.65	0.0471	MIMAT0005848	chr10
12M vs 20M	*mmu-mir-7017*	2.41	0.0478	MI0022866	chr4

Figure 1

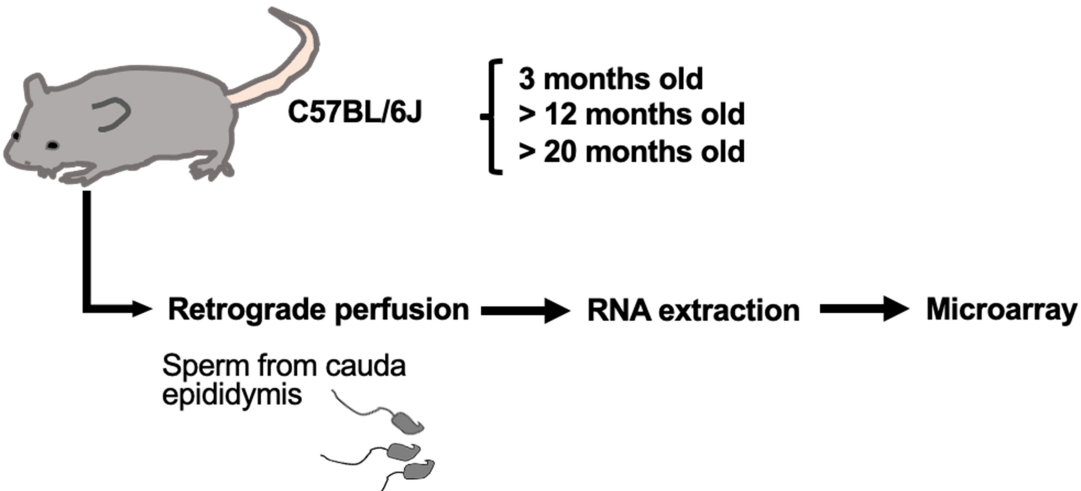

C57BL/6J
3 months old
> 12 months old
> 20 months old
Retrograde perfusion
RNA extraction
Microarray
Sperm from cauda epididymis

Figure 2

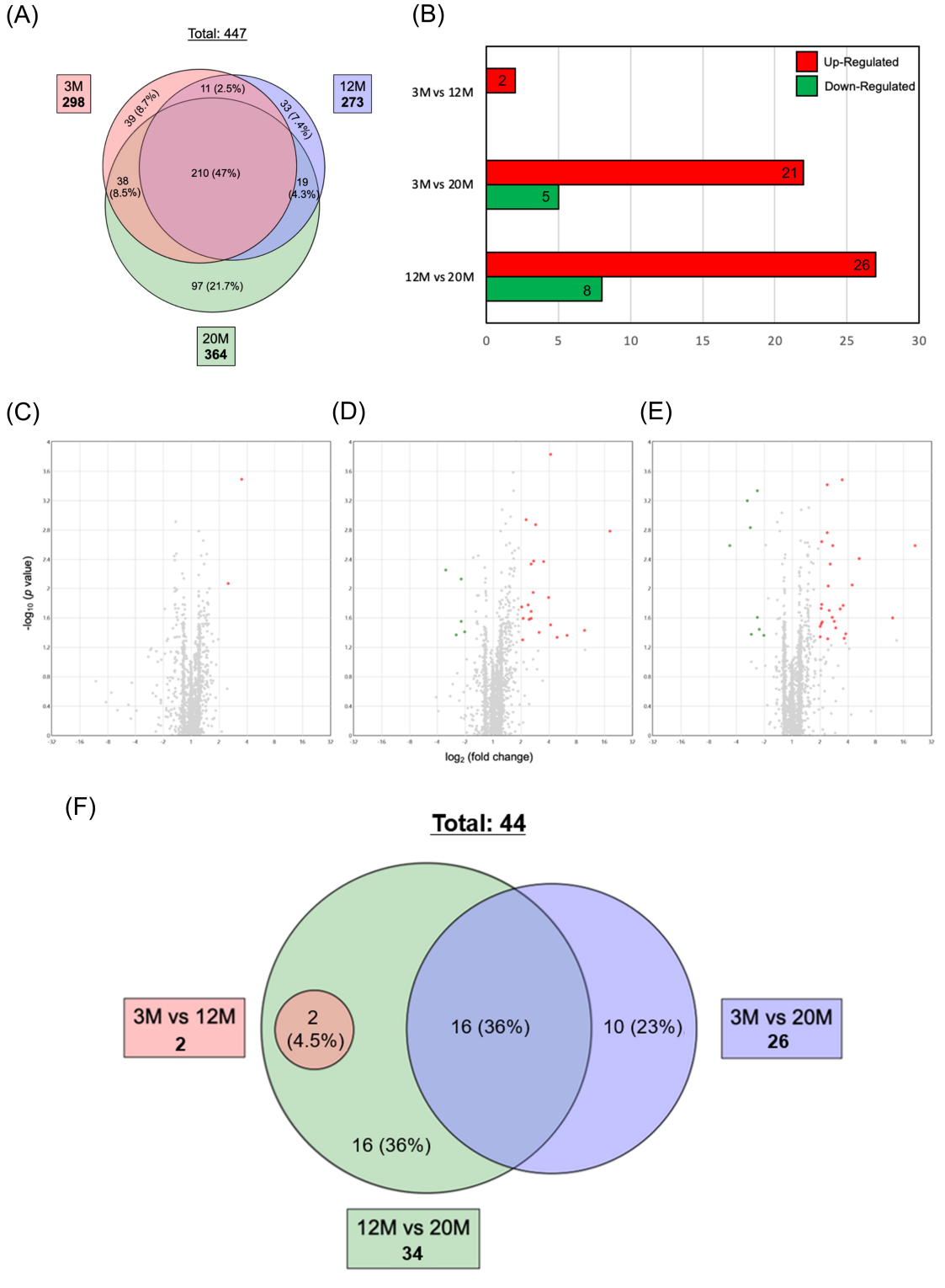
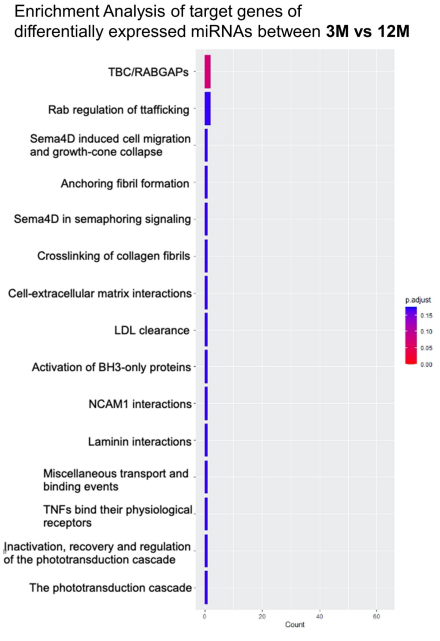
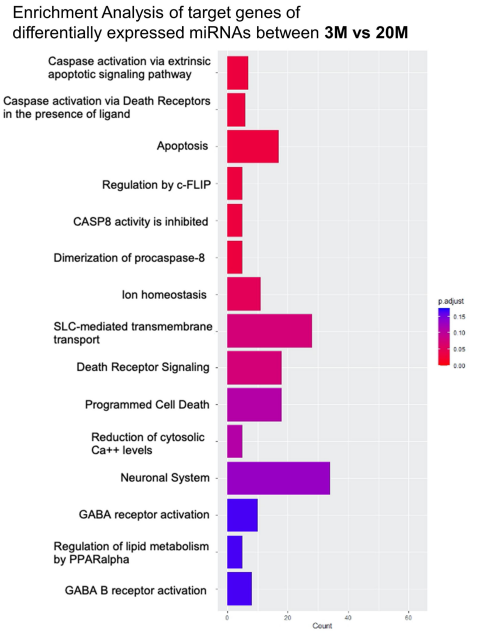

(A)
Total: 447
3M
298
12M
273
20M
364
39 (8.7%)
11 (2.5%)
33 (7.4%)
210 (47%)
38
(8.5%)
19
(4.3%)
97 (21.7%)
(B)
Up-Regulated
Down-Regulated
3M vs 12M
2
3M vs 20M
21
5
12M vs 20M
26
8
0
5
10
15
20
25
30
(C)
(D)
(E)
-log10 (p value)
log2 (fold change)
(F)
Total: 44
3M vs 12M
2
2
(4.5%)
16 (36%)
10 (23%)
3M vs 20M
26
16 (36%)
12M vs 20M
34

Figure 3

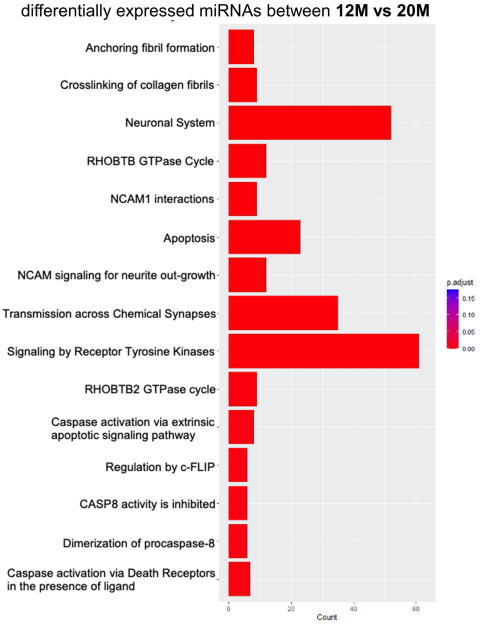
(A)



(B)



(C)



(D)

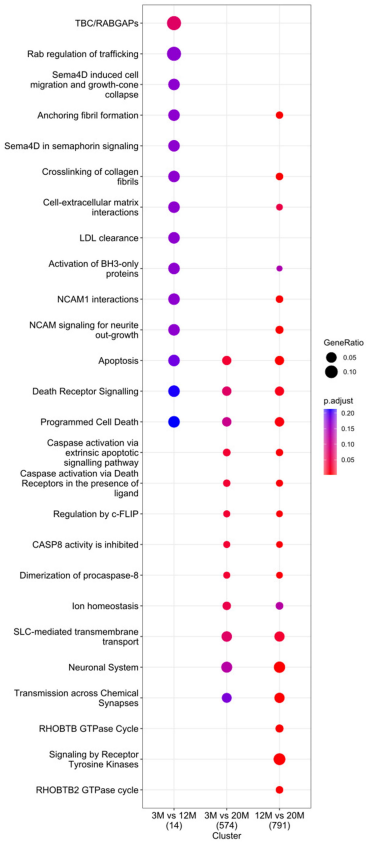
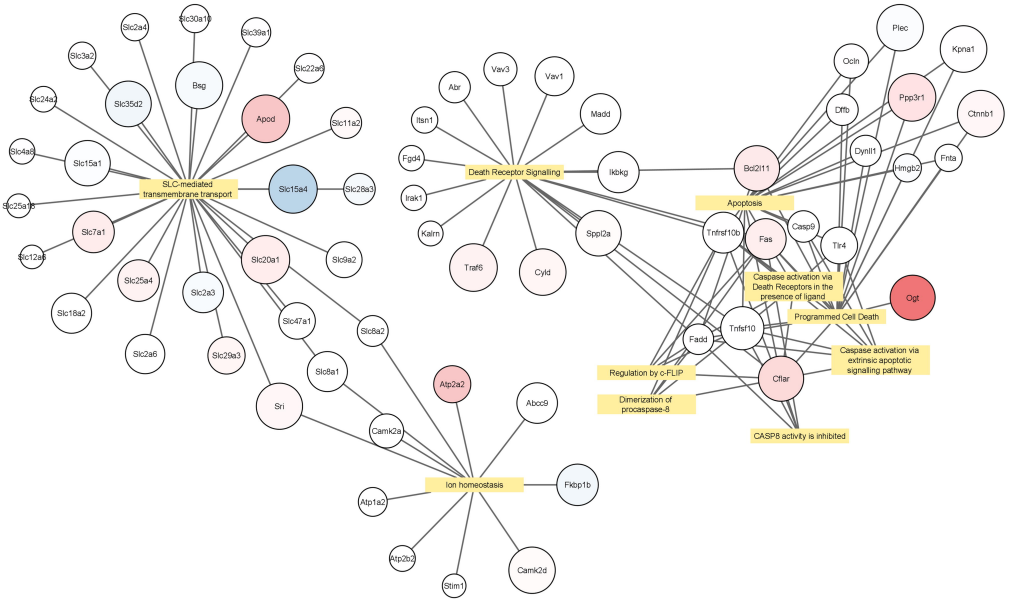


Figure 4

(A)


SLC-mediated transmembrane transport
Slc15a4
Apod
Slc35d2
Bsg
Slc15a1
Slc7a1
Slc20a1
Slc9a2
Slc25a4
Slc2a3
Slc47a1
Slc8a2
Slc8a1
Sri
Slc2a4
Slc30a10
Slc39a1
Slc3a2
Slc22a6
Slc24a2
Slc11a2
Slc4a8
Slc25a36
Slc12a6
Slc18a2
Slc2a6
Slc29a3
Slc28a3
Death Receptor Signalling
Vav3
Vav1
Abr
Madd
Itsn1
Fgd4
Ikbkg
Irak1
Kalrn
Sppl2a
Traf6
Cyld
Plec
Kpna1
Ocln
Ppp3r1
Ctnnb1
Dffb
Dynll1
Hmgb2
Fntb
Bcl2l11
Apoptosis
Tnfrsf10b
Fas
Casp9
Tlr4
Caspase activation via Death Receptors in the presence of ligand
Ogt
Programmed Cell Death
Tnfsf10
Fadd
Caspase activation via extrinsic apoptotic signalling pathway
Regulation by c-FLIP
Cflar
Dimerization of procaspase-8
CASP8 activity is inhibited
Atp2a2
Abcc9
Camk2a
Ion homeostasis
Fkbp1b
Atp1a2
Atp2b2
Stim1
Camk2d

(B)

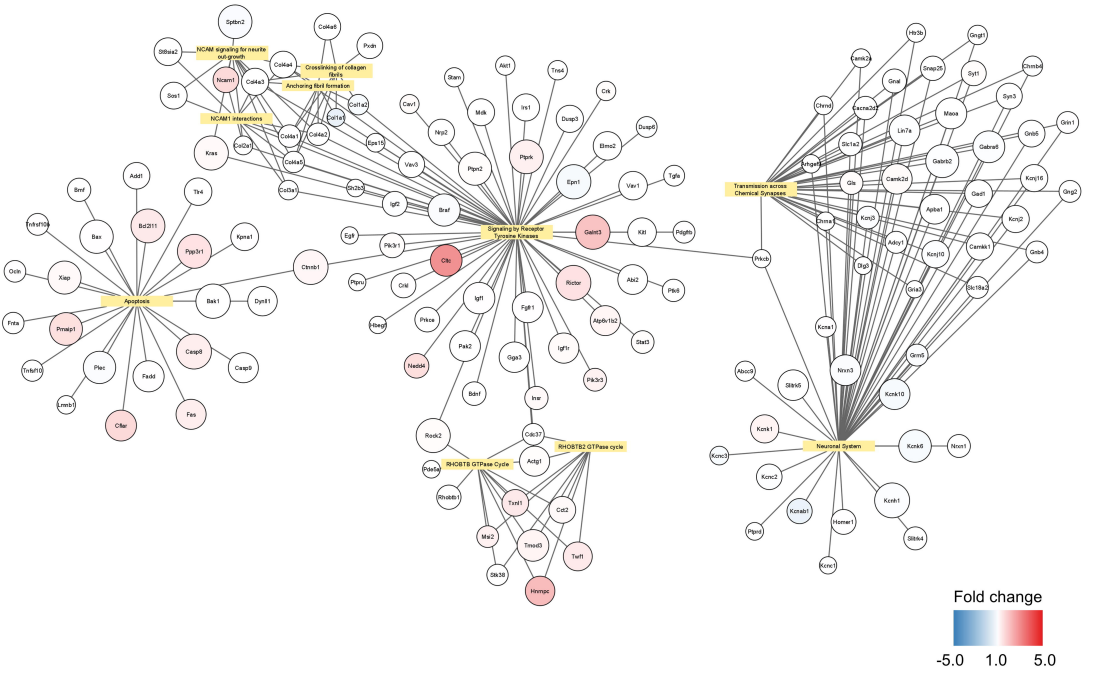

NCAM signaling for neurite outgrowth
Crosslinking of collagen fibrils
Anchoring fibril formation
NCAM1 interactions
Spsbn2
Col4a6
Pxdn
Slitrk2
Col4a4
Ncam1
Col4a3
Sos1
Col1a2
Col18a1
Col4a1
Col4a2
Col4a5
Col5a1
Col26a1
Kras
Eps15
Vav3
Ptpn2
Nip2
Mlk
Stam
Akt1
Trio4
Irs1
Dusp3
Crk
Dusp6
Ptprk
Elms2
Epn1
Vav1
Tgfb
Igf2
Braf
Signaling by Receptor Tyrosine Kinases
Galnt3
Kitl
Plgfb
Egf
Pik3r1
Ctbp
Ptpru
Crtl
Rictor
Akt2
Pik3
Igf1
Fgfr1
Alpdx1b2
Stat3
Prkce
Pak2
Gga3
Igf1r
Pik3c3
Nedd4
Bdnf
Inar
Rhebp
Redx2
Cdc37
RHOBTB2 GTPase cycle
Rhobtb2
RHOBTB GTPase Cycle
Actg1
Rhobtb1
Tsnd1
Cct2
Mia2
Tmed3
Twf1
Stk38
Hnrnpc
Apoptosis
Bcl2l11
Tlr4
Kpna1
Tnfsf10b
Ocln
Xiap
Ppp3r1
Ctnnb1
Bak1
Dynll1
Fntb
Prnicp1
Casp8
Casp9
Tnfsf10
Plec
Fadd
Lmnb1
Cflar
Fas
Bmf
Bax
Addf1
Transmission across Chemical Synapses
Hk2b
Grgt1
Camk2a
Gnal
Snap25
Syt1
Ctnnb1
Chrna6
Kcna2b
Syn3
Moea
Lin7a
Grb5
Grin1
Slc1a2
Gabrat
Gabrb2
Kcnj18
Gla
Camk2d
Gad1
Kcnq2
Apba1
Kcnj3
Kcnj10
Camk1
Grb4
Adcy1
Dlg3
Slc18a2
Gsa3
Kcna1
Gnrl5
Nrxn3
Kcnk10
Abcc9
Slitrk5
Kcnk1
Kcnc2
Neuronal System
Kcnk6
Nrxn1
Kcnc2
Kcnk3
Kcnh1
Homer1
Pipn2
Slitrk4
Kcnc1
Fold change
-5.0
1.0
5.0

Figure 5

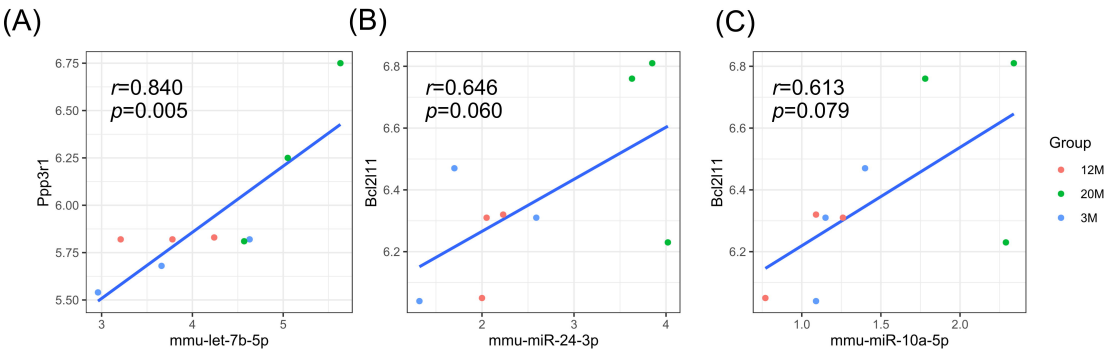

(A)
r=0.840
p=0.005
Ppp3r1
mmu-let-7b-5p
(B)
r=0.646
p=0.060
Bcl2l11
mmu-miR-24-3p
(C)
r=0.613
p=0.079
Bcl2l11
mmu-miR-10a-5p
Group
12M
20M
3M

Figure 6

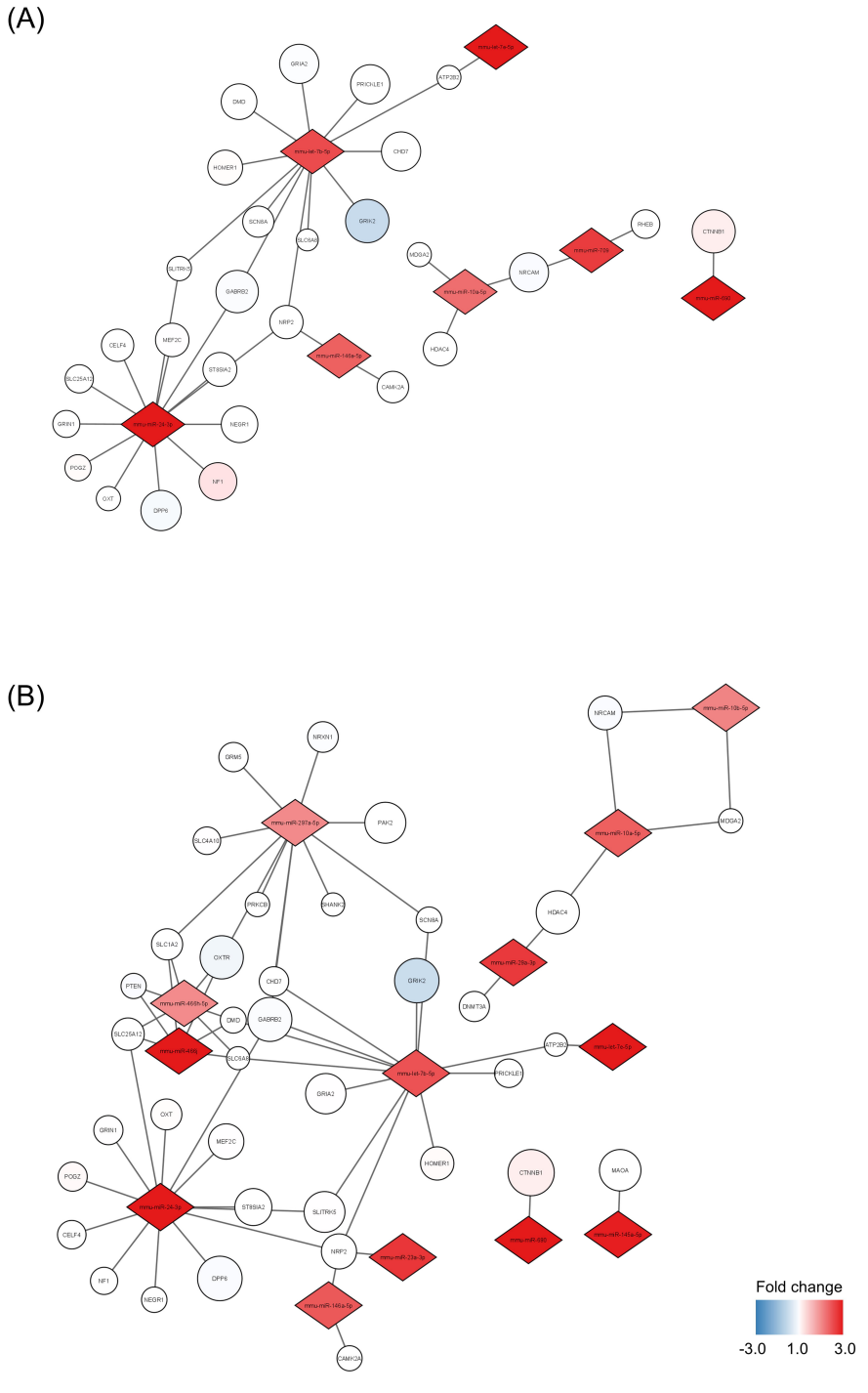
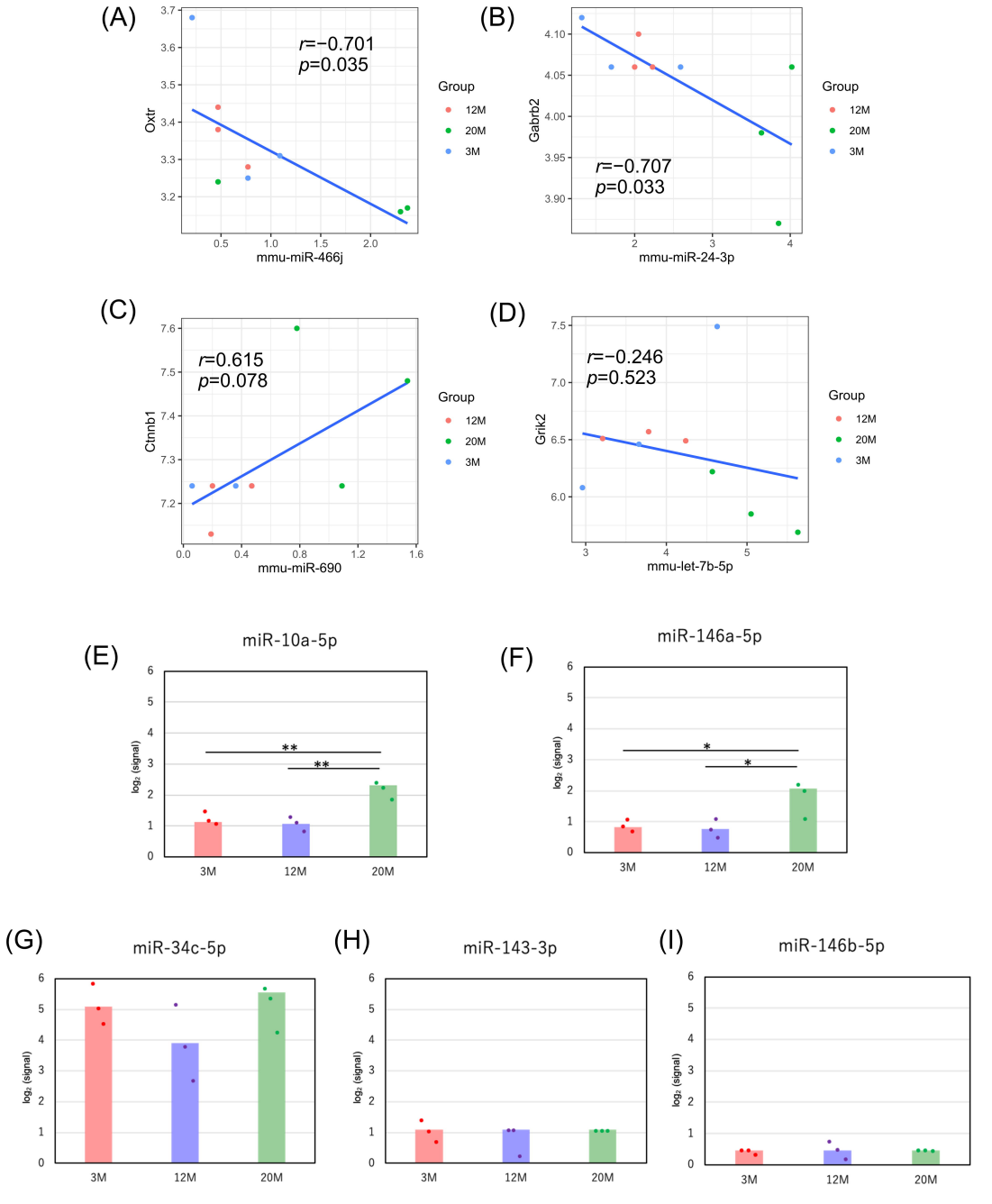

(A)
mmu-let-7a-5p
GRIA2
PRICKLE1
ATP2B2
DMD
mmu-let-7b-5p
CHD7
HOMER1
GRIK2
SCN8A
SLC6A8
RHEB
CTNNB1
mmu-miR-759
MDGA2
NRCAM
mmu-miR-10a-5p
mmu-miR-690
SLITRK5
GABRB2
NRP2
HDAC4
CELF4
MEF2C
mmu-miR-145a-5p
ST8SIA2
SLC25A12
CAMK2A
GRIN1
mmu-miR-24-3p
NEGR1
POGZ
NF1
OXT
DPP6
(B)
NRCAM
mmu-miR-10b-5p
NRXN1
GRM5
mmu-miR-297a-5p
PAK2
mmu-miR-10a-5p
MDGA2
SLC4A10
PRKCB
SHANK2
SCN8A
HDAC4
SLC1A2
OXTR
mmu-miR-29a-3p
PTEN
CHD7
GRIK2
mmu-miR-466h-5p
DNMT3A
DMD
GABRB2
SLC25A12
ATP2B2
mmu-let-7c-5p
mmu-miR-466j
SLC6A8
mmu-let-7b-5p
PRICKLE1
GRIA2
OXT
GRIN1
MEF2C
HOMER1
CTNNB1
MAOA
POGZ
mmu-miR-24-3p
ST8SIA2
SLITRK5
CELF4
mmu-miR-690
mmu-miR-145a-5p
NRP2
mmu-miR-23a-3p
NF1
DPP6
NEGR1
mmu-miR-146a-5p
CAMK2A
Fold change
-3.0
1.0
3.0

Figure 7


(A)
r=−0.701
p=0.035
Oxtr
mmu-miR-466j
Group
12M
20M
3M
(B)
r=−0.707
p=0.033
Gabrb2
mmu-miR-24-3p
(C)
r=0.615
p=0.078
Ctnnb1
mmu-miR-690
(D)
r=−0.246
p=0.523
Grik2
mmu-let-7b-5p
(E)
miR-10a-5p
(F)
miR-146a-5p
log2 (signal)
3M
12M
20M
(G)
miR-34c-5p
(H)
miR-143-3p
(I)
miR-146b-5p