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Prenatal maternal stress in rats alters the epigenetic and transcriptomic landscape of the maternal-fetal interface across four generations

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Prenatal maternal stress (PNMS) determines lifetime mental and physical health. Here, we show in rats that PNMS has consequences for placental function and fetal brain development across four generations (F0-F3). Using a systems biology approach, comprehensive DNA methylation (DNAm), miRNA, and mRNA profiling revealed a moderate impact of PNMS in the F1 generation, but drastic changes in F2 and F3 generations, suggesting compounding effects of PNMS with each successive generation. Both maternal and placental miRNA gene targets included de novo DNA methyltransferases, indicating robust PNMS-induced disruption in the complex epigenetic regulatory network between miRNAs and DNAm. Transgenerational programming mainly involved genes and biological pathways associated with neurological and psychiatric diseases which were linked to maternal-fetal crosstalk facilitated by the placenta. The highly correlated placenta-brain profiles support the use of placenta as a noninvasive biomarker resource to predict pathological changes in the neonatal brain. The transgenerational persistence of critical DNAm, miRNA and mRNA signatures may explain familial non-genetic disease risks.

Prenatal maternal stress (PNMS) is a major driver of adverse pregnancy outcomes and a risk factor for chronic illness in adulthood, including neuropsychiatric, cardiovascular, and metabolic diseases[1,2]. Exposure to PNMS can promote adult-onset diseases by influencing fetal development through epigenetic modifications including DNA methylation (DNAm) and expression of short noncoding RNAs, such as microRNAs (miRNAs)[3]. These dynamic epigenetic changes can regulate the transcriptional activity of genes as cells acquire specific functions[4]. Although select epigenetic marks are relatively malleable throughout development, a subset of epigenetic states is programmed in utero and remains stable into adulthood[5]. Both DNAm and miRNA expression profiles may affect brain development and neuropsychiatric disease risk in adulthood[1,2]. The molecular mechanisms behind these pathologies are not well-characterized, however, the placenta may play an important role in their etiology[6–8].

The placenta acts as an active neuroendocrine organ in fetal programming[9], providing a source of predictive biomarkers for health outcomes[10]. PNMS may dysregulate the in utero environment by altering placental expression of 11β-hydroxysteroid dehydrogenase-2 (11β-HSD2), a barrier enzyme that prevents fetal exposure to maternal corticosteroids[11,12]. Hence, excessive maternal corticosteroid exposure from PNMS may have potentially significant implications for long-term health in the offspring[1,9,13].

In addition to increasing the susceptibility to non-communicable diseases in the F1 generation, PNMS might affect future generations by inducing epigenomic changes that become heritable[8,14–16]. If PNMS occurs during the critical time of primordial germ cell development, aberrant epigenomic patterns can be reprogrammed into the germline and propagate across several generations[17]. Transgenerational epigenetic inheritance involves the transmission of epigenetic modifications and effects that manifest after ancestral exposure, without subsequent direct exposure[3]. Thus, if a gestating female (F0) is exposed to an environmental insult capable of bypassing the placental barrier, the developing embryo (F1) experiences direct exposure[18]. When this exposure occurs during germline development, the germline, which will later contribute to the F2 generation, is also considered directly exposed. Consequently, the F3 generation is

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recognized as the first generation that is entirely unexposed and, therefore, suitable for assessing transgenerational epigenetic inheritance[18].

Due to epigenetic inheritance, remote ancestral PNMS may change cellular metabolism and organ function[14,19], cause preterm birth and adverse newborn outcomes[8] and program brain development and aging trajectories[20–22]. Moreover, ancestral stress can promote behavioral changes, such as increased anxiety-like behaviors[14,19,21], risk taking [23,24], depression-like phenotypes[15,16] and motor hyperactivity [25]. Multigenerational PNMS has been shown to generate new behavioral traits[20] at the expense of accelerating brain aging and greater risk of tumors, respiratory and kidney diseases[14]. These studies highlight an urgent need to identify biomarkers linked to ancestral stress to improve lifetime health outcomes through better risk prediction and prevention.

The present study builds upon our finding that transgenerational and multigenerational PNMS causes adverse pregnancy outcomes and impaired offspring development[8]. While PNMS did not significantly affect gestational length in the F0 mothers, transgenerationally stressed F1 and F2 generation mothers experienced reduced gestational length and altered maternal behaviors[8,26]. Both transgenerational and multigenerational stress lineage F3 pups were characterized by reduced body weight on postnatal day (P) 1 and delayed sensorimotor development[8]. Key miRNA and mRNA markers in uterus and placenta of F1 and F2 rats resembled critical changes observed in human preterm birth[27,28], suggesting epigenetic regulation of pregnancy and developmental outcomes. Here, we investigated neuroplacental effects of PNMS across four generations of rats using a comprehensive systems biology approach of DNAm, miRNA, and transcriptomic analyses for placenta and fetal cortex. The study involved the functionally well-characterized transgenerational and multigenerational PNMS cohort described by ref. 8 where PNMS was delivered on embryonic days E12-E18 during a critical window for transgenerational epigenetic programming [29]. The results revealed cumulative changes in the F2 and F3 generations that were substantially increased from the F1 generation, demonstrating compounding consequences of PNMS across generations. The data provide valuable insights into the molecular etiology of developmental changes following PNMS and propose the placenta as a potential surrogate source of predictive biomarkers associated with neurodevelopmental health.

Results

Distinct profiles characterize fetal cortex and placenta DNA methylation

The number of differentially methylated regions (DMRs) in fetal cortex declined across generations, while placental DMRs grew larger in number in both experimental groups. Declines occurred in fetal cortex of the transgenerational lineage [499 DMRs in F1 (S) to 109 DMRs in F2 (SN) to 36 DMRs in F3 (SNN)] and the multigenerational lineage [499 DMRs in F1 (S) to 87 DMRs in F2 (SS) to 42 DMRs in F3; Fig. 1A. Little overlap was found between groups, with 8 genes overlapping out of a total of 36 SNN (22%) DMRs being similar between SNN and SSS groups (Fig. 1A). In an opposite trend, the transgenerational lineage F1 generation (S) placenta revealed 3 DMRs, the F2 generation (SN) 16 DMRs, and the F3 generation (SNN) 602 DMRs. In the multigenerational lineage, the F2 generation (SS) placenta showed 76 DMRs and the F3 generation 607 DMRs (Fig. 1B). The specific DMRs between each generation did not appear to show major overlaps. In most of the group comparisons in placenta, there was little overlap between DMRs, however, one-third of all DMRs were similar when comparing SNN and SSS groups, with 208 DMRs overlapping out of a total of 602 SNN placenta DMRs (Fig. 1B). A complete list of DMRs for each treatment group and tissue can be found in Supplementary Data 1–10.

DMR-associated genes underwent enrichment analysis and pathway association. The most significant biological processes appeared to be associated with signal transduction (Fig. 1C). Several biological processes relevant to neurodevelopment and placental health included telencephalon development, brain development, sensory organ development, and multicellular organism development. The top altered KEGG pathway was Pathways in Cancer and several other relevant pathways including Wnt signaling, MAPK signaling, insulin secretion, oxytocin signaling pathway, GABAergic synapse, and dopaminergic synapse pathways (Fig. 1D).

Differentially expressed miRNAs in fetal cortex and placenta regulate critical developmental pathways

Differentially expressed miRNAs were identified (Supplementary Data 11–20) and compared between generations with Venn diagrams. There were no significant changes in miRNA expression in either the F1 generation fetal cortex or placenta. There were also no significant changes in the transgenerational or multigenerational stress F2 fetal cortex (SN and SS). In the transgenerational model, rno-miR-409-5p was significantly altered in the F2 placenta (SN), and 14 total miRNAs were differentially expressed in the F3 generation (SNN). Rno-miR-409-5p was found to be differentially expressed in both SN and SNN placentas (Fig. 2A). In the multigenerational model placentas, there were 6 differentially expressed miRNAs in the F2 generation (SS) and 18 miRNAs in the F3 generation (SSS). There were two overlapping miRNAs between the F2 and F3 multigeneration placentas which were identified as rno-miR-409-5p and rno-miR-331-3p (Fig. 2B). The transgenerational and multigenerational stress F3 cortices were compared and revealed 5 significantly differentially expressed miRNAs in SNN and 3 miRNAs in SSS. All SSS cortex significantly differentially expressed miRNAs were also differentially expressed in SNN cortices (Fig. 2C). A complete list of miRNAs for each treatment group and tissue can be found in Supplementary Data 11–20.

Gene target analysis was performed on each significantly differentially expressed miRNA in the dataset. Gene targets were analyzed for functional and pathway enrichment. The top biological process identified was tissue remodeling. Several relevant most enriched biological processes also included autophagy, regulation of neurotransmitter levels, hindbrain development, exocytosis-related processes, branching morphogenesis, regulation of circadian rhythm, and stress-activated protein kinase and MAPK signaling (Fig. 2D). The top KEGG pathway for differentially expressed miRNA targets was the cAMP signaling pathway. Other relevant pathways included Wnt signaling, PI3K-Akt signaling, MAPK signaling, FoxO signaling, autophagy, and extracellular matrix (ECM)-receptor interaction (Fig. 2E).

Differentially expressed genes in fetal cortex and placenta are involved in organ development and aging

Differentially expressed genes were compared between generations for the transgenerational and multigenerational fetal cortex and placenta (Supplementary Data 21–30). The F1 generation (S) cortex provided 56 differentially expressed mRNAs. The transgenerational lineage revealed 355 differentially expressed mRNAs in the F2 (SN) cortex and 1022 mRNAs in the F3 (SNN) cortex (Fig. 3A). In the F1 generation placenta, there was only 1 differentially expressed mRNA. The transgenerational lineage, however, revealed 291 differentially expressed genes in the F2 placentas and 953 differentially expressed genes in the F3 generation. In the multigenerational lineage, there were 749 and 926 differentially expressed genes in the F2 and F3 generation placentas, respectively (Fig. 3B). Due to the small number of differentially expressed genes of both the cortex and placenta in the F1 generation, few genes were identified that were common to all generations in these tissues. However, there was a large percentage of overlapping genes identified when comparing the F2 and F3 generations within the cortex and placenta separately (Fig. 3A, B). In the cortex, 189 out of 355 genes (53%) of SN cortex differentially expressed genes overlapped with the SNN cortex and 151 out of 269 genes (~56%) of the SS cortex differentially expressed genes overlapped with the SSS cortex (Fig. 3A). In the placenta, 237 out of 291 (81%) SN differentially expressed genes overlapped with the SNN placentas and 413 out of 749 (55%) SS placenta genes overlapped with the SSS placentas (Fig. 3B). A complete list of mRNAs for each treatment group and tissue can be found in Supplementary Data 21–31.

Gene category and pathway analysis was performed on the differentially expressed genes. The top biological processes identified were related to translation and concerned apoptotic processes, autophagy, signal

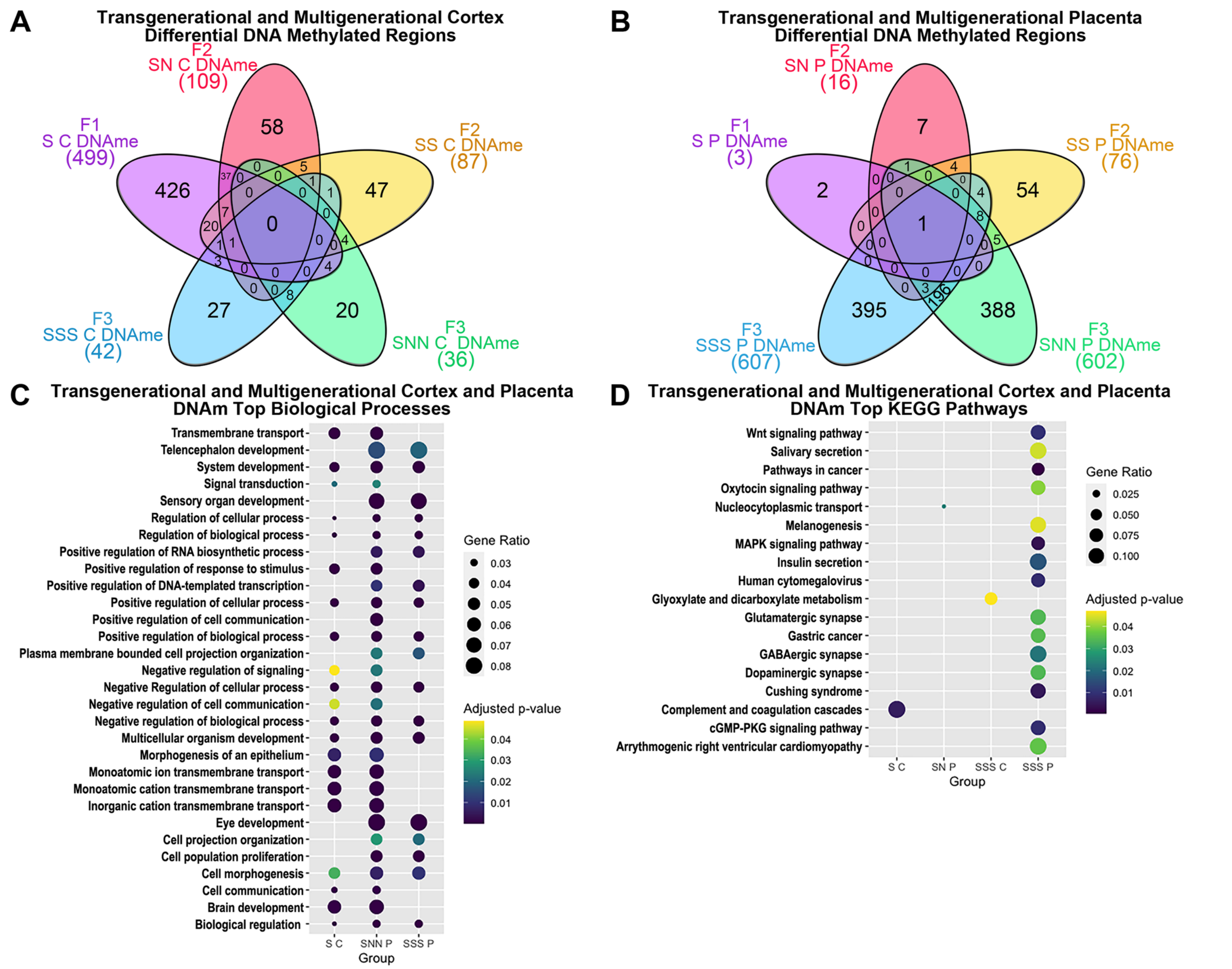

Fig. 1 | DNA methylation overlaps and gene associations. **A** DMR overlap Venn diagram for transgenerational and multigenerational fetal cortex differential DNA methylated regions. **B** DMR overlap Venn diagram for transgenerational and multigenerational placenta differential DNA methylated regions. **C** Top biological processes of gene associations containing DMR-associated genes. Treatment groups without statistically significant enrichment results are not displayed. **D** Top KEGG pathways containing DMR-associated genes. Treatment groups without statistically significant enrichment results are not displayed. “C” denotes cortex, “P” denotes placenta. Gene ratio was calculated by relating the number of genes found in the specific biological process/pathway in the comparison group to the total number of genes in this biological process/pathway.

transduction, cell cycle processes, and *in utero* embryonic development (Fig. 3C). The top KEGG category was spinocerebellar ataxia and other relevant pathways included neurodegenerative diseases (Alzheimer, amyotrophic lateral sclerosis, Huntington, Parkinson, and general pathways of neurodegeneration), autophagy, lysosome and protein processing, and nucleocytoplasmic transport (Fig. 3D).

Similarities in epigenetic and gene expression changes in fetal cortex and placenta

Changes in epigenetic processes and gene expression were compared between the fetal cortex and placenta. The majority of SNN cortex miRNAs were also differentially expressed in the SNN placenta (Fig. 4A). All SSS cortex miRNAs were also differentially expressed in the SSS placenta (Fig. 4B). A $\log_2$-fold change heatmap was generated to compare miRNA expression profiles between all generations. All miRNAs with statistically significant changes in the expression are represented and non-significant expression level reads are included in order to identify potential trends (Fig. 4B). Differential expression analysis revealed distinct overlapping patterns for miRNAs in the F3 generation SNN and SSS cortices and placentas. Specifically, rno-miR-409-5p and rno-miR-331-3p exhibited differential expression in both tissues for both F3-SNN and F3-SSS treatment groups. In the transgenerational animals of F3-SNN, rno-miR-99b-5p and rno-miR-100-5p displayed differential expression in the cortex and placenta, while in multigenerational F3-SSS, rno-miR-30a-3p was differentially expressed in both tissues. Notably, all shared common miRNAs between the cortex and placenta within their respective treatment groups. Generations demonstrated similar patterns of fold change, as illustrated in Fig. 4C. The trends of expression through F1-F3 generations for common miRNAs in cortex and placenta are charted in Fig. 5A–G. Figure 5A–D highlights that rno-miR-409-5p and rno-miR-331-3p were found in both the F3-SNN and SSS treatment groups, and expression levels were well-orchestrated between fetal cortex and placenta. DMRs from the F3 generation cortex and placenta were compared to identify a similar trend. In the transgenerational model, 11 out of 36 (31%) of the SNN cortex DMRs overlapped with the SNN placentas (Fig. 4D). In the multigenerational model, 16 out of 42 (38%) of SSS cortex DMRs overlapped with the SSS placentas (Fig. 4E).

Comparison of differentially expressed genes in fetal cortex and placenta revealed that 388 out of 953 (41%) SNN cortex mRNA overlapped with the SNN placenta mRNA in the transgenerational lineage. The pooled

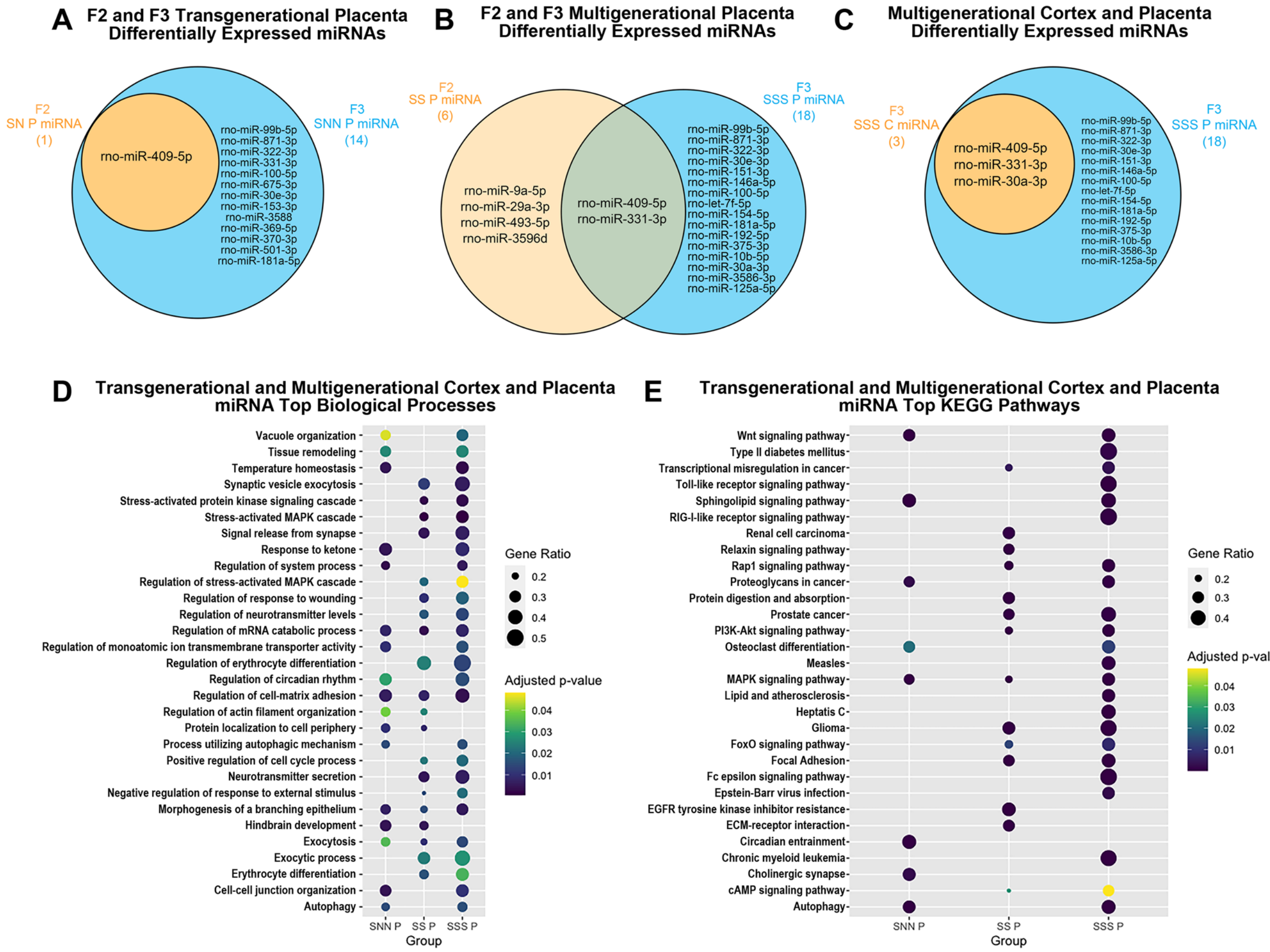

Fig. 2 | miRNA overlaps and miRNA target gene associations. A Venn diagram for transgenerational prenatal stress model differentially expressed placenta miRNAs. **B** Venn diagram for multigenerational prenatal stress model differentially expressed placenta miRNAs. **C** Venn diagram for transgenerational prenatal stress model differentially expressed fetal cortex miRNAs. **D** Top biological processes gene associations containing miRNA target genes. Treatment groups without statistically significant enrichment results are not displayed. **E** Top KEGG pathways containing miRNA target genes. Treatment groups without statistically significant enrichment results are not displayed. Note that miRNA target genes involved pathways with important roles in brain development and plasticity. "C" denotes cortex, "P" denotes placenta.

variance of the $\log_2$ fold change was calculated to be 0.15 (Fig. 4F). When comparing the multigenerational lineage, 357 out of 926 (39%) of SSS cortex differentially expressed genes overlapped with the SSS placenta. The overlapping mRNA had a $\log_2$ fold change pooled variance of 0.16 (Fig. 4G). A $\log_2$-fold change heatmap was generated from all genes that overlapped between the cortex and placenta. The grand majority of genes had similar patterns of expression with the exception of the genes *Fbxo8* and *Tuba1a* with decreased expression in the fetal cortex and increased expression in the placenta (Fig. 4H). A list of genes and expression levels used to generate Fig. 4H can be found in Supplementary Data 31.

Potential maternal miRNA influence on fetal epigenetics and gene expression

A previous study was performed on the F2 generation mothers of the F3 fetuses represented in this study[8]. This dataset showed 10 differentially expressed miRNAs in the F2 generation prefrontal cortex and 2 miRNAs in the maternal uterus (Fig. 6A). In order to further investigate the potential influence of maternal miRNA crosstalk on fetal development, F2 generation maternal miRNA targets were identified and compared to F3 generation fetal mRNAs. There were 18 common genes identified between all groups potentially modulated by maternal miRNAs, including rno-miR-200b-3p and rno-miR-429 which are present in both the maternal uterus and prefrontal cortex: *Spyrd7*, *Mprip*, *Ywhag*, *Tceb1*, *Serinc1*, *Bag6*, *Cnot7*, *Zfr*, *Zfp330*, *Uba3*, *Zc3h15*, *Mex3c*, *Ube2i*, *Eif2s1*, *Ier5*, *Nrbf2*, *Ythdf3*, *Map4k4* (Fig. 6B). Maternal miRNA targets were compared to the fetal miRNA targets, DNAm, and differentially expressed genes. In the fetal cortex, maternal miRNA targets overlapped with 221 out of 868 (25.4%) of the fetal cortex miRNA targets, 8 out of 42 (19%) of DNAm-associated genes, and 186 out of 1139 (16.3%) of cortex differentially expressed genes (Fig. 6C). In the placenta, 1017 out of 2387 (42.6%) maternal miRNA targets overlapped with the fetal miRNA targets, 106 out of 608 (17.4%) of the DNAm associated genes, and 162 out of 926 (17.5%) of differentially expressed genes (Fig. 6D). No known miRNA family classifications were shared between the identified maternal miRNAs and fetal miRNAs according to miRBase[30].

Discussion

Maternal stress represents a critical determinant of offspring lifetime health across generations. This study investigated the effects of PNMS on the maternal-fetal interface in transgenerational and multigenerational stress models of a functionally well-characterized rat cohort[8,26]. DNAm, miRNAs, and mRNA expression profiles were considered in a systems biology approach to assess multiple layers of molecular changes in the gestational day 21 fetal cortex and placenta. Each category of molecular changes was assessed for overlapping genes or associated genes to identify stress-induced heritable regulatory pathways that persist across generations. While there were significant changes in DNAm and mRNA expression in each

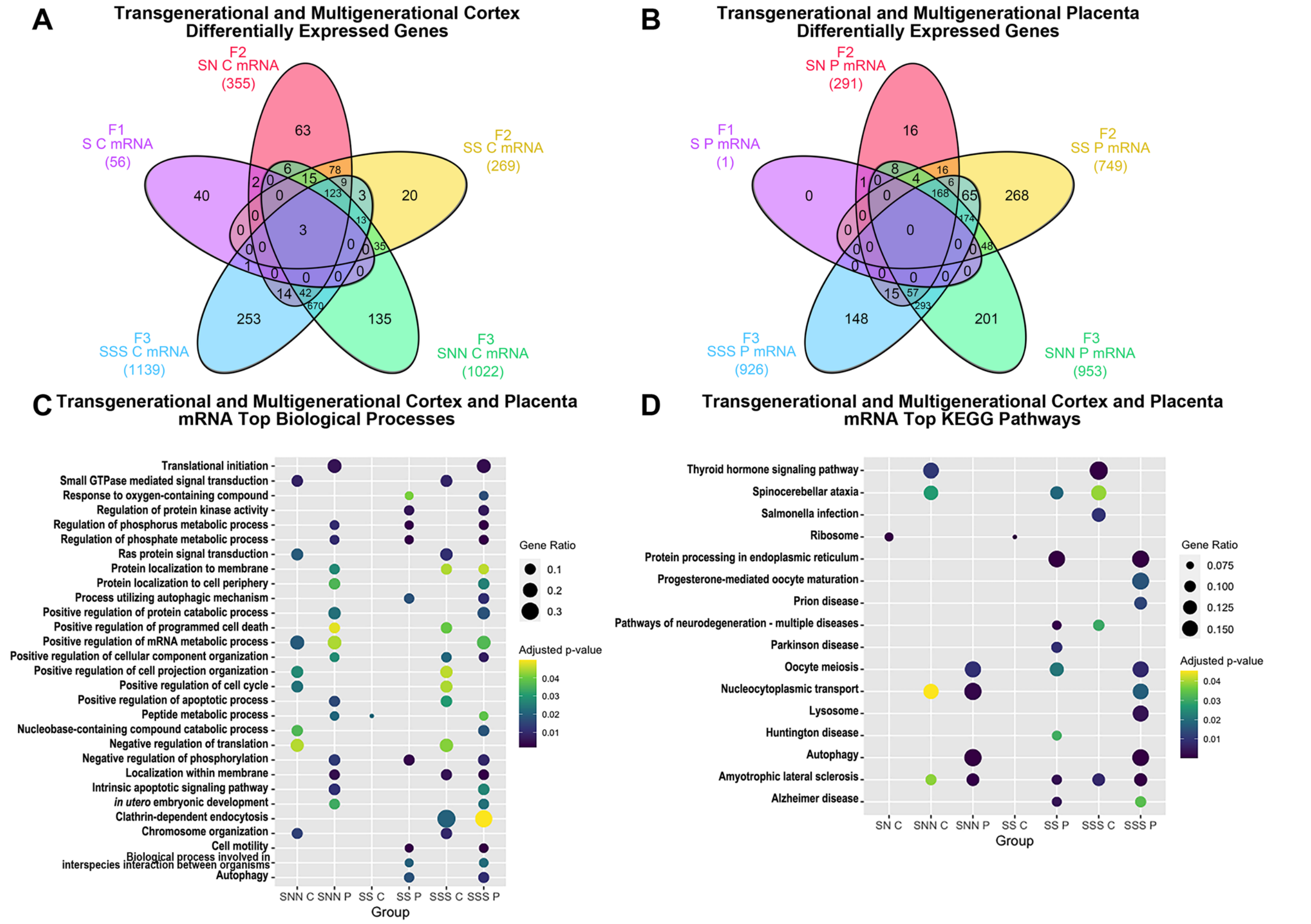

Fig. 3 | mRNA overlaps and gene associations. A mRNA overlap Venn diagram for transgenerational and multigenerational fetal cortex differentially expressed genes. **B** mRNA overlap Venn diagram for transgenerational and multigenerational placenta differentially expressed genes. **C** Top biological processes gene associations containing differentially expressed genes. Treatment groups without statistically significant enrichment results are not displayed. **D** Top KEGG pathways containing differentially expressed genes. Treatment groups without statistically significant enrichment results are not displayed. Note that differentially expressed genes involve pathways with significant pathophysiological roles in major neurological and neurodegenerative diseases. “C” denotes cortex, “P” denotes placenta.

generation as well as characteristic miRNA changes in F2 and F3 generations, the data indicated no major overlaps in common associated genes or miRNAs in each generation. Differential methylation in the fetal cortex and placenta revealed opposing patterns with an increase in differentially methylated genes in the fetal cortex that declined in the F2 and F3 generations. By contrast, the placenta revealed a smaller number of differentially methylated genes in the F1 generation that increased dramatically in the F3 generation. As DNAm patterns have a tissue-specific regulation of inheritance[31], the opposing patterns of DNAm may indicate a different etiology of transgenerational inheritance in the two tissues, facilitating the differential expression of mRNAs and miRNAs in the F2 and F3 generations. However, as a trend, the levels of miRNA and mRNA expression changes increased in the F2 and F3 generations compared to the direct exposure effects observed in F1, demonstrating a compounding effect of PNMS with each successive generation.

The timing of exposure may be very relevant to the response of F1 generation tissues. At gestational day 12, the placenta is already well-established, therefore revealing a lower response to stress by low initial levels of DMRs. Gestational days 12-18, however, are a highly vulnerable period for brain development, therefore potentially showing an immediate response to stress through high initial levels of DMRs in the F1 generation cortex[32,33]. The fetal cortex demonstrated a decrease in DNA methylation levels through successive generations, indicating a tissue-specific plastic response that may involve unique epigenetic modifications. Brain as a somatic tissue may adapt to adversity and thus decrease the number of DMRs across generations[1,2]]. Conversely, the observed increase in DNA methylation levels in the placenta across generations may be influenced by distinct regulatory mechanisms specific to this tissue. Placenta, also being somatic tissue, may develop an increased response to circulating maternal influences like ncRNAs in the form of accumulating DMRs.

The DMRs, miRNA target genes, and transcriptome of the fetal cortex and placenta were integrated for functional analysis and pathway association. Functional analysis identified a group of genes involved with developmental biological processes including brain development, cell differentiation, cell proliferation, and embryonic development as well as signaling pathways regulating pluripotency of stem cells. Abnormal regulation of these developmental processes is detrimental to neurodevelopmental health, increasing the susceptibility to neuropsychiatric disorders[34]. Functional analysis identified various cellular processes including apoptosis, autophagy, tissue remodeling and cell cycle. Apoptosis is vital in the regulation of neurodevelopment to prevent aberrant neural migration and targeting[35] and autophagy is essential for neuronal development, including neurite outgrowth, synaptic pruning, and axonal remodeling[36]. Autophagy plays a crucial role in maintaining cellular homeostasis by removing damaged or dysfunctional cellular components and promoting cellular survival during stress conditions[37]. Moreover, PNMS and ancestral stress also affected regulation of dopaminergic synapse and neurotransmitter secretion pathways. Aberrant dopaminergic signaling plays a role in the etiology of several neurodegenerative disorders[38]. Dopaminergic signaling can stimulate brain remodeling and plasticity through TrkB neurotrophin

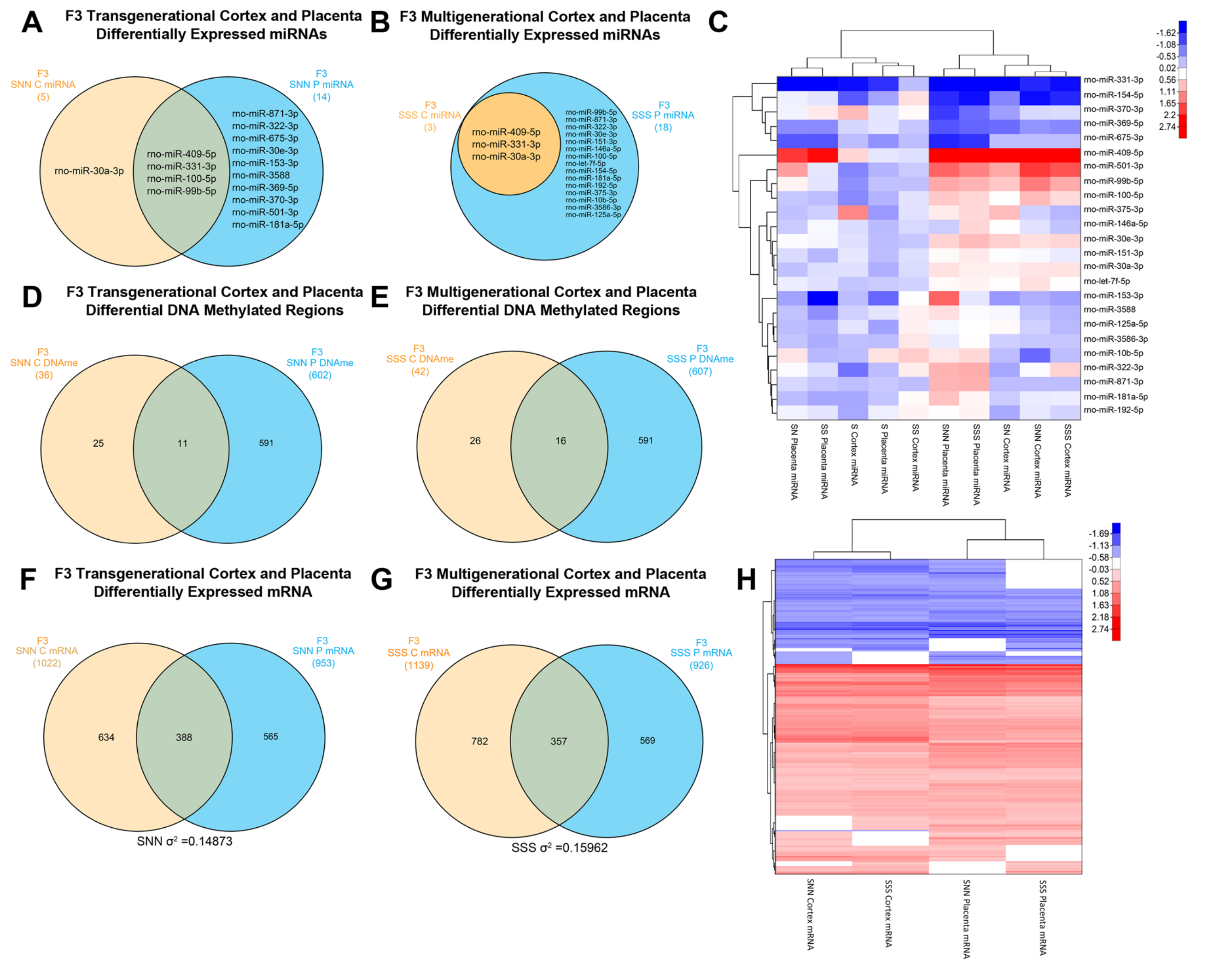

Fig. 4 | Overlaps in DMR and miRNA expression between the fetal cortex and placenta. **A** miRNA overlap Venn diagram for transgenerational differentially expressed fetal cortex and placenta miRNAs. **B** miRNA overlap Venn diagram for multigenerational differentially expressed fetal cortex and placenta miRNAs. **C** Heat map shows log2 fold change for miRNA expression in the transgenerational and multigenerational models fetal cortex and placenta. **D** DMR overlap Venn diagram for transgenerational F3 generation fetal cortex and placenta differential DNA methylated regions. **E** DMR overlap Venn diagram for transgenerational F3 generation fetal cortex and placenta differential DNA methylated regions. **F** mRNA overlap Venn diagram for transgenerational differentially expressed fetal cortex and placenta genes. Pooled variance of the log2 fold change of overlapping genes is represented below. **G** mRNA overlap Venn diagram for multigenerational differentially expressed fetal cortex and placenta genes. Pooled variance of the log2 fold change of overlapping genes is represented below. **H** Heat map shows log2 fold change for mRNA expression for overlapping genes in the F3 transgenerational and multigenerational models for fetal cortex and placenta. Note that the grand majority of genes had similar patterns of expression.

signaling[39]. These alterations in developmental, apoptotic and inflammatory functions, and neural signaling suggests an increased susceptibility to neurodevelopmental disorders following ancestral stress.

At least some of the differential gene expression patterns identified during fetal neurodevelopment may persist into adulthood which indicates the potential for long-term neurological consequences. In a previous study from our lab, female P120 F3 multigenerationally stressed rats (SSS) revealed reduced neural density in several brain areas[22]. The differential expression of genes *Epha5*, *Fgf12*, *Negr1*, and *Snap25* previously shown in the prefrontal cortex of adult females[22] was also found in the current study's SSS fetal cortex samples. McCreary et al.[22] demonstrated that the down-regulation of these genes was associated with reduced brain volume, increased corticosterone levels, and heightened stress sensitivity. Gene expression changes are brain region-specific but different sites could not be considered in the present study due to the small size of fetal brains.

In addition to pathways regulating neural plasticity, inflammation-related pathways revealed differential regulation in FoxO, PI3K-Akt, Wnt, MAPK, Rap1, Ras, cAMP, Fc epsilon RI, Toll-like receptor, and RIG-I-like receptor signaling pathways. Excessive maternal inflammatory signaling can affect fetal neurodevelopment across the placenta[40] and influence behavioral and cognitive development[41,42], raising the susceptibility to psychiatric disorders later in life[40,43]. The alterations of these pathways identified in the cortex and placenta of rats ancestrally exposed to PNMS may explain the increased susceptibility to anxiety-like behaviors identified in similar transgenerational and multigenerational stress models[14,19,21] and transgenerational programming of depression-like behaviors[15,16]. Moreover, ongoing activation of inflammatory pathways by PNMS may create an intrauterine pro-inflammatory state, leading to adverse pregnancy outcomes, such as preterm birth[44], even in future generations[8,45,46]. Notably, preterm birth itself also represents a significant risk factor for psychiatric and mental health problems in later life[47].

The present findings demonstrate a surprising degree of overlap in gene regulation patterns between the fetal cortex and placenta even though heritable epigenetic and transcriptomic changes occur in a tissue-specific manner[31]. The placenta is a neuroendocrine organ that actively facilitates neurodevelopment[9] and both, placenta and fetus, can secrete extracellular

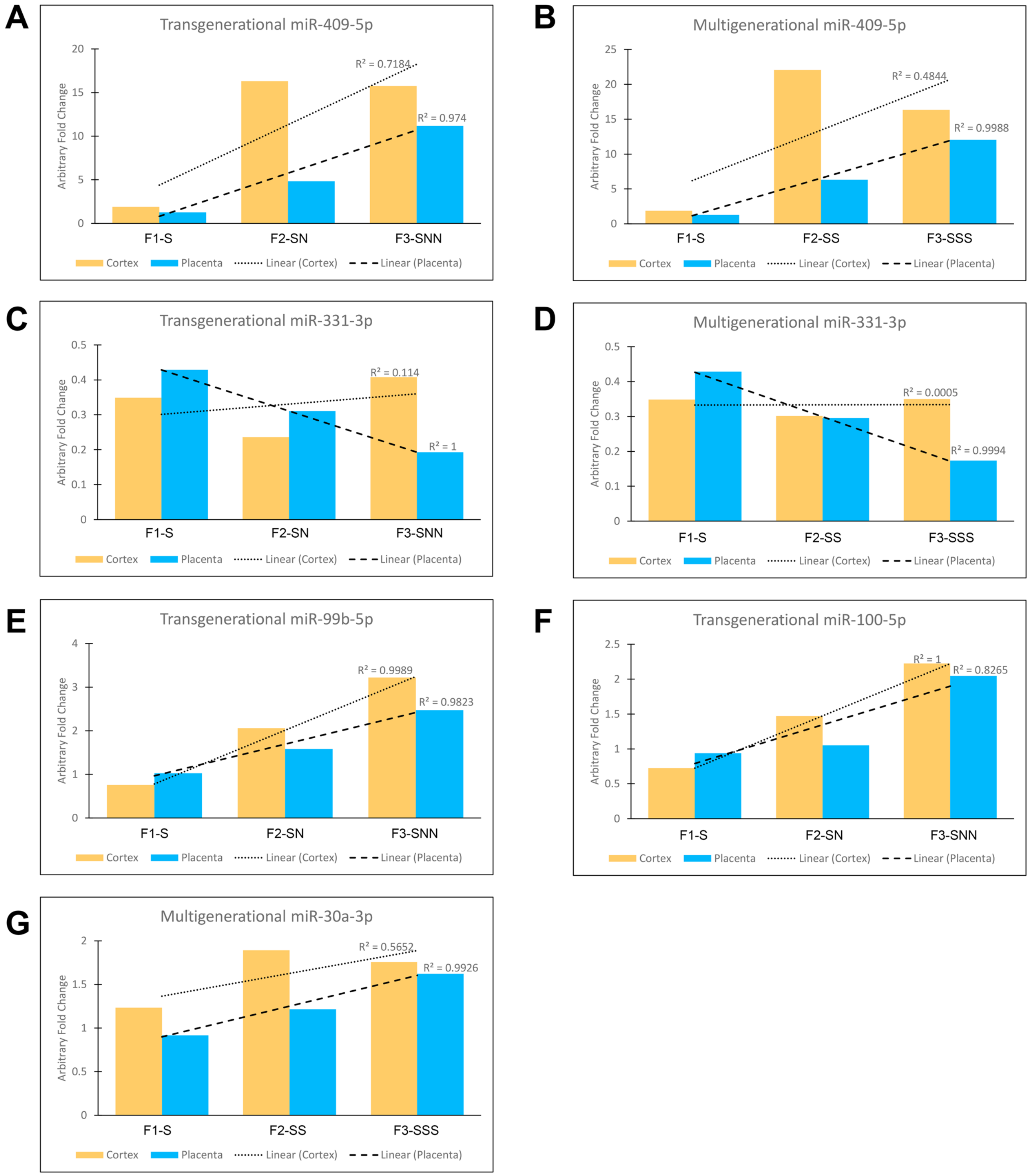

Fig. 5 | Trends of arbitrary fold change in the overlapping fetal cortex and placenta miRNAs. **A** Transgenerational differential expression of rno-miR-409-5p in the fetal cortex and placenta. **B** Multigenerational differential expression of rno-miR-409-5p in the fetal cortex and placenta. **C** Transgenerational differential expression of rno-miR-331-3p in the fetal cortex and placenta. **D** Multigenerational differential expression of rno-miR-331-3p in the fetal cortex and placenta. **E** Transgenerational differential expression of rno-miR-99b-5p in the fetal cortex and placenta. **F** Transgenerational differential expression of rno-miR-100-5p in the fetal cortex and placenta. **G** Multigenerational differential expression of rno-miR-30a-3p in the fetal cortex and placenta. Note the generally well-orchestrated expression patterns between fetal cortex and placenta.

signaling exosomes that contain growth factors, mRNA, and miRNAs as a form of intercellular communication[48,49]. Additionally, several biological processes and pathways relevant to exosome or extracellular vesicle release were altered including exocytosis and exocytic processes, extracellular matrix-receptor interaction, nucleocytoplasmic transport pathways. Hence, it is possible that the overlap in differentially expressed genes and miRNAs may be facilitated by exosomal transfer between the placenta and the fetus.

In addition to placental-fetal crosstalk, exosomal miRNAs and other signaling molecules can be exchanged into and from the maternal circulatory system[48,49]. To investigate this phenomenon, miRNAs identified from

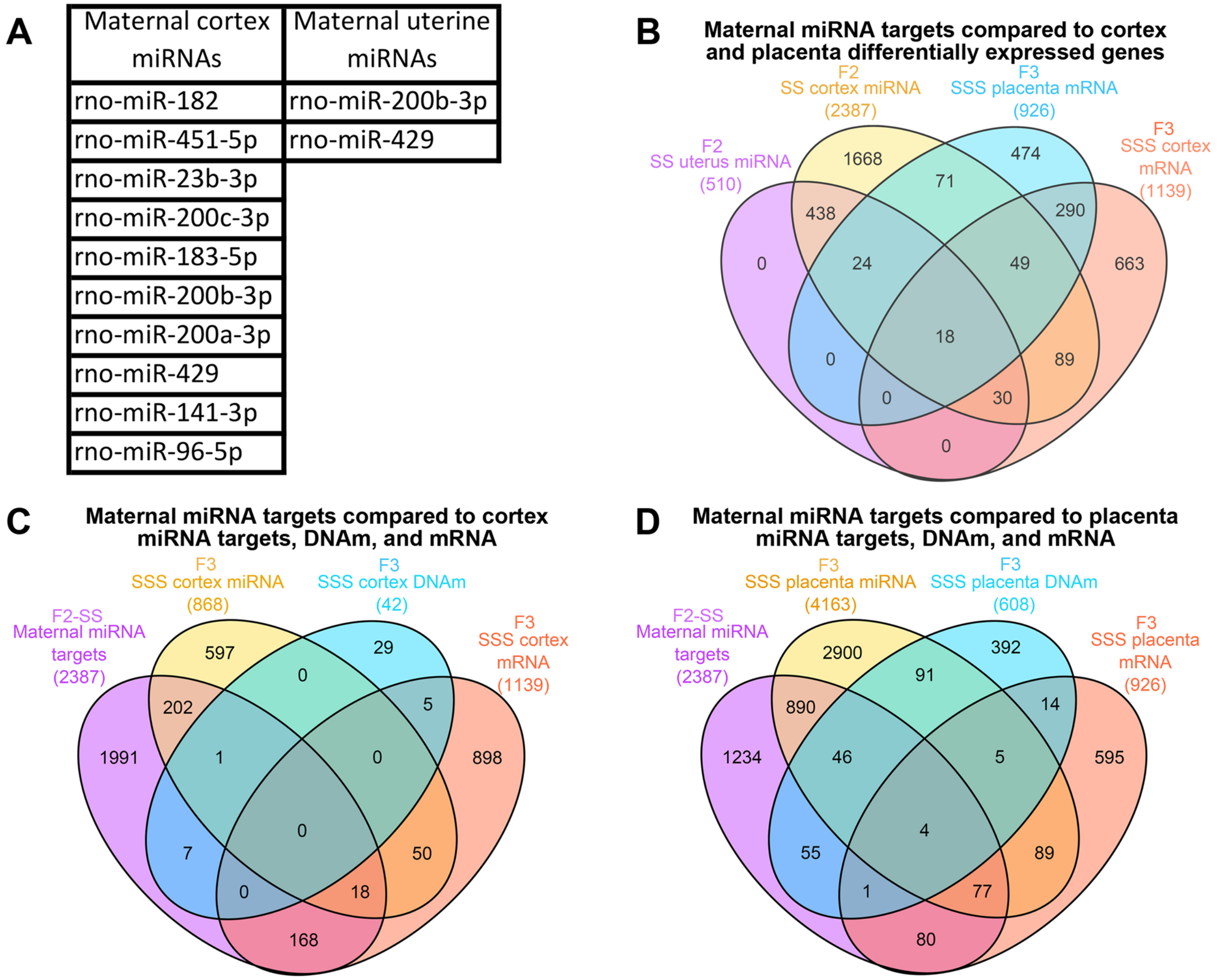

Fig. 6 | Maternal miRNA targets compared with fetal cortex and placenta differentially expressed genes. A Maternal F2 generation miRNAs were shown to be significantly altered in the prefrontal cortex and uterus from a previous study performed by this group[8]. **B** Maternal miRNA targets from the F2 generation uterus and cortex compared with F3 generation fetal cortex and placenta differentially expressed genes. **C** Maternal miRNA targets compared with F3 generation fetal cortex miRNA targets, DNAm, and mRNA. **D** Maternal miRNA targets compared with F3 generation fetal placenta miRNA targets, DNAm, and mRNA. Note that maternal miRNA targets showed similar or sometimes increased percentages of overlap with fetal DNAm and mRNA when compared to identified fetal miRNA targets.

the SN and SS F2 generation mothers of the SNN and SSS F3 generation fetal pups were cross referenced to a list from the F3 generation fetal cortex and placenta (see Fig. 6). Maternal miRNA targets were identified and compared to fetal miRNA, DNAm, and mRNA data to determine potential maternal impact on the three different molecular levels in the fetus. Maternal miRNA targets had similar or in some cases increased percentage overlap with fetal DNAm and mRNA when compared to identified fetal miRNA targets (Fig. 6 and Fig. S1).

When comparing the DNAm-associated genes with other distinct levels of multi-omic analyses, many DMR in the F3 generations did not show overlap with those markers or with previous generations. However, 34% of placenta F3 DMR overlapped between transgenerational SNN and multigenerational SSS treatment groups, indicating a specific change common to the F3 generation. Upon further investigation, several F2 generation maternal and F3 generation placental miRNAs were identified as having an important role in miRNA/DNAm feedback loops, targeting the de novo DNA methyltransferase (DNMT) genes[50]. DNMTs catalyze the addition of a methyl group to CpG dinucleotide residues and certain DNMTs are imperative for the establishment of new or de novo DNAm patterns during early development[51]. The maternal miRNAs miR-200b/c-3p and miR-429 differentially expressed in both the F2 maternal uterus and frontal cortex can target DNMT 3 alpha (*Dnmt3a*)[52–55]. Placental miR-322-3p can target DNMT 3 beta (*Dnmt3b*) and placental miR-375-3p can target the DNMT 3-like (*Dnmt3l*) gene in addition to *Dnmt3b* [Bi et al., 2018[52,53,55];]. During epigenetic reprogramming of the developing embryo, *Dnmt3a*, *Dnmt3b*, and the coactivator *Dnmt3l* re-establish DNAm patterns essential for development[56]. *Dnmt3l* in particular is necessary for maternal lineage gene imprinting of DNAm[57]. These three de novo methyltransferase genes are critical for the DNAm remodeling of placental tissue[58], however, their activity is heavily time-dependent, peaking at earlier developmental stages[59]. It is possible that both maternal and fetal miRNAs work in tandem to shape fetal development transgenerationally and multigenerationally, a phenomenon already reported in F0 to F1 generation studies[49,60]. Disruption of maternal-fetal miRNA expression may therefore have significant consequences on de novo DNAm patterns in the fetus and further investigation must follow to determine if these genes are altered in this model during earlier stages of fetal development.

In order to explore potential therapeutic targets, gene concordance between maternal and fetal datasets was investigated. Eighteen genes were identified in common with maternal miRNA targets and differentially expressed genes in the fetal placenta and cortex, potentially indicating the genes most likely affected by increased levels of the overlapping maternal prefrontal cortex and uterine miRNAs, rno-miR-200b-3p and rno-miR-429. Functional analysis indicates that these genes are involved in responses to endoplasmic reticulum stress, autophagy, mitophagy, ubiquitination, apoptosis, brain development, and aging. Further investigation is needed to determine if these genes continue to be affected later in development and if therapeutic targeting of miR-200b-3p and miR-429 could attenuate the effects of transgenerational and multigenerational maternal stress.

Because fetal development following adverse environmental exposures is often accompanied by inflammatory and immune changes, the present data provide insight into the molecular etiology of non-communicable and chronic diseases and potential therapeutic targets[61]. Such long-term consequences were confirmed by the emergence of new behavioral traits[20], accelerated brain aging and higher incidence of non-communicable disease prevalence, such as heart and respiratory diseases and renal failure, along with metabolomic reprogramming in multigenerationally stressed rats using the present stress protocol[14,19,21].

As opposed to previous studies that investigated intergenerational effects of maternal environment on fetal development and placental health mainly in the F1 or F2 generations[62], this is the first study to investigate the impacts of maternal miRNAs on fetal epigenetics and transcriptomics in both transgenerational and multigenerational models. While the field of transgenerational epigenetic inheritance has primarily focused on germline-mediated epigenetic inheritance[3], placenta-mediated exosomal transfer of epigenetic factors should be investigated as an additional mechanism of epigenetic inheritance. The present study is the first to consider genomic responses in different tissues across generations. Recognizing that different cell types have unique epigenomic and transcriptomic signatures[63], subsequent studies should explore the impacts of ancestral exposure to PNMS on distinct brain regions. Nevertheless, the present study demonstrates transgenerational persistence of many DNAm, mRNA and miRNA markers that offers new insights into the origins of non-communicable disease risks and their early diagnosis. The use of multi-level systems biology approaches will not only aid in the discovery of mechanisms in developmental programming but identify new noninvasive biomarkers for predictive and preventive precision medicine approaches.

In summary, the present study demonstrated that transgenerational and multigenerational PNMS has profound, compounding effects on the fetal cortex and placenta at three different regulatory levels. The significant overlap between these molecular marks in the cortex and placenta implies the potential to utilize the placenta as a proxy for early biomarkers indicative of neurodevelopmental health and aging. The discovery of early predictive and prognostic biomarkers associated with prenatal stress is critical for early-life therapeutic interventions to prevent and potentially mitigate psychological and neurological diseases. Additionally, the overlaps between maternal miRNA targets and fetal differentially expressed genes suggest the potential for exosomal intracellular communications between the two tissues.

Methods

Animal model and stress paradigm

Lineages of Long-Evans hooded rats and their ancestors were continuously bred locally to avoid transportation stress. Nulliparous, pair-housed female rats, 90–120 days old, were paired with naïve breeder males for one hour per day until pregnancy was confirmed[8,26]. On gestational days 12–18, which coincides with periods of epigenetic reprogramming (demethylation and remethylation) of the germline[29,64], the pregnant females (F0 generation) were administered a daily semi-random stress paradigm including 20 min of restraint stress and 5 min of swimming[8]. For restraint stress, gestating mothers were placed individually into transparent, ventilated, and adjustable-length Plexiglas tubes for 20 min. During swim stress, gestating rats were individually placed in a tub filled with room temperature water for 5 min. Stress procedures were performed in separate rooms to avoid bystander stress. All rats were handled and weighed daily. All procedures were performed in accordance with the Canadian Council for Animal Care guidelines and approved by the University of Lethbridge Animal Welfare Committee (#0803 and #1212). We have complied with all relevant ethical regulations for animal use.

Treatment lineages were bred to the F3 generation, and the generations were defined based on whether they received stress (S) or no stress (N). The transgenerational lineage received the stress paradigm in the F0 generation, and the F1 generation (S) was directly exposed to PNMS in utero. The F2 (SN) and F3 (SNN) generations did not receive any subsequent stress exposure. The multigenerational lineage received the same stress paradigm in the F0 (S), F1 (SS), and F2 (SSS) generation gestating females to investigate the impact of cumulative ancestral exposure to stress on the F3 generation. The model of multigenerational stress may help to identify potentially adaptive or resilient traits to a family history of continuous stress[1,22]. The control lineage did not receive any experimental stress in any generation (N, NN, and NNN). A lineage map is provided in Fig. 7.

Cortical and placental tissue collection

On gestational day 21, pregnant dams were euthanized and fetuses and placentas were extracted via cesarean section. Each fetus was rapidly decapitated and the whole brain was extracted and dissected to isolate the fetal cortex. The same cortex and placenta samples were used for all three of the following mRNA, miRNA, and methylated DNA analyses.

Statistics and reproducibility

In this study, biological replicates were utilized to verify the reproducibility of the experimental findings. The sample collection of these replicates was randomized in every aspect of the study. Three pregnant dams were randomly selected from a group of 10 pregnant dams per generation. From these three pregnant dams, one pup was randomly selected for the analysis, generating $n = 3$ per group per generation. A total of 24 animals were used with $n = 3$ for each treatment (F0 control, F0 stress, F1-S, F1-N, F2-SN, F2-SS, F2-NN, F3 SNN, F3 SSS, F3-NNN). For the DNAm, RNA, and miRNA analysis, extensive measures were taken to ensure the reproducibility of the findings. Quality control steps, which were implemented during the DNAm and RNA-seq processes, aimed to minimize technical variability and enhance the reliability of the data. These measures included consistent DNA and RNA extraction, library preparation, and sequencing protocols across the biological replicates.

DNA extraction and bisulfite sequencing

Genomic DNA from cortex and placenta was extracted and digested with restriction enzyme MspI (Thermo Scientific™, Waltham, MA, USA) that cuts at CCGG sites; digestion was done for 18 h at 37 °C. The fragments were blunt-ended and phosphorylated and a single A nucleotide was added to the 3' ends using Klenow enzyme and T4 DNA polymerase (Thermo Scientific™, Waltham, MA, USA). The fragments were ligated to methylated adapters, resolved on 2% gel prepared with Low Range Ultra Agarose (BioRad®, Hercules, California, USA) and size selected to include fragments 200 to 300 nt in length. Ligated fragments were purified from the gel using QIAquick® gel extraction kit (Qiagen®, Hilden, Germany) and underwent bisulfite conversion using EZ DNAm-Gold™ kit (Zymo Research®, Irvine, CA, USA). Bisulfite-converted libraries were purified and sequenced on an Illumina® Genome Analyzer IIx platform (Illumina®, San Diego, CA, USA). Base calling and demultiplexing was performed using Illumina CASAVA 1.8.2 with default settings following the developer's manual (Illumina®, San Diego, CA, USA). Quality control was performed using NGS QCtoolkit (v. 2.3)[65]. Sequencing reads were trimmed of adapters and low quality ends using Trim Galore! (v. 0.2.7).

DNA methylation analysis

Alignment files were analyzed using the methylKit Bioconductor package[66]. Detection of differential DNA methylated regions (DMRs) in cortex and placenta was performed using BiSeq Bioconductor package (v. 2.14.1)[67]. Treatment samples were compared with control samples, that is F1-S with

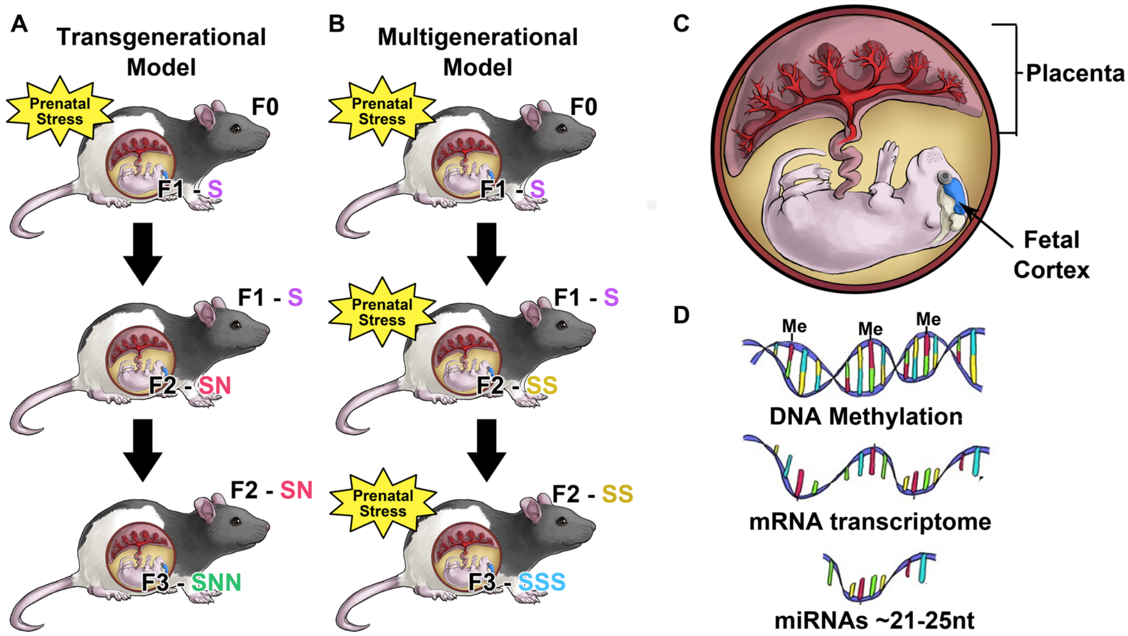

Fig. 7 | Breeding protocol and methods. **A** Transgenerational breeding protocol. **B** Multigenerational breeding protocol. **C** Specific tissues utilized in the current study. **D** Specific genomic assays included in the present study.

F1-N, F2-SS with F2-NN, F2-SN with F2-NN, F3 SSS with F3-NNN and, finally, F3 SNN with F3-NNN for each type of tissue separately. CpG clusters containing at least one differentially methylated CpG site were trimmed of non-significant CpG sites. Significantly changed CpG sites located ≤ 100 base pairs away from an initial CpG site were aggregated into DMRs. The DMR was divided if methylation changes inside the region vary in methylation direction, ensuring that each DMR is either hypermethylated or hypomethylated. Genomic coordinates of DMRs detected were checked for overlaps with promoters and CpG islands using the ChIPpeakAnno Bioconductor package (v. 1.4.2). Coordinates of promoters and CpG islands were identified by adding 2000 base pairs up or downstream of transcriptional start sites using the UCSC table browser[68].

RNA extraction and mRNA analysis

Total RNA isolation from cortex and placenta was performed using TRIzol™ reagent (Invitrogen™, Carlsbad, CA, USA). The samples were treated with DNase I (Invitrogen™, Carlsbad, CA, USA), resuspended in RNase-free water (New England Biolabs®, Ipswich, MA, USA) and stored at −80 °C. In order to perform mRNA expression analysis, cRNA was created using the Illumina® TotalPrep™ RNA Amplification Kit from Ambion® (Applied Biosystems®, Carlsbad, CA, USA) according to manufacturer's protocol with 500 ng total RNA input per sample. The Illumina® direct hybridization assay kit (Illumina®, San Diego, CA, USA) was used to hybridize cRNA to Illumina® Rat-Ref[52]. Whole Genome Expression BeadChip (Illumina®, San Diego, CA, USA). The arrays were scanned on the iScan and the data were normalized using the Illumina® BeadStudio software (Illumina®, San Diego, CA, USA).

Data normalization and statistics were performed using GenomeStudio software (Illumina®, San Diego, CA, USA). Gene expression was compared between groups (similar to the comparison described for DNA methylation analysis) using the Illumina® custom model employing a false discovery rate (FDR) threshold of $p < 0.05$. The detection p-value threshold of 0.01 was used to ensure that a given transcript was expressed above the background defined by negative control probes. Gene expression is presented as a log2 fold change over matching control. Changes in gene expression between the fetal cortex and placenta were compared using pooled standard deviation.

miRNA expression analysis

Small noncoding RNA libraries were generated from total cortical and placental RNA using the TruSeq™ small RNA library construction kit (Illumina®, San Diego, CA, USA). Single end multiplexed sequencing was performed using the Illumina® Genome Analyzer IIx platform (Illumina®, San Diego, CA, USA). Sequencing demultiplexing was performed using Illumina® CASAVA 1.8.1 software with default settings. Adapter trimming was performed using cutadapt software with options to only retain sequences between 17-27 nt in length. Quality of the sequencing libraries was performed using FastQC software. Trimmed sequences were aligned to the reference genome with bowtie 0.12.7[69]. Trimmed reads were collapsed into unique tags using groupReads.pl from miRAnalyzer software[70]. Obtained tags were aligned to all human mature miRNAs present in miRbase (v .18) using MicroRazerS (v. 1.0) aligner[71]. Differential expression between treatment groups (similar to the comparison described for DNA methylation analysis) was analyzed using statistical routines implemented in DESeq Bioconductor (v. 2.10) package (v. 1.8.3) as described in the user manual [Anders and Huber, 2010]. miRNAs with <0.1 False Discovery Rate were considered statistically significant. miRNA targets were determined using a threshold of ≥0.2 cumulative weighted context score from TargetScan[55] combined with a target score threshold of ≥60 from miRDB[52,53].

Gene functional enrichment and pathway analysis

Gene pathway and functional enrichment analysis was performed using g:Profiler (v. e111_eg58_p18_30541362) with g:SCS multiple testing correction method using a p-value threshold of 0.05[72]. Dot plots were generated to visualize the top 30 most significantly enriched biological processes and pathways. Treatment groups that did not yield significant results within these top 30 were excluded from these figures. Gene ratio was calculated by relating the number of the genes found in the specific biological process/pathway in the comparison group to the total number of genes in this biological process/pathway. Heat maps for mRNA and miRNA expression data were developed using the Next-Generation Clustered Heat Map Viewer[73,74]. InteractiVenn was utilized to create Venn diagrams for comparisons of gene expression and DNAm data[75].

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

This study incorporates three layers of data: mRNA, miRNA, and DNA methylation (DNAm). Unfortunately, raw sequencing data for mRNA could not be provided due to its age and subsequent loss. Processed sequencing data for mRNA, along with the numerical and source data used for all figures in this study, are included in the supplementary data files (#1 through #31). The miRNA and DNAm data used in this study are accessible through the Gene Expression Omnibus (GEO): miRNA data: GEO accession GSE276226; DNA methylation data: GEO accession GSE276382.

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Competing interests
The authors declare no competing interests.

Additional information

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