Preimplantation Embryos Amplify Stress-Induced Changes in Sperm-Derived miRNA Content to Mediate Transgenerational Epigenetic Inheritance

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Abstract- Chronically stressing male mice can alter the behavior of their offspring across generations. This effect is thought to be mediated by stress-induced changes in the content of specific sperm miRNAs that modify embryo development after their delivery to oocytes at fertilization. A major problem with this hypothesis is that the levels of mouse sperm miRNAs are much lower than those present in preimplantation embryos. This makes it unclear how embryos could be significantly impacted without an amplification system to magnify changes in sperm miRNA content, like those present in lower organisms where transgenerational epigenetic inheritance is well established. Here, we describe such a system for Chronic Social Instability **(CSI)** stress that can explain how it reduces the levels of the miR-34b,c/449a,b family of miRNAs not only in sperm of exposed males but also in preimplantation embryos (**PIEs**) derived from their mating, as well as in sperm of male offspring. Sperm-derived miR-34c normally positively regulates expression of its own gene and that of miR-449 in PIEs. This feed forward, auto-amplification process is suppressed when CSI stress reduces sperm miR-34c levels. Its suppression is important for the transmission of traits to offspring because restoring miR-34c levels in PIEs from CSI stressed males, which also restores levels of miR-449 in them, suppresses elements of elevated anxiety and defective sociability normally found specifically in their female offspring, as well as reduced sperm miR-34 and miR-449 levels normally found in male offspring, who pass on these traits to their offspring. We previously published that the content of sperm miR-34/449 is also reduced in men raised in highly abusive and/or dysfunctional families. We show here that a similar miRNA auto-amplification system functions in human embryonic stem cells. This raises the possibility that PIEs in offspring of these men also display reduced levels of miR-34/449, enhancing the potential translational significance of these studies.

Main

The concept that the environment's effects on an organism can be transmitted to offspring through epigenetic changes in germ cells is well established in lower organisms like C. *elegans*[1], drosophila[2] and plants[3]. More recently, evidence has emerged for this process occurring in male rodents, where many environmental exposures including diet, drugs, temperature, enriched environment, and chronic stress have been shown to affect the next generation(s) in the absence of any male input on rearing offspring[4]. Evidence for it also exists in humans, but it is mostly based on epidemiology[5-8]. The mechanisms underlying many of these phenomena in mammals are emerging, such as a role for environmentally induced changes in the content of specific sperm miRNAs in the transmission of stress-related traits across generations[9]. For example, studies on the effects of maternal separation[10], chronic unpredictable stress[11] and chronic mild stress[12] have shown phenotypes observed in offspring of these males can be mimicked by injection of pooled miRNA sample from stressed but not control mice into normal zygotes, which necessarily detects only miRNAs whose activities rise.

A major unexplained problem with these studies, as well as others that implicate sperm miRNAs in epigenetic inheritance, is that mammalian sperm do not contain enough miRNAs for environmentally induced changes in their levels to significantly alter their content expressed in early embryos, and thus influence their development. *C. elegans* and plants overcome this obstacle involving heritable small RNAs that mediate RNA interference (RNAi) by amplifying them with an RNA-dependent RNA polymerases[1], and Drosophila use a ping-pong amplification system for pi-RNAs[13]. However, an amplification system analogous to either of these mechanisms has not been reported yet in mammals. In fact, the lack of such evidence has promoted skepticism that transgenerational epigenetic inheritance exists in mammals[5,14]. At best, a recent study showed that artificially manipulating DNA that leads to methylation changes can be transmitted to offspring. However, this effect disappeared in early embryos only to reappear later, but the amplification system involved was not revealed[15].

We noticed this dilemma in our studies investigating how exposure of male mice to **C**hronic **S**ocial **I**nstability (CSI) stress affects subsequent generations. We showed that this form of stress leads to increased anxiety and defective sociability specifically in female offspring for at least three generations through the male lineage[16]. Subsequently, we implicated CSI stress-induced reduction in the levels of members of the miR-34/449 miRNA

family (miR-34b,c and miR-449a,b) in this process because their levels were all reduced not only in sperm of male mice exposed to this stress but also in sperm of their F1 male offspring who, while never being exposed to stress, transmit the anxiety and sociability defects specifically to their female offspring[17]. miR-34/449 miRNA family members are attractive candidates to transmit environmentally induced traits to offspring because they represent two of only 14 sperm miRNAs (out of hundreds) that are not expressed in oocytes[18,19]. And we detect suppression of members of both miRNA families, which is important because they serve highly redundant functions[19,20], such that both genes have to be knocked out in mice to observe a phenotype. Also, family members inhibit expression of a common set of target mRNAs, predicted to include p53, CDK6, c-MYC, HDAC1, and BCL-2[21-23], key regulators of gene expression. We also showed previously that the decrease in the content of members of both miRNA families in sperm persists after mating in preimplantation embryos, such that their levels are reduced ~5-fold through at least the morula stage[17]. Thus, we hypothesize that when their introduction into oocytes by sperm is suppressed when CSI stressed males' mate, subsequent development is likely altered in a way that generates the stress related phenotypes we detect in female offspring as well as reduced sperm miR-34/449 levels found in male offspring.

The problem with this model, and others described in the introduction, became apparent when we found that the levels of members of this miRNA family in sperm were >50 fold lower than those found in preimplantation embryos, even though the 5-10-fold reduction in the small amounts of these miRNAs in sperm from CSI stressed males that are delivered to oocytes was associated with a similar magnitude change in the much larger amounts in preimplantation embryos[17]. Thus, the large drop in total miR-34/449 levels observed in these embryos is very likely due to suppression of their genes' early embryos that occurred either during spermatogenesis when gene expression in sperm is still regulatable and maintained, or after fertilization.

CSI stress reduces miR-34c in the *cauda* epididymis after sperm leave the testes.

Sperm are known to acquire miRNAs from exosomes secreted by the *cauda* (tail end of) epididymis[24], and this appears to be the case for miR-34c, as its content increases significantly after sperm leave the testes and migrate from the *caput* to *cauda* epididymis while its own genome is inert[25]. We find that levels of miR-34c in sperm are normal in the *caput* epididymis from CSI stressed mice but reduced in the *cauda,* implying that

they fail to acquire this bolus of miR-34c once there (**Fig. 1A and B**). Indeed, the level of miR-34c in *cauda* epididymosomes is also reduced (**Fig. 1C**). This implies that suppression of expression of the gene for miR-34c found in early embryos derived from CSI stressed males is not the consequence of its inhibition in sperm during spermatogenesis but after fertilization. Additional evidence for this concept emerges from the following experiments.

Elevation of miR-34c levels in preimplantation embryos derived from mating CSI stressed males increases expression of members of its own gene and those of miR-449.

To reveal how reduced levels of miR-34c detected in preimplantation embryos derived from CSI stressed males contribute to the transgenerational effects of CSI stress, we generated transgenic CD-1 mice in which we could test whether restoring miR-34c levels to normal specifically in preimplantation embryos prevents these effects. To this end, the miR-34c gene locus (miR-34b was deleted) was cloned distal to the tetracycline-on promoter (see Material and Methods and **Supplemental Fig. 1**). Transgenic miR-34c inducible males were stressed or not and then mated to both wild-type females who had been either fed normal chow or transiently fed doxycycline (**Dox**) for 2 days before mating (**Fig 2A,** left). In this way, the male genome is transiently activated in transgenic embryos beginning at the 2-cell stage, along with the initiation of endogenous embryo gene expression, when doxycycline already circulating in the female promotes miR-34c expression only in the embryo since the male is heterozygous for the transgene, a benefit of this approach is that WT littermate controls are generated along with transgenic offspring for behavior and sperm analysis (**Fig. 2A**, right).

To test the validity of the system, we first showed that morula stage embryos derived from mating CSI stressed transgenic males display a 5-10-fold reduction compared to unstressed transgenic males in the levels of miR-34/449 family members (**Fig. 2B**), similar to what we found previously studying WT mice[17]. Then, we showed that, as expected, pre-feeding females mated to CSI stressed WT males had no significant effect on the levels of miR-34/449 family members in morula derived from them (**Fig. 2C**). In contrast, Dox pre-feeding of females increased the level of miR-34c in morula derived from their mating to CSI stressed transgenic males back toward normal levels (**Fig. 2D**). In this case, we mated each of 5 independent male transgenic mouse lines to both normally fed females and Dox pre-fed mothers, and then quantified the miR-34c induction effect

in each set of morula. Each transgenic male displayed elevated levels of miR-34c, albeit to varying degrees (mean ~5-fold). Thus, the system worked as expected. A similar induction was also observed, as expected, in 2 and 4 cell embryos, and it was maintained at least through the blastocyst stage of embryogenesis (**Supplemental Fig. 2).**

Strikingly, while validating this system we noticed that levels of its relatives, miR-449a and miR-449b, encoded by a separate genetic locus, also rose in the same morula derived from transgenic males (mean ~ 5-10-fold) (**Fig. 2D**). To test whether miR-34c also enhances levels of the products of its own gene we could not measure miR-34c itself since we induced it. However, the miR-34 gene co-expresses miR-34b from the same transcript (see **Supplemental Fig. 1C**), and we find the levels of the two variants are coordinately regulated wherever we have looked, including in sperm and early mouse embryos in response to CSI stress, as well as in sperm of men in response to early life adversity[17]. While miR-34b has the same mRNA inhibitory sequence as miR-34c, it does have unique sequences to distinguish it[26]. miR-34b expression was also enhanced in every sample (mean~5-fold) (**Fig. 2D**). Not all miRNAs were affected, as the expression of the internal standard for these experiments, miR-192 did not vary nor did miR-152 and miR-352, miRNAs we showed previously are not altered in sperm (and embryos) of stressed mice[17] (**Supplemental Fig. 3**). Furthermore, this type of auto-amplification effect in PIEs is not typical of most tissues since the levels of a redundant gene normally changes in the opposite direction of its counterpart to maintain homeostasis. For example, knockout of miR-449 in the testes increases the levels of miR-34c[21]. Moreover, Tet-induced induction of miR-34c in lymphocytes from male transgenic mice led to suppressed levels of miR-34b (**Supplemental Fig. 4).**

Overall, these data reveal the basics of a feed-forward amplification mechanism that when inhibited by CSI stress can explain, at least in part, how reductions in very small amounts of sperm miR-34c generate similar fold reductions in much larger amounts of miR-34 and miR-449 family members present in preimplantation embryos. The data also implied we could use this system for its original purpose; to test whether this decline in miR-34/449 levels in early embryos is necessary for the appearance of stress related traits in offspring. This is because the induction of each member of the miR-34/449 family in morula embryos from CSI stressed transgenic males returned to levels close to those normally found in morula derived from mating unstressed males (**Fig. 2E**).

miR-34c also increases expression of its own gene products and those for miR-449 in human embryonic stem cells.

Since we found that extreme early life stress also reduces levels of miR-34b/c and miR-449a,b in sperm of men[17], we tested whether a similar amplification system may exist in human early embryos by transfecting a miR-34c mimic into CHB-04 human embryonic stem (ES) cells[27]. Raising miR-34c levels in these ES cells also significantly increased levels of miR-34b, miR-449a, and miR-449b, but not miR-34a, a related family member expressed from a different gene locus nor miR-152 a gene reported to be elevated in sperm after a different form of chronic stress[10] (**Fig. 2F).**

miR-34c regulates the expression of its own gene products and those for miR-449 in both mouse and human early embryo cells at a posttranscriptional level.

To begin to understand how miR-34c influences the expression of its own gene and that for miR-449 in early embryo cells, we compared the levels of the primary miRNA transcripts (Pri-miRNA) of miR-34 and miR-449 that generate miR-34b,c and miR-449a,b respectively, in morula from control and CSI stressed WT fathers (see **Supplemental Fig. 1C**). The levels of neither of these Pri-miRNAs changed despite mature levels declining ~5-fold (**Fig. 3A).**

Moreover, the Pri-miRNA levels did not rise in morula derived from stressed transgenic fathers mated to dox pre-fed mothers (**Fig. 3B),** despite the mature levels rising ~5-fold (**see Fig. 2D**). Thus, miR-34c normally positively regulates members of its own gene and that of miR-449 in PIEs at a step downstream of transcription, and this effect is suppressed when males are exposed to CSI stress. This finding also confirms the idea presented earlier that suppression of the expression of the miR-34c and miR-449a genes by CSI stress in early embryos does not occur in sperm before or during transit down the epididymis and then maintained after fertilization, but after fertilization. Levels of miR-34c are not always regulated at the post-transcriptional level since in testes, where expression in known to be high and liver where it is known to be low, the levels of the primary form of this miRNA match the fully with processed levels (see **Supplemental Fig. 5**).

Strikingly, the same result was obtained when miR-34c levels were elevated in human embryonic CHB-04 stem cells by transfection. Despite dramatically increasing levels of miR-34b and miR-449a (see **Fig. 2F**), no

effect on the levels of their Pri-forms was observed (**Fig. 3C**). Thus both in mouse and human early embryo cells miR-34c regulates members of its own gene's expression and that of its close relative miR-449 at a posttranscriptional level that may include miRNA processing, which has been detected as an auto-amplification mechanism in lower organisms[28], or miRNA stability possibly via target directed degradation[29].

Reduced levels of miR-34/449 detected in PIEs derived from mating CSI stressed males are necessary for the transmission of indicators of elevated anxiety and defective sociability to their female offspring.

Our original goal of generating dox-inducible miR-34c mice, which led to the discovery of the positive feed forward amplification system described above, was to test whether reduced PIE levels of miR-34c contribute to stress phenotypes we previously observed in female offspring of CSI stressed males[16]. Since we could rescue miR-34/449 levels in morula from CSI stressed transgenic males (see **Fig. 2B-E**), in a new set of experiments we used the same induction protocol described in **Fig. 2A** but let offspring of the set of unstressed and CSI stressed transgenic males develop into adults, and separately assayed offspring by sex and genotype (WT vs transgenic). We then compared WT and transgenic adult female littermates of unstressed and stressed transgenic fathers for: i) the % time they spent in the open arms of the EPM (as an indicator of anxiety), and ii) their interaction time with a juvenile (SIJ as an indicator of sociability) **(Fig. 4A** and see females in **Fig 2A** right for description of mice)**.**

As expected from our previous findings[16], WT female offspring of CSI stressed transgenic fathers mated to control-fed females spent less time in open arms (**Fig. 4B,** first two bars), and interacted less with a juvenile (**Fig. 4C,** first two bars) than WT females from unstressed transgenic fathers, and pre-feeding mothers with doxycycline (**Dox**) had no effect in either case (**Fig. 4B and C**. second and third bars). Also as expected, transgenic female offspring of CSI stressed transgenic fathers mated to control-fed mothers also spent less time in the open arms of the Elevated Plus Maze (**Fig. 4B,** fourth and fifth bars), and interacted less with a juvenile than those from unstressed transgenic fathers (**Fig. 4C,** fourth and fifth bars). However, both stress-related phenotypes returned to normal levels in transgenic female offspring derived from mating the same CSI stressed transgenic fathers to Dox pre-fed mothers (**Fig. 4 B and C**, fifth and sixth bars in each) (see females in right side of **Fig. 2A**).

Reduced levels of miR-34/449 detected in PIEs derived from mating CSI stressed males are necessary for the transmission of reduced sperm miR-34/449 to their male offspring.

To test whether restoring levels of miR-34/449 in these PIEs prevents the appearance of reduced levels of these miRNAs found in their male offspring, we compared the sperm miR-34c (**Fig. 4D and F**) and 449a (**Fig. 4E and G**) levels in the same sets of offspring as described for females above, but this time in males (see males **Fig. 2A** right). As expected from our previous results, sperm of WT male littermates derived from mating CSI transgenic stressed males to control-fed mothers contained reduced levels of both miR-34c and miR-449a, compared to sperm of WT male offspring derived from mating these mothers with non-stressed transgenic fathers, and this was not altered when these CSI stressed transgenic males were mated with Dox pre-fed females (first through third bars in **Fig. 4D and E**). In contrast, while transgenic male littermates derived from CSI stressed transgenic fathers mated to control fed mothers also showed reduced sperm levels of both miR-34c and miR-449a, levels of both miR-34c and miR-449a returned, at least in part, to control levels when the same fathers were mated to Dox pre-fed mothers (first and third bars in **Fig. 4F and G**).

Discussion

These studies reveal how environmentally induced changes in the very small amounts of miRNAs present in sperm can, after fertilization, promote significant changes in the content of much higher levels of miRNAs in preimplantation embryos. Analogous amplification mechanisms solving this dilemma have been identified and shown to be necessary for epigenetic inheritance in lower organisms, but until now no analogous systems have been revealed in mammals[15,30]. In fact, the lack of such evidence has promoted skepticism that transgenerational epigenetic inheritance exists in mammals[5,14]. Importantly, we also show that suppression of this system is critical for CSI stressed males to transmit the stress-related phenotypes of elevated anxiety, as detected using the Elevated Plus Maze, and defective sociability, as detected using Direct Interaction Time with a Juvenile, to their female offspring. We also showed it is critical for the transmission of reduced levels of sperm miR-34/449 to the male offspring, which explains how we find that these F1 males transmit these same phenotypes to their F2 offspring to generate a truly transgenerational epigenetic inheritance

phenomenon[16]. The mechanism we describe is based on the observation that the miR-34/449 levels normally present in preimplantation embryos are dependent upon the delivery from sperm of miR-34c because it enhances levels of expression from both miR-34 and miR-449 genes in these early embryo cells. Interestingly, we find the regulation of both by miR-34c is post-transcriptional, either at the miRNA processing step or at the level of miRNA stability, an issue presently under investigation.

How do these results relate to studies on other forms of chronic stress that induce different phenotypes in offspring that are mediated by changes in different sperm miRNAs? These include chronic variable stress that induces a suppressed HPA axis response in offspring[11], early maternal separation that yields depressive-like phenotypes[10] and chronic mild stress[12] that promotes depressive like phenotypes in offspring. Each of these paradigms have common features that are distinct from those of CSI stress, which suggests they may require distinct amplification mechanisms. First, although many environments besides CSI stress also generate reduced levels of specific sperm non-coding RNAs[12,31,32, 33,34], the stress studies mentioned above all implicate sperm miRNAs whose levels rise in response to stress[15,16,17] . Second, those studies have not shown that miRNA changes detected in sperm persist in preimplantation embryos derived from them. Finally, while one of the three paradigms, maternal separation[15], generates multigenerational behavior effects, it does not show consistent sperm changes in F1 males as we do for CSI stress.

Others have speculated that the increased levels of miRNAs they detect in sperm from stressed mice can nonetheless impact zygotes significantly by excessively suppressing maternal mRNAs. This is because while the maternal versions of these miRNAs are present in oocytes, they are inactive until the 2-cell stage when their endogenous counterparts begin being expressed[35]. However, changes in maternal mRNAs in zygotes from stressed mice have yet to be documented to play a role in the process. Another possibility is that these miRNAs are covalently modified in sperm to magnify their activities above those of their endogenous early embryo counterparts upon fertilization, a phenomenon proposed for tRNA fragments involved in mediating the effects of diet across generations[36-38].

Reduced insertion of sperm miR-34/449 into oocytes after mating CSI stressed males could also potentially impact maternal RNAs in zygotes before endogenous versions of these miRNAs are expressed. However, this

cannot be sufficient for the offspring phenotypes we observe. This is because in our miR-34c transgenic mouse system restoration of miR-34/449 levels in preimplantation embryos from CSI stressed transgenic mice only begins at the 2-cell stage when endogenous genes, including the miR-34c transgene, become activated. Yet it prevents the appearance of the altered phenotypes investigated here in both female and male offspring. Thus, maintained suppression of the miR-34/449 family while endogenous miRNAs are actively expressed in preimplantation embryos beginning at the 2-cell stage is necessary for the phenotypes induced by CSI stress in both male and female offspring to fully appear.

Of potential translational significance is our finding of a similar post-transcriptional miRNA amplification mechanism in human embryonic stem cells. This is because we showed previously that the levels of miR-34/449 are also reduced in sperm from men exposed to severe adverse childhood experiences[17]. Thus, findings presented here raises the possibility that preimplantation embryos derived from these men also display reduced levels of these miRNAs. Since we showed here that this perturbation contributes to the transmission of stress-related phenotypes from CSI treated male mice to their female offspring, it may also contribute to the negative effects these types of experiences are known to have on their offspring[39,40], in this case females. And because the perturbation also contributes to reduced sperm miR-34/449 levels in F1 male mice, it may also cause epigenetic inheritance to be transgenerational in humans.

Further investigation of this possibility is of particular interest because susceptibility to mental health disorders contains a significant inherited component[41]. And a recent mathematical model based on existing clinical evidence supports the idea that a fraction derives from epigenetic inheritance[42], which theoretically is easier to reverse than genetic inheritance.

Figure Legends

Figure 1: CSI stress reduces miR-34c levels in sperm and epididymosomes isolated from the *cauda* epididymis but not sperm isolated from the *caput* epididymis.

miR-34c levels in sperm isolated from *caput* (A) and *cauda* (B) regions of the epididymis from control and CSI stressed males. C) miR-34c levels in epididymosomes isolated from the *cauda* epididymis of control and CSI stressed males, n=5 for *caput* and *cauda* and n=4 for *cauda* epididymosomes. Expression was normalized to that for miR-192. Data are expressed as mean ± S.E.M; Mann-Whitney test, Two-tailed, *p< 0.05. WT: wild-type, CSI: Chronic Social Instability.

Figure 2: Elevation of miR-34c levels in morula stage mouse embryos, as well as human embryonic stem cells, increases the levels of miR-34b and miR-449a and b in them.

A) Timeline describing the Dox induction of miR-34c specifically in preimplantation embryos used in following panels and a scheme representing its consequence in male and female WT and transgenic offspring.
B) miR-34/449 family levels in morula stage embryos derived from mating either control or CSI stressed miR-34c inducible transgenic (Tg) males with WT unstressed females fed with regular chow.
C) miR-34/499 family levels in morula stage embryos derived from mating WT CSI males mated with WT unstressed females pre-fed or not with doxycycline (Dox or no Dox).
D) 5 independently derived CSI stressed transgenic (Tg) males were mated with WT unstressed females pre-fed doxycycline (Dox) or regular chow (no Dox) and miR-34/449 family members levels were measure in morula stage embryos derived from these mating.
E) Control levels of miR-34/449 members were restored in morula stage embryos by mating stressed transgenic males with Dox pre-fed females (compare with panel 2B).
F) CHB-04 human embryonic stem cells were transfected with a miR-34c mimic 48 hrs. later the levels of miRNAs indicated were assayed.

A-F) The same CSI F0 fathers were mated to normal and dox fed females no dox. Data are expressed as mean ± S.E.M; Mann Whitney test, two-tailed, ** p< 0.01, * p<0.05, Dox: doxycycline, Tg: Transgenic, WT:

wild-type, CSI: Chronic Social Instability. Control Tg n=8, CSI Tg No Dox n=5, CSI Tg Dox n=5, CSI WT No Dox n=7 and CSI WT Dox or n=3 for 2/4 cells and blastocysts. G) n=4. Data are expressed as mean ± S.E.M; Mann-Whitney test two-tailed, *p<0.05.

<u>Figure 3:</u> miR-34c alters the level of miR-449a,b in both morula stage mouse embryos and human embryonic stem cells at the post-transcriptional level.

A) Levels of Pri-34bc and Pri-449ab were measured in morula stage embryos derived from the mating of unstressed and stressed WT, Mann-Whitney test, Control WT n=8, CSI WT No Dox n=6 for Pri34bc and Control WT n=8, CSI WT No Dox n= 6 for Pri449ab, WT: wild-type, CSI: Chronic Social Instability. B) Levels of Pri-34bc and Pri-449ab were measured in morula stage embryos derived from the mating of unstressed, stressed and stressed inducible miR-34c transgenic males. In the experiments, the CSI F0 fathers are the same between no dox and dox groups. Expression was normalized to that for GAPDH. Data are expressed as mean ± S.E.M, Kruskal-Wallis test with multiple comparisons. Dox: doxycycline, Tg: Transgenic, CSI: Chronic Social Instability. Control Tg n=8, CSI Tg No Dox n=5 and CSI Tg Dox n=5. C) Pri-34bc and Pri-449ab levels were measure 48 hrs after transfecting CHB-04 human embryonic stem cells, Expression was normalized to that for ACTB. Data are expressed as mean ± S.E.M. Mann-Whitney test two-tailed, n=3 .

<u>Figure 4:</u> Preventing the decline of miR-34c/449 levels in morula derived from mating CSI stressed males prevented the appearance of elevated anxiety and defective sociability in F1 female offspring, as assessed by performance on the Elevated Plus Maze (EPM) and social interaction time with a juvenile (SIJ) respectively, and at least partially prevented reduced miR-34c and miR-449a levels in sperm of F1 male offspring.

A) Timeline for assessing stress-related phenotypes in F1 wild-type and transgenic female and male littermates of control and CSI stressed males. Measurement of the B) % time mice spent in the open arms of the EPM for WT (left three bars) and transgenic (right three bars) littermates derived from Control or CSI stressed

transgenic males mated to either control-fed or dox pre-fed females. C) Interaction time in SIG assay of the same mice used in A. Control WT n=50, CSI WT No Dox n=39, CSI WT Dox n=44, Control Tg n=20, CSI Tg No Dox n=19 and CSI Tg Dox n=24. Data are expressed as mean ± S.E.M. Ordinary one-way Anova with multiple comparisons (Tukey correction), F value 5.144 and degrees of freedom 2.025 (5, 188) for the EMP and F value 25.76 and degrees of freedom 5.531 (5, 190) for SIJ.

Measurement of miR-34c and miR-449a in sperm of male WT (D, E) and transgenic (F, G) littermates of females analyzed in B and C above. Control WT n=8 , CSI WT No Dox n=9 , CSI WT Dox n=7, Control Tg n=8, CSI Tg No Dox n=10 and CSI Tg Dox n=9. Expression was normalized to that for miR-192. Data are expressed as mean ± S.E.M; Kruskal-Wallis test with multiple comparisons, ****< 0.0001, ***<p0.001 ** p< 0.01 Dox: doxycycline, Tg: Transgenic, WT: wild-type.

Materials and Methods

Animals and care facilities

All mice included in this study are of the CD-1 strain, obtained from Charles River Laboratories. Males used for CSI stress began the protocol at 28 days postnatal age, and control females used for breeding were 8 weeks of age. All animals are housed in temperature, humidity, and light- controlled (14 h on/10 h off LD cycle) rooms in a fully staffed dedicated animal core facility led by on-call veterinarians at all hours. Food and water were provided *ad libitum*. All procedures and protocols involving these mice were conducted in accordance with and approved by the Institutional Animal Care and Use Committee of the Tufts University School of Medicine, Boston, MA. Sample sizes for the mouse work were based upon our previous experience with these experiments in the lab to yield adequate concentration of RNA for downstream work, and no animals were excluded from use in this study. No randomization procedures were needed to separate mice into groups.

Tet-inducible miR-34c mouse

Using mouse genomic DNA, we synthesized custom primers to clone out the miR-34c gene, including ~100 bp of flanking gDNA on either side of the ~90 nt locus (which excludes miR-34b sequences) incorporating EcoRI and BamHI restriction enzyme sites to the 3' and 5' ends, respectively (**Supplemental Fig. 1**). This paradigm has been previously employed to express miR-34c for use in bovine embryos[43]. Using these unique

restriction enzyme sites, the miR-34c locus was ligated into the multiple cloning site (MCS) of the pTetOne plasmid (Takara Bio).

Next, we needed to ensure with using this strategy that: i) cloning in the entire miR-34c locus, with extra flanking DNA, would produce functional, mature miR-34c, ii) that culturing in doxycycline would induce robust overexpression of miR-34c compared to untreated cells, and iii) that the baseline levels of miR-34c in 34c-pTetOne transfected, but untreated cells, was not significantly higher than that of untransfected cells. To investigate these concerns, the 34c-pTetOne plasmid was transfected into HEK293T cells and cultured with and without the presence of doxycycline (1µg/mL) for 48 hours. Untreated cells transfected with 0.5υg of plasmid showed only a 1.5-fold increase in miR-34c expression over untransfected cells, and doxycycline induced a close to ~200- fold increase in expression over baseline. As our qPCR kit only detects the mature form of miRNA, we confirmed that this construct design creates functional miR-34c with a robust activation in the presence of doxycycline. A large quantity of this plasmid was grown, carefully purified, and linearized using the SspI restriction enzyme site. We partnered with Dr. Lucy Liaw's group at the MMCRI mouse transgenic core to inject the linearized version of this plasmid into CD-1 mouse embryos to create our transgenic line. 10 founding animals were created from these injections, one of which displayed aggressive behavior incompatible with mating, however 6 of the remaining 9 successfully transmitted this locus to the next generation of offspring, demonstrating germline integration of the transgene.

Chronic social instability (CSI) stress

Starting at P28, the composition of each mouse cage (four mice per cage) was randomly shuffled twice per week, for 7 weeks, such that each mouse was housed with three new mice in a fresh, clean cage at each change as described[6,19]. This shuffling was randomized to reduce the chance that any mouse would encounter the same mouse twice. Control mice were housed four mice per cage with the same cage mates for the duration of the protocol. After 7 weeks, mice were housed in pairs with a cage mate from the final cage change and left for 2 weeks to remove acute effects of the final change. Mice were then either sacrificed for sperm collection or mated with control female mice overnight to generate "F1" animals, with successful mating confirmed via presence of copulation plug the following morning. We have previously shown that stressed males can be

mated multiple times and still transmit stress phenotypes to their offspring[16], so male mice were used for mating, embryo collection, or sperm collection as needed.

Doxycycline treatment

After the CSI stress protocol was complete, males were mated with unstressed control WT females to generate "F1" offspring or embryos at various stages. To avoid activating the gene in all cells by feeding the male transgenic mice, we fed only females doxycycline (40mg/kg, Bio-serv, NJ, USA) 2-days before mating with the male.

Mouse sperm collection

Mature, motile mouse sperm (from the cauda epididymis) was isolated via the swim-up method[44]. Briefly, male F0 and F1 mice were anesthetized under isoflurane and sacrificed via cervical dislocation. The caudal epididymis and vas deferens were dissected bilaterally and placed in 1 mL of warm (37 °C) M16 medium (Sigma-Aldrich, M7292) in a small Petri dish. Under a dissection microscope, sperm were manually expressed from the vas deferens using fine forceps, and the epididymis was cut several times before incubating at 37 °C for 15 min to allow mature sperm to swim out, then large pieces of tissue were removed. The remainder of the extraction took place in a 37 °C warm room. The sperm-containing media was centrifuged at 3.000 RPM for 8min, supernatant was withdrawn and discarded, and 400 µL of fresh, warm M16 medium was then carefully placed on top of the pellet. The tubes were then allowed to rest at a 45° angle for 45 min to allow the motile sperm to swim-up out of the pellet into the fresh medium. The supernatant containing the mature sperm was then carefully withdrawn and centrifuged again for 5 min at 3.000 RPM to pellet the motile sperm. Supernatant was withdrawn and discarded, and the pellet was frozen on dry ice for later processing.

To collect caput sperm[45], two small incisions were made at the proximal end of caput and using a 26G needle holes were poked in the rest of the tissue to let the caput epididymal fluid ooze out. Sperm-containing media was incubated for 15 minutes at 37°C, then transferred to a fresh tube and the sperm-free epididymis tissues

were directly frozen in liquid nitrogen and stored at -80°C. The sperm were incubated for another 15 minutes at 37°C, after incubation sperm were collected by centrifugation at 2000 x g for 2 minutes, followed by a 1X PBS wash, and a second wash with somatic lysis buffer (low SLB buffer, 0.01% SDS, 0.005% Triton X-100 in PBS) for 10 minutes on ice to eliminate somatic cell contamination. Somatic lysis buffer treated sperm were collected by centrifugation at 3000xg for 5 minutes, and finally washed with 1X PBS before freezing down.

Epididymosome isolation by ultracentrifugation and density-gradient.

After pelleting cauda sperm following the swim-up protocol in M16 medium (Sigma, M7167), the supernatant was centrifuged at 2,000xg for 10min, 10,000xg for 30min and then ultracentrifuged at 120,000xg at 4°C for 2h. The epididymosomal pellet was then washed in PBS 1X at 4°C and ultracentrifuged at 120,000xg at 4°C for 2h. The resulting pellet was resuspended in 50µl of PBS[46].

Mouse embryo collection

To obtain sufficient numbers of embryos, a superovulation protocol was employed. Control female mice were injected with 5 U of pregnant mare serum gonadotropin (PMSG, nor272-Prospecbio, Israel) and 46 h later injected with 5 U of human chorionic gonadotropin (HCG, C1063 Sigma-Aldrich) and placed into the home cage of the male to mate overnight. In the morning, female mice were checked for copulation plugs and returned to their own cages. If morula-stage embryos were desired, female mice were sacrificed about 2 days later. On the day of collection, mice were sacrificed via cervical dislocation. Under a dissection microscope, the uterus was removed, and a very high-gauge needle was inserted into the opening of the fallopian tubes and the embryos were flushed out using warm 37 °C M2 medium (Sigma-Aldrich, M7167). The embryos were separated according to cell number, and any unfertilized ova or embryos displaying unusual morphology were discarded. The embryos of interest were pooled by cell stage and individual, and snap frozen on dry ice. As the females are sacrificed during the collection process, this entire protocol was repeated with new females for every embryo stage collection to generate one pool each of one specific stage embryo for both F0 control fathers and F0 stressed fathers CSI WT and Tg and with or without doxycycline. Dox: doxycycline, Tg: Transgenic, WT: wild-type.

CHB-4 human embryonic stem cells and miRNA mimic transfection

hESC line CHB-04[47], on the NIH registry of approved hESC lines, were transitioned from MEF-dependent culture to feeder-free culture using mTeSR® medium (Stem Cell Technologies) and 6-well plates coated with hESC-qualified Matrigel® (Corning) for 1 h at 37 °C. One passage prior to transfection, the cells were single-cell passaged using TrypLE Select Enzyme (Gibco) and replated onto Matrigel in mTeSR® supplemented with 10 µM Y-26732 (Tocris) for 24 hrs. to improve cell survival and attachment. Cells were grown with 5% CO2 at 37 °C. Cells were tested for mycoplasma regularly.

For transfection, CHB-04 cells were cultured to 70% confluency in a 6-well plate. Each miRNA (mimic miR-34c, 50pmole, #MC11039, or mimic negative control, #4464058, Thermofisher) was added to 5 ul R buffer (Invitrogen) and incubated for 10 minutes prior to transfection. CHB-04 cells were transfected using the Neon electroporator (Invitrogen) using the Neon 10 µL transfection kit. About 400,000 single-cell dissociated CHB-04 cells were resuspended in 35 µl R buffer and added to the miRNA suspensions. The CHB-04 cells were then loaded into a 10 µL Neon tip and electroporated using the following pulse conditions: 1200 V 30 ms 1 pulse. Cells were replated into one well of a Matrigel®-coated 6-well plate in mTeSR® medium as before and cultured for 2 days after the initial transfection.

RNA lymphocytes collection and doxycycline treatment

Under isoflurane anesthesia, a small incision in the end of the tail of mice is made and collect the blood into a tube containing EDTA. Then, add 1 ml of lysis buffer ACK is added and let incubate at room temperature for 5 min. The solution is transfer in a 15 ml canonical tube and add 13 ml of PBS containing 0.4% EDTA and 0.1% BSA. Centrifuge at room temperature at 350xg for 5 min. Remove the supernatant and add 200µl complete media at 4°C. Then split the solution in 2 tubes of 100µl. Each will be treated by doxycycline 1.5µg or PBS 1X for 24h at 37°C. The next day, total RNA was extracted from mouse embryos using the Norgen Single-Cell RNA Isolation kit (#51800, Norgen Biotek Corp.) according to the manufacturer's protocol. Concentration of RNA was determined using a NanoDrop-1000 (Thermofisher Scientific).

RNA Tissue collection

The total RNA for the liver and testis from male and uterus are extracted following the miRNEasy Micro kit (#1071073, Qiagen) according to the manufacturer's protocol. For the uterus, the females are feed or not with

doxycycline (40mg/kg, Bio-serv, NJ, USA) for 2-days before mating with the male and sacrifice 3 days after the plug (morula stage).

RNA extraction and quantitative real-time PCR

Total RNA was extracted from both mouse sperm samples and human embryonic stem cells, using the miRVana miRNA Isolation Kit (Invitrogen, #AM1561) according to the manufacturer's protocol. Total RNA was extracted from mouse embryos using the Norgen Single-Cell RNA Isolation kit (#51800, Norgen Biotek Corp.) according to the manufacturer's protocol. Concentration of RNA was determined using a NanoDrop-1000 (Thermofisher Scientific). Relative gene expression for all miRNAs samples was determined using the Taqman Advanced cDNA synthesis and qPCR system (Applied Biosystems) purchased from Thermofisher Scientific.

An aliquot of 10 ng of total RNA from sperm, of total RNA from embryos was used in the initial cDNA synthesis step. Real-time PCR was performed for each target and sample in duplicate on a StepOnePlus PCR System (Applied Biosystems) with primers from TaqMan™ Advanced miRNA Assay (#A25576, Thermofisher, miR-34c-5p , #478052_miR, miR-449a, #478561_miR, miR-34b, #478049_miR, miR-449b, #479528_miR, miR-34a, #478048_miR, miR-152, #477921, miR-375 #mmu481141_miR). Relative gene expression for all Pri- forms was determined using the High-Capacity cDNA Reverse transcription kit and qPCR system (Applied Biosystems) purchased from Thermofisher Scientific and the primers Pri- differentially from mouse or human (TaqMan™ Pri-miRNA Assay, #4427012, mouse Pri-34bc Mm03306660_pri, mouse Pri-449ab Mm03308638_pri, human Pri-34bc Hs03295169_pri, human Pri-449ab Hs03304853_pri). **All data were analyzed using the Comparative ΔΔCT method to generate relative expression data using miR-192-5p (#A25576, Thermofisher, #478262_miR) as the internal control for miRNAs since its levels did not change significantly after of any of our experimental manipulations**, and GAPDH Forward 5'-CATGTTCCAGTATGACTCCACTC-3', Reverse 5'-GGCCTCACCCCATTTGATGT-3'; for mouse Pri- and ACTB (Forward 5-'AGAAAATCTGGCACCACAC-3', Reverse 5'-TAGCACAGCCTGGATAGCAA -3') for human ES cells samples.

Mouse Behavior

F1 female mice from control and stressed with or without doxycycline groups, were tested in a series of behavioral assays in the following order: elevated plus maze (EPM), direct social interaction with a juvenile. These tests were done 1 week apart. All behavioral tests were carried out during the light phase of the cycle, and mice were acclimated to the testing room 1 h prior to any behavioral procedure.

Elevated Plus Maze (**EPM**) Test

Mice were placed in the center of a plus-shaped maze elevated 40 cm from the floor, composed of two open and two closed arms, each 35.5 cm long and 5 cm wide (Campden Instruments Ltd, Lafayette, IN). General mouse activity was analyzed for 5 min, and percent of time spent in the open arms was recorded using the Ethovision XT (Noldus, Leesburg VA, USA).

Direct Social Interaction with a Juvenile (**SIJ**) Test

Direct social interaction was assessed in a clean cage, similar to the home cage in which the animals were regularly housed, with bedding on the floor, and covered by a plastic lid to avoid direct light exposure. Each experimental mouse was placed into this cage and left to habituate for 15 min. Then, an unfamiliar same-sex juvenile (26-28 days old) was introduced and left there for 3 min. Testing sessions were recorded and videos were analyzed by an observer (blind to the treatments) taking only the experimental animal into account. During the 3 min test, time spent performing the following affiliative and social behaviors was measured: sniffing the juvenile, grooming it, following it, crawling over or under it, doing passive physical contact with it, and walking side by side.

Statistical analysis

GraphPad Prism 9.0 software package was used for analyzing the behavioral and qPCR data. For all experiments comparing two groups, unpaired t-test was used. For all experiments comparing more than two groups, ordinary one-way ANOVA with multiple comparisons with Tukey test were used. $p < 0.05$ was used as a cutoff for significance

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Contribution of Authors

Alexandre Champroux- Performed most of the experiments presented.

Yang Tang- Performed transfections of human embryonic stem cells

David Dickson- Generated DNA constructs for transgenic mouse used in paper and characterized mice

Alice Meng- Generated DNA constructs for transgenic mouse used in paper

Ann Harrington- Generated transgenic mouse used in paper

Lucy Liaw- Generated transgenic mouse used in paper

Thornsten Schlaeger- supervised ES cell transfection experiments

Francesco Nicassio- helped design experiments

Matteo Marzi- helped design experiments

Larry Feig- Supervised entire project

Supplemental figures legends

<u>Supplemental Figure 1:</u> Map Of the pTetOne plasmid containing the miR-34c locus and flanking genomic DNA.

A) Mmu-miR-34c genomic locus and ~100bp of flanking genomic DNA on either side of the locus were cloned into the pTetOne vector using the BamHI and EcoRI restriction enzyme sites. TREGs-insert-FWD and Tet-insert-REV primers used for sequencing to original confirm successful integration of the miR-34c locus into the plasmid and are also used for genotyping the transgenic animals. B) Genomic region of miR-34c in the mouse genome on the chromosome 9. C) Expression of miR-34c and miR-34b. The miRNAs are produced together in a primary transcript, Pri-34b/c, from the region chromosome 9 (51,014,134:51,015,000:-1). Then the Pri-34b/c is cleaved into Pre-34b and Pre-34c before being exported to the cytoplasm, where final maturation into miR-34c and miR-34b takes place (See[26]).

<u>Supplemental Figure 2:</u> miR-34c induction from the 2-4 cells stage through the blastocyst stage of preimplantation embryos derived from CSI stressed, Tet-inducible transgenic males

CSI stressed transgenic males were mated to either females pre-fed a control or dox-containing chow and pools of embryos were isolated at the indicated times. n=3 for 4 cells and blastocysts and n=5 for morula. Data are expressed as mean ± S.E.M; Mann-Whitney test two-tailed, *$p<0.05$.

<u>Supplemental Figure 3:</u> Levels of miRNAs unrelated to CSI stress are not altered by CSI stress nor miR-34c induction in morula stage embryos derived from CSI stressed transgenic males

A) Levels of miR-375 and miR-152 in morula derived from mating unstressed and stress transgenic males.

B) 5 different CSI stressed transgenic (Tg) males were mated with WT unstressed females pre-fed doxycycline (Dox) or with regular chow (no Dox) and measure of miR-375 and miR-152 in morula

stage embryos derived from these mating. n=5. Data are expressed as mean ± S.E.M; Mann-Whitney test two-tailed, *p<0.05.

<u>Supplemental Figure 4:</u> miR-34b is suppressed in transgenic lymphocytes cells when miR-34c is elevated by their exposure to doxycycline.

Lymphocytes were extracted from Tet-inducible miR-34c transgenic blood, and then exposed to either doxycycline or saline for 24h. While miR-34c content was increased by doxycycline, miR-34b content decreased while and miR-449a/b was undetectable in both cases. n=5. Data are expressed as mean ± S.E.M; Unpaired t test two-tailed, *p<0.05

<u>Supplemental Figure 5:</u> Levels Pri- and mature forms of miR-34b,c and miR-449a,b are consistent in testis and liver.

A) Levels of Pri-34b/c and miR-34b and miR-34c in liver vs testis.

B) Levels of Pri-449a/b and miR-449a,b in liver vs testis.

N=2, two-way Anova, multiple comparisons, ***<0.0001

Internal standards for the Pri-miRNAs were GAPDH, and miR-192 for the mature form.

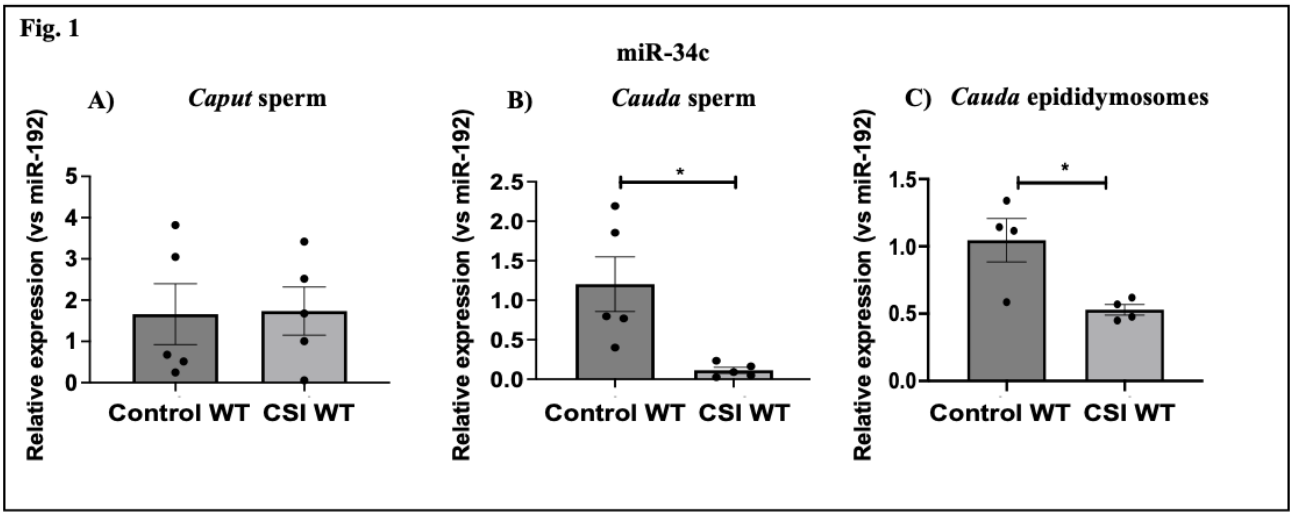


Fig. 1
miR-34c
A) Caput sperm
B) Cauda sperm
C) Cauda epididymosomes
Relative expression (vs miR-192)
Control WT
CSI WT
*

Fig. 2

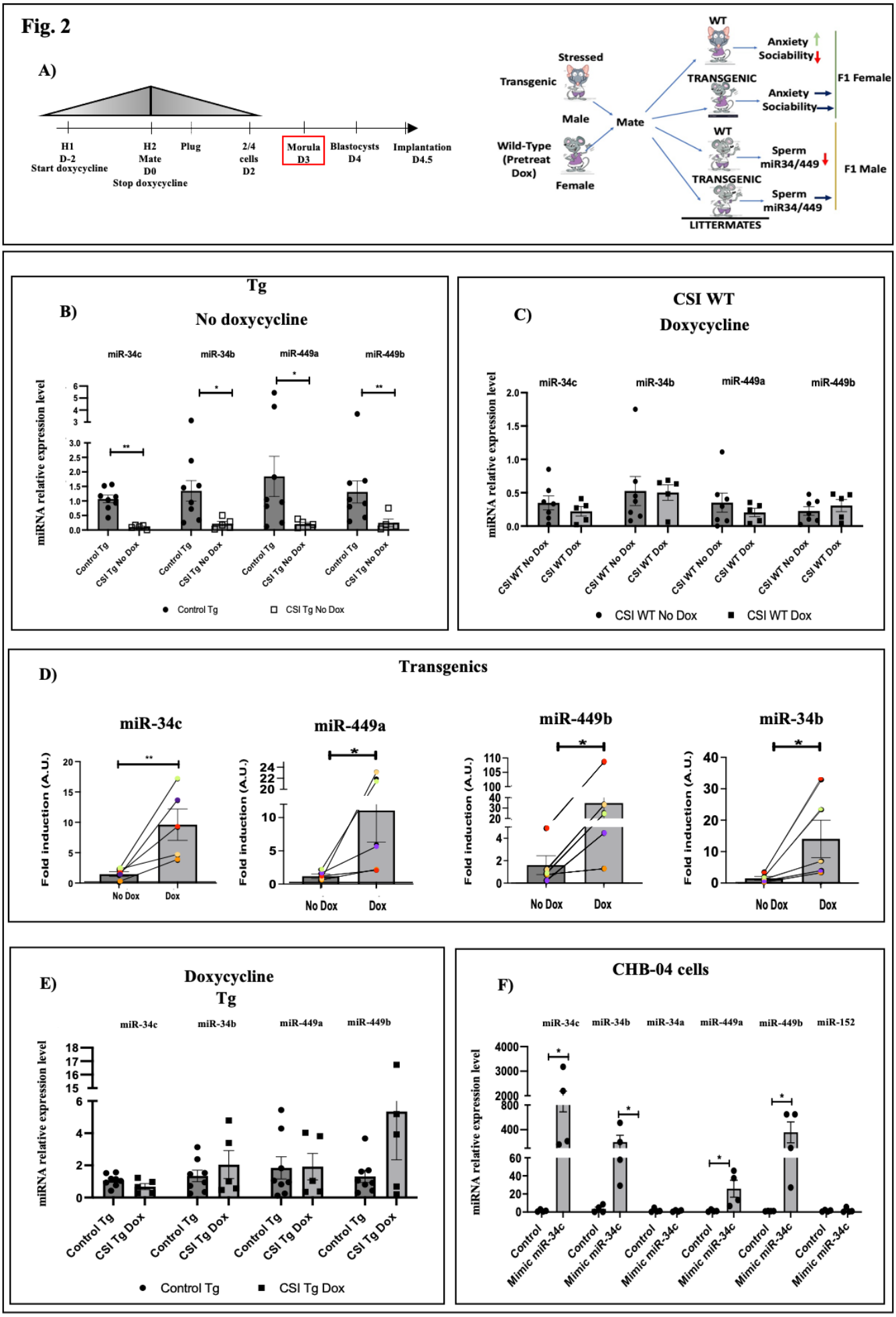

A)
H1
D-2
Start doxycycline
H2
Mate
D0
Stop doxycycline
Plug
2/4
cells
D2
Morula
D3
Blastocysts
D4
Implantation
D4.5
Stressed
Transgenic
Male
Mate
Wild-Type
(Pretreat
Dox)
Female
WT
TRANSGENIC
WT
TRANSGENIC
LITTERMATES
Anxiety
Sociability
Anxiety
Sociability
Sperm
miR34/449
Sperm
miR34/449
F1 Female
F1 Male
Tg
B)
No doxycycline
miR-34c
miR-34b
miR-449a
miR-449b
miRNA relative expression level
Control Tg
CSI Tg No Dox
CSI Tg No Dox
CSI WT
C)
Doxycycline
CSI WT No Dox
CSI WT Dox
D)
Transgenics
miR-34c
miR-449a
miR-449b
miR-34b
Fold induction (A.U.)
No Dox
Dox
E)
Doxycycline
Tg
CSI Tg Dox
F)
CHB-04 cells
miR-34a
miR-152
Control
Mimic miR-34c

Fig. 3

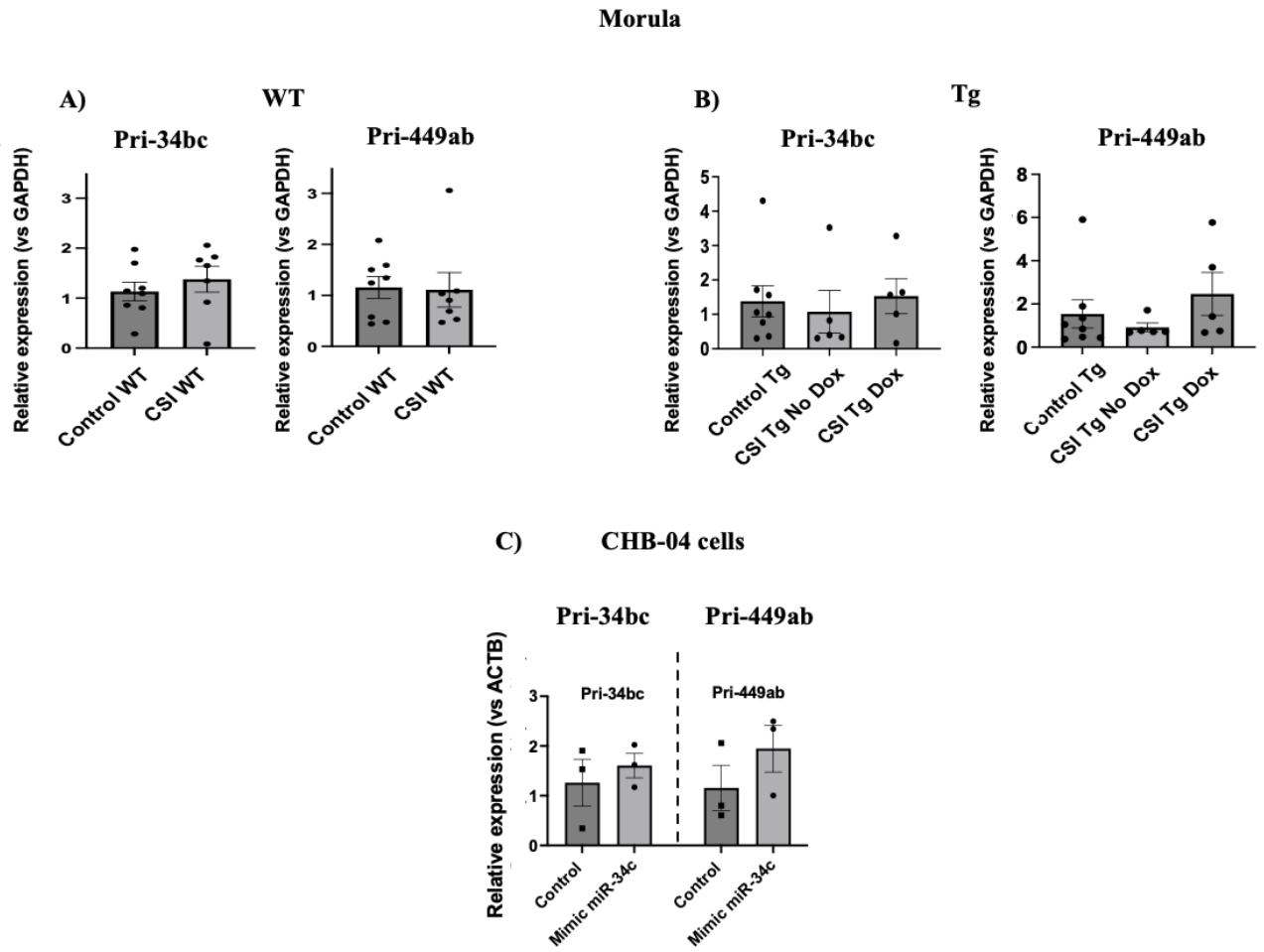
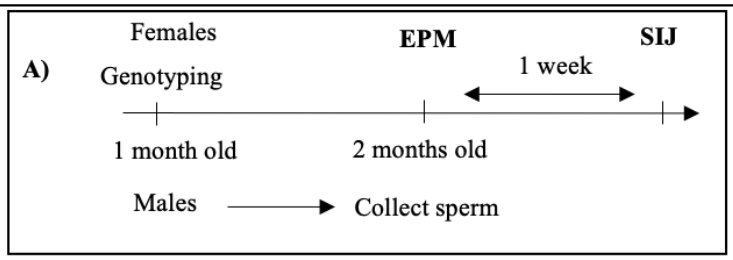
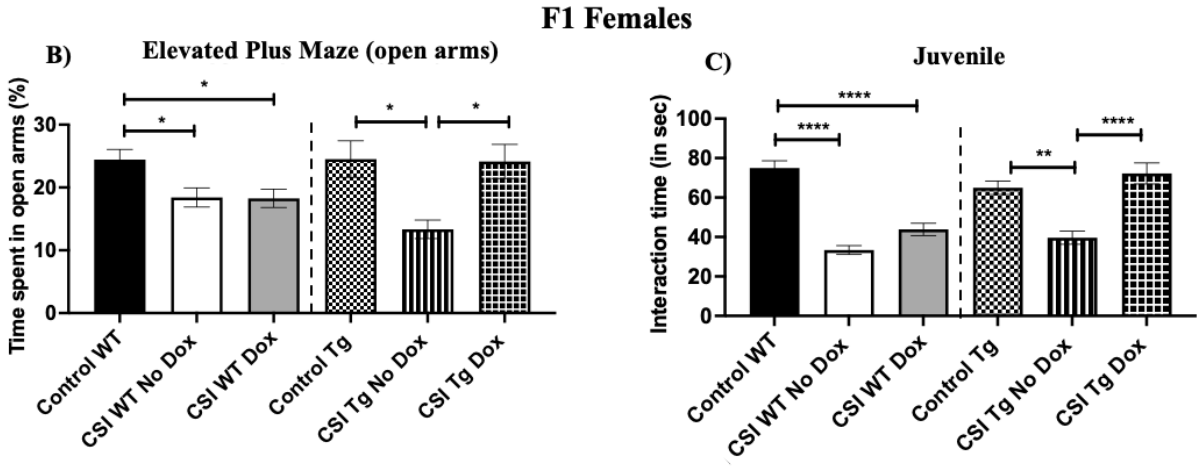
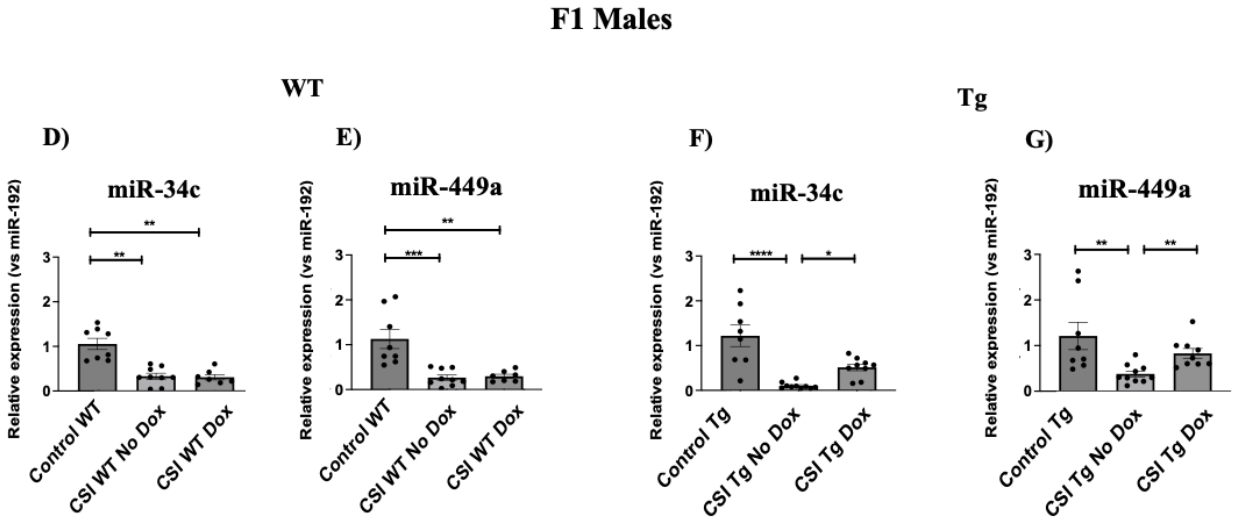

Morula
A)
WT
Pri-34bc
Pri-449ab
Relative expression (vs GAPDH)
Control WT
CSI WT
B)
Tg
Pri-34bc
Pri-449ab
Relative expression (vs GAPDH)
Control Tg
CSI Tg No Dox
CSI Tg Dox
C)
CHB-04 cells
Pri-34bc
Pri-449ab
Relative expression (vs ACTB)
Control
Mimic miR-34c

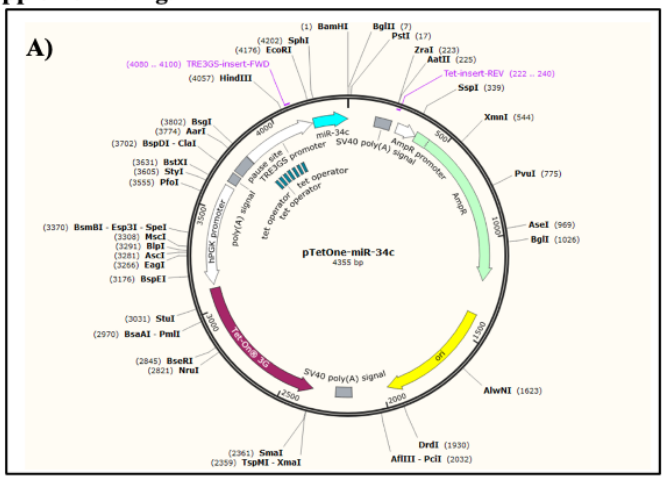
Fig. 4


A)
Females
Genotyping
EPM
SIJ
1 week
1 month old
2 months old
Males
Collect sperm

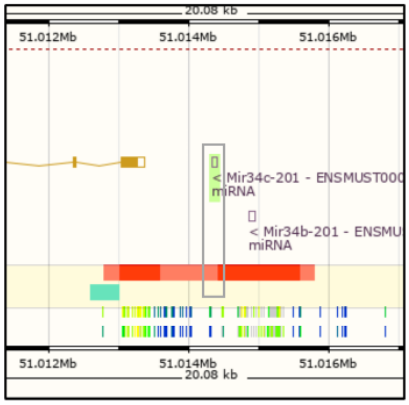

F1 Females
B)
Elevated Plus Maze (open arms)
Time spent in open arms (%)
Control WT
CSI WT No Dox
CSI WT Dox
Control Tg
CSI Tg No Dox
CSI Tg Dox
C)
Juvenile
Interaction time (in sec)

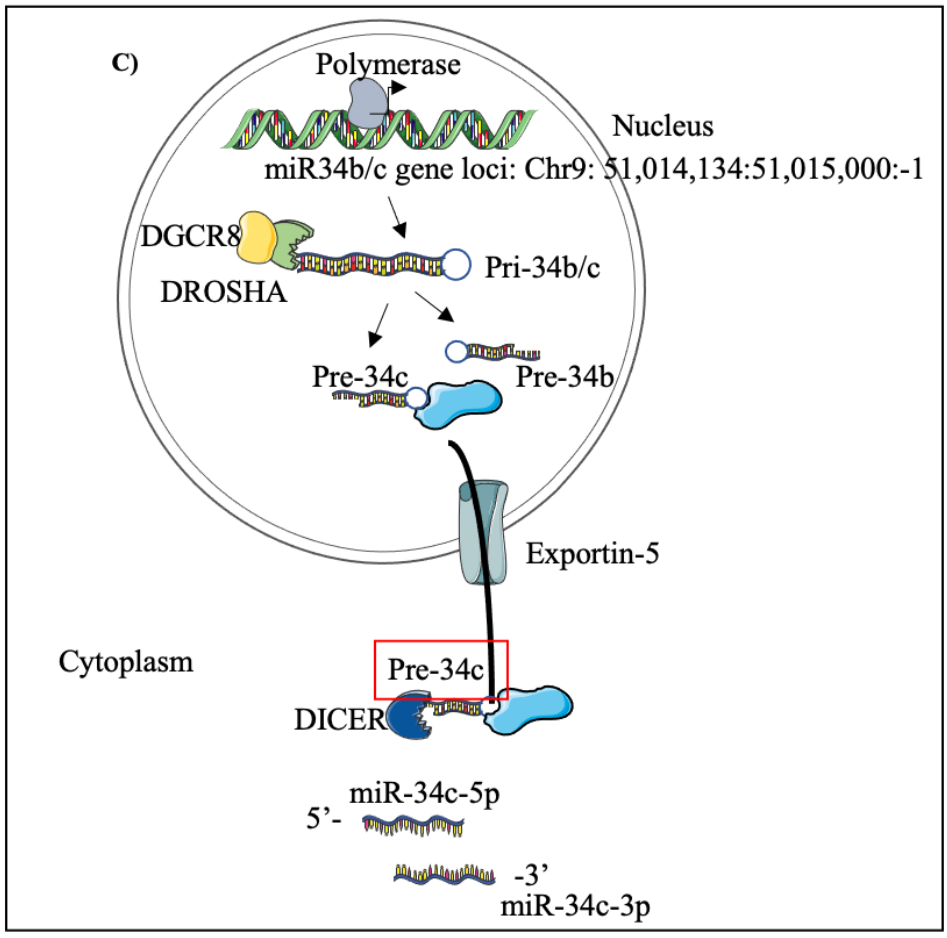

F1 Males
WT
Tg
D)
miR-34c
E)
miR-449a
F)
miR-34c
G)
miR-449a
Relative expression (vs miR-192)
Control WT
CSI WT No Dox
CSI WT Dox
Control Tg
CSI Tg No Dox
CSI Tg Dox

Supplemental Fig. 1

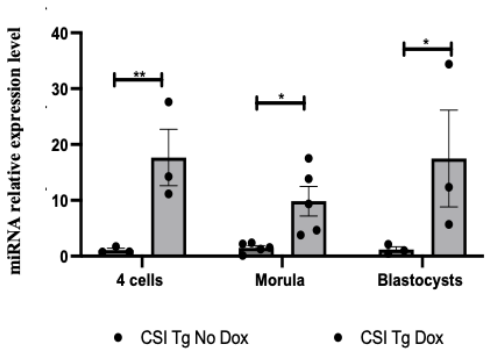


B)

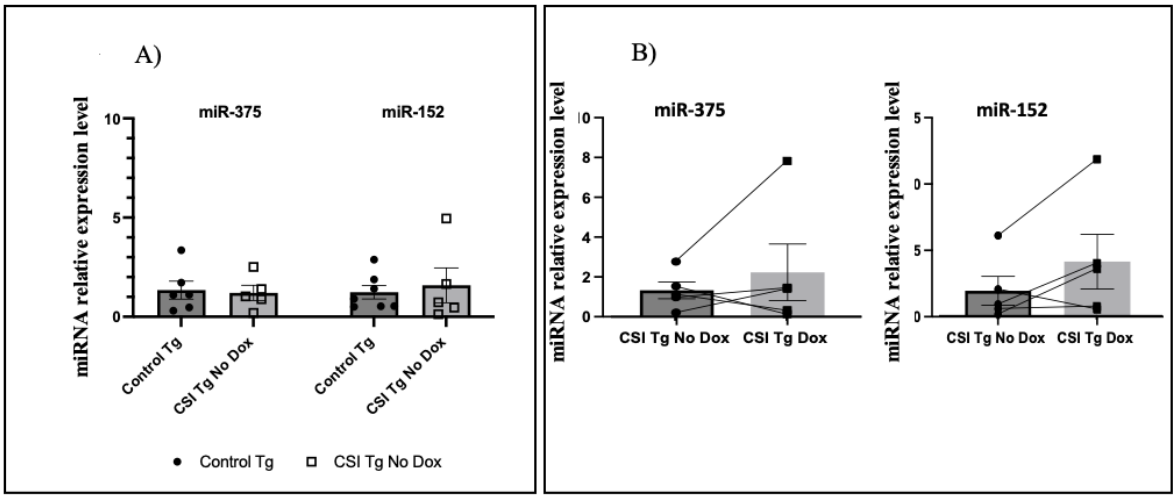




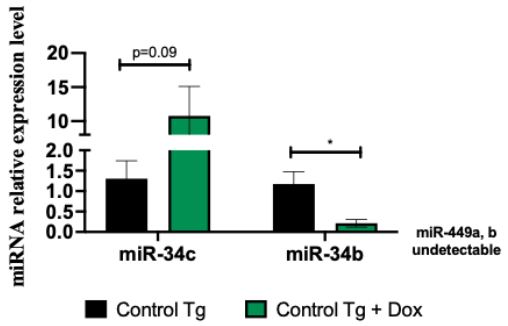
Supplemental Fig. 2



Supplemental Fig. 3

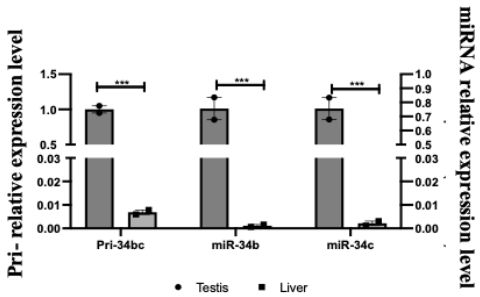
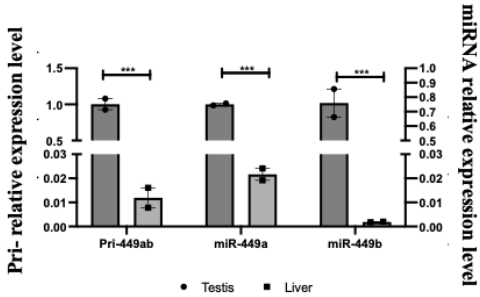


Supplemental Fig. 4



Supplemental Fig. 5

A)



B)