

SMALL RNAs

Sperm tsRNAs contribute to intergenerational inheritance of an acquired metabolic disorder

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Increasing evidence indicates that metabolic disorders in offspring can result from the father's diet, but the mechanism remains unclear. In a paternal mouse model given a high-fat diet (HFD), we showed that a subset of sperm transfer RNA-derived small RNAs (tsRNAs), mainly from 5' transfer RNA halves and ranging in size from 30 to 34 nucleotides, exhibited changes in expression profiles and RNA modifications. Injection of sperm tsRNA fractions from HFD males into normal zygotes generated metabolic disorders in the F₁ offspring and altered gene expression of metabolic pathways in early embryos and islets of F₁ offspring, which was unrelated to DNA methylation at CpG-enriched regions. Hence, sperm tsRNAs represent a paternal epigenetic factor that may mediate intergenerational inheritance of diet-induced metabolic disorders.

Increasing lines of evidence from worms to mammals suggest that parental environmental exposure can affect the germ line and influence future generations through epigenetic mechanisms (1, 2). Specifically, diet-induced metabolic changes in mammals are transmitted from father to offspring (3, 4), suggesting sperm-mediated epigenetic inheritance (5). DNA methylation is affected (6, 7), yet a causal relationship in transgenerational inheritance has not been established. Small noncoding RNAs (sncRNAs) regulate DNA methylation, histone modifications, and mRNA transcription (8) and can induce non-Mendelian transgenerational

inheritance in mammals (9–13). Altered sperm microRNA (miRNA) profiles have been observed after paternal exposure to dietary changes or trauma (14, 15); however, because mammalian sperm harbors a diversity of sncRNAs (16), the specific population of RNAs that mediate intergenerational epigenetic memory remains unknown (17, 18). Here we report that a subset of sperm tRNA-derived small RNAs (tsRNAs), mainly from 5' tRNA halves and about 30 to 34 nucleotides (nt) in size (19), showed alterations in expression profiles and RNA modifications after paternal exposure to a high-fat diet and transmitted certain metabolic disorders from father to offspring.

To establish a model of intergenerational transmission of paternal diet-induced metabolic disorder (4, 7, 14), we continuously fed F₀ male mice with a high-fat diet (HFD, 60% fat) or a normal diet (ND, 10% fat) for 6 months beginning at 5 weeks of age. As expected, males fed a HFD became obese, glucose intolerant, and insulin resistant, whereas males in the ND group did not (fig. S1). The sperm heads of ND and HFD mice were injected into normal mouse oocytes, and the embryos were transferred into surrogate mothers. Male offspring resulting from the HFD- and ND-group sperm were fed a ND and exhibited no obvious differences in body weight over 16 weeks (fig. S2A). However, offspring produced by the HFD-group sperm exhibited the onset of impaired glucose tolerance and insulin resistance as early as 7 weeks of age (fig. S2, B and C), which became more severe at 15 weeks, as revealed by a glucose tolerance test (GTT) and an insulin tolerance test (ITT) (fig. S2, D and F). Although embryo manipulation procedures may induce epigenetic alterations, our parallel sperm-head injection experiments have eliminated the potential influence of male-female contact and semen factors during natural mating (20), suggesting that the sperm itself contains sufficient information

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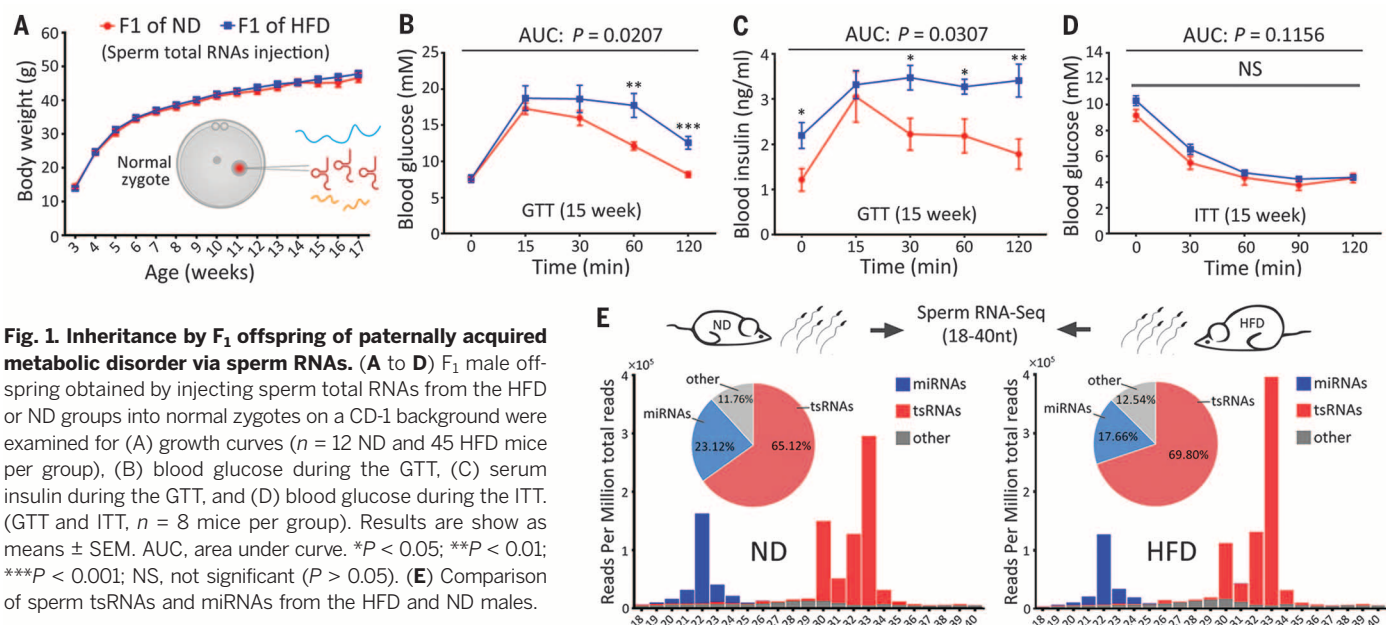
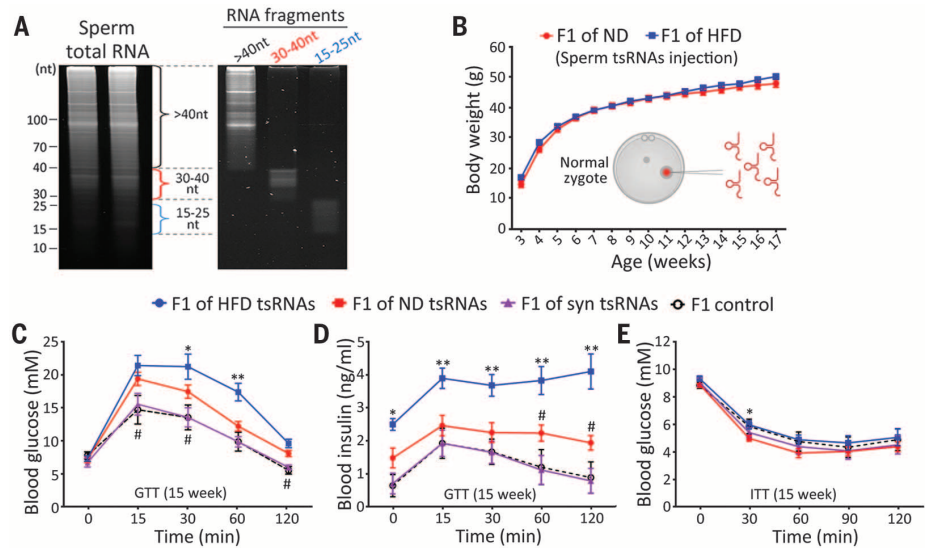


Fig. 1. Inheritance by F₁ offspring of paternally acquired metabolic disorder via sperm RNAs. (A to D) F₁ male offspring obtained by injecting sperm total RNAs from the HFD or ND groups into normal zygotes on a CD-1 background were examined for (A) growth curves ($n = 12$ ND and 45 HFD mice per group), (B) blood glucose during the GTT, (C) serum insulin during the GTT, and (D) blood glucose during the ITT. (GTT and ITT, $n = 8$ mice per group). Results are shown as means \pm SEM. AUC, area under curve. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not significant ($P > 0.05$). (E) Comparison of sperm tsRNAs and miRNAs from the HFD and ND males.

Fig. 2. Sperm tsRNAs conferred paternally acquired metabolic disorder to F₁ offspring.

(A) PAGE image of sperm total RNAs and isolated RNA fragments. (B) Growth curves for F₁ male offspring generated by injecting sperm tsRNAs (30- to 40-nt RNAs) from the HFD or ND group ($n = 12$ ND and 32 HFD mice per group). (C to E) F₁ male offspring generated by injecting HFD-group sperm tsRNAs, ND-group sperm tsRNAs, or synthetic (syn) tsRNAs into CD-1 zygotes were examined, relative to F₁ control mice, for (C) blood glucose during the GTT, (D) serum insulin during the GTT, and (E) blood glucose during the ITT (GTT and ITT, $n = 8$ to 14 mice per group). Results are shown as means \pm SEM. * $P < 0.05$; ** $P < 0.01$ (HFD versus ND tsRNAs); # $P < 0.05$ (ND versus syn tsRNAs).



to transmit an acquired metabolic disorder to offspring.

To assess whether sperm RNAs can induce intergenerational phenotypes (15), we purified total RNAs from the sperm of both HFD and ND mice and injected them into normal zygotes (RNA injection was normalized to about the amount of 10 sperm). Again, although the male offspring from both groups exhibited similar body-weight growth (Fig. 1A), the offspring from the HFD group developed impaired glucose tolerance, showing significantly higher blood glucose and serum insulin levels during the GTT than offspring from the ND group at both 7 and 15 weeks of age (Fig. 1, B and C, and fig. S3). However, the insulin sensitivity of the HFD group's offspring, as determined by the ITT test, was comparable to that of the ND group's offspring (Fig. 1D and fig. S3), which differs from the results produced by sperm-head injection (fig. S2, D and F). These data demonstrate that total RNAs from the sperm of HFD males contain the information to induce glucose intolerance, but not insulin resistance, in the F₁ offspring, suggesting the involvement of other layers of regulations such as DNA methylation and histone modifications (7, 21).

To identify the subpopulation of sperm RNAs that mediates paternally acquired metabolic disorder, we examined the sperm sncRNA profiles of the HFD and ND F₀ males by small RNA-seq (18 to 40 nt) (fig. S4). Analysis of the sequencing data reinforced our recent discovery (19) that, in addition to the well-known miRNA population, mature mouse sperm carry tsRNAs (Fig. 1E). Comparative analysis of sperm small RNAs from HFD and ND mice showed that a larger proportion of tsRNAs (11.53%) exhibited significant differences compared with miRNAs (2.28%) (fig. S5 and tables S1 and S2), suggesting that the population of sperm tsRNAs is more sensitive to HFD exposure.

To further investigate whether sperm tsRNAs or other sperm RNA fragments are able to in-

duce intergenerational transmission of acquired traits, we ran sperm RNAs on a 15% PAGE (polyacrylamide gel electrophoresis) gel and separately collected RNA fragments at sizes of 30 to 40 nt (predominantly tsRNAs), 15 to 25 nt (predominantly miRNAs), and >40 nt from both HFD and ND males; this was followed by RNA extraction and confirmation by polymerase chain reaction and RNA-seq (Fig. 2A and figs. S6 and S7). The three types of RNA fragments were injected separately into normal zygotes, with the same concentration as that of the injected sperm total RNAs.

We found that injecting 15- to 25-nt RNAs at this concentration resulted in embryo lethality, whereas injecting 30- to 40-nt or >40-nt RNAs did not (table S3). The embryonic lethal effect in the former case was probably due to the knock-down effects of miRNAs on multiple mRNAs, which would interfere with normal embryonic development. Injection of a 20 \times dilution of the 15- to 25-nt RNAs did not cause embryo lethality, nor did it cause metabolic disorder in F₁ offspring (fig. S8, A and B). Injection of >40-nt RNAs also did not cause a metabolic phenotype in F₁ offspring (fig. S8, C and D). However, injection of 30- to 40-nt RNAs from HFD and ND groups led to phenotypes that mimicked those in the F₁ offspring that were produced by injecting sperm total RNAs: There was no significant difference in body-weight growth between HFD and ND offspring (Fig. 2B), but male offspring of the HFD group developed glucose intolerance (evident in the GTT; Fig. 2, C and D, and figs. S9 and S10) compared with those of ND group, though with mild or no insulin resistance evident in the ITT (Fig. 2E and figs. S9 and S10). These results demonstrate that sperm 30- to 40-nt RNAs (predominantly tsRNAs) are necessary to reproduce the effect of sperm total RNAs in inducing acquired metabolic disorder in offspring.

We next synthesized a combination of the most highly expressed tsRNAs in the sperm

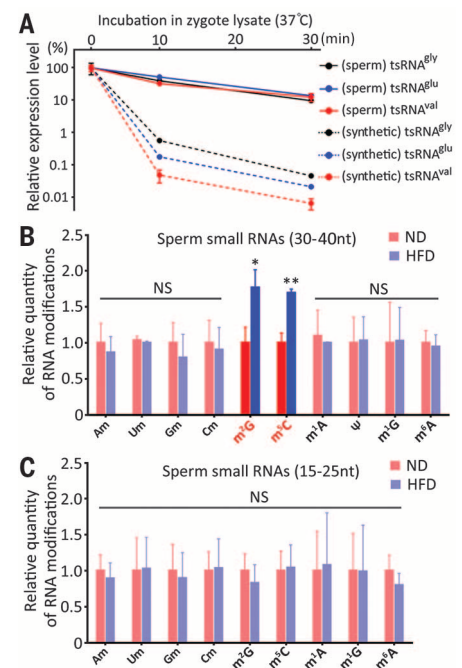


Fig. 3. HFD alters RNA modifications in the sperm tsRNA fraction.

(A) Comparison of RNA stability between sperm tsRNAs and chemically synthesized tsRNAs without RNA modifications (gly, glycine; glu, glutamic acid; val, valine). (B and C) HFD-group sperm shows significantly increased m⁵C and m²G in (B) the tsRNA fraction (30- to 40-nt) but not in (C) the miRNA fraction (15- to 25-nt) of RNAs, as compared with ND-group sperm ($n = 3$ independent samples per group). Results are shown as means \pm SEM.

(accounting for ~70% of sperm tsRNAs; table S4) to investigate whether they could resemble the function of endogenous sperm tsRNAs. However, injecting synthetic tsRNAs into normal zygotes with the same protocol that was

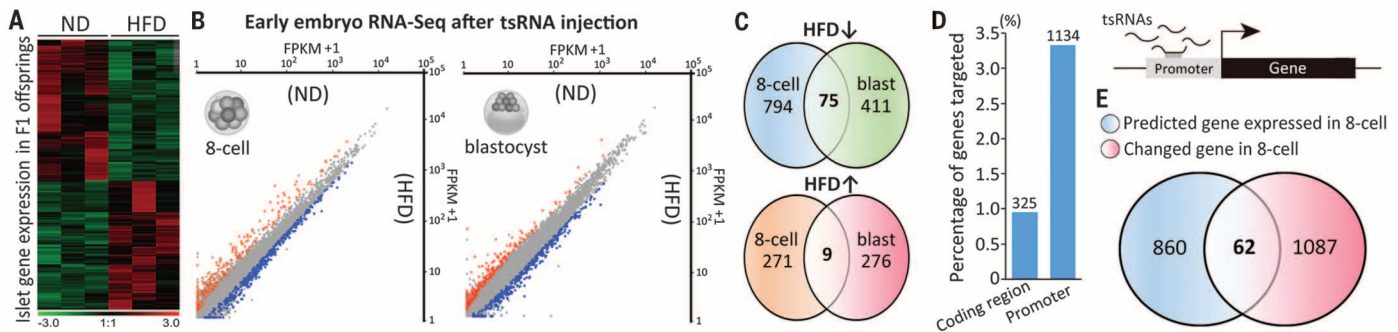


Fig. 4. tsRNAs from HFD-group sperm dysregulated gene expression in early embryos and islets of F_1 offspring. (A) Heat map of differentially expressed genes in islets of F_1 offspring. (B) Scatterplot analysis for differential gene expression in eight-cell embryos and blastocysts after injecting ND- or HFD-group sperm tsRNAs (FPKM, fragments per kilobase of transcript per million mapped reads). (C) Venn diagrams showing number of genes with

significant changes at the eight-cell and blastocyst stages (upward- and downward-pointing arrows indicate up- and down-regulation, respectively). (D) Sperm tsRNA sequences preferentially match to gene promoter regions rather than to mRNA coding regions. (E) Venn diagrams of tsRNA-matched genes that are expressed in eight-cell embryos (blue) and significantly changed genes (red) in eight-cell embryos after injecting sperm tsRNAs.

used for sperm tsRNAs did not induce metabolic disorder in the offspring (Fig. 2, C to E). One explanation for this result could be that the chemically synthesized tsRNAs are not as stable as physiologically derived sperm tsRNAs (22). The synthetic tsRNAs showed faster degradation rates in zygote lysates than did the sperm-derived tsRNAs (Fig. 3A). In contrast to the unmodified synthetic tsRNAs, sperm tsRNAs might harbor various RNA modifications (inherited from their tRNA precursors) that influence RNA stability.

To systematically analyze the RNA modification profiles of HFD and ND sperm tsRNAs, we applied our recently developed high-throughput quantitative approach, which is based on liquid chromatography–tandem mass spectrometry (fig. S11) (23) and which simultaneously identifies and quantifies multiple types of RNA modifications (table S5) in one RNA sample. With this approach, we stably detected and quantified 10 types of RNA modifications in 30- to 40-nt sperm RNAs (predominantly tsRNAs) and found that two RNA modifications, 5-methylcytidine (m^5C) and N^2 -methylguanosine (m^2G), were significantly up-regulated in HFD-group sperm compared with ND-group sperm (Fig. 3B), whereas no significant differences in 15- to 25-nt sperm RNAs (predominantly miRNAs) were found using the same protocol (Fig. 3C). Although the function of m^2G on RNAs in mammals remains unknown, the presence of mammalian m^5C has been reported to contribute to tRNA stability (24) and to be related to RNA-mediated transgenerational epigenetic inheritance (25). Thus, the elevated levels of m^5C and m^2G modifications in the tsRNAs of HFD-group sperm provide another clue in explaining the function of tsRNAs in mediating paternally acquired traits, pointing to a new direction for future investigations.

To uncover the potential causes of glucose intolerance in F_1 offspring produced by injecting HFD-group sperm tsRNAs, we isolated the F_1 offspring islet and performed RNA-seq and RRBS (reduced representation bisulfite sequencing) analyses for a genome-wide comparison of both

transcriptome and DNA methylation. RNA-seq analysis revealed that differentially expressed genes are dominantly enriched in metabolic pathways (including ketone, carbohydrate, and monosaccharide metabolisms) for down- but not up-regulated genes in the HFD group's offspring, as indicated by gene ontology analysis (Fig. 4A and tables S6 and S7). These changed transcription profiles in the F_1 islet could explain the observed metabolic disorder in F_1 offspring. On the other hand, genome-wide RRBS analysis revealed differentially methylated regions (DMRs) between the ND and HFD offspring in 28 genes (table S8). There were no overlaps between the DMR-associated genes and those showing transcriptional changes, suggesting that differential DNA methylation is not directly responsible for the changed transcriptional activity of the F_1 islet.

To test the alternate possibility that injecting sperm tsRNAs into the zygote might cause a transcriptional cascade change in the early embryo that ultimately guides altered gene expression in the islet of F_1 offspring, we collected eight-cell embryos and blastocysts after injection of ND- or HFD-group sperm tsRNAs for comparative RNA-seq analysis (Fig. 4B). Both eight-cell embryos and blastocysts showed that more genes were down-regulated (869 and 486, respectively, with 75 overlaps) than up-regulated (280 and 285, respectively, with nine overlaps) in the group injected with HFD sperm tsRNAs, compared with the group injected with ND sperm tsRNAs. (Fig. 4C and table S9). Embryonic genes showing down- but not up-regulation in the HFD group were enriched in metabolic regulation pathways, in addition to other essential cellular processes (e.g., protein transport and localization) (tables S10 and S11). These early-embryo transcriptional changes might cause profound downstream effects that result in reprogrammed gene expression in the islet of F_1 offspring, hence leading to metabolic disorder.

To explore how the injection of tsRNAs could potentially affect embryonic gene expression, we analyzed sequence matches throughout the

genome and found that sperm tsRNAs (differentially expressed between ND and HFD males) preferentially match to gene promoter regions (~2 kb from the transcription start site) rather than coding regions (Fig. 4D and table S12). Among the 1134 genes with a tsRNA-matching promoter, 922 of them are expressed in the eight-cell embryos, and 62 of them showed differential expression in the eight-cell embryos (Fig. 4E). Biological pathway analysis showed that these deregulated genes have regulatory potential for diverse cellular and molecular events, including apoptosis, autophagy, oxidative stress, glucose input, and others (fig. S12). The genes *Maes*, *Ccnc*, and *Deptor* have been reported to be involved in pancreatic β -cell function or to be associated with diabetic conditions (26–28). This correlation-based evidence suggests that sperm tsRNAs might affect metabolic gene expression through embryo to adulthood via a transcriptional cascade effect and that the deregulation of this process can affect F_1 offspring phenotypes (fig. S13).

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S13
Tables S1 to S12
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GENE EDITING

Postnatal genome editing partially restores dystrophin expression in a mouse model of muscular dystrophy

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CRISPR/Cas9-mediated genome editing holds clinical potential for treating genetic diseases, such as Duchenne muscular dystrophy (DMD), which is caused by mutations in the dystrophin gene. To correct DMD by skipping mutant dystrophin exons in postnatal muscle tissue in vivo, we used adeno-associated virus-9 (AAV9) to deliver gene-editing components to postnatal *mdx* mice, a model of DMD. Different modes of AAV9 delivery were systematically tested, including intraperitoneal at postnatal day 1 (P1), intramuscular at P12, and retro-orbital at P18. Each of these methods restored dystrophin protein expression in cardiac and skeletal muscle to varying degrees, and expression increased from 3 to 12 weeks after injection. Postnatal gene editing also enhanced skeletal muscle function, as measured by grip strength tests 4 weeks after injection. This method provides a potential means of correcting mutations responsible for DMD and other monogenic disorders after birth.

Duchenne muscular dystrophy (DMD) is a fatal muscle disease affecting 1 in 3500 to 5000 boys. Cardiomyopathy and heart failure are common, incurable, and lethal consequences of DMD. The disease is caused by mutations in the gene encoding dystrophin, a large intracellular protein that links the dystroglycan complex at the cell surface with the underlying cytoskeleton, thereby maintaining integrity of muscle cell membranes during contraction (1, 2). In the absence of dystrophin, muscles degenerate, causing weakness and myopathy (3). Many therapeutic approaches for DMD have failed, at least in part because of the size of the dystrophin protein and the necessity for lifelong restoration of dystrophin expression in the myriad skeletal muscles of the body as well as the heart.

The CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated

protein 9) system allows precise modification of the genome and represents a potential means of correcting disease-causing mutations (4, 5). In the presence of single guide RNAs (sgRNAs), Cas9 is directed to specific sites in the genome adjacent to a protospacer adjacent motif (PAM), causing a double-strand break (DSB). When provided with an additional DNA template, a precise genomic modification is generated by homology-directed repair (HDR), whereas in the absence of an exogenous template, variable indel mutations are created at the target site via nonhomologous end joining (NHEJ) (6). Previously, we used CRISPR/Cas9 to correct a single nonsense mutation in *Dmd* by HDR in the germ line of *mdx* mice, which allowed the restoration of dystrophin protein expression (7). However, germline genomic editing is not feasible in humans (8) and HDR does not occur in postmitotic adult tissues, such as heart and skeletal muscle (9), necessitating alternative strategies of gene correction in postnatal tissues. Here, we devised a method to correct *Dmd* mutations by CRISPR/Cas9-mediated NHEJ (termed “Myoediting”) in postnatal muscle tissues after delivery of gene-editing components by means of adeno-associated virus-9 (AAV9), which displays high tropism for muscle (10, 11).

The dystrophin protein contains several domains (fig. S1), including an actin-binding domain

at the N terminus, a central rod domain with a series of spectrin-like and actin-binding repeats, and WW and cysteine-rich domains at the C terminus that mediate binding to dystroglycan, dystrobrevin, and syntrophin (12). The actin-binding and cysteine-rich domains are essential for function, but many regions of the protein are dispensable (3). It has been estimated that as many as 80% of DMD patients could benefit from exon-skipping strategies that bypass mutations in nonessential regions of the gene and partially restore dystrophin expression (13). This approach has been validated in vitro by CRISPR/Cas9-mediated correction of *Dmd* mutations in patients’ induced pluripotent stem cells (14) and immortalized myoblasts (15). Similarly, adenovirus-mediated gene editing was shown to restore dystrophin expression in specific muscles of *mdx* mice after intramuscular injection (16), but adenoviral delivery is not therapeutically favorable (17).

Shown in Fig. 1A is the strategy whereby CRISPR/Cas9-mediated NHEJ can create internal genomic deletions to bypass the premature termination codon in exon 23 responsible for the dystrophic phenotype of *mdx* mice, potentially allowing reconstitution of the *Dmd* open reading frame. In principle, this approach could be applied to many mutations within the gene, including large deletions, duplications, and pseudoexons. An advantage of this approach is that it does not require precise correction of the disease-causing mutation. Instead, imprecise deletions that prevent splicing of mutant exons are sufficient to restore dystrophin protein expression.

To test whether Myoediting could be adapted to skip the *Dmd* mutation in exon 23 in *mdx* mice, we first evaluated a pool of sgRNAs that potentially target the 5’ and 3’ ends of exon 23 (supplementary materials, fig. S2, and table S1). We co-injected Cas9 mRNA with sgRNA-*mdx* (directed toward the mutant sequence in exon 23) and either sgRNA-R3 or sgRNA-L8 (targeting the 3’ and 5’ end of exon 23, respectively) into *mdx* zygotes without a HDR template (fig. S3A). Strikingly, ~80% of progeny mice lacked exon 23 (termed *mdx*-ΔEx23) (fig. S3, B and C, and table S2), representing an increase in the efficiency of *mdx* editing relative to HDR (7). Genomic polymerase chain reaction (PCR) products from the target sites of exon 23 and reverse transcription PCR (RT-PCR) products of *mdx*-ΔEx23 mice were cloned and sequenced, confirming the skipping of exon 23 (fig. S3, D to F). As a result of skipping exon 23, the open reading frame of *Dmd* was restored, allowing dystrophin protein expression

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Sperm tsRNAs contribute to intergenerational inheritance of an acquired metabolic disorder

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Offspring affected by sperm small RNAs

Paternal dietary conditions in mammals influence the metabolic phenotypes of offspring. Although prior work suggests the involvement of epigenetic pathways, the mechanisms remains unclear. Two studies now show that altered paternal diet affects the level of small RNAs in mouse sperm. Chen *et al.* injected sperm transfer RNA (tRNA) fragments from males that had been kept on a high-fat diet into normal oocytes. The progeny displayed metabolic disorders and concomitant alteration of genes in metabolic pathways. Sharma *et al.* observed the biogenesis and function of small tRNA-derived fragments during sperm maturation. Further understanding of the mechanisms by which progeny are affected by parental exposure may affect human diseases such as diet-induced metabolic disorders.

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