University Grenoble Alpes
Master 2 Neurobiology Neurosciences
S4 Internship

Analysis of RNA fragments (tRFs) in a transgenerational mouse model of early trauma

Anastasiia Efimova

Supervised by Gretchen van Steenwyk

Head of Laboratory: Prof. Isabelle Mansuy

Laboratory of Neuroepigenetics

Brain Research Institute

Zurich, Switzerland

Abstract

Increasing evidence suggests that life-time experiences can be transmitted across generations, but the mechanisms remain unclear. The transgenerational mouse model of early life trauma (MSUS) is used to study such mechanisms. Previous work has shown the involvement of sperm short RNAs in transmitting the behavioral phenotype to the offspring of mice that undergo stressful treatment. In this project, sequencing of sperm and serum of MSUS and control mice has demonstrated the implication of recently discovered tRNA fragments (tRFs) in epigenetic inheritance of the MSUS phenotype. This hasn't been confirmed in the individual testing by RT-qPCR. However, an alteration in the level of full-length tRNAs has been detected, which might suggest the involvement of tRNA modifications. Additionally, optimisations of transfection conditions have been performed for elucidating the role of tRFs in the regulation of gene expression. These will later be used if the change in MSUS sperm tRNA modifications is confirmed in further experiments.

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Introduction

Accumulating evidence strongly suggests that consequences of life-time experiences can be transmitted to future generations via non-genetic mechanisms in a variety of species, from plants to mammals, including humans (Heard and Martienssen 2014). These far-reaching effects are thought to play a role in better adaptation of species to the environment conditions. However, these alterations might affect offspring's health, leading to metabolic disorders of progeny following paternal diet abnormalities (Veenendaal et al. 2013), or to psychiatric disorders after ancestral adverse experiences (Yehuda et al. 2000).

In order to study the phenomenon of epigenetic inheritance, a mouse model of early postnatal trauma has been established in the Laboratory of neuroepigenetics (Brain Research Institute, Zurich, Switzerland). The paradigm consists in **M**aternal **S**eparation combined with **U**npredictable maternal **S**tress (MSUS). During postnatal days 1-14 of pups (F1), mothers (F0) are separated daily for 3h and subjected to highly stressful treatments – forced swim or forced restraint. The behavioral effects of such treatment are transmitted up to F4 generation and include depression-like and anxiety symptoms (Franklin et al. 2010) (Weiss et al. 2011) (Gapp et al. 2014), antisocial behavior (Franklin et al. 2011) and cognitive deficits (Bohacek et al. 2015).

Although the mechanisms of such inheritance aren't clear to date, several contributing epigenetic factors have been established. Among them, a change in the level of short non-coding RNAs has been observed in the F1 sperm and F1-F2 brain and blood serum of MSUS mice (Gapp et al. 2014). Injecting total purified F1 sperm RNAs to the fertilized oocytes reproduced the behavioral and metabolic alterations in the resulting offspring. However, since mammalian sperm harbors a diversity of non-coding RNAs, the specific population of RNAs that mediate epigenetic memory remains unknown.

Recently, a new mechanism potentially responsible for epigenetic inheritance has emerged. The **tR**NA **f**ragments (tRFs), have been shown to play a role in the transmission of metabolic disorders to the next generation in mice fed with high-fat (Chen et al. 2016) or low protein diet (Sharma et al. 2016). These fragments of various length are produced by the cleavage of full-length tRNAs at different sites (Fig. 1). In sperm, the most prominent species are 5'-halves, generated through the cleavage in the anticodon loop. The sperm of mice fed with high-fat diet (HFD) exhibited abundant changes in expression profiles of 5'-halves (Chen et al. 2016). Injections of sperm RNA fraction containing 5'-halves of HFD mice into normal zygotes was sufficient to replicate the metabolic alterations observed in natural HFD offsprings.

A prominent feature of tRNAs is that they have multiple nucleotide modifications. A recent follow-up study on epigenetic inheritance of HFD phenotype stresses the role of tRF modifications (epimutations) rather than of tRFs *per se* in the transmission of the metabolic disorder (Zhang et al. 2018).

The study of sperm of mice fed with low-protein diet (LPD) found that tRF-GlyGCC (fragment derived from tRNA binding to aminoacid Glycin with GCC triplet in its anticodon sequence) plays a particular role in transmitting the metabolic phenotype to the offspring (Sharma et al. 2016). This fragment represses retrotransposon MERVL in embryonic stem cells, regulating early pre-implantation development.

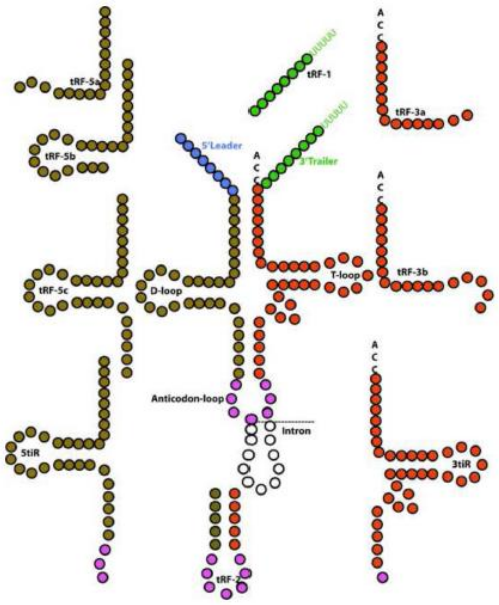

Figure 1. Classification of tRNA derived fragments (tRFs). Depending on the cleavage site, tRFs of various length are produced. 5'-halves are the most abundant species in mature mouse sperm and are implicated in epigenetic inheritance of metabolic alterations **(Kumar, Kuscu and Dutta 2016)**

Establishing the role of short RNAs in the epigenetic inheritance raises a question of their origin. Emerging evidence suggests that instead of being expressed in the precursors of germ cells they can be deliverd from other tissues in the form of extracellular RNAs, circulating in the blood stream in vesicles or ribonucleoprotein particles (Sharma 2014). In favour of this hypothesis, injecting serum collected from MSUS mice can reproduce MSUS metabolic phenotype in control mice (unpublished results from our laboratory). Additionally, it has been shown that dysregulation of circulating microRNAs is implicated in epigenetic inheritance in mammals (Sharma 2014, Gapp et al. 2014). However, no studies of circulating tRFs have been carried out so far.

We hypothesized that tRFs might play a role in transmitting epigenetic memory of early trauma in MSUS mice. The **goal** of the project was to study the abundance and role of tRFs in adult F1 MSUS mouse tissues. The **objectives** were the following:

1. Determine if tRFs are altered in adult tissues by exposure to early postnatal trauma.
2. Manipulate the level of tRFs in cultured germ cells and assess the effects on the activity of candidate genes.

Materials and Methods

Animals

C57Bl/6J mice were maintained under a reverse light-dark cycle in a temperature and humidity-controlled facility with food and water *ad libitum*. All experimental manipulations were performed during the animals' active cycle in accordance with guidelines and regulations of the cantonal veterinary office, Zurich. For unpredictable maternal separation combined with maternal stress (MSUS), C57Bl/6J dams (F0) and litters (F1) were subjected to daily 3h separation from postnatal day 1 to 14. During separation, dams underwent stressing treatment – either forced swim in cold water (18°C) for 5 min or forced restraint in a plexiglas transparent tube for 20 min. Control animals were left undisturbed apart from a cage change once a week until weaning (postnatal day 21). Once weaned, pups were reared in social groups (4-5 mice/cage) composed of animals subjected to the same treatment but from different dams to avoid litter effects. F1 males were sacrificed for tissue collection at the age of 3 months.

Serum injections

2 months old mice received 9 tail vein injections of 90 µL of serum from adult control or MSUS mice respectively over the course of 4 weeks.

Serum and sperm collection

Mice were anaesthetized by isofluorane, decapitated, and trunk blood was collected into non-coated 2 mL Eppendorf tubes. Clotting was allowed overnight at 4°C. After centrifugation for 10 min at 2000 g at 4°C, serum-containing supernatant was collected and kept at -80°C until used.

Cauda epididymis (Fig. 2), the structure containing mature sperm, was dissected bilaterally from adult F1 males and placed in M2 medium at 37°C for 15 mins so that the functional motile spermatozoa can be transferred into the medium («swim-up method»). The tube contents were then placed on cell strainer, washed with 10 mL of M2 medium, and centrifuged at 2000 g for 5 min at 4°C. The pellet was resuspended in 1 mL Somatic Cell Lysis Buffer (0.1% SDS, 0.5%

Triton X-100 in MilliQ water), followed by 2000 g centrifugation for 5 min at 4°C and 2 consecutive washes with 1 mL PBS. The resulting pellet was kept at -80°C until used.

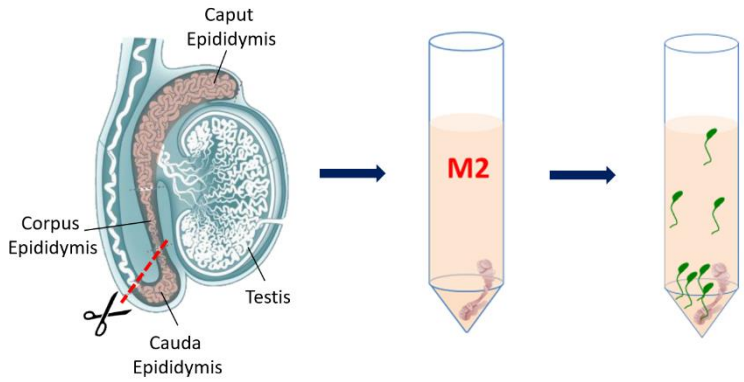

Figure 2. Collection of mature sperm from cauda epididymis. Cauda epididymis is excised bilaterally from anaesthetized mice and placed in M2 medium to allow the transfer of functional motile spermatozoa into the medium. The resulting suspension is used for further processing.

RNA extraction

For sperm samples, spermatozoa were first lysed in Qiagen TissueLyser II for 30 sec at 25 Hz with the help of 5 mm stainless steel beads in a solution containing 180 µL RLT Plus RNeasy lysis buffer (Qiagen) and 20 µL TCEP (tris(2-carboxyethyl)phosphine) (Sigma) per sample. This was followed by a standard RNA phenol-chloroform extraction protocol using TRIzol (Invitrogen).

For serum samples, total RNA was prepared using a standard Trizol LS protocol. Since there is no conventional endogenous control for qPCR normalization in serum samples, an exogenous spike-in control – Cel-miR-39 from *C.elegans* (Qiagen)– was added proportional to the sample volume before RNA extraction.

The quality of RNAs was determined by Agilent 2100 Bioanalyser (Agilent Technologies). The concentration was measured by Qubit fluorometer (Life Technologies).

Small RNA libraries construction, sequencing and data analysis

Small RNA libraries were prepared according to Illumina v1.5 protocol. Briefly, small RNAs of <50 nt were purified on an acrylamide gel. Universal miRNA cloning linker (New England Biolabs) instead of 3′ adapters and then 5′ Illumina adapters were single-stranded ligated with T4 truncated RNA and T4 ligase respectively. The constructs were purified on an acrylamide

gel to remove empty adapters then reverse-transcribed and PCR-amplified. The primers used for cDNA synthesis and PCR were designed to insert an index in the 3′ adapter. This index enables assignation of a specific read to the corresponding library, among the multiplexed libraries of one sequencing lane. High-throughput sequencing was performed on a Genome Analyzer HiSeq 2000 for 50 cycles plus 7 cycles to read the indexes.

After demultiplexing and adapter removal, sequence reads were sorted based on length (number of nucleotides) and only 15-44bp reads were used for analysis. The number of reads of each size was counted and normalized to the total number of reads. The obtained counts were averaged across control libraries. Reads were aligned to the mouse genome mm10 (http://hgdownload.cse.ucsc.edu/goldenPath/mm10/bigZips/).

RNA size separation for RT-qPCR

Total sperm or serum RNA was denatured in 2x Gel Loading Buffer II (Ambion) at 95°C for 15 min, loaded onto a 15% polyacrylamide gel, containing 7M urea, and run at 15W in 1xTBE buffer. DynaMarker prestain marker for small RNA (BioDynamics Laboratory) was used to determine the location of 30-40 nt fraction. The gel slices corresponding to 30-40 nt fraction were excised and ground using RNase-free plastic pestles. The RNAs were then washed out of the ground gel in 0.3M NaCl-TE pH7.5 by shaking for 3h at room temperature. The gel debris was separated by two rounds of centrifugation at 10 000 g for 5 min. RNA-containing supernatant was mixed with 1x volume of isopropanol and 10 µL glycogen, and left overnight at -20°C. RNA was precipitated by centrigugation at 12 000 g for 30 min at 4°C, followed by 2 washes with 75% ethanol, and resuspended in RNAse-free water.

RT-qPCR

Total RNA or RNA containing 30-40 nt fraction, was used for cDNA conversion using miScript reverse transcription kit (Qiagen) with HiSpec buffer (specific for non-polyadenylated RNAs). RT-qPCRs were performed in a LightCycler 480 System (Roche) using miScript SYBR Green PCR kit (Qiagen). Cycling conditions: 15 min at 95°C, 45 cycles with denaturation (15 sec at 94°C), annealing (30 sec at 55°C) and elongation (30 sec at 70°C). For normalization of Ct values in 30-40 nt fraction, the mean of tRF-CysGCA, tRF-GluTTC and tRF-GluCTC was used for both sperm and serum samples. For normalization in total fraction, ribosomal Rrnu6 was used for sperm samples, and exogenous spike-in Cel-miR-39 (Qiagen) – for serum samples. The forward primers were as follows:

tRF-ValCAC 5'-GTTTCCGTAGTGTAGTGGTTATCAC-3',

tRF-GlyGCC 5'-GCATTGGTGGTTCAGTGGTAGAATT-3',
tRF-GluCTC 5'-TCCCTGGTGGTCTAGTGGTTAGGAT-3',
tRF-ProCGG 5'-GGCTCGTTGGTCTAGGGGTATGATT-3',
tRF-CysGCA 5'-GGGGGAATAGCTCAGTGGTAGAGCA-3',
tRF-GluTTC 5'-TCCCACATGGTCTAGCGGTTAGGAT-3'. Universal reverse primer (Qiagen) was complementary to the Universal tag added to RNA during cDNA conversion (Fig. 4E). For Cel-miR-39 and Rnu6, miScript Primer Assays (Qiagen) were used.

Cell transfections

GC1 cells were grown in high-glucose DMEM medium (supplemented with 10% FBS, 0.01% gentamicin), seeded in 12-well plates and transiently transfected with fluorescent Alexa Fluor oligonucleotides (ThermoFischerScientific) or synthetic tRF mimics using Lipofectamine 2000 (Invitrogen) following the protocol recommended by the supplier. Various conditions were assayed to optimize transfection conditions (see results for details).

Results

1. Sequencing of sperm, serum and sperm of mice injected with MSUS serum

In order to study the involvement of tRNA fragments in epigenetic inheritance, we carried out sequencing of short RNAs extracted from sperm and serum of MSUS and control mice. To further establish the role of circulating factors in blood, control mice were injected with MSUS or control serum, and their sperm was collected and used for short RNA sequencing. The analysis of RNA composition revealed that the most represented small RNA species in all three datasets are fragments of tRNAs (Fig. 3A). They comprise 45.7%, 64.6% and 31.0% in sperm, serum and sperm of serum-injected mice, respectively. No full-length tRNAs are captured in this sequencing, since only the fraction containing RNAs under 50 bp was used for sequencing. Among reads mapping to tRNAs, the vast majority belong to the 5'-half of tRNAs (Fig. 3B), which are produced by cleavage of full-length tRNAs in the anticodon loop (Fig. 1). Interestingly, samples contain high number of reads mapping to 5', but not to 3'-halves (Fig. 3C), which might indicate that the 5'-halves are protected from degradation after cleavage and might have functional relevance.

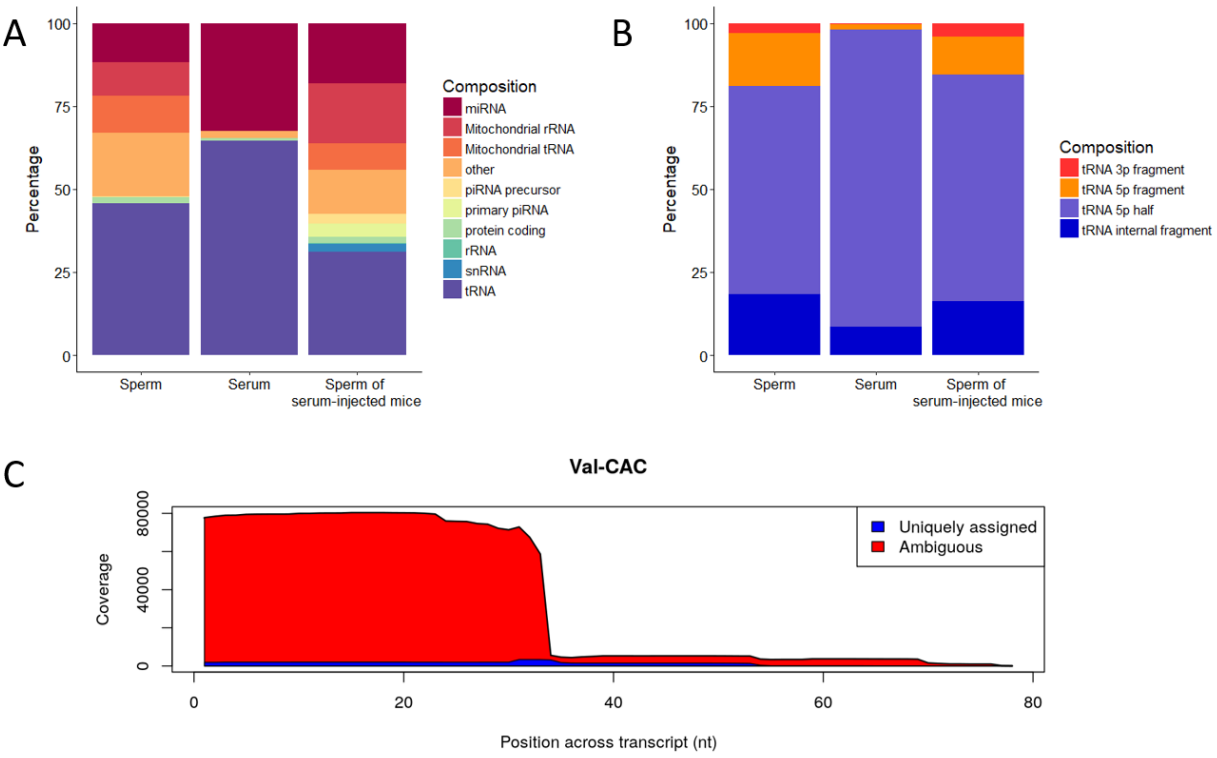

Figure 3. RNA composition of sequenced samples. A. Proportion of RNA types in short fraction of RNAs extracted from sperm (Control, n=3, MSUS, n=3), serum (Control, n=4, MSUS, n=4), and sperm of serum-injected mice (Control, n=4, MSUS, n=5), averaged across all samples. B. Proportion of fragments of tRNAs cleaved at different sites. C. Majority of ValCAC fragments are represented by 5'-halves.

Previous studies have shown the dysregulation of multiple 5'-halves in sperm of mice transmitting metabolic disorders to their offspring, among which tRF-ValCAC and tRF-GlyGCC were the most prominent (Chen et al. 2016, Sharma et al. 2016). Interestingly, these fragments tended to be altered in MSUS tissues, and the change went in the same direction (upregulated in MSUS), although the difference was only significant before applying correction for multiple comparisons (Table 1). Additionally, we detected that tRF-ProCGG was upregulated in MSUS sperm and in sperm of MSUS-serum-injected mice (significant before multiple comparisons). However, the lack of significance under stringent analysis accounting for multiple comparisons might've been caused by a small sample size. Hence, taking into account previously published results, we decided to test each of these fragments individually in MSUS tissues.

	tRF-ValCAC			tRF-ProCGG			tRF-GlyGCC		
	Log2FC	p-value	FDR	Log2FC	p-value	FDR	Log2FC	p-value	FDR
Sperm	1.189	**0.039**	0.825	1.834	**0.002**	0.280	0.409	0.480	0.968
Serum	0.303	0.707	0.995	-	-	-	0.239	0.411	0.678
Sperm of serum-injected mice	2.528	**1.76E-05**	**0.003**	1.663	**0.006**	0.137	1.307	**0.035**	0.331

Table 1. Relative levels of tRNA 5'-halves in tissues of MSUS compared to Controls. tRF-ValCAC, tRF-ProCGG and tRF-GlyGCC are upregulated in MSUS tissues. FC – fold change. FDR – False Discovery Rate.

2. Individual testing of tRNA 5'-halves

After choosing potential targets, RT-qPCRs were performed. The experimental workflow is shown in Fig 4A. Since sperm cells are very robust and resistant to treatment, they had to be lysed before RNA extraction using TCEP and RLT Plus buffer in Tissue Lyser. Standard protocols using Trizol or Trizol LS were used for RNA extractions. Due to the lack of conventional endogenous control for qPCR analysis in serum, an exogenous miR-39 from *C. Elegans* (Qiagen) was added proportional to the sample volume. Integrity of extracted RNA was assessed using Bioanalyzer. The observed RNA profile corresponds to the expected RNA size distribution in mouse sperm (Fig. 4B) and serum (Fig. 4C) samples, with the majority of RNAs having size under 200 nt.

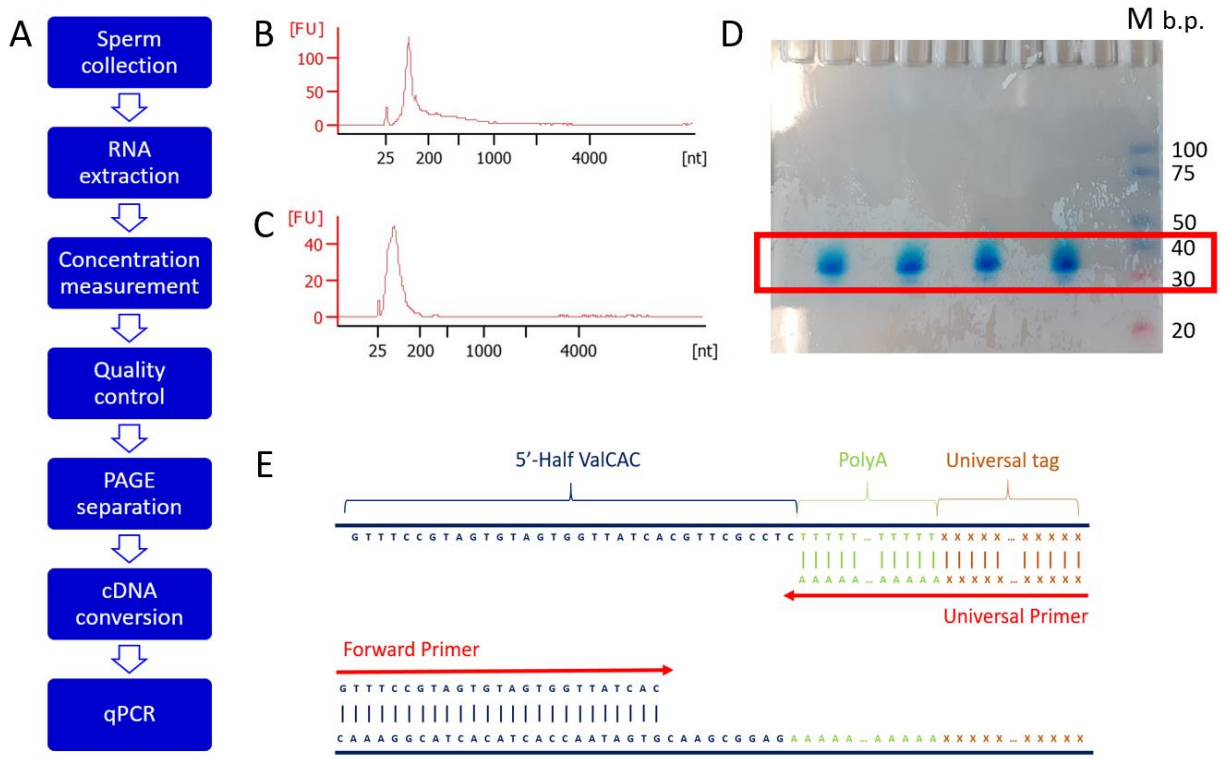

Figure 4. Processing of RNA for RT-qPCR. A. Schematic representation of experimental workflow B. Sperm RNA profile. C. Serum RNA profile. D. Photo of PAGE gel. Slices between 30 and 40 nt were excized for RT-qPCR. E. Primers used for RT-qPCR. Universal reverse primer is complementary to PolyA tail and Universal tag, added to RNAs during cDNA conversion.

100 ng of total sperm or serum RNA were separated on 15% Urea-PAGE gel, and fragments corresponding to 30-40 nt fraction were excised (Fig. 4D), which was followed by washing RNAs out of gel into TE buffer and subsequent isopropanol precipitation. Then, RNAs were reverse-transcribed using miScript II RT kit with HiFlex buffer, which is specific for non-polyadenylated RNAs. During cDNA conversion with this kit, a Poly-A tail, as well as a Universal Tag are added to the 3' end of RNAs (Fig. 4E). qPCRs were performed using MiScript SYBR Green PCR kit on cDNA obtained from 30-40 nt fraction or total RNA.

One of the methodological caveats of using qPCR for tRNA fragments is that 30-40 nt fraction doesn't contain RNA species that are conventionally used as an endogenous control for normalization. In sperm, commonly used Rnu6 has a size of 107 bp, which is above the full-length tRNAs, and has to be cut away during PAGE separation (Fig. 4D). Another generally accepted strategy is to use miRNA-101b (Gapp et al. 2014). However, apart from being out of 30-40 nt size range, it also has a significantly lower level of expression (Ct value for tRF-Val-

CAC is ~20, for miR-101b – ~33), which makes it not suitable for normalization. Here we propose to use a double normalization strategy:

1. For 30-40 nt fraction (mainly containing tRNA halves), normalize the tRF of interest to the mean of 3 other tRFs (CysGCA, GluTTC, GluCTC), found to be expressed at similar levels in MSUS and control samples.
2. For total RNA (additionally containing tRNA fragments shorter than halves, as well as full-length tRNAs), normalize to the conventionally used endogenous control, RNU6 for sperm, and spike-in Cel-miR-39 for serum.

Taking into account results obtained from both 30-40 nt fraction and total RNA would lead to making more reliable conclusions.

tRF-ValCAC wasn't altered in 30-40 nt fraction (when normalized to the mean of CysGCA, GluTTC, GluCTC) in either sperm (Fig. 5A) or serum (Fig. 5B). However, it was found to be significantly upregulated in total RNA fraction of sperm (when normalized to Rnu6) (Fig. 5C). This surprising effect might possibly be explained by the presence of modifications on full-length tRNAs, which are known to interfere with reverse transcription (Zheng et al. 2015, Cozen et al. 2015). This way, if MSUS tRNA-ValCAC has more methylation in anticodon loop, it will lead to the production of truncated cDNA, uncapabe of being amplified by qPCR using primer specific for 5'-end, and hence would skew the qPCR results. However, this hypothesis needs to be tested.

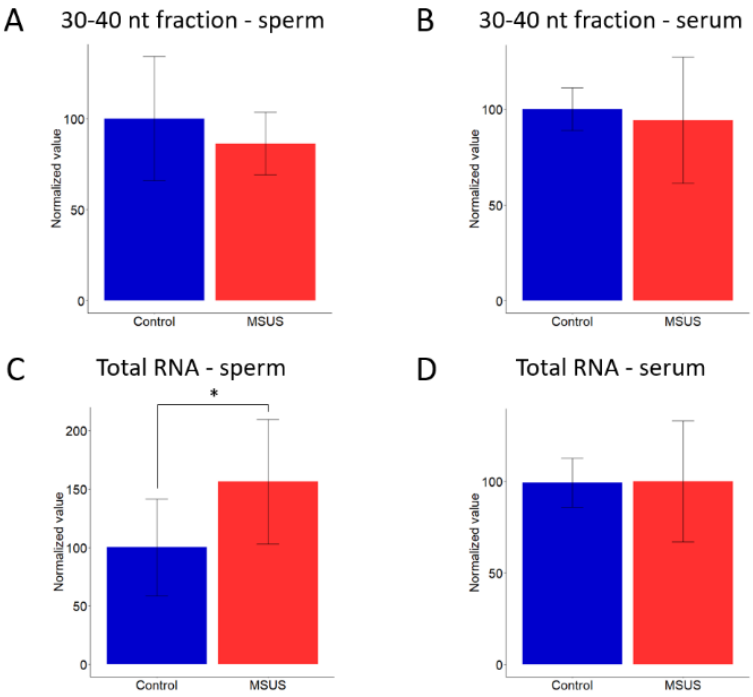

Figure 5. Expression of tRF-ValCAC. A. Sperm 30-40 nt fraction (Control, n=10, MSUS, n=10). B. Serum 30-40 nt fraction (Control, n=5, MSUS, n=5) C. Sperm total fraction (Control, n=10, MSUS, n=10). P-value = 0.03. D. Serum total fraction (Control, n=5, MSUS, n=5). Statistical analysis was performed by two-tailed Student's t-test.

Further, we tested tRF-ProCGG and tRF-GlyGCC, which were altered in sequencing and previous studies (Chen et al. 2016, Sharma et al. 2016), but didn't demonstrate any significant changes in either sperm or serum MSUS samples in RT-qPCR experiments (data not shown).

Taken together, the obtained results must be validated with a method that doesn't involve reverse transcription (Northern blot). The presence of tRNA modifications must be tested using pyrosequencing of cDNA, produced after CT-convertion of tRNAs.

3. Optimization of transfection conditions

The initial plan consisted in studying the function of tRNA fragments, potentially altered in MSUS tissues by sequencing GC1 cells transfected with synthetic mimics of the altered fragments. Given the short time of the internship, the optimizations of transfection conditions were run in parallel with experiments described in the previous section.

Since it's not possible to culture mature sperm cells, we chose to perform experiments on the mouse GC1 spg cell line, which are mouse type B spermatogonia cells, immortalized by transfection with pSV3-neo (a plasmid containing coding sequences for the SV40 large T antigen and neomycin resistance) (Hofmann et al. 1992).

First of all, optimization using fluorescent Block-it Alexa Fluor oligonucleotides (ThermoFischerScientific) was carried out in order to determine the optimal type and amount of transfecting agent, as well as the amount of oligonucleotides. Cells were seeded in 12-well plates. As a transfection agent, we compared Lipofectamine 2000 and Lipofectamine Stem Transfection Reagent in various concentrations. The later is used for difficult to transfect stem cells, without triggering their differentiation. However, in our experiment, transfecting GC1 cells with Alexa Fluor oligos using Lipofectamine Stem Transfection Agent didn't result in any transfected cells (not shown). On the contrary, Lipofectamine 2000 was efficient in all tested concentrations, with slightly better results for 1.5 µL compared to 1.0 µL/well (Fig. 6A).

In literature, final concentrations of tRF mimics from 25nM to 100nM were used. We decided to compare 50nM with 100nM, but no detectable difference between the two concentrations was observed (Fig. 6A). Hence, transfection with 50nM mimics and 1.5 µL Lipofectamine 2000 were chosen as optimal.

Further, we designed tRF-ValCAC mimic. In sperm sequencing data, the majority of reads mapping to Val-CAC 5'-half were 34 nt long (Fig. 3C). Additionally, tRNA halves are known to be cleaved from full-length tRNAs by Rnase Angiogenin (Kumar et al. 2016), resulting in atypical phosphate group at 3' end of produced fragment. Moreover, modifying tRF mimics with 2'-O-Methyl mark has been shown to be beneficial for the fragment stability (Schorn et al. 2017). Consequently, we ordered the mimic of tRF-ValCAC and its scrambled control, which were 34nt long , had 2'-O-Methyl modifications on all nucleotides and a phosphate group at 3' end.

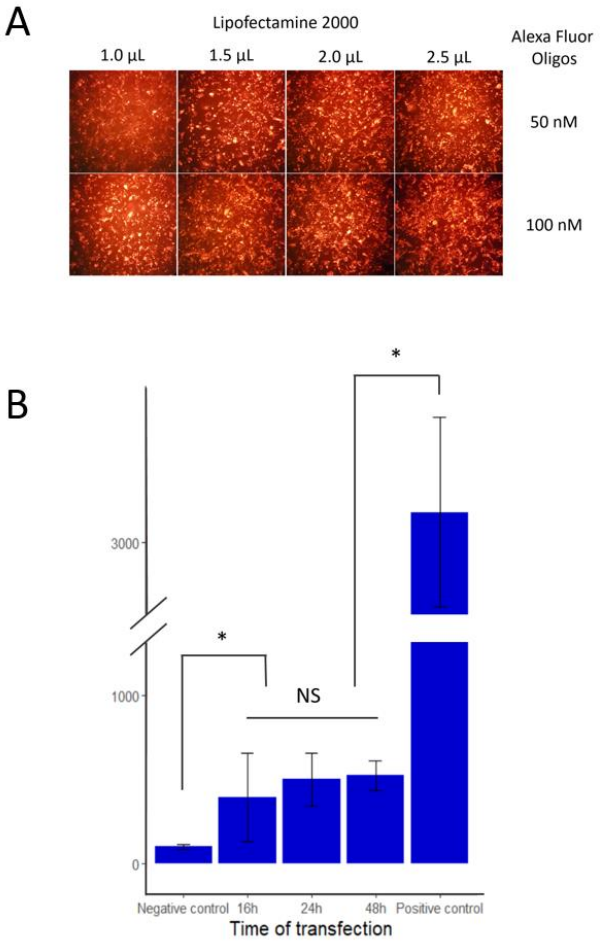

Figure 6. Optimization of transfection conditions. A. Representative images of GC1 cells transfected with fluorescent Block-it Alexa Fluor. B. Efficiency of transfection of GC1 cells with tRF-ValCAC mimic at different time points. P-value < 0.05. Statistical analysis was performed by two-tailed, one-way ANOVA test.

Finally, it was necessary to define the optimal time of transfection, which depends on the transfection efficiency and stability of tRFs in cells. We transfected GC1 cells with synthetic tRF-ValCAC mimics for 16h, 24h or 48h using conditions described above, then extracted RNAs, purified short fraction using mirVana kit (Ambion), separated 30-40nt fraction by PAGE, and measured the amount of tRF-ValCAC by RT-qPCR (Fig. 6B). As a negative control, RNA extracted from non-transfected cells was used, allowing to detect the level of endogenously expressed tRF-ValCAC. As a positive control, we used RNA extracted from non-transfected cells, with the addition of tRF-ValCAC mimic to cell lysate right before RNA extraction. Normalization to endogenous tRF-GlyGCC was used, since transfection with exogenous tRF-ValCAC is unlikely to affect its level of expression.

We observed that already after 16h the level of tRF-Val CAC is significantly increased in transfected cells compared to non-transfected control. Transfection for 24h tends to slightly increase the efficiency compared to 16h. However, transfection for 48h doesn't seem to have any beneficial effect compared to 24h, possibly due to the start of degradation of tRFs in cells. Consequently, 24h seems to be the optimal time for transfection with synthetic tRF-ValCAC mimic.

As described in previous part, there is not enough evidence to conclude that the studied tRFs are altered in the sperm of F1 MSUS mice. At this point, it is not reasonable to carry out an initially planned experiment of sequencing GC1 cells transfected with synthetic tRF-ValCAC mimic. We made a decision to put this experiment on hold until further evidence concerning possible tRF modifications is available.

Discussion

The goal of this internship project was to study the abundance and role of tRFs in adult F1 MSUS mouse tissues. First, we analyzed the short RNA sequencing data from F1 MSUS and control sperm, serum and sperm of MSUS-serum-injected mice. The analysis revealed sevral fragments, which tended to be upregulated in these tissues. Interestingly, the same fragments have been found to be implicated in epigenetic inheritance of metabolic disorders in previous studies (Chen et al. 2016, Sharma et al. 2016). Individual testing of these fragments using RT-qPCR didn't detect any changes in 30-40 nt fraction. One limitation of this study design is that both RNA sequencing and RT-qPCR involve reverse transcription, which might affect results due to the presence of RNA modifications. In order to obtain more reliable results, Northern blot could be used, which allows to detect changes in specific RNAs directly without cDNA conversion.

Surprisingly, we detected a significant upregulation of ValCAC in MSUS sperm when tested in total RNA fraction. The possible explanation of this phenomenon is that tRNAs have abundant modifications, some of which serve as hard-stop signals for reverse transcription. Consequently, if there's a change in the methylation at a particular nucleotide in full-length tRNA-ValCAC between two groups (MSUS and control), reverse transcription would result in the production of truncated cDNA species, skewing the RT-qPCR results, which we observed in our experiment. These modifications wouldn't be able to skew the results of experiments

carried out on fractions containing tRFs, but no full-length tRNAs (30-40 nt for RT-qPCR, <50 nt for sequencing).

A paper that came out in May 2018 demonstrated the importance of tRNA modifications (epimutations) in the intergenerational transmission of metabolic phenotype (Zhang et al. 2018). These modifications play a role in tRNA secondary structure (folding) and stability. They represent another layer of epigenetic information carried by sperm and potentially play a role in regulation of gene expression and developmental program of embryo after fertilization. Hence, this would be of particular interest to use mass spectrometry to test whether tRNA modifications are indeed altered in MSUS sperm. If any changes are detected with this high-throughput approach, a testing of individual methylation sites might be undertaken, using tRNA CT-conversion followed by pyrosequencing.

At this stage, the initially planned experiment of transfecting GC1 cells with synthetic tRFs was deemed to be not rational. However, if the tRNA modifications are found to be altered in MSUS, this would be important to elucidate their role. With this goal, endogenous RNAs harboring modifications might be used for transfections in order to compare the effect produced by RNAs extracted from MSUS and control sperm.

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