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Identification of antisense transcripts of the microsomal triglyceride transfer protein genes in humans and mice

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Abstract

Microsomal triglyceride transfer protein (MTTP) is essential for the assembly and secretion of apoB-containing lipoproteins. Here, we report the presence of genes on the anti-sense strands of the human *MTTP* and mouse *Mttp* genes. The gene on the anti-sense strand of the human *MTTP* gene is called *MTTP-AS1*. It consists of 5 exons and 4 introns and codes for two different transcripts MTTP-AS1-Long and MTTP-AS1-Short. Exons 3 and 5 of the *MTTP-AS1* gene are ancient and evolutionary conserved whereas exons 2 and 4 are primate specific. MTTP-AS1-Long is mainly in the liver and is in the cytoplasm of human hepatoma cells. MTTP-AS1-Short is in the testis. The MTTP-AS1-Long transcript shows complementarity with two different exons of the MTTP transcript. The gene on the opposite strand of the mouse *Mttp* gene is named as *Mttpos*. It consists of 2 exons and one intron and codes for one transcript. Partial sequence of the *Mttpos* exon 2 is homologous in several species from rodents to primates. Mttpos transcript is present in mouse liver, small intestine and testis. The Mttpos transcript shows significant complementarity with the corresponding mouse Mttp mRNA sequences. Further, we identified a conserved sequence in the human MTTP-AS1-Long and mouse Mttpos transcripts indicating for possible evolutionarily conserved regulatory function for these long noncoding RNAs. It is likely that these newly identified long noncoding RNAs interact with their complementary sequences in MTTP mRNAs and affect their stability or translation.

Keywords

Microsomal triglyceride transfer protein; Anti-sense transcript; MTTP-AS1; Mttpos

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Introduction

Microsomal triglyceride transfer protein (MTTP) is required for the assembly and secretion of apolipoprotein B (apoB)-containing lipoproteins, chylomicrons and very low-density lipoprotein, by the liver and intestine, respectively. Because of this essential role in the biogenesis of apoB-containing lipoproteins, MTTP is critically involved in systemic and tissue lipid metabolism [1]. MTTP consist of two subunits, one large MTTP subunit and a smaller protein disulfide isomerase. The large subunit is transcribed from the *MTTP* gene [2, 3]. Loss of function mutations in the *MTTP* gene in abetalipoproteinemia subjects are associated with fat-soluble vitamin deficiency, acanthocytosis, retarded growth and loss of vision [4, 5, 6]. Polymorphisms in the *MTTP* gene have been associated with increased risk of non-alcoholic fatty liver and ischemic heart disease [7, 8, 9]. Thus, understanding various physiologic factors that control the expression of MTTP gene would be useful in finding new ways to combat cardiovascular diseases associated with high plasma lipids.

It is known that MTTP expression is regulated at the transcriptional, post-transcriptional, translational and post-translational levels [10]. There are several regulatory sites in the promoter of the *MTTP* gene [10][11]. HNF4A is a nuclear receptor that plays a key role in the regulation of the *MTTP* gene [12]. Dai *et al.* found that the *MTTP* gene expression was suppressed by the binding of NR2F1 to DR1 [13]. Circadian clock genes regulate MTTP expression at the transcriptional level [14]. In addition, the previous work from our laboratory has shown that microRNA-30c interacts with the 3′ UTR of the MTTP mRNA and induces post-transcriptional degradation [15]. Further, MTTP mRNA is post-transcriptionally degraded in the intestine by IRE1β [16]. Thus, MTTP gene is highly regulated and there is a possibility of additional mechanisms regulating its expression.

Long noncoding RNA (lncRNA) is the most abundant class of ncRNAs [17]. According to the genomic position relative to a protein-coding gene, lncRNA are divided into five groups: antisense lncRNA, lincRNA, intronic lncRNA, bidirectional lncRNA and overlapping sense transcript [18]. LncRNAs control gene expression via multiple mechanisms, such as acting as sponge of miRNA, regulating post-transcriptional mRNA decay, mediating inter-chromosomal interaction [19]. Herein, we asked whether there are any antisense lncRNAs associated with the *MTTP* gene. This study provides evidence for the presence of lncRNAs transcribed from the anti-sense strands of the MTTP genes in humans and mice. We have studied their evolutionary conservation, tissue expression and subcellular localization. These anti-sense transcripts could potentially affect MTTP mRNA stability and play an important role in lipid metabolism.

Materials and Methods

Human Tissue Samples

Twelve human tissue RNAs (heart, liver, spleen, kidney, white adipose, brown adipose, skeletal muscle, brain, lung, testis, pancreas, and ovary) were purchased from Amsbio LLC (Cambridge, MA, USA) and stored at −80°C until use. After comparing data for two housekeeping genes, actin and 18S, only six tissue RNAs (liver, spleen, kidney, testis,

pancreas, and ovary) were considered appropriate for the expression analysis of human MTTP and MTTP-AS1 transcripts.

Mouse Tissue Samples

C57BL/6J mice (male, 12 weeks of age, pathogen and virus free) were purchased from the Jackson Laboratory. The mice were housed one per cage in an air-conditioned room (22 ± 2 °C, 45–55% humidity, and 12 hours light/dark cycle) prior to experiments. The mice were given general anesthesia for the collection of different tissues. Dissected tissues were quickly frozen and stored in liquid nitrogen. Animal experiments were performed in accordance with the “Guide for the care and use of laboratory animals” [20] and were approved by the Institutional Animal Care and Use Committee of the New York University Winthrop Hospital.

Cell culture

Human hepatoma Huh7 and mouse hepatoma AML12 cells (American Type Culture Collection, Manassas, USA) were cultured as described previously [21, 22, 23]. Briefly, Huh7 cells were grown in high-glucose Dulbecco’s modified Eagle’s medium (DMEM, Thermo Scientific™, USA, #11965084) supplemented with 10% fetal bovine serum (FBS, Thermo Scientific™, USA, #10091130), 1% L-glutamine and 1% penicillin-streptomycin in a 37 °C, 5% CO_2 cell culture incubator. AML12 cells were maintained in Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, Thermo Scientific™, USA, 11320–033) supplemented with 10% FBS, 1% penicillin-streptomycin, 1% insulin-transferrin-selenium and 40 ng/ml dexamethasone in a 37 °C, 5% CO_2 cell culture incubator.

Nomenclature of the antisense lncRNAs associated with human and mouse MTTP genes

The human MTTP antisense gene was named *MTTP-AS1* in accordance with the guidelines [24] and agreement with the Human Gene Nomenclature Committee. The mouse Mttp opposite strand gene was named *Mttpos* following Guidelines for Nomenclature of Genes, Genetic Markers, Alleles, and Mutations in Mouse and Rat http://www.informatics.jax.org/mgihome/nomen/gene.shtml.

Phylogenetic analyses

We obtained different sequences from NONCODE (htttp://www.noncode.org/) and Ensembl (http://asia.ensembl.org/index.html). The whole *MTTP-AS1* (AC083902.1; ENSG00000248676) and *Mttpos* genes (Gm43691; ENSMUSG00000104776) were used for Blast (UCSC, NCBI) analyses. Multiple sequence alignments of partial DNA sequences were made using CLUSTAL W in MUSCLE3.8 (https://www.ebi.ac.uk/Tools/msa/muscle/).

RNA extraction and quantitative real-time PCR

Trizol reagent (Thermo Scientific™, USA, #15596018) was used to isolate total RNA from cells and tissues. RNA concentrations were measured by Nano Drop ND-2000 instrument (Thermo Scientific™, USA, #ND-2000). Isolated RNA was reverse-transcribed by Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific™, USA, #4368813) according to the manufacturer’s instructions. qPCR was performed on the ABI

Prism 7000HT Sequence Detection System (Applied Biosystems) as described previously [25]. The specific quantitative primers are listed in Table S1. All qPCR data were normalized to 18S and actin using the 2−ΔΔCt method. Data reported are those obtained after normalization with 18S. Similar results were obtained when actin was used as a housekeeping gene. Each experiment was performed in triplicate.

RNA fluorescence *in situ* hybridization (FISH)

FAM and Cy3 labeled human MTTP-AS1-Long, MTTP-AS1-Short and negative control FISH probes (Table S2) were designed and synthesized by Genepharma (Shanghai, China). Nuclei were counter stained with 4,6-diamidino-2-phenylindole (DAPI). All the procedures were conducted according to the instructions provided in the test kits (Genepharma, China, # F03402). Briefly, Huh7 cells were grown to the logarithmic growth phase and were 90% confluent. The cells were washed twice with phosphate-buffered saline (PBS) and fixed for 15 min in a 4% solution of paraformaldehyde. Following fixation, the cells, in turn, were incubated with buffer A (15 min), buffer B (30 min), 70% ethanol (3 min), 85% ethanol (3 min), and 100% ethanol (3 min). The probes were diluted to 100 μM with sterilized DEPC water before use. The hybridization mixture included buffer C (70 μl), DEPC water (27.9 μl), and the probe specific to MTTP-AS1-Long (0.7 μl + 0.7 μl + 0.7 μl) or to MTTP-AS1-Short (2.1 μl) or to negative control (2.1 μl). Then the cells were hybridized using the above mixture at 37°C overnight. All images were analyzed using a confocal fluorescence microscope (SP8, Leica, Mannheim, Germany).

Statistical analysis

The statistical differences were analyzed using the Graphpad Prism 5 software. The data were shown as means ± SEM. Student's *t*-test, one-way ANOVA and two-way ANOVA analysis were used to compare the qPCR results. Values $P < 0.05$ were considered significantly different.

Results

Anti-sense transcripts from the human *MTTP* gene

With the advent of rapid sequencing of RNA, several long noncoding transcripts have been identified in the mammalian genomes [26, 27]. While browsing through several databases for the human *MTTP* gene, we came across two transcripts that were derived from one gene present on the anti-sense strand of the *MTTP* gene. In accordance with the guidelines proposed for naming lncRNAs [24], we named this as the *MTTP-AS1* gene after approval from the Human Gene Nomenclature Committee. The human *MTTP-AS1* gene (AC083902.1; ENSG00000248676) is located on chromosome 4:99,594,799 – 99,625,913 (31,114 base pairs (bp)) on the anti-sense strand of the *MTTP* gene. Fig 1A shows the genomic organization of the human *MTTP* gene with introns and exons. In addition, we show exons and introns in the *MTTP-AS1* gene and explain origins of two different MTTP-AS1 transcripts. The human MTTP-AS1-Long transcript (491 bp) is derived from 5 exons whereas the MTTP-AS1-Short (359 bp) is derived from 4 exons of the *MTTP-AS1* gene. There is no transcript ID for the long transcript in NCBI and Ensembl. We got this from NONCODE. The transcript ID for MTTP-AS-Short is ENST00000508578.1. The human

MTTP-AS1-short transcript does not contain exon 3. The first exon of the *MTTP-AS1* gene is 1914 bp away from the 3′-end of the MTTP gene. Fig 1B and Fig 1C show complete DNA sequence of the long and short transcripts, respectively. Sequence comparisons between *MTTP* and *MTTP-AS1* genes show that exon 5 of the *MTTP-AS1* gene is complementary to exon 8 of the *MTTP* gene (Fig 1D). In addition, exon 3 of the *MTTP-AS1* gene is complementary to exon 9 of the MTTP gene (Fig 1E). In short, there is a *MTTP-AS1* gene on the reverse strand of the *MTTP* gene that gives rise to two different transcripts. Further, two *MTTP-AS1* exons are complementary to two exons in the *MTTP* gene.

Evolutionary conservation of the human *MTTP-AS1* gene

We used the *MTTP-AS1* gene sequence (AC083902.1) for Blast to obtain homologous sequences from different species. The alignment of the different sequences showed that the gene was conserved in human, chimp, rhesus monkeys, and gorilla (Fig S1A). The homology amongst these species was ~90%. The human *MTTP* gene also showed similar homology in these primates (Fig S1B). Next, we blasted individual exons of the *MTTP-AS1* gene. Exon 1 is small (32 bp). Blast yielded several homologous sequences to draw any meaningful conclusions. Exon 2 had 52.6% homology in humans, monkeys and baboons (Fig S1C). The exon 3 was more evolutionarily conserved and homologous sequences were found in cats, horses, rodents and other species (Fig S1D). The exon 4 sequence was 46.3% homologous among humans, monkeys and baboons (Fig S1E). Exon 5 was highly conserved with ~88% homology amongst different species from rodents to primates and was identical in primates (Fig S1F). Thus, exons 3 and 5 appear to be more ancient and evolutionary conserved whereas exons 2 and 4 are more recent and primate specific.

Tissue specific expression of MTTP-AS1 long and short transcripts

MTTP-AS1-Long (Fig 2A) and MTTP-AS1-Short (Fig 2B) were abundantly expressed in the liver and testis, respectively. In these tissues, liver contained the highest levels of the MTTP mRNA (Fig 2C). These studies indicate that the long and the short transcripts show differential tissue expression and may serve different functions in the liver and testis. Next, we quantified these transcripts in human hepatoma cells and compared them to their expression in the human liver. The expression of human MTTP-AS1-Long transcript is significantly higher than MTTP-AS1-Short in the liver and hepatoma cells (Fig 2D). The human hepatoma cells express significantly lower levels of the MTTP-AS1-Long transcript compared to human liver. Further, Huh7 cells express more MTTP-AS1-Long transcript than HepG2 cells. These studies indicate that the MTTP-AS1-Long transcript is the major form expressed in human liver and in hepatoma cells.

Sub-cellular localization of MTTP-AS1 transcripts in hepatoma cells

Various long non-coding RNAs play different roles based on their sub-cellular localization [28]. Hence, we determined subcellular localization of these transcripts using RNA FISH. For this purpose, specific probes were designed. Probes for MTTP-AS1-Long were made to recognize exon 3 as this exon is absent in the MTTP-AS1-Short. Probes for MTTP-AS1-Short were designed to hybridize across exon 2 and exon 4. This probe is not expected to bind to the MTTP-AS1-Long transcript. Negative controls did not show any staining (Fig 3A). However, MTTP-AS1-Long was easily detectable in the cytoplasm of Huh7 cells (Fig

3B). In contrast, we barely detected MTTP-AS1-Short in these cells (Fig 3C). Thus, the major MTTP-AS1-Long transcript is in the cytoplasm of Huh7 cells.

Presence of a long non-coding RNA on the opposite strand of the mouse *Mttp* gene

Database searches also revealed the presence of one antisense transcript complementary to the mouse *Mttp* gene (Fig 4A) that we called *Mttpos* (Mttp other strand) according to the accepted norm. The mouse *Mttpos* gene (Gm43691; Ensemble ID: ENSMUSG00000104776; 10,801 bp) is located on chromosome 3:138,103,916–138,114,717 and contains two exons (Fig 4A). As opposed to the human *MTTP-AS1* gene that starts beyond the 3´-UTR of the human MTTP gene, the entire mouse *Mttpos* gene is within the complementary strand of the mouse *Mttp* gene. The first exon of the mouse *Mttpos* is within the intron 4 of the mouse *Mttp* gene whereas the exon 2 is complementary to exon 11 and part of the intron 10 of the mouse *Mttp* gene. The first exon is short and codes for 60 bp whereas the second exon is longer and codes for 637 bp. The mouse Mttpos transcript (ENSMUST00000196625.1) is of 697 bp (Fig 4B). A partial sequence of the *Mttpos* exon 2 and mouse *Mttp* exon 11 are complementary to each other (Fig 4C). Homology studies revealed that part of the exon 2 sequence is homologous (>80% homology) in several species from rodents to primates (Fig S2). These studies point to the presence of a *Mttpos* gene on the opposite strand of the mouse *Mttp* gene that codes for one lncRNA.

Homology between human MTTP-AS1-long and mouse Mttpos

Overall, we did not find sufficient homology between these two transcripts. However, part of the mouse *Mttpos* exon 2 showed significant homology (83% homology) with human MTTP-AS1-long exon 3 (Fig 4D). These studies suggest that this sequence might have a common function across species.

Tissue-specific expression of Mttpos transcript

RT-qPCR analysis showed that Mttpos transcript is highly expressed in the liver, small intestine and testis (Fig 4E). Further, Mttpos transcript levels are quantifiable in kidney, adipose tissue, muscle and brain. In contrast, the Mttp mRNA was abundant mainly in the liver and small intestine (Fig 4F). Other tissues such as kidney, adipose tissue, heart, pancreas, brain and testis had measurable Mttp mRNA levels (Fig 4F). The Mttpos transcript was detectable in mouse hepatoma AML12 cells (Fig 4G). The expression of Mttpos in mouse liver was 20 times higher than that is in AML12 cells. Thus, Mttpos is expressed in more tissues than the MTTP-AS1-Long.

Discussion

We report the presence of *MTTP-AS1* and *Mttpos* genes in the anti-sense strands of human and mouse genes that code for MTTP. The *MTTP-AS1* gene consists of 5 exons and 4 introns, whereas the *Mttpos* gene consist of 2 exons and one intron. The *MTTP-AS1* gene gives rise to two independent transcripts most likely arising from differential splicing. By contrast, only one transcript from the *Mttpos* gene was recognized. The *MTTP-AS1* gene is highly conserved in primates. However, two exons have sequence homologies in other

mammals. The *Mttpos* gene also has homologous sequences in other mammals beyond rodents. Thus, some exons of these lncRNA genes might have evolved earlier and later became rodent and primate specific.

The 5′-end of the first exon of the *MTTP-AS1* gene is ~ 2 kilobases away from the 3′-end of the *MTTP* gene suggesting that the transcription start site, the promoter and other *cis*-regulatory elements are further downstream of the *MTTP* gene. Therefore, it is likely that the *MTTP-AS1* gene is regulated independently of the MTTP gene. In this regard, identification of the 5′-end of the MTTP-AS1 transcripts and the promoter of the *MTTP-AS1* gene may provide novel information about its regulation.

The *Mttpos* gene contains 2 exons that are in the middle of the anti-sense strand of the mouse *Mttp* gene. Therefore, it is likely that the expression of the *Mttpos* and the mouse *Mttp* gene expression may be co-regulated. However, the *Mttpos* gene could be regulated independently of the mouse *Mttp* gene due to the presence of its own promoter and regulatory elements in the complementary strand of the mouse Mttp gene.

The MTTP-AS1-Long transcript is in the cytosol of human hepatoma cells (Fig 4). Cytosolic lncRNAs serve as competing endogenous RNAs to compete for miRNA binding and affect mRNA stability [29, 30]. Preliminary analysis using algorithms that predict translation of lncRNAs suggested that the identified transcripts do not code for small peptides. Similarly, these lncRNAs do not appear to have complementary sequences to known microRNAs to act as sponges. The exons 3 and 5 of the *MTTP-AS1* gene are complementary to the human *MTTP* exons 5 and 8. Similarly, Mttpos exon 2 is complementary to mouse *Mttp* exon 11. These significant complementarities suggest that MTTP mRNAs may pair with these lncRNAs and affect their stability. Conversely, these lncRNAs may interact with MTTP mRNAs to affect their stability and/or translation. In addition, these lncRNAs might interact with other mRNAs to affect metabolism.

We found that MTTP-AS1-Long and Mttpos are highly expressed in the liver. Based on RT-qPCR analysis, we found MTTP-AS1-Short and Mttpos are also in the testis. Whether tissue-specific expression is due to differential tissue-specific transcriptional control or due to differential post-transcriptional degradation remains to be determined. MTTP is not expressed to a significant extent in the testis and its role in this tissue is unknown. Thus, the short transcript may be involved in other functions beyond MTTP mRNA stability or translation.

Although the expression, size and sequence of the MTTP-AS1 long and Mttpos transcripts appear very different, we identified a sequence that is highly conserved in these two transcripts (Fig 4D). This sequence in the MTTP-AS1-Long is complementary to exon 9 of the human MTTP gene (Fig 1E) and the Mttpos sequence is complementary to the mouse *Mttp* gene exon 11 (Fig 4C). The conservation of these sequences in both the strands of the human and mouse genes suggest that this sequence might be functionally important and carry out similar function across different species.

In short, these studies provide the first evidence for the presence of genes on the antisense strands of the MTTP genes in humans and mice. These genes give rise to different

transcripts in the liver and testis. They are likely to play novel roles in the regulation of MTTP and lipid metabolism. Report of these novel lncRNAs may encourage future research focusing on discovering the function of these lncRNAs and modulation of their function for therapeutic use.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations used

ApoB	apolipoprotein B
Bp	base pairs
LncRNAs	Long noncoding RNAs
(MTTP)	Microsomal triglyceride transfer protein

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Highlights

- The anti-sense strand of the human *MTTP* gene transcribes two noncoding RNAs
- The MTTP-AS1-Long noncoding RNA is in the cytosol
- The anti-sense strand of the mouse Mttp gene contains one noncoding RNA

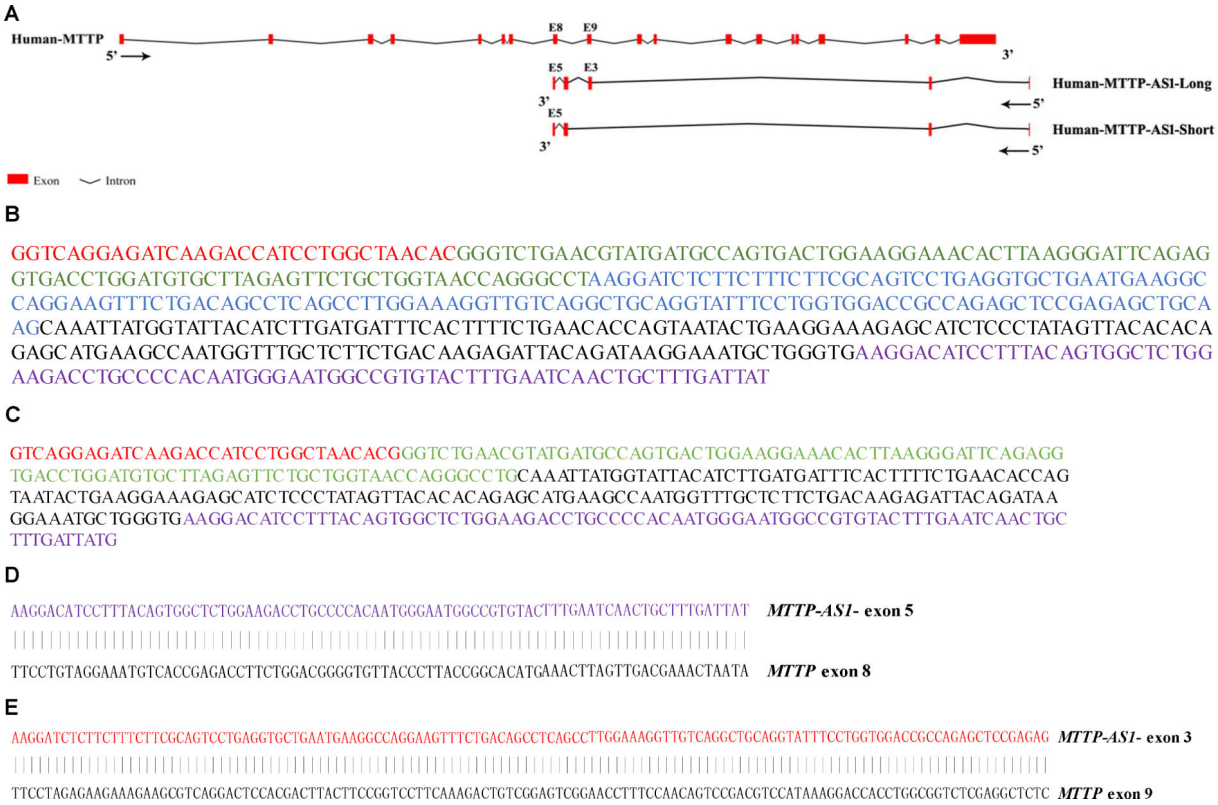

Fig 1: Characterization of the human *MTTP-AS1* gene and its transcripts and their comparison with the human *MTTP* gene.

(A) Genomic organization of the human *MTTP* and *MTTP-AS1* genes. (**Top**) A schematic diagram of the human *MTTP* gene. (**Middle and bottom**) In different databases there is evidence for the presence of two different size transcripts derived from the *MTTP-AS1* gene. The middle and bottom drawings show how two different transcripts could be derived from the *MTTP-AS1* gene using different exons most likely due to differential exon splicing. Arrows show the directions of gene transcription. Exons are depicted as red vertical bars. Lines represent introns. The human *MTTP* gene contains 19 exons and 18 introns. The human *MTTP-AS1* gene contains 5 exons and 4 introns. The MTTP-AS1-Long transcript is derived from 5 exons and MTTP-AS1-Short transcript contains 4 exons.

(B-C) DNA sequences of the long (B) and short (C) MTTP-AS1 transcripts. Different colors represent different exons in the two transcripts. MTTP-AS1-Long has an extra exon 3 (blue). These transcripts were from NONCODE AC083902.1 (Ensembl: ENSG00000248676).

(D-E) Complementary sequences between MTTP and MTTP-AS1 transcripts: *MTTP* exon 8 (82 bp) is complementary with *MTTP-AS1* exon 5 (D) and *MTTP* exon 9 (126 bp) is complementary with *MTTP-AS1* exon 3 (E).

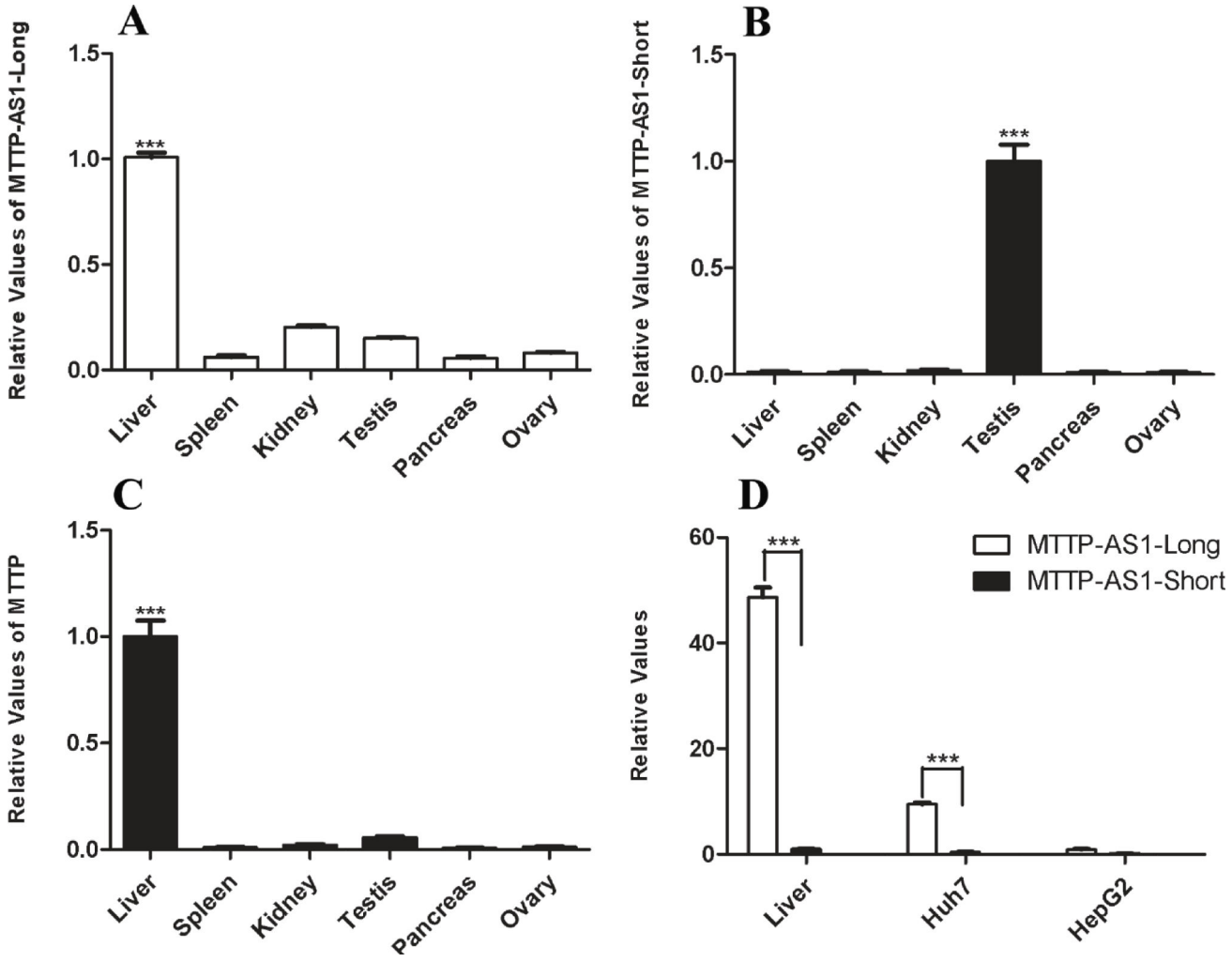

Fig 2: Expression of the MTTP-AS1-Long and MTTP-AS1-Short transcripts in human tissues and hepatoma cells:

(A-C) Relative expression of the MTTP-AS1-Long **(A)**, MTTP-AS1-Short **(B)** and MTTP **(C)** transcripts in six human tissues determined by RT-qPCR.

(D) Relative expression of the MTTP-AS1-Long and MTTP-AS1-Short in human liver and human hepatoma Huh7 and HepG2 cells. *** $P<0.001$.

One-way ANOVA analysis compared panels A, B, and C; two-way ANOVA analysis followed by Bonferroni post-tests was used for panel D.

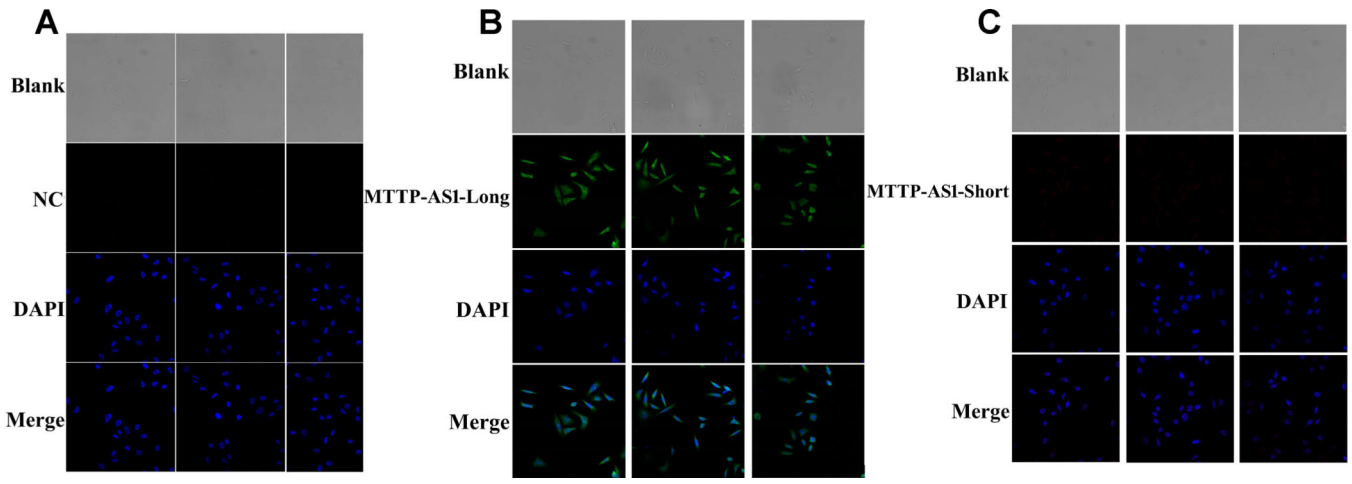

Fig 3: Subcellular localization of MTTP-AS1-Long transcript in human hepatoma Huh7 cells using RNA-FISH.

(A) Negative Control was used for FISH

(B) MTTP-AS1-Long specific probes were used for FISH.

(C) MTTP-AS1-Short specific probes were used for FISH. Green, MTTP-AS1-Long; Red, MTTP-AS1-Short; Blue, DAPI; Merge, the merge of MTTP-AS1-Long and DAPI or MTTP-AS1-Short and DAPI.

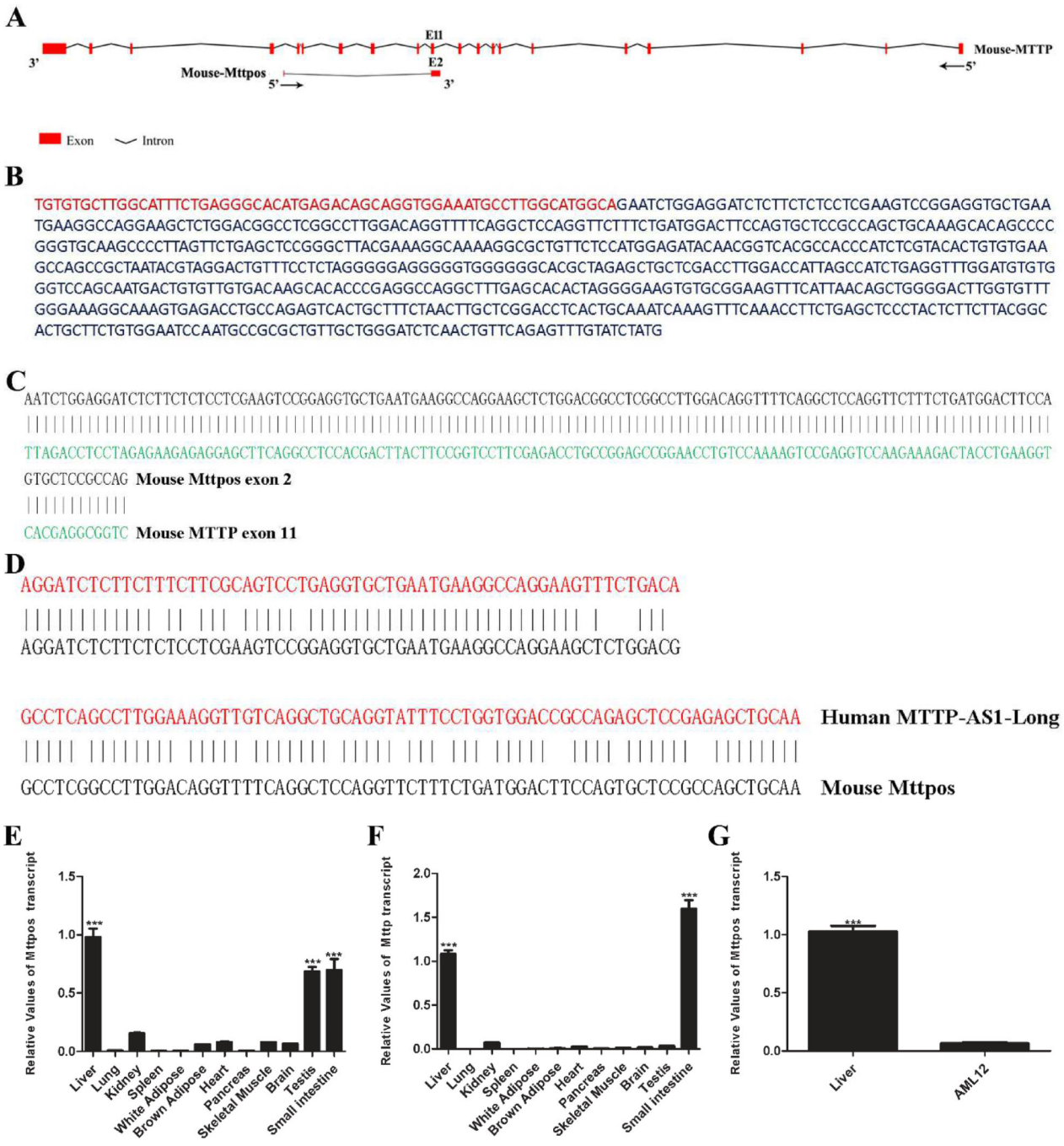

Fig 4: Presence of a mouse *Mttpos* gene in the opposite strand of the mouse *Mttp* gene:

(A) Genomic organization of the mouse *Mttp* and *Mttpos* genes:

(B) DNA sequence of the Mttpos transcript. Red and blue sequences are from exon 1 and exon 2, respectively.

(C) Complementarity between *Mttpos* exon 2 and *Mttp* exon 11.

(D) Homology between human MTTP-AS1-Long and mouse Mttpos transcripts.

(E-G) Expression of mouse Mttpos and Mttp transcripts in different tissues and in mouse hepatoma AML12 cells: Different tissues were collected from three different C57Bl6 mice

(male, 3-month-old, *ad libitum* chow fed). RNA was extracted and used to measure Mttpos **(E)** and Mttp **(F)** transcripts via RT-qPCR. RNA from mouse liver and AML12 cell line was used to quantify Mttpos transcript **(G)**. 18S was used as control. Expression profiles were similar when actin was used as housekeeping gene. One-way ANOVA analysis was used to compare panels A and B; panel C was analyzed using the Student's *t* -test analysis.