

Presence and Characterization of Cell-Free Seminal RNA in Healthy Individuals: Implications for Noninvasive Disease Diagnosis and Gene Expression Studies of the Male Reproductive System

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BACKGROUND: We recently detected cell-free seminal RNA (cfsRNA) and set out to study its concentration, integrity, stability in healthy individuals, and mechanisms for its protection from ribonucleases.

METHODS: We quantified cfsRNA by reverse-transcription quantitative real-time PCR (RT-qPCR) targeting of the 5' region of the *ACTB* (actin, beta) transcript. cfsRNA integrity was analyzed by microcapillary electrophoresis and by amplification of full-length *ACTB* and *DDX4* [DEAD (Asp-Glu-Ala-Asp) box polypeptide 4] transcripts, including measurement of the relative amounts of different regions of *ACTB* and *DDX4* transcripts. Stability of cfsRNA was measured by time-course analysis of different regions of *ACTB* and *DDX4* transcripts. To investigate whether cfsRNA was protected in complexed forms, we processed seminal plasma in 2 ways: filtration through pores of different sizes and Triton X-100 treatment before RNA recovery.

RESULTS: cfsRNA concentrations varied from 0.87–3.64 mg/L [mean (SD), 1.75 mg/L (0.92 mg/L)]. Most cfsRNA was present in partially degraded forms, with smaller amounts of middle and 3' amplicons compared with 5' amplicons. Although the 3' region of the *DDX4* transcript was degraded completely by 90 min, the 5' regions of *ACTB* and *DDX4* transcripts were stable up to 24 h. Filtration through 0.22- μ m pores reduced *ACTB* and *DDX4* mRNA concentrations by 72% and 61%, respectively. Nearly all seminal *ACTB* and *DDX4* mRNA disappeared after Triton X-100 treatment.

CONCLUSIONS: Although cfsRNA was partially degraded, it represented diverse transcript species and was abundant, fairly stable, and associated with particles in healthy individuals. cfsRNA may represent a po-

tential noninvasive biomarker of the male reproductive system and of germline epigenetics.

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Diseases and disorders of the male reproductive system are common and diverse, but their diagnosis remains problematic. For instance, 75% of cases of male infertility are diagnosed as idiopathic (1), and some unknown genetic defects may be transmitted to offspring during assisted-reproduction treatment. The rate of misdiagnosis of seminal vesicle diseases remains high, and earlier diagnosis of prostate cancer and germ cell tumors remains a clinical challenge. These diagnostic difficulties may be attributed in part to the small size of male reproductive organs and their complex series of ducts, which make detailed examinations challenging. Additionally, prostate biopsy may produce complications, such as urethral injury and sexual dysfunction, whereas testicular biopsy may delay semen production or cause androgen deficiency. However, a minimally invasive technique, fine-needle aspiration, often provides limited samples for cytologic and histologic diagnosis and for gene expression studies evaluating etiology and epigenetics. Therefore, approaches that are more reliable and noninvasive are needed for the diagnosis of disease and for carrying out gene expression analysis of the human male reproductive system.

It is reasonable to search for noninvasive biomarkers in human semen, a viscous mixture of spermatozoa and fluid from seminiferous tubules, the epididymis, and accessory glands (seminal vesicles, the prostate, and bulbourethral glands). Compared with the peripheral blood, semen likely contains higher concentrations of chemicals and substances originating from the male reproductive organs, which can be regarded as potential biomarkers

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for disease diagnosis and evaluating function. For example, metabolites such as glycerophosphorylcholine, choline, citrate, and lactate can be used to differentiate spermatogenic failure from obstructive azoospermia and to detect prostate cancer (2, 3). The concentrations of seminal fructose and protein C inhibitor have been used as markers of seminal vesicular function; acid phosphatase, citric acid, and zinc have been used as prostate markers; and choline glycerophosphatide has been used as an epididymis marker (4). Unfortunately, few of these chemical markers have proved to be highly specific and sensitive, diagnostically. Recently, spermatozoal RNA has been used as a noninvasive means to identify germline epimutations (5), but it was found to be unsuitable in individuals with azoospermia (complete absence of sperm). Although immature germ cells and somatic cells also can be shed into semen, the rarity of these cells make them inaccurate predictors of spermatogenic status or tumor formation.

We recently detected cell-free RNA (cfRNA)⁴ in human ejaculates in an initial exploratory investigation (unpublished data). Much interest has been focused in recent years on the applications of cell-free nucleic acids in prenatal diagnosis (6, 7), oncology (8, 9), and the etiopathology of other pathologic disorders (10). Considering previous results regarding cfRNA in human plasma (11–13), saliva (14), and bronchial lavage fluid (15), and the rapid development of molecular biology techniques, we believe cell-free seminal RNA (cfsRNA) may represent a new source of noninvasive biomarkers for the male reproductive system and germline epigenetics. Moreover, it is tempting to think that cfsRNA could serve as a forensic marker for semen stains, especially in the presence of azoospermia; however, knowledge of the fundamental biology of cfsRNA is still essential before applications can be developed.

In this study, we sought to investigate whether the characteristics of cfsRNA can meet the basic requirements of molecular diagnostic assays. For this purpose, we primarily report the isolation of cfsRNA and the characterization of its concentration, integrity, and stability in healthy individuals, and we investigate the possible mechanisms for its protection from seminal ribonuclease activity.

Materials and Methods

PARTICIPANTS AND PROCESSING OF SEMINAL SAMPLES

The study was approved by the institutional review board at our facility, and informed consent was ob-

tained from all of the healthy participants included in this study. The mean age of the participants was 31 years (range, 26–38 years). The healthy participants were selected from infertile couples who underwent treatment with assisted-reproduction technology for diagnosed female factor infertility and in which the man was normozoospermic according to WHO guidelines (16) and had no hyperpyrexia, malignancy, autoimmune disorders, sexually transmitted diseases, inflammation of the reproductive organs, or family history of genetic diseases. Individuals who worked in high-temperature environments or had occupational exposure to toxic chemicals or radiation were also excluded.

Semen samples were obtained by masturbation after 3–5 days of sexual abstinence and were allowed to liquefy within 30 min at 37 °C. The liquefied semen was then centrifuged at 16 000 g for 10 min at 4 °C, and the supernatant was carefully collected for subsequent RNA isolation.

RNA ISOLATION

The concentration of cfRNA is affected by centrifugal force (17); consequently, there may be a bias with respect to cfRNA quantity in aliquots obtained by aspirating the supernatant from the top to the bottom of the centrifuge tube. To avoid such bias, we routinely mixed semen supernatants before cfsRNA isolation.

cfRNA was isolated by a modified protocol, as previously described (18). In brief, 0.4 mL of seminal plasma was mixed with 0.5 mL TRIzol LS reagent (Invitrogen) and 0.15 mL chloroform. The mixture was centrifuged at 11 900g for 15 min at 4 °C, and the aqueous layer was transferred to a new tube. One volume of 700 mL/L ethanol was added to 1 volume of the aqueous layer. The mixture was then applied to an RNeasy minicolumn (RNeasy Mini Kit; Qiagen) and processed according to the manufacturer's recommendations. Total RNA was eluted with 30 μ L of RNase-free water and stored at –80 °C until further processing. To remove any contaminating DNA, we carried out DNase treatment [Recombinant DNase I (RNase-free); Takara Bio] according to the manufacturer's instructions.

RNA SIZE DISTRIBUTION AND MICROARRAY

RNA isolated from 12 healthy individuals was mixed, and the distribution of RNA sizes was measured by capillary electrophoresis on an Agilent Technologies 2100 Bioanalyzer, followed by microarray analysis with the Affymetrix Human Gene 1.0 ST Array.

REVERSE-TRANSCRIPTION PCR (RT-PCR) AND QUANTITATIVE REAL-TIME PCR (RT-qPCR)

cDNA was synthesized with the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to

⁴ Nonstandard abbreviations: cfRNA, cell-free RNA; cfsRNA, cell-free seminal RNA; RT-PCR, reverse-transcription PCR; RT-qPCR, reverse-transcription quantitative real-time PCR; SNK, Student–Newman–Keuls.

the manufacturer's instructions. In brief, we used 7.5 μL of purified RNA in a reaction volume of 20 μL . Random hexamers were used during the reverse-transcription step. As the negative control, we also subjected samples to this procedure with the exception that we replaced RevertAid™ M-MuLV Reverse Transcriptase with RNase-free water.

To characterize the concentration, integrity, and stability of cfsRNA, we selected the transcripts of 2 genes: *ACTB* (actin, beta)⁵ and *DDX4* [DEAD (Asp-Glu-Ala-Asp) box polypeptide 4], a germ cell-specific gene. Primers were designed to amplify the full-length transcript, as well as the 5', middle, and 3' regions of each transcript. All primers were designed to span the intron. Primer sequence information is given in Table 1 in the Data Supplement that accompanies the online version of this article at <http://www.clinchem.org/content/vol55/issue11>.

Nonquantitative RT-PCR was performed to validate the presence of both intact and partial cfsRNAs, whereas RT-qPCR was performed to measure the concentration, integrity, and stability of cfsRNA. We used 2 μL of the 20- μL reverse-transcription products as the initial templates for all PCR reactions in our study. Nonquantitative RT-PCR was carried out in the Mastercycler gradient thermal cycler (Eppendorf 5531), and RT-qPCR was performed as described previously (19) with an Mx3000P thermocycler (Stratagene) and with SYBR Green I used for detecting the fluorescence of amplified products. For the RT-qPCR, we performed triplicate measurements per sample with the following 4-step experimental protocol (20): 95 °C for 10 min (initial denaturation) and 35 cycles of 25 s at 95 °C (denaturation), 30 s at the annealing temperature (see Table 1 in the online Data Supplement), 30 s at 72 °C (elongation), and 8 s at 81 °C (fluorescence measurement). A melting curve was generated at the end of every run to ensure product uniformity. We used full-length PCR amplicons of human *ACTB* and *DDX4* transcripts as calibrators to quantify their different regions. The PCR product of rat *Actb* [actin, beta (*Rattus norvegicus*)] mRNA was cloned into a TA vector, which was used as the calibrator for quantification of rat *Actb* mRNA added to seminal plasma. Calibration curves were constructed by use of serial dilutions. For cfsRNA quantification, calibration curves were constructed by use of serial dilutions of cDNA reverse-transcribed from a cfsRNA preparation, the concentration of which was high and easily measured with an ultraviolet spectrophotometer (Biometra).

QUANTIFICATION OF cfsRNA

The concentration of cfsRNA from 10 healthy individuals was measured by RT-qPCR targeting of the 5' region of the *ACTB* transcript.

MEASUREMENT OF DIFFERENT PARTS OF *ACTB* AND *DDX4* mRNAs

In addition to measuring the size distribution of cfsRNA and detecting full-length transcripts, we further investigated the integrity of cfsRNA by quantifying the amounts of 5', middle, and 3' regions of *ACTB* and *DDX4* transcripts via RT-qPCR analysis of 9 semen samples. The amounts of RNA for other regions of the transcript were expressed relative to the amount for the 5' region. A discrepancy in the measured quantities or detection rates of the 3' regions would suggest the presence of incomplete mRNA fragments.

ANALYSIS OF cfsRNA STABILITY

We incubated 5 seminal samples at room temperature for 0, 20, 45, and 90 min and for 24 h. We extracted RNA from 0.4 mL seminal plasma at each time point and then quantified each region of the *ACTB* and *DDX4* transcripts by RT-qPCR. As a control, we evaluated the effect of seminal ribonucleases in a lability experiment that involved the addition of free RNA. We added 5 μg of total RNA purified from rat testicular tissue to 0.4 mL of seminal plasma and incubated the plasma for 0 s and 15 s at room temperature. TRIzol LS reagent was added immediately after the incubation to stop any ribonuclease activity.

To observe the effect of Triton X-100 on the stability of cfsRNA, we also added Triton X-100 (Sigma-Aldrich) to 5 other seminal samples and quantified the 5' region of *ACTB* and *DDX4* mRNAs at different time points. In brief, we added Triton X-100 to the samples to a final concentration of 10 mL/L and incubated them at room temperature for up to 20 min. In the control group, Triton X-100 was added after protecting RNA with the lysis reagent at each time point. This method has previously been described (21).

FILTRATION OF SEMEN

Seminal plasma from another group of 5 healthy individuals was divided into 4 aliquots. Three aliquots were individually passed through 3 Millex-GV filters (Millipore) of different pore sizes (5, 0.45, and 0.22 μm). The remaining aliquot was not filtered. We then extracted the RNA from 400 μL of the filtered and unfiltered aliquots of seminal plasma and quantified the 5' region of the *ACTB* and *DDX4* mRNAs.

STATISTICAL ANALYSIS

Statistical analyses were performed with SigmaStat software (version 2.03; SPSS). The Friedman test was

⁵ Genes: *ACTB*, actin, beta; *DDX4*, DEAD (Asp-Glu-Ala-Asp) box polypeptide 4; *Actb*, actin, beta (*Rattus norvegicus*).

Table 1. Quantification of cfsRNA in semen of normozoospermic men (n = 10).

Sample	Age, years	Abstinence, days	Liquefy time, min ^a	cfsRNA concentration, mg/L
1	31	4	30	1.20
2	34	5	20	1.17
3	32	4	20	1.19
4	24	5	15	0.87
5	30	4	15	1.64
6	26	4	30	1.95
7	34	5	20	1.43
8	30	3	30	3.64
9	37	5	25	3.12
10	29	4	30	1.26
Mean (SD)	30.70 (3.86)	4.30 (0.67)	23.50 (6.26)	1.75 (0.92)

^a The liquefy time includes the duration of sample storage at 37 °C before cryopreservation.

used to analyze experiments involving integrity, stability, and filtration. When this test gave statistically significant results, it was followed with the Student–Newman–Keuls (SNK) test. For the Triton X-100 experiments, we used a 2-way ANOVA with the SNK test. A *P* value <0.05 was considered statistically significant.

Results

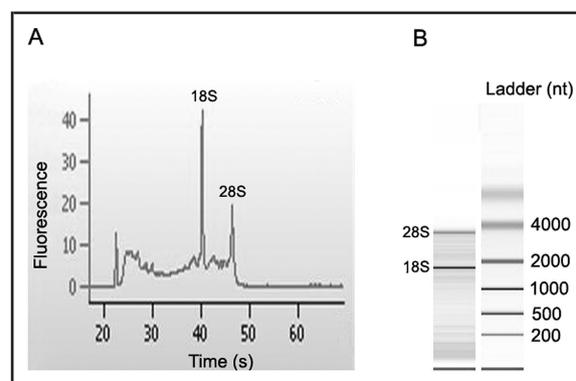
THE QUANTITY AND SIZE DISTRIBUTION OF cfsRNA

cfsRNA concentrations in seminal plasma from the 10 healthy individuals varied from 0.87 mg/L to 3.64 mg/L with a mean (SD) of 1.75 mg/L (0.92 mg/L). Information about these healthy donors is summarized in Table 1. Microarray analysis detected 20 106 different transcripts in the mixed cfsRNA of 12 healthy participants. The chromatogram of the cfsRNA size distribution showed 3 distinct peak signals, which correspond to the 3 predominant RNA classes (28S rRNA, 18S rRNA, and “small RNAs,” such as 5S rRNA, tRNAs, and microRNAs), as well as molecules with a broad distribution of molecular weights and relatively weak signals (Fig. 1). Also evident, however, were abundant bands with sizes consistent with 28S degradation products, which have been reported previously for RNA from apoptotic cells (22). These results indicated that cfsRNA was abundant and that apoptosis might be a major source of its release.

AMPLIFICATION AND INTEGRITY ANALYSIS OF cfsRNA

Short PCR amplicons (<240 bp; see Table 1 in the online Data Supplement) targeting different regions of the *ACTB* and *DDX4* transcripts were detected in

cfsRNA samples from all 9 volunteers; however, we detected long PCR amplicons of *ACTB* mRNA (1499 bp) in 2 of the 9 volunteers but detected no long amplicons

**Fig. 1. Size distribution of cfsRNA by capillary electrophoresis.**

(A), Chromatogram of the cfsRNA size distribution with the Agilent 2100 Bioanalyzer system. The chromatogram showed 3 distinct peaks, which correspond to 28S rRNA, 18S rRNA, and small RNAs, as well as molecules with a broad distribution of molecular weights and relatively weak signals. The quality of the isolated RNA was checked (A_{260}/A_{280} ratio, 2.07; RNA integrity number, 6.4; 28S/18S ratio, 0.6). (B), Electropherogram of total cfsRNA obtained with the Bioanalyzer 2100 instruments. The size distribution of cfsRNA fragments was approximately 25–4000 nucleotides (nt). Although 2 bands representing 18S and 28S rRNA were clearly observed, a series of degradation products was also evident.

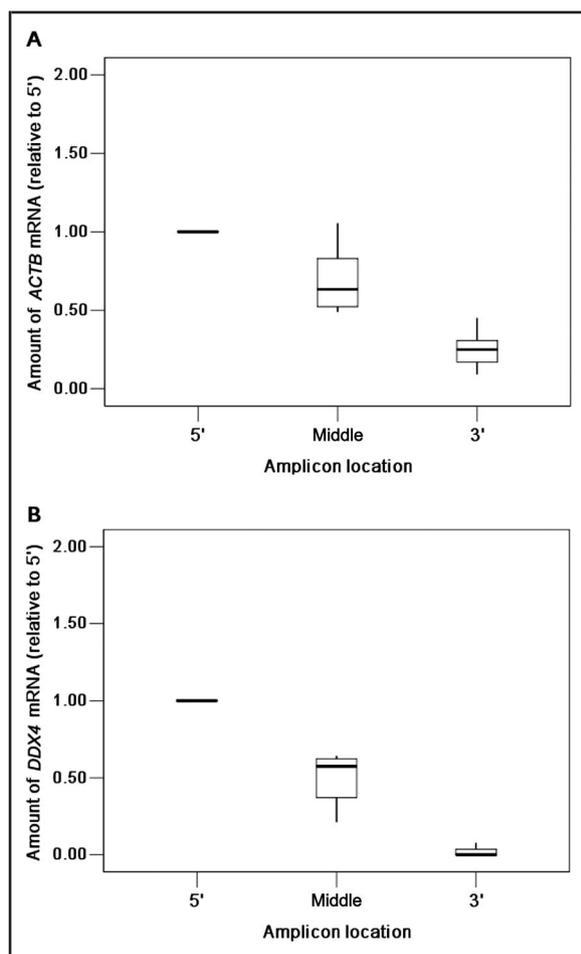


Fig. 2. Relative amounts of different regions of *ACTB*- and *DDX4*-transcripts in cfsRNA.

(A), Amounts of middle and 3' regions of *ACTB* mRNA relative to the amount of the 5' region, as determined by RT-qPCR analysis ($n = 9$). (B), Amounts of middle and 3' regions of *DDX4* mRNA relative to the amount of the 5' region, as determined by RT-qPCR analysis ($n = 9$). The horizontal lines within the boxes denote the median, the boxes represent the interval between the 25th and 75th percentiles, and the whiskers represent the interval between the 2.5th and 97.5th percentiles.

for *DDX4* mRNA (1909 bp). Our data suggest that some transcripts remained intact, although most cfsRNAs existed as fragments of larger molecules.

We further evaluated the integrity of cfsRNA by quantifying the amounts of the 3 different regions of *ACTB* and *DDX4* transcripts. Such an analysis has previously been done in studies of cRNA integrity in serum and saliva (21, 23, 24). As is shown in Fig. 2A, compared with the 5' region of *ACTB* mRNA, we detected 71.0% of the middle region ($P < 0.05$, SNK test),

and 24.2% of the 3' region amplicon ($P < 0.001$, SNK test). Analysis of *DDX4* mRNA revealed much less of the middle and 3' amplicons (middle-to-5' ratio = 0.484; 3'-to-5' ratio = 0.02; $P < 0.001$, SNK test), compared with the 5' amplicon (Fig. 2B). These data suggest preferential degradation of the 3' region of cfsRNAs; consequently, further research or clinical use of cfsRNAs will likely focus on the 5' region.

EVALUATION OF cfsRNA STABILITY

Given that cfsRNA exists mainly in partially degraded forms and that human semen is known to contain various ribonucleases (25), we tested the stability of cfsRNA by a time-course analysis of different regions of the *ACTB* and *DDX4* transcripts. We incubated seminal plasma at room temperature for different times (0, 20, 45, and 90 min, and 24 h). As shown in Fig. 3, A–C, we observed no significant degradation of any region of *ACTB* mRNA at any of these serial time points ($P > 0.05$, Friedman test). For *DDX4* transcripts, although the 5' region was still stable up to 24 h, the amount of the middle region was slightly reduced after 90 min and was reduced to 25.8% after a 24-h incubation (Fig. 3E; $P < 0.01$, SNK test). In contrast, the amount of the 3' region was reduced by 72.6% at 20 min and was completely degraded after a 90-min incubation (Fig. 3F; $P < 0.001$, SNK test), compared with the amount present at time 0. In the control experiment, exogenous rat RNA added to seminal plasma was immediately degraded: 6.6×10^{12} copies of rat *Actb* mRNA were detected per milliliter at the beginning of the experiment, but *Actb* mRNA was totally absent after only a 15-s incubation, confirming the high ribonuclease activity in semen.

RNA-MACROMOLECULE INTERACTION IS IMPORTANT FOR cfsRNA STABILITY

A recent study showed that 95% of serum RNA does not pass through a 0.22- μm filter (26), indicating that serum RNA exists in some kind of macromolecular complex. After observing the stability of cfsRNA, we hypothesized that it was protected from degradation by inclusion in some macromolecule-associated forms. We measured the amount of the 5' region of *ACTB* mRNA after filtering samples through filters with 0.22- μm and 0.45- μm pore sizes. We first filtered samples through 5- μm filters to confirm that the mRNA did not originate from residual cells present in the seminal supernatant. Furthermore, we filtered purified cfsRNA through filters with different size pores and recovered 85% of the *ACTB* mRNA (data not shown), a result that excludes the possibility that these filters bind RNA nonspecifically. The data in Fig. 4, A and B, revealed no significant changes in seminal *ACTB* mRNA or *DDX4* mRNA concentrations after filtration through a 5- μm filter, suggesting that these mRNA

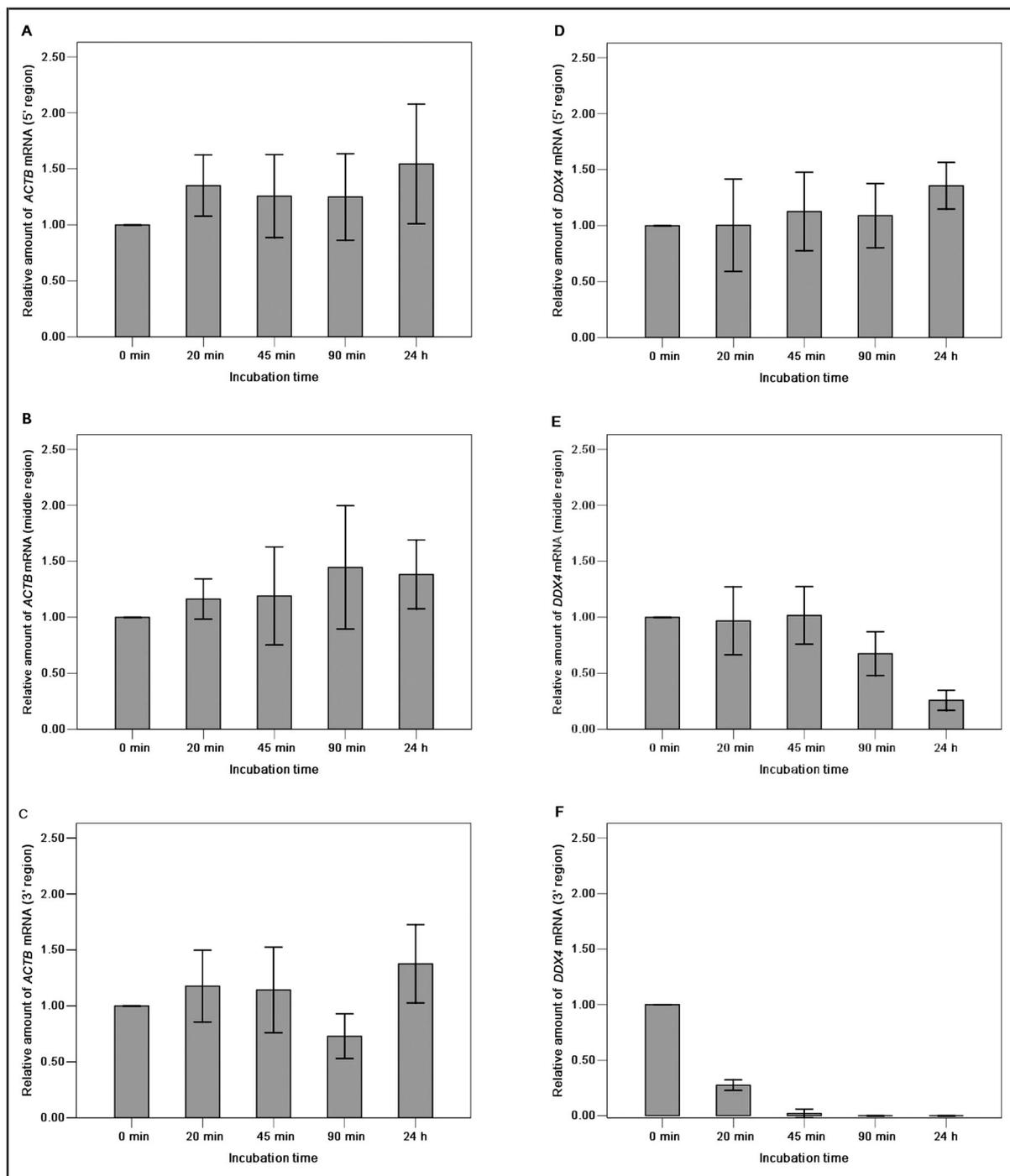
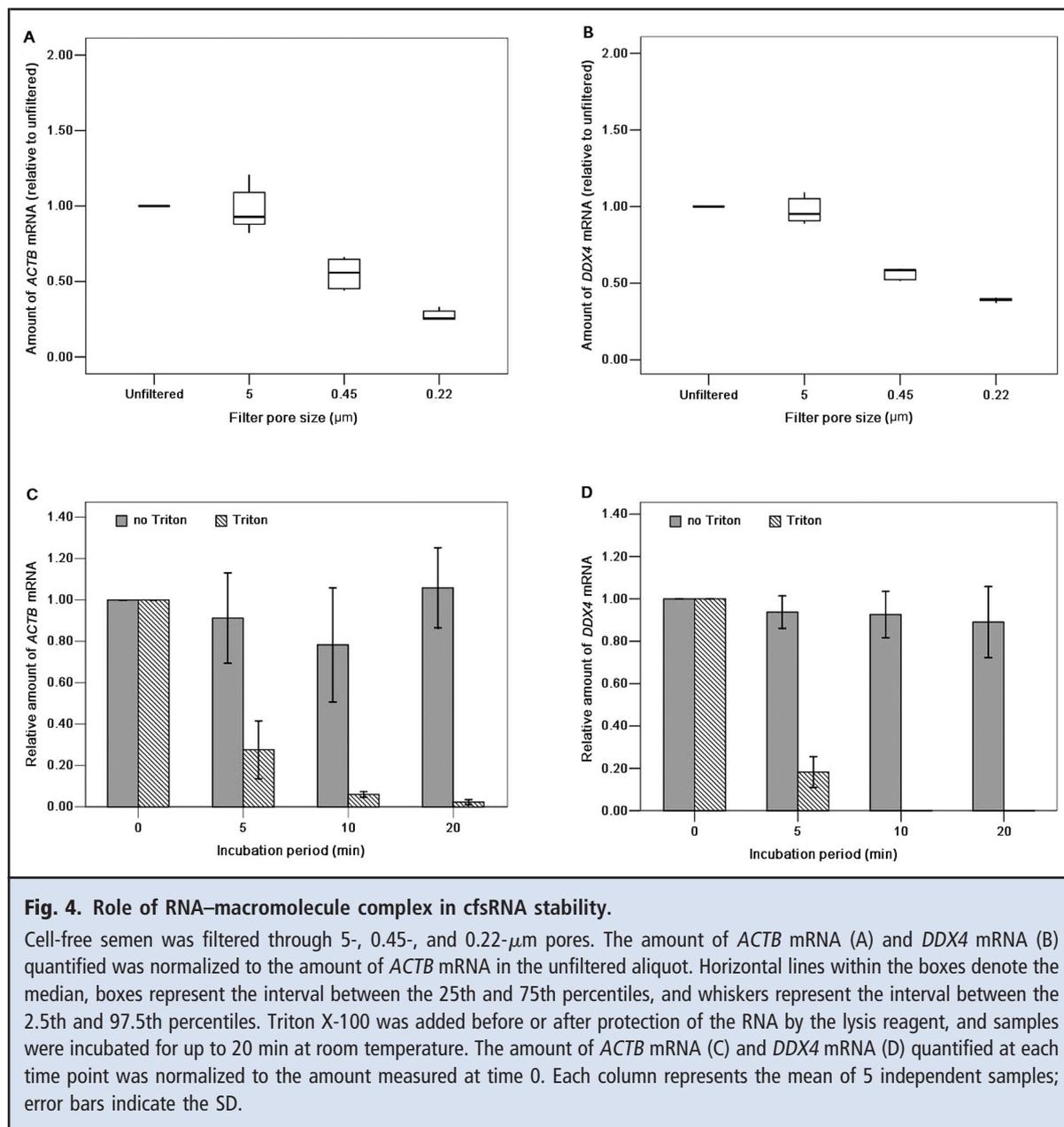


Fig. 3. Stability of cfsRNA.

Seminal plasma was incubated at room temperature for 0–90 min and for 24 h. Amounts of 5' (A), middle (B), and 3' (C) regions of *ACTB* mRNA and 5' (D), middle (E), and 3' (F) regions of *DDX4* mRNA were measured by RT-qPCR at each time point and normalized to the amounts at time 0. Each column represents the mean of 5 independent samples; error bars indicate the SD.

transcripts were not contained within intact cells. Compared with unfiltered samples, 55.2% and 27.7%

of *ACTB* mRNA and 56.0% and 39.4% of *DDX4* mRNA were detected in samples filtered through



0.45- μm and 0.22- μm filters, respectively ($P < 0.05$, SNK test). These data showed that 0.45- μm and 0.22- μm filtration clearly reduced the concentration of seminal *ACTB* mRNA, indicating that the majority of cfsRNA was associated with macromolecules and that the remainder might exist in some other forms.

To further verify that cfsRNA stability was primarily due to binding to complex organic molecules, we added Triton X-100 to seminal samples to disrupt any RNA-protein or RNA-lipid complexes. We observed a dramatic degradation of cfsRNA after treatment with Triton X-100. After 10 min and 20 min, we detected

6.1% and 2.3%, respectively, of the 5' region of *ACTB* mRNA and could not amplify any *DDX4* mRNA (Fig. 4, C and D; $P < 0.001$, SNK test), suggesting that this RNA-macromolecule interaction is important for cfsRNA stability, a conclusion similar to that reported for plasma and saliva (21, 27).

Discussion

A full understanding of seminal constituents is required to discover specific biomarkers for the male reproductive system. A recent report has shown the pres-

ence of cell-free DNA in human semen (28). In the present study, we demonstrated that cfrRNA could readily be detected in human semen and, surprisingly, found the cfrRNA concentration to be much higher in semen than in other body fluids [1.75 mg/L in semen vs <0.1 mg/L in saliva (29) and plasma (30)]. The amounts of cfsRNA available in a single ejaculate (2.40–7.28 μg) sufficed for almost all routine RNA analysis techniques, including microarray, northern blot, electrophoresis, and RT-PCR. Therefore, such abundant quantities and species of cfsRNA should be able to provide comprehensive cell- and tissue-specific information. A noninvasive transcriptome profiling of male reproductive organs could be carried out via cfsRNA analysis. cfsRNA in high concentrations is more readily isolated than spermatozoal RNA and is likely a better candidate to screen for heritable germline epimutations, especially in cases of insufficient sperm production. cfsRNA also has potential as a replacement biomarker of spermatogenic activity and malignant diseases and may thereby overcome the drawbacks and misdiagnoses associated with testicular biopsy.

Given the characteristics of spermatogenesis and the electropherogram of the cfsRNA size distribution, the high concentration of cfrRNA observed in semen may be primarily due to the prominent apoptosis of germ cells, which has been estimated to lead to the loss of up to 75% of potential mature spermatozoa in the testis (31). Moreover, secretions from the seminal vesicles, the prostate, and the bulbourethral glands make up >90% of the volume of semen and likely represent the other sources of cfsRNA. Relatively longer intervals between ejaculations (intercourse) may also affect the relative abundance of cfsRNA in ejaculated semen.

Most cfsRNA was present in partially degraded forms. Some degradation products were apparent in the cfsRNA electropherogram. Detection of full-length transcripts was rare, and the quantitative analysis of multiple regions of seminal mRNAs showed preferential degradation of the 3' region for both *ACTB* and *DDX4*, indicating that exonucleases were primarily involved in the degradation of cfsRNA. Nevertheless, because a 28S/18S ratio of 0.6 and an RNA integrity number of 6.4 indicated the integrity of cfsRNA to be adequate (Fig. 1), most cfsRNA fragments containing epimutations might be identified from the semen of patients with genetic disorders.

The stability of cfsRNA is critical for further research and clinical use. Our results showed that cfsRNA in healthy individuals, especially the 5' region, was relatively stable for 24 h. The 3' region of *DDX4*, however, was scarcely detectable and underwent more rapid degradation. Our data suggest that the stability of cfsRNAs varies, depending on the microenvironment and the properties of the transcripts (e.g., sequences, secondary

structure, and functions before being released). Further research on or clinical use of cfsRNAs will likely focus on the 5' region. Despite the stability of cfsRNA, we propose that samples be stored at $-80\text{ }^{\circ}\text{C}$ immediately after collection and be processed as soon as possible.

The high stability of cfsRNA, like that of cfrRNA in other body fluids, may partially be a consequence of binding with complex organic molecules. We examined this hypothesis by targeting the 5' region of cfsRNA because of its implausible stability. Although we eliminated the effects of residual cells or residual bodies of spermatids with high-speed centrifugation (16 000g for 10 min), the *ACTB* mRNA concentration was still nearly halved after a 0.45- μm filtration step. We presumed that the majority of cfsRNA was associated with macromolecules, because cfsRNA concentrations decreased further after filtration through 0.22- μm pores. Because free RNA, RNA in a RNA–DNA hybrid (26), and RNA contained in microparticles (32) are expected to pass through a 0.22- μm filter, we propose that the cfsRNA that passed through 0.22- μm filters in our study may exist in these forms rather than as macromolecular complexes. We also observed that nearly all of the seminal *ACTB* and *DDX4* mRNA disappeared after Triton X-100 treatment, supporting the idea that the form of the RNA–macromolecule complex may represent one mechanism of cfrRNA protection.

The RNA-associated macromolecules present in some body fluids or culture media may be lipoprotein vesicles that are either actively secreted or released during apoptosis (33). In semen, cfrRNA sequestered within microvesicles derived from apoptotic spermatogenic cells may be the primary macromolecule-associated form present. Additionally, there may be some other important RNA-associated macromolecules in semen, such as free messenger ribonucleoprotein particles (34) and RNA-binding proteins (including translin, Y box-binding protein 2, and polypyrimidine tract-binding protein 2 (35)), a subset of which function as global stabilizers/translational suppressors of mRNAs in male germ cells; however, future research is needed to elucidate the nature of such particle-associated cfrRNA in semen.

On the other hand, the presence of stable and abundant cfsRNA may have biological importance. Considering the spontaneous ability of sperm to take up exogenous nucleic acid and the suggested model for sperm-mediated reverse gene transfer (36), cfsRNA around sperm in the male reproductive tract and in the semen may have latent effects on reproduction. Moreover, both inadvertent transgenesis by rescue intracytoplasmic sperm injection with a semen sample contaminated with bacteria (37) and epigenetic inher-

itance associated with zygotic transfer of RNA molecules in mice (38) have been reported. Therefore, we assumed a latent risk of cfsRNA transgenesis introduced by associated reproductive technology.

To our knowledge, this study represents the first study of cfrRNA in human semen. We characterized seminal cfrRNA as partially degraded, fairly stable, and particle associated, and we found cfrRNA transcript species in semen to be much more abundant and diverse than in other body fluids. Consequently, cfsRNA represents a potential noninvasive biomarker for the male reproductive system and germline epigenetics; however, the latent effects of cfsRNA in reproduction and associated reproductive technology warrant further research.

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acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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