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BACKGROUND: Mature spermatozoa contain thousands of mRNA transcripts. It has been recently shown that human sperm can deliver RNA into the oocyte, suggesting that mRNAs might have a role before or after fertilization. Human embryos express PSG1 (pregnancy-specific β-1-glycoprotein 1) and HLA-E (human leukocyte antigen-E), molecules playing a role in implantation and early development. We compared PSG1 and HLA-E sperm mRNA levels in fertile and infertile men and we tested the hypothesis that these transcripts are selectively retained in the newly formed zygote.

METHODS: Real-time RT–PCR was used to analyze sperm mRNA levels (n = 11 fertile, n = 31 infertile patients) of PSG1, HLA-E and PRM2 (protamine 2). The presence of PSG1 and HLA-E proteins was evaluated by western blot in sperm protein extracts (n = 3). Using ICSI of human sperm into hamster oocytes we evaluated the permanence of these mRNAs at different time points (n = 10 for each time) after fertilization.

RESULTS: PSG1, HLA-E and PRM2 transcripts were demonstrated in ejaculated sperm. The fertile group showed significantly higher levels of PSG1 and HLA-E mRNA (both P < 0.05) than the infertile group, whereas PRM2 levels were not significantly different. However, PSG1 and HLA-E proteins were not found in ejaculated sperm. Following ICSI, PRM2 was undetectable after fertilization; conversely, PSG1 and HLA-E transcripts remained detectable for at least 24 h of zygotic development.

CONCLUSIONS: We provide new evidence that indicates that human sperm deliver transcripts that may have a role in early embryo development and decreased levels of these transcripts may be associated with infertility.

Key words: embryo development / human / mRNA / sperm / male infertility
Introduction

Previous dogma established that the spermatozoon’s single fertilization function is to deliver the male haploid genome to the mature oocyte. The discovery that the spermatozoon introduces a centriole, a prerequisite for the formation of the first cleavage spindle in the fertilized oocyte (Albertson, 1984), as well as a putative oocyte activating factor, responsible for the resumption of the oocyte’s meiosis (i.e. phospholipase C-zeta or PAW-P-post-acrosomal sheath WVV domain-binding protein according to the studied species), have changed our view of the spermatozoon as a simple supplier of the paternal genome (Saunders et al., 2002, 2007; Wu et al., 2007a, 2007b).

Since it has been generally accepted that spermatozoa are terminally differentiated cells that are transcriptionally inactive, the finding that mature spermatozoa carry a variety of mRNA molecules was initially believed to represent a non-functional leftover of the final stage of spermatid gene expression (review in Miller and Ostermeier, 2006). Only recently it has been accepted that sperm RNA can potentially affect embryo development (Cummins, 2001). Whereas the presence of RNA in mature spermatozoon is now established, its functional significance, if any, is still a matter of debate. Any role of these RNA molecules in fertilization and early development has, until recently, seemed unlikely, since the oocyte contains large stores of maternal RNAs known to be required for early embryonic development prior to zygotic genome activation (reviewed in Miller, 2007).

Ostermeier et al. (2004) reported, for the first time, that human spermatozoa can deliver mRNA to the oocyte during fertilization. Furthermore, a more recent report demonstrated that some mRNAs can be translated de novo supporting the hypothesis that mature spermatozoa may selectively retain particular mRNAs, and that at least some transcripts might have a function during or beyond the process of fertilization (Gur and Breitbart, 2006). As there is no evidence that proteins encoded by the majority of mRNAs found in mature spermatozoa are also present (Dadoune et al., 2005), these mRNAs accumulated in the sperm nucleus might be viewed as potential contributors to early embryogenesis. Additionally, micro RNAs have also been detected in human spermatozoa, also raising the possibility that these molecules might play a role in early fertilization events (Ostermeier et al., 2005).

On the other hand, the concept that sperm mRNA present in ejaculated mature spermatozoa might be used as a genetic fingerprint of normal fertile men has been approached by different strategies. Recently, a microarray study defined the differential molecular signatures which could discriminate between normozoospermic and teratozoospermic semen samples (Platts et al., 2007). In addition, semi-quantitative evaluation of the mRNA profiles from ejaculated human spermatozoa provided some correlation with progressive motility and capacitation dynamics of different sperm subpopulations (Lambard et al., 2004). Several studies have also shown significant differences in the mRNA content in sperm exhibiting different biochemical/molecular parameters (Lambard et al., 2003; Steger et al., 2008). Micro-array and real-time PCR analyses detected changes in the levels of several mRNAs related to motility of the sampled sperm populations, facilitating a comparison between fertile and infertile men (Wang et al., 2004).

Although low quantities of functionally stable sperm mRNA can be potentially transferred into the oocyte, it has been speculated that most of these specific sperm mRNAs [like protamine 1 (PRM1) or protamine 2 (PRM2)] will be degraded shortly after fertilization. Conversely, some RNA may persist until the activation of the embryonic genome, or even beyond. These mRNAs could therefore translate proteins involved in critical process related to stress response, embryogenesis and implantation (Boerke et al., 2007). PSG1 (pregnancy-specific B-1-glycoprotein 1) and the non-classical histocompatibility class I antigen, HLA-E (human leukocyte antigen-E), are proteins expressed in the human embryo and have an important immunomodulatory function during embryo implantation and early pregnancy (Motran et al., 2002; Ishitani et al., 2006). It has been shown that mRNA transcripts of PSG-1 and HLA-E are present in human mature spermatozoa as examined by micro array and serial analysis gene expression (SAGE) technology (Ostermeier et al., 2002; Zhao et al., 2006). Conversely, an analysis of the transcriptome of the human oocyte using micro array did not demonstrate the presence of PSG-1 mRNA and showed down-regulation of HLA-E mRNA (Kocabas et al., 2006).

We speculated that following delivery of the sperm mRNA molecules into the oocyte, the newly formed zygote maintains selective mRNA transcripts, which could translate proteins supportive of the early stage of embryogenesis and/or implantation. The specific aims of the present study were: (i) to confirm the presence of PSG-1 and HLA-E mRNA in human ejaculated sperm using real-time RT–PCR; (ii) to test the hypothesis that sperm quality might be related to the levels of these transcripts by comparing ejaculates of fertile and infertile men and (iii) to analyze the presence and temporal variation of PSG-1, HLA-E and PRM2 mRNA levels following heterologous fertilization of hamster oocytes with human spermatozoa through ICSI.

Materials and Methods

Subjects

The Institutional Review Board of Eastern Virginia Medical School approved the study and all participants gave written informed consent. The participating individuals were classified into two groups: (i) Fertile group, which comprised healthy fertile male volunteers whose partners had conceived and delivered a child within the last two years (sperm donors); and (ii) Infertile group, that included the male partners of infertile couples consulting for infertility (defined as inability to achieve a pregnancy in more than one year of unprotected intercourse), presenting an altered semen analysis, without evidence of female factors following the basic female infertility work up, and having failed intrauterine insemination therapy (Oehninger, 2001).

Sperm preparation

A total of 46 semen samples were examined. Unless otherwise noted, semen samples were collected by masturbation into sterile cups, after 2–4 days of sexual abstinence. The samples were allowed to liquefy for 30 min at room temperature followed by assessment of semen characteristics and sperm parameters. Sperm concentration and progressive motility were assessed with an HTR-Ivos semen analyzer (version GS 771: Hamilton Thorne Research, Beverly, MA, USA) with fixed parameter settings and manually monitored as appropriate (Oehninger et al., 1990). Motion parameters were examined after mixing the sperm suspension and loading a 5 μl aliquot into a Makler chamber (Mid Atlantic Diagnostics Inc., Mt. Laurel, NJ, USA). Sperm morphology was examined at ×1000 oil immersion microscopy by strict criteria after staining with STAT III
In order to recover a purified population of highly motile spermatozoa, free of somatic and germ cell contaminants, a swim-up separation was effected in Human Tubal Fluid (HTF; Irvine Scientific, Santa Ana, CA, USA) supplemented with 3 mg/ml Human Serum Albumin (HSA; Irvine Scientific). The spermatozoa were washed twice with HTF–HSA and centrifuged for 10 min at 300 g. After the second wash, the supernatant was removed and fresh HTF–HSA was layered over the pellet and incubated for 60 min at 37°C. In order to retrieve the highly motile fraction, the volume from the top layer was removed, and the concentration was adjusted to 1 × 10^6 sperm/ml. The swim-up fractions were immediately treated according to the experimental design as follows: (i) stored at −196°C without cryoprotectant until RNA extraction, (ii) capacitated by 6 h and injected into oocytes by ICSI, (iii) subjected to protein extraction for immunoblotting studies or (iv) capacitated by 6 h followed by protein extraction. The lack of contamination of the swim-up samples with somatic and germ cells was confirmed by examination of molecular markers, CD45 for leucocytes, E-cadherin (E-cad) for epithelial cells and confirmed by examination of molecular markers, CD45 for leucocytes, E-cadherin (E-cad) for epithelial cells and c-kit for germ cells by RT–PCR (Lambard et al., 2004). Human semen samples contaminated by either leucocytes or testicular germ cells were used as positive controls for CD45 and c-kit, respectively. Endometrial epithelial cells were used as positive marker of E-cad.

### Sperm RNA extraction

The cryopreserved swim-up fractions (n = 42 semen samples, from 11 fertile and 31 infertile men) were thawed in a 37°C water bath for 3 min immediately before RNA extraction. Total RNA was extracted from each cryopreserved-thawed swim-up sample using a combination of the TRIZol reagents methods (Invitrogen, Carlsbad, CA, USA) and RNeasy Micro kit (Qiagen). Briefly, a total of 1 × 10^6 sperm was mixed with TRIZol reagents and centrifuged for 10 min at 300 g. After the second wash, the supernatant was removed and fresh HTF–HSA was layered over the pellet and incubated for 60 min at 37°C. Total RNA was extracted was quantified by spectrophotometry, and a typical yield ranged from 10–20 ng/μl.

### Real-time RT–PCR

Complementary DNA (cDNA) was generated from 3 μl total RNA in a total volume of 20 μl containing: 2.5 μM random hexamers, 2.5 U/μl Murine Leukemia Virus Reverse Transcriptase, 1 U/μl RNase inhibitor, 1 × PCR buffer, 1 mM each deoxy-NTP and 5 mM MgCl₂ (Applied Biosystems, Foster City, CA, USA). RT parameters were as follows: 23°C for 10 min, 42°C for 15 min (RT reaction), 99°C for 5 min (transcriptase deactivation) and 5°C for 5 min in an iCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). cDNA solutions were then stored at −20°C. Preparations without reverse transcriptase were used as negative controls (NoRT), in which the absence of PCR products indicated a complete lack of contaminating genomic DNA.

Real-time RT–PCR was performed using Lightcycler FastStart DNA Master Plus SYBR green I and a Lightcycler 2.0 instrument (Roche Applied Science, Indianapolis, IN, USA) in a 20 μl total reaction volume, containing 3 μl cDNA and 0.5 μM of each sense and antisense primer. The primers were designed using Oligo Explorer or Primer 3 software and purchased from Invitrogen. HLA-E and PSG have no homolog in rodents, therefore the primers were human specific. In addition, the primers were designed on two different exons (to avoid possible signals originating from DNA) and a BLAST search was used to verify the sequence specificity. The primer sequences, annealing temperature and the expected lengths of the resulting PCR products are summarized in Table I.

Before amplification, samples were denatured at 95°C for 10 min. The template was amplified by 45 cycles of denaturation at 95°C for 10 s, annealing of primers at the specific temperature as given in Table I for 5 s, and extension at 72°C for 10 s, followed by a final extension at 72°C for 10 min. The melting protocol consisted of heating the samples to 95°C followed by cooling to 65°C for 15 s, and slowly heating at 0.1°C per second to 95°C while monitoring fluorescence. Melting curve analysis was performed after each run to verify specific amplifications.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequences</th>
<th>Annealing temperature (°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PSGI</strong></td>
<td>Fw: 5′-AATGCTTCTAGGCGGACTATG</td>
<td>56</td>
<td>126</td>
</tr>
<tr>
<td></td>
<td>Rv: 5′-CTCCAAACGACTGCTTCAC</td>
<td>58</td>
<td>126</td>
</tr>
<tr>
<td><strong>HLA-E</strong></td>
<td>Fw: 5′-GCCCGTCACCCCTAGATGGA</td>
<td>62</td>
<td>205</td>
</tr>
<tr>
<td></td>
<td>Rv: 5′-GCTGTGAGACCTAGCACCTCCT</td>
<td>62</td>
<td>205</td>
</tr>
<tr>
<td><strong>PRM2</strong></td>
<td>Fw: 5′-CGAGGCTCAGGAGGAC</td>
<td>55</td>
<td>161</td>
</tr>
<tr>
<td></td>
<td>Rv: 5′-TGTTTCGCGAGGCTTCGC</td>
<td>55</td>
<td>161</td>
</tr>
<tr>
<td><strong>ACTB</strong></td>
<td>Fw: 5′-AATGCTTCTAGGCGGACTATG</td>
<td>56</td>
<td>191</td>
</tr>
<tr>
<td></td>
<td>Rv: 5′-CTCCAAACGACTGCTTCAC</td>
<td>56</td>
<td>191</td>
</tr>
<tr>
<td><strong>CD45</strong></td>
<td>Fw: 5′-ACCACAAGTATTAACGCCAAG</td>
<td>55</td>
<td>126</td>
</tr>
<tr>
<td></td>
<td>Rv: 5′-TTTGAGGGGGATCCAGGTAAT</td>
<td>55</td>
<td>126</td>
</tr>
<tr>
<td><strong>E-Cad</strong></td>
<td>Fw: 5′-CGAGAGCTACAGTCCAGG</td>
<td>56</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td>Rv: 5′-GTGTCGAGGAAAATAGGTG</td>
<td>56</td>
<td>117</td>
</tr>
<tr>
<td><strong>c-kit</strong></td>
<td>Fw: 5′-GCCGACAGATTAGGCTGTGA</td>
<td>56</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>Rv: 5′-GGCTTTTTCCGTGATCCATCA</td>
<td>56</td>
<td>110</td>
</tr>
</tbody>
</table>
quantified by the ΔΔCt method (Livak and Schmittgen, 2001). The expression values obtained were normalized against the housekeeping gene β-actin (ACTB) to account for differing amounts of starting material. All determinations were performed in duplicate.

Protein extraction, electrophoresis and immunoblotting

For these experiments, three different samples (ejaculates) were obtained from fertile donors; the swim-up fractions were recovered and separated in two portions. Half of the sample was immediately washed with phosphate-buffered saline and stored at −80°C and the second portion was capacitated for 6 h in HTF-HSA medium at 37°C, and then treated in the same way as mentioned above. For each condition (non-capacitated and capacitated), the three sperm samples were pooled and incubated on ice for 30 min in RIPA lysis buffer [50 mM Tris (pH 7.5), 1% Triton X-100, 1% deoxycholic acid-sodium salt, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl and 2 mM EDTA] supplemented with protease inhibitor cocktail (Pierce Chemical Co., Rockford, IL, USA). Insoluble components were removed by centrifugation at 14 000g for 20 min. An aliquot was saved for protein concentration determination using the bicinchoninic acid method according to the manufacturer’s instructions (Pierce Chemical Co.), and the remainder was stored at −80°C for later immunoblotting analysis. Placental protein extract (Clontech, Mountain View, CA, USA) was used as positive control in the western blotting.

A 10% polyacrylamide-SDS gel with a 3.5% stacking layer was cast and run in a mini-Protan II device (Bio-Rad Laboratories). To each lane, 60 μg of sperm proteins or 15 μg of placental proteins in 4× gel loading buffer (final composition: 141 mM Tris base, 2% lithium dodecyl sulfate, 10% Glycerol, 0.51 mM EDTA, 0.22 mM SERVA Blue G250, 0.175 mM Phenol Red, pH 8.5) were added. The electrophoresis was performed at 120 V for 1.5 h and the electrotransfer to a nitrocellulose membrane was performed using the mini-transblot cell (Bio-Rad) at 300 mA for 45 min. The membrane was blocked with 5% non-fat dry milk in TBST buffer [10 mm Tris (pH 7.5), 100 mM NaCl, 0.1% Tween 20] for 30 min. Immunoblotting was performed by incubating with a monoclonal mouse anti-PSG1 antibody 1 μg/ml (sc-51621, Santa Cruz Biotechnology), and with an equivalent concentration of non-immune mouse immunoglobulin (IgG) (isotype control), in TBST overnight at 4°C. The membrane was washed with TBST three times for 5 min each time and then incubated in horse-radish peroxidase-conjugated goat anti-mouse IgG (Zymed Laboratories, South San Francisco, CA, USA) for 60 min at room temperature in blocking buffer and washed three times as described above. MagicMark XP (Invitrogen) was used as Western protein standards. The bands were then detected by exposure to Chemiluminescence Reagent Plus reagent (Perkin Elmer, Waltham, MA, USA) and the image was captured in a Kodak Image Station 440CF (Eastman Kodak Co., New Haven, CT, USA).

Microinjection of a single human spermatozoon into hamster oocytes

Highly motile human spermatozoa from a fertile patient were selected by swim-up and capacitated in HTF medium with 3 mg/ml HSA for 6 h at 37°C in 5% CO2. ICSI was carried out as previously described (Palermo et al., 1992). Briefly, 1 μl of sperm suspension was diluted with 4 μl of 10% polyvinyl pyrolidone (Irvine Scientific) and placed in the center of an injection dish. Each frozen-thawed mature (metaphase II) hamster oocyte (Conception Technologies, San Diego, CA, USA) was placed in 5 μl of P1 medium (Irvine Scientific) supplemented with 10% SSS (Synthetic Serum Substitute, Irvine Scientific) surrounding the central drop containing the sperm suspension and covered with mineral oil. A total of 10 metaphase II oocytes were micro-injected for each experimental time (total n = 50 injected oocytes) using Narishige micromanipulators (Narishige, Tokyo, Japan) mounted on a phase-contrast inverted microscope (Olympus, Tokyo, Japan) at 400× magnification. The selected spermatozoon was aspirated into the injecting micropipette and introduced through the zona pellucida into the ooplasm. The micropipette was then slowly withdrawn and the injected oocytes were kept at 37°C in 5% CO2 for 0, 3, 6, 12 and 24 h in P1 medium with 10% SSS. After the respective incubation times, degenerate zygotes (swollen or dark) were discarded. Only injected viable oocytes (n = 6 per each study time) were immediately and individually stored at −80°C until used for RNA extraction. Extraction of RNA was performed using RNeasy Micro Kit (Qiagen). The RNA was extracted following the manufacturer’s instructions and eluted in 14 μl of water RNase free. RNA extraction and RT–PCR was performed for each individual zygote as described above with the modification of the number of cycles for template amplification increased to 55. Non-injected oocytes were tested to ensure that no artifacts were created during PCR amplification.

Statistical analysis

Data are presented as mean ± SEM. Comparisons were performed using two-way analysis of variance analysis to identify significant differences between groups. Correlations between semen parameters and relative expression of mRNA transcript levels were performed with Pearson’s correlation coefficient. A P < 0.05 was considered statistically significant.

Results

Semen samples parameters

The semen samples were classified into two groups: fertile (n = 11) and infertile (n = 31) group according to the criteria described in Materials and Methods section. The basic semen parameters (sperm concentration, progressive motility and normal morphology) for the fertile and infertile groups are presented in Table II. There was no significant difference between fertile and infertile group for any of the semen parameters.

Presence and quantification of PRM2, PSG1 and HLA-E mRNA levels in spermatozoa from fertile and infertile men

To evaluate the presence and levels of PRM2, PSG1 and HLA-E transcripts, RNA was extracted from a purified population of highly motile spermatozoa, free of somatic and germ cell contaminants, recovered by swim-up separation from each sample. The sperm mRNA samples were analyzed for the presence of specific molecular

### Table II Semen parameters according to groups (mean ± SEM)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fertile (n = 11)</th>
<th>Infertile (n = 31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm concentration (× 10^6/ml)</td>
<td>131.1 ± 26.1a</td>
<td>114.5 ± 13.1a</td>
</tr>
<tr>
<td>Progressive motility (%)</td>
<td>79.3 ± 4.3b</td>
<td>53.4 ± 2.8b</td>
</tr>
<tr>
<td>Normal forms (%)</td>
<td>10.2 ± 0.6c</td>
<td>7.5 ± 0.8d</td>
</tr>
</tbody>
</table>

a,b: P > 0.05
markers of leukocytes, germ cells and epithelial cells (CD45, c-kit and E-cad, respectively). Absence of RNA from contaminating cells is shown in Fig. 1A.

The presence of mRNA for ACTB, PRM2, PSG1 and HLA-E were confirmed by RT–PCR in human ejaculated spermatozoa (Fig. 1B). The mRNA levels of these genes were evaluated in fertile and infertile men by real-time RT–PCR. The quantification was expressed as relative transcript levels using ACTB as reference gene. There was a significant difference between fertile and infertile group for PSG1 (2.81 ± 1.39 and 0.53 ± 0.08, respectively, P < 0.05), as well as for HLA-E (6.36 ± 4.81 and 0.59 ± 0.12, respectively, P < 0.05) (Fig. 1C). However, no significant difference was found for PRM2 between fertile donors and infertile patients (0.39 ± 0.12 and 0.96 ± 0.66, respectively, P > 0.05).

No significant correlation was found between any of the semen parameters (sperm concentration, motility or morphology) and the relative mRNA expression levels of PRM2, PSG1 or HLA-E (P > 0.05).

Examination of the presence of PSG1 and HLA-E protein in capacitated and non-capacitated spermatozoa by immunoblotting

To investigate the possible expression of PSG1 and HLA-E proteins in human spermatozoa, SDS-polyacrylamide gel electrophoresis followed by western blotting was performed. A placental protein extract was used as positive control for the presence of PSG1 and HLA-E. Sperm proteins were extracted from pooled spermatozoa obtained from three fertile donors by swim-up to ensure the absence of immature spermatic cells and leukocytes.

A positive signal was observed for PSG1 (72 kDa) and HLA-E (40 kDa) in the lanes corresponding to the placental extract (positive control), while none of the proteins was detected in the sperm protein samples before or after capacitation (Fig. 2).

Detection of sperm mRNA after fertilization of hamster oocytes with human sperm using microinjection by ICSI

To evaluate the possibility that selected sperm mRNA transcripts may persist after fertilization, the presence of PRM2, PSG1 and HLA-E mRNA was evaluated in hamster eggs at different times after fertilization by ICSI. Hamster eggs were injected with a single human capacitated spermatozoon, and incubated in development medium.

As it is well known that the rate of decondensation of the head of the sperm after the injection in hamster eggs varies between samples (Gvakharia et al., 2000), pilot experiments were performed to establish the percentage of sperm decondensation after ICSI in our model. An average of 70% injected oocytes typically demonstrated sperm head decondensation (range 60–80%) when the same sample donor was used. This was confirmed at 3, 6, 12 and 24 h after microinjection, when zygotes were stained with DAPI (4',6-diamidino-2-phenylindole).

Figure 1 PSG1, HLA-E and PRM2 mRNA in spermatozoa from fertile and infertile men. Representative agarose gels of PCR analysis. (A) PCR products for specific markers of leukocytes (CD45), germ cells (c-kit) and epithelial cells (E-cadherin), respectively, used as positive controls in lines 1, 3 and 5, respectively; a representative sperm sample purified by swim-up showing the absence of RNA from contaminating cells in lines 2, 4 and 6; line M: size-calibration ladder. (B) PCR products of ACTB, PRM2, PSG1 and HLA-E from sperm RNA in lines 1, 3, 5 and 7, respectively; NoRT control (with the same primers in absence of the RT enzyme) to confirm that the signals originate from RNA in lines 2, 4, 6 and 8, respectively. (C) Relative mRNA levels of PSG1, HLA-E and PRM2, respectively in purified swim-up fractions obtained from fertile (n = 11) and infertile (n = 31) men.

Figure 2 Electrophoresis and western blot analysis of sperm protein extracts. (A) Coomassie Brilliant Blue stained protein profile obtained by 10% SDS–polyacrylamide gel electrophoresis. M, molecular weight standards (kDa); Pla, placental proteins extract; Sp, sperm protein extract. (B) Western blot with anti-PSG1 and anti-HLA-E antibodies, respectively. M, molecular weight standards; Pla, placental proteins extract (positive control); CaSp, capacitated sperm protein extract; Sp, non-capacitated sperm protein extract. An empty line was left between the placental and sperm protein extract to avoid signals from possible protein diffusion.
to evaluate sperm DNA decondensation. A representative photomicrograph is shown in Fig. 3A.

As expected, none of the sperm mRNA was detected in non-injected hamster eggs. When the presence of PRM2 (Fig. 3B) mRNA was evaluated, no signal was detected in the hamster oocytes at any time after fertilization. However, PSG1 and HLA-E mRNA were detectable immediately after injection and also at 3, 6, 12 and 24 h post-injection (Fig. 3C and D).

**Discussion**

Human PSG1, a major placental glycoprotein, is synthesized in large amounts by placental trophoblasts and can be detected as early as in the culture medium of preimplantation human embryos (Jurisicová et al., 1999). It has been speculated that PSG1 protein may play a crucial role in supporting early gestation and fetus protection against the maternal immune system. This is supported by the fact that PSG1 is able to modulate the monocyte/macrophage metabolism to regulate T-cell activation and proliferation. Also, it induces the secretion of anti-inflammatory cytokines by monocytes (reviewed in Motran et al., 2002).

HLA-E is a human major histocompatibility complex class Ib (non-classical) antigen. It binds peptides derived from other HLA class I signal sequences, interacts with CD94/NKG2A or CD94/NKG2C on natural killer (NK) cells, and inhibits the killing activity of NK cells (Lee et al., 1998). The extra villous trophoblast expresses HLA-E (Ishitani et al., 2003) and can avoid maternal NK cell surveillance.

The presence of PSG-1 and HLA-E transcripts in human mature ejaculated spermatozoa has been previously reported by micro array and SAGE studies. Here, we validated those results by the identification of the specific PCR products and the quantification of mRNA expression by real-time RT–PCR (Fig. 1).

One of the major findings of the present study was the difference in the relative mRNA expression of PSG1 and HLA-E between fertile and infertile men. The transcripts levels were significantly higher in the swim-up fractions of fertile men when compared with those of infertile men (Fig. 1C). On the other hand, PRM2 mRNA levels were slightly higher in infertile patients but not statistically different among the groups.

These results suggest a possible association between sperm mRNA expression and fertility potential. Some sperm mRNA transcripts do not appear to represent a leftover material from spermatogenesis and may therefore have a function after fertilization. It is possible to speculate that decreased levels of a particular mRNA involved in the early stages of embryo development, such as PSG1 and HLA-E, could be related to the infertile condition, even when the semen parameters were only moderately altered. We propose that the mRNA levels could be reflecting sperm defects at the molecular level which cannot be identified microscopically during the basic semen analysis. Higher levels of these transcripts in fertile men may suggest a role during normal embryo development, which could be impaired due to decreased mRNA levels in the infertile group. In addition, the lack of detection of the corresponding proteins in spermatozoa suggests that their function(s) are required by the embryo but not by the spermatozoa.

Previous studies have reported differences in the amount of certain transcripts between different groups of men. A decreased PRM1/PRM2 ratio has been shown when comparing fertile (1:1) and infertile

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**Figure 3** Detection of human sperm mRNA after fertilization following ICSI into hamster oocytes. (A) Representative photomicrography of human sperm head decondensation at different time points (3, 6, 12 and 24 h) after injection into hamster oocytes. A phase contrast image merged with DNA staining (blue). ON, oocyte nucleus; MP, male pronucleus; FP, female pronucleus; PB, polar body. (B) Representative agarose gel showing the PCR products corresponding to PSG1, HLA-E and PRM2, respectively, detected in fertilized hamster oocytes at different time points after human sperm injection. M, size-calibration ladder (bp); Sp, human sperm (positive control); Oo, non-injected hamster oocyte; 0, immediately post-injection; 3, three hours post-injection; 6, six hours post-injection; 12, 12-h post-injection; 24, 24-h post-injection; NoRT, no reverse transcription control.
Our findings strengthen the possibility that some of the paternal mRNAs may be functional at the time of early embryo development, before the beginning of new mRNA synthesis is directed by the embryo’s own genome. On the other hand, they confirm that paternal transcripts that could be deleterious, or are not supportive of the first stage of development, might be rapidly degraded.

In summary, PSG1 and HLA-E mRNA were present at higher levels in spermatozoa of fertile men than in infertile individuals, while PRM2 mRNA, without an apparent function in the fertilized oocyte (and even a deleterious one), did not differ among groups. PSG1 and HLA-E proteins were not detected in sperm extracts, suggesting that the protein products are not required in mature spermatozoa. These findings, in addition to the persistence of sperm PSG1 and HLA-E mRNA (but not PRM2) in the egg after fertilization, lead to the conclusion that PSG1 and HLA-E could belong to the group of mRNAs delivered by the spermatozoon at fertilization having a functional role during early embryo development and/or implantation.

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**References**


Possible role of human sperm mRNA transcripts


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