Expression of human oocyte-specific linker histone protein and its incorporation into sperm chromatin during fertilization

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Objective: To investigate the expression of oocyte-specific linker histone protein (hH1FOO) in human ovaries and its incorporation into sperm chromatin after intracytoplasmic sperm injection (ICSI).
Design: Laboratory study.
Setting: University hospital.
Patient(s): Human ovarian tissues were obtained from patients at oophorectomy. Human oocytes and embryos were obtained from infertile patients undergoing IVF and ICSI.
Intervention(s): A polyclonal rabbit antibody targeting the predicted hH1FOO protein was used for immunohistochemical analysis. Western blot analysis and the reverse transcriptase–nested polymerase chain reaction were done to detect hH1FOO in chromatin of germinal vesicle–stage oocytes through to two-cell embryos.
Main Outcome Measure(s): The hH1FOO antibody reactivity of oocytes, ovarian tissues, and sperm chromatin after ICSI.
Result(s): hH1FOO protein was localized in all oocytes from primordial to Graafian follicles. In unfertilized oocytes after ICSI, the chromatin of injected sperm was condensed without hH1FOO incorporation in 45.5% of oocytes, condensed with hH1FOO incorporation in 9%, and decondensed with hH1FOO incorporation in 45.5%. None of the oocytes contained decondensed sperm chromatin without hHFOO incorporation.
Conclusion(s): hH1FOO protein was expressed by human oocytes from primordial follicles to early embryogenesis. Sperm nuclei that were still condensed after ICSI could be separated into two categories by hH1FOO incorporation, which might provide valuable information regarding failed fertilization. (Fertil Steril® 2010;93:1134–41. ©2010 by American Society for Reproductive Medicine.)

Key Words: H1FOO, oocytes, fertilization, histones, decondensation, human

Eukaryotic nuclear DNA associates with histone proteins to form chromatin, which is composed of nucleosomes connected by linker DNA. A nucleosome has a protein core, which is an octamer containing two molecules each of histones H2A, H2B, H3, and H4, with DNA wound around its surface. It is believed that histone H1, also termed a linker histone, binds to the linker DNA and plays an important role in the development and stabilization of higher-order chromatin structure and also possibly is involved in regulating gene expression (1–4).

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Although all histones are encoded by multiple genes, H1 histones show greater variability than any of the core histones. Eleven different linker histone variants have been identified in mammals, which include seven somatic variants (H1.1–H1.5, H10, and H1x), three spermatogenic variants (H1t, H1T2, and HILS1), and an oocyte-specific variant (H1FOO; H1 histone family, member O, oocyte-specific) (5). In mice, the oocyte-specific histone protein H1FOO is expressed by oocytes from the germinal vesicle (GV) stage through to the two-cell embryo stage (6). After intracytoplasmic sperm injection (ICSI) is performed in mice, H1FOO replaces protamine on sperm chromatin and then is replaced by somatic H1s at the two- or four-cell embryo stage (7). A similar developmental transition also occurs in *Xenopus laevis* (8). Such findings suggest a significant role of H1FOO in the regulation of gene expression during oogenesis and early embryogenesis (9–11).

We recently characterized the transcript encoding human oocyte–specific H1 (hH1FOO) protein, by using direct single-cell reverse transcription followed by a nested polymerase chain reaction (RT-nested PCR) (12). As well as being expressed exclusively by oocytes, this gene product has

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several distinctive features that are shared by oocyte-specific linker histones from other species. First, it encodes the longest H1 histone (347 amino acids). Second, it is located on chromosome 3q21-22 and contains five introns, whereas the somatic H1 genes are located as a large cluster on the short arm of chromosome 6 and lack introns. Third, its messenger RNA (mRNA) shows polyadenylation, whereas somatic H1 mRNA lacks a poly-A tail. However, the expression and localization of the protein encoded by this mRNA have not yet been determined.

Intracytoplasmic sperm injection has proved to be an effective treatment for severe male factor infertility (13). However, fertilization is achieved for only 60% to 70% of injected oocytes, resulting in a significant wastage of oocytes (14, 15), and poor fertilization (failure to detect two pronuclei [PN]) sometimes occurs after ICSI (16, 17). Sperm chromatin is mainly associated with a sperm-specific nuclear protein, which is termed protamine, instead of with histones. It is six times more compact than metaphase chromosomes and represents the most highly condensed chromatin found in eukaryotes (18). To achieve fertilization, the penetrating sperm heads must undergo various morphologic and biochemical changes, including chromatin decondensation and the replacement of protamine by histones (19). Visualization of sperm chromatin decondensation along with protamine-histone replacement might provide useful information about the process of fertilization, as well as about the reasons for failure of PN formation after ICSI.

In this study, we investigated the expression and localization of hH1FOO protein in human oocytes, ovaries, and fertilized embryos by using a specific anti-hH1FOO antibody. The relationship between morphologic changes of the sperm head after ICSI and the dynamics of histone transfer into sperm chromatin also were investigated by immunohistochemical analysis with use of the same antibody.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of Keio University Hospital. All samples used in this study were obtained from patients who had given written informed consent.

Human Oocytes, Embryos, and Ovarian Tissues

Human oocytes were retrieved from infertile women undergoing IVF or ICSI at the Infertility Clinic of Keio University Hospital. Oocytes that did not display 2PN and had not initiated cleavage by 48 hours after ICSI were considered to be unfertilized. The oocytes and embryos that served as samples for this study, including GV-stage oocytes, 3PN oocytes, arrested 2PN oocytes, and arrested two-cell embryos all were scheduled to be discarded. Specimens of normal human ovarian tissue were obtained from women of reproductive age who underwent oophorectomy during surgery for cervical cancer or uterine leiomyoma.

Polyclonal Anti-hH1FOO Antibody

Anti-hH1FOO antiserum was raised in rabbits that were immunized with a mixture of two synthetic oligopeptides corresponding to the amino acid sequences SRSPESEKPGPSHG and KASSSKVSSQRAEA of the predicted hH1FOO protein (12). These oligopeptides were selected by using Epitope Adviser and DNASIS software (Hitachi, Tokyo, Japan) and were conjugated to keyhole limpet hemocyanin. The antiserum thus obtained was purified by affinity chromatography (Takara, Shiga, Japan).

Western Blotting of Ovarian Tissue and Oocytes

To obtain samples for blotting, 10 mg of ovarian tissue was ground in sodium dodecyl sulfate (SDS) sample buffer, as described previously (20). After centrifugation at 800 $\times$ g for 5 minutes, the supernatant was applied to 15% SDS–polyacrylamide gel for electrophoresis (1.0 μg of protein per lane). To obtain oocyte protein samples, a total of 30 oocytes were subjected to three freeze-thaw cycles in SDS sample buffer and applied to 15% SDS–polyacrylamide gel for electrophoresis. Proteins were transferred from the gel to a polyvinylidene difluoride membrane, which was blocked overnight in TBST buffer (10 mmol/L tris[hydroxymethyl]aminomethane [Tris; Sigma Aldrich, St. Louis, MO] HCl, pH 7.5; 150 mmol/L NaCl; and 0.1% polysorbate 20 [Tween 20; Sigma Aldrich]) containing 5% skim milk powder (wt/vol). Next, the membrane was incubated with anti-hH1FOO antibody (diluted 1:500) for 1 hour at room temperature. After washing three times in TBST for 10 minutes each, the membrane was incubated with a peroxidase-conjugated antibody for rabbit immunoglobulin (diluted 1:30,000; GE Healthcare Bio-Sciences, Piscataway, NJ). Signals were detected by using an enhanced chemiluminescence Western blot detection kit (GE Healthcare Bio-Sciences).

Immunohistochemical Analysis of Ovarian Tissues

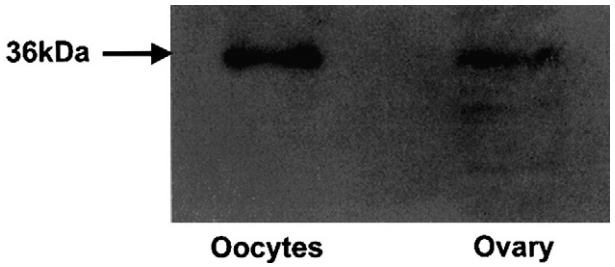
Ovarian tissue specimens were frozen in optimal cutting temperature compound, sections (6 μm thick) were cut on a cryostat, and these sections were fixed in acetone for 15 minutes at room temperature. After blocking with 5% normal swine serum for 30 minutes at room temperature, the sections were incubated with the anti-hH1FOO antibody (diluted 1:500) overnight at 4°C. As a negative control, the antibody was replaced by preimmune serum. Sections then were incubated with peroxidase-conjugated porcine anti-rabbit immunoglobulin (diluted 1:100; DakoCytomation, Glostrup, Denmark). Color was developed with 3,3′-diaminobenzidine tetrahydrochloride (0.2 mg/mL; Dojindo Laboratories, Kumamoto, Japan) in a solution containing 50 mmol/L Tris-HCl (pH 7.4) and 0.003% hydrogen peroxide, after which the sections were counterstained with hematoxylin.

Reverse Transcription–nested PCR of hH1foo mRNA

About 1 μL of phosphate-buffered saline solution (PBS) containing a single unfertilized oocyte that had been previously

FIGURE 1

Western blotting of extracts from unfertilized human oocytes (*left lane*) and human ovarian tissue (*right lane*). An identical band is seen at 36 kDa in each lane (*arrow*), corresponding to the molecular weight of hH1FOO protein.



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washed three times in drops of PBS was added to 1.0 μL of 0.2% sarcosyl solution. After brief vortexing, the lysate was immediately frozen in liquid nitrogen and stored at −28°C until use. The lysate was subjected directly to RT-nested PCR as described previously (12), with use of primer pairs that flanked an intron to avoid amplification of genomic DNA.

Fluorescence Immunohistochemical Analysis of Oocytes and Embryos

Unfertilized oocytes and arrested embryos were fixed for 20 minutes at room temperature in a freshly prepared solution of 2% paraformaldehyde in PBS (pH 7.4). Fixed samples were then incubated for 1 hour at room temperature in blocking solution (PBS with 10% goat serum and 0.5% Triton X-100). After blocking, the oocytes or embryos were incubated for 1 hour at room temperature with the anti-hH1FOO antibody (diluted 1:1,500 with a solution of 0.5% Triton X-100 in PBS). Next, the specimens were washed three times in PBS for 15 minutes each at room temperature and incubated with Alexa Fluor 488 goat anti-rabbit IgG conjugate (diluted 1:1,000; Molecular Probes, Eugene, OR). After the specimens were washed again as above, DNA was stained with use of Hoechst 33342 dye (Molecular Probes). After another wash in PBS, each cell was pipetted into a drop of PBS and placed on a glass slide for examination with an Olympus AX70 microscope (Tokyo, Japan) that was equipped with the appropriate filter sets for epifluorescence studies.

RESULTS

Specificity of the Anti-hH1FOO Antibody

The specificity of the polyclonal anti-hH1FOO antibody was investigated by Western blotting of protein extracts from 30 unfertilized human oocytes and from ovarian tissues. In both the oocytes and the ovarian tissue samples, a single band was detected at 36 kDa (Fig. 1), which was consistent with the molecular weight calculated from the predicted amino acid sequence of hH1FOO.

Ovarian Localization of hH1FOO

Immunohistochemical analysis of ovarian tissue specimens with the polyclonal anti-hH1FOO antibody is shown in Fig. 2. The protein was localized in oocytes at all stages from primordial follicles to Graafian follicles, whereas preimmune serum did not react with any ovarian structures. There was no reactivity of the anti-hH1FOO antibody with granulosa cells or theca cells, confirming its specificity for hH1FOO as opposed to other histones. Although staining was more intense in the nucleus, the cytoplasm also was stained in all oocytes examined, indicating that cytoplasmic synthesis of hH1FOO is active from a very early stage of oogenesis and that this protein persists in the cytoplasm.

Expression of hH1foo mRNA and Protein During Oogenesis and Early Embryogenesis

Unfertilized human oocytes and arrested embryos were analyzed by RT-nested PCR and by fluorescence immunohistochemical analysis. As a result, hH1foo mRNA was detected at all stages from GV-stage oocytes to two-cell embryos as specific bands with a similar intensity (Fig. 3A). We confirmed that the nucleotide sequence of these bands was identical to that previously reported for hH1foo mRNA (12) (data not shown).

The protein also was detected in the chromatin of oocytes from the GV stage through to two-cell embryos (Fig. 3B). In these experiments, at least three oocytes or embryos at each stage were examined by RT-nested PCR and immunohistochemical analysis, with consistent results being obtained.

Replacement of Protamine by hH1FOO in Unfertilized Oocytes After ICSI and Sperm Decondensation

Thirty-three unfertilized oocytes were obtained after ICSI and were classified according to the reactivity of sperm chromatin with the hH1FOO antibody and the occurrence of sperm chromatin decondensation (from slightly to totally decondensed) (Fig. 4): [1] no immunoreactivity for the hH1FOO antibody and persistence of condensed chromatin (15 oocytes, 45%), [2] immunoreactivity for hH1FOO with persistence of condensed chromatin (3 oocytes, 9%), and [3] immunoreactivity for hH1FOO with decondensed chromatin (15 oocytes 45%). Interestingly, none of the oocytes contained sperm that showed decondensation but were nonreactive to the hH1foo antibody.

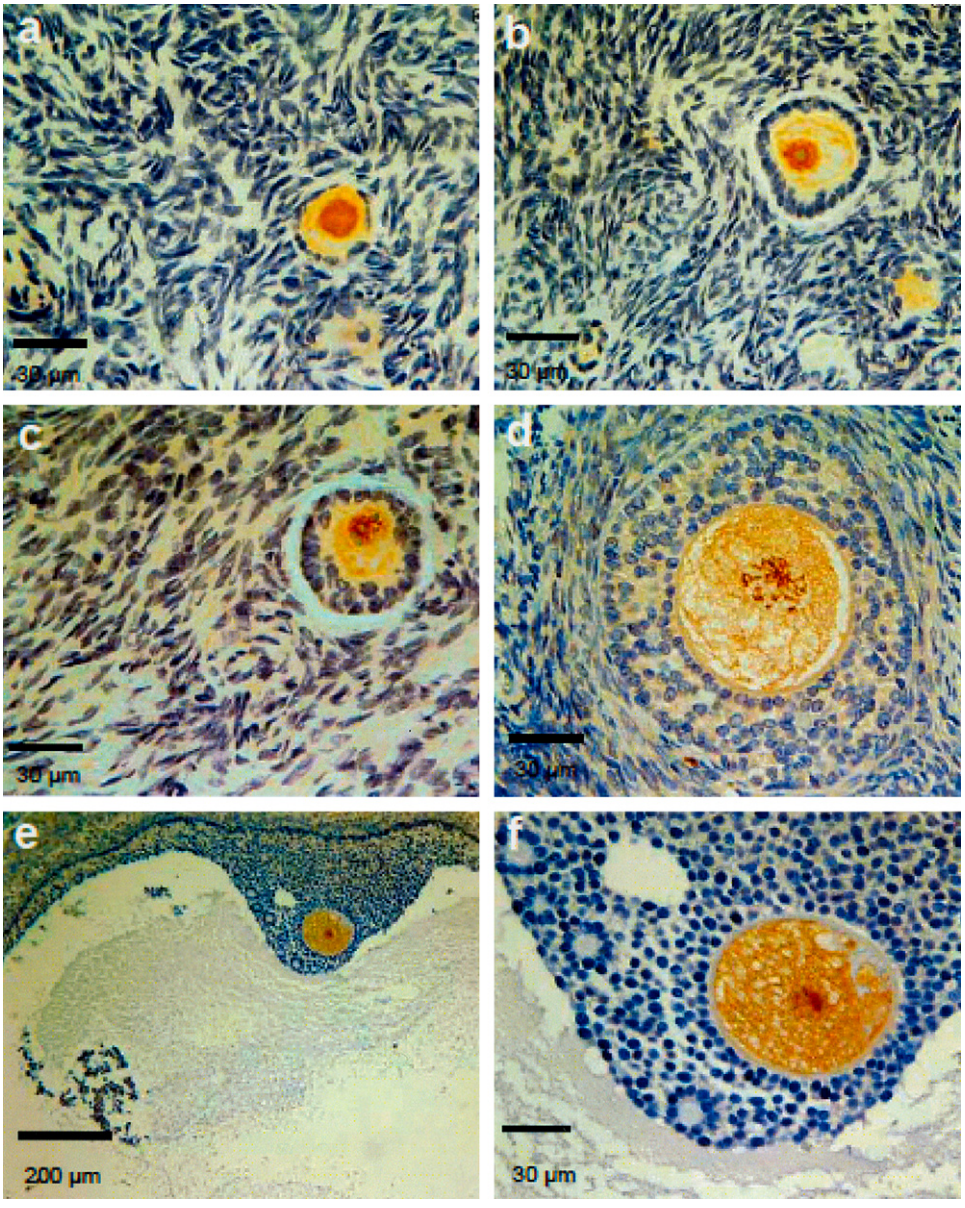
DISCUSSION

Role of H1FOO in Oocytes

H1FOO is thought to be the predominant H1 histone associated with chromatin during oocyte growth, oocyte maturation, and early embryogenesis. On entry of a sperm into an oocyte, protamine bound to sperm chromatin is replaced by maternal histones, including H1FOO, in a process that is referred to as protamine-histone replacement (18, 19). Concurrently, the highly condensed sperm chromatin undergoes

FIGURE 2

Immunohistochemical analysis for hH1fOO in ovarian sections. Human ovarian follicles are shown at various stages, including a primordial follicle (*a*), primary follicle (*b*), secondary follicle (*c*), preantral follicle (*d*), and Graafian follicle (*e, f*). Staining is detected in both the nucleus (orange or dark yellow) and the cytoplasm (yellow) of the oocytes, with its intensity being greater in the nucleus.



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extensive decondensation to form the male PN. In fact, animal studies have shown that development of the male PN from sperm chromatin requires the presence of maternal histones (21). In mice, H1FOO then is replaced by somatic H1 at the two- to four-cell stage of embryogenesis (6). Because this transition of H1 subtypes takes place simultaneously with the initiation of zygotic development, H1FOO is thought to be involved in the transition from maternal to embryonic gene expression, resulting in remodeling of the two PN and the onset of DNA replication (7).

To our knowledge, the present study is the first to demonstrate the expression of H1FOO protein in human oocytes. Immunohistochemical analysis of human ovarian tissues revealed hH1FOO protein expression in oocytes throughout oogenesis, even as early as the primordial follicle stage. This finding differs from a previous report that H1FOO immunoreactivity was limited to oocytes after the primary follicle stage in mice, with no immunoreactivity being detected in the oocytes of primordial follicles (although a few H1foo transcripts were noted in these oocytes) (22).

FIGURE 3

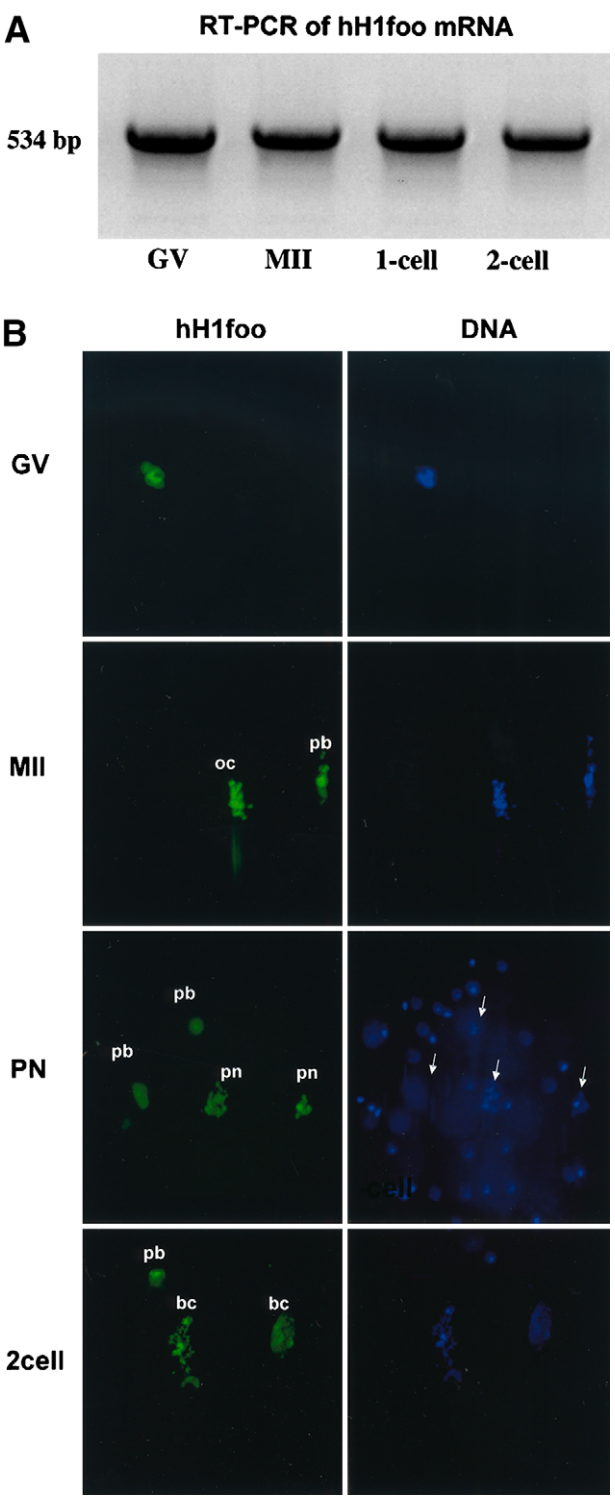

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FIGURE 3 Continued

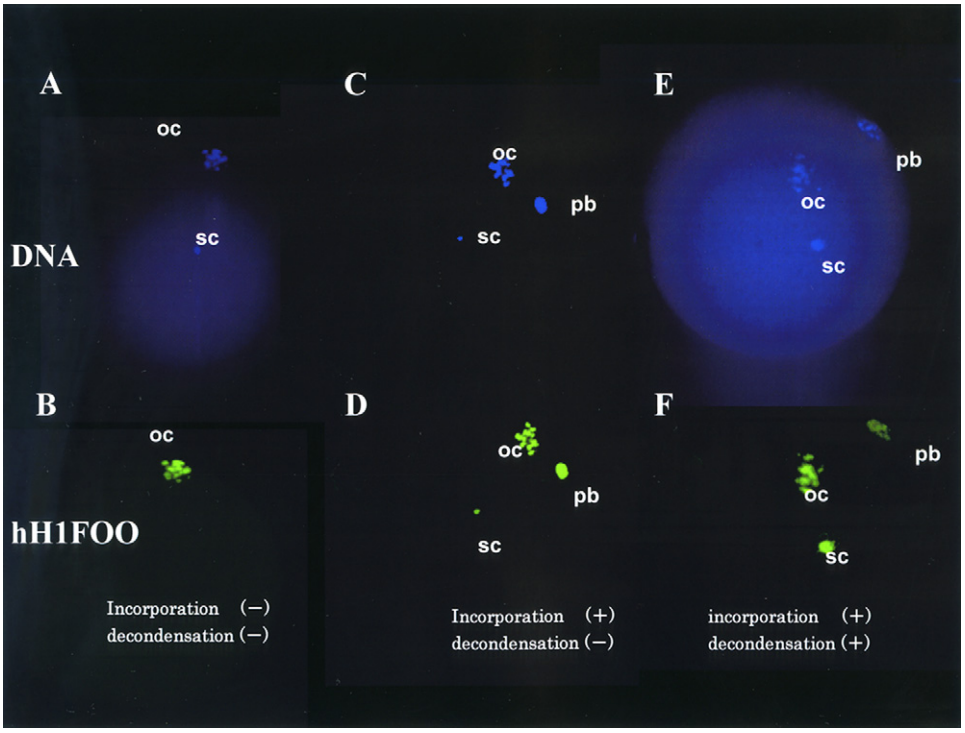
(**A**) Reverse transcription–nested PCR of hH1foo mRNA during oogenesis and early embryogenesis. hH1foo mRNA was detected at all stages from GV-stage oocytes to two-cell embryos as specific bands with a similar intensity. bp = base pair. (**B**) Fluorescence immunohistochemical analysis of an oocyte at the GV stage, an oocyte in MII, a PN oocyte, and a two-cell embryo (*2-cell*). The PN oocyte was obtained from IVF and ICSI, and the arrested 2PN oocyte was obtained after ICSI. The GV and MII oocytes were not exposed to sperm by either conventional IVF or ICSI. Deoxyribonucleic acid was visualized by Hoechst 33342 staining, and hH1FOO was detected by indirect immunofluorescence. hH1FOO protein can be detected clearly in the nucleus of the GV oocyte, MII oocyte, PN oocyte, and two-cell embryo. All polar bodies also were positive for both anti-hH1FOO antibody and Hoechst 33342. All of the sperm surrounding the PN oocyte, four-cell embryo, and morula were unstained by the anti-hH1FOO antibody (some are indicated by *arrows*). oc = oocyte chromatin; pb = polar body; pn = pronucleus; bc = blastomere chromatin.

On the basis of such findings, those authors proposed that expression of the H1foo gene might be coupled to the process of primordial follicle recruitment. On the other hand, Clarke et al. (23) reported that somatic H1 immunoreactivity, which is absent in growing oocytes, was very weak in nongrowing oocytes within the primordial follicles of adult mouse ovaries. Although the report of Clarke et al. suggested that the subtype transition from somatic H1 to oocyte-specific H1 may occur shortly after birth in all oocytes of the ovary (including those in the primordial follicles), further studies will be necessary to clarify the ontogeny and role of hH1FOO during oogenesis.

In the present study, preovulatory oocytes in human ovarian tissue samples showed hH1FOO immunoreactivity in both the nucleus and cytoplasm (Fig. 2), although staining of the nucleus was much stronger than that of the cytoplasm. It has been suggested that mouse H1FOO is mainly localized inside the germinal vesicles, because mouse H1FOO protein contains nuclear localization sequences but has no nuclear export sequences (22). On the other hand, McGraw et al. (24) found that H1FOO immunoreactivity not only was associated with the chromatin in bovine GV-stage oocytes but was also detected in the ooplasm, although staining of the cytoplasm was less intense compared with that of the chromatin. It also has been reported that after removal of the GV nucleus, H1FOO was detected in the somatic nucleus of oocytes after nuclear transfer (7), suggesting the existence of H1FOO in the ooplasm. On the basis of these findings, it seems that H1FOO protein is primarily localized in the nucleus in oocytes, although there is also a relatively abundant cytoplasmic pool.

FIGURE 4

Fluorescence immunohistochemical analysis of oocytes that were not fertilized by ICSI. These oocytes display three patterns of sperm chromatin immunoreactivity with the hH1foo antibody: [1] no immunoreactivity and no decondensation (*A, B*), [2] immunoreactivity without decondensation (*C, D*), and [3] immunoreactivity with decondensation (*E, F*). oc = oocyte chromatin; pb = polar body; sc = sperm chromatin.



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Early Incorporation of H1FOO Into Sperm Chromatin After Penetration of the Oocyte

Studies in animals have shown that H1FOO is assembled on sperm chromatin almost immediately after fertilization. For example, H1FOO was detected on condensed sperm chromatin only 5 minutes after ICSI in metaphase II (MII)–arrested mouse oocytes (6). In pigs, incorporation of H1FOO into the sperm chromatin has been reported to precede decondensation after penetration of the ooplasm (25, 26). The assembly of H1FOO on sperm chromatin during decondensation also seemed to occur very quickly in humans, because all swollen sperm heads (even those that were only slightly swollen) revealed hH1FOO immunoreactivity in this study. We found a small number of unfertilized oocytes that contained condensed sperm heads with hH1FOO immunoreactivity (3/18 unfertilized oocytes containing condensed sperm heads), which also indicated that hH1FOO assembly might occur very early in the decondensation process.

A previous cytogenetic study of unfertilized human oocytes after ICSI revealed various outcomes of the injected sperm: [1] the entire sperm was missing or was ejected outside the ooplasm, [2] sperm chromatin was undecondensed, or [3] there was variable premature chromosome condensation (27). Among these outcomes, failure of decondensation may arise from incomplete immobilization of sperm due to insufficient damage to the sperm plasma membrane. However, there has been no way to determine whether sperm have reacted with the ooplasm. Therefore, we have been unable to assess whether condensed sperm heads had not reacted because the plasma membrane was not sufficiently damaged or had reacted but then failed to decondense. This latter situation is thought to arise from chromatin anomalies (28) or intrinsic oocyte defects. In this context, the performance of immunostaining for hH1FOO might provide valuable information about the cause of failed fertilization after ICSI. Because hH1FOO exclusively exists in oocytes and not in the sperm nucleus, hH1FOO immunoreactivity might be able to distinguish sperm that have started to react with the ooplasm and then failed to proceed to decondensation from membrane-intact and totally unreactive sperm, although our sample size was too small to draw a firm conclusion.

There are two possible explanations for the existence of hH1FOO-positive, condensed sperm nuclei. The first is that hH1FOO has been assembled, but decondensation has not occurred because the fertilization process was arrested at a very early stage. The second possibility is that these nuclei

underwent decondensation, but then recondensed. In mice, natural fertilization leads to initial enlargement and dispersion of protamine, after which the decondensed sperm head decreases in size (recondensation) and then gradually enlarges to form the male PN. It has been proposed that the recondensation of histone-containing sperm chromatin is due to a high level of metaphase-promoting factor activity and that oocyte activation causes this activity to decline to its basal level, resulting in PN formation (29). Indeed, in nonactivated oocytes of pigs, there is a time-dependent increase in the percentage of recondensed sperm nuclei (30). Sperm may fail to decondense because the oocyte lacks decondensing activity. It has been shown that mammalian oocytes possess sperm decondensing activity for only a short period after activation, and sperm introduced into mouse oocytes at any time later than the first cell cycle do not undergo decondensation (31–33). Presumably, the ability of human oocytes to promote the decondensation of sperm is also time limited, because Dozortsev and De Sutter (34) reported a high incidence of slightly swollen sperm in the cytoplasm of 1PN (i.e., activated) oocytes. Although there currently is no way to decide which of these two explanations is correct, further observation of sperm chromatin in nonactivated (MII) oocytes, activated (1PN) oocytes, and chemically (e.g., puromycin) activated unfertilized oocytes might help to clarify the reason for the existence of condensed and histone-positive sperm nuclei.

In the present study, we did not find any decondensed sperm head without H1FOO immunoreactivity. However, it would be interesting to search for such an alternative mechanism of failed fertilization after human ICSI in a study with a larger sample size, along with performing experiments on H1FOO knockout mice. So far, there have been no reports about the detection of decondensed sperm heads without H1FOO immunoreactivity in animals, but such sperm might represent an alternative mechanism of failed fertilization after ICSI.

In conclusion, we demonstrated the exclusive expression of H1FOO protein in human oocytes from those in primordial follicles through to early embryogenesis. During the process of fertilization after ICSI, hH1FOO seemed to be rapidly incorporated into the injected sperm nucleus. Because sperm that remained condensed after ICSI could be separated into two categories based on H1FOO incorporation or nonincorporation, immunostaining for H1FOO might provide valuable information about the factors leading to failed fertilization after ICSI.

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