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# Structure and expression of the human oocyte-specific histone H1 gene elucidated by direct RT-nested PCR of a single oocyte

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**Abstract**

Oocyte-specific histone H1 is expressed during oogenesis and early embryogenesis. It has been described in mice and some nonmammalian species, but not in humans. Here, we identified the cDNA in unfertilized human oocytes using direct RT-nested PCR of a single cell. Sequencing of this cDNA indicated an open reading frame encoding a 347-amino acid protein. Expression was oocyte-specific. Homology was closest with the corresponding gene of mouse (H1oo; 42.3%), and, to lesser extent, with that of *Xenopus laevis* (B4; 25.0%). The gene, named osH1, included five exons as predicted by the NCBI annotation project of the human genome, although the actual splicing site at the 3′ end of exon 3 was different by 48 nucleotides from the prediction. The presence of polyadenylation signals and successful amplification of cDNA by RT-PCR using an oligo(dT) primer suggested that the osH1 mRNA is polyadenylated unlike somatic H1 mRNA. Our technique and findings should facilitate investigation of human fertilization and embryogenesis.


*Keywords:* Human; Oocyte; Oocyte-specific histone H1

Histones are the major protein constituents of chromatin in eukaryotic cell nuclei. Two types of histone proteins exist: the core histones H2A, H2B, H3, and H4 and the linker histone H1. An octamer of the core histones intertwined with 146 bp of DNA forms a component of the nucleosome core particle. The linker histone H1 is involved in sealing DNA joining adjacent nucleosome core particles to maintain higher-order packaging of chromatin. In mammals, each of these classes except for H4 is subdivided into several subtypes [1]. The most divergent class of histones is the H1 protein family, which consists of eight subtypes in mammals: the somatic variants H1a, H1b, H1c, H1d, H1e, and $H1^0$; the testis-specific variant H1t; and the oocyte-specific variant H1oo [2–6].

Oocyte-specific H1 is characteristic in a number of ways. First, it is expressed only during oogenesis and early embryogenesis and then replaced by somatic H1 subtypes shortly after fertilization [6–8]. This replacement may loosen the condensed chromatin structure, resulting in a change in transcriptional pattern from the oocyte to the embryo [9–11]. Second, unlike most other subtypes, it contains both introns and a poly(A) tail, and lacks characteristic motifs in the promoter regions that are shared by the other H1 genes [12]. Finally, in comparison with other subtypes, sequence homology of oocyte-specific H1 genes between different organisms are relatively low.

All H1 subtypes except for oocyte-specific H1 have been characterized at the levels of protein and gene in both humans and mice [5,6]. Oocyte-specific H1 has been identified in four species; csH1 in the sea urchin [13], B4 in *Xenopus laevis* [7,14], H1M in the zebrafish [15], and H1oo in the mouse [6]; but it has not yet been identified in humans. Oocyte-specific H1 may play an important role in forming the male pronucleus during fertilization, since remodeling of sperm chromatin requires replacement of protamine by oocyte-specific H1. Thus, identification and characterization of the human

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oocyte-specific H1 gene would be prerequisite for elucidation of human infertility.

Both clinical and technical constraints limit genomic and protein investigation in human oocytes. Since oocytes usable for research are limited to those failing to initiate cleavage for more than 2 days after retrieval, the amount of available sample is absolutely insufficient for the conventional analysis of gene expression at the molecular level. Therefore, we developed a new method for investigation of gene expression in a single oocyte and used it to identify and characterize the human oocyte-specific histone H1 gene.

## Materials and methods

All procedures were approved by the Ethics and Legal Committee of Keio University Hospital. Specimens used in our research were obtained from informed and consenting patients with written agreements.

*Cells*. Human oocytes were retrieved from infertile women by routine methods to carry out intracytoplasmic sperm injection (ICSI) according to the standard procedure at the Infertility Clinic of Keio University Hospital. After incubation for a few hours, a spermatozoon was injected into each oocyte. If cell cleavage was not initiated after at least 48 h, an oocyte was considered unfertilized and used in our program if the donor couple approved.

Specimens of normal human ovarian tissue were obtained from consenting women of reproductive age who were undergoing oophorectomy in the course of surgery for uterine cervical cancer or uterine leiomyoma at Keio University Hospital. Human ovarian granulosa cells were collected from aspirated follicular fluid during the oocyte retrieval procedure. Briefly, the fluid was centrifuged at 1500 rpm for 5 min. The cell pellet was washed twice by suspension and centrifugation.

MOLT-4 cells (a human T-cell line) were maintained at 37 °C in RPMI 1640 medium supplemented with 10% fetal bovine serum, 0.1 M L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin in an atmosphere containing 5% $CO_2$.

Human semen was obtained with consent from healthy men who had come to the outpatient clinic to have their sperm examined. The semen was processed using polyvinylpyrrolidone-coated colloid media for purification of sperm.

*RNA preparation*. RNA in a single human oocyte was analyzed directly by our newly established method. An unfertilized oocyte selected from sperm-injected oocytes by aspiration was transferred into 1.0 μl of 0.2% sarcosyl solution to give a final volume of 2.0 μl. This concentration of sarcosyl was enough to break down cell membrane of oocytes without inhibiting reverse transcription after a 10-fold dilution. After suspending and agitation, the cell lysate was immediately frozen in liquid nitrogen and then stored at −28 °C in a deeper freezer until use. The lysate was directly subjected to RT-nested PCR as described below. Total RNA was isolated from human ovarian tissue, human ovarian granulosa cells, MOLT-4 cells, and sperm using the RNAeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Isolated RNAs were stored at −28 °C. RNAs from other human tissues (breast, colon, kidney, liver, lung, skeletal muscle, and spleen) were purchased from Stratagene (CA, USA).

*RT-nested PCR*. Sixteen primers were designed according to the genomic sequence [AC080007; *Homo sapiens* 3q BAC RP11-529F4 (Roswell Park Cancer Institute Human BAC Library)] and the mRNA sequence [XM_067747; the gene similar to the H1 histone family (oocyte specific)] predicted by the NCBI (National Center for Biotechnology Information) annotation system (Table 1). The RNA was converted to cDNA using ThermoScript reverse transcriptase (Invitrogen, CA, USA) and RNAsin (Promega, WI, USA) according to the manufacturer's instructions. The cDNA was then amplified by nested PCR using AmpliTaq (Applied BioSystems, NJ, USA) and PCRx Enhancer System (Invitrogen) to attain efficient amplification of GC-rich sequences according to the manufacturer's instructions. The amplification steps involved 30 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min. PCR products were analyzed by electrophoresis in 1.5% agarose gels. All the primers listed in Table 1 were used for each oocyte. For RNAs of ovarian granulosa cells, sperm, MOLT-4, and human tissues, primers used were GM1D for RT; GM1D and MR3U in first-round PCR; and GM2D and MR4U in second-round PCR. As a control, RT-PCR of β-actin was performed with primers, 5′-CACTCTTCCAGCCTTCC TTCC-3′ and 5′-CGGACTCGTCATACTCCTGCTT-3′, which were designed according to the previously reported data [16]. The second-round PCR was not performed for the β-actin controls.

Table 1
Sequences of primers designed for RT-nested PCR

| Primers | Sequence |
|---|---|
| MR3U | 5′-GGTGAGGGGTCTGCTGGCTG-3′ |
| MR4U | 5′-GTCTGCTGGCTGCACCTGTC-3′ |
| GM2U | 5′-GGAGGCCCGAGCCACAGCAG-3′ |
| GM2BU | 5′-TCCCGGTGGGACGCCGCCAC-3′ |
| GM2D | 5′-CGAGGAGGCCACGGCGCATG-3′ |
| GM1D[a] | 5′-CAGTGGCCCCCTGGCTTTG-3′ |
| MR5U | 5′-AATCCAGCCCAGGAAGATGG-3′ |
| MR6U | 5′-CTCCCAGGAGAGCGGGTGAG-3′ |
| MR4D | 5′-CACCTTGCCCACGTTGGGAG-3′ |
| MR3D[a] | 5′-CTTTGCTGGCCTCTTGGCTG-3′ |
| MR7U | 5′-GGCAGGGGCCAAACACCAAG-3′ |
| MR8U | 5′-TGCTCCTGCTAAGGGCAGTG-3′ |
| MR2D | 5′-GATGAGGCCTTGATGGGCAG-3′ |
| MR1D[a] | 5′-CTTCAGCCCTCTGGCTGGAC-3′ |
| MR8D | 5′-TAGGGCAGAGGCTCGGTCTC-3′ |
| MR7D[a] | 5′-AGAGCAGTGGTTAGTTGAAG-3′ |

[a] Primers GM2D, MR1D, MR3D, and MR7D were used for reverse transcription.

*Sequencing*. PCR products were sequenced by Takara (Shiga, Japan) using a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, NJ, USA).

*Accession Number*. AY158091 for the oocyte-specific histone H1 gene.

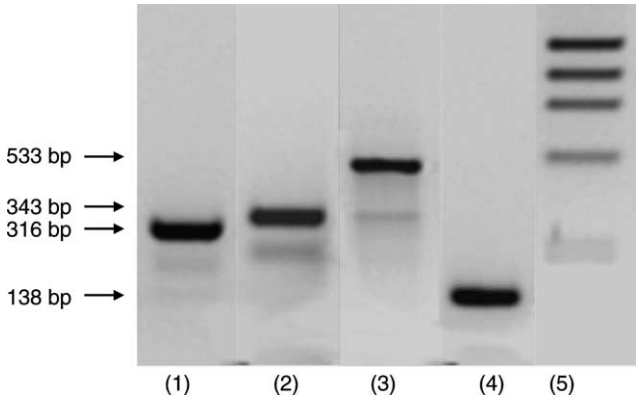


Fig. 1. Products of RT-nested PCR of a single human oocyte. Primer pairs used (Table 1) are: (1) MR3U/GM1D, MR4U/GM2D; (2) GM2U/MR3D, GM2BU/MR4D; (3) MR5U/MR1D, MR6U/MR2D; and (4) MR7U/MR7D, MR8U/MR8D. An *Hae*III digest of ΦX DNA is used for molecular weight markers (5). A contig of cDNA sequence was established from the DNA sequences of the four products.

## Results

### *Identification of H1 mRNA from a single human oocyte*

Since few human oocytes are available for our experiments, we chose RT-nested PCR rather than Northern blotting in order to identify oocyte-specific histone mRNA. The lysate of a single oocyte was directly subjected to RT-nested PCR using four sets of primers, whose sequences were determined based on a putative human oocyte-specific gene (Table 1). Discrete bands with expected lengths were detected from any sets of primers (Fig. 1). When either of sequence-specific primer MR7U and oligo(dT) primer was used for reverse transcription, the primer set of MR7D/MR7U and MR8D/MR8U produced an identical cDNA sequence (data not shown). The DNA bands obtained were considered to have been derived from mRNA but not from genomic DNA because each of a primer set is located in each of an exon pair flanking a long intron. A contig was constructed from DNA sequences of the PCR products, giving a 1067-bp molecular sequence with a 1041-bp open reading frame (Fig. 2). We designated this gene as osH1. The initiation codon was assigned as shown in Fig. 2 because it is the first ATG downstream of the TATA box on the putative genomic DNA. The osH1 gene encoded a 35.8-kDa protein of 347 amino acids. According to the GenBank genomic sequence data, the gene includes five exons and is located on 3q21-22 in contrast with the somatic H1 genes which are located in a large cluster on the short arm of chromosome 6.

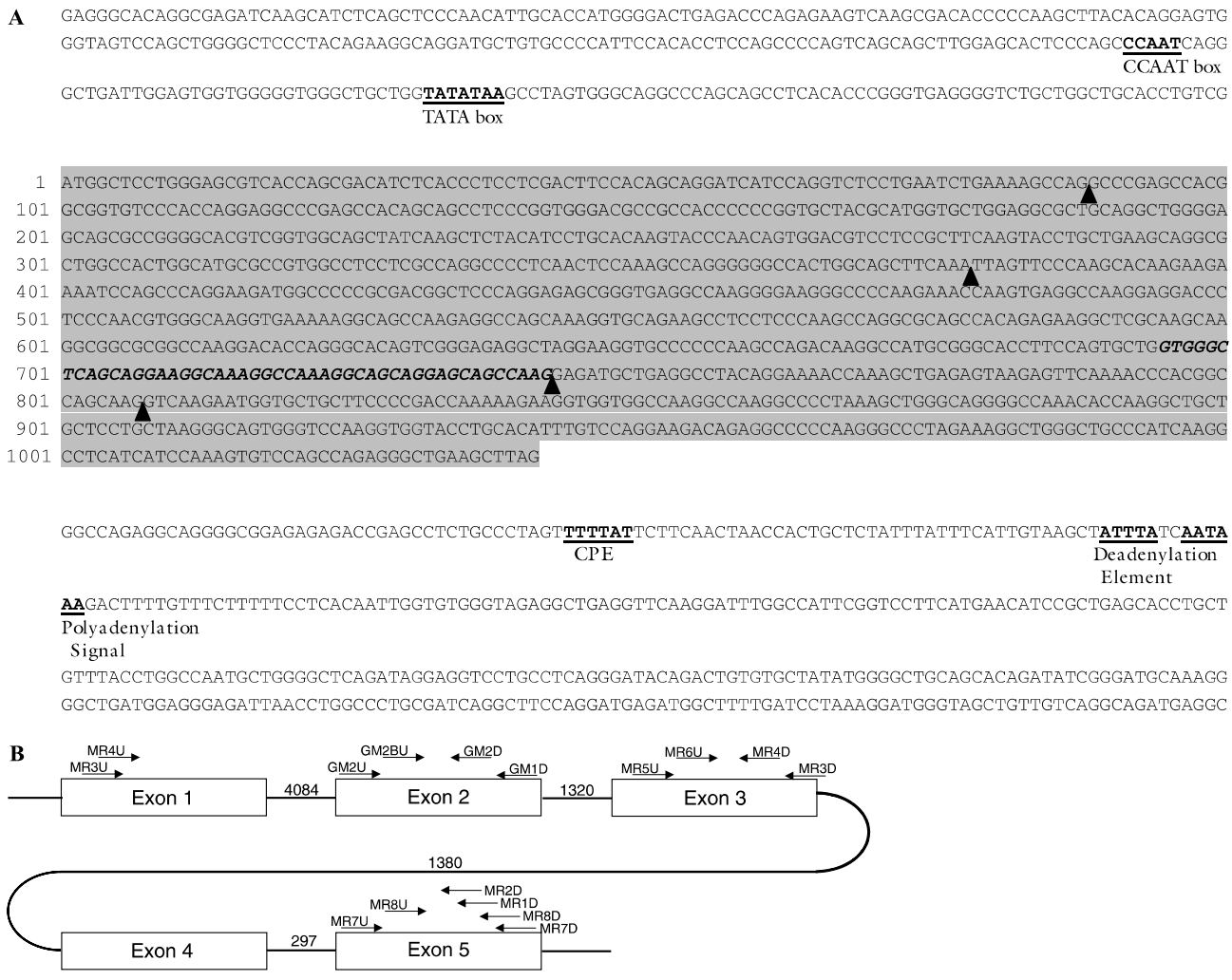


Fig. 2. The osH1 gene. (A) Sequence of the osH1 cDNA. Numbering starts from the initiation codon. The gray-shaded sequence is the open reading frame. The unshaded sequences are the UTR at both ends and the promoter region. Splicing sites are shown in triangles. A sequence of 48 nucleotides not present in the NCB-predicted version is shown in bold italics. The CCAAT box, the TATA box, the cytoplasmic polyadenylation element (CPE), the deadenylation element, and the polyadenylation signal are indicated as bold letters with underlines. (B) Schematic presentation of exons and introns of osH1 in the genomic sequence. Exons are indicated as open boxes. Numbers between exons indicate length of introns. Locations of all primers employed for RT-nested PCR are indicated.

*Homology with oocyte-specific H1 of other species*

Alignment of amino acid sequences of oocyte-specific H1 of human and other species was performed with the assistance of CLUSTAL W software (version 1.8) [17] (Fig. 3). The osH1 gene exhibited a 42.3% identity score with H1oo (mouse), 25.0% with B4 (*X. laevis*), 25.1% with H1M (zebrafish), and 22.5% with csH1 (sea urchin). In contrast, homology of osH1 with H1b (somatic subtype) and H1t (testis-specific subtype) was only 18.5% and 15.3%, respectively (Table 2). In general, linker histones of the H1 variety possess a three-domain structure including a central globular domain (consisting of Helix I, II, and III) flanked by amino- and carboxy-terminal domains. Sequence homology was greatest in the central globular domain, consistent with the observation that linker DNA interacts with the central globular domain of H1 protein [5,6,12]. When homology analysis was limited to the globular domain, osH1 shared 74.3%, 57.1%, 45.1%, and 38.9% identity with the corresponding regions of H1oo, B4, H1M, and csH1, respectively.

*Oocyte-specific expression of osH1*

RT-nested PCR was performed for human ovarian tissue and ovarian granulosa cells. In the ovarian tissue, osH1 cDNA was detectable up to a 25-fold dilution, while β-actin was detectable up to a $5^6$-fold dilution. In ovarian granulosa cells, however, osH1 cDNA was not

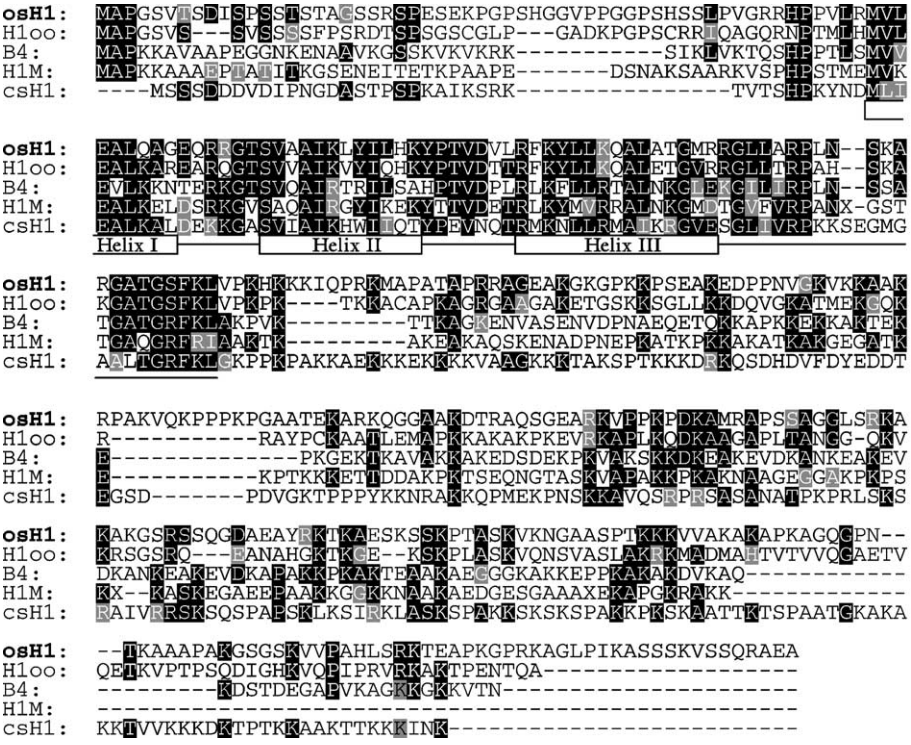


Fig. 3. Amino acid sequence alignment of osH1 with other oocyte-specific H1s. The amino acid sequences of other oocyte-specific H1s were extracted from the GenBank database (for Accession number, see Table 2). Identical amino acid residues are shaded black; similar amino acids are shaded bright gray; and unrelated residues are unshaded. The globular domains are underlined (Helix I, II, and III).

Table 2
Sequence similarity of osH1 protein to oocyte-specific H1 of other species and other subtypes of human H1

| Protein | Sequence identity (%) | Gaps | Amino acid overlap |
|---|---|---|---|
| H1oo (mouse) | 42.3 | 47 | 148 |
| H1M (zebrafish) | 25.1 | 87 | 85 |
| B4 (*Xenopus laevis*) | 25.0 | 74 | 87 |
| csH1 (sea urchin) | 22.5 | 47 | 78 |
| H1t (human) | 15.3 | 129 | 52 |
| H1b (human) | 18.5 | 127 | 64 |
| $H1^0$ (human) | 19.6 | 116 | 68 |

Alignment was performed with the assistance of CLUSTAL W software (version 1.8). Accession numbers for each proteins are: AY007195 (H1oo), X13855 (B4), AF499607 (H1M), AAB48830 (csH1), NP_005314 (H1t), and NP_005312 (H1b).

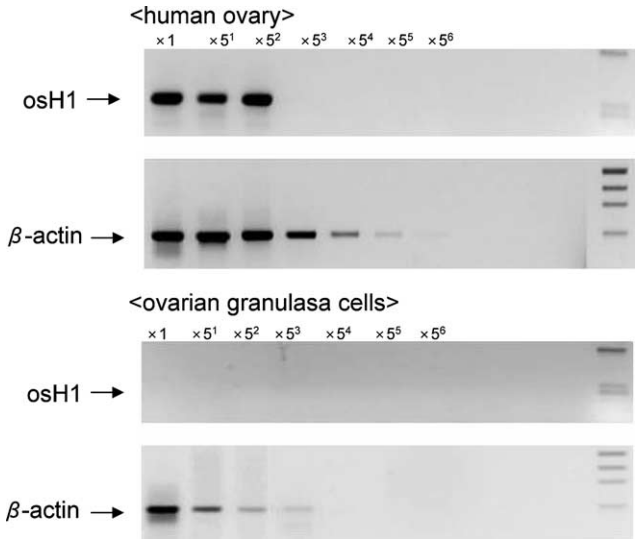


Fig. 4. Expression of osH1 and β-actin mRNA in human ovary and ovarian granulosa cells. RT-nested PCR for osH1 was performed using the primer pairs GM1D for reverse transcription, GM1D/MR1U for the first-round PCR, and GM2D/MR2U for the second-round PCR. RT-PCR for β-actin mRNA was performed as described in Materials and methods.

detected even in the undiluted RNA sample, while β-actin was detectable up to a 125-fold dilution (Fig. 4). The sequence of the cDNA product was identical to that of osH1 (data not shown).

No osH1 mRNA was detected by RT-nested PCR in 10 μg of total RNAs from any human tissues tested (breast, colon, kidney, liver, lung, skeletal muscle, spleen, lymphocytes, and sperm). In contrast, β-actin mRNA was detected up to a 125- or 625-fold dilution of 10 μg of total RNA by RT-PCR (Fig. 5). It must be noted that the second-round PCR was performed for osH1 but not for β-actin; therefore, the former assay was much more sensitive than the latter.

## Discussion

Heterogeneity of histone H1 was first described in 1966 [18]. At the present time, eight subtypes of histone H1 have been identified in mammals. Five of these are collectively called the somatic types, which are designated H1a, H1b, H1c, H1d, and H1e [2–6]. Subtypes from H1b to H1e are present in all somatic cells, while H1a is restricted to the thymus, testis, spleen, and possibly lymphocytes and neurons [19]. They share a highly conserved central globular domain and exhibit variation in their amino- and carboxy-terminal domains [20]. The sixth subtype, known as $H1^0$ or H5, is differentiation stage-specific and has a divergent sequence in the globular domain and the tails compared with somatic subtypes [20]. The remaining two subtypes are known as tissue-specific; testis-specific H1 (H1t) and oocyte-specific H1 (H1oo in mouse) [6,21,22]. H1oo shows little homology with other somatic subtypes over the full length, while H1t exhibits some homology in the globular domain [23].

To date, the oocyte-specific histone H1 gene has been reported in four species of organisms; csH1 in the sea urchin, B4 in *X. laevis*, H1M in the zebrafish, and H1oo in the mouse [6,7,13,14]. Presence of a human homo-

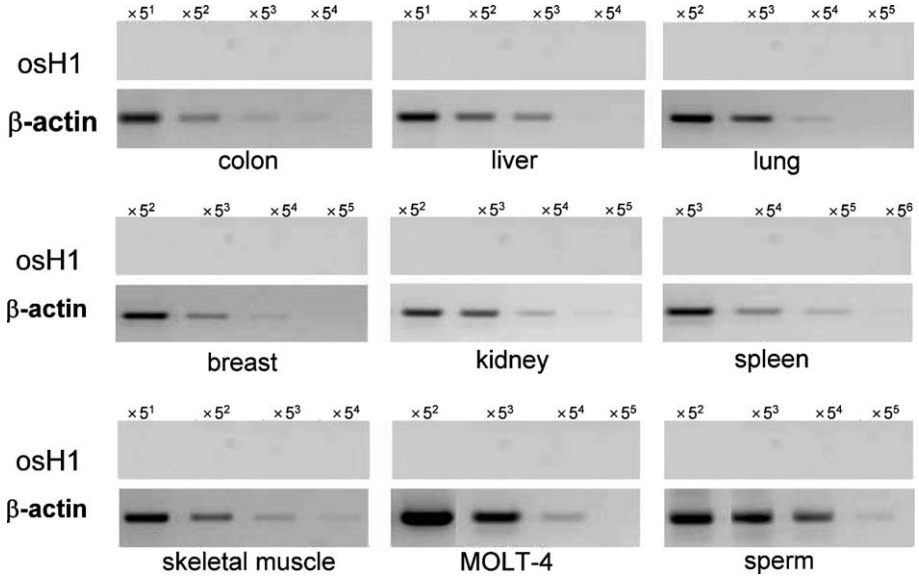


Fig. 5. Tissue localization of osH1 transcripts studied by semi-quantitative RT-nested PCR. Total RNAs from human colon, liver, lung, breast, kidney, spleen, skeletal muscle, MOLT-4 cells, and sperm were diluted fivefold sequentially. All original solutions contained 10 μg of total RNA. β-Actin was detectable up to a $5^4$ dilution in colon, $5^4$ in liver, $5^5$ in lung, $5^5$ in breast, $5^5$ in kidney, $5^6$ in spleen, $5^4$ in skeletal muscle, $5^4$ in MOLT-4 cells, and $5^4$ in sperm. osH1 was undetectable in any of these samples.

logue of the mouse oocyte-specific H1 gene was recently predicted by the NCBI annotation system, which had not been proved in vivo, however. In the present study, using our method for single-cell analysis, we found transcripts of the predicted gene encoding an oocyte-specific linker histone H1 (osH1), as a 347-amino acid protein with a calculated molecular mass of 35.8 kDa. We believe that the osH1 gene belongs to the gene family of oocyte-specific histone H1 for the following reasons. (1) The mRNA was expressed only in the human oocyte and ovary. (2) The encoded protein had the closest homology with H1oo in the mouse, and to lesser extent, with B4 in *X. laevis*. The oocyte-specific H1s are the longest H1 histones described to date [6]; even among these, osH1 appeared exceptionally long (cf. H1oo, 304 amino acids; B4, 273; and csH1, 299).

We identified cDNA of the human oocyte-specific H1 gene with PCR primers designed according to the gene structure that had been predicted by the NCBI annotation project from the human genomic sequence through a gene prediction algorithm, GenomeScan. The cDNA that we obtained was longer than the predicted cDNA by 48 nucleotides at the 3′ end of exon 3 (Fig. 2A). In other words, the actual 5′ splice site of intron 3 is located 48 nucleotides downstream of the predicted one. GenomeScan was developed in 2001; its sensitivity and specificity both have been estimated to exceed 80%, which are better than those of any other gene prediction programs [24,25]. Howsoever the accuracy of programs, our result indicated that exact sequence of a computer-predicted gene should be determined by experiments at the molecular level.

Generally, tissue-specific H1 subtypes are less conserved among organisms than somatic H1 subtypes. For instance, in comparisons between the human and mouse genes, H1b (somatic subtype) and H1t (testis-specific subtype) exhibited homologies of 70.1% and 59.7%, respectively [21,22]. Even when considering these scores, the homology score of 42.3% between mouse and human oocyte-specific H1 (osH1 and H1oo) determined in this study was remarkably low. Oocyte-specific H1 is thought to bind less tightly to linker DNA than the other H1 subtypes because its net charge is less basic [12,14]. Stronger binding of oocyte-specific H1 with linker DNA could interfere with its replacement by somatic H1 during embryogenesis. This need for less stringent interaction may allow oocyte-specific H1 to acquire greater variation.

The cDNA sequence of oocyte-specific H1 involved only 6 and 21 bp in the 5′ and 3′ untranslated regions (UTR), respectively. The remainder of both the UTR and the promoter region, however, can be deduced from the genomic sequence in GenBank. Somatic H1 genes generally harbor TATA boxes, CCAAT boxes, H1-boxes (AAACACA), and TG boxes (TGTGT/CTA) in the promoter [26,27]. Although the oocyte-specific H1 genes of nonhuman species lack such promoter sequences [12], the 5′ flanking region of the osH1 gene contains two of these promoter elements, the TATA box at position −67 and the CCAAT box at −108, with position 1 corresponding to the initiation of translation. On the other hand, the 3′ UTR of both osH1 and H1oo contain the consensus polyadenylation signal (AAUAAA), the cytoplasmic polyadenylation element (UUUUAU), and the deadenylation element (AUUUA) (Fig. 2A). In our analysis, osH1 cDNA was detected by RT-nested PCR using an oligo(dT) primer for reverse transcription. These findings indicated that the osH1 mRNA is polyadenylated like other oocyte-specific H1 mRNAs, in contrast with a lack of polyadenylation in somatic H1 mRNAs.

In the process of normal fertilization, the condensed state of the sperm chromatin is loosened by replacement of protamine by oocyte-specific H1, followed by formation of the male pronucleus, which eventually leads to cleavage [28]. In the clinical practice of in vitro fertilization for infertile couples, some oocytes do not initiate cleavage even after intracytoplasmic sperm injection, thus remaining unfertilized. Morphological studies have shown that some of the unfertilized oocytes terminate the fertilization process at the stage of chromatin decondensation of sperm, suggesting that inadequate replacement of protamine by osH1 is a cause of fertilization failure [29]. Accordingly, further investigation of osH1 may provide new understanding of human fertilization and embryogenesis.

In conclusion, cDNA encoding a human oocyte-specific linker histone H1, osH1, was identified for the first time in human oocytes using a novel method for single-cell analysis of gene expression that was developed in the present work.

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