H1foo is Indispensable for Meiotic Maturation of the Mouse Oocyte

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Abstract. Oocyte-specific linker histone H1foo is localized in the oocyte nucleus, either diffusely or bound to chromatin, during the processes of meiotic maturation and fertilization. This expression pattern suggests that H1foo plays a key role in the control of gene expression and chromatin modification during oogenesis and early embryogenesis. To reveal the function of H1foo, we microinjected antisense morpholino oligonucleotides (MO) against H1foo into mouse germinal-vesicle stage oocytes. The rate of in vitro maturation of the antisense MO group was significantly lower than that of the control group. Eggs that failed to extrude a first polar body following injection of antisense MO arrested at metaphase I. Additionally, co-injection of in vitro synthesized H1foo mRNA along with antisense MO successfully rescued expression of H1foo and improved the in vitro maturation rate. There was no difference in the rate of parthenogenesis between the antisense MO and control groups. These results indicate that H1foo is essential for maturation of germinal vesicle-stage oocytes.

Key words: H1foo, Linker histone, Meiotic maturation, Morpholino, Mouse, Oocyte

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In eukaryotes, the basic chromatin unit is commonly defined as the nucleosome, which is composed of DNA wrapped in two turns around an octamer of core histone proteins [1]. Association of DNA with the histone octamer to form nucleosomes is the first level of chromatin condensation and results in a configuration commonly referred to as “beads on a string”. The DNA between the beads is referred to as linker DNA. Linker histones, termed H1, bind to linker DNA and facilitate a shift in equilibrium towards more condensed, higher order forms of chromatin. Mammals express eight H1 subtypes, including H1fa through H1fe and H1f0, which are found in somatic cells in addition to germ cell-specific H1ft and H1foo [2–4]. Expression of each mammalian H1 subtype depends on the tissue, phase of the cell cycle and developmental stage. Knockout of individual H1 subtype genes in mice is compensated for by increased expression of the remaining subtypes [5]. However, simultaneous inactivation of multiple H1 subtype genes causes embryonic death and therefore linker histones are essential for mammalian development [6].

H1foo is a linker histone specifically expressed in murine oocytes [4]. Oocyte-specific linker histones...
have been found across a wide range of species, including the sea urchin, frog, zebrafish, mouse and human, and they are very well conserved in all species. We have previously shown that mouse H1foo is localized to the nucleus of oocytes at the germinal vesicle (GV) stage, the chromatin during metaphase-II (MII), and the first polar body. After fertilization, H1foo is also detected in the swollen sperm head and second polar body. Expression of H1foo is somewhat reduced in two-cell embryos and is not detectable in four-cell embryos. In Xenopus, oocyte-specific linker histone B4 is replaced by H1 during the midblastula transition [7, 8]. The expression pattern of H1foo in preimplantation embryos is similar to that of Xenopus B4. Furthermore, replacement of the somatic linker histone with H1foo occurs rapidly during nuclear transfer in the mouse [9, 10]. The greater mobility of H1foo compared with H1 is thought to contribute to this rapid replacement, which provides flexibility for the chromatin structure. These findings suggest that oocyte-specific linker histones play a key role in control of gene expression and chromatin modification during oogenesis, early embryogenesis and nuclear remodeling. However, the function of H1foo remains to be elucidated.

Reverse genetic approaches have become increasingly attractive in developmental biology because they represent an efficient means of identifying the roles of interesting candidate genes from genomic databases. Recently, one strategy for inhibiting gene function was shown to be extremely effective. Antisense morpholino oligonucleotides (MO) were first developed for clinical therapeutic applications, an area in which previous antisense approaches have proven to be seriously flawed [11]. They were first introduced into developmental biology early in 2000 [12]. Antisense MOs block translation by interfering with the binding of ribosomes to target mRNA. The use of antisense MOs has proven to be a valuable tool for investigating gene function in zebrafish and xenopus embryos [13]. However, few studies have utilized this technology for the study of mammalian oocytes [14, 15]. In this work, we investigated the feasibility of using antisense MOs to suppress mouse gene function and revealed the function of H1foo by microinjection of an antisense MO against H1foo into mouse GV-stage oocytes.

Materials and Methods

Morpholino antisense oligonucleotides
Morpholino oligonucleotides (MO) were made to order (Gene Tools; LLC, Corvallis, OR, USA). The complementary MO for H1foo was designed to target a sequence containing the initiation codon. The sequence of this MO was 5'-ACTGGAGAC-ActCCCCAGGAGCCATG-3'. Another MO with the inverse of the antisense sequence, 5'-GTACCGAGGACCCTCACAGAGGTCA-3', was used as a control for nonspecific MO toxicity. Stock solutions of the antisense MO and control MO were prepared in sterile water. The MO stock solutions were diluted to a concentration of 500 µM and heated to 65 C for 5 min prior to use.

Oocyte collection and microinjection of MOs
B6D2F1 female mice, age 8 to 12 weeks from Sankyo Labo Service (Tokyo, Japan), were injected with 5 IU of eCG (Teikokuzoki, Tokyo, Japan) and sacrificed 48 h after eCG administration. Fully grown, GV-intact oocytes were obtained by puncture of antral follicles in M2 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 240 µM dibutyryl cyclic AMP (dbcAMP; Sigma). After the oocytes were collected, the surrounding cumulus cells were mechanically removed using a glass pipette. The denuded oocytes were microinjected with 10 pl of the MO solutions in M2 medium containing 240 µM dbcAMP at room temperature. Following microinjection, the injected oocytes were incubated for 10 min in M2 medium containing 240 µM dbcAMP at room temperature. Oocytes that were judged to be morphologically normal after incubation were used for further experiments.

Culture and in vitro maturation of GV oocytes
For protein analysis, the injected and non-injected GV oocytes were cultured in 100 µl droplets of TYH medium (Mitsubishi, Tokyo, Japan) supplemented with 5% heat inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 240 µM dbcAMP under paraffin oil at 37 C in an atmosphere of 5% CO2 in humidified air. Twenty or 44 h after culture, oocytes were used for immunoblotting and immunofluorescence staining. For analysis of meiotic maturation, injected and non-injected GV oocytes were cultured in 100 µl
droplets of TYH medium containing 5% FBS without dbcAMP. Twenty hours after culture, the rate of meiotic maturation was calculated based on the presence or absence of a first polar body.

Parthenogenesis of oocytes
The injected and non-injected oocytes that matured in vitro were selected for evaluation of developmental capacity. After 3 washes in Ca\textsuperscript{2+}-free KSOM medium, the eggs were transferred into Ca\textsuperscript{2+}-free KSOM medium containing 10 mM SrCl\textsubscript{2} and activated for one hour. Following further culture in KSOM medium for 5 h, the rate of parthenogenesis was calculated by formation of pronuclei.

Immunoblotting of H1foo and cyclin B1
Twenty-five oocytes from each group were washed in PBS, extracted in 4 \( \mu l \) of Laemmli sample buffer (Bio-RAD Laboratories, Hercules, CA, USA) with 5% \( \beta \)-mercaptoethanol and boiled for 5 minutes. The extracts were resolved on 12% SDS-PAGE gels and blotted onto a polyvinylidene fluoride membrane. The membrane was blocked with 5% non-fat milk in TTBS (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween20) for 1 h and incubated with 1 \( \mu g/ml \) of rabbit anti-H1foo polyclonal antibody (the kind gift of Dr. E. Y. Adashi) or monoclonal mouse anti-cyclin B1 IgG (Dakocytomation, Carpinteria, CA, USA) at room temperature for 1 h. After washing in TTBS, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1/5,000 in 5% non-fat milk/TTBS for 1 h at room temperature. The signal was detected using the ECL system (Amersham Pharmacia, Piscataway, NJ, USA).

Immunofluorescence staining of H1foo, microtubules and nuclei
Oocytes were fixed for 20 min at room temperature in 2% paraformaldehyde in PBS (pH 7.4). After blocking for 1 h at room temperature in blocking solution (10% goat serum and 0.5% TritonX-100 in PBS), the oocytes were incubated for 1 h at room temperature with rabbit anti-H1foo polyclonal antibody at a concentration of 1 \( \mu g/ml \) and mouse anti-\( \alpha \)-tubulin monoclonal IgG (Sigma) diluted 1/1000 in dilution buffer (0.5% TritonX-100 in PBS). Following 3 washes in PBS with 0.1% TritonX-100, the oocytes were incubated with Alexa Fluor 488 goat anti-rabbit IgG conjugate and Alexa Fluor 546 goat anti-mouse IgG conjugate for 1 h at room temperature. The oocytes were washed again and transferred into a drop of Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and covered with a glass slip. The samples were observed with an AX-70 fluorescence microscope (Olympus, Tokyo, Japan), and images were captured using a SPOT RT Monochrome Digital Camera (Diagnostic Instruments, Sterling Heights, MI, USA).

Histone H1 kinase activity
Histone H1 kinase activity was determined in HK buffer comprised of 80 mM \( \beta \)-glycerophosphate, 20 mM EGTA (pH 7.3), 15 mM MgCl\textsubscript{2}, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 \( \mu g/ml \) leupeptin, 10 \( \mu g/ml \) pepstatin, 10 \( \mu g/ml \) aprotinin using exogenous histone H1 (Sigma) as described previously [16]. Samples containing oocytes in M2 medium were lysed by freezing and thawing three times, diluted twice in 2X concentrated HK buffer and incubated for 30 min at 37ºC in the presence of 3.3 mg/ml histone H1, 1 mM ATP and 30 \( \mu Ci/ml \) \([\gamma,32P]\)ATP. The reaction was stopped by incubation for 2 min at 90ºC immediately following the addition of concentrated sample buffer. The samples were then electrophoresed on 12% SDS-polyacrylamide gel. After drying, the gels were exposed to x-ray film at –80ºC.

In vitro synthesis and microinjection of H1foo-GFP mRNA
The constructed plasmid containing cDNA encoding H1foo and EGFP without the MO recognition sequence was used as a template for in vitro transcription. RNA was synthesized using an SP6 Message Machine Kit (Ambion, Austin, TX, USA).

Synthesized RNA was polyadenylated with a Poly(A) Tailing Kit (Ambion). To avoid folding of the RNA, the polyadenylated RNA was heated at 90ºC for 1 min and cooled on ice. A mixture of MO and the RNA (final concentration 1 \( \mu g/\mu l \)) was used for microinjection.
Results

Antisense MO injection completely abolishes H1foo expression in GV oocytes

Immunocytostaining and immunoblotting were performed to test whether microinjection of antisense MO into GV-stage oocytes suppressed the expression of H1foo protein. After injection of MO, oocytes were cultured in the presence of dbcAMP for inhibition of GV breakdown (GVBD). Expression of H1foo was compared among oocytes that maintained the germinal vesicle. Twenty hours after microinjection, immunocytostaining showed that the expression of H1foo protein in the oocytes injected with antisense MO was markedly reduced compared with those injected with control MO, and after 44 h the signal was no longer detectable (Fig. 1A). To quantitatively compare the expression of H1foo protein, immunoblotting analysis was performed using lysates from 50 oocytes for each lane. A significant reduction in H1foo expression was observed at 20 h and 44 h after injection in the antisense MO treatment group compared with the control MO group (Fig. 1B). These results demonstrated that the antisense MO successfully produced a specific reduction of H1foo in the mouse oocytes.

Knockdown of H1foo impairs meiotic maturation

H1foo transcript and protein is expressed in GV- and metaphase II-stage oocytes and one-cell-stage embryos. However, H1foo expression is mostly extinguished by the two-cell stage. We investigated whether knockdown of H1foo by antisense MO impaired oocyte maturation and development. Following culture for 20 h after microinjection of antisense or control MO, the rate of in vitro maturation (IVM) was calculated by scoring eggs for presence/absence of a first polar body. Only 33.1% of H1foo-depleted oocytes successfully extruded a first polar body and progressed into meiosis II, compared with 81.1% of the control MO treatment group oocytes and 89.0% of the non-injected oocytes. Bars represent means ± the standard deviation of five independent experiments. Student’s t-test revealed significant differences (*: P<0.05).
H1FOO is indispensable for IVM

MO treatment group oocytes and 89.0 ± 5.3% of the non-injected oocytes (Fig. 2). Eggs that matured after microinjection of MO were selected and activated by strontium chloride to trigger parthenogenesis in order to evaluate the developmental capacity of H1foo-depleted oocytes.

The rate of parthenogenesis, as calculated by the formation of pronuclei, was 88.9% (24/27) for the antisense MO group, 93.8% (15/16) for the control MO group and 89.9% (71/79) for the non-injection group. These results suggest that H1foo plays an important role during first meiosis but not in the formation of pronuclei.

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Eggs that failed meiotic maturation following injection of antisense MO were fixed and immunocyto-stained for further evaluation. Immunocyto-staining of α-tubulin and nuclear staining revealed that treatment with antisense MO and transcibed RNA. Noe that H1foo-EGFP protein could be clearly visualized. The rate of IVM was markedly higher in oocytes co-injected with antisense MO and RNA compared with oocytes injected with antisense MO. The bars represent means ± the standard deviation of four independent experiments. (3) Student's t-tests revealed significant differences (*: P<0.05).

H1foo-depleted oocytes arrest at metaphase I

Eggs with failed meiotic maturation following injection of antisense MO were fixed and immunocyto-stained for further evaluation. Immunocyto-staining of α-tubulin and nuclear staining revealed that treatment with antisense MO caused the eggs to arrest at metaphase I (Fig. 3A). However, depletion of H1foo had no effect on chromatin condensation or spindle organization (Fig. 3A). Considering that no morphological defects were observed in the metaphase I-arrested H1foo-depleted oocytes, the observed decreased IVM rate could be the result of functional defects in other factors that would be necessary for progression of meiosis in the absence of H1foo. Maturation promoting factor (MPF), which is a complex of cdc2 and a B-type cyclin, is responsible for driving oocytes through meiotic maturation. We evaluated the activity of MPF and the expression of
cyclin B1 in eggs that arrested at metaphase I due to injection of antisense MO. Cyclin B1 was undetectable and MPF activity was low in the control prophase oocytes (Fig. 3B). Expression of cyclin B1 began just before Gon and MPF was activated until metaphase I (Fig. 3B). Subsequently, MPF activity fell during the metaphase I and anaphase I transition due to temporal degradation of cyclin B1 and then rose again leading to entry into meiosis II with concomitant accumulation of cyclin B1 (Fig. 3B). In eggs injected with antisense MO, cyclin B1 expression was maintained, but MPF activity remained low as revealed by the H1 kinase assay (Fig. 3B). This discrepancy between cyclin B1 expression and MPF activity suggests that suppression of H1foo might prevent the activation of MPF during first meiosis after GVBD or induce inactivation of MPF without degradation of cyclin B1.

**Exogenous H1foo rescues the phenotype produced by the H1foo antisense MO**

To determine the specificity of the effect of the H1foo antisense MO, we attempted to rescue knockdown of H1foo by the simultaneous injection of exogenous H1foo mRNA. The in vitro synthesized mRNA lacked the first 24 nucleotides of the H1foo ORF (so that it would not be targeted by the antisense MO) and included a sequence of enhanced green fluorescent protein (EGFP) conjugated at the 3' end to aid in recognition of expression and localization of exogenous H1foo (Fig. 4A). The H1foo-EGFP mRNA was efficiently translated into protein that was detectable roughly 4 h after injection and was localized on chromatin during meiotic maturation in a similar manner to endogenous H1foo (Fig. 4B). When H1foo-EGFP mRNA was injected along with antisense MO, significant rescue of the IVM rate was observed (Fig. 4C). Thus, the phenotype induced by the H1foo antisense MO was purely attributable to its designed activity and was not attributable to a non-specific secondary effect.

**Discussion**

In the present study, we demonstrated that antisense morpholinos could specifically block the translation of H1foo in mouse oocytes. Suppression of H1foo did not have any influence on GVBD, but did impair extrusion of the first polar body leading to a decrease in the IVM rate. To confirm the specificity of the antisense MO, we rescued this phenotype by co-injection of H1foo mRNA lacking the MO recognition sequence, leading to improvement of IVM. These results show that oocyte-specific linker histone H1foo is involved in the progression of meiosis.

Linker histones are known to be involved mainly in chromatin organization, with ensuing consequences on transcription. However, the biological functions of linker histones are more varied than previously proposed. Recently, some reports have indicated that linker histones may play a role in aging, DNA repair and apoptosis [17–19]. In tobacco, deficiency of the linker histone subtypes H1A and H1B is linked with male sterility resulting from disturbances in correct pairing or segregation of homologous chromosomes during meiosis [20]. Furthermore, in mitosis, overexpression of H1f0 leads to transient inhibition of both G1 and S phase progression [21], and immunodepletion of histone H1 resulted in a lengthening of chromosomes that prevents their proper alignment and segregation [22]. Our results also indicate a relationship between linker histones and the cell cycle. Eggs lacking H1foo arrested at metaphase I despite exhibiting normal compaction, alignment of chromosomes and spindle formation. There is a possibility that H1foo may play a functional rather than structural role in the segregation of chromosomes.

MPF is a heterodimer consisting of a kinase, cdc2, and its regulatory partner, cyclin B1 [23, 24]. Increased MPF activity leads to GVBD and entry into the first meiotic metaphase [25, 26]. After metaphase I, segregation of chromosomes requires a transient decline of MPF activity resulting from anaphase-promoting complex (APC)-mediated destruction of cyclin B1 [27]. The MPF activity of the injected oocytes could not be maintained for enough time before the MI-anaphase I transition. It is possible that this leads to arrest at the MI stage. The H1foo-depleted oocytes exhibited low MPF activity despite the persistent presence of reasonable amounts of cyclin B1. This suggests that H1foo may be essential for maintenance of MPF activity by cyclin B1 and for destruction of cyclin B1 by APC. It is notable that the phenotype resulting from injection of H1foo antisense MO bears a close resemblance to that produced by injection of c-Mos.
antisense oligonucleotides. Following microinjection of c-Mos antisense oligonucleotides, oocytes undergo GVBD but are blocked at or about the first meiosis [28]. The c-Mos protein kinase is an efficient activator of mitogen-activated protein kinase (MAPK) kinase that in turn activates MAPK [29, 30]. During each phase of meiotic maturation, a dynamic cross-talk exists between MPF activity and the c-Mos/MAPK pathway [31]. Thus, reduced MPF activity in H1foo-depleted oocytes may interfere with the c-Mos/MAPK pathway. Moreover, antibody array assays have suggested that H1foo may interact with many kinds of cell cycle-related proteins including CDC25, an activator of MPF (data not shown). Recent studies of fluorescence recovery after photobleaching (FRAP) revealed that H1foo has high mobility [10, 32], potentially facilitating effective recruitment of cell cycle-related factors on chromatin. It is reasonable to speculate that H1foo has greater mobility than somatic linker histones within oocytes, which have significantly more cytosol space than somatic cells. Our results are consistent with H1foo having a role in the regulation of MPF, although it is still unknown whether H1foo directly interacts with the c-Mos/MAPK pathway.

In this report, we demonstrated that translation of H1foo could effectively be blocked in mouse oocytes via microinjection of a specific MO. It is important to note that use of antisense technology such as MOs requires care to avoid serious nonspecific toxic side effects. Particularly, the concentration of antisense MO to be used requires serious consideration. In our experiments, we injected the antisense MO to a final concentration of about 10 µM, which is a typical dose used for the microinjection of zebrafish and Xenopus oocytes. Although there are few published reports on use of this or similar technology for mouse oocytes, this concentration matched well with those reports. In addition, we prepared a control MO with the inverse sequence of the antisense MO and injected it at the same concentration in order to rule out nonspecific toxic side effects. Since there was no difference between the control MO group and the noninjected group with regard to the expression of H1foo, the dose was appeared to be appropriate for analyzing specific knockdown of H1foo. This study clearly establishes the usefulness of antisense MOs for investigating gene function in the mouse. Herein, we have successfully knocked down the expression of oocyte-specific linker histone H1foo in mouse oocytes using a specific antisense MO and revealed the critical role played by this protein in oocyte maturation. Further studies are necessary to fully analyze the relationship between H1foo and the cell cycle machinery.

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