Rapid replacement of somatic linker histones with the oocyte-specific linker histone H1foo in nuclear transfer

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Abstract

The most distinctive feature of oocyte-specific linker histones is the specific timing of their expression during embryonic development. In Xenopus nuclear transfer, somatic linker histones in the donor nucleus are replaced with oocyte-specific linker histone B4, leading to the involvement of oocyte-specific linker histones in nuclear reprogramming. We recently have discovered a mouse oocyte-specific linker histone, named H1foo, and demonstrated its expression pattern in normal preimplantation embryos. The present study was undertaken to determine whether the replacement of somatic linker histones with H1foo occurs during the process of mouse nuclear transfer. H1foo was detected in the donor nucleus soon after transplantation. Thereafter, H1foo was restricted to the chromatin in up to two-cell stage embryos. After fusion of an oocyte with a cell expressing GFP (green fluorescent protein)-tagged somatic linker histone H1c, immediate release of H1c in the donor nucleus was observed. In addition, we used fluorescence recovery after photobleaching (FRAP), and found that H1foo is more mobile than H1c in living cells. The greater mobility of H1foo may contribute to its rapid replacement and decreased stability of the embryonic chromatin structure. These results suggest that rapid replacement of H1c with H1foo may play an important role in nuclear remodeling.

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Keywords: Linker histone; H1foo; Nuclear remodeling; Fluorescence recovery after photobleaching (FRAP)

Introduction

Successful generation of cloned animals by nuclear transfer of somatic cells has been reported in several species of mammals (Wakayama et al., 1998; Wilmut et al., 1997). These reports proved that somatic nuclei could reverse their differentiated state to recover totipotency when introduced into oocyte cytoplasm. Transferred nuclei change their gene expression pattern to that of early embryonic nuclei to permit successful development. This change is generally termed reprogramming, and many researchers have tried to elucidate the mechanism by which nuclear reprogramming occurs (Kikyo and Wolffe, 2000; Shi et al., 2003; Wade and Kikyo, 2002). Accumulating evidence shows that the initial transcriptional activity of the donor cell nucleus is controlled predominantly by the egg cytoplasm so that appropriate chromatin remodeling occurs (Reik et al., 2001; Rideout et al., 2001). Epigenetic modification of the genome ensures proper gene activation during development and involves (i) genomic methylation changes, (ii) the assembly of histones and histone variants into nucleosomes, and (iii) remodeling of other chromatin-associated proteins, such as polycomb group proteins, nuclear scaffold proteins, and transcription factors (Latham, 1999). Whereas DNA methylation has been extensively studied in mammalian development (Reik et al., 2001; Rideout et al., 2001), the molecular mechanisms involved in remodeling of chromatin-associated proteins remains to be elucidated.

DNA is tightly assembled with histone and nonhistone proteins to form chromatin in eukaryotic cells. Compaction
of DNA into chromatin affects a large number of essential nuclear processes, including DNA replication, repair, recombination, and transcription. The fundamental subunit of chromatin is the nucleosome, which contains 146 bp of DNA wrapped around an octamer core composed of two each of the histone proteins H2A, H2B, H3, and H4 (Wolffe, 1995). Because it links adjacent nucleosomes, H1 is often referred to as a linker histone. H1 has many subtypes, with mammalian somatic cells having six subtypes (H1a, H1b, H1c, H1d, H1e, and H1j). The reason why so many subtypes of linker histones exist remains uncertain, but the expression level of each subtype seems to be associated with differentiation (Zlatanova and Doenecke, 1994). According to studies knocking out a subtype(s) of linker histone, no one subtype is essential as members can compensate for each other’s absence (Fan et al., 2001). The association of histone H1 with DNA may stabilize the interaction between the core histone octamer and DNA, and facilitate assembly of the nucleosome array into a higher order structure (Felsenfeld and McGhee, 1986; Thoma et al., 1979). It is currently accepted that H1 plays a regulatory role in transcription through modulation of higher structure of the chromatin. Indeed, phosphorylation of H1 has been shown to regulate chromatin remodeling enzymes (Horn et al., 2002). In Xenopus oocytes and embryos, oocyte-specific linker histone B4 exists rather than somatic type H1 during the first divisions after fertilization. B4 is replaced by somatic H1 at the midblastula transition (Dworkin-Rastl et al., 1994; Smith et al., 1988). Accumulation of H1 is a rate-limiting factor for the loss of mesodermal competence (Steinbach et al., 1997). The major difference between H1 and B4 lies in the stability with which these proteins are incorporated into chromatin (Ura et al., 1996). In the mouse, histone synthesis is also developmentally regulated (Clarke et al., 1992; Weikowski et al., 1997). These findings suggest that linker histones play an important role in early development.

Recently, during the course of a differential screening project, we discovered a mammalian oocyte-specific linker histone, H1foo, which is homologous to B4. H1foo is localized to the nucleus of germinal vesicle stage oocytes, metaphase II (MII) arrested oocytes, and the first polar body. Early one-cell stage embryos displayed H1foo immunoreactivity in condensed maternal metaphase chromatin, but not in the sperm head. However, following the extrusion of a second polar body, H1foo was detected in the swollen sperm head. Nuclear staining was somewhat reduced in two-cell embryos and was no longer detectable in four-cell embryos (Tanaka et al., 2001). The expression pattern of H1foo in preimplantation embryos is developmentally regulated, as is Xenopus B4. An experiment of nuclear transfer in Xenopus showed that somatic type H1 in a donor cell is replaced by oocyte-type B4 soon after transplantation into an oocyte, and that replacement is mediated by nucleoporin, a molecular chaperone that contributes to the acquisition of transcriptional competence.
(Dimitrov and Wolffe, 1996). H1 removal after nuclear transfer also has been observed in mammals (Adenot et al., 2000; Bordignon et al., 1999); however, replacement of H1 with oocyte-type linker histone has not yet been reported.

Another molecular characteristic of linker histones has been clarified. Recent studies of fluorescence recovery after photobleaching (FRAP) show that histone H1 is continuously and rapidly exchanged between chromatin segments (Lever et al., 2000; Misteli et al., 2000). In contrast to this rapid exchange of H1, the association of core histones, especially H3 and H4, with DNA is stable (Kimura and Cook, 2001). Thus, the rapid removal of H1 in nuclear transfer may result from the greater mobility of H1. As the immobile fraction of linker histone stabilizes chromatin structure, the different mobilities among linker histones may alter the chromatin structure.

This study sought to determine whether replacement of the somatic linker histone with H1foo occurs during nuclear transfer in the mouse. To clarify the difference in molecular dynamics between somatic and oocyte-type linker histones in vivo, we used FRAP to examine whether the mobility of linker histones contributes to this replacement machinery and the stability of chromatin structure.

Materials and methods

Oocyte collection and nuclear transfer

The procedure for oocyte collection and nuclear transfer has been described previously (Kono et al., 1996; Ono et al., 2001). Briefly, micromanipulations were performed in M2 medium containing 5 μg/ml cytochalasin B and 0.4 μg/ml nocodazole. After removal of the MII plate, a fibroblast cell arrested in metaphase was introduced into the perivitelline space of an enucleated oocyte using inactivated Sendai virus. Following brief culture, the oocytes were artificially activated with 10 mM strontium for 6 h and then placed in CZB medium.

Indirect immunofluorescence

At various time points after successful fusion with a donor cell, oocytes were fixed with freshly prepared 2% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min. The fixed oocytes then were stained with anti-H1foo antibody and counterstained with Hoechst 33342 as previously described (Tanaka et al., 2001).

Plasmids

Mouse full-length H1foo cDNAs were synthesized by reverse transcriptase–polymerase chain reaction (RT-PCR) and inserted into pcDNAII (Invitrogen, Carlsbad, CA). All PCR primers are listed in Table 1 and annealed at 68°C. Full-length H1c cDNA was synthesized by nested PCR and also inserted into pcDNAII. The correct sequences of the desired inserts were confirmed by sequencing. The pcDNAII/H1foo was subcloned into pEGFP-N3 (Clontech, Palo Alto, CA) via the NheI and EcoRI sites. The constructed pEGFP/H1foo plasmid was excised with NheI and NotI and ligated into the NheI and NotI sites of plasmid pTRE2hyg (Clontech). The pcDNAII/H1c was excised with BamHI and ligated into the NheI and NorI sites of plasmid pTRE2hyg (Clontech). The pcDNAII/H1c was excised with BamHI and ligated into the BamHI site of pTRE2hyg/EGFP. pTRE2hyg/EGFP was constructed as follows: pEGFP-N3 was excised with NheI and NorI, and ligated to the same site of pTRE2hyg. To express C-His-tagged recombinant protein, pET24b/H1foo was generated by inserting full-length H1foo, obtained by cutting pEGFP/H1foo with NheI and EcoRI, into pET24b (Novagen, Madison, WI). The pET24b/H1c was produced by insertion of newly synthesized full-length H1c that contained the terminal EcoRI and SalI site into pET24b.
Cell culture, transfection, and selection of stable clones

Mouse MEF/3T3 Tet-off cells (Clontech) were grown in Dulbecco’s modified Eagle’s medium, supplemented with 10% Tet system-approved fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 100 μg/ml G418, and maintained at 37°C under 5% CO₂. Cells were grown in 10-cm dishes to 50% confluence and transfected with lipofectamine (Gibco-BRL, Rockville, MD) using 24 μg of plasmid and 36 μl of lipofectamine per dish. Twenty-four hours after transfection, 4.5 × 10⁴ cells were re-plated in the presence of doxycycline (1 μg/ml) and hygromycin B (250 μg/ml) in a 10-cm dish. After 2 weeks, hygromycin-resistant colonies were expanded in a 24-well plate. In the absence of doxycycline, H1foo or H1c gene expression was detected by the presence of enhanced green fluorescent protein (EGFP). The integrated plasmid and induced protein expression were confirmed by PCR of genomic DNA and Western blotting, respectively.

Isolation of total histone proteins and immunoblotting

Total histone proteins were isolated by the method of Brown et al. (1996) with slight modifications. Crude histones were subjected to SDS-PAGE, using 15% resolving gels and 4% stacking gels. The gels were stained with GelCode Blue Stain Reagent (Pierce, Rockford, IL) or transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% non-fat milk in TTBS (10 mM Tris–HCl, 150 mM NaCl, 0.05% Tween 20) for 1 h. Primary antibody, mouse monoclonal antibody against green fluorescent protein (GFP; Santa Cruz Biotechnology, Santa Cruz, CA), was diluted 1/200 in

![Fig. 3. Effects of H1-GFP fusion expression on total histone proteins. Total histones were extracted from either parental 3T3 cells or a cell line stably expressing H1foo-GFP or H1c-GFP, and separated by SDS-PAGE. (A) Coomassie blue staining. (B) Immunodetection of GFP-tagged proteins with anti-GFP monoclonal antibody. The H1c-GFP protein level was less than 10% of total H1 protein calculated by coomassie blue staining. H1foo-GFP protein was not visible by coomassie blue staining.](image)

Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Additional restriction enzyme (underlined)</th>
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<tr>
<td>H1foo forward</td>
<td>5'-GCTAGCCATAGCTGTCAGTTGGAGTGTTCAG-3'</td>
<td>NheI</td>
</tr>
<tr>
<td>H1foo reverse</td>
<td>5'-GCCGAGTGGTCTAGCGGTTTGTCAG-3'</td>
<td>None</td>
</tr>
<tr>
<td>H1c forward</td>
<td>5'-GTTAAACACATGCAATTGAGGGTGTAC-3'</td>
<td>None</td>
</tr>
<tr>
<td>H1c reverse</td>
<td>5'-ACGAGCAGGACACAAACAAACAAACAGGC-3'</td>
<td>None</td>
</tr>
<tr>
<td>H1c forward for nesting</td>
<td>5'-GCTAGCCATAGCTGTCAGTTGGAGTGTTCAG-3'</td>
<td>NheI</td>
</tr>
<tr>
<td>H1c reverse for nesting</td>
<td>5'-GCTAGCCATAGCTGTCAGTTGGAGTGTTCAG-3'</td>
<td>BamHI</td>
</tr>
<tr>
<td>H1c forward for protein expression</td>
<td>5'-GCAATCTGTCAGTTGGAGTGTTCAG-3'</td>
<td>EcoRI</td>
</tr>
<tr>
<td>H1c reverse for protein expression</td>
<td>5'-GCTAGCCATAGCTGTCAGTTGGAGTGTTCAG-3'</td>
<td>SaI</td>
</tr>
</tbody>
</table>

To subclone the cDNA into the vector in-frame, the underlined restriction enzyme site was added to the 5' end of the primer.
PBS containing 1% bovine serum albumin (BSA). Horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology) was diluted 1/5000 in 1% non-fat milk/TTBS. The signal was detected using the ECL system (Amersham Pharmacia Biotech, Piscataway, NJ).

**Protein expression and purification**

C-His-tagged protein was expressed in *Escherichia coli* BL21 (DE3) (Novagen) in LB medium. Protein expression was induced by adding isopropylthio-β-D-galactoside (IPTG) to a final concentration of 1 mM and growing the bacteria for 3 h. The bacterial pellets were resuspended in BugBuster reagent (Novagen). Cell suspension was incubated on rotating mixer at room temperature for 20 min and centrifuged at 12,000 × g for 20 min. The insoluble pellets were resuspended in buffer A (50 mM Tris–HCl pH 7.4, 4 M urea, 1 M NaCl, and 5% glycerol) including 20 mM imidazole and incubated on ice for 20 min with frequent vigorous vortexing. Cell debris was removed by centrifugation at 12,000 × g for 10 min. For protein purification, the supernatant was added to Ni-NTA resin (Novagen) that was equilibrated with buffer A. After gentle mixing for 30 min at 4°C, the supernatant was discarded, and the resin was washed three times with buffer A. His-tagged proteins were then eluted in buffer A containing 250 mM imidazole. The eluted sample was transferred to a dialysis bag and dialyzed against TEP buffer (5 mM Tris–HCl pH 7.5, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride) at 4°C overnight.

**H1-depleted chromatin, reconstitution of chromatin, and micrococcal nuclease digestion**

H1-depleted chromatin was prepared as described previously (Ura and Kaneda, 2001). Purified C-His-tagged H1c or H1foo, NaCl, and BSA were added to H1-depleted chromatin to a final concentration of 4 μM, 0.6 M, 0.5 mg/ml, respectively. To create stable reconstituted chromatin, the salt concentration of the dialysis buffer was decreased stepwise to 0.45 M, 0.3 M, and finally 0.15 M (Higurashi and Cole, 1991). A 1/100 volume of 0.3 M CaCl2 was added to the reconstituted chromatin, and the samples were pre-incubated at 37°C for 5 min. Micrococcal nuclease (MNase) (Worthington Biochemical, Lakewood, NJ) was added (0.1 unit/μl), and digestion was carried out for 5, 10, or 30 min. The reaction was stopped by adding 100 mM EDTA, 10% SDS, and proteinase K to a final concentration of 10 mM, 0.5%, and 100 μg/ml, respectively. Total DNA was purified by three extractions with phenol/chloroform/isoamyl alcohol and one extraction with chloroform, and was collected by ethanol precipitation. MNase digested DNA was electrophoresed on 2.2% agarose gel and stained with ethidium bromide.

**FRAP**

FRAP experiments were performed using a Laser Confocal Microscope LSM 510 (Carl Zeiss, Jena, Germany).
H1foo-EGFP and H1c-EGFP stable transfectants (1.5 × 10^5 cells) were plated on a poly-l-lysine-coated 35-mm cover glass bottom dish. Cells were imaged and photobleached using 488 nm argon laser with a Plan-Apochromat 63 × oil objective. The heterochromatin region of the nucleus was photobleached at a high laser power, which resulted in 80% reduction of fluorescence intensity. Fluorescence recovery was observed during 7-s interval scanning at a low laser power, 20 images obtained after photobleaching were stored and analyzed with software controlling the LSM510. Fifteen cells of both H1foo-EGFP and H1c-EGFP were photobleached, the relative fluorescence intensity (RFI) was calculated as described (Misteli et al., 2000), and the mean time for 50% recovery of fluorescence (t50) was determined. The mobile fraction was calculated by comparing the fluorescence in the bleached region after full recovery (Ff)

Fig. 5. Micrococcal nuclease (MNase) digestion of reconstituted chromatin. (A) H1-depleted chromatin, purified recombinant H1s, and the dialyzed mixture of H1-depleted chromatin and H1c or H1foo were subjected to SDS-PAGE. (B) H1-depleted chromatin, reconstituted chromatin with H1c or H1foo were digested with MNase for the indicated time. Extracted DNA was electrophoresed on 2.2% agarose gel which was subsequently stained with ethidium bromide. H1foo was incorporated into nucleosome as well as H1c.
with the fluorescence before bleaching \( (F_i) \) and just after bleaching \( (F_0) \). The mobile fraction was defined as \( R = (F_i - F_0)/(F_i - F_0) \) (Reits and Neefjes, 2001).

Results

**H1foo is rapidly incorporated into the donor nucleus after nuclear transfer**

To investigate the possible involvement of the oocyte-specific linker histone H1foo during nuclear transfer, we analyzed H1foo immunoreactivity of reconstructed embryos at different stages of development. Enucleated oocytes and embryonic fibroblasts arrested in metaphase were fused using the standard micromanipulation procedure as described elsewhere (Kono et al., 1996; Ono et al., 2001). After fusion of the fibroblast with an enucleated oocyte, a single metaphase plate was reassembled in the oocyte cortex. The detection of H1foo in the nuclear-transferred embryos is illustrated in Fig. 1. H1foo was localized to the metaphase-arrested donor nucleus as soon as 10 min after fusion. H1foo was detected in a polar body and pronucleus (PN), as soon as the artificially activated, reconstructed oocytes showed extrusion of the polar body and PN formation. The polar body remained brightly fluorescent throughout early embryogenesis. Nuclear staining, however, was somewhat less in two-cell than one-cell embryos. Nuclear staining was no longer detectable at the four-cell stage of embryonic development, but a strong signal persisted in the polar body (Fig. 1).

**Construction of stable transfectants**

To visualize linker histones in living cells, we fused the coding region of the EGFP to that of mouse H1foo or somatic H1c, and established stable cell lines using mouse 3T3 fibroblasts. Fluorescence microscopy showed that H1foo-GFP binds to chromatin throughout the cell cycle (Fig. 2). H1c-GFP bound to chromatin in the same manner as H1foo-GFP (data not shown). On the basis of SDS-PAGE analysis of total histone protein extracted from cells stably expressing H1foo-GFP or H1c-GFP, we estimated that the cell lines overexpress less than 10% H1-GFP in addition to endogenous H1 (Fig. 3A). H1foo-GFP and H1c-GFP were detected in an acid-soluble nuclear fraction (Fig. 3B). However, neither protein was identified in the other fraction by Western blotting (data not shown). These results clearly demonstrate that the two GFP-H1 fusions were properly incorporated into chromatin. In addition, the expression of neither H1-GFP altered histone protein composition, based on coomassie brilliant blue staining (Fig. 3).

**Release of somatic linker histone H1c from the donor chromosome**

The removal of somatic linker histones after nuclear transfer has been previously shown by indirect immunofluorescence using anti-H1 antibody (Adenot et al., 2000). To visualize this phenomenon directly in vivo, MII-arrested oocyte and H1c-GFP transfectant were fused as described except for enucleation of the recipient nucleus, and subsequent release of H1c-GFP was observed. As shown in Fig. 4, most of the H1c was released within 30 min after fusion. In addition to this rapid release, faint fluorescence was still detectable for 3 h. Thus, the reduction in H1c was biphasic.

**H1foo is incorporated into nucleosome**

To determine whether the exchange of linker histones influences chromatin structure, we investigated the effect of MNase digestion of reconstituted chromatin obtained from the addition of recombinant H1s to H1-depleted chromatin. The purified recombinant H1s were of good quality, and an...
equal molar concentration of purified H1 was added to H1-depleted chromatin (Fig. 5A). The nucleosomal repeat length of the reconstituted chromatin which contained H1c or H1foo was clearly greater than that of H1-depleted chromatin (Fig. 5B). H1foo was incorporated into the nucleosome and exerted a similar constraint on MNase digestion as on H1c.

**H1foo is more mobile than somatic type H1c in living cell nuclei**

To understand the molecular basis of the rapid exchange of linker histone and the differences between H1c and H1foo, we investigated the dynamics of GFP-tagged histone in unperturbed chromatin by FRAP, which can be used to define the mobility of molecules in living cells (Lever et al., 2000; Misteli et al., 2000). H1foo-GFP and H1c-GFP presented the same distribution pattern in the nucleus, and the movement of H1foo-GFP and H1c-GFP occurred from the unbleached to the bleached region (Fig. 6).

Upon bleaching the heterochromatin area, H1foo-GFP fluorescence recovered relatively rapidly and reached a plateau after 100 s. This finding clearly shows that H1foo-GFP is continuously exchanged in the chromatin regions of the cell nucleus in a similar manner to somatic linker histone H1s. The recovery of H1foo-GFP was faster than that of H1c-GFP (Fig. 7), and the mean time for 50% recovery of H1foo-GFP fluorescence was less than that of somatic H1c (Table 2). In addition, the immobile fraction of H1c was greater than that of H1foo. These results indicate that H1foo is more mobile than somatic H1c in the nucleus in vivo.

**Discussion**

This report shows that H1foo is rapidly accumulated into the donor nucleus and persists there until the two-cell stage embryo, then disappeared during the four-cell stage. We also have shown that H1foo is readily detected in the swollen sperm head shortly after fertilization in normal preimplantation embryos, and that nuclear staining of H1foo is somewhat reduced in two-cell embryos and is no longer detectable in four-cell embryos (Tanaka et al., 2001). The developmentally regulated presence of H1foo in a cloned embryo is therefore similar to that of a normal preimplantation embryo. Significantly, H1foo was detected in the donor nucleus 10 min after fusion of the donor cell. In *Xenopus*, the midblastula transition and the activation of zygotic gene expression are associated with a dramatic decrease in B4 content and a simultaneous increase in somatic H1 (Dimitrov et al., 1993; Dworkin-Rastl et al., 1994). In nuclear transfer, the uptake of oocyte-type B4 into donor chromatin and the release of H1 is rapid, taking as little as 15 min from the time when it is mixed with the egg extract (Dimitrov and Wolffe, 1996). In the mouse, zygotic gene activation occurs immediately after formation of a two-cell embryo, a point when H1foo expression begins to decrease. Simultaneous zygotic gene activation and the transition from oocyte-type linker histone to a somatic one strongly suggest that linker histones play an important role in early development.

H1 is thought to be a general repressor of transcription (Paranjape et al., 1994), but recent reports suggest that H1 has selective function in transcriptional regulation (Dou and Gorovsky, 2000; Dou et al., 1999; Shen and Gorovsky, 1996). The replacement of B4 with somatic H1 leads to dominant and specific repression of oocyte 5S rRNA gene transcription (Bouvet et al., 1994). Somatic H1 subtypes have been shown to regulate specific gene expression in the early period of *Xenopus* development (Bouvet et al., 1994; Steinbach et al., 1997). The zygote remodels the paternal genome shortly after fertilization.

### Table 2

<table>
<thead>
<tr>
<th>Experiment</th>
<th>$t_{50}$ (s) ± SE</th>
<th>Mobile fractions, % ± SE</th>
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<tr>
<td>H1foo</td>
<td>21.6 ± 2.0*</td>
<td>91.7 ± 1.8*</td>
<td>15</td>
</tr>
<tr>
<td>H1c</td>
<td>29.4 ± 1.6</td>
<td>85.0 ± 1.3</td>
<td>15</td>
</tr>
</tbody>
</table>

The mean time for 50% recovery of fluorescence and the percent mobile fraction during the course of the experiment were determined. Statistical significance was determined by Student’s $t$ test (*$P < 0.01$). $t_{50}$: the time for 50% recovery of fluorescence, SE: standard error, n: cell numbers.

![Fig. 7. Quantitative FRAP analysis of H1foo-GFP and H1c-GFP. The values are mean ± SE. The recovery kinetics of H1foo-GFP is significantly faster than H1c-GFP.](image-url)
before embryonic genome activation occurs (Rideout et al., 2001). During the cloning process, the somatic nuclei transferred into an oocyte must be quickly reprogrammed to express genes required for early development. Thus, H1foo might be one of the oocyte-derived factors required for genetic reprogramming in mammals, as with B4 in Xenopus.

In FRAP, fluorescent molecules are irreversibly photo-bleached in a small area of the cell by a high-powered focused laser beam. Subsequent diffusion of surrounding non-bleached fluorescent molecules into the bleached area leads to a recovery of fluorescence, which is recorded at low laser power. FRAP experiments provide information concerning the mobility of fluorescent molecules in a defined compartment. In control experiments in which whole nuclei were bleached, no recovery of fluorescence was detected (data not shown). This eliminates the possibility that de novo synthesis of H1foo-GFP or a cytosolic counterpart of H1foo accounts for any of the fluorescence recovery in these experiments. Two parameters can be deduced from FRAP: the mobile fraction of fluorescent molecules and their rate of mobility (Reits and Neefjes, 2001). Despite the similar constraint of MNase digestion in vitro, we have established the in vivo difference in mobility between somatic and oocyte-type linker histones by FRAP. In Xenopus, somatic H1 binds to nucleosomal DNA with an excess affinity relative to B4 (Ura et al., 1996). Our FRAP data, which show that the immobile fraction of H1c is greater than that of H1foo, are consistent with this previous report. A higher immobile fraction implies tight assembly into chromatin with enhanced stability. We suggest that the replacement of linker histones in the donor nucleus may destabilize the chromatin assembly, facilitating assembly of embryonic chromatin structures that are more easily replicated and transcribed when zygotic gene activation occurs.

On the other hand, an intriguing feature of this study was the finding that the reduction in H1 in the reconstructed oocyte which was fused with H1c-GFP transfected was biphasic. FRAP, which demonstrated that 85% of H1c-GFP was mobile and 15% was immobile, provides a compelling explanation to account for the biphasic reduction pattern. Most of the H1 that is lost was from the mobile fraction that can be replaced rapidly with H1foo or diffused into the oocyte cytoplasm, whereas residual H1 represents the immobile fraction. Interestingly, the time interval between the injection of the somatic cell nucleus into the enucleated oocyte and oocyte activation affects the rate of development (Wakayama et al., 1998, 2000). Activation immediately after nucleus injection resulted in significantly less progression to the morula/blastocyst stage in vitro than when activation followed a delay of 1 to 6 h (Wakayama et al., 1998). This interval is consistent with the H1 removal time. Therefore, remaining somatic H1 may disturb appropriate gene expression and lead to failure of development.

We demonstrated replacement of oocyte-type linker histone with a somatic histone occurs rapidly. The greater mobility of H1foo suggests its involvement in linker histone replacement. However, other mechanisms may participate in this replacement. The large amount of H1foo and the difference in volume between donor and recipient cells should be taken into consideration. H1foo rapidly accumulates in the donor nucleus transplanted into the enucleated oocyte, albeit the removed nucleus has a considerable amount of H1foo. As the nuclear envelope of the recipient oocyte disintegrates in metaphase, nuclear proteins may diffusely relocate in the oocyte cytoplasm. H1foo has been identified clearly by Western blot analysis using only 50 oocytes, which suggests that the oocyte originally has an excess amount of H1foo (Tanaka et al., 2001). Thus, the large amount of H1foo dispersed in the reconstructed oocyte cytoplasm, and it may easily attach to the externally derived donor nucleus. On the other hand, somatic histone preferentially diffuses into oocyte cytoplasm, because an oocyte has about a 1000-fold greater volume than a fibroblast cell.

Histone modification in an oocyte alters histone mobility. Lever et al. (2000) have shown that kinase inhibitors change the mobility of H1. Although it has not been corroborated that H1foo can be phosphorylated, there are numerous serine and threonine moieties in H1foo that are putative phosphorylation sites. The phosphorylation state of H1foo in an oocyte may affect its mobility. Another possibility is that proteins regulate H1foo replacement directly. In Xenopus, nucleoplasmin has been shown to regulate the selective removal of somatic linker histones from erythrocyte nuclei (Dimitrov and Wolffe, 1996), and recently, mammalian nucleoplasmin has been demonstrated in mouse oocytes (Burns et al., 2003). Oocyte-specific mouse nucleoplasmin 2 (NPM2) has been found in the nucleus in oocytes and eight-cell embryos, and it is crucial to heterochromatin formation in early embryos. On the basis of the common features of oocyte-specific linker histones in frogs and mice, NPM2 is thought to regulate the removal of somatic H1 and cooperate with H1foo to remodel nuclear function. Alekseev et al. (2002) have suggested that NASP (nuclear autoantigenic sperm protein) might be one of the H1 regulating proteins. Certainly, other yet to be determined proteins may participate in the replacement process. Further investigation is needed to clarify the mechanism responsible for this phenomenon.

In conclusion, we demonstrated that somatic type linker histone H1 in a donor nucleus transplanted into an oocyte is rapidly replaced with oocyte-type H1foo. The greater mobility of H1foo, compared with H1, may contribute to this rapid replacement and the instability of chromatin structures. These findings suggest that the rapid replacement of H1 with H1foo may play an important role in nuclear remodeling.
Acknowledgments

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