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Rapid H1 linker histone transitions following fertilization or somatic cell nuclear transfer: evidence for a uniform developmental program in mice

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Abstract

H1 linker histones (H1s) are key regulators of chromatin structure and function. The functions of different H1s during early embryogenesis, and mechanisms regulating their associations with chromatin are largely unknown. The developmental transitions of H1s during oocyte growth and maturation, fertilization and early embryogenesis, and in cloned embryos were examined. Oocyte-specific H1FOO, but not somatic H1s, associated with chromatin in oocytes (growing, GV-stage, and MII-arrested), pronuclei, and polar bodies. H1FOO associated with sperm or somatic cell chromatin within 5 min of intracytoplasmic sperm injection (ICSI) or somatic cell nuclear transfer (SCNT), and completely replaced somatic H1s by 60 min. The switching from somatic H1s to H1FOO following SCNT was developmentally regulated. H1FOO was replaced by somatic H1s during the late two- and four-cell stages. H1FOO association with chromatin can occur in the presence of a nuclear envelope and independently of pronucleus formation, is regulated by factors associated with the spindle, and is likely an active process. All SCNT constructs recapitulated the normal sequence of H1 transitions, indicating that this alone does not signify a high developmental potential. A paucity of all known H1s in two-cell embryos may contribute to precocious gene transcription in fertilized embryos, and the elaboration of somatic cell characteristics in cloned embryos.

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Introduction

Fertilization unites, within a single cell, two haploid genomes with markedly different chromatin structures. The chromatin of growing and maturing oocytes is presumed to contain a somatic set of core histones and a recently discovered (Tanaka et al., 2001) oocyte-specific H1 linker histone referred to as H1FOO (H1 histone family, oocyte-specific; formerly known as H1oo). The highly compact paternal chromosomal complement, in turn, is largely ordered by representatives of the protamine family. Inevitably, synkar-

yogamy requires that the maternal and paternal chromatin be comparably ordered, thereby permitting the genesis of a totipotent embryonic genome. To this end, the fertilizing paternal chromatin undergoes extensive decondensation as protamines are replaced by maternally derived core histone octamers and by H1FOO. The maternal chromosomes, likewise, decondense upon the completion of meiosis. The resultant zygotic pronuclei display early differences in the vigor of gene transcription and in other characteristics before uniting at the time of the first mitotic division (Aoki et al., 1997; Henery et al., 1995; review, Latham and Schultz, 2001). These events are followed by sequential changes in the transcriptional competence of the developing embryonic genome (Bouniol et al., 1995; Christians et al., 1995; Clegg and Piko, 1982; Davis et al., 1996; Latham et al., 1992; Matsumoto et al., 1994; Ram and Schultz, 1993; Sawicki et al., 1981; Thompson et al., 1995; Van Blerkom and Brockway, 1975; Vernet et al., 1992). Initially, the embryonic

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genome lacks transcriptional control mechanisms. By the end of the two-cell stage, however, a transcriptionally repressive state becomes established, at which point transcriptional enhancers become necessary for transcription, as they are at later stages of development (Majumder et al., 1993; Martinez-Salas et al., 1989).

Genome activation and the development of the need for transcriptional enhancers involve changes in histone content and posttranslational modification (Adenot et al., 1997, 2000; Clarke et al., 1992; Fu et al., 2003; McLay and Clarke, 2003; Stein and Schultz, 2000; Stein et al., 1997; Wade et al., 1997; Wiekowski et al., 1997; Worrall et al., 1995). Prominent amongst these changes is the replacement of H1FOO with somatic H1s (Clarke et al., 1992, 1998; Fu et al., 2003; Tanaka et al., 2001). The degree of coordination between the loss of H1FOO and the acquisition of somatic H1s during normal development has not been characterized. It is likewise unknown whether the relative timing of H1FOO loss and that of somatic H1 acquisition might affect transcriptional activity.

In amphibians, the oocyte-specific H1 linker histone, B4, assembles onto decondensing sperm DNA, thereby replacing sperm-specific basic proteins to become the predominant H1 of embryonic chromatin through the mid-blastula transition (Dimitrov et al., 1993, 1994). The subsequent switch to somatic H1 at the time of gastrulation is associated with transcriptional changes, including the repression of the oocyte 5S rRNA genes. As such, B4 replacement may contribute to the transition from maternal to embryonic gene expression patterns (Bouvet et al., 1994). Similarly, somatic cell nuclear transfer (SCNT) in the *Xenopus* is followed by the loss of somatic H1 from the transferred nucleus, a change that is coupled to the acquisition of HMG1 and of B4 (Dimitrov and Wolffe, 1996). These transitions are presumed to be mediated, at least in part, by nucleoplasmin, a histone binding protein that may also promote transcription factor binding to chromatin by disrupting core histone octamers (Dimitrov and Wolffe, 1996; Kikyo et al., 2000; Okuwaki et al., 2001).

The mechanisms underlying the H1 linker histone transitions witnessed during mammalian gametogenesis and early embryogenesis may also prove relevant to the reprogramming of transferred somatic nuclei during cloning procedures. Thus far, changes in the histone complement of mammalian somatic cell nuclei following SCNT have not been described. In related studies, transfer of bovine blastomeric nuclei to activated [spindle–chromosome complex (SCC)-depleted] early interphase oocytes was followed by the loss of somatic H1 from the transferred nuclei over a period of ≥ 6 h (Bordignon et al., 1999). Somatic H1 loss occurs within 2–4 h after the transfer of bovine blastomeric nuclei to metaphase II (MII)-arrested or telophase-stage (SCC-depleted) oocytes (Bordignon et al., 1999). The loss of somatic H1 was accelerated following the transfer of murine blastomeres into MII-arrested oocytes as compared with activated oocytes (Bordignon et al., 2001). The speed

and extent of loss of somatic H1 in murine SCNT studies was also affected by the cell cycle stage of the donor nucleus and by the presence or absence of maternal chromosomes (Bordignon et al., 2001). The loss of somatic H1 was independent of DNA replication and transcription, but was sensitive to treatment with inhibitors of protein synthesis and of protein kinases (Bordignon et al., 2001). Interestingly, microinjected somatic H1 could be incorporated into bovine pronuclei (Lin and Clarke, 1996). Somatic H1 reappeared in the nuclei of bovine clones at the 8- to 16-cell stage, a time when transcriptional activity is increasing (Bordignon et al., 1999). In so doing, cloned bovine embryos recapitulated the timeline of somatic H1 acquisition noted for fertilized counterparts (Smith et al., 1995).

While changes in the association of somatic H1 with chromatin have been observed following blastomere nuclear transfer in cattle and in mice, no comparable data have been reported for H1FOO, the recently discovered pre-embryonic murine H1 linker histone (Tanaka et al., 2001). Moreover, H1 transitions following the transfer of adult (as distinct from blastomeric) nuclei, for which term development is very low, have not been examined. Similarly, the impact of the type of donor cell on H1 transitions in cloned constructs has not been addressed. Thus, key factors in the success or failure of mammalian cloning procedures have not been studied with respect to transitions in the H1 linker histone composition.

We describe herein the results of SCNT studies in the mouse, in which adult cumulus or myoblast nuclei were transferred into SCC-depleted /MII-arrested oocytes, parthenogenetically activated oocytes, or zygotes. These constructs were analyzed at periodic intervals after SCNT for the presence of somatic H1 and H1FOO in the cognate chromatin. Our results reveal the existence of an active mechanism for the rapid and visibly complete replacement of somatic H1 by H1FOO within minutes of SCNT, followed by recapitulation of the normal loss of H1FOO and the assembly of somatic H1 onto embryonic chromatin at the two to four-cell stages. The ability of the oocyte to drive the switch to H1FOO is developmentally regulated, and H1 switching appears to be an active process. Interestingly, the transition from somatic H1 to H1FOO occurs in all of the cloned constructs (MII-arrested recipients) examined. The relevance of these observations to understanding the mechanisms of H1 linker histone regulation of chromatin structure in the early embryos, and in the biology of cloned embryos, is discussed.

Materials and methods

Collection of MII-arrested oocytes and the preparation of cumulus cells

Female (B6D2)F₁ mice (8–10 weeks old) were superovulated by the sequential injection of 5 i.u. each of equine chorionic gonadotropin (eCG) and human chorionic gonad-

otropin (hCG). Cumulus-enclosed oocytes were collected 13–14 h after the administration of hCG. Cumulus cells were removed from oocytes by treatment with hyaluronidase as described (Chung et al., 2002). Cumulus-free oocytes were then washed and cultured in CZBG medium (Chatot Ziomek Bavister medium supplemented with 5.5 mM glucose) (Chatot et al., 1989; Kuretake et al., 1996) before micromanipulation. Cumulus cell suspensions were washed with HEPES-CZB medium, resuspended in a small amount of CZBG medium containing 3% PVP, and kept on ice before nuclear transfer. All studies adhered to procedures consistent with the *National Research Council Guide for the Care and Use of Laboratory Animals*.

Removal of SCCs from MII-arrested oocytes followed by nuclear transfer

To generate diploid cloned constructs by SCNT, SCCs of MII-arrested oocytes were first removed as described (Gao et al., 2003). Groups of 25–30 oocytes were transferred to a drop of HEPES-CZB medium containing 2.5 $\mu\text{g/ml}$ of cytochalasin B (CB). The SCC was removed using a pipette with an inner diameter of 8–10 μm assisted by piezo-drill pulses. The time to remove the SCC was typically limited to 10 min per group of oocytes. After SCC removal, oocytes were kept in CZBG medium until injection. Groups of 15 SCC-free oocytes were transferred to HEPES-CZB for injection. A pipette with an inner diameter of 5 μm was used to aspirate

cumulus cells, breaking the cell membrane, and allowing most of the cytoplasm to be removed by two or three rounds of gentle pipeting. After injection of the cumulus nucleus, the cloned constructs were allowed to recover for several minutes before transfer back to CZBG medium. For myoblast nuclear transfer, electrofusion was employed in recognition of the larger size of the nuclei. Electrofusion was achieved using 90 V/mm pulses in fusion medium containing 275 mM mannitol, 0.05 mM CaCl_2 , 0.10 mM MgSO_4 , and 0.3% bovine serum albumin (BSA). Most myoblasts fused to the recipient oocytes after a single pulse, but if needed one or two additional pulses were applied at approximately 30-min intervals. The myoblasts employed were cultured from the hindlimb muscle of (C57BL/6 \times *Mus m. castaneus*) F₁ mice as described (Donaghue et al., 1992; Springer et al., 1997). Removal of the SCC typically begins within 20 min after oocyte isolation, and SCNT is typically completed within 2 h.

Activation of oocytes and of cloned constructs

Reconstructed oocytes were cultured in CZBG medium for approximately 1–2 h before activation as described (Chung et al., 2002; Gao et al., 2003). Activation was achieved during 6 h of culture in calcium-free CZBG medium containing 10 mM of strontium and 5 $\mu\text{g/ml}$ of CB. The CB stock was prepared in ethanol at 1000 \times concentration as described. Activated constructs were thoroughly washed and cultured in MEM- α medium (Sigma-Aldrich, St. Louis, MO)

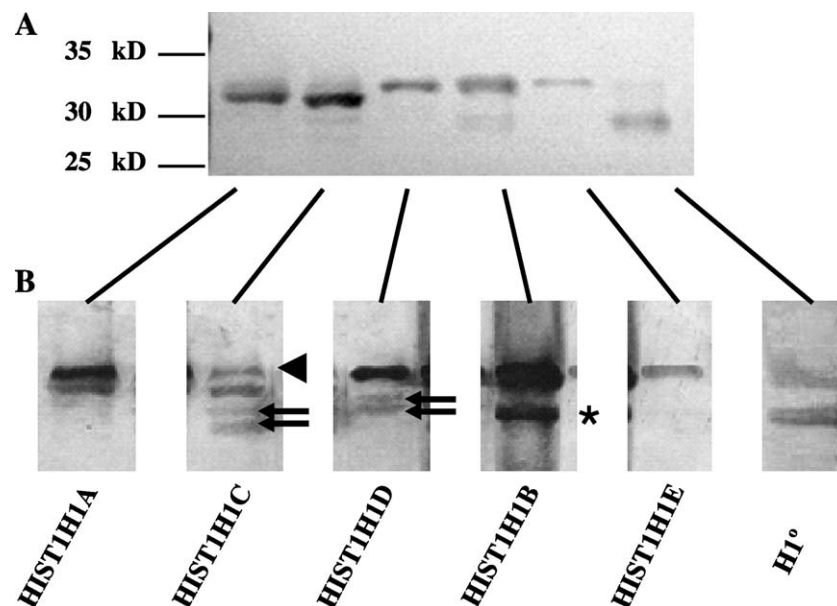


Fig. 1. Immunoblot of mouse histone H1 subtypes to illustrate the reactivity of the anti-somatic H1 antibody. The anti-somatic H1 antibody was originally generated against the first 35 amino acids of the amino-terminal tail for HIST1H1B (also known as H1^s-3; Parseghian et al., 1994). For panels A and B, 0.5 μg samples of the subtypes were loaded in each lane. (A) Immunoblot stained with a reversible general protein stain (FastStain) according to the manufacturer's instructions. (B) Immunoblot stained with anti-somatic H1 at a dilution of 1:100 and then stained with alkaline phosphatase-conjugated goat anti-rabbit antibody as a secondary antibody at a dilution of 1:1000. Cross-reactivity occurs with each of the subtypes; however, greatest cross-reactivity is seen against the mouse HIST1H1B, HIST1H1D, and HIST1H1A homologs (also known as H1^s-3, H1^s-2, and H1a). The anti-somatic H1 is also capable of detecting minute quantities of degradation products, as denoted by the black arrows. The (*) highlights the detection of a common HIST1H1B degradation product that is barely detectable with the general protein stain; similarly, the arrowhead highlights the detection of HIST1H1D present in the HIST1H1C sample lane.

supplemented with 5 mg/ml BSA and glutamine. It should be noted that viable cloned progeny have been produced in the Latham laboratory using the procedures described here for cumulus cell nuclear transfer (S. Gao and K. Latham, unpublished).

Collection of early stage oocytes and fertilized embryos

For analysis of H1 expression in growing oocytes, oocytes were isolated from 8-day-old females using colla-

genase digestion as described (Eppig and O'Brien, 1996), and then fixed immediately for analysis (see below). For analysis of expression in germinal vesicle (GV)-intact oocytes, oocytes were recovered from the ovaries of eCG-primed 4- to 6-week old mice and cultured in MEM α medium in the presence of 0.2 mM isobutylmethylxanthine (IBMX; a potent inhibitor of the resumption of meiosis). Some of these GV-stage oocytes were fixed for analysis. For yet other experiments, the GVs were removed and the oocytes permitted to progress to the equivalent of an MII

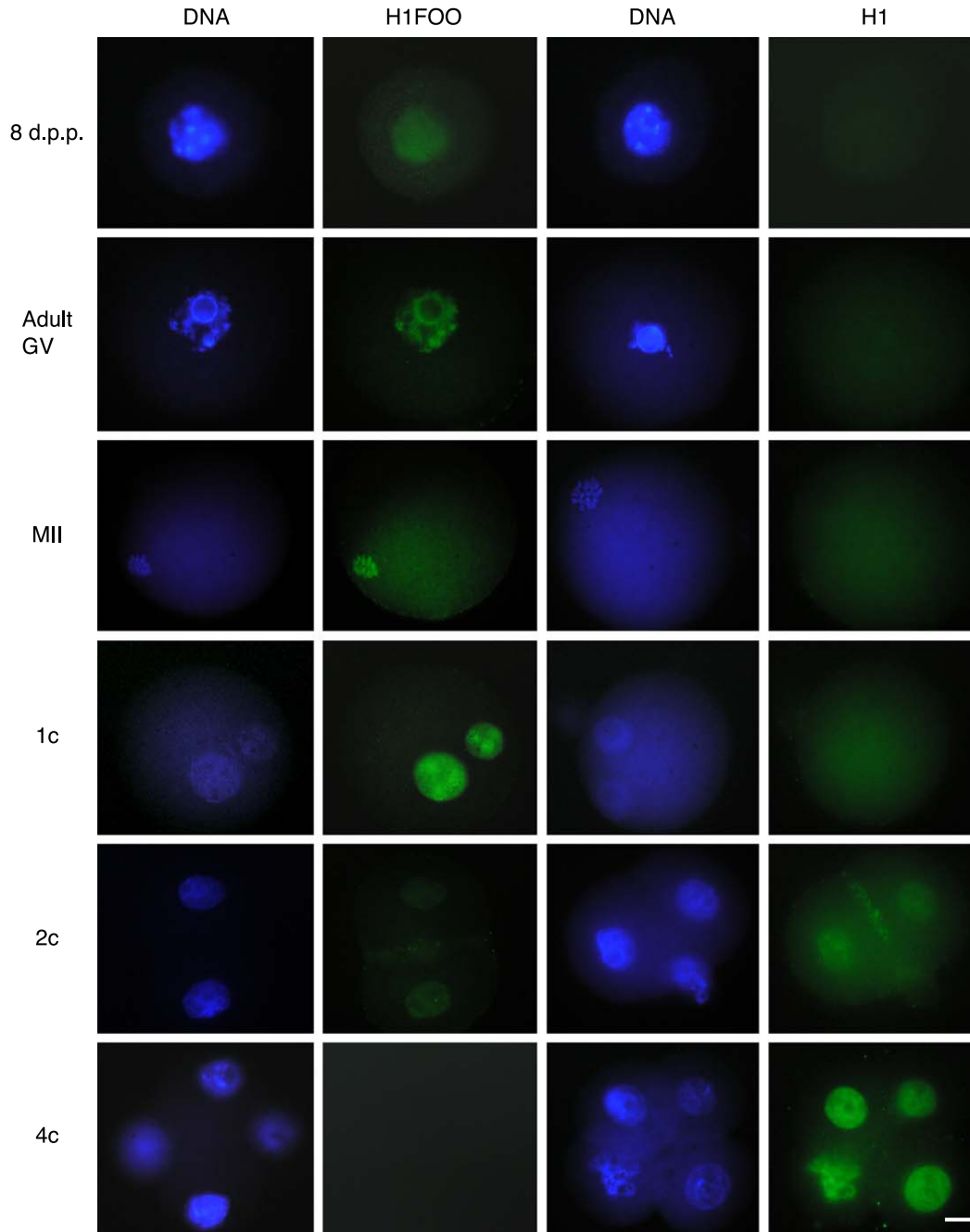


Fig. 2. Nuclear ontogeny of H1FOO and somatic H1s. Oocytes and embryos were fixed and analyzed at the stages indicated, beginning with the growing oocyte (8 days postpartum) through the four-cell embryo stage (4C). DNA was visualized by DAPI staining and H1FOO and somatic H1s by indirect immunofluorescence. Note that in nuclei of the two-cell stage embryo (2C), neither H1 linker histone exists in high abundance. The bar equals 20 μ m.

stage by overnight culture in the absence of IBMX (Gao et al., 2002). Confirmation of ooplasmic maturation was accomplished by documenting changes in MAP-kinase activity (data not shown), as described (Sun et al., 1999). For studies requiring zygotes, successfully mated (B6D2)F₁ female mice were sacrificed 20 h post-hCG and zygotes were collected in M2 medium. After repeated washing, the retrieved zygotes were cultured in MEM- α medium. This medium supports efficient development of cumulus cell-cloned constructs as well as fertilized embryos (S.G. and K.E.L., unpublished). Finally, for some studies, fertilized embryos were obtained by intracytoplasmic sperm injection (ICSI) as described (Kimura and Yanagimachi, 1995). For this purpose, sperm were frozen in liquid nitrogen, and then thawed immediately before injection. Sperm heads were injected and the oocytes activated spontaneously by the endogenous sperm components. The resulting embryos were then fixed at 5, 30, and 60 min after ICSI.

Immunofluorescent detection of H1FOO and of somatic H1

Preantral stage, GV-stage, and MII-stage-arrested oocytes, as well as control fertilized embryos, parthenogenones, and nuclear transfer constructs were fixed in 2% paraformaldehyde for 30 min followed by permeabilization in PBS containing 0.1% Triton X-100 for 30 min. Fixed samples were then treated with PBS containing 10% goat serum and 0.5% Triton X-100 for 1 h at room temperature and then incubated with rabbit polyclonal antibodies (anti-H1FOO or anti-somatic H1) for 1 h at room temperature. After three washes in PBS containing 0.3% BSA and 0.1% Triton X-100, the samples were incubated with the Alexa Fluor 488 goat anti-rabbit IgG conjugate (Molecular Probes, Eugene, OR; diluted 1:1000) for 1 h at room temperature, washed, and stained with 4',6-diamidino-2-phenylindole (DAPI) for 5 min. The samples were then transferred to a drop of anti-fade mounting medium (Molecular Probes) previously placed on

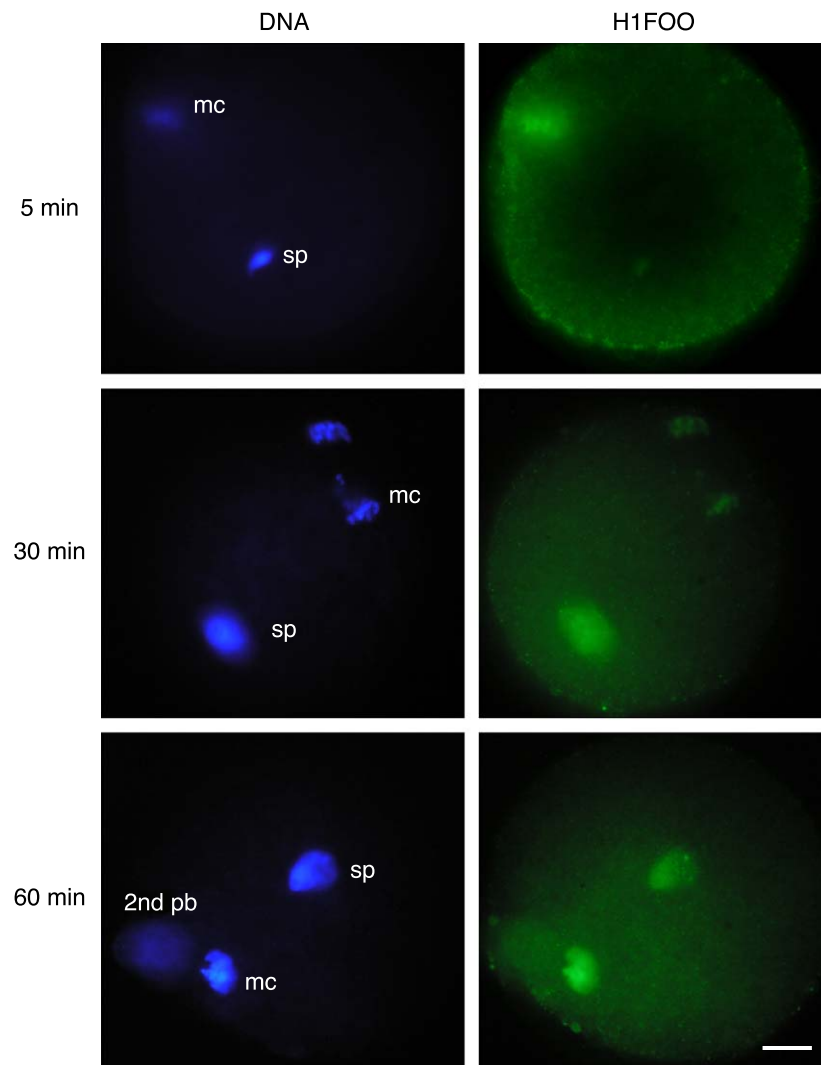


Fig. 3. Acquisition of H1FOO by the paternal chromatin following ICSI. Sperm heads were injected into MII-arrested oocytes. The resultant zygotes were fixed at the times indicated. Note faintly positive H1FOO staining of the injected sperm DNA at 5 min post-ICSI, followed by progressive signal intensification. mc, maternal chromosomes. sp, sperm DNA. pb, polar body. The bar equals 20 μ m.

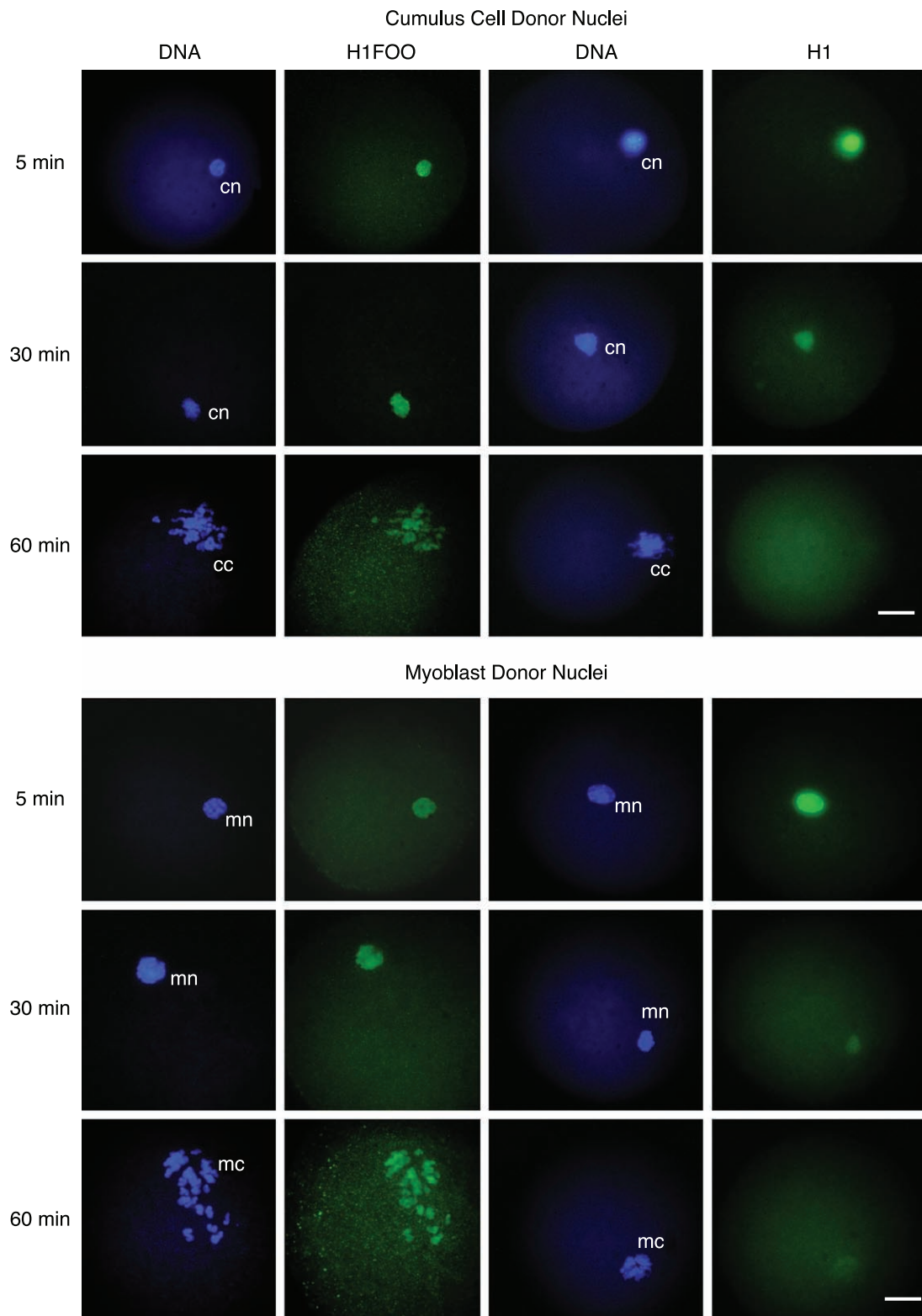


Fig. 4. H1 linker histone transitions in the chromatin of somatic cell nuclei following SCNT. Cumulus cell nuclei were injected into MII-arrested ooplasts, or myoblast nuclei were introduced by electrofusion. The resultant cloned constructs were fixed at the times indicated. Note that H1FOO assembles on the donor nuclei within 5 min. Somatic H1s, in turn, are progressively lost from the donor nuclei by 60 min postinjection. cn, cumulus nucleus; cc, condensed cumulus chromosomes; mn, myoblast nucleus; mc, condensed myoblast chromosomes. The bar equals 20 μ m.

glass slides and mounted under coverslips. Fluorescence was examined using an Olympus microscope equipped for epifluorescence with appropriate filter sets.

The H1FOO-directed antibody may recognize two isoforms, which are expressed in the developing oocyte and in the one-cell stage embryo (Tanaka et al., 2001). The anti-somatic H1 antibody was originally generated in rabbits against a synthetic peptide consisting of the first 35 amino acids of the amino-terminal tail for human HIST1H1B (also known as H1^{S-3}) (Parseghian et al., 1994). The antibody cross-reacts with all known somatic H1s and its cross-reactivity was demonstrated against the mouse somatic H1 subtypes by Western blotting (Fig. 1). The histone H1 subtypes were isolated from mouse liver according to the method in Parseghian et al. (1993). Electrophoresis of the linker histones was performed on a 14% SDS-polyacrylamide gel for 1.5 h at 200 V. Transfer onto a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) was conducted at 25 V for 1.5 h and employed a 12 mM Tris, 96 mM

glycine, 20% methanol transfer buffer. The success of protein transfer was assessed with a reversible general protein stain, known as FastStain, according to the manufacturer's instructions (Genotech, St. Louis, MO). After destaining, the membranes were soaked for 30 min in blocking buffer [2% BSA in TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.5)] followed by incubation in a 1:100 dilution of anti-somatic H1 in blocking buffer for 1 h at 25°C. After three 5-min washes in wash buffer [TBST (0.1% Tween 20 in TBS)], the membranes were incubated with a 1:1000 dilution of alkaline phosphatase-conjugated goat anti-rabbit antibody (Sigma, St. Louis, MO) in blocking buffer for 1 h at 25°C. Following three more 5-min washes with wash buffer, and one 5-min wash with TBS, the membranes were incubated with NBT/BCIP substrate until color development (NBT/BCIP ready-to-use tablets: Roche, Mannheim, Germany). Due to the highly basic nature of these proteins, the H1s appear to run at a molecular weight of approximately 31 kDa; however, their actual molecular weight is 0.67× smaller at 21–22 kDa.

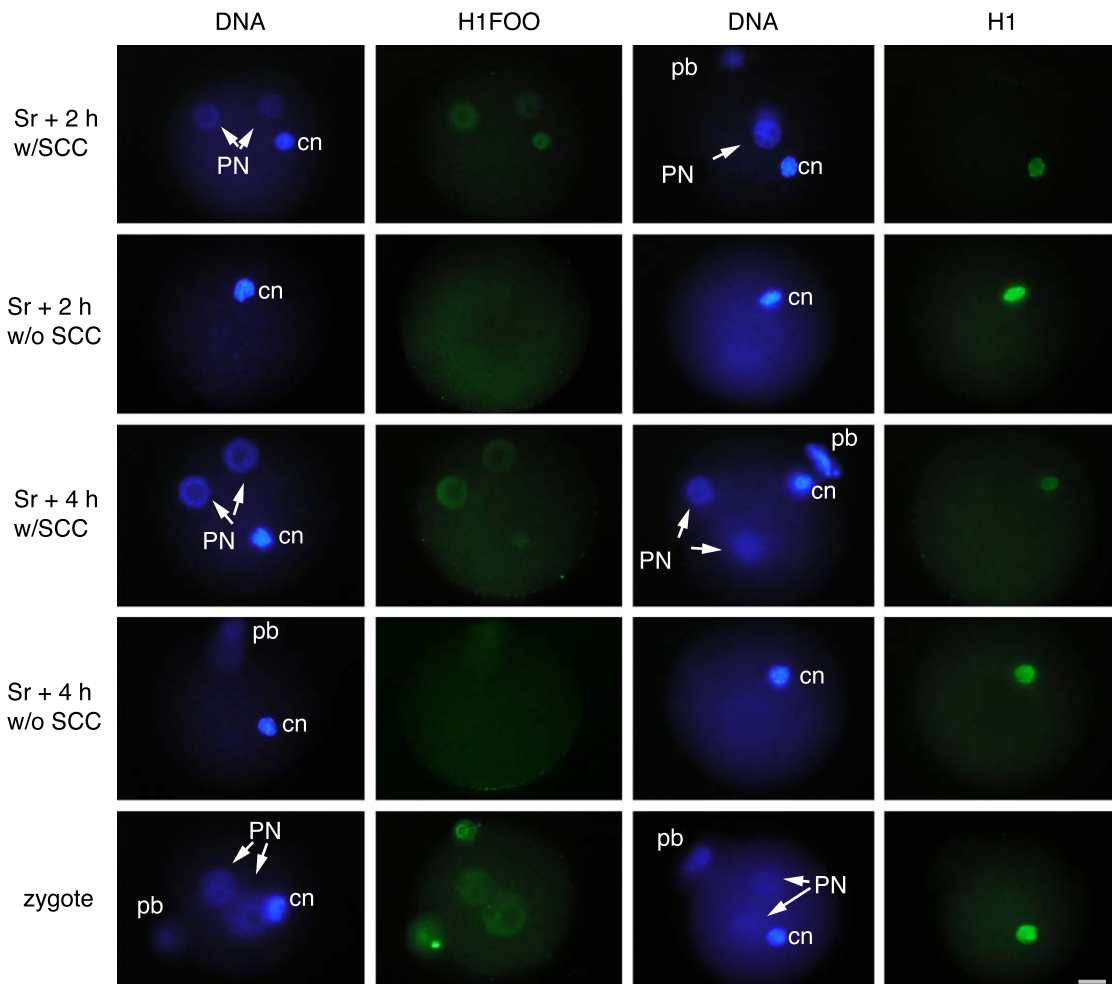


Fig. 5. Effect of the developmental stage of the recipient ooplasm and of the SCC on H1 linker histone transitions following SCNT. Cumulus cell nuclei were injected into parthenogenetically activated oocytes with or without SCC at either 2 or 4 h postactivation (addition of SrCl₂, abbreviated as Sr), as indicated. The injected constructs were fixed at 1 h postinjection. The bottom row depicts the injection of cumulus nuclei into fertilized zygotes, and then fixed after 1 h of culture. PN, pronuclei. pb, polar body. cn, cumulus nucleus. The bar equals 20 μm.

Results

H1 linker histone transitions during oocyte growth, oocyte maturation, fertilization, and early embryogenesis

Studies of H1 linker histone transitions during normal mammalian development have been limited. Previous studies focused on the expression of somatic H1 representatives during oocyte growth and maturation and on their marked enhancement during early embryonic cleavage (Bordignon et al., 1999, 2001). The simultaneous documentation of H1FOO dynamics has not been undertaken, nor has the coordination of the developmental transitions of somatic H1 and H1FOO been characterized. To understand the timing and extent of switching between somatic H1s and H1FOO during oogenesis and early development of fertilized and cloned embryos, the temporal patterns of expression of somatic H1 and H1FOO were studied in detail.

Growing oocytes from 8-day-old mice displayed moderately intense H1FOO immunoreactivity (Fig. 2). Immunofluorescent analysis of fully grown/meiotically competent GV-stage, MII-arrested oocytes, and fertilized embryos (Fig. 2) revealed abundant immunoreactive H1FOO associated with the attendant chromatin, including polar bodies and zygotic pronuclei. However, H1FOO staining was greatly diminished in the two-cell stage embryo and virtually absent by the four-cell stage. A reciprocal pattern of staining was observed when using a somatic H1-directed antibody, which did not stain early (day 8 postpartum) growing, GV-stage, or MII-stage oocytes, polar bodies, or zygotic pronuclei. Instead, faint staining corresponding to somatic H1 was noted at the two-cell stage, a markedly increased signal being noted by the four-cell stage. Interestingly, the slow acquisition of somatic H1 staining relative to the timing of loss of H1FOO staining suggests that a unique transitional state may exist during the two-cell stage wherein neither H1FOO

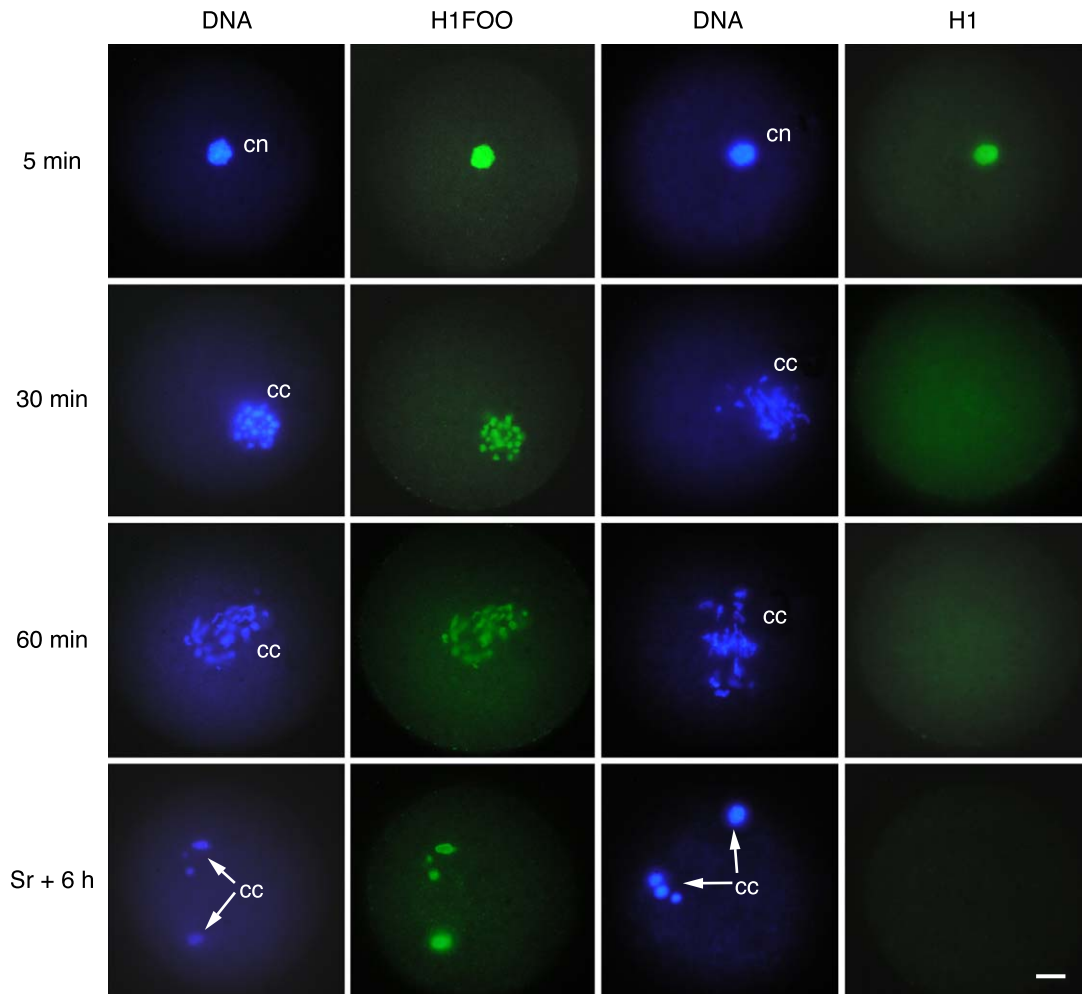


Fig. 6. Lack of effect of GV removal before cytoplasmic maturation on H1 linker histone transitions following SCNT. Germinal vesicles were removed before the *in vitro* cytoplasmic maturation of enucleated ooplasts. Cumulus nuclei were then injected, and the constructs fixed at 5, 30, and 60 min postinjection, and at 6 h post-oocyte activation in Sr^{2+} -containing medium. cn, cumulus nucleus. cc, cumulus chromosomes. Note that pronucleus formation does not occur in these ooplasts, as documented (Gao et al., 2002). The bar equals 20 μ m.

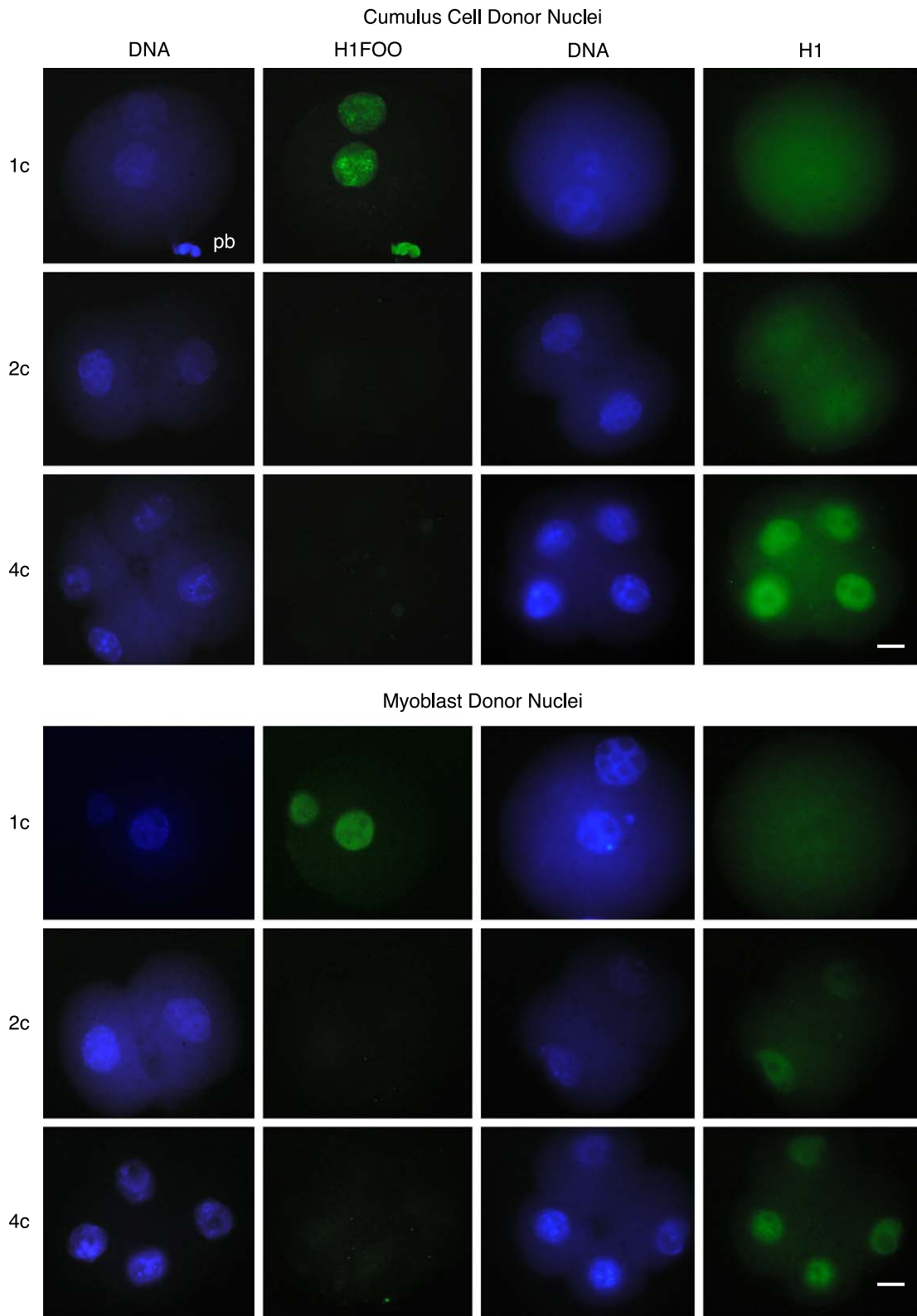


Fig. 7. H1 linker histone transitions following activation of the oocytes after somatic cell nuclear transfer. Cumulus cell nuclei were injected into MII-arrested ooplasts. Myoblast nuclei were introduced into ooplasts by electrofusion. Constructs were activated and then fixed at the one-cell (1C), two-cell (2C), and four-cell (4C) stages. pb, polar body. The bar equals 20 μ m.

nor its somatic H1 counterpart are abundantly associated with the attendant chromatin.

To examine the kinetics with which H1FOO assembles onto the chromatin of the paternal genome after fertilization, MII-arrested oocytes were fertilized by ICSI, fixed at 5, 30, and 60 min after injection, and analyzed for H1FOO association with sperm chromatin (Fig. 3). Within 5 min of ICSI, H1FOO was detectable on the condensed sperm chromatin. By 30 and 60 min postinjection, H1FOO was clearly visible on decondensed sperm chromatin.

Acquisition of H1FOO by somatic nuclei following transfer

To determine whether the oocyte-specific H1FOO is acquired by somatic nuclei following SCNT, as are HMG1 and B4 in amphibians (Dimitrov and Wolffe, 1996), and to characterize the timing of this process, cumulus cell nuclei were injected into MII-arrested oocytes from which the SCC had been removed (Fig. 4). It may be noted that within 30 min of SCNT, the nuclear envelope breaks down and the chromosomes condense and assemble onto a spindle. During karyokinesis, the chromosomes are allocated in a random fashion to either spindle pole (Tateno et al., 2003). Suppression of second polar body extrusion with CB prevents chromosome loss, thereby maintaining a combined diploid chromosome complement within the two pseudopronuclei.

Cloned constructs were fixed 5, 30 and 60 min after SCNT and processed for immunofluorescent microscopy. As early as 5 min after injection, more than 80% of the transferred cumulus cell nuclei displayed intense positive staining for H1FOO. Staining was further increased in intensity by 30 min, at which time all transferred cumulus nuclei were positively stained. At 1 h post-SCNT, the nuclear envelope of the transferred cumulus nuclei had broken down, thereby revealing individual H1FOO-bearing chromosomes. These data indicate that H1FOO is not only capable of entering the transferred donor nucleus but is also able to assemble onto the corresponding chromatin.

To determine whether the donor cell type was of relevance to this series of events, myoblast nuclei were electrofused into SCC-free/MII-arrested oocytes (Fig. 4). Once again, intense nuclear staining for H1FOO was seen in the myoblast nucleus within 5 min of SCNT, a further increase in intensity being noted by 30 min of incubation. Intense staining of individual somatic chromosomes for H1FOO was again apparent after nuclear envelope breakdown (60 min incubation).

Loss of somatic H1s by somatic nuclei following transfer

To examine the corresponding dynamics of somatic H1s, cloned constructs were subjected to immunofluorescent staining using the somatic H1-directed antibody. Intense staining for somatic H1 was still evident in cumulus or myoblast nuclei 5 min post-SCNT (Fig. 4).

By 30 min post-SCNT, however, the staining intensity for somatic H1s was greatly reduced in all embryos. By 60 min post-SCNT, staining for the somatic H1s had all but disappeared.

Effect of the developmental stage of the recipient oocyte on H1 linker histone transitions post-SCNT

To determine whether the developmental stage of the recipient oocyte affected the acquisition of H1FOO or the loss of somatic H1s, cumulus nuclei were transferred into parthenogenetically activated intact (i.e., the SCC is present) oocytes, activated SCC-free oocytes, and fertilized one-cell stage zygotes (Fig. 5). It may be noted that parthenogenetic activation and suppression of second polar body extrusion will lead to karyokinesis and to the formation of two pronuclei. This allows for parallel staining of endogenous maternal chromatin and of exogenous somatic chromatin when the SCC is not removed. Cumulus cell nuclei transferred into parthenogenetically activated oocytes (2 and 4 h postactivation) acquired H1FOO staining relatively promptly with the SCC present. Because somatic cell nuclear envelope breakdown does not occur with this stage of activated oocyte recipient, these findings suggest that H1FOO can transit through the intact nuclear envelope of the transferred cumulus cell nucleus. The overall H1-to-H1FOO exchange was greatly inhibited, however, as evidenced by the persistence of somatic H1s in the transferred cumulus cell nuclei. Interestingly, transfer of cumulus nuclei into SCC-free/parthenogenetically activated oocytes was associated with complete failure to acquire H1FOO by the transferred cumulus cell nuclei. Thus, the absence of the SCC further diminished or accelerated the loss of the ability of the ooplasm to mediate H1 switching. Cumulus cell nuclei transferred into zygotes failed to acquire H1FOO (Fig. 5), providing additional evidence for a stage-dependent decline in the oocytic ability to effect H1 switching.

Effect of GV removal on the acquisition of H1FOO by somatic nuclei post-SCNT

Previous studies revealed that the removal of the GV before the maturation of the ooplasm precluded pronucleus formation following the transfer of embryonic stem cell nuclei (Gao et al., 2002). In that the H1FOO protein contains a nuclear localization signal and is preferentially localized to the nuclear compartment, the removal of the GV may give rise to a relative paucity of H1FOO, and thus to failure of pronucleus formation. To examine this hypothesis, cumulus cell nuclei were transferred into ooplasts that were at a stage equivalent to that of MII-arrested oocytes, having been matured in vitro after the removal of the GV. As shown (Fig. 6), the acquisition of H1FOO and the loss of somatic H1s proceeded efficiently, despite the failure of pronuclei to form after oocyte activation (Fig. 6; bottom

row), much as it did following the standard SCNT protocol (Fig. 4). Interestingly, the loss of somatic H1s from the transferred cumulus cell nuclei occurred more rapidly (within 30 min versus 60 min) in these ooplasts than in MII-arrested oocytes from which the SCC was removed after maturation.

The H1FOO-to-somatic H1 transition during the early cleavage stages of cloned embryos

To determine whether cloned embryos recapitulated the temporally regulated loss of H1FOO and the reciprocal re-appearance of somatic H1, as noted for fertilized counterparts (Fig. 2), the transferred cumulus or myoblast cell nuclei were examined for the presence of H1FOO and of somatic H1s in one-, two-, and four-cell stage embryos (Fig. 7). H1FOO was undetectable by the two-cell stage in cloned constructs derived from cumulus or myoblast cell nuclei. Nuclear staining for somatic H1s was apparent, albeit at a low intensity at the two-cell stage, a significant increase in intensity being noted by the four-cell stage. As such, the temporal pattern of the transition from H1FOO to somatic H1s in cloned embryos closely recapitulated the pattern observed in fertilized embryos.

Discussion

The observations described herein, combined with earlier observations (Tanaka et al., 2001), reveal that H1FOO is the predominant H1 linker histone associated with chromatin during oocyte growth, oocyte maturation, and early embryogenesis. It is equally apparent that H1FOO can direct the rapid reordering of chromatin from a variety of sources, displacing somatic H1s in the process. Although other studies suggest that some somatic H1s, such as HISTH1F0 (also known as H1^o), exist in the mammalian oocyte and early embryo (Fu et al., 2003), our data indicate that the ooplasmic H1FOO content may vastly exceed that of somatic H1s, as the somatic H1s are acquired rather slowly and do not reach high abundance in chromatin until the four-cell stage, a while after H1FOO is lost from the chromatin. As such, our results are compatible with those obtained in the *Xenopus* model, in which the maternally expressed oocytic H1 content also vastly exceeds somatic H1s (Dworkin-Rastl et al., 1994; Hock et al., 1993).

H1FOO assembles rapidly onto paternally derived chromatin of the incoming sperm, presumably in lieu of protamines. Likewise, H1FOO rapidly and completely displaces somatic H1s from transferred somatic cell nuclei introduced during cloning procedures. Remarkably, the acquisition of H1FOO initiates within 5 min of either SCNT or ICSI. The total replacement of somatic H1s by H1FOO appears complete by 1 h after SCNT. Qualitatively similar results were obtained when using two different

donor cell types, thereby indicating that the H1 linker histone transitions observed are likely donor nucleus-independent. The apparent uniformity of H1 linker histone transitions in both normal and clonal development indicates that the mechanisms responsible for these transitions constitute an inherent property of the oocyte. Indeed, the oocyte appears capable of using any chromatin as its substrate, an ability that surely plays an integral role in creating a developmentally potent embryonic genome during either normal or clonal development.

The replacement of somatic H1s of the donor chromatin likely begins before nuclear envelope breakdown. Indeed, the nuclear envelopes of donor cell nuclei appeared intact for most of the time period relevant to the H1 linker histone transitions. Equivalent results were obtained when using either electrofusion or piezo-mediated injection. Although the possibility of piezo-mediated mechanical damage to nuclear envelopes has not been formally excluded, there should be no mechanical disruption of the nuclear envelope after electrofusion. It is unlikely, therefore that the rapid entry of H1FOO into the donor nucleus is dependent upon nuclear envelope rupture, indicating that H1FOO can traverse the nuclear envelope of the somatic nucleus. This conclusion is in agreement with studies of cultured somatic cells, wherein H1FOO-GFP fusion proteins readily assembled onto somatic chromatin (Tanaka et al., unpublished). Thus, while other aspects of nuclear reprogramming may require nuclear envelope breakdown (Czolowska et al., 1984; Kim et al., 2002; Szollosi et al., 1986, 1988), the assembly of H1FOO onto chromatin clearly does not.

The oocytic capacity to direct H1 linker histone replacement is developmentally regulated. Indeed, the ability to deplete somatic H1 variants from transferred somatic nuclei is diminished rapidly, within 2–4 h post-SCNT. Because H1FOO can incorporate into intact somatic nuclei, this loss in ooplasmic ability to direct the somatic H1-to-H1FOO transition cannot be attributed to the persistence of somatic nuclear envelopes in activated oocytes. Removal of the SCC before SCNT further accelerates the loss of the oocytic ability to mediate H1 linker histone switching. This indicates that the H1 linker histone exchange is a developmentally regulated process rather than one that is simply dependent upon the presence or absence of a nuclear envelope. Additionally, these observations provide important insight into the inability of activated oocytes to support efficient cloning.

These results argue for an active mechanism directing the removal of somatic H1s from the transferred somatic nuclei of cloned constructs, one reminiscent of the active mechanisms directing the remodeling of sperm chromatin following normal fertilization. The rapid acquisition of H1FOO within 5 min of nuclear transfer could be attributed to occupation of available (i.e., previously unoccupied) nucleosomal sites. Alternatively, active removal of somatic H1s from transferred somatic nuclei may begin immediately after transfer, thereby rapidly creating “uncoupled” regions of

chromatin for H1FOO to occupy. Finally, it is possible that H1FOO “displaces” somatic H1s by virtue of its higher affinity for nucleosomal DNA (Becker et al., unpublished). The rapid loss of the ability to remove somatic H1 after egg activation could contribute to the inability of recipient stages beyond MII to support efficient clonal development in mice (Czolowska et al., 1984; Kim et al., 2002; Szollosi et al., 1986, 1988). The results of experiments employing in vitro-matured ooplasts indicate that the ooplasm constitutes a rich source of H1FOO by way of a substantial cytoplasmic reservoir or through ongoing translation of maternally inherited mRNA. Indeed, removal of the GV before the resumption of maturation was not sufficient to curtail the apparent non-rate-limiting supply of H1FOO. This result also indicates that the inability of pronuclei to form after SCNT to ooplasts obtained by maturation in the absence of a GV is not attributable to rate-limiting availability of H1FOO.

Cloned constructs recapitulated the developmentally regulated pattern of exchange of H1FOO for somatic H1 linker variants seen in fertilized embryos. Interestingly, H1FOO almost completely disappeared by the two-cell stage, whereas somatic H1 intensified noticeably between the two- and four-cell stages. This transitional pattern was observed in both cloned constructs and fertilized embryos. To the best of our knowledge, this report is the first to study thoroughly the transitional developmental dynamics of both H1FOO and somatic H1. This report is also the first to reveal that the two-cell stage may be characterized by an overall deficiency of H1 linker histones at a time when H1FOO has disappeared but has not, as yet, been fully replaced by somatic H1s. Previous studies revealed that early embryonic genes may be lacking in transcriptional control mechanisms (Majumder et al., 1993; Martinez-Salas et al., 1989; Wiekowski et al., 1997). Because H1 linker histones are believed to play an integral role in transcriptional regulation via a chromatin-repressive effect (Dimitrov and Wolffe, 1996; Dimitrov et al., 1993, 1994), our observations raise the interesting possibility that the “immature” nature of gene expression in the early embryo may be due, in part, to transient deficiency of H1 linker histone in the attendant chromatin when transcription first begins.

Because cloned embryos follow a pattern of H1 transitions comparable to that of fertilized embryos, the preceding observations may have significant implications for the health and viability of cloned embryos. We have shown previously that cloned embryos display dramatically altered culture medium requirements and molecular characteristics of somatic cells. We have suggested that this reflects the continued expression of the donor cell program in the early cloned embryo, as a result of slow or inefficient nuclear reprogramming (Chung et al., 2002; Gao et al., 2003). The apparent delay between the loss of H1FOO and complete assembly of somatic H1s could contribute to the expression of somatic cell characteristics by early cloned constructs by allowing a more open chromatin configuration and thus increasing the

tendency to transcribe those genes that were active in the donor nucleus.

Lastly, the loss of somatic H1s and the rapid acquisition of H1FOO occurred in all of the cloned constructs examined. It was previously suggested that such transitions could provide useful indicators of nuclear reprogramming (Bordignon et al., 2001). However, the occurrence of such transitions in 100% of cloned constructs, of which only 2–3% may be competent to develop to term, casts doubt on this suggestion. While the transition in chromatin-associated H1s may contribute to the process of reprogramming and the eventual correct regulation of gene transcription, it is not likely to constitute a useful indicator of successful nuclear reprogramming or of high developmental competence.

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