Architectural defects in pronuclei of mouse nuclear transplant embryos

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Summary

Reprogramming somatic nuclear function by transplantation of nuclei into recipient oocytes is associated with a morphological remodeling of the somatic nucleus. Successful cloning of animals by nuclear transplantation (NT) demonstrates that reprogramming somatic cell function is possible. However, low pregnancy rates and high frequencies of lethal abnormalities in animals born suggest that reprogramming is rarely complete. To address this issue, we tested the hypothesis that nuclear transplantation leads to nuclear remodeling deficiencies. We report the identification of several markers of morphological remodeling, or lack thereof, of mouse cumulus cell nuclei after transplantation into oocytes. Notably, nuclear transplant mouse embryos exhibit nuclear assembly of the differentiated cell-specific A-type lamins at the one-cell stage, as a result of misregulation of *lamin A* gene expression. The transplanted nuclei also display enhanced concentration of the nuclear matrix-associated protein NuMA as a result of translation from maternal mRNA and de novo transcription. The A-kinase anchoring protein 95 (AKAP95), a marker of the nuclear envelope-chromatin interface, is of somatic origin. Furthermore, greater resistance of AKAP95 and DNA to in situ extractions of one-cell stage NT embryos with non-ionic detergent, DNase, RNase and NaCl reflects an enhanced proportion of heterochromatin in these embryos. Passage through first embryonic mitosis does not rescue the defects detected in one-cell stage embryos. We propose that somatic nuclear reprogramming deficiencies by NT might emanate from, at least in part, failure to remodel the somatic nucleus morphologically into a functional embryonic nucleus.

Key words: Chromatin, Embryo, Mouse, Nuclear envelope, Nuclear transplantation

Introduction

Successful development to term of mammals cloned by somatic cell nuclear transplantation (NT) demonstrates that complete reprogramming of a differentiated somatic nucleus is possible (Oback and Wells, 2002). Functional nuclear reprogramming is believed to occur through a morphological remodeling of the donor nucleus following introduction into a recipient oocyte cytoplast. Nuclear remodeling includes a phase of nuclear envelope breakdown as donor chromosomes condense (Barnes et al., 1993; Collas and Robl, 1991). Upon activation of the oocyte, decondensation of the donor chromatin and reformation of a nuclear envelope take place, reconstituting a nucleus that is expected to function as that of a fertilized embryo (Stice and Robl, 1988). Nevertheless, current limitations to the application of mammalian cloning technology include low rates of embryonic development, high occurrence of pregnancy loss and low survival of the cloned offspring (Kasinathan et al., 2001a; Kasinathan et al., 2001b). These limitations are presumably caused by failure to reprogram the donor nucleus completely. Previous studies have emphasized the characterization of defects in cloned fetuses and placentas (Cibelli et al., 2002; Lanza et al., 2001; Hashizume et al., 2002). The objective of this study was to identify early nuclear markers of morphological remodeling of somatic nuclei by NT.

We have chosen to examine molecular markers of the nuclear envelope, of a nonionic detergent-, nuclease- and salt-resistant ‘nuclear matrix’ structure, and of the interface between the nuclear envelope and chromatin. There is increasing evidence that the nuclear envelope is more than just a barrier around chromosomes: it also plays a crucial role in maintaining the integrity of genome function via interactions with the nuclear matrix and chromatin.

The nuclear envelope consists of two concentric membranes, nuclear pores and the nuclear lamina, a meshwork of intermediate filaments called A- and B-type lamins (reviewed in Gruenbaum et al., 2000). A-type lamins include lamins A and C, which are splice variants of the *LMNA* gene in humans and are expressed in differentiated somatic cells (Guilly et al., 1990). B-type lamins include lamins B1 and B2, products of the *LMNB1* and *LMNB2* genes, respectively, and are ubiquitously expressed (Gruenbaum et al., 2000). Lamins mediate interactions between the inner nuclear membrane and chromatin or DNA and play a functional role in the nucleus. Disorganization of the lamina with dominant negative lamin mutants alters replication (Ellis et al., 1997; Spann et al., 1997; Moir et al., 2000) and improper assembly of the lamina at the end of mitosis leads to cell death (Steen and Collas, 2001). Intranuclear lamin foci also co-localize with RNA splicing factors, suggesting that lamins might contribute to organizing

the RNA processing machinery (Jagatheesan et al., 1999). Moreover, the discovery that mutations in the *LMNA* gene cause life-threatening hereditary disorders affecting skeletal, cardiac and adipose tissues (reviewed in Vigouroux and Bonne, 2002) suggests a role for the nuclear envelope in the regulation of gene expression. Interestingly, *Lmna* null mice display phenotypes reminiscent of those created by lamin A/C mutations in humans (Sullivan et al., 1999).

The nuclear and mitotic apparatus (NuMA) protein is a ~240-kDa intranuclear protein that is distributed into each daughter cell during mitosis by association with the spindle apparatus (Zeng, 2000; He et al., 1995; Compton and Cleveland, 1994). In interphase, NuMA is a major structural component of the nuclear matrix (Harborth and Osborn, 1999; Compton and Cleveland, 1994). Mutational analyses have shown that functional NuMA is required during mitosis for the terminal phases of chromosome separation and/or nuclear reassembly to occur (Compton and Cleveland, 1993;Compton and Cleveland, 1994).

A-kinase anchoring protein 95 (AKAP95) is a 95-kDa protein that binds the cAMP-dependent protein kinase at mitosis (Coghlan et al., 1994; Eide et al., 1998) and is implicated in recruiting components required for chromosome condensation in human cultured cells (Collas et al., 1999) and in mouse female pronuclei (Bomar et al., 2002). In interphase, AKAP95 is a nuclear protein that primarily associates with the nuclear matrix, although a small proportion also co-fractionates with nuclease-soluble chromatin (Collas et al., 1999). The role of AKAP95 in the nucleus remains elusive but recent data localizing AKAP95 preferentially to transcriptionally silent (hypoacetylated) chromatin in HeLa and mouse cumulus cells (P.N.M. et al., unpublished) suggest that AKAP95 might interface the chromatin with the nuclear envelope. The growing evidence that AKAPs can anchor several signaling molecules (Feliciello et al., 2001; Smith and Scott, 2002; Tasken et al., 2001) raises the possibility that AKAP95 might function in integrating multiple signaling pathways in the nucleus.

Here, we characterize the dynamics of A- and B-type lamins and NuMA, and variations in intranuclear anchoring properties of AKAP95 following NT in the mouse. We show that some of these structural and functional proteins constitute markers of incomplete somatic nuclear remodeling by NT. Partial remodeling is manifested by the identification of structural abnormalities in nuclei of NT embryos.

Materials and Methods

Antibodies

Anti-lamin-A/C monoclonal antibodies (mAbs) and goat anti-lamin-B polyclonal antibodies were from Santa Cruz Biotechnology. Anti-NuMA mAbs were from Transduction Laboratories. Rabbit anti-rat AKAP95 antibodies were from Upstate Biotechnologies (Coghlan et al., 1994; Collas et al., 1999). Affinity-purified rabbit polyclonal antibodies against a peptide of human lamin B were from J.-C. Courvalin (Institut Jacques Monod, Paris, France) (Chaudhary and Courvalin, 1993). These antibodies do not distinguish between lamins B1 and B2, and so B-type lamins are referred to as 'lamin B' in this paper.

Oocyte and embryo collection

Metaphase II (MII) oocytes and fertilized pronuclear stage embryos were collected from superovulated B6D2F1 mice 14 and 20 hours after injections of human chorionic gonadotrophin (hphCG) injection, respectively. Cumulus cells were dispersed with 300 U ml^{-1} hyaluronidase and oocytes or embryos were cultured in Potassium Simplex Optimized Media (KSOM; Specialty Media) at 38°C in an atmosphere of 5% CO_2 in air. Oocytes were micromanipulated at ~15 hphCG.

Intracytoplasmic sperm injection

Intracytoplasmic sperm injection (ICSI) was performed as described (Bomar et al., 2002; Wakayama et al., 1998b). Briefly, sperm heads thawed after storage in liquid nitrogen were washed in PBS containing 3 mg ml^{-1} bovine serum albumin (BSA) and 10% polyvinyl-pyrrolidone. ICSI was performed at 19°C in PBS containing 3%BSA using a piezoelectric drill. After a 15-minute recovery at 35°C, oocytes were cultured in KSOM.

NT and oocyte activation

NT with cumulus cell donor nuclei was done as described (Wakayama et al., 1998a) with minor alterations. Cells obtained from four to eight cumulus-oocyte complexes were washed in α-Modified Eagle's Medium (α-MEM; Gibco-BRL), resuspended in 100 μl α-MEM and cultured until use. MII oocyte enucleation was carried out by aspiration of a translucid cytoplasmic area containing the metaphase plate in Flushing Holding Medium (FHM; Specialty Media) containing 5 μg ml^{-1} cytochalasin B. Enucleated oocytes were washed and returned to culture. After ~2 hours, cumulus cells were transferred to FHM containing 10% polyvinyl pyrrolidone, lysed in a pipette attached to a piezoelectric micromanipulator and a single nucleus was injected into an enucleated oocyte. Oocytes were cultured in KSOM for at least 1 hour before artificial activation.

Recipient oocytes were activated for 6 hours with 10 mM $SrCl_2$ in Ca^{2+}-free CZB medium (Specialty Media) containing 5 μg ml^{-1} cytochalasin B to prevent polar body extrusion. For inhibition of transcription or protein synthesis in NT embryos, activation medium contained 5 μg ml^{-1} actinomycin D (ActD; Sigma) or 20 μg ml^{-1} cycloheximide (CHX; Sigma), respectively. At the end of treatment, embryos were washed and cultured in KSOM. Parthenogenetic activation of MII oocytes was carried out as for NT embryos in the presence of 5 μg ml^{-1} cytochalasin B to maintain diploidy.

Immunological procedures

Proteins from cells, oocytes and embryos (n>200) were resolved by 10% SDS-PAGE, transferred to nitrocellulose (Bomar et al., 2002) and probed with the following antibodies: anti-AKAP95 (1:250 dilution) and rabbit anti-lamin-B (1:1,000), anti-lamin-A/C (1:500) and anti-NuMA (1:500). Blots were incubated with peroxidase-conjugated secondary antibodies and developed by enhanced chemiluminescence (Amersham). For immunofluorescence analysis, cells, oocytes and embryos were settled onto poly-L-lysine-coated coverslips. Samples were either fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton X-100 (Bomar et al., 2002) or extracted with 0.1% Triton X-100, 1 mg ml^{-1} DNase I, 100 μg ml^{-1} RNase A containing 100 mM NaCl or 300 mM NaCl in Tris-HCl buffer (pH 7.2) for 30 minutes before fixation (Martins et al., 2000). Proteins were blocked in PBS containing 2% BSA and 0.01% Tween-20. Primary antibodies (1:100 dilution) and TRITC- or FITC-conjugated secondary antibodies were incubated each for 30 minutes. DNA was stained with 0.1 μg ml^{-1} Hoechst 33342 or 0.1 μg ml^{-1} propidium iodide as indicated.

Microscopy and image analysis

Observations were made on an Olympus BX60 microscope and

photographs were taken with a JVC CCD camera and processed using Adobe Photoshop. Quantification of fluorescence signals was performed with AnalySIS and data expressed as the mean±s.d. of relative fluorescence intensities.

Results

Immunolocalization of A- and B-type lamins, and AKAP95 in mouse cumulus cells and preimplantation embryos

Using cross-reactive antibodies, the distributions of lamins A/C, lamin B and AKAP95 were examined in mouse cumulus cells that were later used as nuclear donors for NT. Immunofluorescence analysis of interphase cells showed that all markers were expressed and distributed in agreement with the literature (Coghlan et al., 1994; Compton and Cleveland, 1993; Gruenbaum et al., 2000). A- and B-type lamins were detected at the nuclear periphery, whereas AKAP95 and NuMA were intranuclear (Fig. 1A). Immunofluorescence labeling was corroborated on immunoblots (Fig. 1D). Single bands were detected by the antibodies at their expected apparent M_r, namely 68 kDa (lamin B), 70 kDa and 60 kDa (lamins A and C, respectively), ~240 kDa (NuMA) and 95 kDa (AKAP95).

Immunofluorescence analysis of preimplantation embryos is shown in Fig. 1B. In fertilized embryos, lamin B was detected at the nuclear periphery at all stages examined. Lamin A/C, however, was not detected up to the blastocyst stage, as expected from a marker of differentiated cells. AKAP95 labeling was restricted to the female pronucleus (FPN), the smaller of the two pronuclei, as detected by Hoechst labeling (Fig. 1C), confirming our earlier observations (Bomar et al., 2002). Intranuclear localization of AKAP95 was observed in nuclei of all blastomeres in subsequent developmental stages. Similar to AKAP95, NuMA labeling occurred only in the FPN at the pronuclear stage (Fig. 1B,C) and in nuclei of all blastomeres thereafter. Detection of lamin B, NuMA and AKAP95, but not of lamin A/C, in pronuclear stage embryos was confirmed on western blots (Fig. 1D). The blotting data indicate that the lack of immunofluorescence labeling of lamin A/C in preimplantation embryos was not due to antigen masking but rather to the absence of the protein. Because A-type lamins are expressed in differentiated somatic cells, we rationalized that they constitute a potential marker of nuclear remodeling by NT.

Dynamics of nuclear lamins, NuMA and AKAP95 following NT

The dynamics of lamin B, lamin A/C, AKAP95 and NuMA were examined during morphological remodeling associated with transplantation of cumulus cell nuclei into enucleated oocytes. Reconstituted embryos were activated with $SrCl_2$ for 6 hours starting ~2 hours after NT. Donor nuclei underwent premature chromatin condensation (PCC) within 3 hours of injection into oocytes in all embryos examined (n=20), as shown by DNA staining with Hoechst 33342 (Fig. 2, PCC). By 7 hours after injection, embryos contained fully expanded nuclei (Fig. 2, NT PN). Notably, in all experiments conducted in this study, 89% of activated NT embryos displayed more than one reconstituted nucleus, but each of these nuclei was similar to those shown in Fig. 2. This observation is largely consistent with previous reports of NT in the mouse (e.g. Wakayama et al., 1998a; Wakayama et al., 1999) but reasons for the formation of multiple nuclei following NT remain unexplored.

PCC and interphase one-cell-stage NT embryos and, as controls, parthenogenetic pronuclei were analysed by immunofluorescence (Fig. 2). AKAP95 was associated with PCC, a property consistent with the chromosome association of AKAP95 in mitotic somatic cells and blastomeres (Collas et

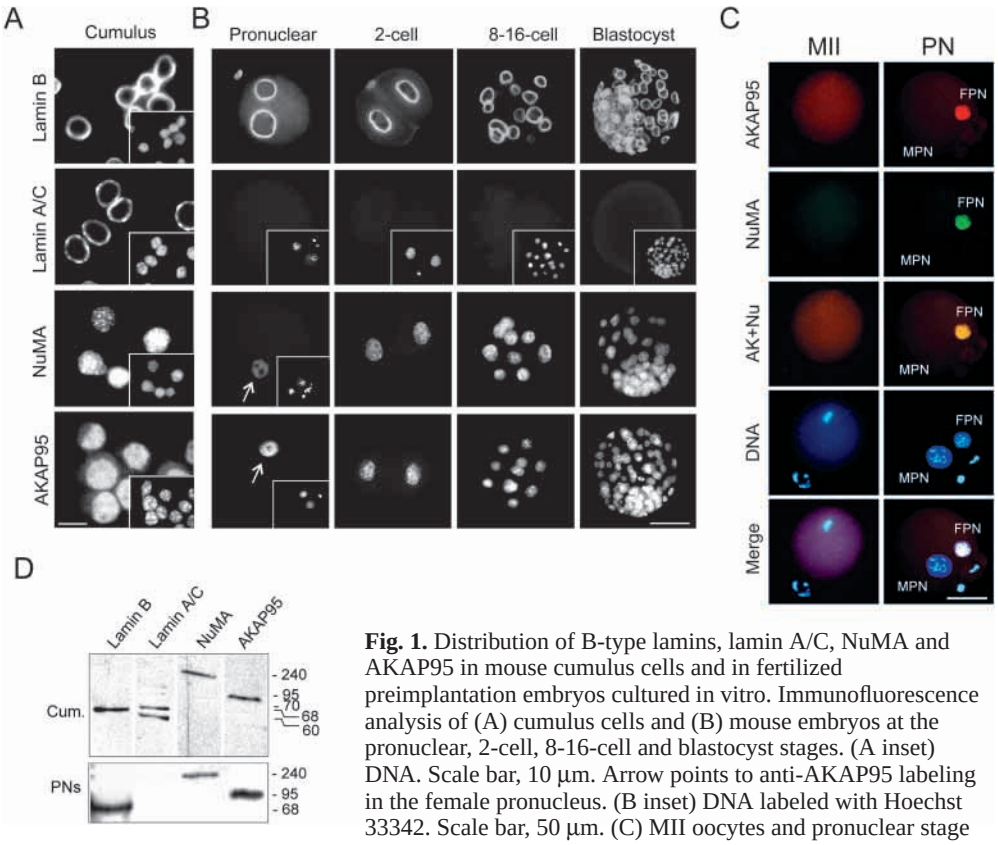

Fig. 1. Distribution of B-type lamins, lamin A/C, NuMA and AKAP95 in mouse cumulus cells and in fertilized preimplantation embryos cultured in vitro. Immunofluorescence analysis of (A) cumulus cells and (B) mouse embryos at the pronuclear, 2-cell, 8-16-cell and blastocyst stages. (A inset) DNA. Scale bar, 10 μm. Arrow points to anti-AKAP95 labeling in the female pronucleus. (B inset) DNA labeled with Hoechst 33342. Scale bar, 50 μm. (C) MII oocytes and pronuclear stage fertilized embryos (PN) were triple-labeled using anti-AKAP95 polyclonal antibodies, anti-NuMA mAbs and Hoechst 33342 to visualize association of AKAP95 and NuMA with the female pronucleus (FPN). MPN, male pronucleus. Scale bar, 50 μm.
(D) Immunoblotting analysis of cumulus cells and fertilized pronuclear stage embryos using antibodies against indicated proteins. Apparent M_r is shown in kDa.

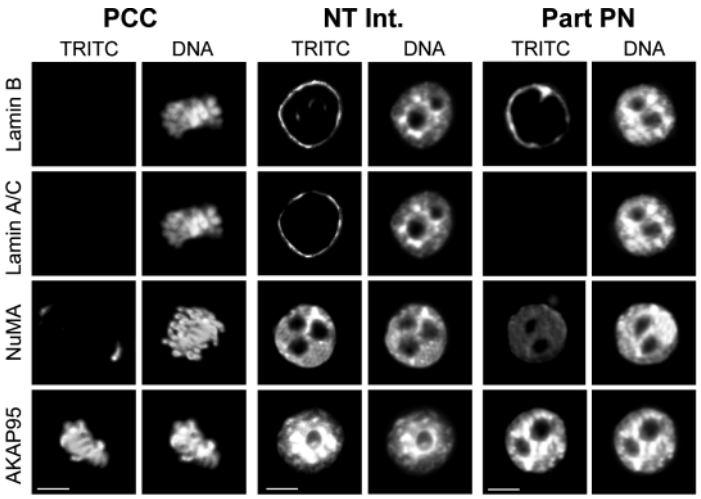

Fig. 2. Dynamics of B-type lamins, lamins A/C, NuMA and AKAP95 following NT. Disassembly of the donor nucleus and reformation of the new nucleus were monitored at the premature chromatin condensation [PCC; 3 hours post-infection (hpi)] and interphase (NT Int.; 7 hpi) stages, using antibodies against indicated proteins. Female pronuclei formed after parthenogenetic activation of MII oocytes were also analysed 6 hours after start of activation treatment (Part PN). TRITC refers to labeling with TRITC-conjugated secondary antibodies. Scale bars, 20 μm. *n*>20 embryos per treatment.

al., 1999; Bomar et al., 2002). Lamins A/C and B were not detected on chromosomes, probably as a result of their dispersal in the egg cytoplasm (data not shown). Likewise, NuMA was absent from the condensed chromosomes and showed signs of association with polar structures on either side of the chromosome plate. At the one-cell stage, all markers were detected in the nuclei of reconstructed embryos (Fig. 2, NT Int.). Lamin B was detected in NT nuclei and parthenogenetic pronuclei. Remarkably, lamins A/C were also detected at the nuclear envelope of NT embryos, which contrasted with their absence from the pronuclear envelope of parthenotes (Part PN) or fertilized embryos (Fig. 1B). NuMA also displayed consistently strong intranuclear labeling in NT embryos, which contrasted with the weak labeling detected in parthenogenetic pronuclei (Fig. 2). Weaker NuMA labeling in parthenogenetic nuclei could not be accounted for by a reduced DNA content, because parthenotes were diploidized with cytochalasin B after activation. Rather, it probably reflected a lower concentration of protein in these nuclei (see below). Lastly, AKAP95 decorated the nuclear interior except nucleoli in nuclei of NT embryos and parthenotes, consistent with its localization in fertilized embryos. We concluded from these observations that reconstituted nuclei of NT embryos express strong lamin A/C and NuMA immunoreactivity, two characteristics of the somatic donor cells.

Misregulation of A-type lamin expression in NT embryos

We determined whether the assembly of lamin A/C in nuclei of NT embryos resulted from (i) retargeting of somatic lamins disassembled upon PCC, (ii) translation and assembly of lamins from a pool of maternal lamin A/C mRNA or (iii) de novo transcription of the somatic *Lmna* gene in reconstituted NT nuclei. To distinguish between these possibilities, mouse NT embryos were activated with $SrCl_2$ as described in Materials and Methods, activated in the presence of the protein synthesis inhibitor cycloheximide (CHX) or activated in the presence of the RNA polymerase II inhibitor actinomycin D (ActD) to inhibit transcription.

The results are shown in Fig. 3. Activation with 20 μg ml^{-1} CHX or 5 μg ml^{-1} ActD, both compatible with nuclear reformation, inhibited lamin A/C assembly. This argues that lamin A/C assembly resulted from transcription of the somatic *Lmna* gene rather than from retargeting from a somatic pool brought into the oocyte during the NT procedure. Lamin B assembly was not affected by CHX or ActD, suggesting that lamin B was retargeted to chromosomes from a disassembled somatic pool and/or from a maternal pool of lamins. Essentially no NuMA was seen after CHX exposure but ~40% NuMA immunoreactivity was detected in NT nuclei after ActD treatment. This suggests that NuMA reassembled as a result of translation from maternal mRNA and of de novo transcription from the transplanted genome. AKAP95 detection was not altered in CHX- or ActD-treated NT embryos, suggesting that

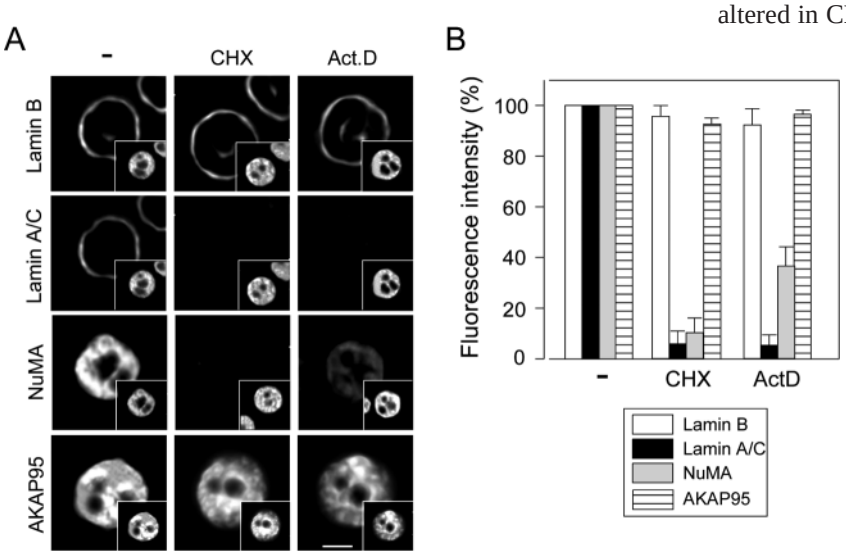

Fig. 3. Misregulation of lamin A/C and NuMA expression in one-cell-stage NT embryos. (A) Reconstructed embryos were activated for 6 hours with $SrCl_2$ and either no supplementation (–), 20 μg ml^{-1} cycloheximide (CHX) or 5 μg ml^{-1} actinomycin D (ActD). At the end of incubation, embryos were analysed by immunofluorescence. Anti-lamin-B (rabbit polyclonal) and anti-lamin-A/C (mAb) antibodies were used on the same preparations. Insets, DNA. Scale bar, 20 μm. (B) Relative immunofluorescence labeling intensities for indicated markers in nuclei of NT embryos treated as in (A). Reference value for each marker (100% fluorescence) represents relative amounts of immunolabeling in untreated embryos. *n*≈15 embryos per marker per treatment.

a large proportion at least of nuclear AKAP95 is presumably of somatic origin. Therefore, because lamins A/C and NuMA appear to be abnormally transcribed in NT nuclei, we propose that they constitute two markers of incomplete nuclear remodeling in the oocyte.

Different anchoring of AKAP95 in nuclei of fertilized and NT embryos

The next marker of nuclear remodeling examined was the intranuclear anchoring properties of AKAP95 in reconstituted nuclei of one-cell-stage NT embryos. AKAP95 was the only marker investigated that was detected in somatic donor nuclei, on PCC chromosomes and in NT nuclei with a labeling intensity similar to that of parthenotes and fertilized embryos, and, as such, it did not appear to be a valuable marker of nuclear remodeling by NT. Nevertheless, the strength of AKAP95 association with intranuclear ligands (the nature of which remains to be explored) was examined by in situ extraction of ICSI embryos, NT embryos, parthenotes and cumulus cells with 0.1% Triton X-100, 1 mg ml^{-1} DNase I and 100 μg ml^{-1} RNase A together with 100 mM NaCl or 300 mM NaCl. In ICSI female pronuclei, nearly all AKAP95 and all detectable DNA were extracted under 100 mM NaCl (Fig. 4, FPN); male pronuclei do not harbor any AKAP95 (Bomar et al., 2002). Similarly, in parthenotes ~90% of AKAP95 and DNA was extracted under 100 mM NaCl, and ~98% of AKAP95 was removed under 300 mM NaCl. By contrast, a significant proportion of AKAP95 and DNA (~50%) was resistant to extraction even under 300 mM NaCl in nuclei of NT embryos (Fig. 4). Resistance of AKAP95 and DNA to extraction in NT nuclei resembled that of cumulus cell nuclei (Fig. 4B). Notably, no detectable lamin B extraction occurred in ICSI, parthenote or NT nuclei regardless of extraction conditions (Fig. 4), suggesting that alterations in AKAP95 and DNA distributions did not result merely from gross changes in nuclear architecture. These results imply that NT nuclei are characterized by tight anchoring of AKAP95 to intranuclear ligands and restricted DNA accessibility to DNase I. They also suggest that nuclei produced by somatic NT display structural abnormalities as a result of incomplete morphological remodeling of donor nuclei and/or transcriptional misregulation of somatic genes.

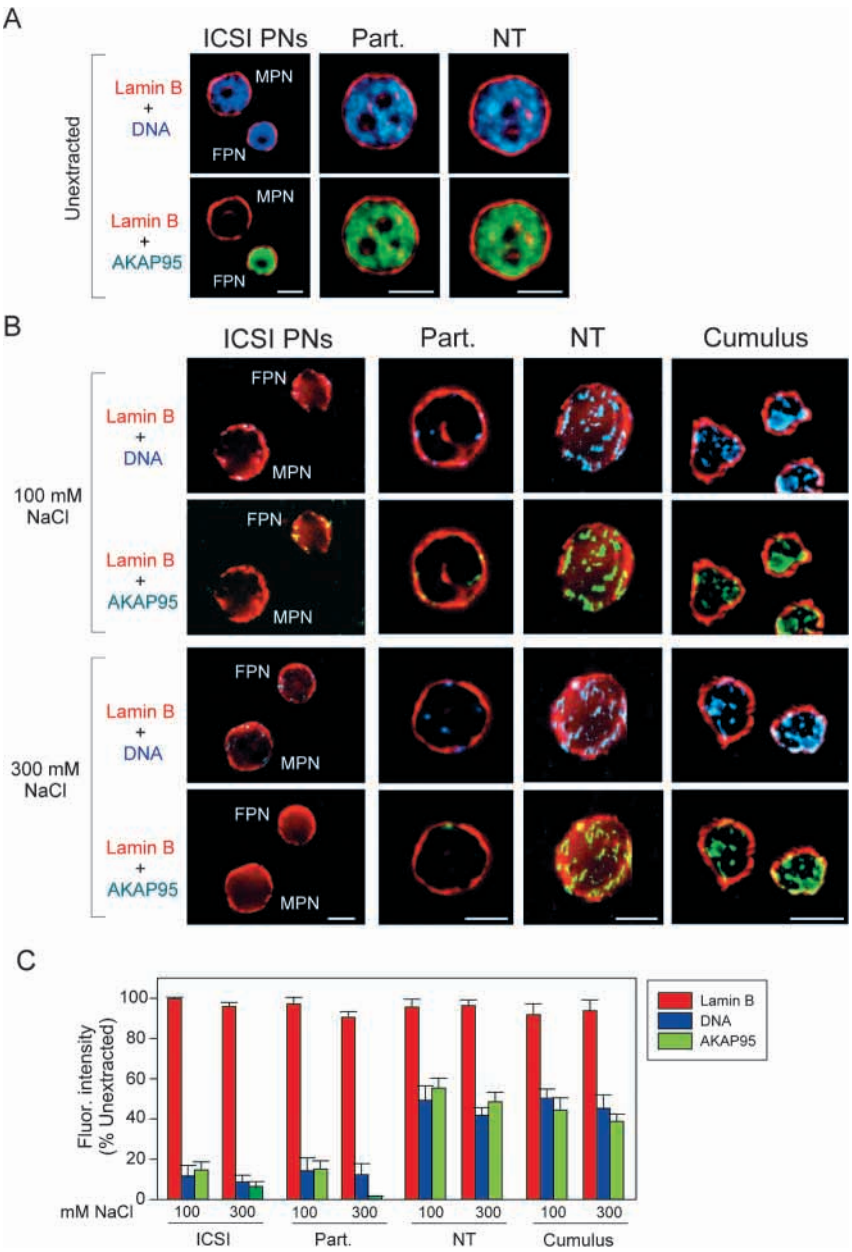

Fig. 4. AKAP95 is more strongly anchored in NT pronuclei than in ICSI or parthenogenetic pronuclei. (A) Mouse one-cell-stage ICSI, parthenogenetic (Part) and NT embryos were double immunolabeled using anti-lamin-B (red) and anti-AKAP95 (green) antibodies. DNA was labeled with Hoechst 33342. Only nuclei are shown. FPN, female pronucleus; MPN, male pronucleus. Scale bars, 20 μm. (B) Mouse one-cell-stage ICSI, parthenogenetic (Part) and NT embryos, and mouse cumulus cell nuclei, were extracted with 0.1% Triton X-100, 1 mg ml^{-1} DNase I, 100 μg ml^{-1} RNase A together with 100 mM (upper two rows) or 300 mM (lower two rows) NaCl prior to fixation and double immunofluorescence analysis of lamin B (red) and AKAP95 (green). DNA is labeled blue with Hoechst 33342. Only nuclei are shown. Scale bars, 20 μm. (C) Relative proportions of unextracted lamin B, AKAP95 and DNA in nuclei of embryos or cumulus cells extracted as in (B). Fluorescence labeling intensity of each marker after in situ extraction as in (B). Reference value (100%) represents immunofluorescence labeling intensity in unextracted embryos. n≈10 embryos per marker per treatment.

Passage through first mitosis does not rescue nuclear anomalies in NT embryos

Mitosis involves extensive morphological remodeling of the nucleus, including breakdown of the nuclear envelope, condensation of chromosomes and reformation of a new nuclear envelope as the separated sets of chromosomes

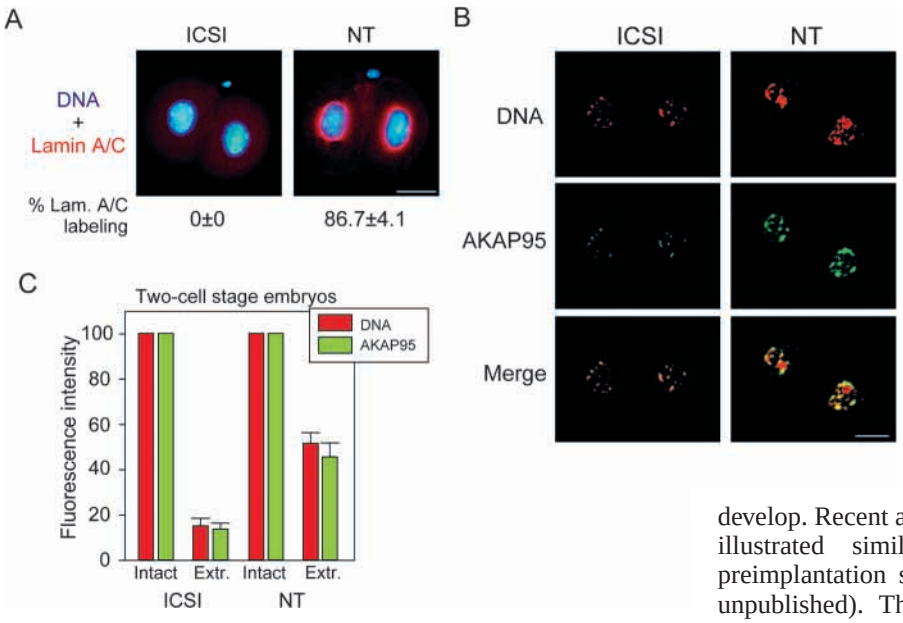

Fig. 5. Passage through first mitosis does not restore abnormalities in lamin A/C labeling and intranuclear anchoring of AKAP95 in NT embryos. (A) Two-cell stage ICSI and NT embryos were analysed by immunofluorescence using anti-lamin-A/C mAbs. (B) Two-cell-stage ICSI and NT embryos were extracted 0.1% Triton X-100, 1 mg ml^{-1} DNAse I, 100 μg m^{-1} RNase A, 300 mM NaCl prior to immunofluorescence analysis using anti-AKAP95 antibodies. DNA was labeled with propidium iodide. Scale bars, 20 μm. (C) Relative DNA and AKAP95 immunolabeling intensities in intact (100%) and extracted two-cell-stage ICSI and NT embryos. *n*>10 embryos per treatment.

decondense. We rationalized that, although passage through PCC did not allow a complete remodeling of the somatic nucleus, passage through mitosis might enable completion of nuclear remodeling. To test this hypothesis, we examined lamin A/C immunolabeling and the resistance of AKAP95 to in situ extraction with 0.1% Triton X-100, 1 mg ml^{-1} DNase I and 100 μg ml^{-1} RNase A in 300 mM NaCl in two-cell-stage NT and ICSI embryos. In contrast to ICSI embryos, two-cell-stage NT embryos displayed lamin A/C labeling in each blastomere (Fig. 5A), whereas lamin B distribution was similar in ISCI and NT two-cell-stage embryos (data not shown). Furthermore, as in one-cell-stage embryos, more AKAP95 and DNA were resistant to detergent, nuclease and salt extraction in nuclei of NT embryos than in ICSI embryos (Fig. 5B,C). In addition, both lamin A/C labeling and enhanced resistance to extraction of DNA and AKAP95 were detected in later-stage preimplantation NT embryos (data not shown). We concluded from these observations that passage of NT embryos through first mitosis does not further remodel donor somatic nuclei, at least based on lamin A/C labeling and intranuclear AKAP95 anchoring properties.

Discussion

We describe four structural and functional markers of incomplete nuclear remodeling by NT in the mouse. These are: (1) nuclear assembly of lamins A/C; (2) enhanced intranuclear NuMA concentration; and increased resistance of (3) DNA and (4) AKAP95 to extraction with non-ionic detergent, nucleases and salt. These markers are easily identifiable by immunofluorescence and extraction analyses. Furthermore, gathering informative results as early as the one-cell stage (i.e. within hours of NT) allows a prompt determination of the extent of nuclear remodeling and might enable a rapid, efficient screen for the efficiency of somatic nuclear remodeling by NT. Despite these advantages, a relationship remains to be established between the markers identified in this study (in particular, pronuclear expression of A-type lamins and strong anchoring of AKAP95) and the failure of NT embryos to develop. Recent additional studies in bovine NT embryos have illustrated similar defects in nuclear architecture in preimplantation stages of development (E.J. Sullivan et al., unpublished). Thus, the phenotypes reported here are not specific to the mouse but might reflect a general feature of somatic nuclear processing by NT.

We tested the hypothesis that passage through mitosis might restore nuclear abnormalities detected and further remodel the donor nucleus. This hypothesis was rejected on the basis that first mitosis did not eliminate perinuclear lamin A/C labeling, nor did it readjust the strength of intranuclear AKAP95 anchoring. Together with the apparent lack of *Lmna* gene inactivation in nuclei reconstituted after PCC, this finding implies that mitotic chromosome condensation per se is not sufficient to elicit inactivation of the *Lmna* gene in the subsequent cell cycle. A second implication is that PCC or first embryonic mitotic chromosome condensation does not significantly reset the overall chromatin organization of the parent cell to remodel it into an embryonic pattern, at least based on nuclease sensitivity of DNA and AKAP95 anchoring. We do not, however, exclude the possibility that progressive remodeling occurs with each blastomere division, such that embryonic chromatin organization is obtained later in development.

A significant observation is that the *Lmna* gene most likely remains active in nuclei of pre-implantation NT embryos, as judged by inhibition of transcription and translation after NT. This resulted in the assembly of differentiated cell-specific A-type lamins. The lack of immunodetection of A-type lamins in fertilized preimplantation embryos contradicts a previous report where these epitopes were recognized in the mouse embryo at least until the 8-cell stage (Prather et al., 1991). Reasons for this discrepancy are unclear but might involve the source of antibodies used. Through interactions with chromatin and components of the RNA processing machinery, nuclear lamins have been suggested to participate in the regulation of transcription (Jagatheesan et al., 1999). Thus, assembly of the correct set of lamins is probably crucial for proper nuclear function in NT embryos. One hypothesis is that aberrant assembly of A-type lamins might lead to sequestration of transcriptional regulators important for the activation of developmental genes. Alternatively, because A-type lamins bind DNA (Stierlé et al., 2003), its untimely presence in the nuclear envelope might affect chromatin modifications, replication or transcription in these areas. Although technically

challenging, it would be interesting to determine whether the low proportion of NT embryos developing to term and remaining healthy after birth are those not harboring lamins A/C during preimplantation development or whether, on the contrary, this anomaly is compatible with normal development and health.

Like *Lmna*, the *NuMA* gene apparently also remains active in nuclei of one-cell stage NT embryos, resulting in apparent NuMA overexpression in NT pronuclei. Owing to its involvement in the formation and maintenance of the mitotic spindle (Compton and Cleveland, 1994; Compton, 1998), NuMA overexpression might lead to abnormal chromosome segregation and aneuploidy. Moreover, the suggested involvement of NuMA with various nuclear functions (Gribbon et al., 2002; Barboro et al., 2002) suggests that misregulated NuMA levels in NT embryos might also affect functions important for normal development. This hypothesis remains to be tested.

We observed that AKAP95, a component of the matrix-chromatin interface in somatic cells, was more strongly associated with its as-yet-unidentified ligands in nuclei of NT embryos than in fertilized embryos or parthenogenetic pronuclei. This association might impose constraints on DNA organization or result from altered chromatin conformation in NT embryos. In any event, because most chromatin-bound AKAP95 remains associated with DNase-I-resistant DNA, which is mostly transcriptionally silent, we propose that increased resistance of AKAP95 to extraction by nucleases and salt reflects an enhanced proportion of heterochromatin in early NT embryos. This, in turn, raises the speculative hypothesis that expression of developmentally important genes might be affected. It will be interesting to identify and investigate the transcriptional regulation of genes involved in placental development, maintenance of late pregnancy and post-natal survival of cloned animals.

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