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The nuclear mitotic apparatus (NuMA) protein is contributed by the donor cell nucleus in cloned porcine embryos

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1. ABSTRACT

The Nuclear Mitotic Apparatus (NuMA) protein is a multifunctional protein that is localized to the nucleus in interphase and to the poles of the mitotic apparatus during mitosis. In unfertilized porcine oocytes, NuMA is localized to the meiotic spindle. NuMA is removed along with the meiotic spindle during the enucleation process before reconstructing the egg by introducing the donor cell nucleus to produce cloned embryos. Questions have been raised regarding the source for NuMA in cloned embryos, as the enucleated oocyte does not contain detectable NuMA in the cytoplasm. To determine the source of NuMA in porcine nuclear transfer (NT) embryos, we conducted an immunofluorescence microscopy study with antibodies against NuMA to investigate the appearance and distribution of NuMA before and after reconstructing NT embryos with porcine skin fibroblasts. We used donor cells from a confluent culture with all cells in interphase. For comparative studies, we also determined the immunofluorescence pattern of NuMA, gamma-tubulin, and alpha-tubulin in porcine fibroblasts, parthenogenetic embryos and *in vitro* fertilized (IVF) embryos. Results show that NuMA was localized in nuclei of 33.5% (163/456) of the serum-deprived fibroblasts used as donor cells. No NuMA staining was detected in enucleated pig oocytes. Immediately after nuclear transfer, NuMA staining was absent in all donor cell fibroblast nuclei (0 h) but staining was detected by 6 h within the reconstructed eggs, at which time the transferred somatic cell nucleus swelled in most cells (19/27) and became a pronucleus-like structure. NuMA was localized exclusively within the pronucleus-like structures (15/27). At 25 h, NuMA was detected inside the nucleus (16/25) either in one-cell or in 2-cell stage embryos. Interestingly, in parthenogenetic embryos, NuMA staining was not detected in all 42 eggs examined at 1 h, and evident NuMA staining was only detected inside a few (4/51 at 6 h; 6/48 at 25 h) of the nuclei. In IVF embryos, NuMA was detected within the nucleus at 6 h (5/20) and 25 h (13/16). These results show that the donor cell nucleus contains NuMA that is contributed to the reconstructed embryo and possibly activated by mechanisms in the oocyte's cytoplast.

2. INTRODUCTION

One of the problems in nuclear cloning is the low cloning efficiency (1-5% in most mammalian species) for which reasons are only little understood. Reprogramming of the donor cell nucleus is central for successful nuclear cloning and a number of recent investigations are focused on genetic, epigenetic, and cell and molecular aspects of nuclear reprogramming. The Nuclear Mitotic Apparatus (NuMA) protein has recently come into focus, as it couples nuclear cycles with centrosome cycles. Centrosomes serve as the main microtubule organizing centers and are crucially important for the translocation of the donor nucleus to the egg center, for establishing the cell axis, cell division, and subsequent symmetric and asymmetric cell divisions and proper embryo development. NuMA becomes a transient centrosome protein during mitosis after translocating from the nucleus into the cytoplasm upon signals that it receives from the cell's cytoplasm which has been linked to phosphorylation by cdc2/cyclin B. The functions of NuMA in the interphase nucleus are still quite unknown but it has been shown that NuMA serves as a nuclear matrix protein and is linked to DNA replication, reorganization, and transcription (reviewed in references 1,2), as well as to apoptosis. During mitosis and cell division, NuMA plays a critical role in the organization of microtubules into the mitotic apparatus. In unfertilized oocytes, NuMA is localized to the poles of the meiotic spindle. NuMA is removed along with the meiotic spindle during the enucleation process before reconstructing the egg by introducing the donor cell nucleus to produce cloned embryos. Questions have been raised regarding the source for NuMA in cloned embryos, as the enucleated oocyte does not contain detectable NuMA in the cytoplasm. Furthermore, improper NuMA regulation and translocation can result in abnormal spindles (3,4,5,6) that will affect development of the embryo.

The present study was conducted to investigate the distribution and remodeling of NuMA during the first cell cycle in porcine nuclear transfer embryos as compared to *in vitro* fertilized embryos and parthenogenetically activated embryos. We show that NuMA is detected in the donor cell nucleus at 6h of nuclear transfer and is redistributed to the nuclei in the dividing daughter cells at 25h of nuclear cloning.

3. MATERIALS AND METHODS

3.1. Cumulus Cell-Oocyte-Complex (COC) Collection and Maturation

Ovaries were collected from pre-pubertal gilts at a local abattoir and transported to the laboratory in 0.9% NaCl solution at 30-35°C. COCs were aspirated from antral follicles (3-5mm) with an 18-gauge needle fixed to a 10-ml disposable syringe. The COCs with uniform cytoplasm and several layers of cumulus cells were selected and washed three times in the culture media, and transferred into 500 µl of culture media covered with 200 µl mineral oil in a four-well dish (Nunc, Roskilde, Denmark). After 42-44 hours cumulus cells were removed from COCs by vortexing COCs 5 min in Tyrode's lactate-Hepes (TL-Hepes) containing 0.1% (w/v) polyvinyl alcohol (PVA) and 0.1% hyaluronidase. Only the oocytes with a clear polar body were counted as matured oocytes and used in the experiments.

3.2. Skin Fibroblast Culture

At one day of age, a skin biopsy was obtained from a a-1,3-galactosyltransferase null piglet (7) and cut into small pieces with fine scissors in PBS containing 0.05% trypsin and 0.02 mM EDTA and incubated for 30 min at 39°C. The suspension was centrifuged at 300 x *g* for 10 min, and the cell pellet was resuspended and cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 2 mM L-glutamine, 0.1 mM sodium pyruvate, 75 µg/ml of penicillin G, 50 µg/ml of streptomycin, and 15% (v/v) fetal calf serum (FCS). After 3 days of culture, when the cells were more than 90% confluent, the cells were digested with 0.05% trypsin and 0.02 mM EDTA. The harvested cells were resuspended in 10% dimethyl sulfoxide in FCS. Then, 50-µl aliquots containing 5000-7000 cells were placed into a freezing container (Nalgene, Rochester, NY) and frozen at -80°C, overnight. Cells were swiftly transferred into liquid nitrogen for long-term storage. For nuclear transfer, cells were thawed at 37°C, and 200 µl of FCS was added and cultured for 30 min. The sample was then centrifuged at 500 x *g* for 5 min. The supernatant was discarded, and 100 µl of TCM-199 with Hepes were added to resuspend the cells. For immunofluorescence microscopy to analyze NuMA, alpha-tubulin and gamma-tubulin, thawed cells were resuspended in 100 µl of DMEM with 15% FCS. Then the cells were cultured on coverslips placed in a small Petri-dish with 3ml DMEM (with 15% FCS). When the cells had reached more than 90% confluency on the coverslips, samples were processed for immunofluorescence microscopy.

3.3. Production of *In vitro* Fertilized (IVF) Embryos, Somatic Cell Nuclear Transfer (SCNT) Embryos and Parthenogenetic Embryos

For the production of IVF embryos, oocytes were inseminated in a 100 µl drop of modified Tris-buffered medium (mTBM) contaning 0.2% BSA and 2 mM caffeine with frozen-thawed ejaculated spermatozoa (5×10^5 sperm/ml) (8). Six hours after insemination, oocytes were removed from the fertilization drop and cultured in embryo culture medium (9,10). For producing SCNT embryos, oocytes were enucleated by aspirating the first polar body and adjacent cytoplasm in micromanipulation medium (Hepes-buffered TCM-199, 0.3% BSA, and 7.5 µg/ml of cytochalasin B) with a glass pipette 25-30 µm in diameter. A single donor cell was injected into the perivitelline space of the oocyte to contact the oocyte membrane. Injected oocytes were placed between two 0.2 mm diameter platinum electrodes 1 mm apart in activation medium (0.3 M mannitol, 1.0 mM $CaCl_2{\cdot}H_2O$, 0.1 mM $MgCl_2{\cdot}6H_2O$, and 0.5 mM Hepes). Fusion was induced with two successive DC pulses of 1.2 kV/cm for 30 µsec on a BTX elector-cell manipulator 200 (BTX, San Diego, CA) (11). For producing the parthenogenetic embryos, oocytes were activated by using the same pulse parameters as for fusion of SCNT. 20-30 IVF embryos, SCNT embryos or parthenogenetic embryos were cultured in 500 µl porcine

zygote medium-3 (11, 12) covered by mineral oil in a four-well dish at 39°C in an atmosphere of 5% CO_2 in air.

3.4. Immunofluorescence Microscopy

After two washes in PBS-PVA, denuded oocytes were fixed in 3.7% paraformaldehyde in PBS-PVA for 2 hours. The fixed oocytes were washed twice in PBS-PVA for 15 min each, and then stored in 1% BSA-supplemented PBS-PVA (BSA-PBS-PVA) at least overnight. The oocytes were permeabilized with 0.2% Triton-X-100 in BSA-PBS-PVA for 30 min at room temperature, and then blocked with 10% goat serum in BSA-PBS-PVA for 30 min at 38°C. For NuMA labeling, oocytes were incubated with the first antibody for 1h at 38°C or overnight at 4°C. Mouse monoclonal anti-NuMA antibody (Transduction Laboratories; 1:50 dilution, IgM) was used, followed by second antibody labeling with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM (Sigma, 1:50 dilution). We also used mouse polyclonal NuMA antibody (Calbiochem, 1:100 dilution, IgG), followed by FITC-conjugated goat anti-mouse IgG (Sigma, 1:100 dilution). For gamma-tubulin labeling, rabbit monoclonal anti-gamma-tubulin antibody (1:1500 dilution) [Sigma, T3559] was used followed by second antibody labeling with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:100 dilution) [Sigma, F6005]. The oocytes were incubated with the first and the second antibodies for 1 hour at 38°C, respectively, and washed three times in PBS-PVA for 15 minutes each. For alpha-tubulin labeling, rabbit polyclonal anti-alpha-tubulin antibody directly conjugated with FITC (1:50 dilution) was used to incubate the oocytes for 1 hour at 38°C. The oocytes were washed 3 times in PBS-PVA and mounted on slides with Vectashield mounting medium [Vector Laboratories Inc. H-1200] containing DAPI to counterstain DNA (13-17).

Somatic cells were washed once with PBS-PVA, then fixed in 3.7% paraformaldehyde in PBS-PVA for 10 min, washed twice in PBS-PVA for 5 min each, and permeabilized with 0.2% Triton X-100-supplemented PBS-PVA for 5 min. After two additional washes, cells were blocked with 10% goat serum in BSA-PBS-PVA and incubated with the first antibodies in BSA-PBS-PVA at appropriate dilutions for 1 h at 38°C. After two washes, cells were incubated with the secondary antibodies, and DNA was counterstained with DAPI in mounting medium and mounted on slides. All samples were analyzed with epifluorescence microscopy [Nikon, Eclipse 800] and images were acquired using Meta Morph software [Universal Imaging Corporation].

3.5. Experimental Design

IVF embryos were fixed at 6-8 h or 25 h post insemination; SCNT embryos and parthenogenetic embryos were fixed at 1 h, 6 h or 25 h post fusion or activation, respectively. MII stage oocytes were fixed in all groups for comparison. Alpha-tubulin, gamma-tubulin and NuMA were analyzed with fluorescence microscopy in all samples. At least 30 oocytes were stained with each antibody at each stage in three replicates. Somatic cell samples were stained with the respective antibody in three replicates. In some trials, eggs treated with DNase I (deoxyribonuclease I) (Sigma) for 1h at 37°C to remove nuclear DNA or treated with both DNase I and microwave unmasking (95-98°C for 10-15min) were used for NuMA staining. The GV stage oocytes and uncultured cumulus cells were used as positive controls.

3.6. Statistical Analysis

Data were subjected to a Generalized Lineal Model procedure (PROC-GLM) of the Statistical Analysis System (SAS Institute, Cary, NC, USA). Differences among groups were considered significant at $P<0.05$.

4. RESULTS

To investigate remodeling of NuMA in the donor cell nucleus, we determined the staining patterns and distribution of NuMA in porcine NT embryos with immunofluorecence microscopy by using antibodies against NuMA, gamma-tubulin to detect centrosomes, and alpha-tubulin to detect microtubules. For comparison, we also determined the immunofluorescence pattern of NuMA, gamma-tubulin and alpha -tubulin in parthenogenetic embryos, porcine fibroblasts, and *in vitro* fertilized (IVF) embryos.

To analyze whether NuMA is present in activated oocytes we used oocytes after electric activation. Figure 1a shows an oocyte before activation. NuMA is localized around the chromosomes. Figures 1b-d display parthenogenetic embryos that were derived from electrically activated oocytes. Embryos were fixed at 1, 6 or 25 h post fusion or activation. In parthenogenetic embryos, evident NuMA was only detected inside a few (0/40 1h, 4/51 6 h; 6/48 25 h) of the nuclei. Figure 1b shows a parthenogenetic oocyte at 1 h of activation without NuMA labeling detected at the nuclear area. At 6 h of activation (Figure 1c) faint NuMA labeling is detected inside the pronuclei of a few embryos (16/51) and no staining was observed in most of the embryos (31/51). At 25 h of activation (Figure 1d) NuMA labeling is detected inside the nuclei of very few embryos (6/48). These experiments show that during parthenogenesis nuclear NuMA staining is lost in most embryos.

To reveal whether the negative nuclear NuMA staining was due to the masking of nuclear NuMA by DNA, we treated the parthenogenetic eggs with DNase or DNase plus microwave exposure. As shown in Figure 2, nuclear DNA was removed, but no nuclear NuMA staining was observed in all 62 eggs treated with either method at 3 h of parthenogenetic activation. The negative staining was not due to the antibody itself, because there was strong NuMA staining in the GVs of oocytes and the nuclei of granulosa cells when the same antibody and staining methods were applied (Figure 2).

We next determined whether NuMA is present in the donor cell nuclei before nuclear transfer. Figure 1e shows cells from a donor cell culture that was confluent with all cells in interphase. Interestingly, NuMA was localized in the nucleus of 33.5% (163/456) of the fibroblast cells while no NuMA staining was detected in

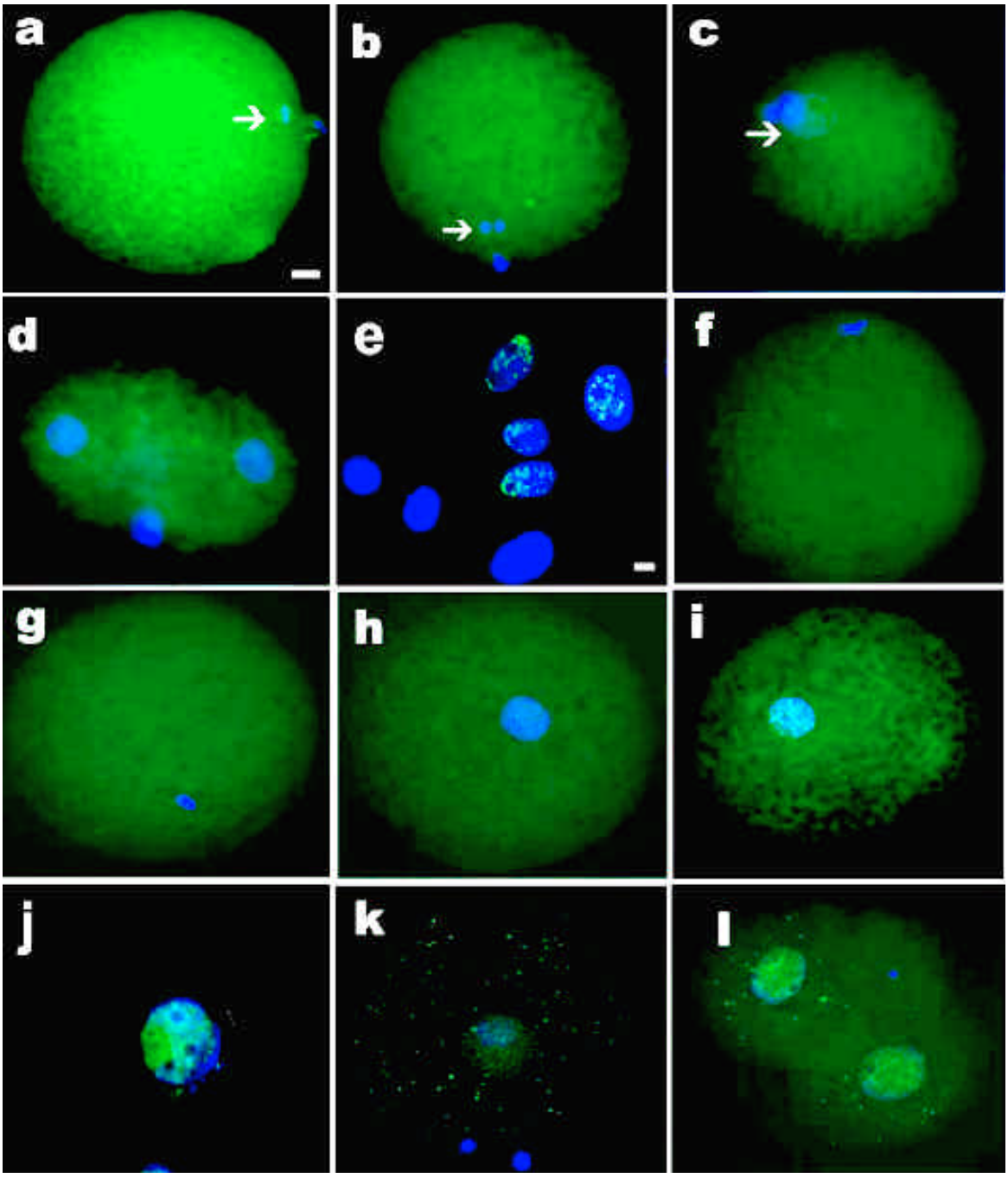

Figure 1. NuMA labeling of parthenogenetic, NT and IVF embryos at different stages. a) MII stage oocyte: NuMA is localized around the chromosomes (arrow); b) Parthenogenesis 1 h: NuMA labeling is not detected in the nuclear area (arrow); c) Parthenogenesis 6 h: faint NuMA labeling (arrow) is detected inside the pronuclei of a few embryos (16/51), while most pronuclei (31/51) were devoid of staining; d) Parthenogenesis 25 h: NuMA labeling is not detected inside the nuclei of most embryos (16/21); e) Fibroblasts: Only 33.5% of fibroblast nuclei (63/456) are labeled with NuMA antibody; f) NT 0 h: No NuMA labeling is detected in somatic cell nuclei immediately introduced into enucleated MII oocytes.; g) NT 1 h: No NuMA labeling is detected at nuclear area; h) NT 6 h: NuMA labeling is not detected inside some of the pronuclei; i) NT 6 h: NuMA staining is detected in more than half (15/27) of the NT nuclear structures; j) NT 25 h: NuMA labeling is detected inside the nuclei of a high percentage of embryos; k) IVF 6 h: NuMA labeling is detected inside the pronuclei of some embryos (5/20); l) IVF 25 h: NuMA labeling is detected inside the nuclei of most embryos (13/16). Images show double-staining for NuMA (green) and DNA (blue). Bar = 15 µm except for e = 10 µm.

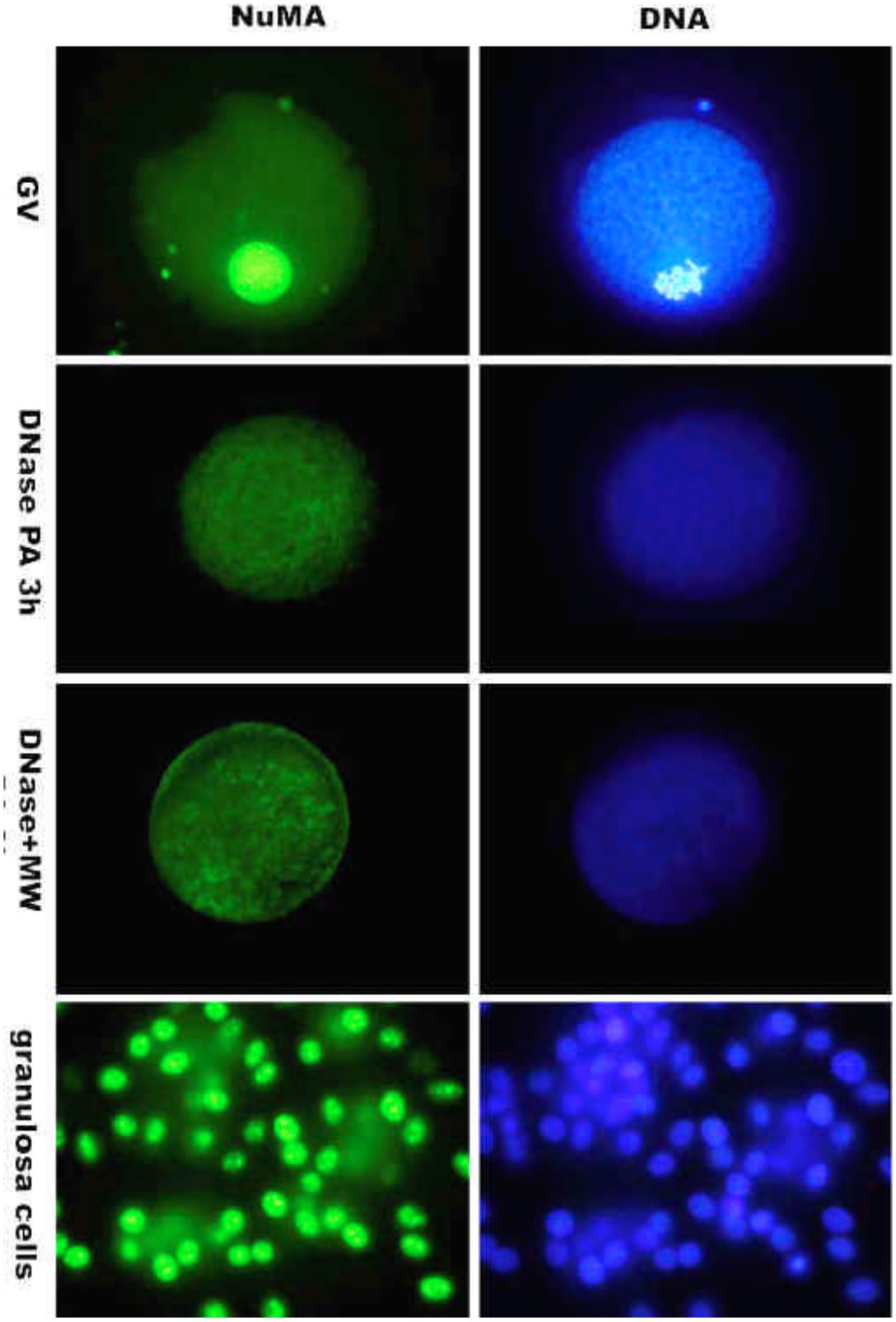

Figure 2. NuMA staining in germinal vesicle (GV) stage oocyte, DNase- or DNase plus microwave-treated eggs collected after parthenogenetic activation, and granulosa cells. The same antibodies and staining method were applied to different groups. Strong NuMA staining was observed in the GV of oocyte and the nuclei of granulosa cells. NuMA staining in nuclear area was not observed after unmasking of NuMA by removing nuclear DNA and microwave treatment at 3 h after parthenogenetic activation (PA). Green, NuMA; Blue, DNA.

66.5% of these fibroblast cells, which indicates a quiescent fibroblast cell population (18).

We then examined whether NuMA will be expressed in nuclei that are transferred into enucleated pig oocytes. Figures 1f-j show NT embryos that were reconstructed with skin-derived fibroblasts. Immediately after transfer of the fibroblast cells into the enucleated oocytes NuMA staining was absent in all donor nuclei (0 h; Figure 1f), and no NuMA staining was detected at 1 h (Figure 1g). After the somatic cells were introduced into the oocytes, the somatic cell nucleus started to swell (19/27) and became a pronucleus-like structure around 6 h. Although pronucleus-like structures lacked NuMA staining in some eggs (Figure 1i), evident NuMA staining was observed in most pronucleus-like structures at 6 h (15/27) (Figure 1i). At 25 h, NuMA was detected inside the nucleus (16/25) in most transferred nuclei when embryos had reached either the one-cell or 2-cell stages. Figure 1j represents oocytes after nuclear reformation at 25 h with NuMA staining detected inside the nucleus. These experiments indicate that the pig oocyte may not have regulatory components for NuMA until about 6 hours after nuclear transfer when more than half of the transfer nuclei displayed NuMA staining.

To compare the NT results with IVF-derived embryos we determined NuMA distribution after IVF at 0, 6 and 25 h post insemination. Figures 1k-l show IVF-derived embryos fixed at 6 h (Figure 1k) and 25 h (Figure 1l) post insemination. In these IVF-derived embryos, NuMA was detected inside the nucleus at 6 h (5/20) and 25 h (13/16).

The presented data show that there is some variability in NuMA staining for NT embryos as compared to IVF embryos. The percentage of embryos displaying NuMA is lower at 25 h after nuclear transfer.

Figure 3 displays cells stained for gamma-tubulin. The images are complimentary to those shown in Figure 1. The letters refer to the same time points, treatments, and cell cycle stages as those shown in Figure 1. Shown in Figure 3a is a control cell at the MII stage with gamma-tubulin localized to the spindle poles. Figure 3b displays a parthenogenetic oocyte at 1 h of activation. Gamma-tubulin is detected at the spindle microtubules. Small additional gamma-tubulin aggregates are seen in the cytoplasm. Figure 3c shows an activated oocyte at 6 h. Gamma-tubulin is detected in the cytoplasm where it forms small punctate aggregates. At 25 h after activation gamma-tubulin is detected as small punctate aggregates in the cytoplasm but no gamma-tubulin is detected at the nuclei (Figure 3d). Figure 3e shows fibroblast cells with gamma-tubulin localized to the nuclei.

When fibroblast cells are transferred into enucleated oocytes no gamma-tubulin staining is detected at 0 h (Figure 3f). At 1 h of nuclear transfer gamma-tubulin staining is detected as small aggregate dots in the cytoplasm (Figure 3g). This pattern is also seen at 6 h after nuclear transfer (Figure 3h) and at 25 h after NT when cells are either at the one-cell stage (Figure 3i) or the 2-cell stage (Figure 3j). After IVF gamma-tubulin is detected in association with sperm nuclei (Figure 3k) and at 25 h after IVF gamma-tubulin is detected in association with the two pronuclei (Figure 3l).

Figure 4 displays cells stained for alpha-tubulin. The images are complimentary to those shown in Figures 1 and 3. The letters refer to the same time points, treatments, and cell cycle stages as those shown in Figures 1 and 3. Shown in Figure 4a is a control cell at the MII stage displaying meiotic spindle microtubule staining. Figure 4b shows a parthenogenetic oocyte at 1 h after activation. Microtubules are detected in the anaphase II meiotic spindle. Figure 4c shows an activated oocyte at 6 h displaying pronuclei. Alpha-tubulin staining is detected in the cytoplasmic asters. At 25 h after activation (Figure 4d) alpha-tubulin is detected in the anaphase spindle of the first cleavage. Figure 4e shows fibroblast cells with heaviest alpha-tubulin staining at the centrosome area.

When fibroblast cells are transferred into enucleated oocytes some microtubule structures are detected within the cytoplasm but not around the somatic cell nucleus at 0 h (Figure 4f), but a small microtubule aster was detected around the transferred somatic cell nucleus and at 1 h (Figure 4g). At 6 h after nuclear transfer (Figure 4h) NT pronucleus-like structures are seen and small microtubule asters are detected in the cytoplasm. At 25 h after NT cytoplasmic microtubules were observed in interphase eggs (Figure 4i), and anaphase spindle microtubule structures of the first cleavage are seen between the two sets of chromatin (Figure 4j). After IVF alpha-tubulin staining is detected in association with the decondensing nuclei of sperm (Figure 4k). An early 2-cell stage embryo at 25 h is shown in Figure 4l displaying the microtubule-containing midbody.

Figures 5-7 are schematic diagrams which summarize the distribution of NuMA during *in vitro* fertilization (Figure 5), nuclear cloning (Figure 6), and parthenogenetic activation (Figure 7).

Table 1 summarizes our results and provides a statistical analysis.

5. DISCUSSION

The studies presented here were aimed at determining whether the nuclear mitotic apparatus (NuMA) protein is remodeled in nuclear-transfer (NT) pig eggs and whether NuMA staining patterns in NT eggs are comparable to those observed after *in vitro* fertilization. Our results revealed for the first time that in this species NuMA is remodeled and the donor cell nucleus contributes NuMA to the developing cloned egg. These results are consistent with recent studies on NuMA in intraspecies and interspecies nuclear transfer embryos in which we showed that the donor cell's NuMA contributes to centrosome formation during mitosis and cell division (19). Our results differ from those reported for non-human primates in which the absence of NuMA may play a role in cloning failures (20,21). However, we do not yet know whether

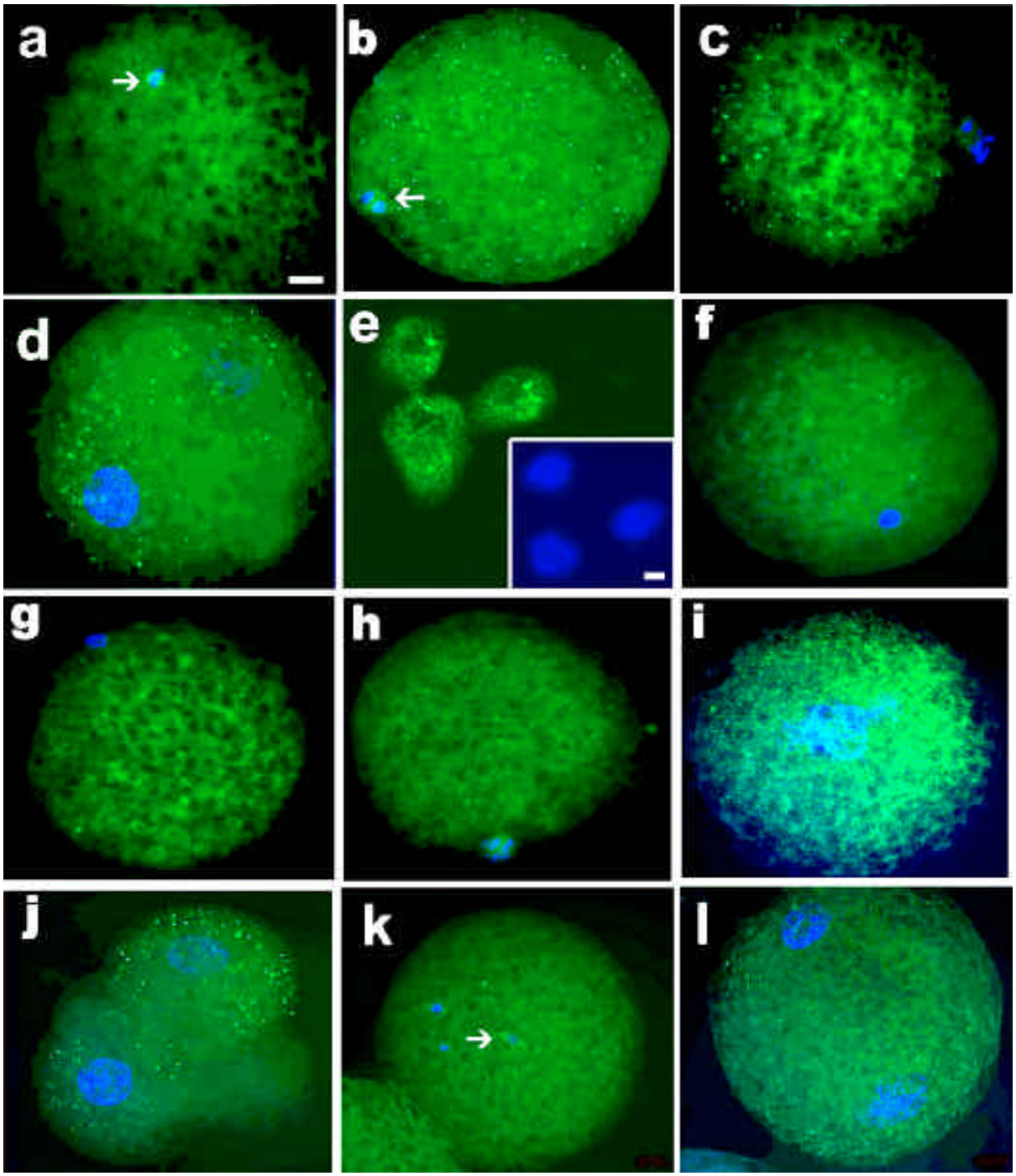

Figure 3. Gamma-tubulin labeling of parthenogenetic, NT and IVF embryos at different stages. a) MII oocytes: Gamma-tubulin is localized to the spindle poles (arrow); b) Parthenogenesis 1 h: Gamma-tubulin is detected at anaphase spindle microtubules (arrow) and additional gamma-tubulin aggregates are seen in the cytoplasm; c) Parthenogenesis 6 h: Gamma-tubulin is detected in the cytoplasm where it forms small punctate aggregates; d) Parthenogenesis 25 h: gamma-tubulin is detected as small punctate aggregates in the cytoplasm but no gamma-tubulin is detected at the nuclei; e) Fibroblasts: Gamma-tubulin is localized as small aggregates associated with the nucleus; f) NT 0 h: No gamma-tubulin staining is detected; g), h), I) and j): gamma-tubulin staining is detected as small aggregate dots in the cytoplasm at NT 1h, 6 h, 25 h (1-cell) and 25 h (2-cell); k) IVF 6 h: Gamma-tubulin is detected in association with sperm nuclei (arrow); l) IVF 25 h: Gamma-tubulin is detected in association with the two interphase nuclei. Images show double-staining for gamma-tubulin (green) and DNA (blue). Bar15 µm except for e = 10 µm.

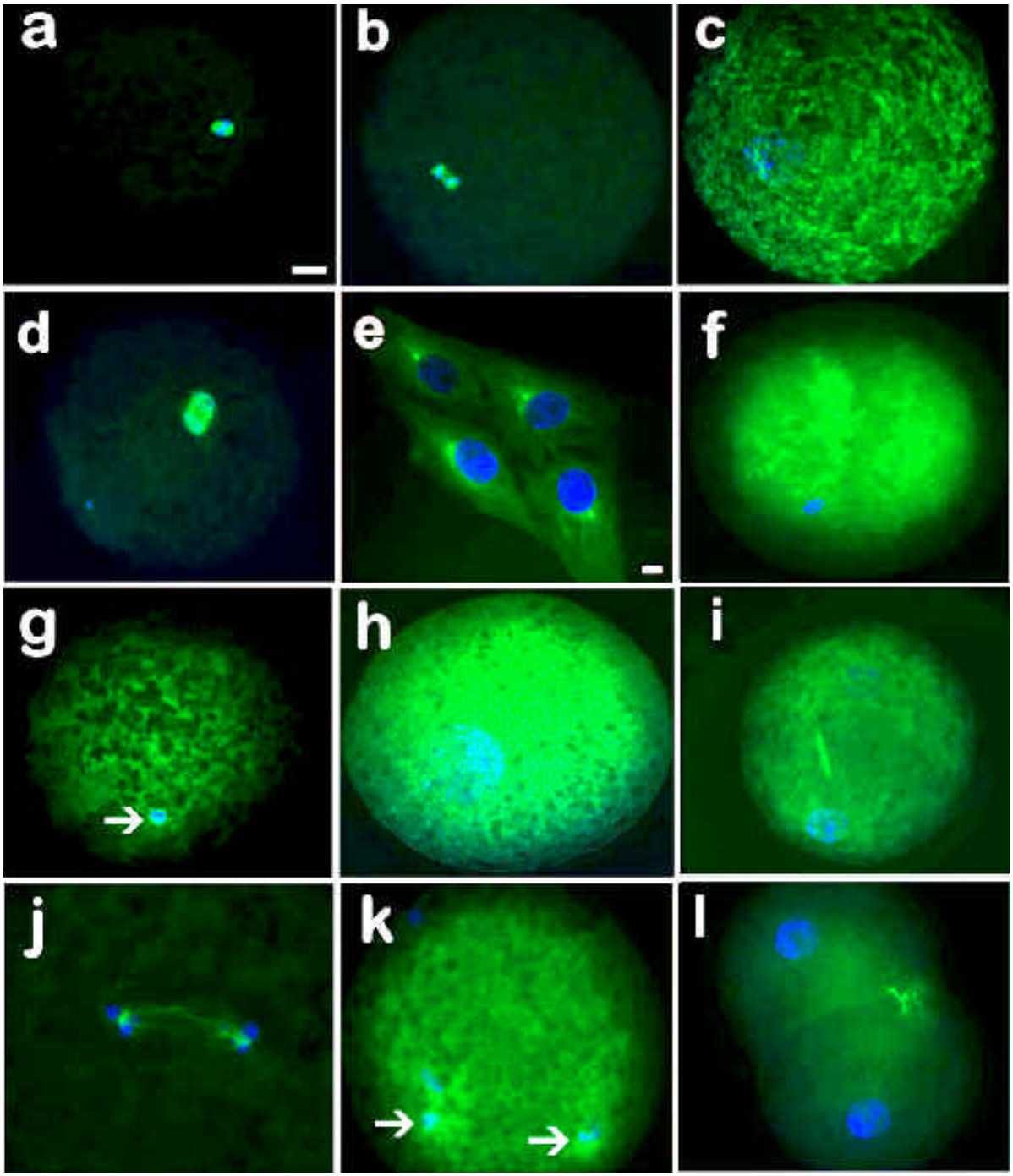

Figure 4. Alpha-tubulin labeling of parthenogenetic, NT and IVF embryos at different stages. a) MII stage oocyte displaying spindle microtubule staining; b) Parthenogenesis 1 h: Microtubules are detected in the anaphase II meiotic spindle; c) Parthenogenesis 6 h: Pronuclei are seen. Alpha-tubulin staining is detected in the cytoplasmic asters; d) Parthenogenesis 25 h: alpha-tubulin is detected in the anaphase spindle of first cleavage; e) Fibroblast cells displaying microtubules with heaviest staining at the centrosome areas; f) NT 0 h: Some cytoplasmic microtubule structures are detected within the cytoplasm but not around the somatic cell nucleus; g) NT 1 h: A small microtubule aster (arrow) is detected around transferred somatic cell nucleus; h) NT 6 h: NT pronucleus-like structures are seen and small microtubule asters are detected in the cytoplasm; i) NT 25 h: cytoplasmic microtubules are seen in the cytoplasm of an interphase egg; j) Anaphase spindle microtubule structure of first cleavage is seen between the two sets of chromosomes; k) IVF 6 h: Alpha-tubulin staining around the decondensing nuclei of sperm are shown (arrows); l) IVF 25 h: Early 2-cell stage embryo displaying microtubule-containing midbody. Images show double-staining for alpha-tubulin (green) and DNA (blue). Bar = 15 µm except for e = 10 µm.

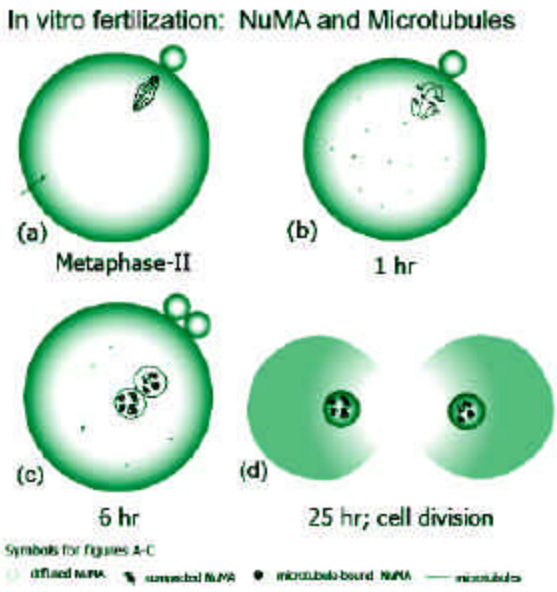

Figure 5. Distribution of NuMA during *in vitro* fertilization.

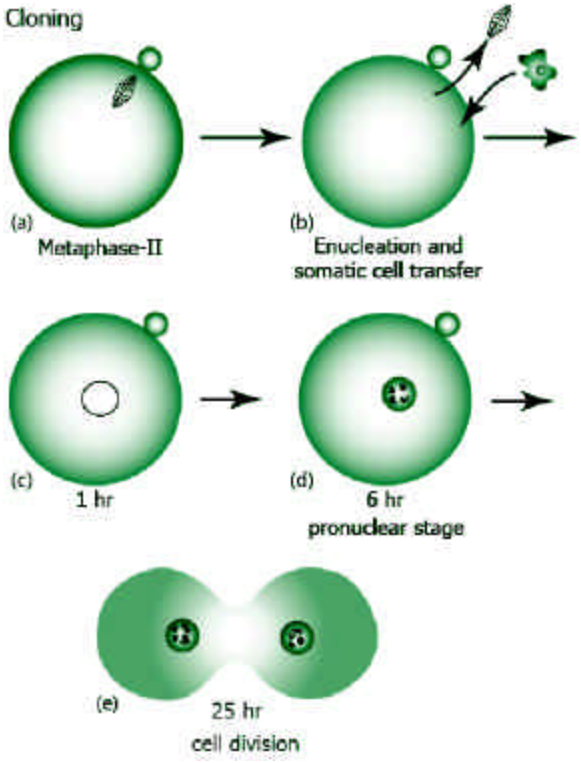

Figure 6. Distribution of NuMA during nuclear cloning.

abnormalities in NuMA remodeling will occur at a later time during development of the reconstructed embryo.

NuMA is an important multifunctional protein that plays significant roles in DNA replication, reorganization, and transcription during interphase (reviewed in reference 1) and in the organization of the mitotic apparatus during mitosis and cell division. In the unfertilized mature pig oocyte NuMA is concentrated at the meiotic spindle (MII) where its functions include organizing and bundling microtubules into the meiotic apparatus (22). The NuMA-containing meiotic spindle is removed before nuclear transfer which leaves the oocyte without evident NuMA. The donor cell nucleus provides most likely the only source for NuMA in cloned embryos.

Nuclear remodeling is a complex process requiring intimate and numerous interactions between the transferred donor cell nucleus and the ooplasm that are only poorly understod. Typically, the donor cell nucleus is derived from somatic cells and is normally regulated by somatic cell cytoplasm. In reconstructed eggs, the somatic cell nucleus needs to be regulated by factors in the oocyte's cytoplasm to participate in embryonic cell cycles and carry out functions that are normally carried out by the egg's zygote nucleus. Several modifications in the donor nucleus are likely to be required for successful nuclear cloning and include epigenetic modifications in the genome and subsequent changes in gene expression. Because NuMA specifically responds to cell signaling and carries out functions that vary with different signals, successful remodeling of NuMA is crucial for the reconstructed embryo. We do not yet know which regulatory systems are required for remodeling of nuclear proteins including NuMA.

Evidence from mammalian tissue culture cells suggests that cyclin B plays a critical role in translocating NuMA from the nucleus to the cytoplasm and from the cytoplasm to the mitotic centrosomes where it forms an insoluble crescent around centrosomes that tethers microtubules precisely into the bipolar mitotic apparatus (23,24). Inactivation of cdc2/cyclin B kinase is important for dissociation of NuMA from the mitotic centrosomes and its relocalization to the nucleus during exit from mitosis (25). The disassembly from the mitotic centrosomes is crucial for NuMA's relocalization to the nucleus to resume its functions as nuclear matrix protein. If NuMA is not translocated properly to the nucleus it may become localized to the cytoplasm and nucleate small asters that may result in embryo fragmentation.

Results show that NuMA is mainly absent in parthenogenetically activated oocytes which raises questions on regulatory mechanisms that are required for the activation of NuMA. The absence of NuMA detection is not related to masking of NuMA by DNA or other components since unmasking methods including microwave unmasking or digestion of DNA still did not result in NuMA detection. NuMA may either exist in a form that is not detectable by antibody staining, similar to the results obtained for quiescent cells (18), or it may be that factors are missing in parthenotes that do not allow aggregation of NuMA in parthenogenetically activated nuclei and therefore prevent visualization by immunofluorescence microscopy. This hypothesis is strengthened by some of our earlier findings in unfertilized invertebrate eggs in which centrosome material could not

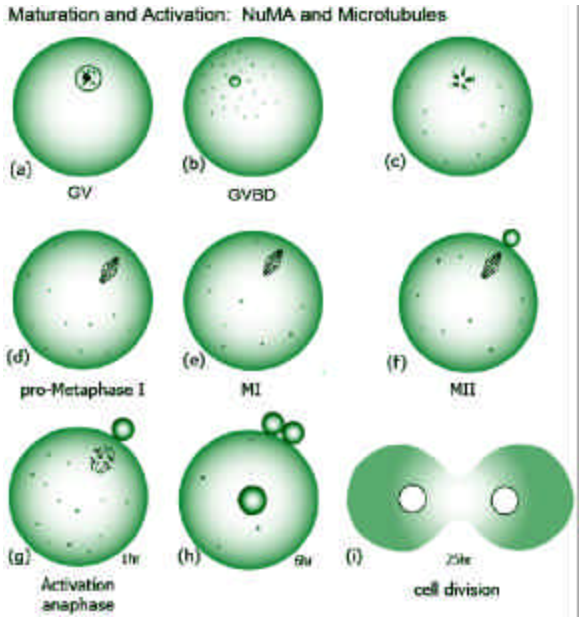

Figure 7. Distribution of NuMA during parthenogenetic maturation and activation.

Table 1. Pronucleus or nucleus with evident staining of NuMA in NT, IVF and parthenogenetic embryos

	6hr (%)	**25hr (%)**
NT	15/27 (55.2)[a]	16/25 (62.3)[a]
IVF	5/20 (25.0)[b]	13/16 (82.2)[a]
Parthenogenesis	4/51 (7.8)[c]	6/48 (12.5)[b]

a,b,c Different superscripts within the same column indicate values which are significantly different (P<0.05)

be visualized by immunofluorescence microscopy unless it was activated through changes in pH or calcium (26). Similarly, NuMA may be present in a latent state in the nuclei but not in compacted form to be visualized. The reasons for the absence of NuMA staining will require further experimental analysis.

Parthenogenetically activated eggs are different from fertilized eggs in many aspects. During normal fertilization sperm induces a cascade of ionic changes associated with pH changes in the fertilized egg that is only partly mimicked in artificially activated oocytes (27-29, 26). The absence of NuMA immunofluorescence detection in the donor cell nuclei directly after transfer may be explained on the basis of factors that need to be acquired from the oocyte's cytoplasm, perhaps related to pH or calcium. NuMA staining is positively detected at 6h after nuclear transfer which indicates an influence of the oocyte's cytoplasm on NuMA in the donor nuclei.

Somatic cells and embryos have different requirements for *in vitro* culture and adjustments are likely to be needed for the somatic cell nucleus to adapt to the oocyte's cytoplasm and to the medium that has been defined for *in vitro* fertilized embryo culture. Under our present culture conditions we know that cell cycle delays occur in reconstructed eggs as compared to *in vitro* fertilized eggs (30). We do not yet know the optimal culture requirements for reconstructed oocytes but studies are underway to improve growth conditions to faithfully reprogram the donor nucleus to match that of the fertilized egg (31,32).

Results on fetal fibroblasts show that only 33.5% (163/456) of the fibroblast cells display NuMA staining while no NuMA staining was detected in 66.5%. We explain this result by the large percent of quiescent cells that are seen when cell cultures are grown to confluency. As shown by Taimen *et al* (18), MCF-7 cells after long-term culture also did not display NuMA staining. NuMA staining of fibroblast cells at various times after culture before reaching confluency resulted in a larger percent of NuMA-stained cells. In granulosa cells, 72.7 % displayed NuMA staining under normal growth conditions (data not shown). NuMA may be present in non-stained cells but in a form that is not detectable by NuMA antibody. We do not yet know whether differences exist between embryos that had been reconstructed with NuMA immunofluorescent positive compared to NuMA immunofluorescent negative somatic cells. Currently is is technically not possible to transfer immunofluorescently stained cells and, based on our experience, we do not have indications whether different-sized cells yield different cloning efficiencies. These studies are in progress in our lab. Several investigators have analyzed the cell cycle stages and concluded that cells in the G0/G1 stage provided optimal nuclear transfer conditions (30) although other investigators have used mitotic cells (33). Recently, cloned calves have been produced from cells with *in vitro* remodeled chromatin before nuclear transfer (34).

The present report is focused only on the first cell cycle to show that NuMA is contributed by the donor cell nucleus in reconstructed pig oocytes but we do not yet know whether NuMA is expressed and regulated accurately throughout development. NuMA is developmentally regulated and can either participate in apoptosis or cell division, depending on the signals that it receives. It is therefore crucial to provide culture conditions that support accurate NuMA regulation. So far several reports have shown that cloned embryos exhibit defects in the expression and regulation of key genes (35) and abnormalities in DNA methylation (36-44). Inefficient changes in chromatin structure, inaccurate posttranslational regulation of key proteins (45,46) and other factors have been suggested to cause abnormalities of nuclear-ooplasmic interactions. The studies of cloned pig embryos are of particular current interest, as cloning of pig embryos is an important new topic in biomedical research with potential for numerous biomedical and agricultural applications. The exceptional physiological similarity of pigs with humans has generated high interest in producing genetically modified pigs as tissue and organ donors for humans and as models for human disease (47,48). Cellular and molecular studies as well as genetic and epigenetic approaches are aimed at understanding the remodeling and subsequent reprogramming of the donor cell nucleus by the enucleated oocyte which is one of the crucial requirements to increase cloning efficiency which at present ranges between 1-2% in reconstructed pig embryos.

The present studies have opened up new avenues to explore nuclear remodeling by using NuMA as one of the important cell and molecular markers that not only plays a significant role in the nucleus but also during mitosis, cell division, cell differentiation, and subsequent embryo development, therefore providing a marker for nuclear reprogramming and developmental analysis. We have determined that NuMA is remodeled after NT in pig oocytes. We do not yet know whether abnormalities in NuMA regulation play a role in the low cloning efficiency of pig oocytes and developmental abnormalities that are commonly seen in cloned animals. Further studies are needed to determine the precise regulatory mechanisms and to evaluate whether misregulation of NuMA during mitosis and during development plays a role in abnormalities encountered after nuclear cloning. These studies will provide steps toward modifying and optimizing culture conditions for NT-derived embryos.

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7. REFERENCES

1. Zeng, C. 2000. NuMA: a nuclear protein involved in mitotic centrosome function. Microsc Res Tech, 49, 467-477 (2000)

2. Sun, Q.-Y. & H. Schatten: Role of NuMA in vertebrate cells: review of an intriguing multifunctional protein. *Frontiers in Bioscience*, 11, 1127-1146 (2006)

3. Wells, R.A., C. Catzavelos & S. Kamel-Reid: Fusion of retinoic acid receptor alpha to NuMA, the nuclear mitotic apparatus protein, by a variant translocation in acute promyelocytic leukaemia. Nat Genet, 17, 109-13 (1997)

4. Sukhai, M.A., X. Wu, Y. Xuan, T. Zhang, P.P. Reis, K. Dube, E.M. Rego, M. Bhaumik, D.J. Bailey, R.A. Wells, S. Kamel-Reid & P.P. Pandolfi: Myeloid leukemia with promyelocytic features in transgenic mice expressing hCG-NuMA-RARalpha. Oncogene, 23, 665-78 (2004)

5. Saunders, S., M. Shuster, X. Huang, B. Gharaibeh, A.H. Enyenihi, I. Petersen & S.M. Gollin: Chromosomal instability and cytoskeletal defects in oral cancer cells. *Proc Natl Acad Sci U S A*, 97, 303-308 (2000)

6. Kammerer, S., R. B. Roth, C.R. Hoyal, R. Reneland, G. Marnellos, M. Kiechle, U. Schwarz-Boeger, L.R. Griffiths, F. Ebner, J. Rehbock, C.R. Cantor, M.R. Nelson & A. Braun: Association of the NuMA region on chromosome 11q13 with breast cancer susceptibility. *Proc Natl Acad Sci U S A*, 102, 2004-2009 (2005)

7. Kolber-Simonds, D., L. Lai , S.R. Watt, M. Denaro, S. Arn, M.L. Augenstein, J. Betthauser, D.B. Carter, J.L. Greenstein, Y. Hao, G.-S. Im, Z. Liu, G.D. Mell, C.N. Murphy, K.-W. Park, R. Rieke, D.J.J. Ryan, D.H. Sachs, E.J. Forsberg, R.S. Prather & R.J. Hawley: Production of α-1,3-galactosyltransferase null pigs by means of nuclear transfer with fibroblasts bearing loss of heterozygosity mutations. *Proc Natl Acad Sci USA*, 101, 7335-7340 (2004)

8. Abeydeera, L.R., W.H. Wang, R.S. Prather & B.N. Day: Maturation *in vitro* of pig oocytes in protein-free culture media: fertilization and subsequent embryo development *in vitro*. *Biol Reprod*, 58, 1316-1320 (1998)

9. Tao, T., A.Z. Boquest, Z. Machaty, A.L. Petersen, B.N. Day & R.S. Prather Development of pig embryos by nuclear transfer of cultured fibroblast cells. CLONING, 1(1), 55-62 (1999)

10. Tao, T., Z. Machaty, L.R. Abeydeera, B.N. Day & R.S. Prather: Optimization of porcine oocyte activation following nuclear transfer. Zygote, 8, 69-77 (2000)

11. Im, G.-S., L. Lai, Z. Liu, Y. Hao, D. Wax, A. Bonk, R.S. Prather: *In vitro* development of preimplantation porcine nuclear transfer embryos cultures in different media and gas atmospheres. *Theriogenology*, 61, 1125-1135 (2004)

12. Yoshioka, K., C. Suzuki, A. Tanaka, I.M. Anas & S. Iwamura: Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. Biol Reprod, 112-119 (2002)

13. Sun, Q.Y., L. Lai, K.W. Park, B. Kühholzer, R.S. Prather & H. Schatten: Dynamic events are differently regulated by microfilaments, microtubules, and mitogen-activated protein kinase during porcine oocyte maturation and fertilization *in vitro*. *Biol Reprod*, 64, 879-889 (2001a)

14. Sun, Q.Y., L. Lai, A. Bonk, R.S. Prather & H. Schatten: Cytoplasmic changes in relation to nuclear maturation and early embryo developmental potential of porcine oocytes: effects of gonadotropins, cumulus cells, follicular size and protein synthesis inhibition. *Mol Reprod Dev*, 59, 192-198 (2001b)

15. Sun, Q.-Y., L. Lai, G. Wu, K.-W. Park, B.N. Day, R.S. Prather & H. Schatten: Microtubule assembly after treatment of pig oocytes with taxol: correlation with chromosomes, γ-tubulin and MAP kinase. *Mol Reprod Dev*, 60, 481-490 (2001c)

16. Sun, Q.-Y., G. Wu, L. Lai, K.W. Park, B.N. Day, R.S. Prather & H. Schatten: Translocation of active mitochondria during porcine oocyte maturation, fertilization and early embryo development *in vitro*. *Reproduction*, 122, 155-163 (2001d)

17. Sun, Q.-Y., L. Lai, R.S. Prather & H. Schatten: Antioxidants stimulate meiosis resumption, but inhibit MAP kinase phosphorylation and further cell cycle progression in porcine oocytes. Reprod Fertil Dev 12 383-389 (2001e)

18. Taimen, P., M. Viljamaa & M. Kallajoki: Preferential expression of NuMA in the nuclei of proliferating cells. *Exp Cell Res*, 256, 140-149 (2000)

19. Zhong, Z.-S., G. Zhang, X.-Q. Meng, Y.-L. Zhang, D.-Y. Chen, H. Schatten & Q.-Y. Sun: Function of

donor cell centrosome in intraspecies and interspecies nuclear transfer embryos. *Exp Cell Res* 306 35-46 (2005)
20. Simerly, C., T. Dominko, C. Navara, C. Payne, S. Capuano, G. Gosman, K.-Y. Chong, D. Takahashi, C. Chace, D. Compton, L. Hewitson, & G. Schatten: Molecular correlates of primate nuclear transfer failures. *Science*, 130, 297 (2003)
21. Simerly, C., C. Navara, S.H. Hyun, B.C. Lee, S.K. Kang, S. Capuano, G. Gosman, T. Dominko, K.Y. Chong, D. Compton, W.S. Hwang, & G. Schatten: Embryogenesis and blastocyst development after somatic cell nuclear transfer in nonhuman primates: overcoming defects caused by meiotic spindle extraction. Dev Biol 276 237-252 (2004)
22. Lee, J., T. Miyano & R.M. Moor: Spindle formation and dynamics of γ-tubulin and nuclear mitotic apparatus protein distribution during meiosis in pig and mouse oocytes. *Biol Reprod*, 62, 1184-1120 (2000)
23. Saredi, A., L. Howard, D.A. Compton: Phosphorylation regulates the assembly of NuMA in a mammalian mitotic extract. J Cell Sci, 110, 1287-1297 (1997)
24. Merdes, A. & D.W. Cleveland: The role of NuMA in the interphase nucleus. *J Cell Sci*, 111, 71-7 (1998)
25. Gehmlich, K., L. Haren & A. Merdes: Cyclin B degradation leads to NuMA release from dynein/dynactin and from spindle poles. *EMBO reports*, Vol. 5, No. 1, 97-103 (2004)
26. Schatten, H., M. Walter, H. Biessmann & G. Schatten: Activation of maternal centrosomes in unfertilized sea urchin eggs. *Cell Motil Cytoskel*, 23, 61-70 (1992)
27. Ruddock, N.T., Z. Machaty, M. Milanick & R.S. Prather Mechanism of intracellular pH increase during parthenogenetic activation of *in vitro* matured porcine oocytes. *Biol Reprod*, 63, 488-492 (2000a)
28. Ruddock, N.T., Z. Machaty & R.S. Prather: Intracellular pH increase accompanies parthenogenetic activation of porcine, bovine and murine oocytes. *Reprod Fertil Dev*, 12, 201- 207 (2000b)
29. Ruddock, N.T., Z. Machaty, R.A. Cabot & R.S. Prather: Porcine oocyte activation: differing roles of calcium and pH. *Mol Repro Dev*, 59, 227-234 (2001)
30. Kühholzer, B., T. Tao, Z. Machaty, R.J. Hawley, J.L. Greenstein, B.N. Day, R.S. Prather: Production of transgenic porcine blastocysts by nuclear transfer. *Mol Repro Dev*, 56, 145-148 (2000).
31. Gao, S., Y.G. Chung, J.W. Williams, J. Riley, K. Moley & K.E. Latham: Somatic cell-like features of cloned mouse embryos prepared with cultured myoblast nuclei. *Biol Reprod*, 69, 48-56 (2003)
32. Rideout, W.M., K. Eggan, R. Jaenisch: Nuclear cloning and epigenetic reprogramming of the genome. *Science*, 293(5532), 1093-1098 (2001)
33. Lee, J.-W., S.-C. Wu, X.C. Tian, M. Barber, T. Hoagland, J. Riesen, K.-H. Lee, C.-F. Tu, W.T.K. Cheng, & X. Yang: Production of cloned pigs by whole-cell intracytoplasmic microinjection. *Biol Reprod*, 69, 995-1001 (2003)
34. Sullivan, E.J., S. Kasinathan, P. Kasinathan, J.M. Robl & P. Collas: Cloned calves from chromatin remodeled *in vitro*. *Biol Reprod*, 70, 146-153 (2004)
35. Boiani, M. S. Eckardt, H.R. Schoeler & K. McLaughlin: Oct4 distribution and level in mouse clones: consequences for pluripotency. *Genes Dev*, 16, 1209-1219 (2002)
36. Humphreys, D., K. Eggan, H. Akutsu , K. Hochedlinger, W.M., III, Rideout, D. Biniszkiewicz, R.Yanagamachi, R. Jaenisch: Epigenetic instability in ES cells and cloned mice. *Science*, 293, 95-97 (2001)
37. Ohgane, J., T. Wakayama, Y. Kogo, S. Senda, N. Hattori, S. Tanaka, R. Yanagamachi & K. Shiota: DNA methylation variation in cloned mice. *Genesis*, 30, 45-50 (2001)
38. Kang, Y.K., D.B. Koo, J.S. Park, Y.H. Choi, A.S. Chung, K.K. Lee, Y.M. Han 2001a. Aberrant methylation of donor genome in cloned bovine embryos. Nat Genet, 28, 173-177 (2001a)
39. Kang, Y.K., D.B. Koo, J.S. Park, Y.H. Choi, H.N. Kim, W.K. Chang K.K. Lee & M. Han 2001b. Typical demethylation events in cloned pig embryos. Clues on species-specific differences in epigenetic reprogramming of a cloned donor genome. *J Biol Chem*, 276, 39980-39984 (2001b)
40. Kang, Y.K., D.B. Koo, J.S. Park, Y.H. Choi, K.K. Lee & Y.M. Han: Influence of oocyte nuclei on demethylation of donor genome in cloned bovine embryos. *FEBS Lett*, 499, 55-58 (2001c)
41. Reik, W., W. Dean & J. Walter: Epigenetic reprogramming in mammalian development. *Science*, 293**,** 1089-1093 (2001)
42. Dean, W., F. Santos, M. Stojkovic, V. Zakhartchenko, J. Walter, E. Wolf, & W. Reik: Conservation of methylation reprogramming in mammalian development: aberrant reprogramming in cloned embryos. *Proc Natl Acad Sci USA*, 98, 13734-13738 (2001)
43. Chen, T., Y. Jiang, Y.-L. Zhang, J-H Liu, Y. Hou, H. Schatten, D.Y. Chen & Q.-Y. Sun: DNA hypomethylation of individual sequences in aborted bovine clones. *Front Biosci*, 10, 3002-3008 (2005)
44. Zhang, Y.-L., T. Chen, Y. Jiang, Z.-S. Zhong, S.-Z. Liu, H. Schatten, D.-Y. Chen & Sun, Q.-Y. Active demethylation of individual genes in intracytoplasmic sperm injection (ICSI) rabbit embryos. Mol Reprod Dev, 2005, in press
45. Chung, Y.G., M.R. Mann, M.S. Bartolomei & K.E. Latham: Nuclear-cytoplasmic "tug-of-war"during cloning: effects of somatic cell nuclei on culture medium preferences of preimplantation cloned mouse embryos. *Biol Reprod*, 6, 1178-1184 (2002)
46. Chung, Y.G., S. Ratnam, J.R. Chaillet, & K.E. Latham: 2003. Abnormal regulation of DNA methyltransferase expression in cloned mouse embryos. *Biol Reprod*, 69, 146-153 (2003)
47. Lai, L. & R.S. Prather Creating genetically modified pigs by using nuclear transfer. *Repro Biol Endocrinol* 1, 82, 1-6 (2003)
48. Lai, L., & R.S. Prather: Cloning pigs as donors for organ transfers in humans: promises and challenges. *IEEE Engineering in Medicine and Biology* (2004)

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