

NuMA distribution and microtubule configuration in rabbit oocytes and cloned embryos

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Abstract

The assembly of microtubules and the distribution of NuMA were analyzed in rabbit oocytes and early cloned embryos. α -Tubulin was localized around the periphery of the germinal vesicle (GV). After germinal vesicle breakdown (GVBD), multi-arrayed microtubules were found tightly associated with the condensed chromosomes and assembled into spindles. After the enucleated oocyte was fused with a fibroblast, microtubules were observed around the introduced nucleus in most reconstructed embryos and formed a transient spindle 2–4 h post-fusion (hpf). A mass of microtubules surrounded the swollen pseudo-pronucleus 5 hpf and a normal spindle was formed 13 hpf in cloned embryos. NuMA was detected in the nucleus in germinal vesicle-stage oocytes, and it was concentrated at the spindle poles in both meiotic and mitotic metaphase. In both donor cell nucleus and enucleated oocyte cytoplasm, NuMA was not detected, while NuMA reappeared in pseudo-pronucleus as reconstructed embryo development proceeded. However, no evident NuMA staining was observed in the poles of transient spindle and first mitotic spindle in nuclear transfer eggs. These results indicate that NuMA localization and its spindle pole tethering function are different during rabbit oocyte meiosis and cloned embryo mitosis.

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Introduction

Since the first cloned mammals were produced (Wilmut *et al.* 1997), many species have been cloned by somatic cell nuclear transfer (SCNT). There were some reports on rabbits cloning, but only until 2002, the cloned rabbits derived by SCNT were successfully born (Chesne *et al.* 2002). After the introduction of G₀-phase somatic cell into a mature oocyte, the reconstructed embryo undergoes nuclear membrane breakdown and chromosome condensation, followed by metaphase spindle-like structure formation, but most of these embryos ceased development afterwards due to possible defective chromatin remodeling and abnormal microtubule organization (Wakayama *et al.* 2000, Mir *et al.* 2003, Zhong *et al.* 2005, Yan *et al.* 2006).

NuMA (a nuclear protein that associates with the mitotic apparatus) is an abundant 235 kDa protein that is localized to the nucleus during interphase and accumulates at the spindle poles during mitosis. NuMA is associated with dynein/dynactin and spindle poles in extracts and cultured cells. Disruption of NuMA function by antibody injection or immunodepletion in extracts demonstrated that NuMA is required for the assembly

and maintenance of spindle poles (Kallajoki *et al.* 1991, Yang & Snyder 1992, Gaglio *et al.* 1995, Merdes *et al.* 1996, Gordon *et al.* 2001). In dividing cells, upon phosphorylation, NuMA disperses into the cytoplasm, associates with cytoplasmic dynein/dynactin to form a complex. NuMA's function in spindle microtubule organization is regulated by RanGTP and Pins-related protein. It has been shown that phosphorylation of NuMA is by cyclin B/cdc2 kinase and NuMA's dephosphorylation is due to the cyclin B degradation. NuMA also functions during meiotic spindle organization in male and female germ cells (see reviews by Zeng 2000, Sun & Schatten 2006).

In previous studies, the researchers analyzed the expression pattern and function of NuMA in mouse and pig oocytes (Lee *et al.* 2000) and early embryos (Tang *et al.* 2004) as well as mouse (Zhong *et al.* 2005, Van Thuan *et al.* 2006) and pig (Liu *et al.* 2006) cloned embryos. But the dynamic changes of NuMA and microtubules during meiosis I–II transition in rabbit oocytes have not been reported. Further, the spindle formation and NuMA distribution in early rabbit cloned embryos have not been observed.

In this study, we observed the dynamic changes of NuMA, chromatin, and microtubules during rabbit oocyte maturation and the first cell cycle progression of cloned rabbit embryos.

Materials and Methods

Animals

Animal care and handling were in accordance with the policy on the Care and Use of Animals of the Ethical Committee, Institute of Zoology, Chinese Academy of Sciences. Female big-eared Japan white rabbits (purchased from Laboratory Animal Center, Institute of Zoology, Chinese Academy of Sciences) were housed in stainless steel cages and were fed with regular rabbit fodder and water *ad libitum*.

Oocyte collection

Mature female rabbits were superovulated by administering pregnant mares serum gonadotrophin (PMSG) and human chorionic gonadotropin (hCG) (Institute of Zoology, Chinese Academy of Sciences). Each rabbit was injected with 100 IU PMSG and 100 IU hCG 96 h later. At 14 h after hCG treatment, the females were laparotomized. Ovaries were then removed and minced in M199 and oocytes were isolated by mouth pipette. Since the rabbits were treated by superovulation, we could get oocytes at different maturation stages from the ovaries without culture *in vitro*. Oocytes without cumulus cells were fixed directly. Matured metaphase II stage (MII) rabbit oocytes were flushed from the oviducts with warm 199 medium (Gibco BRL, NY).

All oocytes were treated shortly with 300 IU/ml hyaluronidase (Sigma Chemical Co) in M199 medium at 37 °C. Cumulus cells were stripped from the oocytes by repeated gentle pipetting. Rabbit oocytes at germinal vesicle (GV) stage were freed of cumulus cells by repeated pipetting with a pipette, whose diameter was a little smaller than the oocyte.

Oocyte enucleation

The cumulus-free MII eggs were transferred to M199 medium containing 7.5 µg/ml cytochalasin B (Sigma) and 10% fetal bovine serum (FBS) for 10 min, and then manipulated under an inverted microscope (Nikon's ECLIPSE TE300, Nikon Corporation, Japan). Removal of the meiotic spindle and chromosomes was accomplished in the following way. The 'squish' enucleation method was used for metaphase II spindle aspiration. A cumulus-free oocyte was held with a holding micropipette (170 µm outer diameter, 20 µm inner diameter) and the zona pellucida was partially dissected with a fine glass needle to make a slit near the first polar body. The first polar body and adjacent cytoplasm containing the metaphase II

spindle were squeezed and extruded with a 20 µm pipette gently. After enucleation, the karyoplast was stained with 1 µg/ml Hoechst33342 (Sigma) and exposed to the u.v. light to confirm the removal of chromosomes.

Donor cell preparation

Fibroblast cells were collected from an ear-skin biopsy of a mature Japanese white female rabbits. Primary cell culture was performed with the same method as described previously (Han *et al.* 2001). Fibroblasts at passages 4–10 were used as donors.

Cloned rabbit embryo reconstruction

A single donor cell was introduced into the perivitelline space of an enucleated oocyte. The couplets were pre-equilibrated in fusion medium consisting of 0.25 M sorbitol, 0.5 mM HEPES, 0.1 mM Ca(CH₃COO)₂, 0.5 mM Mg(CH₃COO)₂, and 1 mg/ml BSA, and then placed between the electrodes of a fusion chamber in fusion medium. Two direct current pulses (1.4 kV/cm, 80 µs each, 1 s apart) were applied with an ECM2001 Electrocell Manipulator (BTX inc., San Diego, CA, USA).

Activation of rabbit oocytes and reconstructed eggs

Cumulus-free rabbit MII oocytes and fused eggs were transferred to the fusion medium and incubated at room temperature for 1 min, and then transferred to a fusion chamber containing 100 µl fusion medium. Activation was achieved by applying double DC pulses of 1.2 kV/cm for 20 µs. The activated oocytes and embryos were washed in M199 + 10%FBS at least thrice, and then transferred into the pre-equilibrated culture medium.

Production of in vivo fertilized rabbit embryos

In vivo fertilized zygotes were collected 18 h post-hCG from the oviduct ampullae of superovulated females that had been mated with the same strain of males just after hCG injection. After removing cumulus cells with 300 IU/ml hyaluronidase in M199 medium, zygotes were cultured in M199 + 10%FBS. At 30 h post-hCG, embryos at different stages of mitosis were collected for observation.

Immunofluorescent confocal microscopy

After removing the zona pellucida in acidic M₂ medium (pH 2.5), eggs were fixed with 4% paraformaldehyde in PBS (pH 7.4) for at least 30 min at room temperature. Oocytes and reconstructed embryos were permeabilized with 1% Triton X-100 for 1 h at 37 °C, followed by blocking in 1% BSA for 1 h and incubation for 1 h at 37 °C or overnight at 4 °C with mouse anti-human NuMA

antibody (Oncogene, EMD Biosciences, San Diego, CA, USA) diluted in the ratio of 1:50 in blocking solution. After washing thrice in PBS containing 0.1% Tween 20 and 0.01% Triton X-100 (washing solution) for 5 min each, the oocytes were labeled with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG diluted in the ratio of 1:100 for 1 h at 37 °C. After extensive washing, DNA of samples was counterstained with propidium iodide. Finally, samples were mounted between a coverslip and a glass slide supported by four columns of a mixture of petroleum jelly and paraffin (9:1). As for microtubule staining, samples were treated exactly the same as those described above except that the first antibody was replaced by monoclonal anti- α -tubulin-FITC antibody (Sigma).

Digested and dispersed rabbit fibroblasts were placed into Petri dishes containing a cover glass slip, then cultured in Dulbecco's modified Eagle's medium (DMEM)-F12 containing 10% FBS. When 60–80% confluence is reached, the cells were fixed. The following immunostaining steps were same as the treatment of reconstructed embryos.

Slides were scanned using Leica TCS4D microscope (Leica Laser Technik, GmbH, Heidelberg, Germany) with an argon/krypton laser at 488 and 563 nm and two-channel scanning for detection of fluorescein isothiocyanate and phosphatidylinositol respectively.

The distribution patterns of the microtubules, NuMA, and chromosomes were observed at 0.5, 2, 3, 4, 6, 8, 10, 12–13, and 15 h after fusion. Each experiment was repeated at least thrice and at least ten randomly selected oocytes were examined each time.

Results

Distribution of NuMA in rabbit fibroblasts

In rabbit fibroblasts, NuMA was not detected in the nucleus during interphase (Fig. 1A). By prometaphase,

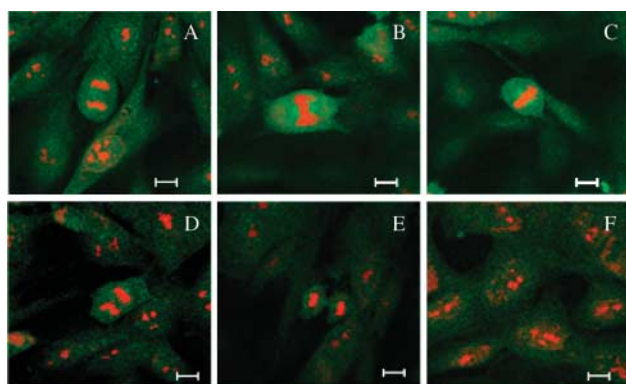


Figure 1 Immunofluorescent localization of NuMA in rabbit during mitosis (A–F). NuMA appears in green and DNA appears in red. Yellow reflects the overlapping of NuMA and DNA images. (A) Interphase, (B) prometaphase, (C) metaphase, (D) anaphase, (E) telophase cytokinesis, (F) serum starvation for 3 days. Bar = 10 μ m.

NuMA appeared in the future spindle poles (Fig. 1B), and located in the spindle poles during metaphase (Fig. 1C). But by late anaphase, the intensity of NuMA staining was decreased in the spindle poles and disappeared from reforming daughter cell nuclei during cytokinesis (Fig. 1D and E). As donor cells in SCNT, fibroblast cells were often treated with serum starvation (0.5% FBS) for 3–5 days. In this situation, little NuMA could be detected in the nuclei (Fig. 1F).

Localization of NuMA during rabbit oocyte meiotic maturation, early cleavage of parthenotes, and fertilized embryos

During meiotic maturation of rabbit oocytes, NuMA was detected either as spots in the nucleus or diffusely spreading across the nucleoplasm (Fig. 2A) at the beginning of germinal vesicle breakdown (GVBD). When chromatin assembled to form chromosome clusters, NuMA distributed as several discrete spots in the vicinity of the condensed chromosomes (Fig. 2B). NuMA surrounded the chromosome clusters in a characteristic crescent shape during prometaphase (Fig. 2C). During both meiotic metaphases I (Fig. 2D) and metaphase II (Fig. 2E) stages, NuMA was localized at the spindle poles.

As for rabbit MII oocyte parthenotes, before the first cell division, NuMA was detected in interphase nucleus 6 h after electrical activation (Fig. 2F). At the first mitotic prometaphase (Fig. 2G), NuMA was connected with the condensed chromosomes (Fig. 2H) and concentrated at the spindle poles during the metaphase. But during the anaphase stage, NuMA disappeared from the poles and scattered into cytoplasm with some small dots (Fig. 2I). When the embryo divides into two cells, NuMA returned to the nucleoplasm (Fig. 2J).

The pattern of NuMA distribution in rabbit fertilized embryos is different from that in parthenotes. NuMA was located in pronucleus (Fig. 2K) or two-cell stage (Fig. 2O) interphase nuclei and then dispersed into cytoplasm during metaphase of the first division (Fig. 2L). However, NuMA cannot be detected during both anaphase (Fig. 2M) and telophase (Fig. 2N).

Distribution of NuMA in rabbit SCNT embryos

Before enucleation, NuMA was located in the MII spindle poles (Fig. 2E). After enucleation, spindle pole NuMA was removed together with the spindle (Yan *et al.* 2006). At the first 4 h post-fusion (hpf), although the donor cell nucleus had undergone premature chromosome condensation (PCC), little NuMA was detected in the reconstructed embryos (Fig. 3A). With the embryo development, NuMA appeared in the newly formed pseudo-pronucleus (Fig. 3B and C) about 5 hpf. After pseudo-pronucleus membrane broke down, NuMA aggregated into some dots near chromosomes (Fig. 3D)

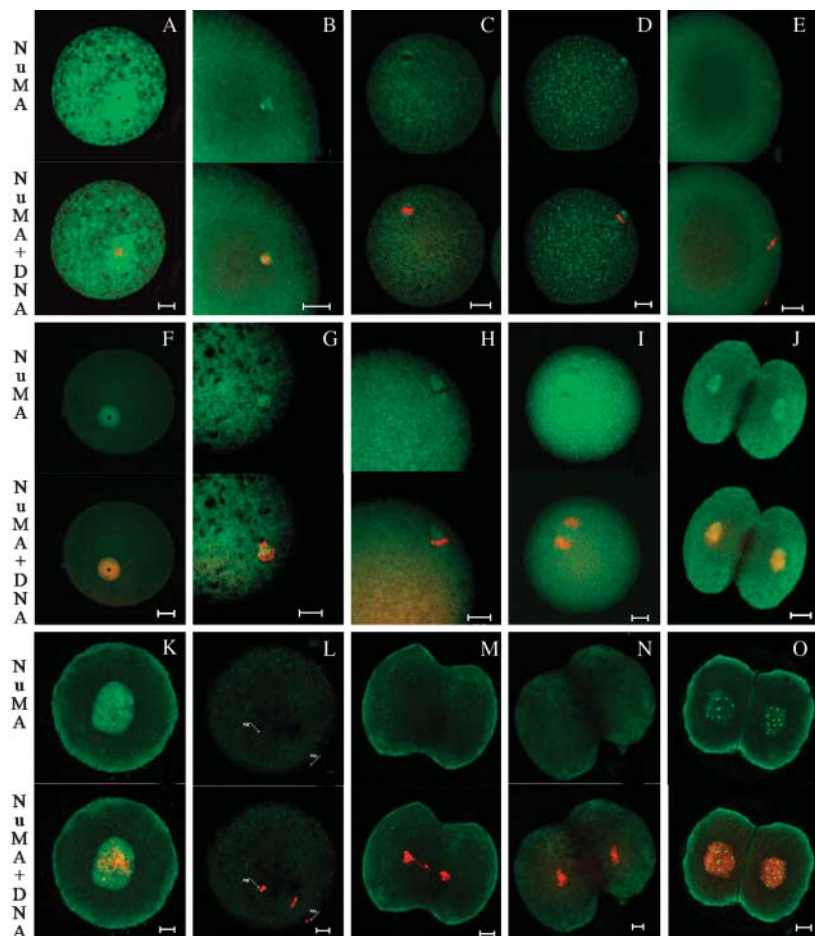


Figure 2 Immunofluorescent localization of NuMA in rabbit meiotic oocytes (A–E), parthenotes (F–J), and fertilized embryos (K–O). NuMA appears in green and DNA appears in red. Yellow reflects the overlapping of NuMA and DNA images. (A) GV stage, (B) GVBD stage, (C) prometaphase I stage, (D) metaphase I, (E) metaphase II, (F) 6 h post-activation, (G) first mitotic prometaphase, (H) first mitotic metaphase, (I) anaphase, (J) two-cell stage, (K) pronuclear stage, (L) metaphase of the first division, (M) anaphase, (N) telophase, (O) NuMA was located in two-cell stage interphase nuclei. Bar=20 μ m.

and gradually moved to the whole cytoplasm in numerous green dots at metaphase, anaphase, and telophase (Fig. 3E–G). When the reconstructed embryo cleaved into two cells, NuMA was relocalized in the whole interphase nuclei (Fig. 3H).

Microtubule and chromatin dynamics in rabbit oocytes and reconstructed embryos

Changes in microtubule assembly during rabbit oocyte meiosis were examined using anti-tubulin antibody (Fig. 4). Cytoplasmic microtubules distributed around the periphery of the GV (Fig. 4A). When chromatin was aggregated into a chromosome cluster after GVBD, randomly arrayed microtubules were tightly associated with the chromatin cluster (Fig. 4B). The microtubules organized into two polarized arrays with the axis parallel to the cell surface as chromosomes became aligned on the metaphase plate (Fig. 4C). After first polar body emission, the formation of the meiosis II spindle followed the same process as that for the meiosis I spindle (Fig. 4D and E). During chromosome segregation at anaphase II (Fig. 4F), the spindle elongated and moved to the cell surface.

Microtubule assembly and DNA changes in rabbit reconstructed eggs were examined by fixing the eggs at

different time points after the somatic cells were introduced into enucleated oocytes. Minimal change in somatic cell DNA and no microtubule assembly were observed within the first 30 min after fusion. Within 3–4 h, the PCC was observed in 87% reconstructed eggs (39/45), and microtubules were assembled into transient spindles (Fig. 5A–C). But at 5–9 hpf, only one pronucleus-like structure could be observed in the majority of embryos (Fig. 5D and E), which was not different from our previous report (Yan *et al.* 2006) in which the embryos constructed by monkey fibroblast and non-activated enucleated rabbit oocyte formed two pronucleus-like structure following the formation of PCC and spindle organization. During 10–13 hpf, a normal mitotic spindle of the first cell cycle established and reconstructed egg began to undergo cytokinesis (Fig. 5F–H).

Discussion

NuMA is a large nuclear protein that accumulates to the spindle poles in mitosis (Lydersen & Pettijohn 1980), and NuMA is required during mitosis for the terminal phases of chromosome separation and/or nuclear reassembly (Compton & Cleveland 1993). As the role in meiosis,

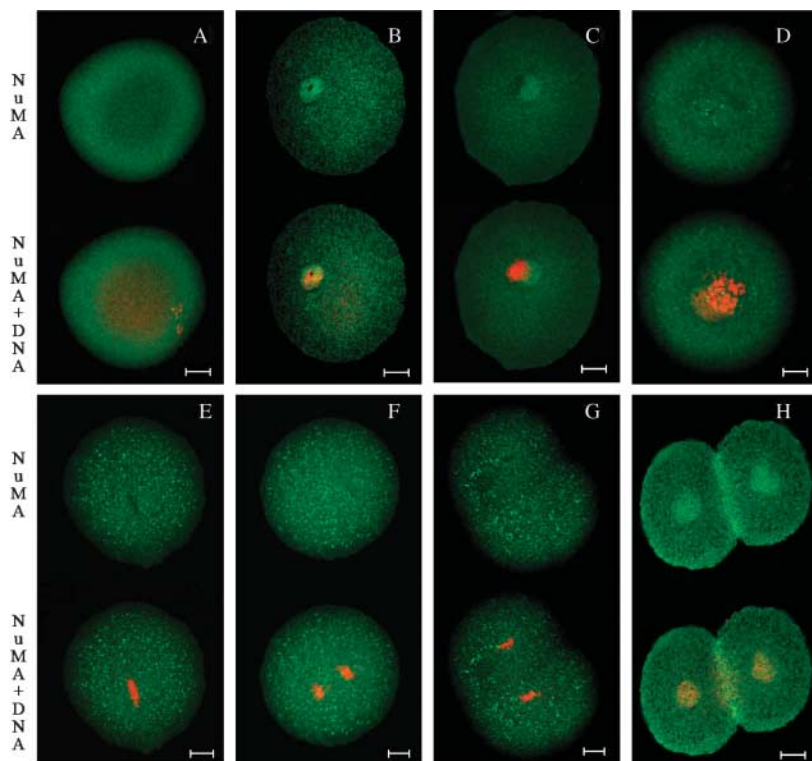


Figure 3 Distribution of NuMA in rabbit SCNT embryos. DNA and NuMA were stained red and green respectively. At 4 h post-fusion (hpf), donor cell nucleus had undergone PCC and NuMA was not detected between the chromosomes, (B and C) 5–9 hpf, NuMA was located in the pseudo-pronucleus, (D) 10 hpf, the membrane of pseudo-pronucleus broke down, (E) metaphase of the first division, (F) anaphase, (G) telophase, (H) NuMA was located in two-cell stage interphase nuclei. Bar=20 μ m.

NuMA appeared to localize to spindle poles of the meiotic pig and mouse oocytes and treatment with nocodazole disrupted the localization of NuMA (Lee *et al.* 2000). In maturing *Xenopus* oocytes, the microinjection of NuMA antibodies perturbed the organization of the transient MT array and the meiotic spindle (Becker *et al.* 2003). But the function of NuMA during interphase is still obscure. There are reports showing that NuMA might organize the chromatin structure (Luderus *et al.* 1994), others thought that NuMA is a non-essential structural protein of the nucleus, which is preferentially expressed in proliferatively active cells (Merdes & Cleveland 1998, Taimen *et al.* 2000). We did not find nuclear localization of NuMA in rabbit fibroblasts whether they were serum starved for 3 days or not. Little NuMA could be observed in interphase nuclei, but a strong expression was observed after the prophase. Evident distribution of NuMA into pole regions of the mitotic spindle was observed. Thus, NuMA may play a role in spindle assembly, but it is not an essential structural nuclear component in rabbit fibroblast cells. In contrast, monkey fibroblasts, even in confluent inhibition status for 3–5 days, contain NuMA in all stages of cell cycle (Yan *et al.* 2006). The absence of NuMA in rabbit fibroblast could be due to a lack of expression, specific post-translational modifications, or by proteolytic degradation.

During meiotic maturation of rabbit oocytes, NuMA was detected as spots in the nucleus spreading across the

nucleoplasm at GV phase or the beginning of GVBD. The NuMA foci were translocated from the condensed chromosomes and became aligned at both poles of the meiotic spindle during the metaphase I and II stages. Comparing with previous studies, we found that NuMA distribution in rabbit species is similar to pig (Lee *et al.* 2000) but different from mouse (Lee *et al.* 2000, Tang *et al.* 2004). In mouse oocytes, NuMA was localized along spindle microtubules (Lee *et al.* 2000) and at cytasters (Tang *et al.* 2004).

Although NuMA had not been detected in both donor cell and enucleated oocyte, as embryo development

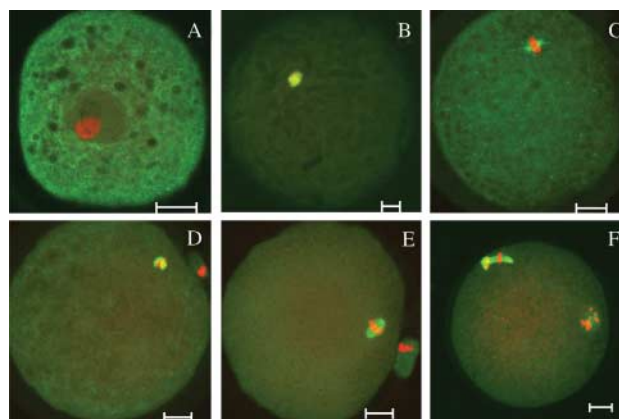


Figure 4 Microtubule organization during rabbit oocyte meiosis. DNA and α -tubulin were stained red and green respectively. (A) GV stage, (B) GVBD stage, (C) metaphase I, (D) prometaphase II, (E) metaphase II, (F) anaphase II. Bar=20 μ m.

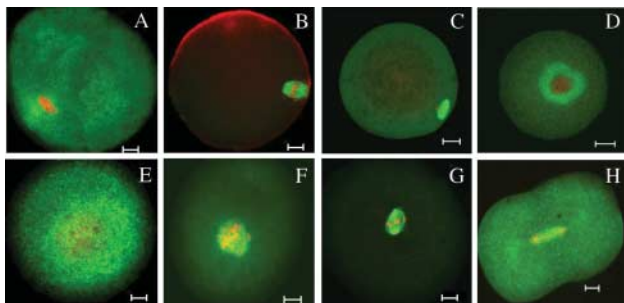


Figure 5 Spindle formation in rabbit reconstructed embryos. DNA and α -tubulin were stained red and green respectively. (A) 2 hpf, (B) 3 hpf, chromosome condensation, (C) 4 hpf, transient spindles formed, (D and E) pseudo-pronucleus formed and a microtubule cluster was assembled around the pseudo-pronucleus at 5–9 hpf, (F) first mitotic prometaphase, (G) first mitotic metaphase 12 hpf, (H) first mitotic telophase. Bar=20 μ m.

proceeded, NuMA reappeared in pseudo-pronucleus during interphase and in cytoplasm during metaphase and anaphase of the first mitotic cell cycle. At first, we regarded the green spots in cytoplasm as non-specific staining. However, such an experiment was repeated eight times and more than 100 cloned embryos 13 hpf were observed, and we found the same phenomenon in all metaphase-stage embryos. In contrast, in other embryos prior to the pronucleus envelope breakdown, little specific NuMA protein was detected in the cytoplasm, although these embryos in different cell cycle phases were fixed at 13 hpf and treated with the same protocol.

These findings raise the question that how NuMA is regulated in the development of rabbit cloned embryos? NuMA expression was influenced by many factors. Spindle pole localization of NuMA requires microtubules and is lost upon treatment of cells with nocodazole (Merdes *et al.* 1996). When disrupting the function of cytoplasmic dynein or dynactin results in the complete lack of organization of microtubules and the failure to efficiently concentrate the NuMA protein despite its association with the microtubules (Gaglio *et al.* 1996). However, Heald *et al.* (1997) thought that dynein is not required for the localization of NuMA to the minus end of MTs.

NuMA is more likely connected to the proliferation phase of the cells (Taimen *et al.* 2004). For successful nuclear transfer and development of the resulting reconstructed egg, the properties of the donor nucleus have to become like those of the normal zygotic nucleus and the normal kinetics of remodeling occurs during the one-cell stage, and this appears to be the determining factor for the normal onset of gene expression (Pinto-Correia *et al.* 1995, Renard 1998). This procedure requires co-operation of the activities of cell cycle regulators (Fulka *et al.* 1996) and maternal stores of protein (Almouzni & Wolffe 1993). It is assumed that the somatic cell nuclei are mysteriously converted to

embryonic genomes, which then passively progress down the developmental path leading to a new individual.

Numerous studies in fertilized embryos, however, have made it clear that the embryonic genome is first formed in the zygote, but is then drastically altered to set it on its path of totipotentiality (reviewed in Latham 1999, Latham & Schultz 2001). Complete reprogramming in cloning seems possible only after remodeling of the donor nucleus, i.e. PCC in non-activated cytoplasm, followed by nuclear swelling upon activation of the oocyte (Bredman *et al.* 1991). Thus the gene of NuMA, which was repressed in G_0 stage rabbit fibroblast, was reactivated and expressed in the pseudo-pronucleus of new reconstructed embryo. Although atypical NuMA distribution in cloned rabbit embryos were observed, normal spindles still formed. The function of NuMA in early cloning embryo development and the relation between NuMA distribution and developmental potential of embryos require further clarification.

Results in this study show that NuMA location is different among rabbit fibroblasts, oocytes, parthenotes, fertilized embryos, and cloned embryos. First, NuMA in meiosis differed within mitosis. It can be observed at every stage during oocyte maturation but disappeared in interphase nuclei of fibroblasts. Second, NuMA in embryonic cells differed with in somatic cell. It exists in interphase nuclei of fertilized and cloned embryos but not in that of fibroblasts. It focuses on the spindle poles of the latter but not on that of the embryos. Finally, the presented data show that there is some variability in NuMA staining for NT embryos as compared with fertilized embryos. We did not observe any NuMA staining in cytoplasm during anaphase and telophase stages in fertilized embryos as in cloned embryos. On the other hand, there are some similarities among them. During the metaphase of fibroblasts, oocytes, or parthenotes, we could detect NuMA at the spindle poles. NuMA may play an important role in spindle pole organization in them. And then, NuMA localization has some similarities between cloning and fertilizing. During metaphase, it did not concentrate on the spindle poles and was absent during anaphase and telophase of fertilized embryos. The reasons for the differences of NuMA distribution, NuMA function, and regulatory mechanisms in the five kinds of objects will require further experimental analysis.

The α -tubulin localization during the rabbit oocyte maturation has not been reported. In the present study, we found that cytoplasmic microtubules first appeared around the periphery of the GV. After GVBD, multi-arrayed microtubules were found tightly associated with the condensed chromosomes. In fully grown G_2 -arrested mouse oocytes, several electron-dense condensations from which microtubules radiate have been observed at the periphery of the GV (Szollosi *et al.* 1972). In pig oocytes, Kim *et al.* (1996) have reported that microtubules

are not detected at the GV stage, and that microtubule asters are first observed near the condensed chromatin after GVBD, and microtubules in the meiotic spindle become apparent only during the later meiotic stages.

Similar to the study of Yin *et al.* (2002), most of the somatic cell nuclei (87%, 39/45) will undergo PCC when they were fused with non-activated enucleated oocytes and chromosomes in two embryos (2/39) orderly array at metaphase plates and formed a normal transient spindle 4 hpf, instead of misaligned chromosome arrays in all embryos as report by Chesne *et al.* (2002). Within 5–9 hpf, 84% embryos (27/32) form a swollen pronucleus-like structure.

Compared with our previous data (Yan *et al.* 2006), following PCC and transient spindle formation, two pronucleus-like structures were found in 68.3% embryos constructed with monkey fibroblasts and non-activated rabbit oocytes. But only 8.2% have two pronucleus-like structures when pre-activated ooplasm was used as recipient. These results indicate that the number of pseudo-pronucleus may be influenced by both the donor cell species and the activation administration of recipient oocytes.

In summary, NuMA is involved in meiotic spindle microtubule assembly and spindle pole tethering in rabbit oocytes. However, mitotic spindles can be organized in the absence of NuMA at the poles in cloned rabbit embryos.

Acknowledgements

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