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Fate of centrosomes following somatic cell nuclear transfer (SCNT) in bovine oocytes

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Abstract

Cloning mammalians by somatic cell nuclear transfer (SCNT) remains inefficient. A majority of clones produced by SCNT fail to develop properly and of those which do survive, some exhibit early aging, premature death, tumors, and other pathologies associated with aneuploidy. Alterations of centrosomes are linked to aberrant cell cycle progression, aneuploidy, and tumorigenesis in many cell types. It remains to be determined how centrosomes are remodeled in cloned bovine embryos. We show that abnormalities in either distribution and/or number of centrosomes were evident in approximately 50% of reconstructed embryos following SCNT. Moreover, centrosome abnormalities and failed 'pronuclear' migration which manifested during the first cell cycle coincided with errors in spindle morphogenesis, chromosome alignment, and cytokinesis. By contrast, nuclear mitotic apparatus protein (NuMA) exhibited normal expression patterns at metaphase spindle poles and in 'pronucleus' during interphase. The defects in centrosome remodeling and 'pronuclear' migration could lead to chromosome instability and developmental failures associated with embryo production by SCNT. Addressing these fundamental problems may enhance production of normal clones.

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Introduction

Cloning by somatic cell nuclear transfer (SCNT) has been successful in a variety of mammalian species yet remains quite inefficient. In addition to embryonic loss, SCNT is also associated with high rates of fetal, perinatal and neonatal wastage, as well as abnormal offspring (Renard *et al*. 1999, Wilmut *et al*. 2002). The fate of cloned embryos is determined by cellular and molecular events occurring within hours of nuclear transfer, but little is known about the nature and consequences of adaptations in the cell cycle that must take place during this critical period of development (Wilmut *et al*. 2002). Research on epigenetic reprogramming of somatic cell nuclei following nuclear transfer shows that aberrant molecular mechanisms may contribute to the inefficiency of cloning (Kang *et al*. 2001, Jaenisch *et al*. 2002, Xue *et al*. 2002, Yanagimachi 2002, Mann *et al*. 2003).

Yet little is known about the nuclear and cytoplasmic changes associated with the integration of donor cell components into the cytoplasm of the recipient ooplasm. Studies on SCNT embryos in the rhesus monkey have suggested that nuclear mitotic apparatus protein (NuMA) fails to reorganize during cell cycle progression (Simerly *et al*. 2003). Also, somatic donor cell centrosomes introduced during SCNT in the mouse appear to be rapidly degraded in activated ooplasts (Zhong *et al*. 2005). However, degradation of centrosomes was not seen in mouse cloning by Van Thuan *et al*. (2006).

Although the efficiency in cloning cattle has been relatively high, and some bovine clones appear healthy, both physically and behaviorally (Lanza *et al*. 2001), many others exhibit high rates of abortion, prenatal and newborn death (Tsunoda & Kato 2002). Some clones of animals with shorter life-spans, such as mouse and sheep, develop chronic diseases, including obesity and diabetes, precocious aging, premature death, and tumors (Kuhholzer-Cabot & Brem 2002, Ogonuki *et al*. 2002, Gou *et al*. 2003). It remains to be determined why some species are more easily cloned, and produce healthier offspring than others,

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or what mechanisms underlie the defective development of cloned embryos. Depletion of NuMA during enucleation of oocytes with removal of spindles may limit the efficiency of cloning some species, particularly primates (Simerly *et al.* 2003, 2004), resulting in aneuploidy. Chromosomal abnormalities are a well-known cause of subsequent developmental failure, pregnancy failure, embryonic loss during implantation, perinatal death, and congenital malformation (Ekins & Shaver 1976, Hassold & Hunt 2001, Racowsky 2002). Because in somatic cells, terminal phases of mitotic chromosome separation and/or nuclear reassembly require NuMA (Merdes & Cleveland 1998) and functional centrosomes, it is imperative that the fate and function of centrosomes should be elucidated in SCNT embryos. In the present study, the spatial and temporal disposition of NuMA and centrosomes were studied in bovine SCNT embryos compared with parthenogenetic embryos and embryos produced by *in vitro* fertilization (IVF) using three-dimensional reconstruction during the first cell cycle.

Materials and Methods

Fetal fibroblasts and adult granulosa cells

Skin biopsy was obtained from a bovine fetus at day 80 of pregnancy, cut into small pieces, and cultured in DMEM (GIBCO) supplemented with 10% fetal bovine serum (FBS) at 37.5 °C in 5% CO_2 and humidified air. After 3–6 days in culture, fibroblast cell monolayers formed around the tissue explants, which were then removed, and fibroblast cells were cultured to confluency. The cells were routinely passaged and maintained until passage 5–10. Bovine granulosa cells were collected by aspiration from ovarian follicles of 2–7 mm in diameter, washed by sedimentation and centrifugation twice, and cultured under the same condition as fetal fibroblasts. For each passage, cells were cultured until confluent, disaggregated by incubation in a 0.1% trypsin and EDTA solution for 1–2.5 min at 37 °C, and allocated to three new dishes for further passaging. For long-term storage, cells at different passages were collected following trypsin treatment and frozen stored in 10% dimethyl sulfoxide in liquid nitrogen.

Recipient oocytes, IVF and embryo culture

Procedures for *in vitro* maturation (IVM) and fertilization (IVF) of bovine oocytes were described previously (Liu *et al.* 1998, Dinnyes *et al.* 2000). Briefly, cumulus–oocyte complexes (COCs) were aspirated from follicles (2–8 mm diameter) and selected based on their morphology. They were washed in maturation medium, which was comprised of tissue culture medium (TCM-199; Life Technologies) supplemented with 10% FBS, 0.01 U/ml bovine follicle-stimulating hormone (bFSH), 0.01 U/ml bovine luteinizing hormone (bLH), 1 μg/ml estradiol-17β and 1% penicillin/streptomycin (Life Technologies). Approximately 50–60 COCs were transferred into 0.5 ml maturation medium in 4-well dishes covered with mineral oil, and cultured at 38.5 °C in 5% CO_2 for 18–20 h.

Frozen–thawed sperm were washed twice by centrifugation in modified Brackett-Oliphant (m-BO) medium supplemented with 10 mM caffeine and 4 mg/ml BSA, and then resuspended in the fertilization medium (m-BO medium supplemented with 10 μg/ml heparin and 4 mg/ml BSA). Sperm concentration was adjusted to 1×10^7 sperm/ml, and approximately 25 oocytes were added to a 100 μl fertilization-medium droplet. After 6 h co-incubation, oocytes were washed and cultured in CR1aa medium (Sims & First 1994), supplemented with 0.1% BSA, for 48 h at 39 °C in a humidified atmosphere of 5% CO_2. After 48 h in culture, IVF eggs were freed of cumulus cells by pipetting, and cleavage was examined.

Somatic cell nuclear transfer

Enucleation of oocytes was achieved by piercing the zona pellucida with a glass needle and gently pushing out the polar body and surrounding cytoplasm (Gong *et al.* 2004). Successful enucleation was confirmed by Hoechst 33342 staining of extruded karyoplasts. Donor cells were starved to enrich them at G_0 phase (Wilmut *et al.* 1997), and cultured for 3–4 days in DMEM/F12 containing 0.5% FBS after confluence. Prior to SCNT, cells were typsinized, centrifuged, and re-suspended with PBS supplemented with 0.5% FBS. Donor cells were then individually transferred into contact with enucleated recipient oocytes. Donor cell–cytoplasts were electrically fused in a chamber filled with Zimmerman cell fusion medium, induced with two DC pulses of 2.5 kV/cm for 10 μs each at 1 s apart, delivered by a BTX2001 Electro Cell Manipulator (BTX, San Diego, CA, USA). Activation was induced by incubation in 10 μg/ml cycloheximide and 2.5 μg/ml cytochalasin D (Liu *et al.* 1998). Following activation, reconstructed embryos were cultured in CR1aa medium supplemented with 0.1% BSA as described above.

Immunofluorescence microscopy

Oocytes or embryos were washed in PBS containing 0.1% polyvinyl pyrrolidone (PBS-PVP), then fixed as described previously (Combelles & Albertini 2001). Oocytes were incubated overnight at 4 °C with mouse anti-NuMA, or mouse anti-γ tubulin (1:100), washed, then labeled with FITC-anti-mouse IgG. Centrosomes were routinely identified by immunocytochemistry using antibodies specific to γ tubulin, a core component of the centrosome (Doxsey *et al.* 1994, Merdes & Cleveland 1997, Combelles & Albertini 2001, Wong & Stearns 2003,

Sluder & Nordberg 2004). The antibody against γ tubulin, appearing as a single dot of labeling, corresponds to one centrosome (Wong & Stearns 2003). Some oocytes were also stained for spindles with rat anti-α tubulin (Yol 34) and cy3 anti-rat IgG (1:100). Oocytes were washed and mounted in Vectashield medium (Vector Laboratories, Burlingame, CA, USA) containing 0.5 μg/ml DAPI (4′,6-diamino-2-phenylindole). Immunofluorescence was detected with a Zeiss Axioplan 2 imaging microscope, equipped with epifluorescence, AxioCam and AxioVision 3.0 software, or with a Zeiss LSM 510 Meta laser scanning confocal microscope. Three-dimensional images were reconstructed and projected from serial optical Z-sections captured by the confocal microscope with its software.

Results

Remodeling of NuMA in parthenogenetic, IVF, and SCNT embryos

NuMA was shown by immunostaining to be weakly expressed as broad bands at both spindle poles in MII oocytes (Fig. 1A). Fertilized eggs exhibited increased NuMA staining in both female and male pronuclei (Fig. 1B and C), and at spindle poles during the first mitosis (Fig. 1D). Parthenogenetically activated oocytes retained NuMA staining at poles throughout the anaphase–telophase II transition (Fig. 1E and F). Between 8 and 24 h post-activation, NuMA labeling was evident in pronuclei (Fig. 1G and H), and finally was restricted to both spindle poles in the first mitosis (Fig. 1I).

The nuclei of donor granulosa cells contained immunodetectable NuMA (Fig. 2A) and the NuMA disappeared in condensing nuclei soon after fusion with recipient oocytes (Fig. 2B). Labeling was readily apparent in enlarging 'pronuclei' at 2 and 4 h after cell fusion (Fig. 2C–E), and dramatically increased in enlarged "pronuclei" at 8 (Fig. 2F) and 18 h post-fusion. These events were followed, 22–24 h after nuclear transfer (NT), by chromosome condensation (Fig. 2G), nuclear breakdown, and the formation of a mitotic spindle, with NuMA accumulation at the spindle poles (Fig. 2H). Also, following cleavage of reconstituted embryos (by 24 h), NuMA was present in the nuclei of blastomeres (Fig. 3).

NuMA staining after SCNT with fibroblasts exhibited patterns similar to those of granulosa cells. However, pronuclei derived from fibroblasts developed more slowly and appeared to contain lower levels of NuMA, being smaller at identical time points when compared with granulosa cells. Quantification of NuMA in granulosa and fibroblast embryos using digital imaging further confirmed that the initial onset and extent of NuMA accumulation was greater in granulosa compared with fibroblast SCNT embryos (Fig. 2I and J). While the significance of differential NuMA accumulation remains unclear, our studies demonstrate that NuMA remodeling during the first cell cycle in bovine nuclear transfer embryos proceeds as in fertilized or parthenogenetic embryos. Further evidence suggesting that NuMA is not a key regulator of initial cell cycle events in cloned embryos includes NuMA retention in nuclei of embryos that are abnormally cleaved or display aberrant pronuclear position (Fig. 3B–E), and the spindle pole staining observed in activated oocytes with dispersed chromosomes (Fig. 3F).

Remodeling of centrosomes in parthenogenetic, IVF, and SCNT embryos

Since centrosomes are crucial for the formation of spindle poles, chromosome segregation and cytokinesis (Doxsey 2001), we reasoned that these might be likely targets for reprogramming, especially as related to the cleavage defects and high rates of aneuploidy so commonly observed in SCNT embryos (Slimane-Bureau & King 2002, Bureau *et al*. 2003, Shi *et al*. 2004). Centrosomes were identified by immunostaining with an antibody to γ tubulin, which is a well-characterized centrosomal component (Nigg 2002, Matsumoto & Maller 2004, Sluder & Nordberg 2004), but would not enable us to identify centrioles. The combination of γ tubulin with α-tubulin and DAPI staining also allowed us to relate centrosome disposition to their cell cycle state and nucleation status respectively. No focal staining with γ tubulin was evident in MII oocytes (Fig. 4A), nor during anaphase–telophase II following activation (Fig. 4B) through pronuclear stages (Fig. 4C). Moreover, activated oocytes organized barrel-shaped spindles at mitosis (Fig. 4D), and completed anaphase (Fig. 4E) and cell division without discernible γ tubulin foci (Fig. 4F). In contrast, embryos produced by IVF exhibited two centrosomes around each blastomere nucleus (Fig. 4G). Interestingly, we found that 46% of activated oocytes ($n=24$) showed abnormal cleavage, which included both anucleate and binucleate blastomeres, indicating a loss of coordination between karyokinesis and cytokinesis.

Prior to fusion with cytoplasts, 75% of granulosa cells exhibited one γ tubulin focus organizing the microtubule network, whereas no comparable foci were seen in oocyte cytoplasts. Within 1 h after fusion, all donor nuclei had one or two γ tubulin foci (Fig. 5A). At 2 h, pronuclei appeared somewhat swollen and enlarged, and most had two γ tubulin foci close to the pronuclei in which a single γ tubulin focus appeared to nucleate microtubule asters (Fig. 5B). By 4–8 h, pronuclei became markedly expanded and contained two perinuclear centrosomes (Fig. 5C). By 18 h, most enlarged pronuclei moved to the center of the reconstituted oocytes and retained two perinuclear centrosomes that were often diametrically opposed at the pronuclear surface (Fig. 5D). By 22–24 h, NT embryos at the first mitosis showed centrosomes localized to each spindle pole as chromosomes aligned at the equatorial region from metaphase (Fig. 5E) through

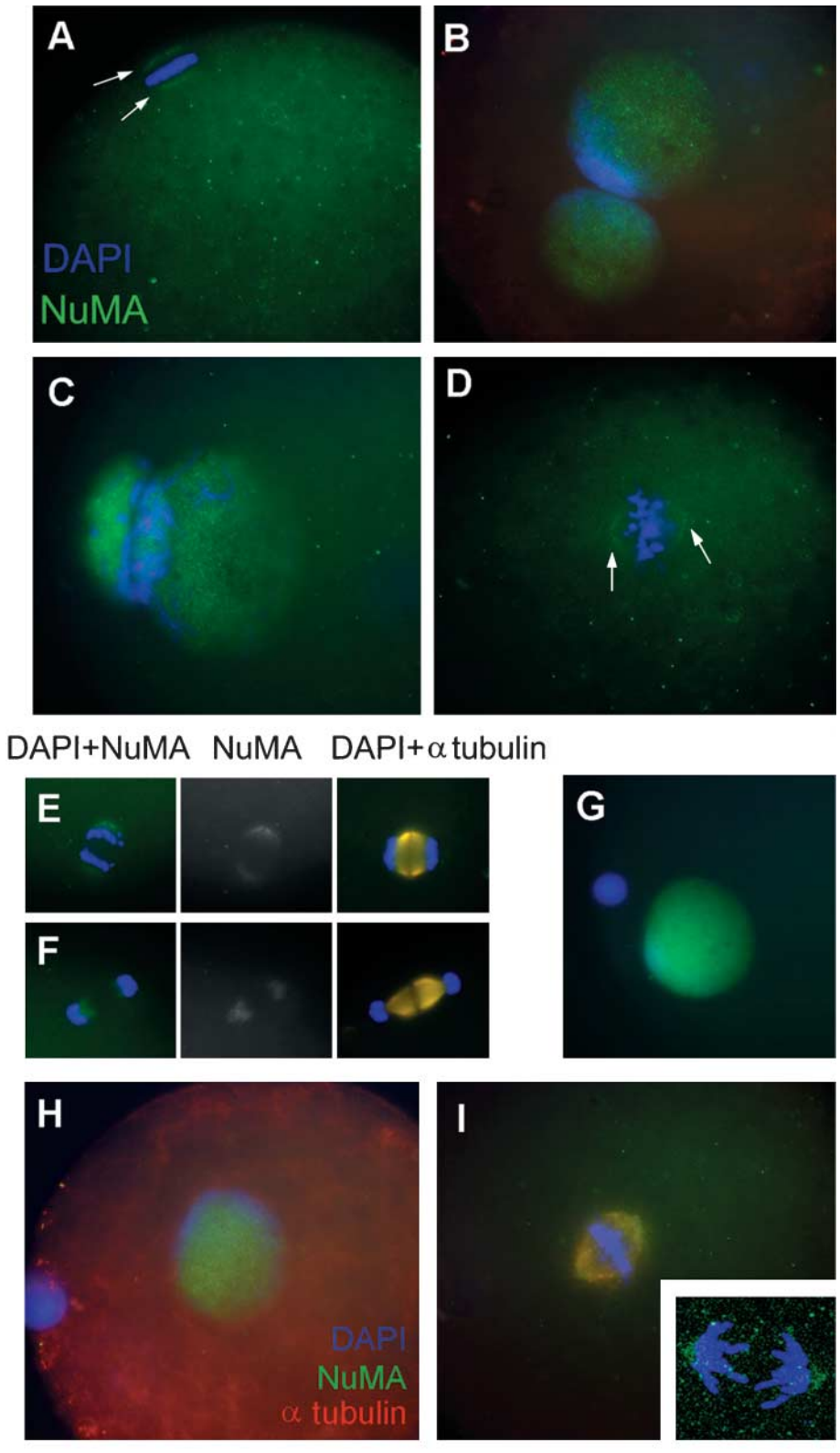


Figure 1 NuMA localization in bovine oocytes following *in vitro* fertilization (IVF) and parthenogenetic activation (PA). (A) MII oocytes. Arrow indicates NuMA. (B) NuMA accumulation in both female and male pronuclei 18 h after IVF. (C) NuMA associated with condensed chromosomes immediately following nuclear envelope breakdown 24 h after IVF. (D) Preferential accumulation of NuMA (arrows) at poles of the first mitosis. (E) Early anaphase II after PA. (F) Late anaphase II after PA. (G) NuMA accumulation in a female pronucleus 8 h after PA. (H) NuMA in a pronucleus 24 h after PA. (I) Preferential accumulation of NuMA at poles of PA embryos at mitotic and early anaphase (insert, 2×). Scale bar, 25 μm.

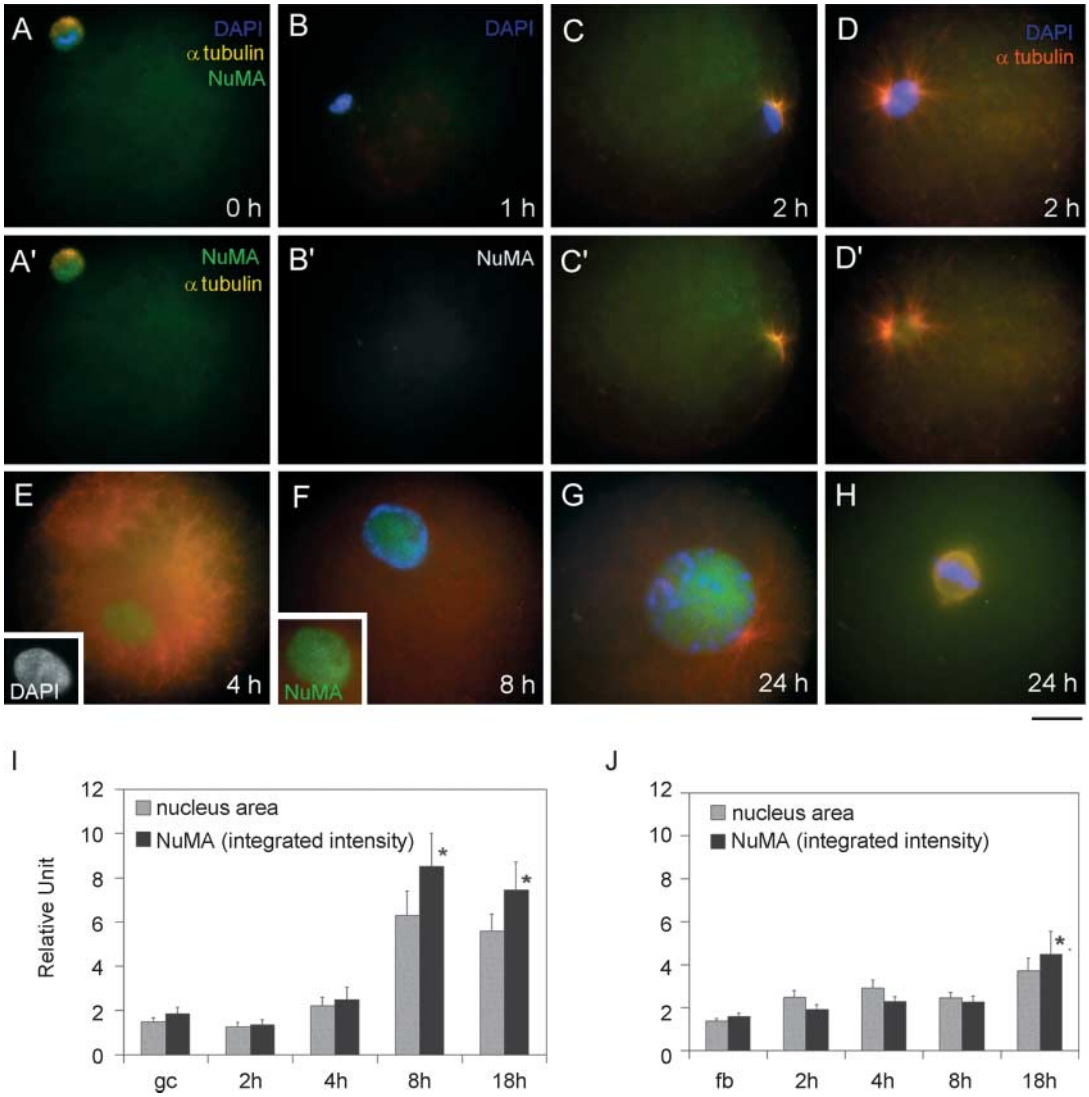


Figure 2 NuMA remodeling in nuclear transplant embryos from granulosa cells (gc) and fibroblasts (fb). (A) NuMA in nucleus of donor granulosa cell. (B) Absence of NuMA from condensed nucleus 1 h following nuclear transfer of granulosa cells. (C) NuMA starts to appear in the pronucleus at 2 h, and one microtubule aster forms. (D) NuMA accumulation with formation of two microtubule asters. (E) Pronuclear swelling and NuMA accumulation at 4 h. (F) Extensive NuMA accumulation at 8 h. (G) Nuclear envelope breakdown and NuMA surrounding the condensed chromatin at 24 h. (H) NuMA localization at poles of the first mitotic spindles. (I) Relative quantity of NuMA in nuclear transfer embryos from granulosa cells. (J) Relative quantity of NuMA in nuclear transfer embryos from fibroblasts. Scale bar, 25 μm.

anaphase (Fig. 5F). Most two-cell NT embryos contained perinuclear centrosomes (Fig. 5G). It is interesting to note that although a solitary centrosome was evident at the spindle pole at metaphase, two centrosomes, presumably produced through splitting, were apparent soon after initiation of anaphase and telophase (Fig. 5F). This pattern persisted in cleaved, two-cell embryos, and was not unlike what was observed in fertilized embryos at a similar stage of development (Fig. 4G).

Abnormalities in the number and distribution of centrosomes (compared with Fig. 5A–G), however, were apparent in a high percentage (47%, $n=45$) of NT embryos produced with granulosa cells. The abnormalities fell into several categories that included (a) multiple centrosome foci that are either dissociated from the pronucleus (Fig. 5H and I), or retain a perinuclear location (Fig. 5J), (b) spindle associated variants with three centrosomes associated with dispersed chromosomes (Fig. 5K) or a solitary ectopic centrosome near a misaligned chromosome when two other centrosomes are apparent at the poles of the metaphase spindle (Fig. 5L), and (c) variants in centrosome number such as solitary centrosomes in monopolar spindles with scattered chromosomes (Fig. 5M) or three centrosome foci surrounding the pronucleus (Fig. 5N). We often noticed that embryos

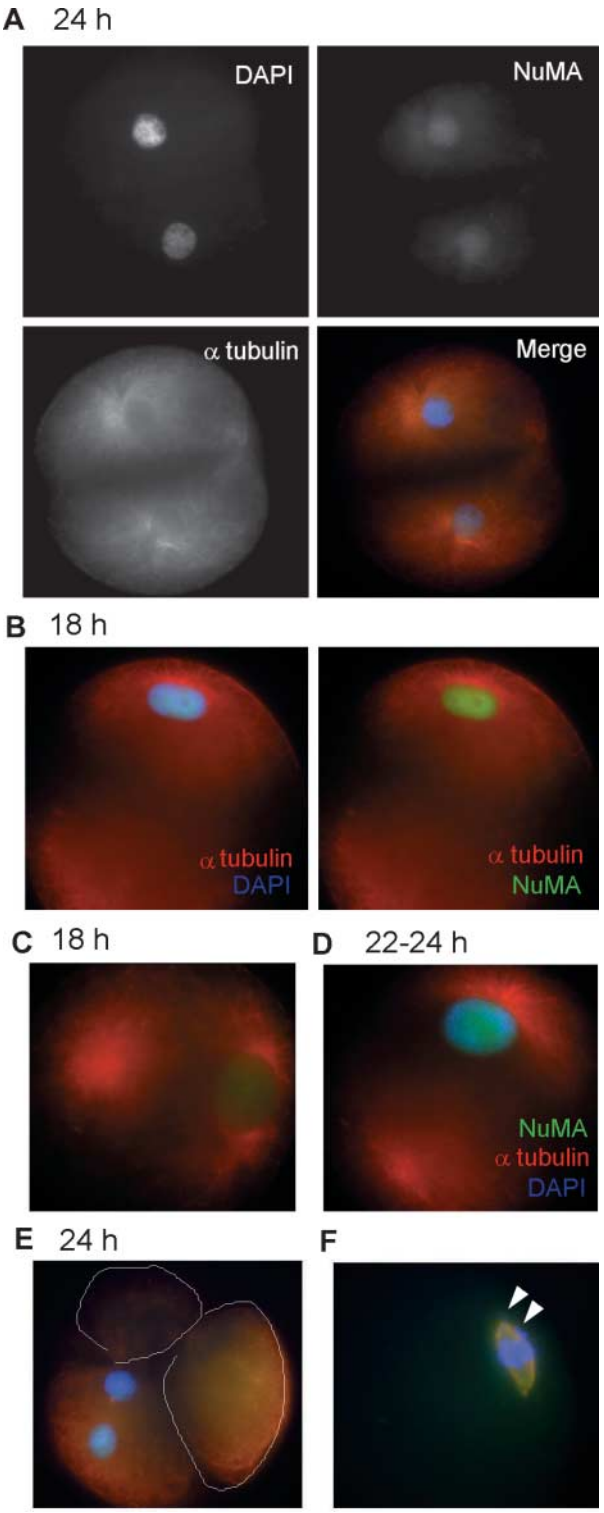


Figure 3 Localization of NuMA in cleaved, cloned embryos derived from granulosa cells. (A) NuMA in nucleus of normally cleaved, 2-cell embryos at 24 h. (B, C) Abnormal cortical localization of nucleus and NuMA at 18 h. (D, E) Abnormal division 22–24 h after reconstitution. (F) Chromosome scattering (Arrowheads) on the spindle with NuMA at the poles.

with cortical pronuclei typically had a solitary perinuclear γ tubulin focus and a second focus distant from the pronucleus. Moreover, abnormally cleaved 3–4 cell embryos either lacked centrosomes in one or more nuclei, or in some cases, centrosomes were present in one anucleate blastomere (Fig. 5O).

Variations in centrosome number (1–6 γ tubulin foci) and localization (perinuclear or not) were also found at various stages of development in NT embryos derived from fibroblasts (data not shown). Briefly, like their granulosa cell counterparts, oocytes fused with donor fibroblasts developed microtubule asters radiating from one or two γ tubulin foci, and at later time points (2–18 h) centrosomes remain perinulcear. Again, the first mitotic M phase was characterized by abnormalities in chromosome alignment, multipolar spindles, and ectopic centrosomes. Thus, both donor cell types contribute centrosomes that are modified soon after fusion, and in roughly 50% of SCNT embryos, the integration of these structures into passage through the first cell cycle results in abnormalities that compromise early development.

Discussion

Aberrant constitution of centrosomes is associated with formation of abnormal spindles and chromosome misalignments

SCNT introduces centrosomes from donor somatic cells into oocyte cytoplasm, mimicking the introduction of centrosomes by sperm during fertilization (Navara *et al.* 1994, Shin *et al.* 2002). We also established that centrosomes in cloned cattle are unlikely to be provided by recipient cytoplasts of maternal origin. Furthermore, we found that the number of centrosomes varied from 2 to 6 in cloned embryos during first mitosis. Also, the centrosomes do not always associate with the pronucleus or perinuclear region; rather some are located away from nucleus.

Variable numbers of centrosomes could result from a duplication of centrosomes, driven by cytoplasmic conditions specific to cell cycle stages (Hinchcliffe & Sluder 2001). Centrosome duplication occurs at G1/S phase, but not at G0 phase. This might explain the original success of somatic cell cloning using synchronization at G0. The centrosome duplicates once in S phase, which is controlled by a centrosome-intrinsic block to reduplication. However, G2 centrosomes are unable to reduplicate in a cellular environment that supports centrosome duplication (Wong & Stearns 2003). In somatic cell nuclear transfer, S phase nuclei may undergo S phase reduplication of centrosomes following oocyte activation, thus resulting in variable numbers of centrosomes. The transferred centrosomes at G2 stage may not be able to duplicate in the subsequent 'S' phase of activated oocyte cytoplasm. Thus, the cell cycle of donor cells influences reproduction of centrosomes following nuclear transfer. However, centrosome

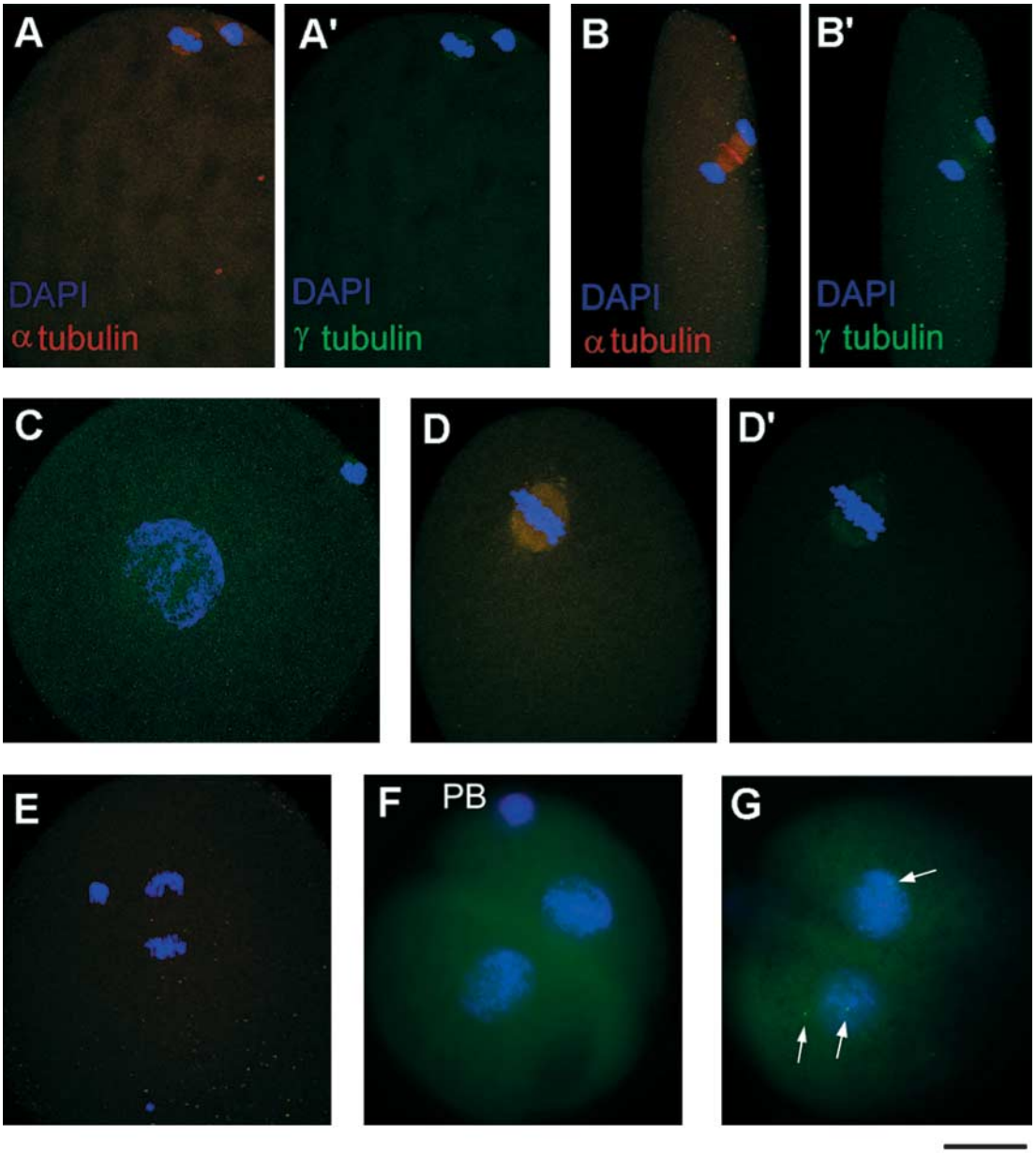
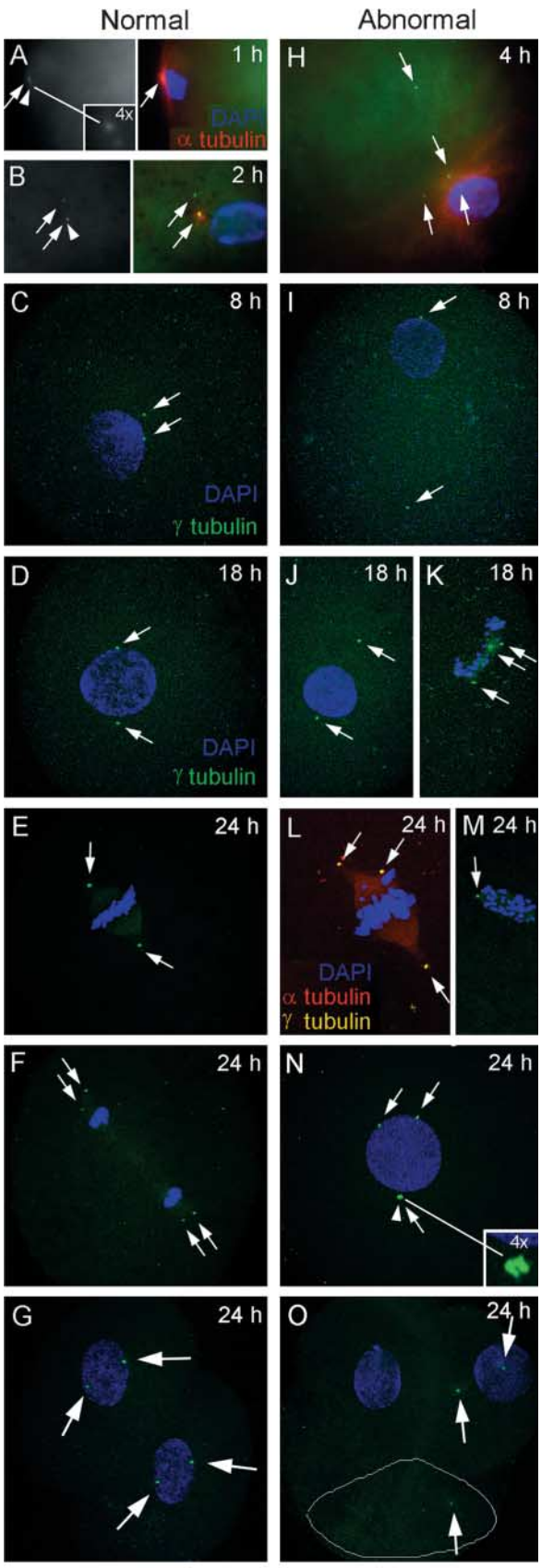


Figure 4 Centrosomal distribution in bovine oocytes following parthenogenetic activation (PA) or IVF. (A–F) MII and (PA) oocytes exhibit no centrosomal foci, but (G) IVF oocytes do. (A,A′) Absence of centrosome foci in MII oocytes. (B,B′) Oocytes 2 h after PA. (C) Pronuclei at 18 h, and absence of centrosomal foci. (D,D′) The first mitosis of PA embryos at 24 h (D) Merged image of microtubule spindle and chromosomes stained by DAPI; (D′) Merged image of γ tubulin staining, and chromosomes stained by DAPI. (E) Anaphase of PA embryos. (F) Cleaved 2-cell PA embryos; PB, polar body. (G) Cleaved 2-cell IVF embryos, showing two centrosomal foci (arrows) surrounding nuclei, a third is out of focus. (A–E) Three-dimensional reconstruction using Z-serial section imaged with confocal microscopy. (F,G) Conventional fluorescence images. Scale bar, 25 μm.

duplication does not require *de novo* protein synthesis, since an inhibitor of protein synthesis, cycloheximide, was present during the first 6 h following nuclear transfer, consistent with previous findings in embryos from other species (Gard *et al*. 1990, Sluder *et al*. 1990). The subunits for complete centrosome assembly can be stockpiled ahead of time, and the properly controlled use of these subunits for centrosome reproduction does not require nuclear transcription or nuclear DNA synthesis at each cell cycle (Sluder *et al*. 1986).

The origin of centrosomes differs among species. In most mammalian species, including primates and bovine, with the exception of mice and other rodents, the sperm contributes a centriole during fertilization and helps organize the assembly of the first mitotic spindle in the zygote (Schatten *et al*. 1985, 1986, Sutovsky & Schatten 2000). Parthenogenetically activated



mouse eggs can assemble functional mitotic spindles and undergo mitosis without participation of sperm derived centrioles and centrosomes (Schatten *et al*. 1991). Bovine embryos appear to be an appropriate model to study centriolar inheritance (Sathananthan *et al*. 1997). In the absence of a paternally supplied centrosome, bovine oocytes can still be activated, and develop parthenogenetically to blastocysts with normal ploidy, at rates comparable to IVF derived embryos (Liu *et al*. 1998, Van De Velde *et al*. 1999), demonstrating that early embryos can develop without centrosomes. However, postimplantation development is severely compromised. Shin and Kim (2003) showed that γ-tubulin foci started to form in 8–16-cell stage bovine parthenotes (Shin & Kim 2003). Centrosomes may enhance the fidelity of chromosome segregation.

Regardless of the origin of centrosomes, centrosome amplification can have serious consequences on subsequent development of clones. Indeed, chromosomal aberrations and aneuploidy are frequently found in cloned preimplantation embryos in cattle, rabbits and monkey (Slimane-Bureau & King 2002, Vogel 2003, Shi *et al*. 2004) and in some embryos, aneuploidy seems not to be derived from the donor cells (Booth *et al*. 2003). Chromosomal abnormalities are also well-known causes of developmental failure, perinatal death, and congenital malformations. The most frequently observed deviation from diploid karyotype was mixoploidy, resulting from aberrant cell division and polyploidy (Slimane-Bureau & King 2002). At the first mitosis, centrosomes migrate to each spindle pole, and normal metaphase chromosome alignment ensues. Lack of centrosomes at one or both spindle poles, or abnormal numbers of centrosomes, promote chromo-

Figure 5 Centrosomal remodeling following nuclear transfer of granulosa cells. Shown are stages of normal (A–G) and abnormal (H–O) cell cycle progressions. (A) Centrosome (arrow) transferred with the nucleus of a donor cell 1 h after fusion. One aster is detected. Arrowhead indicates two closely associated γ tubulin foci. (B) Two γ tubulin foci surround the nucleus 2 h after fusion. (C) Two γ tubulin foci surround the swollen pronucleus at 8 h. (D) Two γ tubulin foci move to opposite poles of the swollen pronucleus at 18 h. (E) One γ tubulin focus (arrow) at each pole of mitotic spindle at 22–24 h. (F) Two γ tubulin foci (arrows) appeared at anaphase–telophase transition. (G) Two centrosomes surround nucleus of normally cleaved 2-cell NT embryos. (H) Four γ tubulin foci near the nucleus, or distant from the pronucleus 4 h following fusion. (I) One γ tubulin focus located distant from pronucleus. (J) One γ tubulin focus remains dissociated from pronucleus. (K) Abnormal chromosome scattering with 3 disorganized γ tubulin foci. (L) γ tubulin foci at the poles, and also one associated with misaligned chromosomes at first mitosis. (M) One γ tubulin focus associated with scattered chromosomes at one pole. (N) Three γ tubulin foci near a swollen pronucleus. (O) Abnormal distribution of centrosomes in abnormally cleaved 3–4-cell embryos at 24 h. White circle indicates a blastomere without a nucleus. Blue, DNA stained with DAPI; green, γ tubulin; red, α tubulin. (C–O) Three-dimensional reconstruction after Z-series section imaged by confocal microscopy. Scale bar, 25 μm.

some misalignment or scattering at metaphase in cloned embryos, demonstrating that centrosomes are critical for normal chromosome segregation and ploidy in clones. Normal constitution and distribution of centrosomes are required to ensure the fidelity of chromosome segregation, and to maintain genomic stability during prenatal and postnatal development. Defects in centrosome remodeling likely lead to subsequent abortion of some clones, although it has not been determined whether these defects lead to chromosome instability and tumorigenesis in other clones.

Abnormal distribution of centrosomes coincides with abnormal cleavage

Normally, cleaved embryos divide equally, with centrosomes surrounding nuclei. Abnormal, unequally cleaved 3–4 cell embryos lacked nuclei in one or two blastomeres, some blastomeres had centrosomes and others did not. Our data demonstrate that abnormal cleavage coincides with aberrant distribution and numbers of centrosomes that may lead to a failure of pronuclei to move to the center in 35% of NT embryos. The centrosome has been known to direct the assembly of bipolar mitotic spindles, and thus determines the location of the cleavage furrow (Stevenson *et al*. 2001).

The formation of a functional mitotic spindle requires coordination between centrosomes and chromosomes (Zhang & Nicklas 1995). Spindles assembled without centrosomes are anastral. However, if centrosomes are present, they become the primary organizer of spindles (de Saint Phalle & Sullivan 1998). Animal cells that normally contain centrosomes can utilize a centrosome-independent pathway to form bipolar spindles (Khodjakov *et al*. 2000, Budde & Heald 2003), but a complete lack of centrosomes impairs cytokinesis (Hinchcliffe *et al*. 2001). Thus, in the absence of centrosomes, bovine parthenotes proceed through the first mitosis but exhibit cleavage abnormalities consistent with this deficiency. Centrosome pathways are thought to be the dominant process in determining spindle polarity (Hinchcliffe & Sluder 2001). Recent evidence suggests that the primary role of the centrosome in somatic cells may not be to form spindles, but rather to ensure cytokinesis and subsequent cell cycle progression (Khodjakov & Rieder 2001). Through astral microtubules, centrosomes determine spindle polarity, spindle position/orientation and cleavage plane. When mammalian somatic cells enter mitosis with extra centrosomes, they are apt to assemble multipolar spindles and divide into more than two daughter cells (Sluder *et al*. 1997). Abnormal localization of centrosomes, therefore, would be expected to lead to abnormal cleavage or fragmentation of NT embryos at first mitosis.

Functions of NuMA in reprogramming and cell replication

The formation of mitotic centrosomes is a complex process in which a number of cellular proteins translocate to mitotic poles and play critical roles in the organization of the mitotic apparatus. The abundant coiled-coil protein NuMA is located in the nucleus during interphase, but when the nuclear envelope disassembles in prometaphase it rapidly redistributes to the developing spindle poles, and becomes transiently associated with centrosomes, and is involved at the spindle poles in mitosis and chromosome segregation (Cleveland 1995, Taimen *et al*. 2000, Du *et al*. 2001).

We observed nuclear organization of NuMA in bovine oocytes during the cell cycle following nuclear transfer of two types of cells: granulosa cells and fetal fibroblasts. NuMA was also was detected in pronuclear stage embryos of mouse clones and in donor cumulus cells (Moreira *et al*. 2003), as well as in normal bovine embryos (Degrouard *et al*. 2004). In bovine clones produced by simultaneous fusion and activation, NuMA was weak or absent in donor nuclei, but strongly expressed in pronuclei following NT. Consistently, weak or absent staining for NuMA was found in the nuclei of granulosa cells and fibroblasts after several passages in serum-starved conditions, consistent with reports by Taimen *et al*. (2000). Oocyte cytoplasm reactivates expression of NuMA in the 'pronucleus'. This dramatic redistribution of NuMA can be critical for nuclear remodeling and molecular events for initial development. These findings differ from those of most monkey clone embryos during the first mitosis, which exhibit depletion of NuMA (Simerly *et al*. 2003), indicative of both lack of nuclear reprogramming and defects in spindle polar function.

In bovine clones, NuMA may result from *de novo* synthesis, since transplanted nuclei lack NuMA staining for a few hours until expanded pronuclei have formed following NT. Cycloheximide also was used as a protein synthesis inhibitor in the present bovine cloning procedure, which may contribute to lack of NuMA in the introduced nuclei for a few hours. After removal of cycloheximide 6 h following NT, extensive translation and expression of NuMA could occur. Consistently, NuMA reassembles as a result of translation from maternal mRNA and of *de novo* transcription from transplanted nuclear genomes in the mouse cloning (Moreira *et al*. 2003). NuMA could be important for spindle pole organization in primates (Simerly *et al*. 2003). Without these proteins, resulting cells become aneuploid. In bovine clones, NuMA was detected at both poles of normal spindles with chromosome alignment at the metaphase plates as well as at poles of abnormally

elongated or disrupted spindles with misaligned chromosomes, indicating that NuMA was not necessarily a key player in organization of mitotic spindle poles in this species. Yet, increased centrosomes were found to coincide with multipolar spindle formation, whereas lack of γ tubulin or centrosome duplication prior to mitosis can lead to monopolar spindle formation and chromosome scattering, demonstrating that centrosomes are critical for maintaining functional mitosis in clones.

NuMA in bovine oocytes was distributed in both cytoplasm and to some extent on the poles of meiotic spindles. The presence of NuMA in cytoplasm after enucleation, possibly *de novo* synthesis of NuMA, or relocalization of NuMA from cytoplasm to the nuclei following nuclear transfer and oocyte activation might contribute to the greater success of bovine cloning and other species. Aberrant centrosome replication could be a major cause of abnormal cleavage of early embryos. It remains to be determined how somatic cell nuclei in NT and sperm in fertilization initiate γ tubulin formation in the oocyte cytoplasm. We speculate that cloning efficiency by SCNT will be greatly improved in most species by assuring normal centrosome remodeling.

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