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# Abnormal chromosome segregation at early cleavage is a major cause of the full-term developmental failure of mouse clones

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ABSTRACT

To clarify the causes of the poor success rate of somatic cell nuclear transfer (SCNT), we addressed the impact of abnormalities observed at early cleavage stages of development on further full-term development using 'less-damage' imaging technology. To visualize the cellular and nuclear division processes, SCNT embryos were injected with a mixture of mRNAs encoding enhanced green fluorescent protein coupled with α-tubulin (EGFP-α-tubulin) and monomeric red fluorescent protein 1 coupled with histone H2B (H2B-mRFP1) and monitored until the morula/blastocyst stage three-dimensionally. First, the rate of development of SCNT embryos and its effect on the full-term developmental ability were analyzed. The speed of development was retarded and varied in SCNT embryos. Despite the rate of development, SCNT morulae having more than eight cells at 70 h after activation could develop to term. Next, chromosomal segregation was investigated in SCNT embryos during early embryogenesis. To our surprise, more than 90% of SCNT embryos showed abnormal chromosomal segregation (ACS) before they developed to morula stage. Importantly, ACS per se did not affect the rate of development, morphology or cellular differentiation in preimplantation development. However, ACS occurring before the 8-cell stage severely inhibited postimplantation development. Thus, the morphology and/or rate of development are not significant predictive markers for the full-term development of SCNT embryos. Moreover, the low efficiency of animal cloning may be caused primarily by genetic abnormalities such as ACS, in addition to the epigenetic errors described previously.



## Introduction

Wilmut and Campbell first cloned a normal, fertile adult mammal – a sheep – by transplanting nuclei from adult mammary cells (Campbell et al., 1996; Wilmut et al., 1997). Soon after this breakthrough, cloning was achieved by somatic cell nuclear transfer (SCNT) in mice (Wakayama et al., 1998) and bovines (Kato et al., 1998). Through careful technical refinements, it has now been achieved in many mammalian species (Cibelli, 2007). However, the success rates of animal cloning by SCNT are consistently low, especially in the mouse: typically fewer than 3% of cloned embryos develop to term (Thuan et al., 2010; Wakayama, 2007; Yang X. et al., 2007).

Recent analyses of SCNT embryos have revealed many abnormalities caused by incomplete epigenetic reprogramming of the donor nucleus as being significant reasons for developmental failure. During the development of naturally fertilized embryos, epigenetic marks are established over long periods during germ cell formation and fertilization. However in SCNT cloning, these processes might be bypassed and an adult somatic pattern of epigenetic modification that is normally very stable must be reversed within a short period before zygotic genome activation (Fulka et al., 2004; Yang X. et al., 2007). In fact, abnormal DNA and histone modification patterns such as heavy methylation of the *Sall3* locus (Ohgane et al., 2001), hypermethylation pattern in the centromeres (Yamagata et al., 2007), or abnormal H3K9 methylation (Santos et al., 2003) has been reported in SCNT embryos. Moreover, histone deacetylase inhibitors dramatically improved the success rate of mouse cloning (Kishigami et al., 2006b, 2007; Ono et al., 2010; Van Thuan et al., 2009). Thus, epigenetic alterations indeed exist at the zygotic stage of SCNT embryo.

In addition to the epigenetic alterations, genetic abnormalities during early cleavage stage, such as chromosomal abnormality may also be a reason for low success rate of cloning. Although not limited in the SCNT embryos, it is now becoming apparent that the chromosomal integrity is not secured more than we have thought. In human, the clinical outcome data of preimplantation genetic diagnosis revealed that surprisingly nearly 80–90% of embryos showed numerical and/or structural abnormalities in chromosomes at blastocyst stage, and this may be a reason for the low pregnancy rate in the

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assisted reproductive technologies (Santos et al., 2010; Vanneste et al., 2009). Especially, as evidenced by the high incidence of chromosome mosaicism, a phenomenon that chromosome constitution differ among the blastomere, the abnormalities may mainly come from abnormal chromosome segregation process during early cleavage stages. Also in mouse, the chromosome mosaicism was found in 25% of cleavage-stage embryos derived from naturally-mated female (Lightfoot et al., 2006). Furthermore, we and other group demonstrated that embryos produced by intracytoplasmic sperm injection (ICSI) frequently showed abnormal chromosomal segregation or constitution during first mitosis, and that this leads to early pregnancy loss (Tateno and Kamiguchi, 2007; Yamagata et al., 2009a). In fact, some reports document that the SCNT embryos from primates (Simerly et al., 2003), rabbits (Yin et al., 2002) and mice (Van Thuan et al., 2006) show structural abnormalities of the nucleus, such as incorrect spindle formation. Improper distribution of chromosomes at the metaphase plate has also been reported (Kawasumi et al., 2007; Yang J.W. et al., 2007; Yu et al., 2007). In addition, structural and numerical aberrations of chromosomes were demonstrated by spectral karyotyping analysis of SCNT 1-cell embryos (Osada et al., 2009). Taken together, it is possible that the chromosome instability during early cleavage may account for the low success rate of the cloning.

Despite many efforts in the study of cloning, fundamental problems remain. Mass analysis in which multiple cloned embryos are intermingled and extracted obscures the truth because the cloned embryos might differ individually, so we cannot know which of them is capable of developing to term. Moreover, staining methods require fixation and/or denaturation steps to prevent the embryos from developing any further. Thus, even if we can find any difference in SCNT embryos compared with normally fertilized embryos, it is difficult to link any effect to further development. For these reasons, although many reports have described abnormalities in SCNT embryos during early embryogenesis, the central causes of the low cloning success rate remain enigmatic.

Recently, we succeeded in developing a 'less-damage' live-cell imaging system optimized for preimplantation mouse embryos. It involves microinjection with mRNAs encoding fluorescent proteins and observations using a particular confocal microscope (Yamagata et al., 2009b). This system allows for long-term observations on molecular dynamics during early embryo development in vitro, such as changes in global DNA methylation status (Yamazaki et al., 2007b), heterochromatin constitution (Yamazaki et al., 2007a) and chromosomal segregation patterns (Yamagata et al., 2009a). The most important point of our system is that embryos do not show any deleterious effects and can develop to term even after repeated fluorescent observations for up to 4 days of culture. Thus, with this imaging technology, we can link the abnormalities in each embryo directly with their developmental ability for the first time. Furthermore, using this methodology, the real causes for the low success rate of cloning and essential prerequisites for full-term development of SCNT embryos will be clarified. Therefore, in the present study, we acquired time-lapse movies of cytogenetic dynamics during early cleavages stages by labeling the spindle and chromosomes. We assessed the effects of the rate of embryo development and of patterns of chromosomal segregation in SCNT embryos during early cleavage on full-term development.

## Materials and methods

### *Mice*

All mice used in this study were purchased from SLC (Hamamatsu, Japan) or bred in our mouse colony. All animal experiments conformed to our Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Committee for Laboratory Animal Experimentation (RIKEN Kobe Institute).

### *Preparation of oocytes*

Cumulus–oocyte complexes were collected from the oviducts of 8- to 12-week-old BDF1 females that had been induced to superovulate by sequential injections of equine chorionic gonadotropin and human chorionic gonadotropin (hCG) as described (Mizutani et al., 2008) and placed in Hepes-buffered Chatot–Ziomek–Bavister (CZB) medium (Chatot et al., 1990) containing 0.1% hyaluronidase. The denuded oocytes were placed in fresh CZB medium and cultured until use. Cumulus cells for nuclear donation were prepared from all females used as oocyte donors. They were immediately mixed in the collection medium, transferred to a drop of HEPES–CZB medium containing 10% polyvinylpyrrolidone (PVP), and used to provide donor nuclei for SCNT.

### *Preparation of mRNA*

mRNAs encoding enhanced green fluorescent protein (EGFP) coupled with α-tubulin (EGFP-α-tubulin) and monomeric red fluorescent protein 1 (mRFP1) fused with histone H2B (H2B-mRFP1) were prepared as described (Yamagata et al., 2009b). Briefly, after linearization of the template plasmid at the *Xba* I sites, mRNA was synthesized using RiboMax™ Large Scale RNA Production Systems-T7 (Promega, Madison, WI, USA). The 5′ end of each mRNA was capped using Ribo m7G Cap Analog (Promega). To circumvent the integration of template DNA into the embryo genome, reaction mixtures from in vitro transcription were treated with RQ-1 RNase-free DNase I (Promega). Synthesized RNAs were treated with phenol–chloroform followed by ethanol precipitation. After dissolution in RNase-free water, mRNAs were subjected to gel filtration using a MicroSpin™ G-25 column (Amersham Biosciences, Piscataway, NJ, USA) to remove unreacted substrates and then stored at −80 °C until used.

### *mRNA injection*

mRNA injection into oocytes was performed as described (Yamagata, 2010). Briefly, each mRNA was diluted to 5 ng/μL using Milli-Q ultrapure water (Millipore Corp., Madison, WI, USA) and an aliquot was placed on a micromanipulation chamber. Collected Metaphase II oocytes were transferred to droplets of Hepes–CZB medium in the chamber and a few picoliters of mRNA solution were introduced into the oocyte cytoplasm using a piezo-activated micromanipulator (Prime Tech, Ibaraki, Japan) with a glass micropipette (1–3 μm diameter).

### *SCNT procedure*

SCNT were performed as described (Kishigami et al., 2006b; Wakayama et al., 1998). The chromosome spindle complex in metaphase II oocytes previously injected with mRNAs was removed and a cumulus cell nucleus was injected into the ooplasm. The reconstructed oocytes were activated by incubation for 6 h in $Ca^{2+}$-free CZB containing 10 mM $Sr^{2+}$, 5 μg/mL CB and 50 nM trichostatin A (TSA) (Kishigami et al., 2006b). After activation, they were further cultured in CZB medium containing 50 nM TSA for 3 h.

### *Intracytoplasmic sperm injection (ICSI)*

Sperm were collected from the cauda epididymidis of BDF1 males (>12 weeks) in 0.2 mL drops of TYH medium (Toyoda et al., 1971) and capacitated by incubation for 2 h at 37 °C under 5% $CO_2$ in air. ICSI was performed according to the original procedures (Kimura and Yanagimachi, 1995). Briefly, oocytes preinjected with mRNAs were transferred to a droplet (about 10 μL) of Hepes–CZB medium in the chamber of a microscope stage. One microliter of capacitated sperm