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# Cloned Mice from Fetal Fibroblast Cells Arrested at Metaphase by a Serial Nuclear Transfer[1]

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## ABSTRACT

**Cloning using $G_0$-arrested somatic cells has led to the suggestion that this stage of the cell cycle is necessary for the success of cloning. In this study we report that cloned mice can be generated from fetal fibroblasts arrested at metaphase of the cell cycle. The procedure involves fusing a metaphase-arrested fetal fibroblast to an enucleated oocyte. After parthenogenetic activation a polar body and single diploid pronucleus were formed. Some of these were allowed to develop to the blastocyst stage, while others were enucleated and the nucleus was transferred to an enucleated fertilized 1-cell embryo. After the single transfer technique, 2 out of 164 developed to late stages of gestation were dead with gross abnormalities. However, after the serial nuclear transfer, 5 out of 272 embryos were recovered live at Day 19.5, and 2 of these went on to develop into apparently normal adults. All of the cloned embryos showed severe placental hypertrophy and defective differentiation of placental tissues. This study illustrates that reprogramming can occur after nuclear transfer at metaphase of the cell cycle.**

*conceptus, developmental biology, embryo, implantation, implantation/early development, oocyte development, ovum, placenta*

## INTRODUCTION

The successful cloning of sheep using cells synchronized in $G_0$ of the cell cycle has demonstrated the capacity for reprogramming the DNA of differentiated cells [1]. Since this original report, production of cloned animals has progressed rapidly in cattle [2–5], sheep, and goats [6–9]. Recently, cloned mice have also been produced from cumulus cells that normally provide somatic support for oocyte growth and differentiation [10]. Cumulus cells are a differentiated population of ovarian granulosa cells that undergo terminal differentiation and arrest in $G_0$ in response to the midcycle surge of LH. These studies have clearly demonstrated that the genome of differentiated somatic cells can be reprogrammed and that this reprogramming requires exposure of the DNA to unknown ooplasmic factors [11]. An intriguing observation is that the initial and subsequent successes of cloning techniques have utilized cells arrested in $G_0$ of the cell cycle. However, there is no direct evidence that the chromatin structure of nuclei arrested in $G_0$ is more favorable than other stages for the induction of reprogramming by ooplasmic activities that are necessary for supporting term development. Many studies have shown that it is possible to produce normal diploid cloned embryos from nuclei arrested at any stage of the cell cycle [12]. Some experiments have suggested that somatic cell cloning can be successful with cells in stages of the cell cycle other than $G_0$, because offspring have been produced from nonsynchronized fetal and adult fibrobrast cells in cows [3, 5].

We have previously developed a technique for cloning mice using embryonic blastomeres arrested at metaphase. In this system a nucleus is first transferred to unfertilized oocytes, and then the resultant pronucleus is again transferred into enucleated fertilized eggs [13]. This procedure allowed the successful production of identical sextuplet mice from single 4-cell embryos synchronizing at metaphase. This work revealed that nuclei arrested at metaphase at the time of transfer can be reprogrammed, and the fertilized cytoplasm contains activities for supporting the further development of the cloned embryos. In order to determine whether somatic cell nuclei also retain the capacity for undergoing reprogramming after transfer at metaphase, we have synchronized fetal fibroblasts at metaphase and used them in our cloning technique. We report that cloned mice have been produced from cultured fetal fibroblast cells arrested at metaphase using the serial nuclear transfer system. In contrast, the original nuclear transfer procedure resulted in stillborn embryos with severe abnormalities.

## MATERIALS AND METHODS

### *Animals*

All mice were maintained and used in accordance with the Guide for Care and Use of laboratory Animals by Japanese Association for Laboratory Animal Science. Mice were kept under controlled temperature and lighting conditions during experiments and were given food and water ad libitum.

### *Oocytes and Embryos*

The oocyte and zygote donors were female B6CBF1 mice (C57BL/6 × CBA). They were superovulated with injections of 5 IU of eCG (Peamex; Sankyo Ltd., Tokyo, Japan) and 5 IU of hCG (Puberogen; Sankyo Ltd.) given 48 h apart. Oocytes at metaphase II were released from the oviducts 14 h after hCG, and the cumulus cells were removed by a brief incubation in 300 U/ml hyaluronidase in M2 medium [14]. Fertilized 1-cell embryos were produced by in vitro fertilization using B6CBF1 females and males as oocytes and sperm donors.

### *Preparation of Donor Cells*

Fibroblast cells were prepared from 15.5 dpc (days postcoitus) fetuses derived from CD-1 × CD-1 and CD-1 ×

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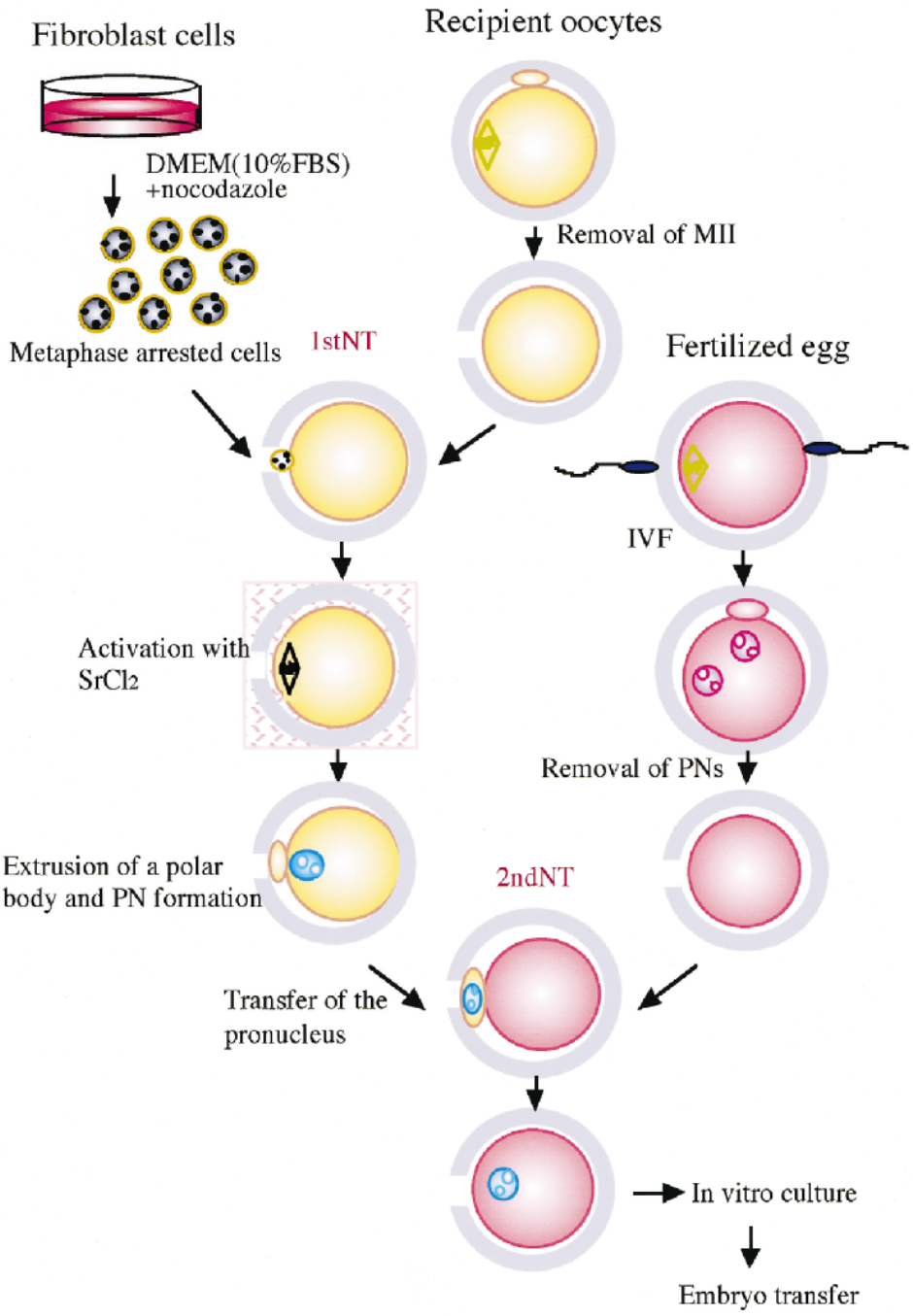


FIG. 1. Construction of cloned embryos by serial nuclear transfer. Fetal fibroblast cells arrested at metaphase were transferred to enucleated MII oocytes. After artificial activation, the resultant MII apparatus was transferred again to enucleated fertilized 1-cell embryos. The constructed embryos were cultured up to the blastocyst stage and then transferred to pseudopregnant females.

C57BL/6 embryos, of which C57BL/6 males were transgenic mice carrying the transgene, CMV-β-actin-enhanced green fluorescent protein (EGFP) [15]. The fetuses were killed by decapitation and sexed by gonadal phenotyping. The internal organs were removed, and the remaining tissue was cut into small pieces (1–2 mm) in preparation for culture. The pieces of tissue were incubated with rotation in Dulbecco minimum essential medium (DMEM) medium contained 0.05% trypsin-EDTA (Gibco) for 10 min. The cell suspension was filtered after adding the same volume of DMEM medium containing 10% fetal calf serum, and the cell suspension was seeded into tissue culture dishes at a concentration of $5 \times 10^5$/ml. The supernatant was removed after 1 h and fresh medium was added, and the remaining cells were allowed to grow to confluency. When confluent, the cells were suspended in medium containing 10% dimethylsulfoxide and frozen in cryotubes. After thawing and culturing, the passage 2 cells were cultured with medium containing 0.4 μg/ml nocodazole (Sigma), a microtubule polymerization inhibitor, for 2 h to induce metaphase arrest. After gentle pipetting, cells floating in the medium were collected and used for nuclear transfer. Using this synchronization procedure, approximately 80% of the collected cells were arrested at metaphase, as determined by Hoechst 33258 (10 μg/ml). Further selection of the metaphase-arrested cells was performed using an Olympus

microscope with Hoffman modulation contrast when the cell was sucked into a micropipette.

### *Nuclear Transfer*

Embryos were constructed by serial nuclear transfer using standard micromanipulation procedures described elsewhere [13]. The protocol used in the current experiment is outlined in Figure 1. All micromanipulations were performed in M2 medium containing 5 μg/ml cytochalasin B and 0.4 μg/ml nocodazole in a micromanipulation chamber. After enucleation of MII chromosomes [16], a fibroblast cell arrested at metaphase, as indicated by the absence of a nuclear membrane and condensation of chromosomes, was introduced with inactivated Sendai virus 2700 (hemaglutinating activity units/ml) into the perivitelline space of the enucleated oocytes. Thus, when sucked into a transfer pipette, only the cells arrested at metaphase are selected and used as nuclear donors. The oocytes that successfully fused with a donor cell were cultured for 2 h in an atmosphere of 5% $CO_2$, 5% $O_2$, and 90% $N_2$ at 37°C. Following brief culture, the oocytes were artificially activated with 10 mM strontium for 6 h and then placed in CZB medium [17]. After 9–12 h the nucleus of the constituted egg was again transferred to previously enucleated fertilized 1-cell embryos obtained by in vitro fertilization.

### *In Vitro Culture and Embryo Transfer*

Embryos were cultured in CZB medium containing 5.56 mM glucose in an atmosphere of 5% $CO_2$, 5% $O_2$, and 90% $N_2$ at 37°C. Blastocysts obtained were transferred to the uterine horns of females on Day 3 of pseudopregnancy (2.5 dpc). To assess development, recipients were killed at 19.5 days of gestation, and pups were recovered from the uterus.

### *Genetic Analysis*

Genomic DNA was extracted from liver tissues from a cloned pup and from CD-1 strain adult mice by phenol chloroform extraction. Twelve loci, *Idh1, Pep3, Akp1, Mup1, G6pd1, Es1, Trf, Es3, Hba, H2-k, H2-D,* and *Cd5* in seven chromosomes, were biochemically examined by the standard procedure [18], and 20 microsatellite markers were analyzed by polymerase chain reaction (PCR) using the specific primers according to a procedure described elsewhere [19].

### *Histological Analysis of Placenta*

Placentas that were collected from live pups at birth were fixed with 4% paraformaldehyde and processed for wax embedding. Serial sections prepared in the cross planes were mounted on slides and stained with hematoxylin and eosin.

### *Statistical Analysis*

Data were analyzed by χ-square analysis and Student's *t*-test analysis.

## RESULTS

After fusion of the metaphase-arrested fibroblast with an enucleated oocyte a single metaphase plate reassembled in the oocyte cortex. Parthenogenetic activation induced the extrusion of a polar body within 4 h of the activation and formation of a single diploid nucleus within 8 h. The rates of oocytes successfully fused with a donor cell and activated forming a pronucleus with the second polar body were 58–69% and 87–91%, respectively. The efficiency for oocyte construction was around 50%, with most of the loss due to a failure of cell fusion in the first nuclear transfer. Development to the morula-blastocyst stage after the second nuclear transfer was 29% and 31% in two donor cell lines (Table 1). The proportion of morulae to blastocysts was significantly higher (37%) when embryos were constructed by the original procedure where the second nuclear transfer is omitted.

To assess the ability to develop to term, a total of 272 the embryos constructed by the serial nuclear transfer were transferred into 38 pseudopregnant females (Table 1). A total of five live pups (2%) were recovered by cesarean section at 19.5 days of gestation. Of these, three pups were successfully fostered to mothers. Two of them grew up normally and matured (Fig. 2, d and e), but one was deserted soon after introduction to the foster mother (Fig. 2f). The two clones that developed to puberty showed normal reproductive performance (Fig. 2h). The other two pups died from an umbilical hernia and respiratory deficiency within 1 h after recovery from the uterus (Fig. 2, i and j). The production of clones from the single transfer technique failed to yield any healthy offspring. A total of 164 morulae-blastocysts derived from embryos constructed by single nuclear transfer were transferred into 18 pseudopregnant females, and two dead fetuses with severe abnormalities were recovered (Fig. 2, k and l) at 19.5 days of gestation.

To confirm that the cloned mouse was derived from the fibroblast cells of CD-1 mice we analyzed 12 loci in 7 chromosomes and 20 microsatellite markers. These studies demonstrated that the cloned mouse was genetically identical to the donor strain mice from which the fibroblast cells were prepared. The other cloned mouse was produced from fetal fibroblast cells derived from transgenic mice carrying EGFP. As shown in Figure 2, a and g, the emission of green fluorescence was detected in the whole body when irradiated with UV. This provides direct evidence for the genomic origin of the clone.

As typical phenotype of the cloned conceptuses, hypertrophy of the placenta, which was over two times heavier (289–362 mg, $n = 7$) ($P < 0.001$) than that of controls (136 mg, $n = 20$, data were used when litter size was less than five), was observed in all cases (Table 2). Histological analysis on the hypertrophic placentas of cloned mice showed several significant anomalies (Fig. 3). Unusually large giant cells were irregularly distributed along with the decidua basalis in the endometrium side. Trophoblastic cells in the spongiotrophoblast were enlarged with a large nucleus. In addition they had undergone proliferation and formed large symplastic colonies that contained few maternal blood vessels. Vasoganglions composed of maternal blood vessels were seen among the trophoblastic giant cell colonies. A large number of endometrial glycogen cells that were vacuolated were also distributed within the layer. The extensive proliferation of spongiotrophoblast tissues led to severe defection of the labyrinthine chemochorial placenta development, such that the border between the spongiotrophoblast and labyrinth layers was severally in disrupted. The chorionic plate was also significantly enlarged and inverted into the labyrinthine layer.

## DISCUSSION

In this study, we described the production of cloned mice from metaphase-arrested fetal fibroblasts, showing that con-

TABLE 1. In vitro and in vivo development of cloned embryos produced by single and serial nuclear transfer.

| Nuclear transfers | Origin of donor cells | No. of oocytes fused at first NT/ manipulated (%) | No. of oocytes activated normally* | No. of oocytes fused at second NT/ manipulated (%) | No. of cloned embryos cultured | No. of oocytes developed to morula and blastocyst (%) | No. of embryos transferred | No. of pregnant recipients (%) | No. of implantation sites (%) | No. of dead fetuses (%) | No. of live pups (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Single NT | CD-1 | 652/930 (69) | 594 (91) | — | 593 | 222 (37)† | 164 | 6/18 (33) | 28 (17) | 2 (1.2)‡ | 0 |
| Serial NT | CD-1 | 650/1049 (62) | 583 (90) | 436/448 (97) | 436 | 133 (31) | 130 | 8/20 (40) | 28 (22) | 0 | 4 (3) |
| Serial NT | CD-1 × B6$^{Tg}$ | 628/1078 (58) | 546 (87) | 502/513 (98) | 502 | 145 (29) | 142 | 3/18 (17) | 17 (12) | 0 | 1 (0.7) |

* Oocytes formed a pronucleus and second polar body.
† $P < 0.05$.
‡ Dead fetuses were recovered at 19.5 days of gestation with gross abnormalities.

densed metaphase chromatin from somatic cells can be reprogrammed to support full-term development. In our previous study we demonstrated the feasibility of this procedure by producing identical sextuplet mice using metaphase-arrested nuclei from blastomeres of a 4-cell-stage embryo [13]. Recently, Wakayama et al. also reported that cloned mice were produced from embryonic stem cells synchronized at metaphase that were selected based on size [20]. The first event after cloning using metaphase nuclei is the formation of a new mitotic spindle, which is diamond-shaped in somatic cells, in response to high maturation-promoting factor (MPF) levels present in the oocyte [12]. After artificial activation, a high proportion of oocytes extruded a second polar body, resulting in a normal diploid 1-cell embryo. Thus the approach of using metaphase nuclei provides a direct line from transferring the chromatin to the production of a normal embryo. In contrast, the use of interphase nuclei in $G_2$ requires that the MPF present in the oocyte induces nuclear membrane breakdown and premature chromosome condensation (PCC) prior to the activation of the oocyte [12]. While it may be argued that this provides an additional opportunity for reprogramming, PCC is also associated with chromatin damage and aneuploidy, resulting in loss of developmental ability of the embryos [21, 22]. The use of nuclei arrested at metaphase as donors may help to alleviate this problem [13].

The process of nuclear reprogramming after cloning is not well understood. It is thought to involve the replacement of factors that control chromatin structure and gene expression [23] with oocyte-derived factors that can redirect chromatin structure and function to the requirements of the oocyte and embryo [24–26]. Thus, in any cloning procedure exposure of the donor chromatin to oocyte-derived factors is necessary for reprogramming. In embryo cloning using interphase nuclei such as those in $G_0$-$G_1$, nuclear swelling and/or nuclear membrane breakdown are induced after fusion with an ooplast. This is thought to be the period when chromatin-associated factors are replaced with materials of ooplasmic origin, and the genome is epigenetically modified to induce reprogramming for term development. Because metaphase chromatin is heavily condensed and generally silent for gene expression, the replacement of chromatin-associated factors necessary for reprogramming must occur during decondensation of the donor chromatin after oocyte activation when cells arrested at metaphase are used as donor nuclei. This implies that the factors necessary for reprogramming of donor chromatin are present during the first cell cycle and are not degraded immediately after oocyte activation.

In this study we have utilized a serial nuclear transfer technique. In this procedure, a donor nucleus is first transferred into an ooplast that is then parthenogenetically activated, forming a single pronucleus. The second transfer removes this nucleus from the parthenogenetic cytoplast into an enucleated in vitro-fertilized 1-cell embryo. We have previously found that this procedure allows cloned mice to be produced from 4- [13] and 8-cell [27] embryo nuclei, showing the efficiency of cloning using embryonic cells as demonstrated by our ability to produce sextuplet clones from a single 4-cell stage embryo [13]. As expected, the present study showed that live pups were produced by serial nuclear transfer, suggesting the efficiency of the serial nuclear transfer in somatic cell cloning of mice. When the second transfer was omitted, only the dead fetuses with severe abnormalities were obtained. Thus, only the serial nuclear transfer produced live cloned pups, but this was not

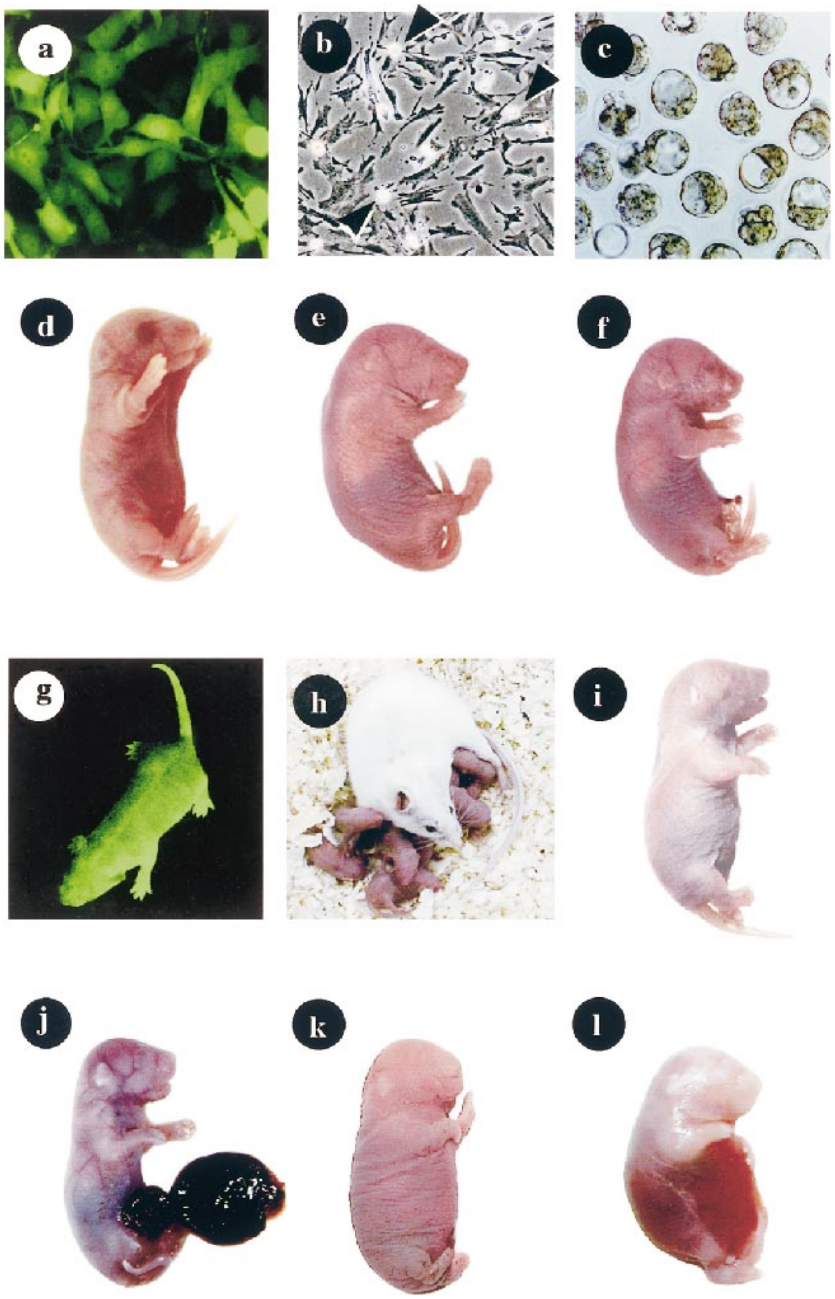


FIG. 2. Cloned mice produced from fetal fibroblast cells. **a**) Fibroblast cells carrying the EGFP transgene. ×400. **b**) Fibroblast cells treated with nocodazole. Arrowheads indicate cells arrested at metaphase. ×200. **c**) Blastocysts derived from constructed oocytes using fibroblast cells arrested at metaphase. ×100. **d**–**f**, **i**, **j**) Cloned live pups produced by serial nuclear transfer. A cloned pup (**d**) derived from a fibroblast cell carrying the EGFP transgene is emitting green fluorescence (**g**). **h**) A cloned mouse nursing her own pups. Cloned pups died soon after recovery due to a respiratory deficiency (**i**) and umbilical hernia (**j**). **k**, **l**) Fetuses obtained from constructed oocytes by single nuclear transfer show severe abnormalities.

TABLE 2. Cloned pups produced from metaphase fibroblasts.

| Pup number | Nuclear transfer | Weight (g) at birth | | Notes |
|---|---|---|---|---|
| | | Pups | Placentas | |
| I | Serial NT | 1.58 | 0.34 | Grew up normally (EGFP), Figure 2d |
| II | Serial NT | 1.59 | 0.31 | Grew up normally, Figure 2e |
| III | Serial NT | 1.25 | 0.34 | Dead after 1 day, Figure 2f |
| IV | Serial NT | 1.45 | 0.29 | Dead with respiratory deficiency, Figure 2i |
| V | Serial NT | 1.59 | 0.33 | Dead with umbilical hernia, Figure 2j |
| VI | Single NT | 1.34 | 0.36 | Dead fetus, Figure 2k |
| VII | Single NT | — | 0.29 | Dead fetus, Figure 2l |

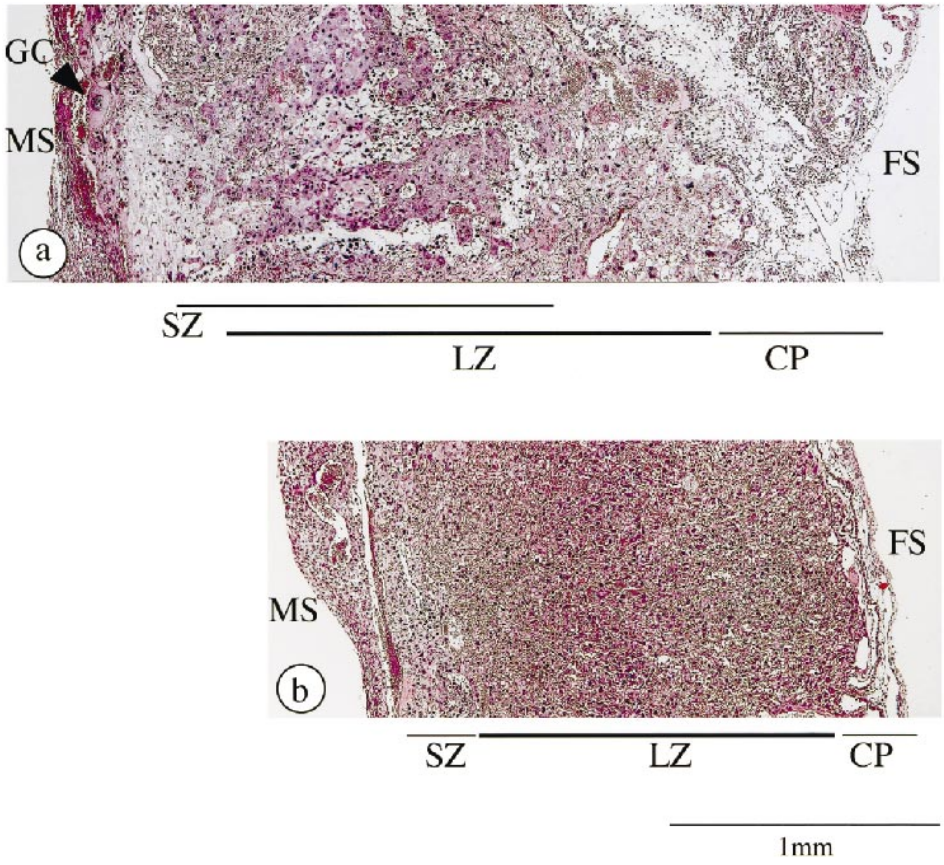


FIG. 3. Comparative histological sections of placentas from cloned (**a**) and control (**b**) live pups at birth. FS, Fetal side; MS, maternal side; GC, giant cell; SZ, spongiotrophoblastic zone; LZ, labyrinthine zone; CP, chorionic plate.

a significant advantage. Although further experiments are required to clarify the beneficial effect of serial nuclear transfer, several lines of possibilities may be suggested. The cytoplasmic environment after fertilization leads to gene expression capable of supporting development to term while that of the parthenogenetic embryo does not. One possible explanation is the recent finding that mRNA synthesis occurs first in the male pronucleus starting in early S phase of the first cell cycle [28]. It may be that these early transcripts are important for directing later development [29, 30]. Otherwise, donor cell-derived products in the constructed eggs are diluted by the second nuclear transfer, by which the ovicidal effect is reduced and does not harm the development.

Although our results show that cloning mice from metaphase-arrested cells is possible, there were a number of serious concerns as to the efficacy and safety of the cloning procedure. One of the most striking observations was the increased placental weight and abnormal placental anatomy of the cloned conceptuses. Given the critical role of the placenta in nutrient exchange and cytokine production important for embryonic growth [31], it appears likely that the disordered state of the placenta would be of functional significance. This is further supported by the histological observation that the severe hypertrophy is caused largely by proliferation of the trophoblastic cells, endometrial glycogen cells, and unusually large giant cells. The defect was accompanied by a limited distribution of maternal blood vessels in the spongiotrophoblast layer and an oppressed development of the labyrinthine layer. These suggest a decrease in uteroplacental circulation that would greatly reduce the functional capacity of the placenta to act as a mechanism of nutrient and gas exchanges. These results suggest that the hypertrophic placenta may be one of the reasons for the high neonatal death in cloned pups derived from somatic cells. On a more speculative note, it has been suggested that placental development is a driving influence on the evolution of imprinted genes—the so-called parent offspring, or tug-of-war hypothesis [30, 32]. If nutrient transfer is important in the evolution of imprinting, it may be that the placental abnormalities described here are a result of disrupted patterns of expression of imprinted genes important for placental development. It is not known how or whether chromatin remodelling during the cloning procedure modifies chromatin in a manner that leaves the gamete-derived imprints intact while removing other chromatin components that direct gene expression.

Embryo cloning results in a relatively high rate of late abortion, abnormal phenotypes, and prenatal and early postnatal death, particularly when somatic cells are used as nuclear donors [2–5]. However, the reason for this is still unclear. It is possible that defective placental function is a major contributor to the high rate of embryo loss. The genes *hand1* [33], *Mash2* [34], and *GCM*1 [35, 36] are known as key genes for governing cell differentiation in the labyrinth, spongiotrophoblast, and giant trophoblast cells of the placenta. Improving the development of somatic cell cloning will require an improved understanding of the molecular basis of nuclear reprogramming and how this may lead to normal fetal and placental development.

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