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[REVIEW]

The Recent Progress on Nuclear Transfer in Mammals

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ABSTRACT—Differentiated mammalian somatic cell nuclei and embryonic nuclei can now be reprogrammed to develop into young if they are introduced into enucleated oocytes. The success rates for cloning are generally low, however, and peri- and postnatal death rates of the young are high. Cloning technology will be useful for the genetic improvement of farm animals, therapeutic human protein production, and organ or tissue transplantation into humans. In addition, the information obtained on nuclear reprogramming will be helpful for understanding the fundamental mechanisms of differentiation and aging.

INTRODUCTION

In 1997, Wilmut *et al.* reported that a lamb, named Dolly, was obtained after nuclear transfer of cultured mammary epithelial cells from a 6 year old pregnant ewe. The first successful nuclear transfer in animals was reported by Briggs and King (1952) who introduced blastula nuclei into enucleated oocytes, which developed into swimming tadpoles. Since then, tadpoles as well as fertile frogs have been obtained after nuclear transfer of nuclei from embryos at various stages and from various somatic cells of tadpoles (Gurdon, 1962; 1986; DiBerardino, 1997). Although the enucleated oocytes receiving nuclei from differentiated somatic cells of adult frogs developed into tadpoles, none of them developed into fertile frogs (DiBerardino, 1997). In spite of the difficulties in adult somatic cloning in frogs, a number of lambs, mice, and calves have been produced after nuclear transfer of somatic cells obtained from various adult tissues. This review article, presents the current status, applications, and future of animal cloning.

Developmental potential of germ line nuclei

Illmensee and Hoppe (1981) published a questionable report that cloned mice were obtained after inner cell mass cells of blastocysts were transferred to enucleated zygotes. This report, however, has not been confirmed. The first reliable report of nuclear transfer in mammals was reported by McGrath and Solter (1983), who obtained young after the exchange of pronuclei between two types of zygotes. The methods included enucleation and discard of pronuclei with a small volume of cytoplasm (pronuclei karyoplast) from recipient zygotes, removal of pronuclei karyoplast from donor zygotes, and fusion of donor pronuclei karyoplasts with enucleated zygote cytoplasm using inactivated Sendai virus. Several laboratories confirmed that a high proportion of pronuclei-exchanged zygotes developed into young. Enucleated zygotes receiving nuclei from 2-cell, 4-cell, and 8-cell stage mouse embryos, however, did not develop *in vitro* or *in vivo* except for a few cases with 2-cell embryos (McGrath and Solter, 1984; Tsunoda *et al.*, 1987). When nuclei from 4-cell and 8-cell stage embryos were fused with enucleated blastomeres of 2-cell embryos, live mice with normal fertility were obtained (Tsunoda *et al.*, 1987). Nuclei were not fully reprogrammed, however, because the compaction of enucleated 2-cell embryos receiving nuclei of 8-cell stage embryos occurred at the 4- to 8-cell stage, not at the 8- to 16-cell stage, as in normal embryos.

As in the case of the frog, the nuclei from mammalian preimplantation embryos could be reprogrammed in the cytoplasm of unfertilized oocytes (Willadsen, 1986). Enucleated oocytes at the second metaphase receiving nuclei from 4-cell, 8- to 16-cell, and morula stage embryos, and inner cell mass cells of blastocysts developed into young in several mammalian species. The developmental potential of embryonic stem cells (ES cells), which were established from the inner cell mass of blastocysts, was limited. Recently, however, cloned mice have been produced by direct injection (Wakayama *et al.*, 1999; Rideout *et al.*, 2000) and membrane fusion (Amano *et al.*, 2000b), as described below. The enucleated oocytes receiving male fetal germ cells developed into blastocysts but did not develop into young. The reason for the failure is probably due to gamete imprinting for the next generation in male germ cells (Kato *et al.*, 1999a). One live calf was born after nuclear transfer of male fetal germ cells on day 50 to 57 (Zakhartchenko *et al.*, 1999a), suggesting that the starting point of gamete imprinting might be different among

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species.

The chromosomes of oocytes at the second metaphase stage that were matured *in vitro* or *in vivo* were removed by a micropipette attached to micromanipulator. Because the metaphase chromosomes of mouse oocytes can be observed under an inverted microscope, they can be easily removed. Although the metaphase chromosomes in bovine and pig oocytes are difficult to observe, a small amount of oocyte cytoplasm near the first polar body where the chromosomes are usually located is removed (Fig. 1). The removed cytoplasm is stained with Hoechst to confirm the presence of chromosomes and the remainder of the oocyte is used as recipient cytoplasm. A single donor blastomere or cell of the preimplantation embryo, fetal germ cell, or embryonic stem cell is inserted into the pervitelline space of recipient oocytes (Fig. 2). The incorporation of the donor nucleus into recipient cytoplasm is achieved by membrane fusion with inactivated Sendai virus or electrical stimulation, or direct injection using a piezoelectric micromanipulator (Wakayama *et al.*, 1998). Recipient oocytes are parthenogenetically activated by chemical or electrical stimulation before, at the time of, or after, fusion.

The timing for the activation in relation to the cell cycle of donor nuclei and condition of recipient oocytes is important for the normal development of nuclear transferred oocytes. Two different combinations can be used for successful nuclear transfer (Campbell *et al.*, 1996; Campbell, 1999). One method is to use non-activated recipient oocytes at the second metaphase when the activity of the maturation promoting factor (MPF) is high. When donor cells are fused with non-activated oocytes, the nuclear membrane is broken down and the chromosomes of the donor nucleus are prematurely condensed (premature chromosome condensation). After parthenogenetic activation, the nuclear membrane reforms and DNA synthesis begins, irrespective of the cell cycle stage of the donor nucleus. Thus , the cell cycle stage of the donor cells before nuclear transfer should be at either G1 or G0. The other method is to use activated oocytes as recipient cytoplasm. In

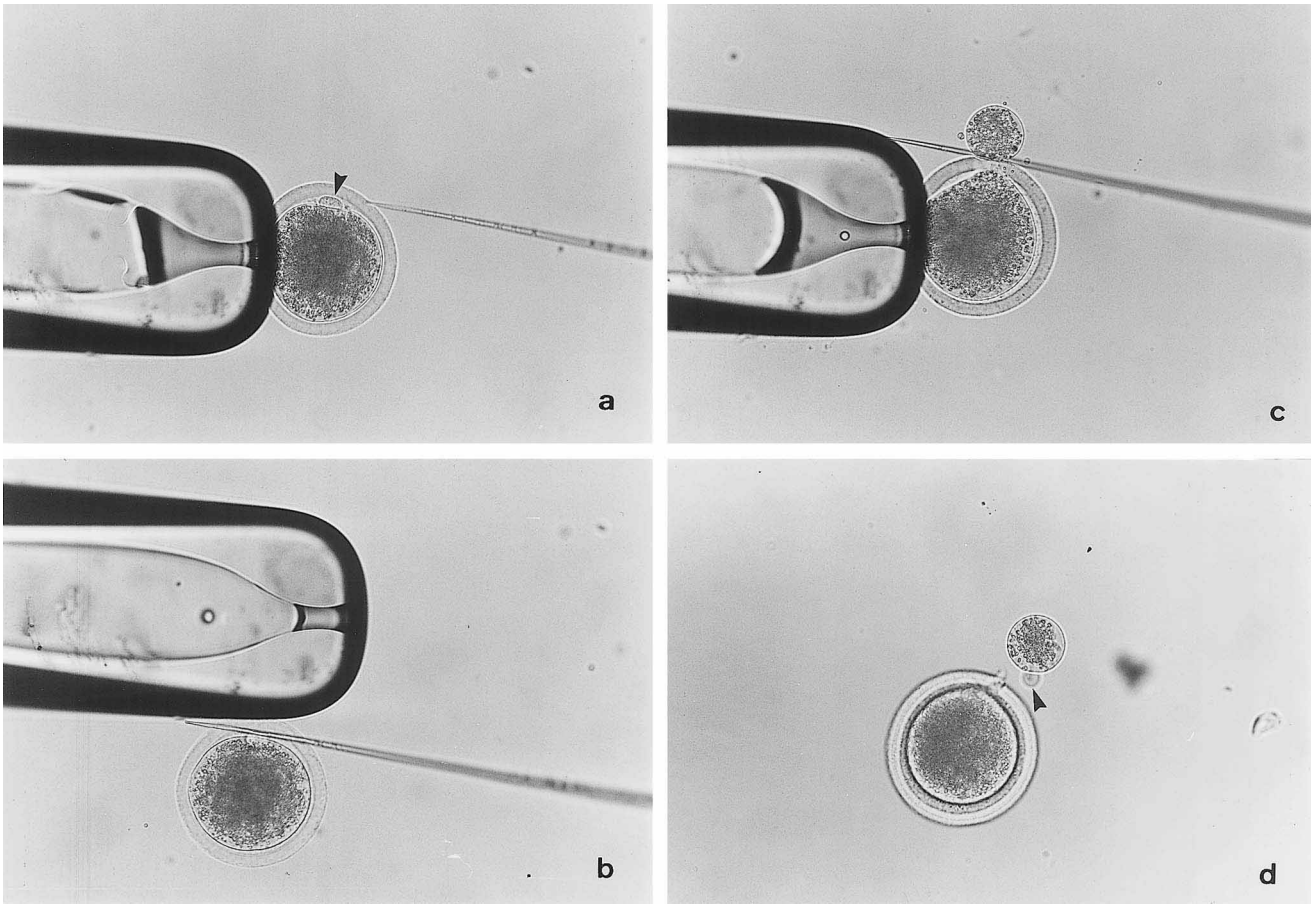

Fig. 1. The procedures for removing the metaphase chromosomes in bovine oocyte
a. The oocyte is sucked with a small bore holding pipette (left) and a fine glass needle (right) is inserted into perivitelline space near the first polar body (arrow).
b. The needle is rubbed against the wall of the pipette to tear the zona.
c. The oocyte is pushed from above using a glass needle and a small amount of cytoplasm near the first polar body was pushed out from the slit on the zona.
d. The small volume of cytoplasm with the first polar body (arrow) removed from oocyte is stained with Hoechst. When metaphase chromosomes are confirmed to be present in the cytoplasm, the rest of oocyte cytoplasm is used as the recipient cytoplasm.

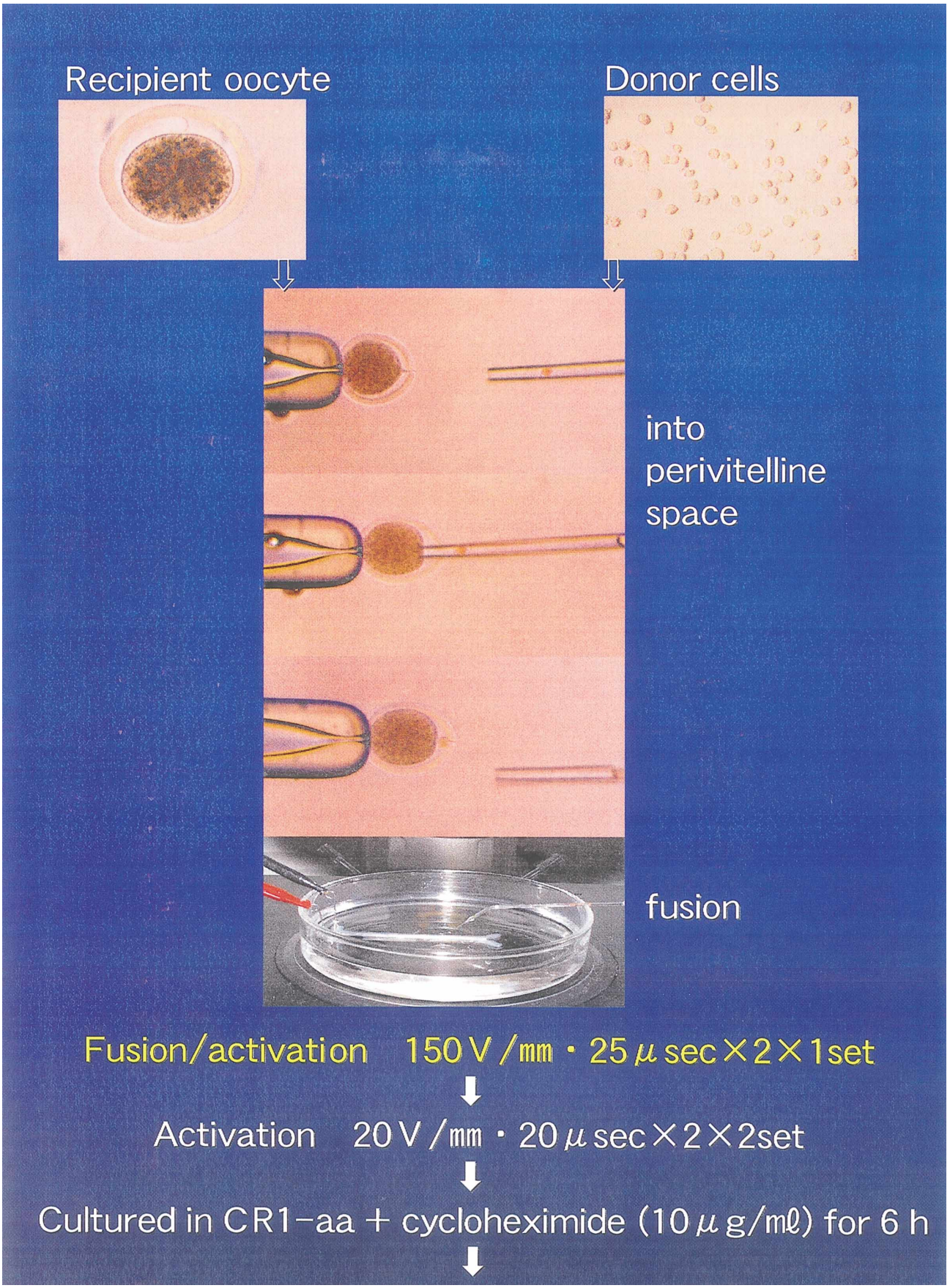

Fig. 2. The procedures for nuclear transfer in bovine

this case, nuclear membrane breakdown and premature chromosome condensation of the donor nucleus do not occur, but DNA synthesis occurs in relation to the cell cycle stage of the donor nucleus at the time of nuclear transfer and normal development of nuclear transferred oocytes is expected, irrespective of the cell cycle of the donor nucleus. Because synchronization of the cell cycle of the donor nucleus of bovine preimplantation embryos to the G1 stage is difficult, the second method is more practical for cloning and a large number of calves have been produced using this method.

Nuclear transfer of embryonic stem cells

Embryonic stem (ES) cells are remarkable because they can be cultured and manipulated relatively easily *in vitro* without losing their developmental potential and behave like normal embryonic cells when they are injected into host blastocysts (Robertson, 1987) or 8-cell stage embryos (Tokunaga and Tsunoda, 1992). Because of these characteristics, ES cells are used for gene targeting to produce transgenic mice. It takes time, however, to use gene targeting methods to obtain homologous transgenic mice because at least two matings are necessary. ES-cell derived mice are advantageous over chimeras because direct analysis of post-implantation embryos developed from gene targeted nuclei is also possible. Entirely ES-cell derived mice have been obtained by aggregating ES cells with carrier tetraploid embryos (Nagy *et al*., 1990; 1993) and heat-treated blastocysts (Amano *et al*., 2000a).

There have been several attempts to produce ES-cell derived mice by nuclear transfer. When ES cells were fused with enucleated oocytes (Tsunoda and Kato, 1993) or 2-cell stage embryos (Modlinski *et al*., 1996), they developed into blastocysts *in vitro* or into a day-16 fetus, respectively, but full term fetuses were not obtained. Recently, Wakayama *et al*. (1999) and Amano *et al*. (2000b) obtained live young after transfer of ES cells to enucleated oocytes. Wakayama *et al*. (1999) directly injected ES cells at the G1 or G2/M phase of the cell cycle, which had been cultured for 1 to 5 d without feeder cells, into enucleated oocytes using a piezoelectric micromanipulator. We (Amano *et al*., 2000b) fused ES cells in which the cell cycle had been synchronized to the M phase after preincubation with nocodazole with enucleated oocytes using inactivated Sendai virus. In both reports, the proportion of nuclear transferred oocytes that developed to full term was low and, moreover, a large proportion of the young died soon after birth. The incidence of postnatal death was different among the ES cell lines (Rideout *et al*., 2000; Amano *et al*., unpublished). Although the reason is not clear, it is possible that the high postnatal deaths is due to insufficient reprogramming of donor nuclei or epigenetic modifications of ES cells, including the occurrence of imprinted genes before or during the establishment and micromanipulation.

Although definitive ES-cell lines have not been established in farm animals, live calves were produced after nuclear transfer of undifferentiated short-term cultured inner cell masses of blastocysts into enucleated oocytes (Sims and First, 1993). Live lambs were also obtained using cell lines established from embryonic disc cells that were induced to differentiate into epithelial cells before nuclear transfer (Campbell *et al*., 1996; Wells *et al*., 1997).

Developmental potential of somatic cell line nuclei

Mammalian embryos first differentiate into two distinct cell lineages at the blastocyst stage; one is the inner cell mass, which forms the embryo proper, and the other is the trophectoderm, which contributes to form the placenta and fetal membranes but does not participate in the formation of the fetus proper. At least some mouse trophectoderm cell nuclei have the same developmental totipotency as inner cell mass cells because fertile mice were obtained after nuclear transfer into enucleated oocytes (Tsunoda and Kato, 1998). Moreover, Wilmut *et al*. (1997) reported that nuclei from fetal and adult somatic cells have the potential to develop into young. Wilmut and colleagues obtained four lambs from cultured embryonic disc cells, three lambs from fibroblast cells of a day-26 fetus, and one lamb, named Dolly, from mammary gland cells of a 6 year old pregnant female. This was the first animal produced from somatic cells of an adult animal since none of the nuclear-transferred oocytes receiving somatic cells from adult frogs developed into frogs. Soon after the success of Wilmut *et al*., cloned mice (Wakayama *et al*., 1998) and calves (Kato *et al*., 1998) were produced from cumulus cells around ovulated eggs, and cultured follicular epithelial cells and oviductal cells from mature females. In the last 3 y, a large number of cloned female and male sheep (Wilmut *et al*., 1997; Schnieke *et al*., 1997), mice (Wakayama *et al*., 1998; Kato *et al*., 1999b; Wakayama and Yanagimachi, 1999), calves (Kato *et al*., 1998; Cibelli *et al*., 1998; Vignon *et al*., 1998; Zakhartchenko *et al*., 1999b, Wells *et al*., 1999; Renard *et al*., 1999; Shiga *et al*., 1999; Kubota *et al*., 2000; Lanza *et al*., 2000), goats (Baguisi *et al*., 1999), and, recently, pigs (Betthauser *et al*., 2000; Onishi *et al*., 2000; Polejaeva *et al*., 2000), have been produced after nuclear transfer of somatic cells cultured from various tissues of fetuses, newborns, and adults (mammary gland, cumulus, oviduct, skin, ear, muscle, liver, and tail). In most studies, nuclei at the quiescent stage of the cell cycle, which was induced by serum starvation or contact inhibition, are fused with enucleated oocytes with high MPF activity. The fused oocytes are parthenogenetically activated, cultured in vitro, and then transferred to recipients. In the studies of Yanagimachi and colleagues on mouse nuclear transfer, donor cells are directly injected after the rupture of cell membrane using a piezoelectric micromanipulator and then activated with strontium. Electrical stimulation, however, has been used for cloning cattle, sheep, and goat.

The procedures for bovine somatic nuclear transfer in our laboratory are shown in Figs. 1 and 2. Donor cells were obtained from various somatic tissues of adult, newborn, and fetal female and male bovine (cumulus, oviduct, uterus, skin, ear, heart, liver, kidney, muscle, lung, gut, mammary gland, testis, epididymis, and tongue; Kato *et al*., 1998; 2000). The cultured somatic cells passaged several times were induced to the quiescent stage by serum starvation or contact inhibi-

tion. The necessity of the quiescence of somatic cells for nuclear reprogramming is questionable and somatic cells at the M phase have been successfully used as nuclear donors (Tani *et al*., 2001). *In vitro* matured oocytes whose chromosomes were removed at the second metaphase are used as nuclear recipients. A single donor cell was electrically fused with enucleated oocytes and fused oocytes were again electrically stimulated to ensure activation (Fig. 2). Fused oocytes were treated with cycloheximide-supplemented medium for several hours and then cultured for 7 to 8 d *in vitro*. Visually normal blastocysts (Fig. 3) were non-surgically transferred to recipient females.

As shown in Table 1, the percentages of blastocysts that developed from various donor cell types were not largely different among donor cells and there were also no difference in the percentages of blastocysts that developed from oocytes containing adult (42%), newborn (37%) or fetal calf (40%) nuclei, or between female (39%) and male (40%) nuclei. Among the 139 recipient cows, 55 (40%) became pregnant but 27 of them aborted and the remaining 28 produced 32 calves (Table 2). Seventeen calves died, however, around or after parturition due to dystocia or morphologic abnormalities. The nuclei from diverse cells and tissue types directed the development of newborn calves, such as cumulus, oviduct, skin, ear, and liver. Cumulus and oviduct cells appeared to be the most suitable nuclear donors for cloning calves because

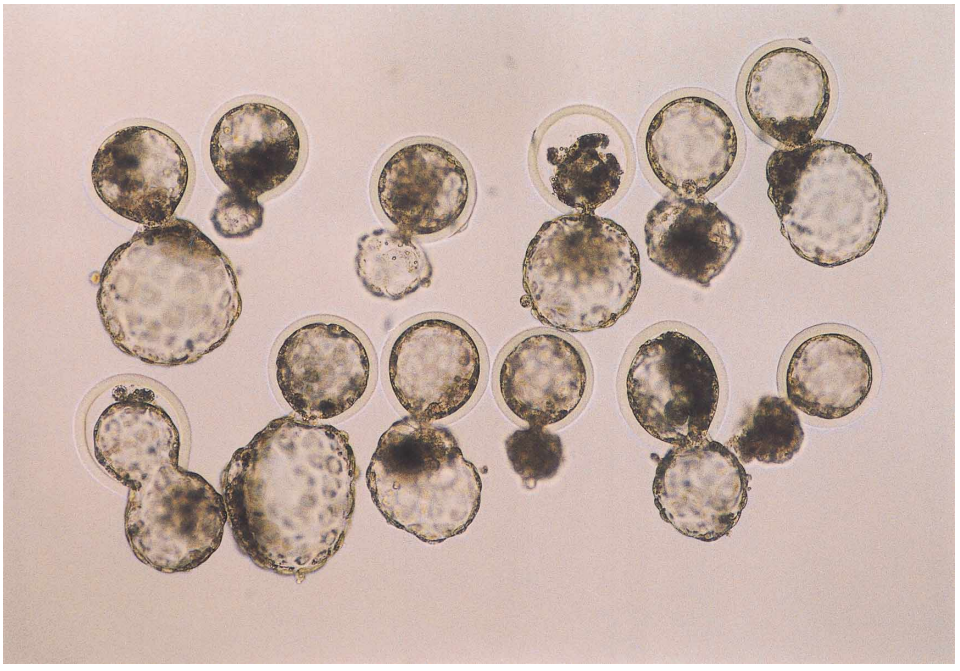

Fig. 3. One example of cloned bovine blastocysts derived from adult somatic cells.

Table 1. Developmental potential of somatic nuclear transplant in vitro

Origin	Adult		New born		Fetus	
	Female	Male	Female	Male	Female	Male
cumulus	30					
oviduct	38					
uterus	50		33			
skin	52	49	39	27	46	41
ear	48	41	44	25		
heart	34	40	41	46		45
liver	44	53	32	47		4
kidney	36	33	28	43	47	38
muscle			36		23	
lung			40			
mammary gland				42		
testis*				38		
epididymis*				30		
gut					41	37
tongue						42

*Only somatic fibroblast cells were used. Kato *et al*. (2000)

Table 2. Developmental potential of somatic nuclear transplants in vivo

Donor cells			No. of pregnants / recipients (%)		No. of calves / No. of blastocysts transferred (%)	
Adult	Female	cumulus	9 / 37	(24)	8 / 47	(17)
		oviduct	6 / 16	(38)	5 / 21	(29)
		uterus	3 / 7	(43)	2 / 14	(17)
		skin	2 / 4	(50)	2 / 4	(50)
		ear	1 / 2	(50)	0 / 2	(0)
Newborn	Female	skin	2 / 5	(40)	1 / 5	(20)
		ear	1 / 2	(50)	0 / 2	(0)
		liver	1 / 5	(20)	0 / 5	(0)
Adult	Male	skin	10 / 19	(53)	5 / 23	(22)
		ear	11 / 23	(48)	5 / 30	(17)
Newborn	Male	skin	1 / 4	(25)	1 / 5	(20)
		ear	1 / 2	(50)	0 / 4	(0)
		liver	3 / 3	(100)	2 / 5	(40)
		testis	1 / 1	(100)	0 / 2	(0)
Fetal	Male	skin	2 / 6	(33)	1 / 7	(14)
		liver	1 / 3	(33)	0 / 6	(0)
Total			55 / 139	(40)	32 / 182	(18)

Kato *et al.* (1998) and (2000)

Fig. 4. One example of cloned bovine developed from adult somatic cells. (Photographed at Ishikawa prefecture livestock experimental station)

8 of 13 (62%) calves survived (Fig. 4), but only 2 of 10 calves produced with bull skin or ear survived.

The interesting question is whether calves obtained by nuclear transfer of somatic cells from aged animals have normal longevity. Although data on clone longevity have not been reported, the telomere length of somatic cells from Dolly were decreased compared with that of age-matched control animals (Shiels *et al.*, 1999). Lanza *et al.* (2000) recently reported that the cell life-span and telomere length of cloned calves obtained from senescent somatic cells were extended. Our study indicates that the changes in telomere length might differ according to donor tissue (Kato *et al.*, 2000).

Nuclear reprogramming mechanism

The nuclear reprogramming mechanism might be different between embryonic and somatic cells. The reprogramming of embryonic nuclei occurs not only in non-activated oocytes but also in activated oocytes (Campbell *et al.*, 1996).

Although the nuclear membrane of donor cells is intact in activated oocytes, DNA synthesis occurs according to the original cell cycle stage at the time of nuclear transfer and nuclear reprogramming occurs during the expansion of the donor nucleus (Stice and Robl, 1988). Normal sheep have also been produced after transfer of activated or non-activated oocytes receiving cultured embryonic disc cells (Campbell *et al*., 1996). These observations suggest that nuclear membrane breakdown and premature chromosome condensation are not necessary for the reprogramming of embryonic nuclei. On the other hand, the direct exposure of donor chromosomes to non-activated oocyte cytoplasm is essential for the reprogramming of somatic cell nuclei (Tani *et al*., 2001).

It has been speculated that the nuclear reprogramming factor for somatic cells might be present in the oocyte cytoplasm where there is high MPF activity. The activity of such cytoplasmic factors disappear soon after parthenogenetic activation but the molecular nature is unknown (Kikyo and Wolffe, 2000).

Future of cloning

It is now possible for sheep, mouse, bovine, goat, and pig clones to be produced by nuclear transfer of a variety of embryonic and somatic cells. Success rates, however, are generally low and, moreover, peri- and postnatal death rates are high in many cases (Hill *et al*., 1999; Kato *et al*., 2000). Such abnormalities might occur due to insufficient reprogramming and/or epigenetic modifications of the nuclei.

If the success rate to produce normal cloned animals is increased, the techniques can be effectively used for genetic improvement of farm animals under the same regulations as for artificial insemination and embryo transfer. Cloning techniques are also effective for production of transgenic animals that yield therapeutic human proteins in milk. Cloning also offers the possibility to produce genetically-modified animal organs, especially in pigs, that are suitable for transplantation into humans. In addition to applications for animal husbandry and for medicine, information obtained from studies on nuclear reprogramming of somatic cells might be helpful in understanding the fundamental mechanisms of differentiation and aging.

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