

Cloning: experience from the mouse and other animals

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Abstract

Cloning mammals has been successful for many years by splitting an early embryo or transferring embryonic cell nuclei into enucleated oocytes. Cloning is now possible with adult somatic cells. At present, cloning efficiency—as determined by the proportion of live offspring developed from all oocytes that received donor cell nuclei—is low regardless of the cell type (including, embryonic stem (ES) cells) and animal species used. In all animals, except of Japanese black beef cattle, the vast majority (>97%) of cloned embryos perish before reaching full term. Even in the Japanese cattle, less than 20% of cloned embryos reach the adulthood. This low efficiency of cloning seems to be due largely to faulty epigenetic reprogramming of donor cell nuclei after transfer into recipient oocytes. Cloned embryos with major epigenetic errors die before or soon after implantation. Those with relatively ‘minor’ epigenetic errors may survive birth and reach adulthood. We found that almost all fetuses of inbred mice die at birth from respiratory problems, while those of hybrid mice do not, suggesting that genomic heterogeneity masks—to some extent—faulty epigenetic errors. Thus far, the majority of cloned mice that survived birth, had a normal life span and were fertile. However, these animals may not be totally free of health problems. Postpubertal obesity in certain strains of mice is one example. A trial and error approach may discover better cells for cloning, but it would be wiser to understand the molecular mechanisms of epigenetic nuclear programming and reprogramming to find the way to make cloning safer and more efficient. The relatively high cloning success rate in the Japanese black cattle may provide us a clue of solving the problem of high mortality of cloned offspring. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Cloning—the production of a group of individuals with the same genotype by asexual reproduction—occurs naturally in many plants and some lower forms of animals such as hydra, sea anemone, planarians and annelids. Although, these animals can multiply asexually by budding or fission of their bodies, for example, they do not totally depend on this form of reproduction. They undergo bisexual reproduction at a certain stage of their life cycle. The majority of animals solely use bisexual reproduction for multiplication. The reason for this is not clear, but sexual reproduction may be instrumental in accelerating the rate of adaptation in evolution, while avoiding irreversible accumulation of detrimental mutations in the face of a competitive and constantly changing environment.

Artificial animal cloning started towards the end of the 19th century when Driesch separated blastomeres of a single 2–4 cell sea urchin embryo and obtained two or four smaller, but complete larvae (Driesch, 1892). The principle of this technique has been applied to various animals including mammals. Mice, rabbits, sheep and pigs were cloned by separating blastomeres of early cleaving embryos (e.g. Willadsen, 1979). Spemann (1938) used another cloning technique. He ligated a recently fertilized newt egg so that cell division occurred in only one of two halves. When the nucleated portion cleaved to 8–16 cells, one of these nuclei was allowed to enter the enclaved portion of the egg before it was completely separated from the rest. This resulted in the development of two normal larvae, indicating that the nuclei of a 8–16 cell embryo retain full developmental potential. Gurdon (1962) cloned adult frogs by injecting enucleated oocytes with the nuclei of endothelial cells of swimming tadpoles. Since, most of the pioneering experiments of cloning were carried out using the frog (Di Bernardino, 1997), it was somewhat

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unexpected that first successful animal cloning using adult somatic cells was achieved in mammals (sheep). This may be due to the preconception, that nuclei of adult somatic cells of the frog (and of all other animals) are differentiated irreversibly and have lost their totipotency. Campbell and his associates—who cloned sheep using an embryo-derived epithelial cell line (Campbell et al., 1996a), believed that cloning with adult somatic cells would be possible (K. Campbell, personal communication). In 1997, they reported the birth of a sheep, which they named Dolly, that was cloned with a mammary gland cell of an adult female sheep (Wilmut et al., 1997). Since Dolly was the only cloned animal for many months, some scientists as well as laymen cast doubt about whether Dolly was really cloned with an adult somatic cell (e.g. Nash, 1998). However, Wakayama et al. (1998) and Kato et al. (1998) confirmed that cloning using adult somatic cells was indeed possible, at least in the mouse and cattle. Subsequently, pigs and goats were cloned with adult somatic cells (Polejaeva et al., 2000; Behboodi et al., 2001).

2. Cloning by nucleus transfer

The transfer of donor cell nuclei into enucleated oocytes has been achieved most commonly by electrofusion of a donor cell with an oocyte (Wilmut et al., 1997). It was also achieved by the fusion of a donor cell with an enucleated oocyte with fusogenic Sendai virus (Tsunoda et al., 1987; Kono et al., 1991), or by injection of a donor cell nucleus into an enucleated oocyte (Wakayama et al., 1998; Onishi et al., 2000; Lacham-Kaplan et al., 2000). Unlike spermatozoa (Swann, 1990; Yanagimachi, 1994; Parrington et al., 1998), embryonic and adult somatic cells lack the ability to activate oocytes, and therefore, nuclear transferred oocytes needed to be activated by artificial means. Electric pulse (single or multiple) has been most commonly used for this purpose, but chemical stimuli or a combination of electric and chemical stimuli can be used as well (Wakayama et al., 1998; Loi et al., 1998; Cibelli et al., 1998; Wells et al., 1999). Perhaps, there is no single protocol for cloning that works for all mammalian species, because, the characteristics of oocytes and donor cells are different from species to species. A protocol that is the best for a given species may not be suitable for other species. Technical details must be worked out for each species.

3. Efficiency of cloning

As the ultimate goal of cloning is to obtain healthy fertile offspring, the efficiency of cloning should be assessed based on the proportion of healthy offspring

produced. In literature the efficiency of cloning has been presented in many different ways. Some investigators calculated the success rate by the proportion of midterm fetuses that developed from the blastocysts transferred into surrogate mothers. Others calculate it from the proportion of live offspring developed from all nuclear-transferred oocytes. Obviously, the former gives a much higher rate than the latter. As some investigators never reported the numbers of nuclear transferred oocytes, it was impossible for this reviewer to calculate the overall success rates in their experiments. To make comparisons easier, I calculated the success rate based on the proportion of live offspring developed from all manipulated (nuclear transferred) oocytes. Obviously, not all the oocytes are successfully enucleated and re-nucleated, so the real success rates of cloning would be lower than described here.

3.1. Cloning using the nuclei of early embryonic cells

We expect that all the nuclei in early embryos, such as those of 2–8-cell embryos, have developmental totipotency. In fact, an enucleated oocyte fused with one of eight blastomeres of an eight cell (sheep) embryo could develop into a normal offspring (Willadsen, 1986), but no one has ever succeeded in producing eight offspring using blastomeres of a single 8-cell embryo. The same is true for 2- and 4-cell embryos (Prather et al., 2000; Kono et al., 1991; Kwon and Kono, 1996). Even the slightest damage to the donor cell (cytoplasm and/or nucleus) may render the nuclei incapable of participating in normal embryo development.

Inner cell mass (ICM) cells and trophectoderm (TE) cells in the expanded blastocysts have been used for cloning, but the success rates were less than 3% (Keefer et al., 1994; Tsunoda and Kato, 1998). Cultured embryonic stem (ES) cells and embryonic germ (EG) cells have also been used for cloning, but success rates were again no more than 3% (Wells et al., 1997; Rideout et al., 2000; Brink et al., 2000).

3.2. Cloning using somatic cell nuclei of fetuses and immature and adult animals

Table 1 shows the success rates of cloning when somatic cells of fetuses and of immature and mature animals were used. It is interesting to note that cloning efficiency is no more than 3%, regardless of the developmental age of the cell (from embryonic to senile adult) and the types of the cell used (see also Table 1 of Solter, 2000). What does this 3% mean? Are only less than 3% of the cells in any tissue developmentally totipotent? If so, can we increase the cloning success rate substantially (ten times or more) by pre-selecting

cells? We may find some tissues in which many cells retain developmental totipotency. We may find some ways to render any type of cell suitable for cloning.

It is important to emphasize that the majority of cloned embryos die before and after implantation (Fig. 1). We noticed that placentae of almost all cloned mouse fetuses at term were unusually large (Wakayama and Yanagimachi, 1999; Ogura et al., 2000; Ono et al., 2001). Abnormal placentae were also noted in the cattle (Cibelli et al., 1998; De Lille et al., 2001). Large fetus syndrome which was first reported in cloned cattle (Cibelli et al., 1998) was also seen in some cloned mice (Eggan et al., 2001). Large offspring syndrome could be due to in vitro handlings of the oocytes and preimplantation embryos (Young et al., 1998), rather than the cloning procedure itself. Some cloned mice died at birth of respiratory problems (Wakayama et al., 1999; Eggan et al., 2001). It is known that many cloned cattle offspring also died of respiratory as well as immunological and multiple systemic dysfunctions (Cibelli et al., 1998; Renard et al., 1999; Wells et al. 1999; Pace et al., 2001; Teneja et al., 2001). Thus far, cloned offspring that survived birth and reached adulthood were the exception rather than the rule. We found that >90% of mouse embryos cloned with cumulus cells had normal chromosome constitutions (Yanagimachi et al., unpublished data). Thus, it is very likely that most cloned embryos/fetuses died of faulty epigenetic reprogramming rather than of genomic problems.

3.3. Exceptional cases

Reports by Kato et al. (1998) and Urakawa et al. (2001) deserve special attention (Table 2). Kato et al. collected cumulus cells or oviductal epithelial cells from a single adult female and introduced their nuclei into enucleated oocytes of Japanese black beef cattle. Of 141 nuclear transferred oocytes, 38 (27%) developed to blastocysts in vitro. They selected 10 of 38 blastocysts and transferred them to ten surrogate females, eight of which delivered eight live calves. Four survived. It is not clear how ten blastocysts were selected for the transfer. If all 38 blastocysts were of the same quality as the selected (transferred) ones and if all were transferred to 38 surrogate females, 30 calves would have been born, 18–19 of which would survive according to my presumptive calculation. This means that about '13%' of all nuclear-transferred oocytes develop into live offspring. It is not clear whether this is an isolated, exceptional case or typical of all experiments they performed. More recently, Urakawa et al. (2001) reported an even higher success rate using the same cattle breed. In a series of their experiments, the nuclei of fetal fibroblasts were introduced into 95 enucleated oocytes. Of these, 27 (28%) developed into compact morulae in vitro. When six morulae were selected and transferred to six surrogate females, four live offspring were obtained. It is not clear whether the authors selected six morulae randomly or chose the 'best-looking' ones. If

Table 1
Comparison of cloning success rates in various animals

Type of donor cell	Species	Total number of reconstructed oocytes	Number (%) of live offspring	Notes	References ^a
<i>Fetal</i>					
Fibroblast	Mouse	3057	5 (0.2)		1
	Bovine	276	4 (1.4)	One died	2
		1896	6 (0.3)		3
	Goat	285	3 (1.1)		4
	Pig	210	1 (0.5)		5
	Sheep	417	14 (3.4)	11 died within 6 months	6
<i>Juvenile</i>					
Skin cell	Bovine	175	1 (0.6)	Died 51 days later	7
Sertoli	Mouse	1190	7 (0.6)		8
<i>Adult</i>					
Mammary gland cell	Sheep	227	1 (0.4)		9
Cumulus cell	Mouse	2468	31 (1.3)		10
	Fibroblast	440	6 (1.4)	2 died	11
		664	8 (1.2)		12
Granulosa	Bovine	552	10 (1.8)		13
	Pig	977	5 (0.5)		14

As many investigators did not describe the original numbers of the oocytes used for nuclear transfer, success rates of cloning shown here are based on the numbers of reconstructed oocytes.

^a (1) Ono et al., 2001; (2) Cibelli et al., 1998; (3) Lanza et al., 2000; (4) Baguisi et al., 1999; (5) Onishi et al., 2000; (6) McCreath et al., 2000; (7) Renard et al., 1999; (8) Ogura et al., 2000; (9) Wilmut et al., 1997; (10) Wakayama et al., 1998; (11) Kubota et al., 2000; (12) Lacham-Kaplan et al., 2000; (13) Wells et al., 1999; (14) Polejaeva et al., 2000.

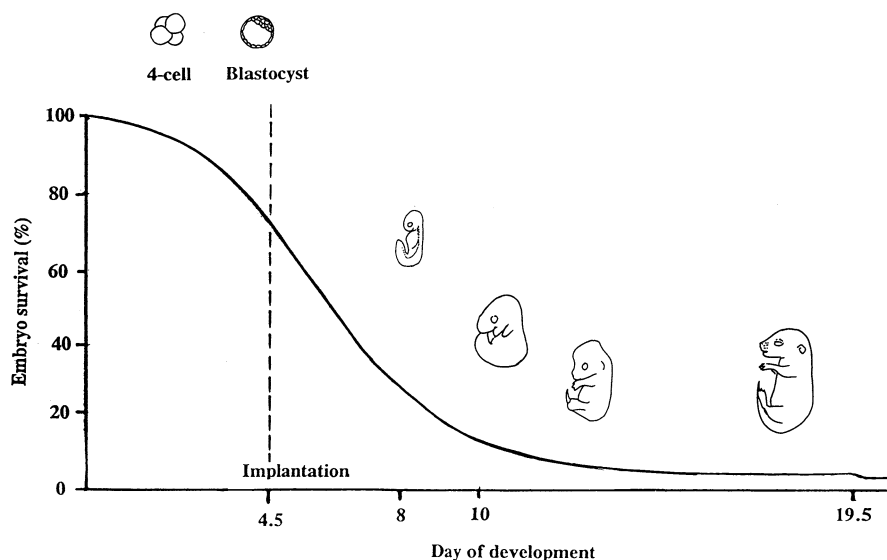


Fig. 1. Development and survival cloned mouse embryos.

all 27 morulae were of the same quality and these authors selected six morulae randomly, we would expect the birth of 18 'healthy' offspring from 27 transplanted morulae. In other words, about 19% of all nuclear-transferred oocytes develop into live offspring. This is the highest cloning success rate as of this writing.

4. Factors contributing to cloning success and failure

4.1. Epigenetic reprogramming

It is well established that differentiating and differentiated cells in embryos and in adults have tissue-specific and cell-specific gene expressions. Genes transcriptionally active in cumulus cells, for example, may be silenced in other types of cells such as skin cells and muscle cells. The fact that enucleated oocytes receiving adult somatic cell nuclei are able to develop into live offspring suggests that molecular functions of the adult somatic cell nuclei were somehow 'reprogrammed' back to the zygotic state. Although the nature and mechanism of the nuclear reprogramming are unknown at present (Di Bernardino, 1997; Kikyo and Wolffe, 2000), we obtained convincing evidence that at least some imprinted genes are reprogrammed. We cloned female mice using tail-tip and cumulus cells. These somatic cells had X chromosomes with genetic markers to distinguish their parental origin. In adult somatic cells, either maternal or paternal X chromosomes were transcriptionally silenced (X-inactivation).

When a cell with a transcriptionally 'inactive' paternal X chromosome was used for cloning, somatic cells of the offspring had paternal X-chromosomes either

transcriptionally active or inactive. Similarly, when a cell with a transcriptionally 'active' paternal X chromosome was used for cloning, paternal chromosomes in somatic cells of this cloned animal were either transcriptionally active or inactive. The results of this and other studies led us to conclude that the genes responsible for X chromosome activation/inactivation were 'reprogrammed' within the oocyte's cytoplasm by nuclear transfer (Eggan et al., 2001).

The fact that the vast majority of cloned embryos die during embryonic development, despite their normal chromosome constitution seems to suggest that epigenetic reprogramming of the reconstructed oocytes is incomplete in most cases. We compared the genome-wide methylation status of CpG islands in placenta, skin and kidney cells of cloned and normal (control) mice and found that in all cloned offspring several CpG islands were aberrantly methylated or unmethylated. CpG islands, with high densities of the dinucleotide CpG, are about 1 kb long and commonly found upstream of many housekeeping genes (Gardiner-Garden and Frommer, 1987). There are CpG islands with tissue- and 'parent'-specifically methylated regions (Ohgane et al., 1998). Interestingly, the extent of aberrant DNA methylation varies among phenotypically normal offspring (Ohgane et al., 2001). Apparently, slight aberrations in DNA methylation in somatic cells are not life threatening. Extensive aberrations, on the other hand, must be fatal to cloned embryos. Although, cloned offspring that survived birth may be genomically identical with the original animal, they may not be phenotypically identical with the original animal, as well as their siblings because of random errors in epigenetic gene expression.

It was somewhat unexpected that the so-called ‘totipotent’ ES cells were not as easy to use for cloning as we originally anticipated. In our experiments, an average of less than 3% of nucleus-transferred mouse oocytes developed into live offspring (Wakayama et al., 1999; Rideout et al., 2000). We found that the epigenetic state of the ES cells cultured in vitro was unstable and varied even between clonally related cells. Imprinted gene expression of ES cell populations seems to be inherently unstable. It is rather amazing that some cloned embryos survived the birth despite a wide range of imprinting errors (Humphreys et al., 2001). We found that the fetuses cloned with ES cells of hybrid mice survived birth, whereas, those cloned with inbred mouse ES cells seldom did (Rideout et al., 2000; Eggen et al., 2001, Tables 3 and 4). Newborns had the best chance of survival when both donor cells and recipient oocytes were from hybrid mice. These facts seem to suggest that epigenetic errors in cloned offspring are overcome, to some extent, by genomic heterogeneity.

4.2. Cell cycle of donor cell and recipient oocyte

The importance of cell cycle coordination between donor cells and recipient oocytes has been stressed (Campbell et al., 1996a; Campbell, 1999). Although, it was once thought that donor cells must be at the G0 stage of a cell cycle during nuclear transfer for successful cloning (Wilmut et al., 1997), subsequent studies revealed that this is not necessarily the case. The cells at the G1 or G2 stage and even those at the M stage can be used as long as the cloned embryos are diploid (Campbell et al., 1996b; Tani et al., 2001; Ono et al., 2001). When we injected cumulus cell nuclei (which were mostly at the G0 and G1 stage) into mouse oocytes and then activated these oocytes, part of cumulus chromosomes (2n) were shed into pseudo-second polar bodies, resulting in the production of hypoploidy embryos which were doomed to die. We avoided this problem by culturing nucleus-injected oocytes in a medium, which activated oocytes and inhibited their cytokinesis simultaneously. The medium we used was a Ca^{2+} -free medium containing Sr^{2+} and cytochalasin B. Sr^{2+} activated the oocytes and cytochalasin B inhibited

cytokinesis of the activated oocytes (Wakayama et al., 1998). Thus all (2n) chromosomes of the cumulus cell were retained within an activated oocyte. After DNA replication and the first cleavage, diploid embryos started to develop. When donor cells at the G2 stage were used, we activated reconstructed oocytes in the normal medium, allowing half of the chromosome (2n) to be shed into the pseudo-polar body. After DNA duplication and the first cell division, each blastomere received 2n chromosomes and the embryos started to develop.

Twenty years ago, Illmensee and Hoppe (1981) reported the birth of mice after injection of ICM nuclei into enucleated pronuclear eggs, but no one else could confirm this report (McGrath and Solter, 1984; Tsunoda et al., 1987). We injected enucleated pronuclear eggs with mouse cumulus cell nuclei, instead of ICM nuclei, and found that chromosomes of donor (cumulus) cells were extensively pulverized before the first cleavage. This did not happen when the nuclei were injected into enucleated Met-II oocytes (Wakayama et al., 2000a). The major chromosomal damages would be catastrophic to embryo development. Even ‘minor’ sub-chromosomal damages would result in developmental arrest or the death of embryos/fetuses. We found that over 90% of mouse embryos cloned with cumulus cell nuclei had normal chromosome constitutions (Yanagimachi et al., unpublished data), yet the majority of them die before and after implantation. It seems that many cloned embryos/fetuses die of problems at the gene level rather than the chromosomal level.

5. Can we increase cloning efficiency

It is natural to speculate that the oocyte’s cytoplasm has ‘magic ingredients’ which reprogram epigenetic imprinting from the somatic state to the zygotic state. Attempts to increase the efficiency of cloning by increasing the exposure time of donor nuclei to the oocyte’s cytoplasm have met with some success (Tsunoda and Kato, 1997; Kato et al., 1999; Wells et al., 1999; Ono et al., 2001), but not in very dramatic ways. It is unknown whether all or only a few cells in a given tissue are potentially reprogrammable.

Table 2
Highly successful cloning in Japanese black cattle

Type of donor cell	Total number reconstructed oocytes	Number (%) of morula/bl.	Number transferred to surrogate (number of surrogate)	Number of live offsprings	Note	References ^a
Adult cumulus and oviductal cell	141	38 (27) Blast.	10 (10)	8	4 died at or soon after birth	1
Fetal fibroblast	92	24 (26) Morula	8 (8)	7	1 still-born	2

^a (1) Kato et al. 1998; (2) Urakawa et al., 2001.

Table 3
‘Hybrid vigor’ in mouse cloning using adult cumulus cells

Cumulus cell donor	Recipient oocyte	Number of reconstructed and active	Number of transferred embryos	Number of live offspring
<i>Inbred</i>				
DBA/2	DBA/2	46	44	0
129/sv	129/sv	19	19 ^a	0
DBA/2	B6D2F1	354	308	1
129/sv	B6D2F1	461	212	5
B57BL/6	B6D2F1	1006	413	0
<i>Hybrid</i>				
B6D2F1	B6D2F1	2924	1563	50
B6C3F1	B6D2F1	663	554	18

Wakayama and Yanagimachi, 2001.

^a Transferred at 2-cell stage; no ability to develop to blastocysts in vitro.

Beyond a doubt, technical skill greatly attributes to the success rate of cloning. For example, we failed to clone mice using adult Sertoli cells (Wakayama et al., 1998), yet Ogura et al. (2000) succeeded by using the same technique, but with Sertoli from immature animals rather than mature animals. Thus, it is possible that the cells with which we cannot clone animals today, may one day become the cells of our choice. As mentioned already, Japanese black beef cattle was exceptional in that as high as 19% of reconstructed oocytes developed into live offspring (Kato et al., 1998; Urakawa et al., 2001). As the methods used in these experiments were not particularly unique, the reason for such an astonishingly high success rate is unknown. At any rate, it should be noted that even with these animals, 80% or more of nuclear transferred oocytes die some time before birth. A trial and error approach may discover better cells for cloning and develop more efficient cloning techniques, but it would be wiser first to understand the molecular mechanisms of epigenetic imprinting and reprogramming before we find a way to make cloning safer and more efficient. If Japanese black beef cattle consistently yield very high rates of successful cloning, this animal may provide some clues to correct or overcome epigenetic errors in cloned animals.

6. Biological safety of cloning

We were confounded when we learned that ‘Dolly’, the first cloned sheep, had shorter telomeres (Shiels et al., 1999). This may mean that Dolly has a shorter life expectancy than aged matched normal sheep. According to Lanza et al. (2000), however, telomeres in the cattle cloned with aged fetal cells were actually longer than those of age-matched normal animals. Tian et al. (2000) also found that the telomere length of ten cloned calves derived from cultured fibroblasts was indistin-

guishable from that of their age-matched controls. We examined the telomere length of the mice sequentially cloned for six generations and found no evidence of telomere shortening (Wakayama et al., 2000b). Although, further studies are needed, the cytoplasm of mature oocytes seems to ‘rejuvenate’ the nuclei of adult donor cells. Over 250 mice cloned with adult cumulus cells, tail–tip cells and embryonic neural cells most had a normal life span of 2–3 years without serious health problems before they died (Yanagimachi et al., unpublished data), except for the postpubertal obesity in some mice (Tamashiro et al., 2000).

It is possible that even successfully cloned animals with normal life span are not entirely normal in their gene expression. Those with relatively minor non-life-threatening errors in gene expression will survive. Unless we find the ways to completely ‘reprogram’ the epigenetic imprinting status of donor nuclei from the somatic to the zygotic type, cloned animals will harbor potential health problems.

We found that offspring of cloned female mice that were mated with cloned male mice were all normal and fertile (Yanagimachi et al., unpublished data). All epigenetic problems in the parents seem to be erased when cell nuclei go through the germ line.

7. Use of cloning

Cloning would be acceptable for the production of medically and pharmaceutically valuable farm animals (e.g. Baguise et al., 1999; Polejaeva et al., 2000) as long they are reasonably healthy and their products (e.g. meat, tissues, organs, special cells and their secretions, including milk) are safe for human use. Cloning pet animals (cats, dogs and horses, for example) is appealing to some people, even though cloned animals may not be 100% identical phenotypically with the original animals. Cloning of highly inbred animals could be

Table 4
'Hybrid vigor' in mouse cloning using ES cells

Donor cell genetic background	Recipient oocyte	Number of reconstructed and active	Number of transferred embryos	Number of live born	Number of offspring survived
<i>Inbred</i>					
129	B6D2F1	418	76	8	0
		830	108	13	0
<i>Hybrid</i>					
129 × C57BL	B6D2F1	227	34	7	7
		66	18	3	2
129 × FVR	B6D2F1	69	19	2	2
129 × M. cast.	B2D2F1	143	27	3	2

Wakayama and Yanagimachi, 2001; Eggen et al., 2001.

difficult as already shown in inbred mice. Cloning endangered species is a noble idea, but may not be as easy as we think. The production of a herd of cloned animals with very limited or no genomic diversity would quickly lead to the second extinction crisis. Cloning of extinct species (such as the woolly mammoth) can be done provided some of their cells have intact DNAs (genomes). Techniques, which repair extensively altered/damaged genomes, are not available at present.

It is possible to allow embryonic and adult stem cells differentiate, in vitro or in vivo, into various cell types (Robertson, 1987; Thompson et al., 1998; McDonald et al., 1999; Fuchs and Segre, 2000; Lumelsky et al., 2001). Someday, it will become possible to 'reprogram' the nucleus of a fully differentiated adult somatic cell such that the cell re-differentiates into an entirely different type of cell. However, we must be fully aware that incomplete epigenetic reprogramming of the donor cell nucleus may lead to the production of cells and tissues with dormant cytopathological problems.

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