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Cloning the laboratory mouse

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A brief account is given of early attempts to clone mammals (mice) by transferring cells (nuclei) of preimplantation embryos into enucleated oocytes, zygotes or blastomeres of two-cell embryos. This is followed by a brief review of recent successes using adult somatic cells: mammary gland cells for sheep, muscle cells for cattle and cumulus cells for mice. We have developed a technique for cloning the laboratory mouse by transferring cumulus cell nuclei into enucleated oocytes. With this technique, we have produced a population of over 80 cloned animals, and have carried the process over four generations. Development and fertility of these appear normal. However, the yield is very low; only approximately 1% of injected oocytes are carried to term. The challenge is now to understand the reason for this high loss. Is it a problem of technique, genomic reprogramming, somatic mutation, imprinting or incompatible cell cycle phases?

Key words: cloning / mouse / nucleus / embryo / cell differentiation

General introduction

RECENT SUCCESS IN CLONING of sheep, mice and cattle using adult somatic cells[1–3] has drawn considerable attention from scientists and laymen alike. Some scientists consider cloning a new research tool to study basic biological phenomena, such as cell differentiation and redifferentiation and cell aging and rejuvenation. Others may consider this a new method of rapidly reproducing animals of economical and/or medical value. Some laymen may see cloning as the way to revive their aging/dying pets. Still others regard cloning as the only way to revive their dying or dead children or even themselves.

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Mice have been the most commonly used laboratory animals because their small size allows housing large numbers in a relatively small space. As a result, the information available on their reproduction, development and genetics[4,5] is unrivalled so that numerous advanced techniques have been developed using mouse embryos.[6,7] The short generation period of approximately 3 months is certainly an advantage for the study of long-term genetic effects of biological manipulation, such as cloning.

Mouse cloning using cell nuclei of preimplantation embryos

Table 1 shows the years when the first live, cloned offspring of mice, sheep, cattle and rabbits were born. The types of donor cells used in these studies are also shown in this table. Illmensee and Hoppe[8] were the first to report the production of cloned mice by mechanical injection of inner cell mass (ICM) cell nuclei into enucleated zygotes. However, other investigators were unable to repeat this experiment using ICM cells[9,10] and embryonic stem (ES) cells,[11] and it was only very recently that Tsunoda and Kato[13] succeeded in cloning mice using ICM cell nuclei, but with a very different method. First, a single ICM cell was fused with an enucleated oocyte using Sendai virus. The oocyte was then activated by an electric shock. When the egg reached the two-cell stage, the karyoplast of each blastomere was fused with each of enucleated blastomeres from another two-cell embryo developed from a normally fertilized egg. Two out of 139 fused couples thus produced developed into live young. Two more young were obtained after fusion of enucleated blastomeres with trophectoderm cells. Thus, the totipotency of ICM cells (as well as trophectoderm cells) is now confirmed.

It was Tsunoda *et al*[10] who first produced cloned mice using cells of early preimplantation embryos. They fused one blastomere of a two- to eight-cell embryo with either an enucleated zygote or one

Table 1. The year when cloning was first reported for different species

Year	Species	Authors	Donor cell	Recipient enucleated cell
1981	Mouse	[8]	ICM cell	Zygote
1986	Sheep	[40]	8-cell embryo	Met II oocyte
1987	Cattle	[41]	9–15 cell embryo	Met II oocyte
1990	Rabbit	[42]	8–16 cell embryo	Met II oocyte

blastomere of another two-cell embryo. Sendai virus was used to mediate cell fusion. The transfer of 160 reconstructed embryos to foster mothers resulted in the birth of a total of eight young. Since then, other investigators, including Tsunoda and his associates themselves, have obtained many cloned mice by transferring the nuclei of two- to eight-cell embryos into enucleated mature (Met II) oocytes, zygotes or the blastomeres of two-cell embryos (Table 2).

According to McGrath and Solter,[9] the nuclei of four- or eight-cell embryos introduced microsurgically into enucleated zygotes or by using Sendai virus never supported the development of embryos to term. However, later investigators found that this was not the case when donor cell nuclei were introduced into oocytes by using electrofusion or Sendai virus.[14–17] It is interesting that nuclei of eight-cell mouse embryos introduced into enucleated zygotes could support only one or two cleavages[18] or at best support embryo development only up to the blastocyst stage.[19] In contrast, the same nuclei introduced into enucleated oocytes or enucleated blastomeres of two-cell embryos supported development till mid-term[17,20] or even to full-term.[10,14] Apparently, the success/failure of cloning experiments is largely dependent on the technical skill of investigators. Furthermore, the cell cycle phases of the donor and host cells at the time of fusion or nuclear transfer seem to contribute greatly to the outcome of the experiments.[21,22] Even though many investigators believe that enucleated zygotes and blastomeres of two-cell embryos are the best recipients of donor nuclei, we think that enucleated mature (Met II) oocytes, first used by Willadsen,[40] are better suited for the production of live, cloned offspring, at least for the mouse (see below) and perhaps for many other species. When enucleated oocytes are used as recipients, donor cells must be at either G0 or G1 phase of the cell cycle in order to obtain normal diploid embryos.[21]

Table 2. Donor and recipient cells used for mouse cloning: only the studies resulting in the birth of live offspring are listed here

Nucleus donor	Recipient	Reference
2–4 cell embryo	Met II oocyte	15,43–45
2–4 cell embryo	2-cell blastomere	46
2–8 cell embryo	Met II oocyte or zygote	14–17
4–8 cell embryo	2-cell blastomere	10
4–cell or morula embryo	Met II oocyte	16

Use of embryonic cells for production of mouse chimeras

ICM cells aggregated with eight- or 16-cell mouse embryos or those injected into blastocysts can contribute to the production of chimeric fetuses or young, suggesting totipotency of ICM cells.[23–25] Totipotency of embryonic stem (ES) cells was demonstrated in the same way.[26,27] According to Kato and Tsunoda,[28] fetal germ cells of 15.5–16.5-day postcoitum (*dpc*) mouse embryos can contribute to the formation of chimeric embryos which, however, are apparently unable to develop to term.

Cloning using fetal somatic cells

Campbell *et al*[29] cloned five sheep by fusing enucleated oocytes with fetal epithelium cell line cultured for six to 13 passages. Cibelli *et al*[30] reported the birth of three male calves following electrofusion of enucleated mature oocytes with fibroblasts collected from a 55-*dpc* fetus. They believed that fibroblasts were at the G1 stage of the cell cycle during fusion with oocytes. Cloning of the mouse using fetal somatic cells has not been reported. Even primordial germ cells have not been used successfully for cloning.[12]

Cloning using adult somatic cells

The sheep 'Dolly' was the first animal ever cloned using adult somatic cells.[1,31,32] Donor cells were mammary gland cells from a 6-year-old ewe. The cells were electrofused with enucleated mature oocytes. Only one of 385 reconstructed couples developed to term. A key to this success, according to the authors, was to bring donor cells to the G0 phase of the cell cycle by 'starving' them before fusion with enucleated oocytes. Recently the mice[2] and cattle[3] were cloned using adult somatic cells. Two calves (one male and one female) were born after fusion of muscle cells with enucleated oocytes.[3]

Mouse clones were obtained using cumulus cells.[2] According to Schultz *et al*,[33] more than 90% of cumulus cells surrounding recently ovulated oocytes are in G0/G1 phases of the cell cycle. The first live clone mouse was born on 3 October, 1997 and was named 'Cumulina'. She proved to be fertile. As of 30 October 1998 we have over 80 mice cloned using cumulus cells, some of them being the 4th generation of the clone (clone of clone of clone of clone). All cloned mice proved to be fertile.

The cloning procedure we used is shown in Figure 1. Donor oocytes (ooplasm donors) were collected from recently superovulated black (B6D2F1) mice. Their Met II chromosome–spindle complexes were removed microsurgically. Meanwhile, cumulus cell–oocyte complexes were collected from recently superovulated agouti (B6C3F1) females. Cumulus cells were dispersed with hyaluronidase, washed, the plasma membranes disrupted and their nuclei injected individually into enucleated oocytes. We used an injection pipette housed in a piezo-impact pipette drive unit. This unit, which drives the pipette a short distance (e.g. 0.5 μm) very rapidly, allows the pipette to drill the zona pellucida easily and penetrate the oocyte's plasma membrane without lysing the oocyte.[34] We think that our success in cloning mice is due, in part, to the use of this special pipette-driving unit.

We used a thin-walled, flush-ended pipette (approximately 7 μm in diameter) for injection. The plasma membrane of the cumulus cell (8–15 μm) was broken when the cell was drawn in and out of the pipette a few times. In most cases the nuclei we injected were devoid of visible cytoplasmic material. Each nucleus was injected within 5 min of its isolation.

The cumulus cell nucleus injected into an enucleated oocyte transformed into disarrayed chromosomes. This disorder reflects an unusual situation in which single, condensed chromatids are each attached to a single pole of the spindle and are therefore not aligned on a metaphase plate. After standing for 1–6 h in the medium, the oocytes were exposed to a medium containing both Sr^{2+} and cytochalasin-B. The former activated the oocytes,[35] while the latter prevented subsequent polar body formation and therefore chromosome expulsion. We chose Sr^{2+} for mouse oocyte activation because, unlike an electric shock, it induces repetitive rises in free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of the mouse oocyte.[36] Repetitive $[Ca^{2+}]_i$ rises are known to be a salient feature of oocyte activation in a wide variety of animals.[37,38] Activated oocytes each contained two or more pseudo-pronuclei in which DNA replication takes place. We transferred developing embryos into albino (CD-1) foster females. Figure 2 shows the original cumulus cell (nucleus) donor and two generations of cloned mice generated in this way. In the procedure illustrated here, the donor (cumulus cell)

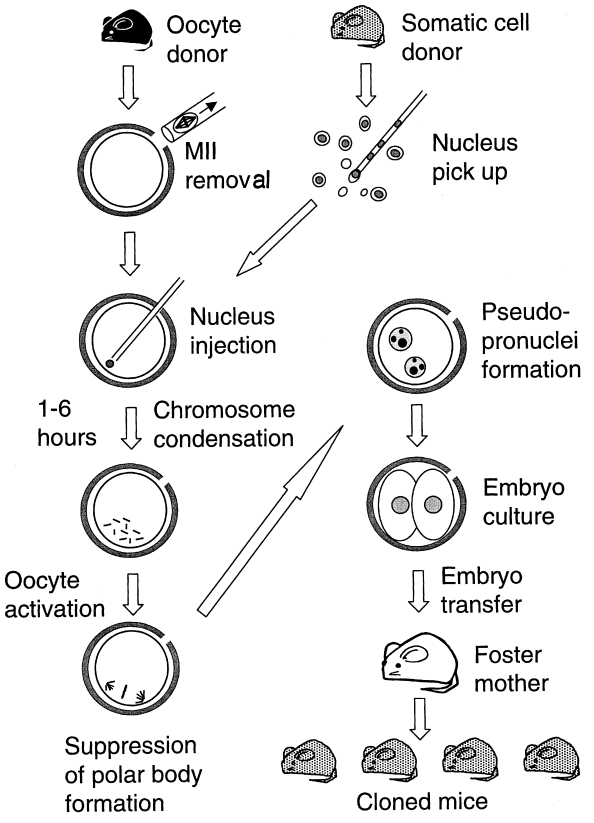

Figure 1. A diagram illustrating the cloning procedure we used[2] and described in the text.

nucleus was first injected into an enucleated oocyte followed by oocyte activation. When we activated enucleated oocytes first, then injected them with the donor nuclei (approx. 1 h later), all reconstituted oocytes fragmented without even developing into normal two-cell embryos (Wakayama and Yanagimachi, unpublished data).

It was thought that cloning mice would be difficult because the mouse embryonic genome begins to be expressed at the two-cell stage or even during the last zygotic stage, thus leaving too little time for the transferred nucleus to be reprogrammed.[39] We left injected (cumulus cell) nuclei in the cytoplasm of unactivated oocytes for 1–6 h. Reprogramming, if it occurs, must be completed at least in the oocytes that successfully developed to live offspring. It is important to note that although large proportions of reconstructed oocytes could develop to blastocysts and implant, most were unable to develop to term (Figure 3). The reasons for this are not at all clear at present. Genomic heterogeneous nature of the donor cells and unpredictable nature of the reprogramming processes[39] could be the causes of this disappointing and puzzling phenomenon.

We found that embryos developed from enucleated oocytes receiving Sertoli cell and neuron nuclei could develop and implant fairly well, but none de-

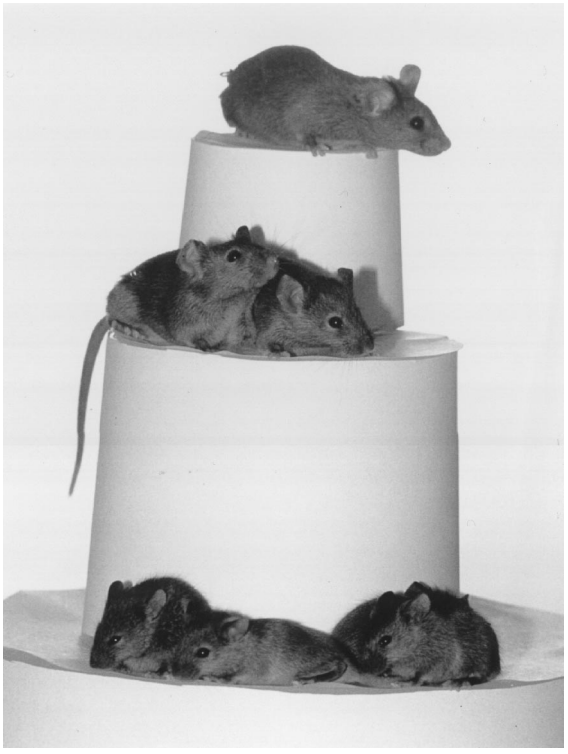

Figure 2. Two generations of cloned mice. The top row is the original cumulus cell donor. The second row is two clones from the above. The third row is four clones of the clones (with permission of ProBio America, Inc.).

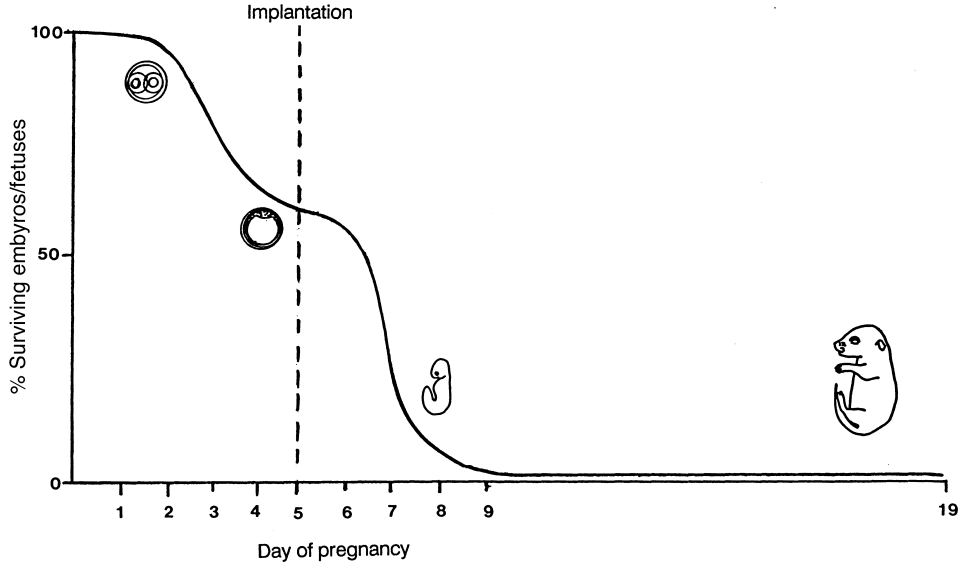

Figure 3. A diagram illustrating a sharp decline in the survival of cloned embryos during their development *in vitro* (before implantation) and *in vivo* (after implantation). Only approximately 1% of cumulus nucleus-injected oocytes develop to term.

veloped to term.[2] These cells were believed to be at the G0 phase of the cell cycle. Apparently G0 phase may be a preferable,[1] but not necessarily the only, condition necessary for successful cloning. In our experiments we used only three types of somatic cells. Thus far cumulus cells were better than Sertoli cells and neurons for cloning purposes, but there is no reason to believe that the latter two are inferior to cumulus cells. Technical improvement may make these cells as efficient as cumulus cells for cloning. It is very likely that there are still other cell types that are better suited for cloning than cumulus cells. Someday any types of cell could be used for cloning. Cloning experiments have just begun.

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