Mice cloned from embryonic stem cells

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Cloning allows the asexual reproduction of selected individuals such that the offspring have an essentially identical nuclear genome. Cloning by nuclear transfer thus far has been reported only with freshly isolated cells and cells from primary cultures. We previously reported a method of cloning mice from adult somatic cells after nuclear transfer by microinjection. Here, we apply this method to clone mice from widely available, established embryonic stem (ES) cell lines at late passage. With the ES cell line R1, 29% of reconstructed oocytes developed *in vitro* to the morula/blastocyst stage, and 8% of these embryos developed to live-born pups when transferred to surrogate mothers. We thus cloned 26 mice from R1 cells. Nuclei from the ES cell line E14 also were shown to direct development to term. We present evidence that the nuclei of ES cells at G_1- or G_2/M-phases are efficiently able to support full development. Our findings demonstrate that late-passage ES cells can be used to produce viable cloned mice and provide a link between the technologies of ES cells and animal cloning. It thus may be possible to clone from a single cell a large number of individuals over an extended period.

Mammalian cloning has been achieved by introducing the nucleus of a donor cell into an enucleated oocyte. There are two strategies for cloning mammals by nuclear transfer. The first to be described is based on cell fusion and has been applied to sheep (1–4), cows (5–8), and goats (9), by using acutely isolated or primary cultured cells. We recently reported a second approach in mice based on nucleus microinjection (10). Our method involved the microsurgical isolation of a nucleus followed by its piezo-electrically actuated microinjection into an enucleated, unfertilized metaphase II oocyte. In this way, viable cloned mice have been produced from the nuclei of cumulus cells freshly isolated from adult females (10) and short-term cultured cells derived from tails of adult males (11). Taken together, all reports of mammalian cloning thus far describe the use of freshly isolated cells, or cells from primary, often ill-defined cell cultures as nucleus donors.

Can mammals be cloned from well-established cell lines? The answer is not obvious. With time, nuclei from cultured cells may lose their ability to direct full and normal embryonic development. This loss may correlate with the accumulation of genetic or epigenetic alterations. Mammalian cells adapted to *in vitro* culture generally undergo mutations with time, including gross karyotypic alterations, and such genomic changes are unlikely to be compatible with normal embryonic development. Epigenetic changes such as loss of imprinting and/or alterations in methylation status may affect the ability of nuclei to direct normal development (12).

Mouse embryonic stem (ES) cell lines exhibit unusual karyotypic stability. ES cell lines are derived from the inner cell mass of embryos at the blastocyst stage and can be cultured *in vitro* for many passages without becoming evidently aneuploid (13–15). These cells exhibit developmental pluripotency: they can be used to generate chimeric mice containing an ES cell contribution that is apparently unrestricted in terms of cell type (15, 16). However, for ES cells to contribute to full development, they must be accompanied by heterologous embryonal cells in the developing embryo (hence, the embryo is chimeric). The heterologous cells are from diploid (16, 17) or tetraploid (18–20) embryos. The requirement for "carrier" cells raises a question as to whether or not it is possible for a single ES cell to direct the clonal development of a normal individual.

Cloning mice from ES cells also has practical implications for manipulating the genome. Gene targeting in ES cells has been widely used to create manifold strains of mice with targeted mutations (21, 22). If nuclei from ES cell lines, even after prolonged *in vitro* culture, could be used to produce viable, fertile cloned animals, they would be a prime choice for engineering the mammalian genome through cloning.

Here, we report that with varied parameters, microinjection of ES cell nuclei into enucleated oocytes enables development to term and beyond. We cloned five live-born offspring from the cell line E14 and 26 from R1; respectively, one and 13 mice survived. A single ES cell nucleus thus can direct the full development of an individual.

Materials and Methods

ES Cell Lines. The ES cell line E14 (17) was derived from the inbred mouse strain 129/Ola in 1985 by Dr. Martin Hooper in Edinburgh, Scotland, and obtained by P.M. from him in 1990 at passage 11. Cells were further expanded in three different laboratories for at least another 10–22 passages (1:3 to 1:4 splits) under a variety of conditions. Thus, the cells used for this study had undergone at least 22–33 passages, corresponding to 33–66 cell divisions from the time the E14 line was established. In our hands, germ-line transmission is obtained with E14 clones carrying targeted mutations at a frequency of at least 80% (23–27). The ES cell line R1 (19) was subcloned by Dr. Markus Stoffel at The Rockefeller University 14 passages after its establishment; an aliquot was obtained from him by P.M. at passage 4, and further grown for another seven passages. Thus, the R1 cells used for these experiments had been cultured for at least 32 passages (with the subcloning occupying an estimated seven passages).

ES Cell Culture Conditions. Cells were grown in DMEM for ES cells (Specialty Media, Phillipsburg, NJ) supplemented with 0.5–15% heat-inactivated FCS (HyClone), 1,000 units of leukemia inhibitory factor/ml (Gibco), and the following reagents (Specialty Media): 1% penicillin-streptomycin, 1% L-glutamine, 1% nonessential amino acids, 1% nucleosides, and 1% β-mercaptoethanol. Cells were split 1:3 or 1:4 every 24 h, reflecting an approximate cell cycle period of 12 h. Routine culture was on a feeder layer of mitomycin-C treated primary embryonic fibroblasts, derived from embryonic day 13.5 mice heterozygous for the T cell antigen receptor-δ knockout mutation (25). Cells were cultured in feeder-free conditions for at least 1 week before micromanipulation; by the time of nuclear transfer no feeder cells were detectable in the culture.

Routine culture was in medium supplemented with 15% FCS and 1,000 units/ml of leukemia inhibitory factor. The concentration of FCS was reduced stepwise. At a concentration of 5% FCS, cells divided almost as vigorously as they did at 15%, with little overt differentiation. However, growth of the cells slowed noticeably when the FCS concentration was 4% or less. Extensive cell death occurred when the cells were cultured in medium

Abbreviations: ES, embryonic stem; dpa, days postactivation.

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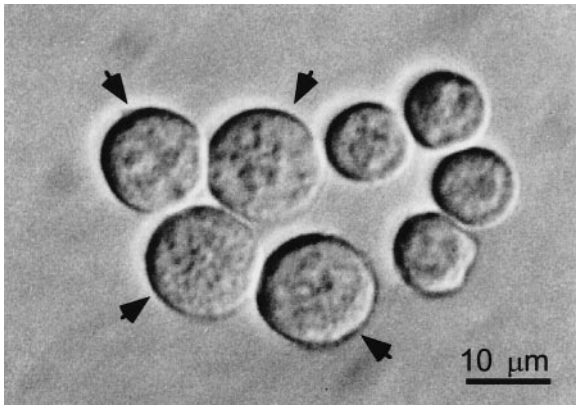

Fig. 1. Selection of E14 cells for subsequent nucleus microinjection into enucleated mouse oocytes. Representatives of small (average diameter 10 μm) and large (arrowhead; average diameter 18 μm) cells were grouped together by using micromanipulators to show contrasting sizes. They are viewed here by Hoffman modulation contrast microscopy at $\times$200 magnification.

with 0.75% or 0.5% FCS, conditions previously reported to "starve" certain cell types and cause them to exit the cell cycle (i.e., enter G_0) (2–4, 6, 8, 9).

Where appropriate, R1 ES cells were exposed to the microtubule disrupting agent nocodazole (Sigma) at 3 μg/ml for 12 h (28, 29). Cultures treated in this way altered dramatically compared with untreated cultures, with the appearance of many rounded and floating cells.

Nuclear Transfer. Nuclear transfer was essentially as described (10). B6D2F1 (C57BL/6 $\times$ DBA/2) oocytes were used as recipients. Cells selected on the basis of their size and plasma membrane integrity were approached with the injection needle (internal diameter 6 μm for small cell experiments and 10 μm for large cell experiments; see *Results*) and suction was applied to break the plasma membrane and isolate the nucleus. After microinjection of nuclei from large ES cells or nocodazole-treated ES cells, cytochalasin B was omitted from the oocyte activation protocol. Microinjections were at room temperature (25–29°C in Honolulu). Culture was in CZB medium (30).

PCR Analysis of Genomic DNA. PCR amplification of the microsatellite markers D7Mit22 and D4Mit204 (31) was performed by using primer pairs (Mappairs) obtained from Research Genetics, Huntsville, AL. DNA was extracted from tail or ear biopsies, or from placentae. For the amplification of *Zfy*, primers ZFY3 and ZFY4 (32) were used. Reactions (20 μl) were subjected to 34 cycles of 1 min 95°C, 1 min 60°C, and 2 min 72°C, and products were separated on a 4% agarose gel (Genamp PCR, Perkin–Elmer) before visualization.

Table 1. Examination of ES cell nuclei 4 h after transfer into enucleated oocytes, without activation by Sr^{2+}

		No. (%) of oocytes with			
ES cells*	No. of oocytes examined	Intact nucleus	Chromosomes	Indistinct chromatin condensation	Pseudo-pronuclei (spontaneous activation)
Small	75	4 (5.3)	38 (50.7)	32 (42.7)	1 (1.3)
Large	59	4 (6.8)	40 (67.8)	11 (18.6)	4 (6.8)

*Average cell diameters are 10 μm (small) and 18 μm (large).

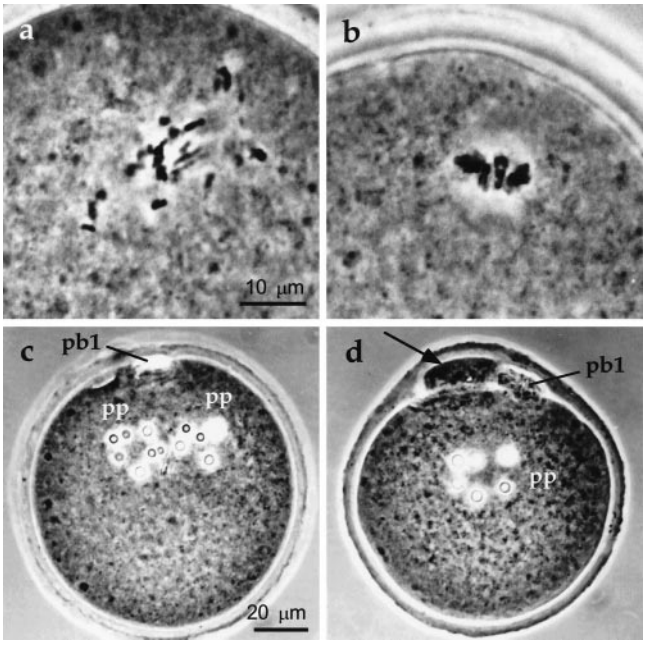

Fig. 2. The behavior of nuclear material from small or large ES cells after microinjection into enucleated mouse oocytes. (*a*) Small cell nuclear components condense to form a disorganized chromosome array 3–4 h after microinjection. (*b*) Large cell nuclear components condense to form an orderly chromosome array (resembling the maternal metaphase plate) 3–4 h after microinjection. (*c*) A one-cell embryo formed by the microinjection of a small E14 cell nucleus into an enucleated oocyte and activation by exposure to Sr^{2+} for 6 h in the presence of cytochalasin B. Two pseudo-pronuclei (pp) are discernible, each containing several nucleoli; remnants of the first polar body (pb1) are also visible. (*d*) A one-cell embryo formed by the microinjection of a large E14 cell nucleus into an enucleated oocyte and activation by exposure to Sr^{2+} for 6 h in the absence of cytochalasin B. A degenerate first polar body (pb1), single pseudo-pronucleus (pp), and pseudo-second polar body (indicated by an arrow) are visible.

Results

The Fate of ES Cell Chromosomes After Nuclear Transfer into Enucleated Oocytes. In initial studies, we opted to use the ES cell line E14 (17), because it is representative of widely used, multiply passaged, pluripotent ES cell lines. These cells are dividing vigorously in our culture conditions. Given that the cell cycle stage is a determinant of cloning efficacy, the choice of nucleus donor in these experiments is problematic; rapidly dividing cell populations are normally asynchronous with respect to the cell cycle. We therefore took the size of the cell as an indicator of its stage in the cell cycle. Typically, cell diameters were approximately 10 μm for "small" cells and 18 μm for cells considered "large" (Fig. 1). We surmised that small cells were in the G_1-phase (2C DNA) while large cells corresponded to those in G_2/M-phases (post-S-phase, 4C DNA).

In a first series of experiments, enucleated oocytes receiving ES cell nuclei were not subjected to an activating stimulus. Such reconstituted oocytes therefore remained in metaphase II. When examined 2–4 h after microinjection of the nuclei of small cells, 51% of reconstituted oocytes possessed condensed chromosomes (Table 1) arranged in a scattered fashion (Fig. 2*a*), which is reminiscent of the chromosomal configuration after injection of cumulus cell nuclei (10). It reflects an unusual situation. Individual univalent chromosomes (transplanted from a putatively G_1-phase nucleus into a metaphase II cytoplasm) each become tethered to only a single pole of the spindle; their chromatids are not aligned with a homologous partner. By contrast, 68% of oocytes injected with nuclei from large cells possessed condensed chromosomes aligned in a regular array (Fig. 2*b*) resembling that of maternally derived chromosomes in mature metaphase II oocytes. Presumably, chro-

Table 2. Examination of ES cell nuclei after transfer into enucleated oocytes, 6 h after oocyte activation by Sr^{2+}

ES cells*	No. of oocytes examined	No. (%) of incoming nuclei transformed to one or more pseudo-pronuclei (pp) with or without extrusion of a pseudo-second polar body (ppb)				
		Not transformed	1pp and ppb	1pp	2pp	3pp
Small	23	0	0	1 (4.3)	18 (78.3)	4 (17.4)
Large	22	1 (4.5)	15 (68.2)	3 (13.6)	3 (13.6)	0

*Reconstructed oocytes were activated in the presence (small cells) or absence (large cells) of cytochalasin B as described in *Materials and Methods*. Cell sizes are as for Table 1.

mosomes in large E14 cells already had completed a duplicative round of DNA synthesis (S-phase) before transfer, to generate sister chromatids that became attached to opposite poles of the spindle within the oocyte (33). This configuration would permit an orderly chromosome array after nuclear transfer (34).

In a second series of experiments, we supplied the reconstituted cells with an activation stimulus (strontium ions, Sr^{2+}) after nuclear transfer. Anticipating potential differences in the DNA content of small and large cells, we accordingly adapted the nuclear transfer protocol used for each cell type. Oocytes reconstructed with the nucleus of a small cell were removed from CZB culture medium ≈4 h after nuclear microinjection and placed into medium containing Sr^{2+} (to activate them) and cytochalasin B (to prevent cytokinesis). We included cytochalasin B because in its absence donor chromosomes would be extruded quasi-randomly into a pseudo-second polar body, generating inviable, hypodiploid embryos (10, 35, 36). Of the embryos we generated from small cell nuclei, 78% examined ≈6 h after activation contained two pseudo-pronuclei (Fig. 2*c*; Table 2), presumably because the 2n (= 40) chromosomes within the cell usually formed two clusters before formation of pseudo-pronuclei (10).

By contrast, activation of each oocyte reconstructed with the nucleus of a large ES cell was in the absence of cytochalasin B because we reasoned that cytokinetic extrusion of a pseudo-second polar body would be expected to re-establish the normal 2C DNA complement of the reconstituted cell in many such cases (33). We noted that after activation in the absence of cytochalasin B, 68% of the oocytes harbored a single pseudo-pronucleus and had emitted a pseudo-second polar body (Fig. 2*d*; Table 2).

Term Development of Mice Cloned from E14 Cells. Table 3 summarizes results obtained after the reconstruction of 1,765 oocytes using nuclei from E14 cells of different sizes and grown in the presence of different concentrations of FCS. We found no evidence for a marked effect of FCS concentration in the culture medium on the ability of ES cell nuclei to direct development to the blastocyst stage.

After small cell nuclear transfer, 17% of activated oocytes produced morulae/blastocysts. After transfer into suitable surrogate mothers, 62% of embryos implanted, giving rise to nine fetuses at 20 days postactivation (dpa). Four offspring were delivered alive by cesarean section, and the other five fetuses were developmentally arrested at 15–17 dpa. One of the live-born pups was euthanized because of lack of a foster mother, and two died within 24 h of delivery. One mouse, eponymously named "Hooper" to indicate the cell line from which he was derived (17), was born on January 23, 1999. Hooper is a male with a chinchilla coat color and pink eyes (Fig. 3*a*). These characteristics were predicted, because E14 is an XY line derived from the 129/Ola strain, which has a chinchilla coat color and pink eyes. All pups that developed to term were also males with nonpigmented eyes. Hooper has sired three litters with a total of 33 apparently normal pups when crossed with CD-1 females.

After the transfer of nuclei from large cells, 37% of successfully activated oocytes developed to the morula/blastocyst stage after 3.5 days of culture *in vitro*. Of the transferred embryos, 67% implanted in the uterus. One full-grown, apparently normal pup and three dead fetuses (developmentally arrested at 15–17 dpa) were removed by cesarean section 20 dpa; the pup died within 1 h of delivery.

Table 3. Development of embryos from enucleated oocytes injected with E14 ES cell nuclei

ES cells*	Conc. of FCS (%) in culture medium	No. of enucleated oocytes injected	No. (%) of activated oocytes	No. (%) of morulae/ blastocysts developed	No. of transferred embryos (recipients)	Postimplantation development			
						Total no. (%) of implantation sites	No. (%) of placentae alone	No. (%) of dead fetuses†	No. (%) of live offspring
Large	15	87	72 (82.8)	32 (44.4)	32 (3)	17 (53.1)	0	0	0
	5	152	135 (88.8)	58 (43.0)	51 (5)	31 (60.8)	1 (2.0)	2 (3.9)	1 (2.0)
	3–4	117	91 (77.8)	22 (24.2)	22 (3)	16 (72.7)	3 (13.6)	0	0
	1–2	93	79 (84.9)	27 (34.2)	27 (3)	24 (88.9)	0	1 (3.7)	0
	0.5–0.75	16	9 (56.3)	3 (33.3)	3 (1)	2 (66.7)	0	0	0
	Subtotal	465	386 (83.0)	142 (36.8)	135 (15)	90 (66.7)	4 (3.0)	3 (2.2)	1 (0.7)
Small	15	48	38 (79.2)	12 (31.6)	12 (2)	0	0	0	0
	5	566	466 (82.3)	65 (13.9)	63 (7)	40 (63.5)	5 (7.9)	3 (4.8)	2 (3.2)
	3–4	154	141 (91.6)	32 (22.7)	32 (3)	18 (56.3)	2 (6.3)	1 (3.1)	2 (6.3)
	1–2	388	326 (84.0)	55 (16.9)	55 (7)	35 (63.6)	4 (7.3)	1 (1.8)	0
	0.5–0.75	144	126 (87.5)	17 (13.5)	17 (2)	17 (100)	0	0	0
	Subtotal	1,300	1,097 (84.4)	181 (16.5)	179 (21)	110 (61.5)	11 (6.1)	5 (2.8)	4 (2.2)
Total		1,765	1,483 (84.0)	323 (21.8)	314 (36)	200 (63.7)	15 (4.8)	8 (2.5)	5 (1.6)

*Cell sizes are as for Table 1.
†Estimated to have died at 15–17 dpa.

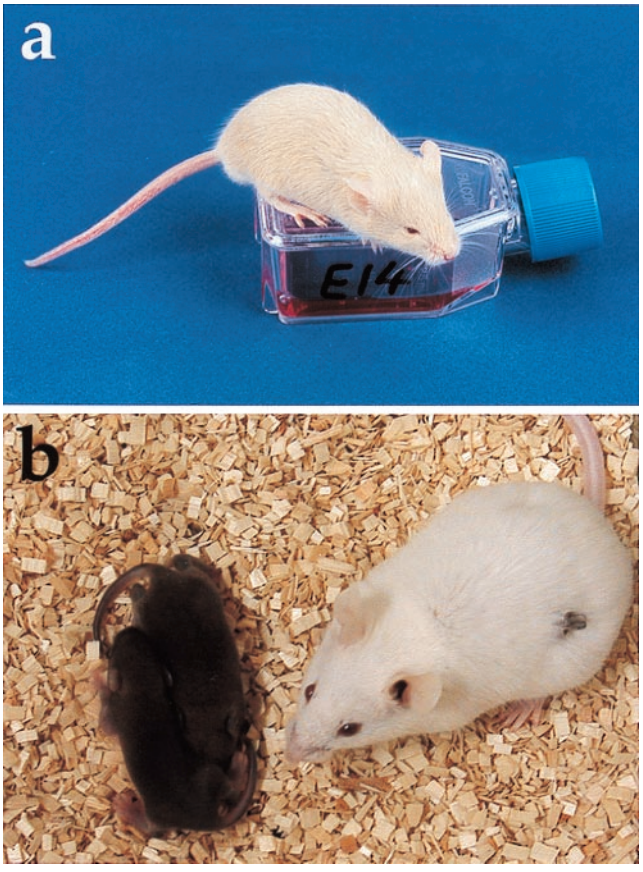

Fig. 3. Mice cloned from ES cells. (*a*) Hooper (born January 23, 1999) 47 days after birth. He was cloned by using the nucleus of a small E14 cell. He possesses the chinchilla coat color and pink eye phenotypes indicative of the 129/Ola strain. (*b*) Two cloned mice derived from small R1 ES cells, 9 days after birth. The pups are agouti. The surrogate mother delivered these pups through natural birth.

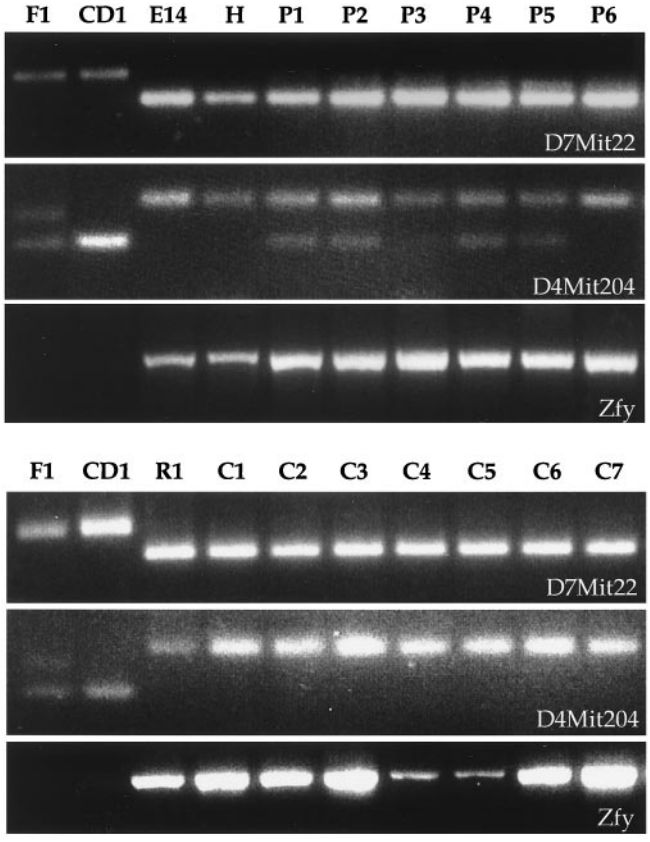

Fig. 4. DNA genotyping corroborates the ES cell provenance of Hooper and other cloned mice. Results for polymorphic DNA markers on chromosome 7 (D7Mit22) and chromosome 4 (D4Mit204) are shown. Confirmation of sex was with PCR primers for the Y-chromosome specific *Zfy* gene (Zfy). The oocyte donor strain is B6D2F1 (F_1), and female surrogates used to carry the pregnancies are of a CD-1 background. (*Upper*) E14 derived clones. Genomic DNA was from placentae for cloned offspring P1–P6. Genomic DNA from an ear-punch biopsy of Hooper is indicated by H. The weak lower band for the D4Mit204 marker is shared between the placental samples of cloned offspring and the CD-1 sample in a manner consistent with the expected presence of both CD-1-derived (maternal) and clone-derived (E14) cells in the placenta. Accordingly, this lower band is absent from the ear-punch biopsy DNA sample of Hooper (H), whereas it is present in the corresponding placental DNA sample (P1). (*Lower*) R1-derived clones. Genomic DNA was from tail biopsies for cloned offspring C1–C7.

We isolated genomic DNA from the placentae of ES cell-derived cloned mice and an ear biopsy from Hooper, and subjected the samples to PCR analysis for polymorphic markers and the presence of the Y chromosome-specific gene, *Zfy* (Fig. 4). These analyses corroborate the E14 provenance of the cloned pups.

We noticed that while the neonatal weight of the E14-derived cloned live offspring was in the normal range (1.65 ± 0.25 g, n = 5), the weight of the placenta of each was approximately twice the normal value (0.23 ± 0.02 g, n = 5) compared with corresponding values for control, noncloned, singleton, term pregnancies in our hands (0.13 ± 0.03 g, n = 21). Placental enlargement previously has been observed for mice cloned from cumulus cells (0.33 ± 0.08 g, n = 23) (10) or tail-tip derived cells (0.30 ± 0.08 g, n = 3) (11).

In preliminary experiments, we tested whether a cell clone of E14 harboring a targeted mutation could be used to produce mouse clones. We chose line T15 (C. Zheng and P.M., unpublished data), because blastocyst injection yielded chimeras with extensive colonization of somatic tissues and the germ line. Of 252 successfully reconstructed oocytes, 36% developed to the morula/blastocyst stage; eight dead fetuses were found and one live-born clone was obtained that died within 1 h after cesarean section (data not shown). In other preliminary experiments, we failed to produce viable cloned mice with the cell lines AB1 (n = 103 reconstructed oocytes, 31% morula/blastocyst development), AB2.2 (n = 193 and 25%), and TL1 (n = 120 and 28%), which are all of inbred 129 origin (data not shown).

Development of Embryos After Nuclear Transfer of R1 ES Cells. Table 4 shows the results of 1,087 nuclear transfers with this cell line, derived from the F_1 hybrid, 129/Sv × 129/Sv-CP (19). There was no pronounced effect of the FCS concentration on cloning outcome. However, the cloning efficiency was markedly higher for R1 cells than for E14 cells. From 312 transferred morulae/blastocysts, 26 live-born cloned pups (8.3%) were obtained, of which 13 survived. Their clonal provenance is supported by PCR analysis shown in Fig. 4. We again noticed that while the weight of the live offspring cloned from R1 ES cells was in the normal range (1.69 ± 0.23 g, n = 25), the weight of the placenta was approximately twice the normal value (0.24 ± 0.06 g, n = 21).

We also experimented with a second F_1-derived ES cell line, TT2, which was derived from C57BL/6 × CBA (37). Although the frequency of development to the morula/blastocyst stage was comparable to that of R1 (28.2%, n = 286 reconstructed oocytes), we did not produce surviving pups (data not shown). This finding suggests that the high cloning efficiency exhibited by R1 cells is not simply a function of their F_1 derivation.

Because the nuclei of large E14 cells could, under appropriate experimental conditions, support full development after transfer, we performed analogous experiments with R1 cells. Instead of simply selecting large R1 cells, we exposed cultures to nocodazole

Table 4. Development of embryos from enucleated oocytes injected with R1 ES cell nuclei

ES cells*	Conc. of FCS (%) in culture medium	No. of enucleated oocytes injected	No. (%) of activated oocytes	No. (%) of morulae/ blastocysts developed	No. of transferred embryos (recipients)	Postimplantation development: Total no. (%) of implantation sites	Postimplantation development: No. (%) of placentae alone	Postimplantation development: No. (%) of dead fetuses†	Postimplantation development: No. (%) of live offspring
Large‡ (Nocod.)	5	197	153 (77.7)	47 (23.9)	47 (6)	36 (76.6)	0	2 (4.3)	3 (6.4)
Small	15§	364	317 (87.1)	129 (35.4)	129 (11)	78 (60.5)	2 (1.6)	5 (3.9)	7 (5.4)
	5	275	212 (77.1)	73 (26.5)	73 (9)	59 (80.8)	5 (6.8)	9 (12.3)	9 (12.3)
	0.5§	251	193 (76.9)	63 (25.1)	63 (7)	44 (69.8)	4 (6.3)	4 (6.3)	7 (11.1)
Total		1,087	875 (80.5)	312 (28.7)	312 (33)	217 (69.6)	11 (3.5)	20 (6.4)	26 (8.3)

*Cell sizes are as for Table 1.
†Estimated to have died at 13–17 dpa.
‡Nocodazole treatment.
§Three recipient females delivered naturally. The uteri of these females could not be observed, such that the number of implantation sites, placentae, and dead fetuses are not included these females' data.

for 12 h before nuclear transfer, to synchronize the cells in culture at M-phase such that they contained 4C DNA (28, 29). The proportion of live offspring from this experimental series did not significantly differ from the corresponding value for small R1 cells. Three live-born clones were born. One of them, named "Noco," was born on July 13, 1999, and is still alive, which further suggests that neither nucleus donor ploidy, nor, *ipso facto*, cell cycle stage are critical parameters in cloning.

Discussion

Cloning Mice from ES Cell Lines. We produced live-born clones from two ES cell lines, E14 and R1, respectively, five and 26 clones, of which one and 13 survived. The first-born survivor, Hooper, was produced from an E14 nucleus. Hooper has sired numerous, apparently normal offspring. The cloning efficiency was markedly higher for R1 cells than E14 cells.

We deliberately chose ES cell lines that had been passaged extensively, to reflect the typical conditions in which these cells are used. Cloned mammals thus far reported have been derived from freshly isolated cells, or from primary, often ill-defined cell cultures that are not widely available (1–11). By contrast, we describe here the cloning of mice with cells from established lines that are well-characterized, widely used, not proprietary, and readily available to scientists worldwide.

Cloning and Stage of the Cell Cycle. Whereas it frequently has been emphasized that the success of cloning is critically and exclusively dependent on nucleus donors being in the G_0-phase of the cell cycle (2, 4), our work provides two additional lines of evidence (5) that this hypothesis is unlikely to be universally true.

First, we have demonstrated that cloning may be achieved with nuclei from large E14 and R1 cells. That these ES cells are likely to be post-S-phase and to contain a 4C genomic complement is corroborated by several indirect arguments. (*i*) Cells with such a relatively large cytoplasmic volume are typical of those that have advanced beyond the G_1-phase, and whose cytoplasmic swelling is in preparation for division (38). (*ii*) Treatment with the microtubule-disrupting agent, nocodazole, would serve to arrest the population of cells at M-phase (with a 4C DNA complement) before nuclear transfer (28). (*iii*) Reconstructed oocytes from nuclei of large E14 or R1 ES cells were activated without preventing cytokinesis; they successfully progressed through development, suggesting that half of the chromosomes were discarded into a pseudo-second polar body, restoring a 2C genomic complement. If the large cell nuclei had had only a 2C complement, some chromosomes

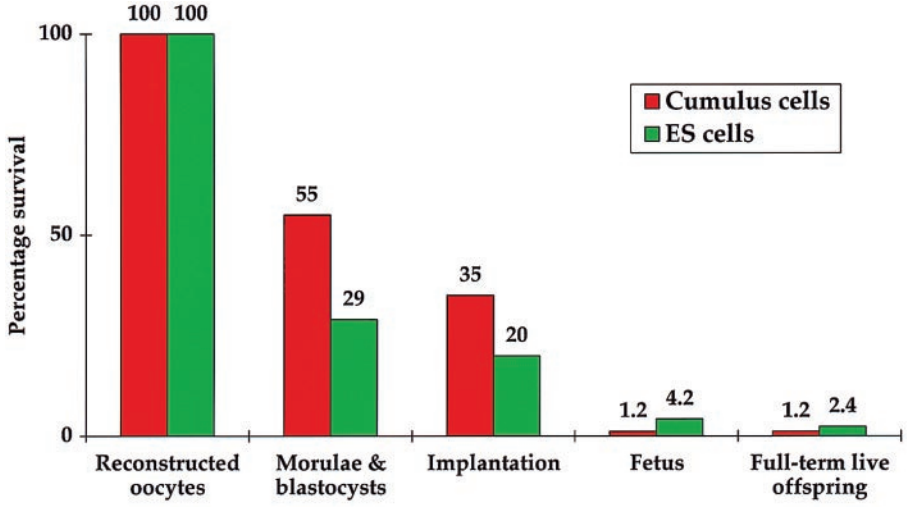

Fig. 5. Survival of cloned embryos at various stages of development. The number of oocytes surviving nuclear transfer is taken as 100%. Progress to the morula/blastocyst stage occurs over 3.5 days *in vitro*. Sixteen days later, cesarean section is performed, and the number of implantation sites, dead fetuses, and live-born pups are counted. The stage ''fetus'' includes both dead fetuses and live-born pups.

would have been extruded into a pseudo-second polar body, resulting in an aneuploid and inviable embryo. Thus, we argue that our success in readily producing term fetuses from nuclei of large E14 or R1 cells without preventing cytokinesis suggests that G_2/M-phase cells are indeed permissive for cloning.

Second, we successfully used cells taken from actively dividing cultures. Moreover, there was no correlation between the cloning efficiency and the rate of cell growth. We cannot exclude the possibility that some of the cloning-competent ES cell nuclei were derived from cells that had exited the cell cycle to enter the G_0-phase (see also ref. 39), for instance by terminally differentiating into postmitotic cells.

Nuclear Totipotency. Production of live young using ES cells required thus far that their low potential to differentiate into extraembryonic (placental) tissue be rescued by generating chimeras with "carrier" cells, which are either diploid (16, 17) or tetraploid (18–20). This need contrasts to our demonstration that the nucleus of a single ES cell can program full development, including placentation. We term the ability of a nucleus to direct full embryonic development as "nuclear totipotency." Our data do not support a link between nuclear totipotency and either growth conditions or the cell cycle stage of the nucleus donating cell. Given that nuclear totipotency is cell cycle independent, other factors must be involved in determining developmental outcome.

Restriction Points in Cloning. When expressed as the fraction of reconstructed oocytes that develops to a live-born cloned pup, the success rate is 1.2% for cumulus cells and 2.4% for R1 ES cells. But disparities emerge when progress to successive developmental stages is examined in detail for cumulus cells compared with R1 ES cells (Fig. 5).

With cumulus cells, 55% of cloned embryos developed *in vitro* to the morula/blastocyst stage. Evaluation of development *in utero* beyond that stage was made at the time of cesarean section, 16 days after embryo transfer (20 dpa); 35% of cloned embryos appeared to have implanted, and 1.2% developed to a live pup, with no dead fetuses.

However, for R1 ES cells, only 29% of cloned embryos completed preimplantation development. Twenty percent showed evidence of implantation, 4.2% developed into a fetus, and 2.4% were alive at term. Many placentae (without fetuses) and dead fetuses arrested at 15–17 dpa were observed on cesarean section.

These findings indicate that the restriction points within a successful cloning process may vary with the source of cells. We believe that the mouse is a preferred organism to study systematically the parameters governing the cloning phenomenon. The present study reports the results of ≈3,500 cloning experiments, a number that would be impractical to achieve for larger animals such as sheep, cows, and goats.

Practical Implications. Cloning animals from established ES cell lines suggests the opportunity to generate large numbers of identical individuals from a single somatic cell, as follows. The nuclei of all somatic cell types tested to date are able to support *in vitro* development to the blastocyst stage after transfer by microinjection into an enucleated oocyte (T.W., unpublished data). Assuming ES cell lines can be established from these cloned blastocysts, they then would permit the production, over a protracted period, of an essentially unlimited number of cloned individuals. Each would contain an identical nuclear genome originating from a single cell of an individual. The nuclear genome of an individual thus would be immortalized in the form of an ES cell line. Such ES cell lines also could be used for engineering the genome and for controlled *in vitro* differentiation for cell or tissue replacement purposes.

Furthermore, cloning from ES cells by nuclear transfer should afford the option to produce, in a single generation, mutant mice with any one of a multiplicity of genetic alterations, including homozygosity for a targeted mutation. After the creation of a desired ES cell genome *in vitro*, it should be possible to produce mutant mice in a single step. This is particularly important for the analysis of complex genetic traits requiring multiple genetic alterations.

ES cells that can contribute to the germ line are known to exist only for mice. But if these findings can be extrapolated to ES-like cells from other mammalian species, cloning would offer the potential of creating in a single generation mutant animals that otherwise would take years to obtain. This is particularly relevant to species with relatively long life cycles such as cows, sheep, goats, and pigs.

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1. Willadsen, S. M. (1986) *Nature (London)* **320,** 63–65.
2. Campbell, K. H. S., McWhir, J., Ritchie, W. A. & Wilmut, I. (1996) *Nature (London)* **380,** 64–66.
3. Schnieke, A. E., Kind, A. J., Ritchie, W. A., Mycock, K., Scott, A. R., Ritchie, M., Wilmut, I., Colman, A. & Campbell, K. H. (1997) *Science* **278,** 2130–2133.
4. Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J. & Campbell, K. H. S. (1997) *Nature (London)* **385,** 810–813.
5. Cibelli, J. B., Stice, S. L., Golueke, P., Kane, J. J., Jerry, J., Blackwell, C., Ponce de Léon, F. A. & Robl, J. M. (1998) *Science* **280,** 1256–1258.
6. Kato, Y., Tani, T., Sotomaru, Y., Kurokawa, K., Kato, J., Doguchi, H., Yasue, H. & Tsunoda, Y. (1998) *Science* **282,** 2095–2098.
7. Renard, J. P., Chastant, S., Chesné, P., Richard, C., Marchal, J., Cordonnier, N., Chavatte, P. & Vignon, X. (1999) *Lancet* **353,** 1489–1491.
8. Wells, D. N., Misica, P. M. & Tervit, H. R. (1999) *Biol. Reprod.* **60,** 996–1005.
9. Baguisi, A., Behboodi, E., Melican, D. T., Pollock, J. S., Destrempes, M. M., Cammuso, C., Williams, J. L., Nims, S. D., Porter, C. A., Midura, P., *et al*. (1999) *Nat. Biotech.* **17,** 456–461.
10. Wakayama, T., Perry, A. C. F., Zuccotti, M., Johnson, K. R. & Yanagimachi, R. (1998) *Nature (London)* **394,** 369–374.
11. Wakayama, T. & Yanagimachi, R. (1999) *Nat. Genet.* **22,** 127–128.
12. Dean, W., Bowden, L., Aitchison, A., Klose, J., Moore, T., Meneses, J. J., Reik, W. & Feil, R. (1998) *Development (Cambridge, U.K.)* **125,** 2273–2282.
13. Evans, M. J. & Kaufman, M. H. (1981) *Nature (London)* **292,** 154–156.
14. Martin, G. R. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 7634–7638.
15. Hogan, B., Beddington, R., Costantini, F. & Lacy, E. (1994) *Manipulating the Mouse Embryo* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed., pp. 173–181.
16. Bradley, A., Evans, M. J., Kaufman, M. H. & Robertson, E. (1984) *Nature (London)* **309,** 255–256.
17. Hooper, M., Hardy, K., Handyside, A., Hunter, S. & Monk, M. (1987) *Nature (London)* **326,** 292–295.
18. Nagy, A., Gocza, E., Diaz, E. M., Prideaux, V. R., Ivanyi, E., Markkula, M. & Rossant, J. (1990) *Development (Cambridge, U.K.)* **110,** 815–821.
19. Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W. & Roder, J. C. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8424–8428.
20. Zang, Z., Kiefer, F., Urbanek, P. & Wagner, E. F. (1997) *Mech. Dev.* **62,** 137–145.
21. Capecchi, M. R. (1989) *Science* **244,** 1288–1292.
22. Ramirez-Solis, R., Davis, A. C. & Bradley, A. (1993) *Methods Enzymol.* **225,** 855–878.
23. Mombaerts, P., Clarke, A. R., Hooper, M. L. & Tonegawa, S. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 3084–3087.
24. Mombaerts, P., Clarke, A. R., Rudnicki, M. A., Iacomini, J., Itohara, J., Lafaille, J. J., Wang, L., Ichikawa, Y., Jaenisch, R., Hooper, M. L., *et al*. (1992) *Nature (London)* **360,** 225–231.
25. Itohara, S., Mombaerts, P., Iacomini, J., Lafaille, J. J., Nelson, A., Clarke, A. R., Hooper, M. L., Farr, A. & Tonegawa, S. (1993) *Cell* **72,** 337–348.
26. Mombaerts, P., Wang, F., Dulac, C., Chao, S. K., Nemes, A., Mendelsohn, M., Edmondson, J. & Axel, R. (1996) *Cell* **87,** 675–686.
27. Rodriguez, I., Feinstein, P. & Mombaerts, P. (1999) *Cell* **87,** 199–208.
28. Kwon, O. Y. & Kono, T. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 13010–13013.
29. Tsunoda, Y. & Kato, Y. (1998) *J. Reprod. Fertil.* **113,** 181–184.
30. Chatot, C. L., Lewis, J. L., Torres, I. & Ziomek, C. A. (1990) *Biol. Reprod.* **42,** 432–440.
31. Dietrich, W., Katz, H., Lincoln, S. E., Shin, H. S., Friedman, J., Dracopoli, N. C. & Lander, E. S. (1992) *Genetics* **131,** 423–447.
32. Kunieda, T., Xian, M., Kobayashi, E., Imamichi, T., Moriwaki, K. & Toyoda, Y. (1992) *Biol. Reprod.* **46,** 692–697.
33. Wakayama, T. & Yanagimachi, R. (1998) *Biol. Reprod.* **59,** 100–104.
34. Rieder, C. L. & Alexander, S. P. (1990) *J. Cell Biol.* **110,** 81–95.
35. Epstein, C. J. (1986) *The Consequences of Chromosome Imbalance* (Cambridge Univ. Press, Cambridge).
36. Kono, T., Ogawa, M. & Nakahara, T. (1993) *J. Reprod. Dev.* **39,** 301–307.
37. Yagi, T., Tokunaga, T., Furuta, Y., Nada, S., Yoshida, M., Tsukada, T., Saga, Y., Takeda, N., Ikawa, Y. & Aizawa, S. (1993) *Anal. Biochem.* **214,** 70–76.
38. Murray, A. & Hunt, T. (1993) *The Cell Cycle* (Oxford Univ. Press, Oxford), pp. 28–41.
39. Wilmut, I. & Campbell, K. (1998) *Science* **281,** 1611 (lett.).