# Clonal Propagation of Primate Offspring by Embryo Splitting

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**Primates that are identical in both nuclear and cytoplasmic components have not been produced by current cloning strategies, yet such identicals represent the ideal model for investigations of human diseases. Here, genetically identical nonhuman embryos were produced as twin and larger sets by separation and reaggregation of blastomeres of cleavage-stage embryos. A total of 368 multiples were created by the splitting of 107 rhesus embryos with four pregnancies established after 13 embryo transfers (31% versus 53% in vitro fertilization controls). The birth of Tetra, a healthy female cloned from a quarter of an embryo, proves that this approach can result in live offspring.**

The promise and perils of cloned animals created from adult somatic cells were made vivid with the birth of the sheep "Dolly" (*1*), followed by equally remarkable achievements in cattle (*2*), mice (*3*), and goats (*4*). Among the most compelling scientific rationales for cloning is the study of disease models in which the genetics are invariable. However, zeal has been tempered by the deaths of fetuses and newborns (*2*, *5*), as well as by reports of shortened telomeres (*6*), which suggest that nuclear transfer does not reverse aging. Furthermore, mitochondrial heterogeneity shows that nuclear transfer results in genetic chimeras (*7*). Nuclear transfer in macaques has succeeded with blastomere nuclei, but not yet with adult, fetal, or embryonic stem (ES) cells (*8*). We have obviated some of the perils of cloning in the formation of identical twin, triplet, and larger sets of identical rhesus monkey embryos by adapting methods successful in other mammals (*9*), resulting in the birth of Tetra, the first nonhuman primate clone produced by embryo splitting.

One hundred and seven rhesus embryos were split to create 368 multiples (*10*). An eight-cell embryo was split to produce a set of identical quadruplet embryos each consisting of two blastomeres (Fig. 1). The zona-free, eight-cell embryo was dissociated into individual blastomeres, of which two were inserted into each empty zona pellucida (Fig. 1A), creating one set of quadruplets (Fig. 1B). After transfer of a pair of the quadruplet embryos into two fertile surrogates, both became pregnant. One (Fig. 1C) gestated a "blighted" pregnancy, i.e., a placental sac devoid of fetal tissue. Pedigree analysis by microsatellite-based polymerase chain reaction (PCR) (*11*) demonstrated that it was genetically identical to Tetra (Figs. 1D and 2).

Tetra was born at 157 days, after an uneventful pregnancy (*12*). The initiation of pregnancy after embryo splitting and transfer occurred at acceptable frequencies: 31% [4/13 versus 53.3% in in vitro fertilization (IVF) controls (*12*)] resulting in two biochemical pregnancies after transferring twin embryos (miscarried <30 days of gestation); one biochemical quadruple pregnancy (Fig. 1C); and one live quadruple offspring (Figs. 1D and 2). Four pregnancies (31%), but only one fetal sac and one live birth (8%), resulted from the 13 transfers. Eight pregnancies (53%), 10 fetal sacs (66%; due to twins), and six live births (40%) occurred in controls. Pregnancy losses could be due to the "donated" ruptured zona (*13*); micromanipulation; dissociation; seasonality; or, perhaps most likely, the presence of fewer cells in the smaller split embryos.

Blastocyst cell allocation is different in splits than controls (Fig. 3). Embryonic cells have one of two fates: trophectoderm (TE; extraembryonic membrane precursors) or inner cell mass (ICM; fetal and extraembryonic membranes). Confocal imaging and three-dimensional (3D) reconstruction (*14*) of blastocysts from splits shows 6 ± 2.6 ICM and 51.2 ± 30.0 TE versus 13.2 ± 4.8 ICM and 122.6 ± 52.1 TE cells in IVF blastocysts (Fig. 3). Remarkably, primate blastocysts display bilateral symmetry, like mice (*15*), suggesting that the first meiotic axis specifies the embryonic plane separating the ICM from the blastocoel, and perhaps also the plane for gastrulation.

ICM and TE reduction result in fewer progenitor cells and may affect implantation and fetal development. Apoptosis (*14*), higher in the splits and highest in the ICM (Fig. 3) (39.9 ± 35.3% versus 13.2 ± 7.7% in controls), contributes to miscarriages, because TE cells participate in implantation, but too few ICM cells reduce fetal viability.

Quadruplets appear as the upper viability limit for offspring, although septuplets formed blastocysts with viable ICM cells. Fifty-nine percent of the split embryos underwent compaction, but only 12% formed blastocysts. Most embryos were dissociated at 40 to 48 hours after insemination, between the 2nd and 4th division, i.e., the 4- to 16-cell stage (*16*).

Compaction and blastocyst success rates

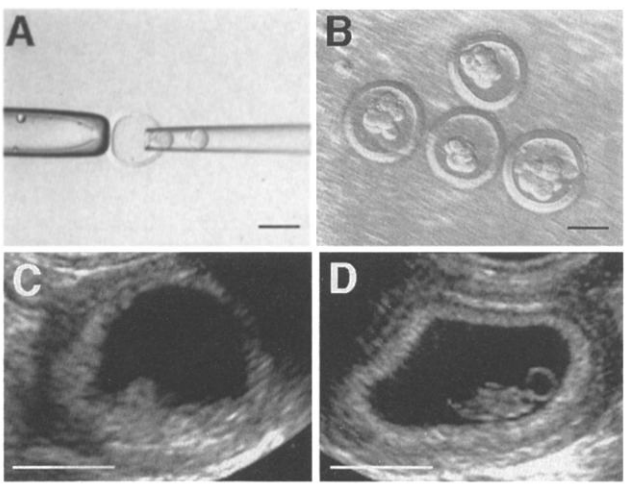


**Fig. 1.** Embryo splitting and development of nonhuman primates after embryo transfer. A zona-free eight-cell stage rhesus embryo, fertilized in vitro, is dissociated into eight individual blastomeres by mechanical disruption in $Ca^{2+}$- and $Mg^{2+}$-free medium. Two dissociated blastomeres are transferred into each of four empty zonae (**A**), thereby creating the four quadruplet embryos, each with two of the eight original cells (**B**). Split embryos are scored daily for development and structural normalcy, and embryos showing signs of compaction are selected for transfer 1 to 3 days after splitting. Endocrine profiles are traced daily and implantation is confirmed by ultrasound on day 31 after transfer. A miscarried pregnancy in which the fetus is absent though the placenta appears normal (**C**), and the quadruplet pregnancy with normal fetal development (**D**) that resulted in the birth of Tetra (Fig. 2) resulted from the transfer of two quadruplet embryos each to two surrogates. Bar in (A) and (B), 120 μm; in (C) and (D), 5 cm.

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**Fig. 2.** Tetra, a nonhuman primate quadruplet cloned from an eight-cell embryo by splitting.

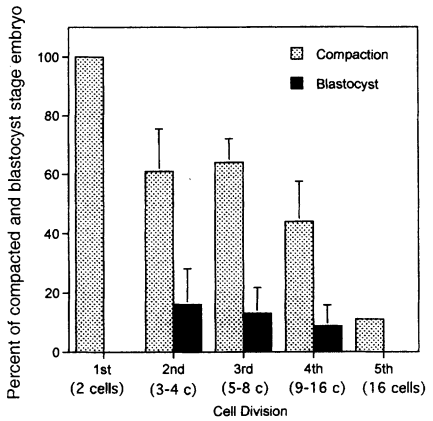


**Fig. 4.** Developmental potential of reconstructed embryos decreases when advanced-stage preimplantation embryos are split. Embryos split into twins display higher rates of compaction and blastocyst formation than embryos separated into triplets and higher orders.

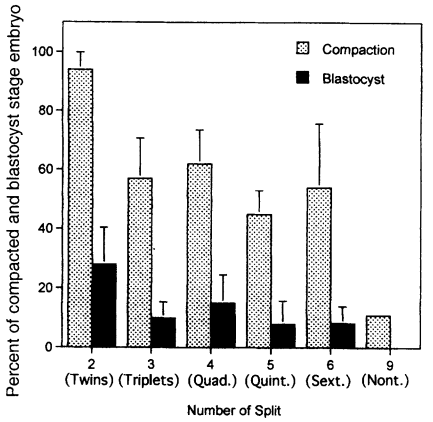


**Fig. 5.** Larger sets of identicals show reduced developmental potential. Compaction rate is maintained when larger sets of identicals are produced. Unlike compaction, blastocyst formation rate is more sensitive to the number of identicals created. Blastocyst rate is reduced by half when triplet rather than twin embryos are created, and development frequently arrests when splitting beyond sextuplets is attempted. Totals do not equal 107 original embryos and 368 splits because some embryos were cryopreserved before compaction.

declined with later stages (Fig. 4). Also, developmental potential decreased when larger sets of identicals were produced (Fig. 5). Twins showed high compaction rates (94%, $n$ = 32) with 28% blastocyst formation ($n$ = 18). Reconstructed embryos compacted slightly faster than controls, suggesting intrinsic chronological and/or cell-cycle clocks.

Cloning by splitting provides genetically identical offspring. Blastomere nuclear transfer has been successful [3.8% live fetuses per embryos transferred (*8*)]. Splitting results in 31% pregnancies and 8% births, and similar results were obtained by using four- to eight-cell blastomere nuclear transfer in rhesus, i.e., 24% (4/17) pregnancies and 12% (2/17) births (*8*). Neither strategy permits cloning with known phenotypes.

Like identicals, transgenic and stem cell–derived primates are well justified for producing disease models and for assessing the efficacy of innovative therapies [e.g., gene and cell therapy (*17*)]. If achieved, the scientific gap between knockout mice and patients would be bridged. Invariable genetics improve accuracy, reducing animals needed while providing perfect controls. Splits have been frozen and transferred successfully; clones with differing birth dates have numerous applications: e.g., phenotypic analysis of an offspring before propagating clones; serial transfer of stem cells to address cellular aging beyond life expectancy; and investigating simultaneous retrospective (in the older twin) and prospective protocols. Identicals implanted sequentially in the same surrogate might prove epigenetic effects (including the maternal environment), such as the Barker hypothesis that birth weight has life-long consequences (*18*) or that maternal hypothyroidism during pregnancy results in lower IQs (*19*). For transgenic primates, favored approaches use modified cells either for nuclear transfer or as stem cells. Since the former is encountering hurdles, the latter might prove feasible, but only if chimeras can be produced. Here, we report all the procedures necessary to evaluate the totipotency of stem cells and other chimeras in primates.

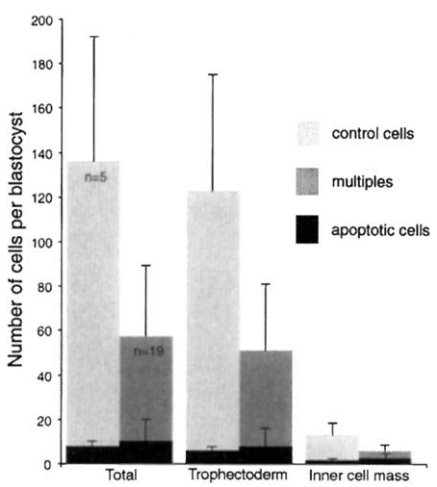


**Fig. 3.** The allocation of embryonic cells to both the trophectoderm and inner cell mass is lower in split embryos than in controls. Controls have twice the cell number of the split embryos at the blastocyst stage. Apoptosis is higher in split embryos than in controls and highest in ICM cells.

In summary, cloning by embryo splitting has efficiently produced identical embryos and resulted in a live birth. While propagation beyond quadruplets may not be possible, blastocysts from quintuplets to septuplets could be used for establishing ES cells. Because it is not yet known if stem cells from outbred populations of humans or primates (*20*) have complete totipotency, like those from selected inbred mouse strains, primate cells can now be evaluated by producing stem cells from one multiple, later tested in its identical sibling. In so doing, we might learn the full potential for primate stem cells and determine whether they might produce cancers like teratocarcinomas. Furthermore, producing identical twin, triplet, or quadruplet macaques would permit essential investigations now.

*Note added in proof*: Four pregnancies, each with a viable fetus, have been established from the last seven embryo transfers of identical twins (implantation rate: 57%). One pregnancy is from the transfer of a single embryo, the other three are singletons resulting from the transfer of two unrelated embryos. If successful, these identical twins will be named Romulus and Rhesus.

### References and Notes

1. I. Wilmut, A. E. Schnieke, J. McWhir, A. J. Kind, K. H. S. Campbell, *Nature* **385**, 810 (1997).
2. Y. Kato *et al.*, *Science* **282**, 2095 (1998).
3. T. Wakayama, A. C. Perry, M. Zuccotti, K. R. Johnson, R. Yanagimachi, *Nature* **394**, 369 (1998).
4. A. Baguisi *et al.*, *Nature Biotechnol.* **17**, 456 (1999).
5. J. B. Cibelli *et al.*, *Science* **280**, 1256 (1998); J. R. Hill, A. J. Roussel, J. F. Edwards, J. A. Thompson, J. B. Cibelli, *Theriogenology* **51**, 1451 (1999); J. P. Renard *et al.*, *Lancet* **353**, 1489 (1999); D. N. Wells, P. M. Misica, H. R. Tervit, W. H. Vivanco, *Reprod. Fertil. Dev.* **10**, 369 (1998); D. N. Wells, P. M. Misica, H. R. Tervit, *Biol. Reprod.* **60**, 996 (1999).
6. P. G. Shiels *et al.*, *Nature* **399**, 316 (1999).
7. M. J. Evans *et al.*, *Nature Genet.* **23**, 90 (1999).
8. D. P. Wolf, L. Meng, N. Ouhibi, M. Zelinski-Wooten, *Biol. Reprod.* **60**, 199 (1999).
9. V. E. Papaioannou, J. Mkandawire, J. D. Biggers, *Development* **106**, 817 (1989); N. Seike, K. Utaka, H. Kanagawa, *Jap. J. Vet. Res.* **38**, 1 (1990); Y. Heyman *et al.*, *Reprod. Nutr. Dev.* 38, 595 (1998); R. Kolberg, *Science* **262**, 652 (1993).
10. Rhesus oocytes recovered by laparoscopy from stimulated females were fertilized by IVF [G.-J. Wu *et al.*, *Biol. Reprod.* **55**, 269 (1996)]. Embryos were cultured

[L. Hewitson *et al.*, *Hum. Reprod.* **13**, 2786 (1998)], zonae removed with pronase, and the zona-free embryos recovered individually for 20 min before splitting. After transferring into calcium- and magnesium-free TALP-Hepes medium, blastomeres were dissociated by aspiration through a 30-μm micropipet. Blastomeres were transferred into empty zonae produced by mechanical removal of oocyte cytoplasm after zona scoring. Each multiple was placed in its own zona to ensure blastomere aggregation. Consequently, zonae were limiting, and additional zonae recovered from bovine oocytes were used successfully. Surrogate females were selected on serum estradiol and progesterone.

11. Parentage assignments were performed by DNA typing for 13 microsatellite loci amplified by PCR with heterologous human primers for loci D3S1768, D6S276, D6S291, D6S1691, D7S513, D7S794, D8S1106, D10S1412, D11S925, D13S765, D16S403, D17S804, and D18S72 (Veterinary Genetics Laboratory, University of California, Davis).
12. L. C. Hewitson *et al.*, *Nature Med.* **5**, 431 (1999); A. F. Tarantal and A. G. Hendrickx, *Am. J. Primatol.* **15**, 309 (1988).
13. J. Cohen, M. Alikani, J. Trowbridge, Z. Rosenwaks, *Hum. Reprod.* **7**, 685 (1992).
14. Terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL; Boehringer Mannheim) was used to assay apoptosis. Blastocysts were fixed (2% formaldehyde; pH 7.4; 30 min), rinsed, and permeabilized with 0.1% Triton X-100–0.1% Na citrate (4°C; 2 min). Hoechst 33258 (Sigma) was used to visualize total DNA. To retain their three-dimensionality, coverglass spacers (170 μm; >130 to 150) were placed alongside the blastocysts. Serial confocal imaging (3 μm) used Leica confocal TCS SP with ultraviolet argon and 488-argon lasers. These generated 3D reconstructions analyzed with Photoshop (Adobe Systems, Mountain View, CA). Reconstructions permitted total cell number by counting nuclei serially. TE cells were distinguished at the periphery, whereas ICM were interior.
15. R. L. Gardner, *Development* **124**, 289 (1997); R. J. Weber *et al.*, *Development* **126**, 5591 (1999).
16. Supplemental Web material is available at *Science* Online at www.sciencemag.org/feature/data/1042714.shl.
17. S. Lehrman, *Nature* **401**, 517 (1998).
18. H. Leese, I. Donnay, J. G. Thompson. *Hum. Reprod.* **13**, 184 (1998).
19. J. E. Haddow *et al.*, *N. Engl. J. Med.* **341**, 549 (1999).
20. M. J. Shamblott *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 13726 (1998); J. A. Thompson *et al.*, *Science* **282**, 1145 (1999).
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# Severely Reduced Female Fertility in CD9-Deficient Mice

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**CD9 is a widely expressed cell surface molecule that belongs to the tetraspanin superfamily of proteins. The tetraspanins CD9, KAI-1/CD82, and CD63 are involved in metastasis suppression, an effect that may be related to their association with β1 integrins. Knockout mice lacking CD9 were created to evaluate the physiological importance of CD9. $CD9^{-/-}$ females displayed a severe reduction of fertility. Oocytes were ovulated but were not successfully fertilized because sperm did not fuse with the oocytes from $CD9^{-/-}$ females. Thus, CD9 appears to be essential for sperm-egg fusion, a process involving the CD9-associated integrin α6β1.**

Sequence analysis of CD9 predicts a structure with four transmembrane domains, two extracellular loops, and short intracytoplasmic amino and carboxyl tails (*1*). This structure is shared by all members of the tetraspanin superfamily of proteins (*2*). CD9 is expressed in multiple tissues but is not ubiquitous (*3*). The tetraspanins CD9, KAI-1/CD82, and CD63 act as metastasis suppressor molecules (*4*). Low expression of these molecules is correlated with an increased invasive and metastatic potential (*2*, *5*). In vitro studies show that tetraspanins function in cell adhesion, motility, proliferation, differentiation, and signal transduction (*2*, *6*). The tetraspanins are physically associated with each other and with several cell surface molecules, including a subset of β1 integrins, which are receptors for extracellular matrix proteins (*7*). Thus, tetraspanins may function as surface organizers or facilitators that group and interconnect specific cell surface proteins in macromolecular complexes and thus increase the formation or the stability of functional signaling complexes, or both (*2*, *7*).

To investigate the role of CD9 in vivo, we generated mice in which the CD9 gene was disrupted by gene targeting in embryonic stem (ES) cells (*8*, *9*). The murine CD9 gene consists of eight exons spanning more than 20 kb (*10*, *11*). The promoter and exon 1 were replaced by the neomycin resistance gene (Web fig. 1) (*12*). The recombination was verified by Southern (DNA) blot (*13*). Splenocytes from homozygous mice displayed no detectable CD9 surface expression as determined by flow cytometry analysis or by immunohistochemistry of kidney sections (*10*).

Heterozygous $CD9^{+/-}$ mice appeared normal and, when interbred, yielded litters of normal size with a Mendelian genetic distribution 25:50:25(%) and with an equal distribution between males and females. Homozygous adult $CD9^{-/-}$ mice showed no obvious abnormalities and appeared healthy. However, when the $CD9^{-/-}$ mice were intercrossed, fertility was severely reduced. Normal fertility (93 to 100% of pregnancies) was observed when $CD9^{-/-}$ males were mated with wild-type or heterozygous females. In contrast, only 50 to 60% of $CD9^{-/-}$ females produced litters after being maintained in the presence of fertile wild-type, heterozygous, or $CD9^{-/-}$ males for up to 2 months. The delay before the beginning of a successful pregnancy was 19 to 30 days on average for $CD9^{-/-}$ females, as compared with 4.5 days for wild-type animals. In addition, the litter size was reduced ($2 \pm 0.6$ pups in $CD9^{-/-}$ females versus $8 \pm 2.3$ pups in $CD9^{+/+}$ females) and the initial mortality rate was increased to 32 to 55% as compared with less than 2% for wild-type females (Web table 1) (*12*).

No difference in the frequency of vaginal plugs was observed between wild-type and CD9-deficient mice, indicating normal mating behavior (16.9%; $n = 46$ for $CD9^{-/-}$ versus 17.4%; $n = 42$ in $CD9^{+/+}$). The infertility was also not due to the absence of sperm at the site of fertilization, as shown by the presence of numerous sperm in the oviduct. Histological examination of ovaries of 6-week-old $CD9^{-/-}$ mice showed that there was no difference when compared to wild-type females (*10*) and that the number of ovulated oocytes was normal (Table 1). However, the naturally ovulated oocytes from $CD9^{-/-}$ mated females collected at day 0.5 did not divide and became fragmented if maintained in cell culture (Fig. 1A), whereas oocytes from $CD9^{+/+}$ mated females divided and 2 days later reached stage 4 (Fig. 1B). Similar fragmented oocytes (Fig. 1C), instead of blastocysts (Fig. 1D), were present in the uterus of $CD9^{-/-}$ females 3.5 days after mating.

The oocytes recovered from nonmated superovulated $CD9^{-/-}$ mice (Fig. 2A) or $CD9^{+/+}$ mice (Fig. 2C) 12 hours after human chorionic gonadotropin (post-hCG) injection had a similar response: 70 and 74%, respectively, showed a polar body (Web table 1)

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