Strategies for the production of genetically identical monkeys by embryo splitting

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Abstract
Genetically identical rhesus monkeys would have tremendous utility as models for the study of human disease and would be particularly valuable for vaccine trials and tissue transplantation studies where immune function is important. While advances in nuclear transfer technology may someday enable monkeys to be cloned with some efficiency, embryo splitting may be a more realistic approach to creating pairs of genetically identical monkeys. Although several different approaches to embryo splitting, including blastocyst bisection and blastomere separation, have been used successfully in rodents and domestic species for production of pairs and sets of identical offspring, efforts to create monozygotic twins in rhesus monkeys using these approaches have not met with similar success. Aggregation of split embryos with other types of blastomeres, such as tetraploid and developmentally asynchronous blastomeres, that could potentially increase their cell numbers and developmental competence without contributing to term development has been investigated as an alternative approach to creating monozygotic twin monkeys. The major challenges encountered with respect to the efficient production of monozygotic twins in rhesus monkeys and potential strategies to overcome these challenges are discussed.

Review
Rhesus macaques are one of the most suitable animal models for studies of human disease because of their genetic and physiological similarity to humans [1]. Genetically identical rhesus monkeys would have tremendous utility as models for the study of human disease and would be particularly valuable for vaccine trials and tissue transplantation studies where immune function is important. Furthermore, use of genetically identical monkeys in biomedical research would substantially reduce the numbers of animals required for generating statistically valid data due to elimination of genetic variation. This is particularly important when considering the experimental limitations often encountered in nonhuman primate research.

Since the birth of Dolly [2], several mammalian species including sheep, cattle, goats, pigs, mice, rabbits and cats have been cloned using somatic cell nuclear transfer [3,4]. Despite the limited success of embryonic blastomere nuclear transfer that led to birth of two unrelated rhesus monkey infants [5], efforts to clone rhesus monkeys using somatic cell nuclear transfer have been unsuccessful [[6-8], Schramm, unpublished]. To date, very few blastocysts (~1%; [6], Schramm, unpublished) and no clinical pregnancies [6] have resulted from somatic cell nuclear transfer in rhesus monkeys. Based upon the relative inefficiency of somatic cell cloning in domestic species, and limited availability of oocytes, the probability of obtaining even a pair of genetically identical rhesus monkeys using this technology is exceedingly low. While
advances in nuclear transfer technology may someday enable monkeys to be cloned with some efficiency, embryo splitting may be a more realistic approach to creating pairs or sets of genetically identical monkeys. Additionally, unlike clones produced by nuclear transfer, which exhibit various degrees of mitochondrial heterogeneity [9], monkeys produced by embryo splitting would be completely genetically identical with respect to nuclear as well as mitochondrial DNA.

**Twinning efforts in rhesus monkeys**

In domestic species, embryo splitting has been accomplished by two different approaches: blastocyst bisection [10] and blastomere separation [11]. Blastocyst bisection has led to the birth of monozygotic twins in several mammalian species [12-16], while blastomere separation has led to the birth of triplets and quadruplets [17,18], as well as monozygotic twins mice; [19,20], sheep; [11,21], cattle; [17,18,22], goats; [23], pigs; [24] horses; [25,26]. Efforts to create monozygotic twins by embryo splitting have not met with similar success in rhesus monkeys. Initial studies on embryo splitting in rhesus monkeys demonstrated that the percentage of split embryos developing into blastocysts was reduced when blastomere separation was performed in more advanced cleavage stage (8–16 cell stage) embryos [27]. However, demiembryos created by blastomere separation at either the 2- or 4-cell stage develop into blastocysts equally as well as nonmanipulated control embryos [28 Schramm, unpublished]. Although the ratios of inner cell mass (ICM) to trophoderm (TE) and ICM to total cells in split rhesus blastocysts are equivalent to those of intact control blastocysts [28], the total cell numbers are reduced by approximately 50% [28 Schramm, unpublished], similar to results reported for other species [18,22,29,30]. Unlike in bisected blastocysts, the total cell numbers in blastocysts derived from blastomere separation were remarkably different within a given demiembryo pair, due perhaps to unequal distribution of cytoplasm among blastomeres at separation or differences in polarity within an embryo [28]. Nevertheless, while bisection of rhesus monkey blastocysts resulted in a higher yield of demiembryos, the yield of clinical pregnancies per oocyte was higher following blastomere separation, which was not limited by the need to culture embryos to the blastocyst stage [28]. Based upon results from the above-described studies, it does not appear that split rhesus monkey embryos created by blastomere separation or blastocyst bisection are less viable per se than those of other species. However, while pregnancies have been established following both blastomere separation and blastocyst bisection, only singleton offspring have resulted, regardless of whether split embryos were transferred together to the same recipient or separately to different recipient monkeys [27,28]. Transfer of 22 pairs of demiembryos created by blastomere separation led to a 33% (7/22) pregnancy rate with 2 twin pregnancies (9%) initiated, but in neither case did both twins develop to term [28]. Similarly, a 33% (4/12) pregnancy rate was established with bisected blastocysts, but all pregnancies were singletons [28].

**Challenges in producing monozygotic twins in rhesus monkeys**

Two of the major issues underlying the difficulty in producing monozygotic twins in rhesus monkeys are the sub-optimal pregnancy rates following embryo transfer and the inherent difficulties in supporting twin gestations in this species. Although twin births have resulted following transfer of nonmanipulated in vitro produced embryos [31,32], rhesus monkeys do not normally carry twins, and in the few cases (0.25%) of naturally occurring twin pregnancies, offspring rarely survive due to undefined complications in the fetal or perinatal period [33]. Pregnancy rates following transfer of two normal (nonmanipulated) IVF-derived embryos typically average 25–40%, with fewer than 15% of those being twin pregnancies ([28,31], Schramm, unpublished). Thus, production of monozygotic twins following transfer of split embryos into either the same or different recipients, would not be expected to be highly efficient.

The establishment of pregnancy following embryo transfer is highly dependent upon the synchrony between the embryo age (stage) and the recipient endometrium, relative to the day of ovulation. Optimal results for uterine transfer of blastocysts are obtained following asynchronous transfer into a Day 4 recipient [28,34,35], while optimal results for oviductal transfer of cleavage stage embryos are obtained by synchronous transfer into a Day 2 recipient [31]. Because rhesus monkeys cannot be synchronized to ovulate on a specific day, optimal timing for embryo transfer generally requires cryopreservation of embryos. This presents an additional problem for split embryos, because the post thaw survival of normal IVF-derived embryos is typically only 56–78% [36], and cryopreservation of split embryos has not generally been successful in any species. Therefore, the efficient production of monozygotic twins is likely to be considerably more challenging in rhesus monkeys than in rodents and domestic species.

**Strategies for creating monozygotic twins in rhesus monkeys**

Due to the difficulties associated with twinning efforts in rhesus monkeys, a nontraditional approach may be required for the efficient production of monozygotic twins in this species. Splitting embryos into quadruplet sets, rather than pairs, would increase the number of identical embryos available for transfer. This would be particularly important if they were to be cryopreserved prior to
transfer. Unfortunately, 4-cell stage embryos split into quadruplet sets are less competent to develop into blastocysts, and exhibit substantially reduced cell numbers in resulting blastocysts compared with demiembryos (Table 1). Interestingly, 16-cell stage rhesus embryos split into quadruplet sets develop into blastocysts at an equivalent rate as nonmanipulated control embryos [Schramm, unpublished]. However, resulting blastocysts have only one quarter of the total cell numbers of control blastocysts and little to no ICM compared to blastocysts derived from 4-cell embryos split into quadruplet sets [Schramm, unpublished]. Similar results were obtained from 8-cell stage sheep embryos separated into octaves. These "eighth embryos" developed into trophoblastic vesicles with little to no ICM [29]. This may be because blastomeres on the outside of the embryo (the large majority) have already been programmed to become trophectoderm cells as early as the 8-cell stage. Thus, creation of larger sets of identicals (quadruplets) may be counter productive for creating monozygotic twins unless cell numbers in resulting blastocysts can be enhanced.

Aggregation of split embryos with other types of blastomeres that could potentially increase their cell numbers and developmental competence without contributing to term development may provide an alternative approach to creating monozygotic twin monkeys. Potential types of blastomeres that could fulfill these criteria include tetraploid, developmentally asynchronous, parthenogenetic and androgenetic blastomeres. Assuming such blastomeres do not contribute to term development, this would potentially provide a means for creating quadruplet sets of embryos having the developmental competence and cell numbers of embryos split into pairs. Aggregation of 4-cell stage rhesus embryos split into quadruplet sets with an equal number of blastomeres from 4-cell stage tetraploid embryos enhanced both their developmental competence and total cell numbers in resulting blastocysts to levels similar to those of embryos split into pairs (Table 1). Although encouraging, the ICM of resulting chimeric blastocysts was consistently comprised of both diploid and tetraploid cells. This was unexpected based upon earlier studies in diploid/tetraploid mouse chimeras, in which the tetraploid blastomeres were either completely excluded from the ICM [37-39] or contributed specifically to the primitive endoderm layer of the ICM, persisting only in extraembryonic tissues [37-41]. Thus, in mice, tetraploid blastomeres have a strong bias against contributing to the ICM when aggregated with normal diploid blastomeres and, with few exceptions, are clearly excluded from the embryo proper in developing concepti. In contrast, tetraploid blastomeres appear to be equally capable of contributing to the ICM and TE in rhesus monkey blastocysts. It is unknown whether tetraploid cells in the ICM of chimeric monkey blastocysts would be allocated to specific lineages or would be preferentially selected against during fetal development.

In similar studies of mouse chimeras produced from aggregates of developmentally asynchronous embryos, the more advanced blastomeres generally formed either the ICM [42-46] or the TE [47], but not both, while the less advanced blastomeres were allocated to the opposite cell lineage. Similar results were obtained in sheep goat chimeras derived from developmentally asynchronous blastomeres [48]. In contrast, when 4-cell stage rhesus monkey embryos were split into quadruplet sets and aggregated with equal proportions (four) of developmentally asynchronous blastomeres from 16-cell stage embryos, resulting blastocysts were derived completely

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**Table 1: Developmental competence in vitro of split monkey embryos following aggregation of individual diploid and tetraploid blastomeres from 4-cell stage embryos**

| Treatment Group                        | n  | Compacted | Blastocyst | Cell # |
|----------------------------------------|----|-----------|------------|--------|
| Quarter (1 diploid blastomere)         | 47 | 48.6a     | 17.4a      | 48.7a  |
| Half (2 diploid blastomeres)           | 53 | 59.5a     | 39.8b      | 81.4b  |
| Half Chimera (1 diploid and 1 tetraploid blastomere) | 49 | 65.9a,b   | 43.2b      | 94.1b  |
| Control (nonmanipulated embryos)       | 41 | 88.0b     | 59.6b      | 165.7c |

*Quarter = single blastomere from a 4-cell stage diploid embryo; Half = two blastomeres from a 4-cell stage diploid embryo; Half Chimera = single blastomere from a 4-cell stage diploid embryo and a single blastomere from a 4-cell stage tetraploid embryo; Control = intact nonmanipulated diploid embryos; Cell # = numbers of nucleated cells in blastocysts on Day 7 post insemination. n = total number of embryos (7 replicates). Different letters within columns denote significant (P < .05) differences among treatments.
from the 16-cell stage blastomeres, with complete exclusion of the 4-cell stage blastomeres [Schramm, unpublished]. Neither the proportion developing into blastocysts nor the total cell numbers in resulting blastocysts were different from those of 16-cell stage embryos split into quadruplet sets. Similar results were obtained following aggregation of developmentally asynchronous cow embryos in which the larger, less advanced blastomeres were excluded at compaction, resulting in blastocyst formation from only the more advanced embryo [42]. Thus, aggregation of developmentally asynchronous blastomeres leads to similar results in the cow and rhesus monkey, which are markedly different from those obtained in the mouse and sheep. In the present study, it is possible that aggregation of different proportions of developmentally asynchronous blastomeres may have led to different results, as previously shown for mouse chimeras derived from aggregation of single 8-cell stage blastomeres with 2-cell stage embryos [47]. However, preliminary studies in rhesus monkeys indicated that aggregation of a single 4-cell stage blastomere with two 8-cell stage blastomeres resulted in blastocysts having chimeric ICMs (Schramm, unpublished). Therefore, due to exclusion of the less advanced blastomeres, aggregation of developmentally asynchronous blastomeres is unlikely to be a viable strategy for creating monozygotic twin rhesus monkeys, although it is possible that aggregation of different proportions of developmentally asynchronous blastomeres may lead to different results.

Conclusions

Recent efforts to create monozygotic twins in rhesus monkeys have been challenging due to suboptimal pregnancy rates following embryo transfer and the inherent difficulties in supporting twin gestations in this species. Future efforts involving transfer of quadruplet sets of diploid/tetraploid chimeras may prove to be an efficient means for creating monozygotic twins, assuming preferential selection against tetraploid cells in the developing conceptus. Similarly, creation of chimeras from aggregation of normal diploid blastomeres with blastomeres from androgenetic [49,50] or nuclear transfer derived [42] embryos might be a viable alternative for creating quadruplet sets of developmentally competent identical embryos. Although these unique strategies, or others, may enhance the number of developmentally competent identical embryos available for transfer, improvements in assisted reproductive technologies, including embryo cryopreservation, synchronization of menstrual cycles, and methods for enhancing implantation rates, will be essential in future efforts to produce genetically identical rhesus monkeys.

Authors' contributions

RDS and AMP carried out the blastomere separation and aggregation studies. RDS performed the statistical analyses and drafted the manuscript.

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A list of references is shown, including titles and authors. The text mentions the distribution of blastomeres in chimeric mouse blastocysts, the influence of ploidy on distribution, and the developmental capacity of blastomeres from four and eight-cell sheep embryos. Other topics include monkey oocytes, rhesus monkey twins, and the development of cytochalasin-B induced tetraploid and diploid/tetraploid mosaic blastocysts.