Journal of Assisted Reproduction and Genetics, Vol. 15, No. 5, 1998

Recent Progress in Mammalian Cloning

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Submitted: December 15, 1997
Accepted: December 19, 1997

Purpose: *Our purpose was to review recent progress in the use of nuclear transfer technology to produce genetically identical mammals.*
Methods: *A literature review was conducted.*
Results: *The reasons for cloning nonhuman mammals are manyfold including commercial, biomedical, and basic research applications. Individual steps in the nuclear transfer process are itemized, along with a detailed description of the specific approaches used in the production of Dolly, NETI, and DITTO. The potential application of nuclear transfer in the treatment of human infertility is also considered, along with bioethical concerns. Finally, insights are provided concerning the future application of cloning technology in rhesus macaques.*
Conclusions: *The cloning of a lamb (Dolly) from an adult, mammary gland cell coupled with the successful production of rhesus monkeys (NETI and DITTO) by nuclear transfer of embryonic cells marks the beginning of a "Golden Age" in the development and application of somatic cell cloning technology in mammals.*

KEY WORDS: mammalian cloning; nuclear transfer technology; literature review.

INTRODUCTION

The verb "to clone" comes from the Greek word "klon," meaning twig (1). That plants can reproduce asexually by simply rooting a cutting provides some insight into the derivation of the term. A medical definition of cloning would specify that somatic cell nuclei (derived from any diploid cell of an embryo, fetus, child or adult) can be transferred into chromosome-free oocytes to produce reconstituted embryos that are capable, upon transfer to suitable hosts, of developing into viable offspring. This definition of somatic cell cloning can be further subdivided into cloning that involves a differentiated, adult cell nucleus—the definition used by the National Ethics Advisory Commission (2)—versus a nucleus derived from a relatively undifferentiated fetal or embryonic cell. Using a broader definition of cloning, that is, to produce a copy, there are actually several approaches available, including embryo splitting or twinning and blastomere separation and culture, in addition to nuclear transfer. In twinning, a two cell– or four cell–stage embryo is separated into halves and cultured before transfer. Alternatively, embryos at the later blastocyst stage can be bisected before culture or transfer. In blastomere separation, early stage embryos are dissociated into component blastomeres; for instance, at the eight-cell stage one could dissociate into eight blastomeres and culture each one separately. However, this approach is limited by problems that may develop secondary to allocation of cells during preimplantation development (3). Because our interest in using cloning technology in nonhuman mammals is dependent on the production of large numbers of genetically identical, cloned animals, attention here is confined to nuclear transfer.

Nuclear transfer is not a new concept. The German developmental biologist, Hans Spemann, suggested in 1938 an experiment that involved nuclear transfer (4). He did not, however, live to see the experiment completed, for it was not until the 1950s that Briggs and King first reported such studies in amphibians (reviewed in Ref. 5), followed some years later by the work of John Gurdon and colleagues in the toad, *Xenopus laevis* (reviewed in Ref. 5). The important conclusions derived from these early studies were that

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1058-0468/98/0500-0235$15.00/0

somatic cell cloning, while possible, was very inefficient and that the developmental potential of a nucleus decreases as a function of age. That is, as cells become differentiated, their nuclei become less capable of undergoing the developmental reprogramming required when transferred to an undifferentiated, enucleated recipient cell. For instance, while nuclei from tadpoles were capable of producing adults following nuclear transfer, those from adult frogs were not. This concept, that somatic cell cloning from adult cells is difficult, if not impossible, persisted for many decades. However, earlier this year, the birth of Dolly, the lamb produced by scientists in Scotland (6) by transferring a nucleus from an adult mammary gland cell into an enucleated oocyte (cytoplast), refuted the older limiting concepts.

In mammals, live offspring have now been born following the transplantation of an embryonic cell nucleus in the mouse, mink, rat, rabbit, pig, sheep, and cow and, most recently, in the rhesus monkey (5,7). All have used enucleated, mature, metaphase II oocytes and whole-cell fusion as the nuclear transfer method. Nuclear transfer success is dependent on access to a reliable source of high-quality, developmentally competent, metaphase II oocytes. Such oocytes can be obtained following the in vitro maturation of ovarian oocytes, by direct follicular aspiration of mature oocytes from gonadotropin-stimulated animals or by recovering ovulated oocytes from the oviduct. Compatiblity between the nucleus and the cytoplast is of utmost importance to success. If these conditions are not met, the incidence of chromosomal damage and aneuploidy (inappropriate number of chromosomes) becomes unacceptably high (8). Reports of successful cloning from fetal and adult cells (6) has led to an increased appreciation for the cell cycle stage of the donor nucleus. Thus, removing the donor nucleus from an active cell cycle by serum starvation, into a quiescent stage termed G_0, may enhance the developmental potential of the nucleus when transferred into a cytoplast (6).

WHY CLONE?

Reasons for cloning nonhuman mammals can be subdivided into commercial, biomedical, and basic research categories. An example in the commercial realm would be the propagation of a prize-winning quarter horse or a highly valuable transgenic animal in order to upgrade a herd or strain rapidly. The biomedical applications of the technology are, likewise, literally unlimited. In general, when the genetic background is held constant, the number of animals required for a given protocol or experimental series can be greatly reduced. Obviously, there are many situations in which clonally derived animals could be uniquely used such as in vaccine development, the preservation of endangered species, and the restocking of an endangered gene pool by cloning a genetically unique (and possibly preserved dead) animal. Finally, cloned animals could contribute to basic research efforts, for instance, in understanding primate development, differentiation, and oncogenesis.

INDIVIDUAL STEPS IN NUCLEAR TRANSFER

Individual steps in the procedure include the preparation of a cytoplast (which involves chromosome removal by micromanipulation and is often referred to as oocyte enucleation), blastomere or donor nucleus isolation and transfer to produce an unfused pair, cytoplast activation, cell fusion to produce a reconstituted embryo, reconstituted embryo culture, and ultimately, embryo transfer. At present, the overall success rate of the nuclear transfer procedure is relatively low when results are expressed as implantations per reconstituted embryo transferred, of the order of 1 in 25 to 50. The basis for this high failure rate is unknown, however, improved success and additional insights will undoubtedly follow over the next several years as experience is accumulated in a variety of species.

PRODUCTION OF RHESUS MONKEYS BY NUCLEAR TRANSFER (NETI AND DITTO)

In 1995, we became interested in producing genetically identical rhesus monkeys by nuclear transfer technology using in vitro fertilization (IVF)–produced embryos as the source of donor nuclei. Initially, we set out to demonstrate that viable term pregnancies could be established by nuclear transfer, with the intent of pursuing the production of clonally derived animals at a later time. In our efforts to establish nuclear transfer technology in the nonhuman primate (7), we first evaluated three sources of potential cytoplasts: (a) metaphase II (MII) oocytes obtained from gonadotropin-treated monkeys 27 hr post-human chorionic gonadotropin administration and cultured until release of the first polar body, (b) aged MII oocytes (oocytes that had failed to fertilize approximately 20 hr after insemi-

nation), and (c) zygotes obtained from IVF. The overall efficiency in producing cleaving, reconstituted embryos was highest with fresh MII oocytes. Of the 101 reconstituted embryos produced from fresh MII cytoplasts and which cleaved in a timely manner and appeared morphologically normal, 53 were transferred into the oviducts of synchronized recipients (n = 17) during spontaneous menstrual cycles. Animals were monitored for pregnancy by measuring circulating levels of estradiol and progesterone as well as by conducting uterine ultrasonography. Four pregnancies resulted, two of which were lost at approximately 30 days of gestation. The remaining two pregnancies culminated in the birth of one male and one female at 166 and 149 days' gestation, respectively. These infants have been named NETI (for nuclear embryo transfer individual) and DITTO (for obvious reasons), despite the fact that they are siblings and not clones. The parentage of both nuclear transfer infants was ascertained by genetic typing with seven unlinked short tandem repeat (STR) markers amplified by polymerase chain reaction. The male allelic contribution to NETI and DITTO genotypes, as revealed on autoradiographs of the STRs, was "subtracted" from that of the nuclear transfer infant, and the remaining maternal allele was used to identify the mother. In this manner, both the host mother and the enucleated oocyte donor mother were definitively eliminated as the maternal parent. We are currently pursuing the production of clonally derived monkeys using embryonic stem cells or fetal/adult fibroblasts as the source of donor nuclei.

NUCLEAR TRANSFER IN SHEEP (DOLLY)

In the studies by Wilmut and co-workers (6), donor nuclei from fetal fibroblasts or adult, mammary gland cells were used. Finn Dorsett sheep were used as nuclear donors and Scottish Blackface ewes provided the oocytes that served as cytoplasts. Primary cultures of donor nuclei cells were maintained in vitro and subjected to serum starvation before use. Reconstituted embryos were created by micromanipulation followed by electrofusion and culture. Such cultured embryos were then transferred to a synchronized host uterus. One of the keys to success in these studies may have been the unique approach to cell cycle staging, as mentioned earlier, wherein serum starvation was used to move cells out of an active cell cycle into a quiescent state. If applicable to other species and other cell types, the discovery that nuclear reprogramming of differentiated cells can occur represents a major breakthrough in cloning technology, as it opens the door to the straightforward production of clones of virtually unlimited number. However, it is important to note that Dolly was the only lamb born from 277 reconstituted embryos created following nuclear transfer of adult mammary cells (or 0.4% of the total). Of these 277 embryos, 29 developed to morulae or blastocysts and were transferred to 13 recipients, resulting in one term birth (1/29; 3.4%). The corresponding numbers when fetal fibroblasts were used were 172 reconstituted embryos and 47 morulae or blastocysts, which when transferred to recipients, produced five pregnancies and three live lambs (3/47; 6.4%).

NUCLEAR TRANSFER IN THE TREATMENT OF HUMAN INFERTILITY

There are, perhaps, a number of applications in which nuclear transfer technology could be advantageous in the treatment of human infertility. In couples in whom advanced maternal age negatively impacts embryo quality or increases the likelihood of chromosomal abnormalities, and therefore the chance of successful pregnancy, one current treatment plan is to engage a younger woman to act as an oocyte donor. The younger oocytes would subsequently be fertilized by sperm from the infertile women's spouse and the resultant embryos implanted into the infertile wife. Unfortunately, in this case, there is no genetic contribution from the wife. Theoretically, however, nuclear transfer could be used to transfer nuclei from the lower-quality oocytes or embryos of this couple into enucleated oocytes (cytoplasts) originating from an oocyte donor. In this case, the wife's genetic contribution to the pregnancy would be maintained along with the reconstituted embryo's increased chances of successful implantation and pregnancy. Indeed, preliminary efforts at conducting nuclear transfer at the germinal vesicle stage in immature oocytes have already been reported (9).

THE ETHICS OF HUMAN CLONING

In 1993, Hall and co-workers (10) received the prize paper award from the American Fertility Society (now the American Society for Reproductive Medicine) for their work on blastomere separation and culture of human polyploid embryos. This work involved abnormal embryos that had been discarded from an IVF program. Embryos were manipulated to remove zonae

before embedding in alginate blocks and culture under standard conditions in an IVF laboratory environment. The authors concluded that such blastomeres can cleave after separation (on average three cleavages per cultured blastomere) and that there was a trend for the faster-cleaving embryos to develop further. Although this work was conducted after the appropriate institutional review, it met with considerable adverse publicity. In a follow-up paper, Jones *et al.* (1) not only offered justification for the experimentation by Hall and co-workers but suggested that it was entirely appropriate to continue such work in the context of institutionally approved research designed to improve the human condition.

In March of 1997, in response to the announcement of Dolly's birth, President Clinton charged a National Bioethics Advisory Committee (NBAC) with the task of reviewing the implications of human cloning and reporting back to him with recommendations within 90 days. The NBAC examined the somatic cell cloning of humans from religious, ethical, and legal perspectives. From the religious point of view, the cloning of a human would be intrinsically immoral for many but not all thinkers, as moral justifications could be envisioned for some, given strict regulation of the practice to prevent abuses. For bioethicists, one overriding concern was the possible physical harms that could occur to a child as a result of the manipulations that comprise the procedure. This consideration alone was viewed as justification for a prohibition on cloning. However, it was also concluded that the practice of cloning, if widespread, could undermine important social values. A similar, overriding argument was presented from a legal perspective, namely, that the use of the cloning technique would be a premature experiment that exposes the developing child to unacceptable risks. Such risks would outweigh any consideration of a fundamental right to attempt to procreate. The NBAC recommendations, presented to the President in June of 1997 (2), were to continue the current moratorium on the use of federal funds to support any attempt to create a child by somatic cell cloning; to extend an immediate request to all firms, clinicians, investigators, and professional societies in the private and non–federally funded sectors to comply with the intent of the federal moratorium; to create federal legislation to prohibit anyone from attempting somatic cell cloning with a sunset clause—a period of 3–5 years before the issues are reconsidered; and to cooperate with other nations and international organizations to enforce a ban on human somatic cell cloning. Legislation that would ban the cloning of humans from adult cells has been passed in several countries and is pending in others including the United States.

The debate about cloning humans has, perhaps, only just begun. Soon after the announcement of Dolly's birth, polls conducted by CNN and *Time* magazine indicated that 7% of those polled would clone themselves if given the chance. Moreover, the Raelian Movement has announced the creation of a Bahamas-based company named Valiant Venture, Ltd., which will offer a service called "Clonaid" to provide technical assistance to would-be parents willing to have a child cloned from one of them. This service claims to offer "a fantastic opportunity to parents with fertility problems or homosexual couples to have a child cloned from one of them."

THE FUTURE

The ultimate success of nuclear transfer in nonhuman mammals is dependent on the ability to produce large numbers of genetically identical animals easily and in a cost-effective manner, the key to which is the selection of a nuclear donor source that is virtually unlimited. One option is to use primary cell cultures, an alternative created recently by the success in sheep with cultured adult, mammary gland cells or fetal fibroblasts. These cultures are relatively easy to maintain in vitro but cannot be immortalized, because they become aneuploid after repeated passages. While this may make homologous recombination impractical, if not impossible, very large populations of such cells can be generated and stored frozen. A second option involves the use of totipotent embryonic stem (ES) cell lines derived from preimplantation-stage embryos. The ability of ES cells to serve as vectors for the transfer of foreign DNA in the production of transgenic animals is a well-established tool by which defined or random mutations of the genome can be propagated (11). ES cell nuclei can be reprogrammed readily in the context of nuclear transfer, and the cells remain undifferentiated and euploid during proliferation in vitro. Such cell lines have been cloned from rhesus monkey embryos that remain undifferentiated in continuous passage (12). Our studies in the rhesus monkey include nuclear transfer experiments with both fibroblasts and embryonic stem cells.

In conclusion, while it would be naive to believe that we now possess all the technological tools and ethical awareness to proceed unhindered in the area of somatic cell cloning, it would be short-sighted to abandon attempts to use our knowledge to better the

human condition. Applications of this technology in nonhuman animal models will undoubtedly contribute in a major way to vaccine development and the unraveling of teratological and genetic problems in human development as well as impacting wildlife conservation efforts.

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