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History and Current Status of Nuclear Transfer Cloning

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Introduction

The desire to produce an identical twin of an animal, especially a valuable agricultural animal or a beloved family pet, is long-standing but, unlike plants, higher animals cannot be cloned from cuttings and, unlike simple invertebrates such as the flatworms, higher animals cannot be reproduced simply by cutting them in half.

Yet, since the creation of Dolly, the first sheep cloned via somatic cell nuclear transfer (SCNT), in 1997 (Wilmut *et al.*, 1997), scientific research has used this cloning methodology and produced cloned animals in at least thirteen mammalian species (Lee *et al.*, 2005) using somatic cells as nucleus donors, and in even more species if we include the amphibians upon which the earliest cloning research was done (Gurdon, 1962).

There are two types of cloning: reproductive cloning, which produces a genetic 'twin' of the organism that donated the cell nucleus, and therapeutic cloning, which does not produce a new whole organism but can produce specific cell types with potential medical applications (Xu and Yang, 2004). Reproductive cloning can improve the genetics of livestock – such as better quality meat and increased milk yields – and show animals, can produce genetically identical animals for biomedical and veterinary research, and can aid in reproduction of endangered species. New technologies will allow improvement in food animals and may be accomplished through selection of animals with the best genetic performance for cloning, producing a nuclear genetic copy of the parent.

Therapeutic cloning of human cells is expected to produce cells, tissues, and organs that will complement or replace damaged tissues or organs in the human body. Possibly even before researchers have successfully used replacement cells

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Abbreviations: NKT cells, natural killer T cells; NT, nuclear transfer; ntESCs, nuclear transfer embryonic stem cells from cloned embryos; SCNT, somatic cell nuclear transfer.

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from cloning in the body or designed replacement organs, therapeutic cloning will yield cellular growth or development factors to test drug development or toxicity that could help in treating diseases. Therapeutic cloning has already entered the marketplace, with companies in Europe selling human embryonic stem cell lines for toxicology testing, replacing the use of animals in some of these tests.

Yet, cloning can be an ethical and legal slippery slope. Most countries have banned or will ban human reproductive cloning, as they should, but there are serious ethical issues that may limit human therapeutic cloning, including a position that is accepted by the current US administration: an embryo is a human life (Fox, 2004). There is also concern in developing countries that allowing cloning will create a commercial market for women's eggs (Fox, 2004). National legislatures and international bodies, such as the United Nations, are and will be facing these complex issues (Galli *et al.*, 2004).

History

Some of the earliest research that created clones was carried out by the great developmental biologist, Hans Spemann, in the early 1900s. He discussed his work, mainly on the European common striped newt, *Triton taeniatus*, in his 1935 lecture upon receipt of the Nobel Prize in Physiology or Medicine (Spemann, 1965). Although forming clones from early amphibian embryos was not the goal of his research, Spemann found that by bisecting newt embryos up to the early gastrula stage, he was able to produce twins. He also produced chimeras by removing pieces from a *T. taeniatus* embryo and exchanging them with pieces from a *T. cristatus* embryo – and even by exchanging pieces of tissue between newt and frog embryos. Spemann hypothesized that to learn whether the nucleus of a differentiated cell was capable of causing the production of a full embryo, one would need to implant such a nucleus into an enucleated oocyte (Yang, 1991).

The next stage in the development of cloning techniques was to do just what Spemann had suggested. Early studies implanted nuclei from blastula cells into oocytes in the frog, *Rana pipiens* (Briggs and King, 1952; King and Briggs, 1955), resulting in the development of tadpoles. Adult frogs developed from embryos produced by the same technique in the African clawed toad, *Xenopus laevis*, and in *R. pipiens* (Gurdon, 1962; McKinnel, 1962). Clearly, the next step was the production of embryos through somatic cell nuclear transfer, in which a nucleus from a somatic cell, not an early embryo cell, is implanted into an enucleated oocyte. Initial studies, again in the frog, used somatic cells from late embryos (Gurdon, 1962) but, eventually, researchers began using somatic cells from tadpoles or adult amphibians (Gurdon *et al.*, 1975). Here, research was moving closer to the dream of cloning a mammal.

Some of the early techniques experimented not only with the types and ages of cells used in nuclear transfer but also on the means of destroying the nucleus in the host oocyte. Although, from Spemann's time onwards, standard methods used micromanipulation of the ovum to remove the nucleus, Gurdon and colleagues destroyed the host cell's nucleus with ultraviolet light before implanting a skin cell nucleus into *Xenopus* eggs (Gurdon *et al.*, 1975).

Despite these rapid accomplishments in early cloning experiments in the mid

1970s, there was some doubt that cloning of an entire animal would be accomplished in mammals, particularly after reported failures of embryonic cloning in mice (McGrath and Solter, 1983). Yet, by the mid 1980s, cloning from embryonic cells had already been successful in sheep and cattle (Yang, 1991).

Sheep were initially cloned via surgically splitting an early embryo harvested from a donor ewe and implanting the embryonic pieces into recipient ewes (Willadsen, 1979). This method is referred to as embryo splitting, which was later used in cattle in the 1980s (Norman *et al.*, 2004). The technique produced genetically identical offspring, as they all originated from cells from the same embryo. By the mid to late 1980s, after a successful report of nuclear transplantation in sheep embryos (Willadsen, 1986), nuclear transplantation techniques began to take hold in the dairy industry (Norman *et al.*, 2004). These were based on methods that used early embryos as the source of the nuclei, which were then transplanted into enucleated oocytes (Willadsen, 1986; Prather *et al.*, 1987; Robl *et al.*, 1987). Some of these oocytes were collected from live cows after oocyte *in vivo* maturation, and others were collected from slaughtered cows, followed by *in vitro* maturation of the oocytes. Norman and colleagues have pointed out that neither method was tremendously popular (Norman and Walsh, 2004; Norman *et al.*, 2004), as a maximum of 1536 cows and 783 bulls created by embryo splitting were registered with the Holstein Association USA between 1982 and October 2002, and the greatest number of these animals (246) was produced in 1985. Nuclear transfer was even less popular, reaching a maximum of 1000 Holstein cows and bulls born in the late 1980s and early 1990s.

Problems with these methods include the possibility that several embryos would have been removed from the original mother and, after embryo splitting, there was no easy way of knowing for certain which animals were clones and which were siblings. Although the Holstein Association required blood typing of offspring produced by embryo splitting, the same blood type did not guarantee that the animals were, indeed, clones and not simply siblings (Norman *et al.*, 2004). Furthermore, nuclei from embryos produce a low rate of calving (Bousquet and Blondin, 2004).

Somatic cell nuclear transplantation (SCNT), in which a nucleus from an adult tissue somatic cell is transferred into an enucleated oocyte, became an additional method of cloning once Wilmut and colleagues had shown that it could be done successfully by producing Dolly the sheep (Campbell *et al.*, 1996; Wilmut *et al.*, 1997) (*Figure 4.1*). SCNT – or, in fact, any cloning – is not without problems: in most species, cloned offspring are very large, embryo loss and perinatal loss are high, and placentas may be abnormal. However, cloned animals that survive to reproductive age appear to be healthy.

We have been successful in producing second generation bull clones (Kubota *et al.*, 2004). Even the second generation cloned bull has normal health and fertility.

Furthermore, the age of the nucleus donor is not a limitation for producing viable clones. We successfully cloned a 17-year-old breeding bull using his ear skin cells in 1998, and the four surviving clones are healthy and normal (Kubota *et al.*, 2000). Also, in another study, muscle cells from an older, infertile bull were used successfully in SCNT to produce four cloned bulls that survived to maturity. The semen of two of these bulls, in turn, was used in *in vitro* fertilization, and one of the bulls sired ten apparently normal calves (Shiga *et al.*, 2005).

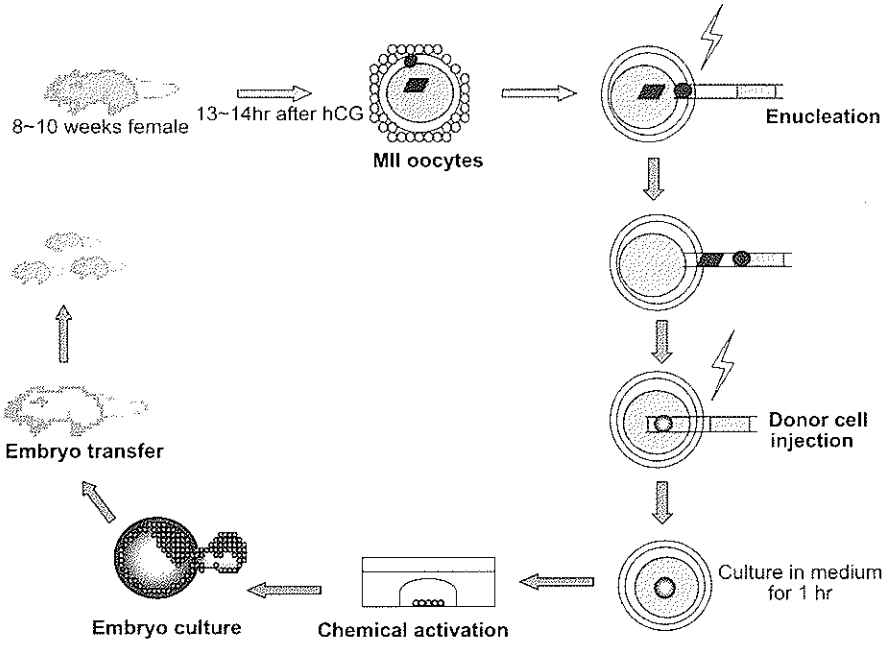

Figure 4.1. Outline of nuclear transplantation (NT) using the mouse as an example. A female mouse is treated with hormones to stimulate development of many mature oocytes. Oocytes in metaphase II (MII) are selected for use in an NT procedure. The oocyte is enucleated and the nucleus of another cell (in somatic cell nuclear transplantation, or SCNT, a somatic cell, such as a fibroblast, cumulus cell, white blood cell, or skin cell may be used) is injected into the enucleated oocyte. The fertilized egg is allowed to develop *in vitro* to the blastocyst stage. At that stage, the blastula may be implanted into the uterus of a female that is hormonally ready to incubate the early embryo.

Although sheep, cattle, pigs, goats, mice, rabbits, gaur, horses, mules, rats, and cats have been cloned (Xu and Yang, 2004), perhaps one of the most technically difficult animals to clone was the dog. Yet, this last summer, Lee *et al.* (2005) staged a coup by cloning an Afghan hound via SCNT.

In humans, unlike farm, research, or companion animals, the goal is to use SCNT for therapeutic, and not reproductive, cloning. The goal, which has not yet been accomplished, is to produce patient-specific embryonic stem cells via SCNT. These cells would be an exact genetic match to the patient.

Recently, we have compared global gene expression between cloned and naturally fertilized embryos to test whether the embryonic stem cells from cloned embryos are as normal as those from naturally fertilized embryos (Smith *et al.*, 2005). When we compared cloned embryonic cells with the adult donor cells used for cloning, 84% of the genes in the cloned embryos were expressed at different levels. Furthermore, the genes in the cloned embryos had switched to an expression pattern 99% similar to that of naturally fertilized embryos. In short, the gene activity in the cloned embryos was more like that of other embryos and less like that of adult cells. This is good news for therapeutic cloning, but bad news for reproductive cloning.

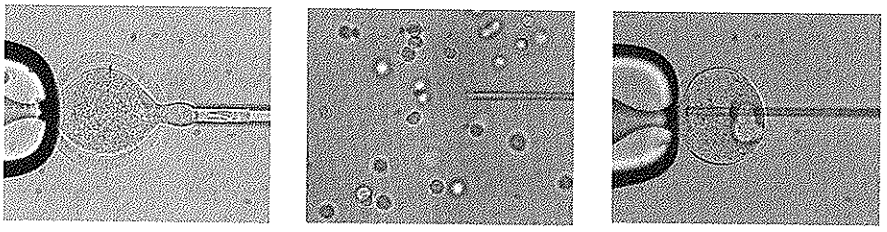

Figure 4.2. Photomicrographs of the nuclear transfer procedure. Left, enucleation of the donor oocyte. Centre, selection of the somatic cell for nuclear donation. Right, injection of the nuclear donor cell into the enucleated oocyte.

One question is whether the cells used successfully in SCNT are, in fact, 'stemness' cells and are not fully differentiated mature adult cells. Inoue and co-workers (2005) have produced mouse clones by implanting the nuclei of natural killer T (NKT) cells into enucleated mouse ova in a one-step cloning procedure. These more-differentiated cells produced a larger percentage of blastulae and morulae than did less-differentiated peripheral blood T cells. In fact, the T cells produced no fetuses or even sole placentae, while SCNT using NKT cells produced four fetuses. In our recent research, we went one step further and used granulocytes, cells that were absolutely terminally differentiated, for SCNT in a one-step procedure in mice (L. Sung and X. Yang, unpublished). These cell nuclei produced more blastulae/morulae than bone marrow-derived haematopoietic stem cells, which may be presumed to be less differentiated.

Persistent rumours that a human has been cloned remain unverified, and their sources have been dubious, although a 4-cell human embryo was produced by SCNT, and implanted, but did not result in a pregnancy (Zavos and Illmensee, 2006).

Methods

The basic nuclear transfer technique has not changed considerably since the early amphibian experiments; particularly, it is similar to the successful technique commonly used for embryonic cell (blastomere) NT. But what has evolved is our knowledge of the appropriate stage of development or reproduction of both the recipient cell (oocyte) and the donor cell.

Depending upon the species to be cloned, oocytes may be taken from ovaries obtained from an abattoir (cattle) or from females who have been pharmacologically superovulated [as in humans (Zavos and Illmensee, 2006), mice (Wakayama *et al.*, 1998), or dogs (Lee *et al.*, 2005)]. Oocytes taken from ovaries need to be maintained in an *in vitro* culture system (Ohkoshi *et al.*, 2003). In fact, what made cloning a dog so difficult was that canine oocytes need to mature *in vivo* and it is difficult to time oocyte harvest so that the cells are at the correct stage for SCNT, which is at the metaphase II stage of meiosis (Lee *et al.*, 2005). The oocyte nucleus is removed by micromanipulation, commonly under an invented microscope.

For SCNT, donor tissue somatic cells are maintained in culture *in vitro*. The cell cycle stage of the donor cell is important in determining the potential success of the cloning process. Donor cells in G0 or G1 tend to be the most effective in producing

viable offspring, although the specific stage and the treatment of the cultured cells may depend upon the tissue type being cultured (Tian *et al.*, 2003).

In a one-step process, the donor cell at the correct stage is introduced into the mature enucleated oocyte and the cells are fused with the enucleated oocytes by subjecting them to an electrical pulse. They then may be activated chemically (Cibelli *et al.*, 1998) and maintained in culture *in vitro* until they develop into blastocysts (Tian *et al.*, 2003).

In a two-stage procedure, as has been used to clone pigs (Polejaeva *et al.*, 2000), the fused cell forms a pronucleus within a karyoplast, which itself is fused with an enucleated zygote from a third donor pig. That new cellular construct undergoes electrofusion, creating a reconstructed zygote.

Whether a one- or two-stage method is used, when the blastocyst is formed, there must be a surrogate mother available for embryo transplantation. Surrogate mothers must be hormonally synchronized so they are at the correct stage of the oestrous cycle to receive the embryo and establish a pregnancy.

For therapeutic cloning, embryos in the blastocyst stage that have totipotent stem cells (i.e. in their inner cell masses) are collected and then maintained in culture *in vitro*. Inner cell mass cells are then removed for culture. Because of their totipotency, these nuclear transfer embryonic stem cells from cloned embryos (ntESCs) can eventually be differentiated into specific cell types (Xu and Yang, 2004).

In some cases, especially when a transgenic clone is desired, the somatic donor cell initially may be transfected with the DNA to be integrated into the clone's genome. Transfection may be carried out via microinjection, liposomes, or electroporation. Early stage embryos can be transfected via a retroviral vector.

DRAWBACKS TO NT OR SCNT

Producing clones by NT or SCNT, despite its great, rapid, and visible successes, still has many drawbacks. It is highly inefficient, resulting in few term pregnancies and even fewer surviving offspring (Wilmut *et al.*, 2002). It is expensive, and labour and animal-use intensive. For example, Lee and his colleagues produced 1095 reconstructed canine embryos and implanted them into 123 surrogate mothers, resulting in two cloned dogs, of which only one survived beyond the age of one month (Lee *et al.*, 2005). Efficiencies of scale must be reached before cloning will be a major means of livestock or companion animal production.

Ethical issues do not only beset the question of human reproductive and therapeutic cloning, but they also affect animal cloning. There are questions of whether nuclear donors may be of one preferred genetic line to the exclusion of other lines, and whether these cloned animals are, indeed, as normal as they currently appear to be.

Questions of whether human therapeutic cloning should be allowed or whether it should be limited need to be addressed, especially as not allowing research on therapeutic cloning may affect a nation's scientific progress and may withhold research on needed therapies from people who are ill.

Yet, despite these thorny questions, there is no doubt that animal reproductive and human therapeutic cloning will go on in some parts of the world.

Potential advantages

There are many potential advantages to cloning research. Cloning is expected to

produce high-genetic-value agricultural animals for developing countries; it can change a country's ability to produce agricultural products, such as meat and dairy. It can produce cell lines that can be used for toxicological and pharmaceutical research, reducing the numbers of animals used in research laboratories. It can produce tissue and organs to replace damaged tissue. Research on creating substitute pancreatic cells for people suffering from type I diabetes is expected to radically change the lives of those affected by the disease by eliminating the need for insulin and tight dietary control and averting the long-term health effects on every system of the body. Tissue that could repair a damaged heart may one day obviate the need for heart transplants. And tissue that could be implanted into the brain to replace tissue damaged by Parkinson's disease will give people with this degenerative disease some hope for a normal life.

And perhaps, more importantly, studying the biochemistry, genetics, cellular biology, and developmental biology of embryonic stem cells and cloned animals and cells will help us understand why organisms develop as they do, and how to prevent abnormal development, as occurs in cancer and many diseases.

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