CHAPTER 1

HISTORICAL PERSPECTIVE

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As we reflect on the transition from nature's ancient unassisted cloning to the modern era of attempting to understand the basic mechanisms of arrays of gene action during development in a complex genome (Oliver *et al.*, 2001; Venter *et al.*, 2001), we should not forget basic ideas and paths that pioneers laid out before us, stimulating modern efforts to clone mammals. Who directed attention to the basic molecules responsible for hereditary characteristics? Avery *et al.* (1944) are often overlooked as the group that discovered DNA as the molecule of life. Soon after, Beadle (1946) described how genes regulate biology. Spemann (1938), and others, established a model in amphibians that served as an excellent guideline to build on to clone mammals. The study of sperm, eggs, and embryos, the development of successful procedures for artificial insemination, and embryo manipulation, culture, and transfer provided a background for cloning research (Heape, 1897; Brackett *et al.*, 1981; Foote, 1998, 1999).

ANCIENT CLONING (ASEXUAL REPRODUCTION)—IN THE BEGINNING

Cloning, or asexual reproduction, a time-honored method of reproduction, is the method by which many extinct and existing species of organisms reproduced. "Clone" comes from the Greek word "klon," meaning twig. In horticulture clones are defined as descendants of a single plant. This clearly indicates an expected genetic identity within a clone. When a particular genotype is well-adapted to a particular environment, cloning is a simple and favorable way to reproduce.

Many plants can propagate by both asexual (vegetative) and sexual reproduction. Many simple one-celled organisms, such as *Escherichia coli*, propagate primarily by a type of asexual reproduction called binary fission. A relatively simple single circular chromosome containing DNA replicates, and by binary fission one bacterium becomes two identical progeny cells. However, even *E. coli* have sex factors and occasionally conjugate. Yeasts have various stages in which they exist with haploid, diploid, or a mixture of one or two copies of DNA. Simple protozoa, such as paramecia, reproduce both asexually and sexually. Thus, in the evolution of plant and animal types, many variations of sexual and asexual reproduction have arisen.

Sexual reproduction permits a continuum of genetic diversity whereby an organism can adapt to survive and thrive in many environments. Even earthworms, which are hermaphrodites, do not self-fertilize. Within a group of organisms, the diversity of genotypes promoted by sexual reproduction likely is responsible for the multitude of types that have adapted to fill various biological niches throughout the evolutionary process.

SPONTANEOUS REGENERATION

Simple multicellular organisms such as *Planaria* (flatworms) can regenerate parts extensively. When cut in half each remaining half of the flatworm can regenerate the missing half to form a complete organism. Starfish can regenerate an arm that has been broken off. Various amphibians can regenerate limbs. However, in more complex animal forms regeneration generally is limited to repair of injury to tissues or organs, such as cut skin and broken bones. Damaged whole organs are not replaced spontaneously. Such repair would require a complex turning on and off of an array of genes in the appropriate sequence, as happens during embryological development, but without triggering action of other developmental genes. This beautiful process of development is poorly understood, but the advances in cloning and related technologies provide a golden opportunity to design studies to reveal more about how the intricacies of nature work.

TYPES OF CLONING

In this brief historical overview the term "cloning" will be used to reflect propagation of replicated biological material in many forms. Emphasis will be on studies designed to produce functional cloned individuals through a controlled series of mitotic divisions and cellular differentiation leading to formation of the fetus and placenta, and finally to the birth of complex individuals. Most of the millions of cells in the individual presumably contain the same genetic material in their nuclei. A cell that can give rise to a complete individual would be totipotent. Many early embryonic cells have the potential to produce cloned individuals. Other cells, such as embryonic stem (ES) cells, have the ability to produce the three germ layers, but, as currently manipulated *in vitro*, they may not result in formation of a normal trophoblast, placenta, and fetus. These cells are pluripotent, unless the nuclei are placed in the cytoplasm of oocytes, wherein they may be totipotent. Restrictions on development, as we currently describe them, may change rapidly as research moves our understanding forward. The manipulation, implications, and applications of pluripotent and totipotent cells will be found in various specialized chapters that follow in this book. With the unfolding of the information and technology that allow us to explore as never before the biology of life and its regeneration, it is an exciting time to live.

EXAMPLES OF NATURAL CLONING IN HUMANS AND ANIMALS

Identical twin embryos occur spontaneously at low frequency in human pregnancies, by mechanisms that are not well understood. One possibility is that the zygote divides into two blastomeres that ultimately form two identical individuals instead of a single individual, but duplication can also occur at somewhat later stages of early embryonic cell divisions. Identical multiplets beyond twins are relatively rare in nature, but it is known that the fertilized egg in the armadillo species divides mitotically, producing multiple blastomeres. These develop individually to produce four or more progeny, all genetically identical.

EXPERIMENTAL CLONING OF NONMAMMALS

AMPHIBIANS

Important discoveries that were made in early studies of amphibians provided guidelines for research with mammals, but not all were heeded. Spemann (1938) demonstrated that nuclei of newt salamanders are pluripotent up to the eight-cell stage.

This eventually led to intensive studies with *Rana pipiens* and *Xenopus laevis*, using nuclear transfer to determine whether a somatic cell underwent stable changes in the genome during morphogenesis, or whether the nuclei in differentiated somatic cells were capable of directing formation of new individuals (for references, see McKinnel, 1981; Gurdon, 1986; Di Berardino, 1997). It was recognized that oocyte cytoplasm had a major effect on activity of somatic cell nuclei. Therefore, nuclear transfer to enucleated oocytes was the method of choice to test pluripotency or totipotency of these nuclei.

Studies by Briggs and King (1952) and King and Briggs (1955) showed that oocytes receiving blastula nuclei in *R. pipiens* could be reared to maturity, but gastrula nuclei from the mesoderm or endoderm injected into oocytes were unable to develop into mature frogs (Briggs and King, 1960). Results with *Xenopus* were somewhat more successful (Fischberg *et al.*, 1958)—nuclei from the endoderm of tail-bud larvae were totipotent. Gurdon (1962b) also produced fertile adult frogs following transfer of nuclei from the intestines of tadpoles.

These studies revealed several significant points. First, there was a difference between the genetic reprogramming in *Xenopus* versus *Rana* under the laboratory conditions used to rear frogs to maturity. Second, some somatic cells could reach a significant stage in development, i.e., the nuclei did not require reprogramming beyond any that might occur immediately following injection into oocytes (Gurdon, 1962b). However, attempts to use nuclei from more advanced cell types demonstrated only pluripotency (see McKinnell, 1981; Gurdon, 1986). Even diploid spermatogenic cells, which might be expected to retain totipotency, were only pluripotent (Di Berardino and Hoffner, 1971). Thus, the view was held by some that nuclei in highly specialized somatic cells had undergone irreversible changes in DNA that precluded them from becoming totipotent.

One of the problems noted was the persistent prevalence of chromosomal anomalies in embryos derived from pluripotent nuclei (Di Berardino and Hoffner, 1970). In some experiments it was shown that this was an experimentally produced artifact because the recipient eggs were not completely enucleated. However, chromosomal anomalies were more likely associated with the differential rates of division between the recipient egg and the slower dividing nuclei of somatic cells (Di Berardino and Hoffner, 1970).

It was known that enucleated amphibian ovarian oocytes as recipients had an advantage over ovulated and fertilized oocytes (eggs). Oocytes do not divide in culture without some stimulus. Gurdon (1986) stated that in amphibians "a single nucleus transplanted into an egg will have increased to 10^4 nuclei in 7 hours, whereas 500 somatic nuclei injected into an oocyte will still be 500 nuclei after several days," because the oocyte does not respond spontaneously to injected nuclei. The oocyte can be maintained in what appears to be an unchanged state for up to 3 weeks.

The results with nuclear transfer in amphibians provided substantial information on likely successful techniques for cloning mammalian embryos, particularly because genetic material important for development has been so highly conserved during evolution. McKinnell (1981) stated "that procedures are available with mammals that are entirely analagous to the methods that have resulted in successful cloning with amphibians." To what extent did we take advantage of all this information in attempting to clone mammals?

Cloning Carrots

During the time that intensive studies on amphibian development using nuclear transfer were taking place, successful cloning of carrots received little attention (Steward *et al.*, 1958; Steward, 1970). The carrot studies illustrated the importance of providing the appropriate culture conditions for the proper expression of the genetic material (nature vs. nurture). The paper by Steward (1970) is extraordinary, and worth reading by anyone who is a student of cloning. In this paper Steward traces the research from the time that Haberlandt predicted that one should be able to grow artificial embryos from somatic cells until they were cloned. He documents in detail the development of special conditions required to isolate viable cells in quantity and to nurture them in an ordered way so that they will recapitulate embryogenesis instead of undergoing random cell proliferation. He pointed out the comparative totipotency of somatic cells nuclei in plants and amphibians, but noted the importance of oocyte cytoplasm in promoting embryogenesis in amphibians. As an example of the scope and vision of this paper the concluding paragraph is quoted:

> Thus, the subject has developed greatly since Haberlandt's bold, but unsupported, prophecy of 1902. However, there is still an outstanding gap to be filled. This involves the precise understanding of the means by which the totipotent cells of the angiosperm plant body (whose nuclei and cytoplasm clearly contain all the necessary information to become a whole plant and also the machinery to transcribe it) are constrained in the orderly course of development to do what they should, when they should and where they are. Thus, the free cell cultures of angiosperms, and the morphogenesis which they may be induced to exhibit, now furnish systems upon which these important problems may be investigated. No problems, not even those of outer space, have greater challenge than the marvelously coordinated journey through time and space of a fertilized egg, or of suitably stimulated somatic cells, as each may develop into a complete organism.

CLONING MAMMALS

Several overviews of the status of cloning have been published in the modern, rapidly moving field of mammalian cloning (Campbell, 1999; Colman, 1999; Chan, 1999; Dominko *et al.*, 1999; Fissore *et al.*, 1999; Polejaeva and Campbell, 2000; Foote, 2001; Peura *et al.*, 2001; Westhusin *et al.*, 2001; Renard *et al.*, 2002). These publications, in addition to the following chapters, should be considered for additional references.

Producing Homozygotes Experimentally

There are several ways that homozygous offspring may be produced, some with nearly identical genetics. Highly inbred mice are used routinely to provide uniformity among test animals, although they are not completely homozygous. A variety of methods have been used to diploidize haploid embryos, but developmental failures are high and they do not lead to multiple clones. These procedures contributed minimally to development of somatic cell cloning by nuclear transfer and will not be outlined here. They were reviewed by Markert (1982) and by Seidel (1982, 1983).

Producing Multiple Clones without Nuclear Transfer to Oocytes

The principle that one blastomere is totipotent in the two-cell embryo was demonstrated by Nicholas and Hall (1942). They derived progeny in rats resulting from half of a two-cell embryo following destruction of one blastomere.

Moustafa and Hahn (1978) divided morulae into halves and succeeded in producing eight sets of twin mice. This principle was independently applied by Ozil *et al.* (1982) and Williams *et al.* (1982) to produce identical twins in cattle. This procedure has also been applied commercially to increase the progeny of embryos collected from superovulated cattle.

Willadsen (1979, 1982) and Willadsen and Fehilly (1983) separated the blastomeres from sheep embryos at the two-, four-, and eight-cell stages, and placed them into surrogate pig zonae pellucidae. Substantial pregnancy rates were obtained with blastomeres from the two- and four-cell embryos, but they were very low when using single blastomeres from eight-cell embryos. The procedure may have been successful because Willadsen cleverly kept the blastomeres in close contact in agar chips, and an *in vivo* culture system was used. These studies also demonstrated that heterologous zonae pellucidae could be used. Also, the procedure has been successful in cattle, horses, and pigs. This advance in cloning demonstrated that individual blastomeres retained totipotency up to the eight-cell stage in sheep.

These researchers found that when combining blastomeres from four-cell and eight-cell embryos, the blastomeres from the four-cell embryos tended to form trophoblast and those from the eight-cell embryos tended to form the inner cell mass in these chimeras. Markert and Petters (1978) had reported that in triply chimeric mice the fetus may be derived from as few as three inner cell mass cells. Although chimeras are a mosaic of cloned cells, they are mentioned here because they contributed to the search for producing true clones.

Chimeras have been useful in understanding normal and abnormal embryogenesis by including appropriate genetic markers. Chimerism also has been used to produce interspecific pregnancies by production of the trophoblast and eventually the placenta of the species serving as a surrogate mother, and a fetus of another closely related species. These various applications have been reviewed by Anderson (1987). Because chimeric animals are characterized by variability in the proportion of cells that comprise the mosaic composition of different tissues, including the germ line, these animals cannot be copied with reasonable fidelity.

Mouse Cloning Limitations and Controversy

Further stimulation of research, seeking to resolve the limited success of cloning mammals, was provided by Illmensee and Hoppe (1981). These researchers reported that three genetically identical (cloned) mice were produced by injecting inner cell mass (ICM) cells into enucleated zygotes. The zygote has already been triggered to undergo further development as a result of fertilization. Although this was a major achievement, most researchers thought at the time that the DNA of adult cells was irreversibly modified, and the report was met with skepticism. Other researchers were unable to repeat these results.

McGrath and Solter (1984b) had reported that both maternal and paternal genomes were required for mouse embryogenesis. A decade later Kimura and Yanagimachi (1995) reported that mouse secondary spermatocyte nuclei injected into oocytes would produce young, indicating "that gametic imprinting of mouse spermatogenic cells is completed either in the testis before the second meiotic division or within the cytoplasm of a mature oocyte after artificial nuclear transfer."

The methylation and demethylation of chromosome proteins and other changes during spermatogenesis were considered to be part of the problem associated with the low efficiency of nuclear transfer as a practical method of producing clones. The roles played by these non-DNA changes in chromosome structure still are not understood. In addition, McGrath and Solter (1984a), from carefully done research, reported that nuclear transfer in mice from one-cell and two-cell stages could support blastocyst formation, but the nuclei from four-cell or later stages did not support blastocyst formation. Based on this collective information these researchers concluded that the differential functioning of the maternal and paternal genomes might be required for early development, and that this condition would not exist in nuclei of adult cells. Consequently, the prevailing view then was that cloning in mammals using somatic cell nuclei was not likely to be successful.

Many years later Cheong *et al.* (1993) were able to obtain newborn mice following injection of nuclei from eight-cell stage embryos into enucleated oocytes, provided the donor nuclei were obtained from the early stage (G1 stage) of the cell cycle. This indicated that complete reprogramming was possible after the embryonic genome was activated, but that chromatin structure changes in later stages of the cycle reduced the totipotentiality of the nuclei. However, somatic cells have since been used successfully as donor nuclei (Wakayami and Yanagimachi, 2001). It is not surprising that different results were obtained by different groups working with different strains of mice, and making small but not so subtle changes in procedures with the different selection of donor nuclei, or oocyte recipients, and varying micromanipulation and other steps through fusion, culture, and transfer. A remarkable beneficial effect of heterozygosity of the donor genome was reported by Eggan *et al.* (2001). All but one of the six strains of F_1 hybrid mice used to form ES cell lines were capable of producing live adult pups by nuclear transfer. Only one pup was produced by similar procedures using four inbred (homozygous) donor strains. This opens new avenues to explore gene expression during development. Most domestic animal donors used to date likely are highly heterozygous.

Cloning Domestic Animals Using Embryonic Cells

Despite the early failures of attempts to clone mice there were reasons to be optimistic about cloning domestic animals. Large-scale attempts to produce cattle clones by using multiple blastomeres of early embryos and recycling cloned embryos (Bondioli *et al.*, 1990) offered an opportunity to disseminate the genes of prize male–female combinations. Also, much could be learned about gene imprinting, cell lineage, etc., to fill large gaps in our understanding of early development. Now genetic programming at early stages of embryonic development in mammals could be studied *in vitro*.

Many techniques needed for successful cloning have been improved in recent years. Extensive research has been done and is continuing on embryo culture media and procedures (Daniel, 1978; Biggers, 1987; Gordon, 1994; Bavister, 1995; Foote, 1999, 2001). Embryo transfer became a standard practice, especially in cattle, and embryos could be preserved by cryopreservation following the work by Whittingham *et al.* (1972), with possible multiplication after some of the clones had been transferred and grown to maturity for phenotypic evaluation (Westhusin *et al.*, 1991). These successful procedures represented major advances in knowledge and application since the first successful embryo transfer in cattle by Willett *et al.* (1951).

In this brief overview on past development of cloning in mammals, examples of problems and progress are given for different species. However, emphasis is on cattle, being the species researched most extensively because of its agricultural importance (Massey, 1990). However, sheep often have served as a less costly ruminant model (Willadsen, 1986). Among domestic animals, pigs have been particularly difficult to clone (Prather *et al.*, 1989; Li *et al.*, 2000; Onishi *et al.*, 2000; Polejaeva *et al.*, 2000), although genetically engineered pigs have been cloned in limited numbers (Bondioli *et al.*, 2001).

Rabbits have been a useful model for nuclear cloning (Yang *et al.*, 1990; Collas and Robl, 1990; Yang, 1991). As indicated previously, the mouse has been difficult to clone (Tsunoda and Kato, 1993; Wakayama *et al.*, 1998; Wakayama and Yanagimachi, 2001), although Tsunoda *et al.* (1987) produced offspring following blastomere transfer to enucleated two-cell embryos.

Tools and Skills for Micromanipulation

Anyone who researched embryo micromanipulation in the 1970s and 1980s will remember the necessary emphasis on producing the appropriate micropipettes and micromanipulators, and learning how to handle embryos gently and deftly. One of the pioneers in this field, T. P. Lin, described equipment used to inject mouse eggs (Lin, 1971), and for intracytoplasmic sperm injection (ICSI) of mouse sperm. Lin described the consistency and size of the egg, and the proper equipment needed to penetrate the zona pellucida, and the vitellus. During microinjection, mouse eggs often shrank without appreciable damage, leaving a perivitelline space. Later Yang and co-workers (Yang *et al.*, 1990) expanded this space in rabbit oocytes by using hypertonic media to facilitate nuclear transfer without mechanically disturbing the cytoplast.

Gardner (1978) used microinjection techniques extensively to produce chimeras. He described the equipment needed in considerable detail, advantages and limitations, and stepwise procedures to follow for one to be successful. He emphasized the need for patience as much as manual dexterity. Finally, Gardner emphasized the need to have spare needles because as he stated "trying to continue with broken needles or partly occluded pipettes leads to a vicious circle of abortive operations and increasing exasperation." Practitioners of the art of micromanipulation can relate to these words of wisdom.

Embryonic Stages of Donor DNA and Totipotency

Based on the earlier amphibian experiments, it seemed clear that during embryogenesis individual cells lost their totipotency during differentiation. Consequently, mammalian cloning was focused on using early embryonic cells as totipotent stem cells. From an agricultural standpoint it was important to devise ways to use the most advanced embryos possible (maximal number of cells) so that the potential yield of clones would be maximal.

Why had the mouse been a poor model? What could be learned from this fact? The mouse genome is active at the two-cell stage. This might be a reason that cloning was so unsuccessful in mice, because there may not have been enough time to reprogram nuclei from more advanced embryonic cells, when they were transferred. Embryonic genomes of other animals normally are activated one to three cycles later than is the case for mice (Table 1). This may have facilitated cloning by nuclear transfer with more advanced embryonic cells from other species. Also, we know now that heterozygous mice can be cloned much more easily than inbred strains, and domestic animals are highly heterozygous.

During development the embryo undergoes a multide of synchronous morphologic changes. These include membrane polarity, cytoplasmic polarity, and changes in the microvilli over the surface of the blastomeres. These changes were reviewed by Ziomek (1987), with permanent cellular polarity established in the mouse by the eight-cell stage. As blastomeres continue to divide, the outer polar cells preferentially form the trophectoderm (TE) layer of the blastocyst, the precursor of the placenta. The inner nonpolar cells preferentially form the inner cell mass, giving rise to the fetus. How advanced could an embryo become before cells were no longer directly totipotent?

Table 1 Species Comparison of Stages of Genome Activation and Maintenance of Nuclear Totipotency before Dolly

	Stages in development by species[a]				
Event	**Mouse**	**Pig**	**Rabbit**	**Cow**	**Sheep**
Genome activation	Two cell	Two cell	Two cell	Eight to sixteen cell	Eight to sixteen cell
Nuclear totipotency	Two cell	Two cell	Thirty-two cell	Thirty-two cell	Sixty-four cell

[a]From the literature. Also, *Xenopus laevis* contains about 4000 cells before embryonic genome transcription, and tadpole intestinal epithelial cells were totipotent when transferred to enucleated oocytes.

Based on this background of events in the mouse, Koyama *et al.* (1994) examined the polarization of microvilli on the blastomeres of rabbit and cattle embryos through six cell cycles with the aid of scanning electron microscopy. In cattle, extensive polarization of the microvilli occurred at the 16-cell stage, whereas in the rabbit it occurred at the 32-cell stage. These polarity results provided further support for the hypothesis that loss of totipotency was a gradual phenomenon, that species differed, and that some cells might retain totipotency after multiple mitoses.

Stice and Robl (1988) were the first to clone rabbits using blastomeres obtained from 8-cell embryos. Later Collas *et al.* (1992) studied the stage of the cell cycle of 8- and 16-cell rabbit embryos and reported that 59, 32, and 3% of the nuclear transfers from donor blastomeres at the early, middle, and late stages of cell division, respectively, resulted in nuclear transplant blastocysts.

A contrast between pluripotency and totipotency is demonstrated in the difficult to clone pig. Prather *et al.* (1989) prepared 88 nuclear transfer embryos using two-, four-, or eight-cell nuclei, and one piglet resulted from the transfer of a four-cell nucleus to an enucleated, activated oocyte in metaphase II. This was the only cloned pig reported for more than 10 years. Recently Li *et al.* (2000) reported that two piglets were born following blastomere transfer from four-cell embryos produced *in vivo* to enucleated oocytes matured *in vivo*. Onishi *et al.* (2000) produced one piglet and Polejaeva *et al.* (2000) produced five piglets following injection of somatic cells into enucleated oocytes. In addition, freshly obtained pig ICM cells transferred to blastocysts have resulted in live chimeric progeny (Anderson *et al.*, 1994), and ICM-derived embryonic stem cells have contributed to a chimeric pig (Wheeler, 1994).

Research on nuclear transfer in cattle was facilitated by using slaughterhouse ovaries as a source of viable cytoplasts. This inexpensive source of oocytes was utilized by Lu *et al.* (1987), and reviewed by Gordon (1994) to produce progeny following fertilization *in vitro* and embryo transfer. Prather *et al.* (1987) studied development *in vitro* of blastomeres obtained from 2- to 32-cell bovine embryos following injection into enucleated oocytes. Development in culture tended to decrease when blastomeres from the more advanced-stage embryos were used. When 23 morula- or blastocyst-stage embryos were transferred to 13 heifers, two calves were produced. It is worth noting that the embryo culture system used at that time caused a block in embryo development at the 8- or 16-cell stage, so the embryos were cultured in ligated sheep oviducts. In contrast, when blastomeres from two-, four-, and eight-cell embryos were transplanted into pronuclear recipient embryos and placed in sheep oviducts for 5 days, no development was observed (Robl *et al.*, 1987). However, control nuclei from pronuclear embryos removed, reinserted, and cultured in sheep oviducts resulted in development to morulae or blastocysts, and normal offspring following transfer.

Many questions remained on the source of nuclei, the recipient cytoplasts, and the techniques to use. With advances in methodology, Keefer *et al.* (1994) produced calves following transfer of bovine ICM cells to enucleated oocytes. Collas and Barnes (1994) also produced progeny using ICM donor nuclei, but no progeny resulted when using granulosa cell donor nuclei. Thus totipotency of ICM cells was demonstrated as improved laboratory procedures became available, but nuclei from differentiated somatic cells were not totipotent under these conditions, or at least at an efficiency that was detected by the number of transfers.

Variability in Donor Nuclei, Recipient Oocytes, and Cell Cycle Synchrony

Because of the variability in results as experimental cloning progressed, it is valuable to examine some of the sources of variability. McGrath and Solter (1984a) used zygotes and Tsunoda *et al.* (1987) used early-stage embryos as recipient cytoplasts, but the earlier work with amphibians (Gurdon, 1962a,b, 1986) indicated that oocytes not yet activated to initiate mitosis were potentially more useful recipients of transferred nuclei. Willadsen (1986) was the first to demonstrate that nuclear transfer to mature oocytes in sheep could lead to successful reprogramming and the production of young.

Considerable advances appeared to be in the making when Granada Genetics, Inc. mounted a major effort to clone cattle commercially (Bondioli *et al.*, 1990; Massey, 1990). This group used both fresh embryos and frozen-thawed embryos that were obtained 5 to 6 days after superovulating donor cows (average number of blastomeres ranged from 28 to 48). There was no difference in development rate using blastomeres from embryos at different stages of development. However, the pregnancy rate was higher when fresh embryos were used as blastomere donors.

In addition to the stage of embryonic development used as a source of donor nuclei, the importance of donor–recipient cell cycle synchrony was recognized, and much early work focused on determining the optimal stage of the cell cycle for the recipient cell at the time of transfer (Robl *et al.*, 1986; Fissore *et al.*, 1999). Initially bovine oocytes were in short supply and were expensive because they came from superovulated cattle (Bondioli *et al.*, 1990). This was a labor-intensive and costly procedure. However, based partly on the success of Lu *et al.* (1987) with slaughterhouse ovaries, oocytes were matured *in vitro* (IVM), and these IVM oocytes were enucleated and used as the recipients for nuclear transfer. Massey (1990) reported pregnancy results with both sources of oocytes. Prather *et al.* (1987) had used oocytes from slaughtered cattle, but the IVM oocytes did not fuse readily with the transferred nucleus. Robl and Stice (1989) noted that further improvement in IVM procedures was needed for these inexpensive oocytes to be used as the cytoplast for accommodating any reprogramming of early embryo-stage donor nuclei. Also, it was important to develop procedures to enucleate completely the recipient oocytes (Westhusin *et al.*, 1992; Bondioli, 1993).

Bondioli *et al.* (1990) attempted to increase the number of clones by making cloned embryos from the previous generation of clones. However, the success rate decreased with repeated generations. Subsequently, others have attempted to increase the number of clones using methodologies that may have improved with time. Stice and Keefer (1993) obtained only 10, 2, and 3% calves with clones produced from one, two, and three recycled donor clones, respectively. Peura *et al.* (2001) compared one, four, and seven rounds of donor nuclei produced by nuclear transfer, and calving percentages resulting from the selected embryos transferred were 25, 4, and 0%, respectively. Whether the reduction in totipotent recycled nuclei is a result of intrinsic genomic modification during reprogramming and/or epigenetic factors during repeated manipulation is unknown.

Stice *et al.* (1994) obtained calves following nuclear transfer of blastomeres obtained from day 5 or 6 embryos that had been produced *in vivo* in superovulated cows as well as from blastomeres isolated from day 5 or 6 embryos produced by *in vitro* maturation, fertilization, and culture. Results were best when the aged recipient oocytes were activated prior to the time of fusion.

Similar studies were in progress in sheep (Smith and Wilmut, 1989). Reconstituted sheep embryos derived from either 16-cell (day 4) embryos or from the ICM of day 6 embryos resulted in 18% becoming lambs. Collas and Robl (1990) tested a variety of procedures in rabbits, including oocyte age, which increased the efficiency of the nuclear transfer procedure. With the best procedures, 23/110 (21%) of the cloned embryos derived from 8- to 16-cell stage donor nuclei developed into young, compared with 4/41 (10%) of the control nonmanipulated embryos.

Many procedures were used to obtain highly viable donor nuclei and recipient cytoplasts, as well as to ensure appropriate synchrony between the cell-cycle stage of the donor and recipient cells (Prather *et al.*, 1987; Robl and Stice, 1989; Smith and Wilmut, 1990; Foote and Yang, 1992; Yang and Anderson, 1992; Campbell *et al.*, 1996b; Heyman and Renard, 1996). Donor nuclei and recipient cytoplasts respond differently to varying culture conditions. It is amazing to note how many published procedures were successful to some extent, and it is not clear what is optimal. Certainly improvements have been made in preparing the donor nuclei (blastomeres) and recipient oocytes, in ensuring complete enucleation of the oocytes with minimal damage during injection of the donor nucleus into the recipient oocyte, in fusing and activating the oocyte more appropriately, and in improved culture and transfer of the manufactured embryos. The development of a more compatible system has allowed nuclei from more advanced-stage embryos to express their totipotency, limited in the beginning by suboptimal handling procedures.

Advances have been made in understanding the biology of cell-cycle regulation. For example, changes in substances critical in cell-cycle regulation, such as meiosis–mitosis phase (maturation)-promoting factor (MPF, $p34^{cdc2}$/cyclin B) and cytostatic factor (CSF), have been identified. Oocyte maturation, germinal vesicle breakdown, and chromosome condensation are promoted by MPF. Oocytes are prevented from exiting metaphase II by CSF, which is inactivated after fertilization or parthenogenetic activation. Addition of various biochemical criteria associated with detailed morphologic changes (Kaňka *et al.*, 1991) will assist in defining oocytes that are competent to perform the role to which they are uniquely fitted for mammalian cloning.

Embryonic Stem Cells

Brief comment on ES cells is in order because they were intensively investigated as a possible means of establishing pluripotent cell lines for domestic animals following earlier success with certain strains of mice (see Yang and Anderson, 1992; Anderson, 1999; Piedrahita *et al.*, 1999). These cells can be multiplied in an undifferentiated state under precise culture conditions. These cell lines were valuable for developmental studies, use of genetic markers to study cell lineage in chimeras, and possible genetic engineering. However, few lines from domestic animals were successfully developed, they were difficult to maintain, and they were of limited value in producing clones because they were not totipotent. With the successful cloning from somatic cells (Wilmut *et al.*, 1997), and the ability to maintain totipotent lines of fibroblasts, the value of ES cells for experimental or practical ends diminished.

Cloning Domestic Animals from Somatic Cells

In 1996 there was a signal (Campbell *et al.*, 1996a,b) that the world of cloning and stem cell biotechnology would never be the same. This was followed with the paper by Wilmut *et al.* (1997) of the production of Dolly, a viable lamb resulting from a cultured cell derived from somatic mammary gland cells harvested from a 6-year-old ewe. Some questions were raised concerning this "impossible" event, but this carefully conducted research has stood the test of subsequent experimentation by others. Only one viable lamb was produced from the original fusion of 277 donor nuclei and enucleated oocytes. However, one was enough to stimulate the scientific community to explore the mechanisms that made this remarkable event possible, and to utilize this knowledge to improve the efficiency and safety of cloning procedures. Needless to say, the world news media flooded the print press and airwaves with fanciful stories; "rubber stamping" and "ditto machine" cartoons spread fear and some thoughtful concern among the public and legislative bodies. My own 1997 files are filled with daily communiques, and requests for mass media interviews, because Dr. Yang in our laboratory had cloned rabbits and cattle from embryonic cells many years earlier (Yang, 1991).

What Wilmut, Campbell, and colleagues had done (Campbell *et al.*, 1996a,b; Wilmut *et al.*, 1997) was to culture cells on a minimal medium that caused the cells to exit the growth cycle and become arrested in the quiescent (G0) stage. These G0 nuclei are diploid. They were transferred to metaphase II (MII)-stage enucleated oocytes, avoiding chromosomal damage due to premature chromosome condensation. Cells maintained in this quiescent G0 stage for a period of time can undergo various changes in chromatin structure with alteration of specific factors that could promote or result in reprogramming. Many of these important changes during embryogenesis are dealt with in various chapters in this book. The expansion of knowledge rather than commercial cloning of animals likely will be the first payoff from cloning and allied research.

Colman (1999) summarized the practical results of live births reported from somatic cell (nuclear) transfer in cattle, sheep, goats, and mice. Production of multiple cattle clones has been encouraging (Kato *et al.*, 1998; Wells *et al.*, 1999). Numerous reviews have been written in the past decade discussing the implications, future avenues for research, and applications of cloning for animal agriculture and medicine (Robl and Stice, 1989; First, 1990; Smith and Wilmut, 1990; Yang and Anderson, 1992; Di Berardino, 1997; Wilmut *et al.*, 1998; Stice *et al.*, 1998; Colman, 1999; Seidel, 2000). Improved techniques for somatic cell processing and donor–recipient synchrony (Stice *et al.*, 1998; Campbell, 1999; Colman, 1999; Dominko *et al.*, 1999; Mohamed Nour *et al.*, 2000) are being investigated in many laboratories. These include the ability to maintain totipotency following limited (Cibelli *et al.*, 1998) or multiple passages of cells (Kubota *et al.*, 2000), important for experimental gene targeting and practical genetic engineering (Murray, 1999; Piedrahita *et al.*, 1999; Polejaeva and Campbell, 2000; Piedrahita, 2000; Georges, 2001; Westhusin *et al.*, 2001). Combining genomic information with the ability to detect selected gene expression in embryos will facilitate marker-assisted selection (Chan, 1999; Band *et al.*, 2000; Bredbaka, 2001; Cross, 2001; Georges, 2001; Roberts, 2001). With new knowledge rapid progress is being made when compared to earlier reports based on theory and less efficient procedures (Van Vleck, 1981; Hammer *et al.*, 1985; Pursel *et al.*, 1989).

An area of major interest not only to cloning, but also to aging and cancer research, is the regulation of cell proliferation and senescence, stimulated by the report on telomere length in Dolly (Shiels *et al.*, 1999). However, telomere length does not seem to be a problem in other studies (Lanza *et al.*, 2000a; Tian *et al.*, 2000; Yang and Tian, 2000; Betts *et al.*, 2001; Xu and Yang, 2001), nor is telomere length the only component affecting the inherent ability of cells to continue to proliferate.

Cloned animals as producers of pharmaceutical products (Wilmut *et al.*, 1990; Wall *et al.*, 1997; Ziomek, 1998; Brink *et al.*, 2000), as organ donors (Stice *et al.*, 1998; Colman, 1999; Bondioli *et al.*, 2001), and as models for studying diseases are other examples of useful applications. Cloning also has an application in preserving endangered species (Wildt, 1992; Wells *et al.*, 1998; Lanza *et al.*, 2000b; Solti *et al.*, 2000). However, there are many unsolved problems, such as the high incidence of anomalies (Garry *et al.*, 1996; Hill *et al.*, 1999) and the inefficiency of the current procedures (Colman, 1999; Dominko *et al.*, 1999). These must be improved before they become cost-effective for most applications.

One of the most exciting fringe benefits is the major increase in research on developing procedures to enable many types of differentiated somatic cells to be reprogrammed and guided into forming specific cells and tissues. This has great potential for improving the quality of life for our aging human population afflicted with age-associated diseases.

This has been and will continue to be a wonderful journey on the road to understanding the biology of, and pondering the meaning of, life (Foote, 1992, 1998, 1999). It has been marked by insight, determination, and serendipity. The latter word, coined by Walpole in 1754 (Remer, 1965), combines sagacity with accidental discovery, and is not correctly defined in the dictionary. The largest room in the world is the room for improvement. Exciting times lie ahead as the mysteries of developmental biology are waiting to be unraveled. The opportunities in this area are as exciting for the twenty-first century as landing on the moon was in the twentieth century. These powerful techniques also put powerful emphasis on us to discern how these technologies might best be applied, especially to our own species. We should all ask and ponder the question, "just because we can do it, should we?" Those who blaze trails should also assume some responsibility for the consequences.

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