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Animal cloning: problems and prospects

D.N. Wells

Reproductive Technologies Group, AgResearch Ruakura, East Street, PB 3123, Hamilton, New Zealand

Summary

An efficient animal cloning technology would provide many new opportunities for livestock agriculture, human medicine, and animal conservation. Nuclear cloning involves the production of animals that are genetically identical to the donor cells used in a technique known as nuclear transfer (NT). However, at present it is an inefficient process: in cattle, only around 6% of the embryos transferred to the reproductive tracts of recipient cows result in healthy, long-term surviving clones. Of concern are the high losses throughout gestation, during birth and in the post-natal period through to adulthood. Many of the pregnancy losses relate to failure of the placenta to develop and function correctly. Placental dysfunction may also have an adverse influence on post-natal health. These anomalies are probably due to incorrect epigenetic reprogramming of the donor genome following NT, leading to inappropriate patterns of gene expression during the development of clones. Whilst some physiological tests on surviving clones suggest normality, other reports indicate a variety of post-natal clone-associated abnormalities. This variability in outcome may reflect species-specific and/or cloning methodological differences. Importantly, to date it appears that these clone-associated phenotypes are not transmitted to offspring following sexual reproduction. This indicates that they represent epigenetic errors, rather than genetic errors, which are corrected during gametogenesis. Whilst this needs confirmation at the molecular level, it provides initial confidence in the first application of NT in agriculture, namely, the production of small numbers of cloned sires from genetically elite bulls, for natural mating, to effectively disseminate genetic gain. In addition to the animal welfare concerns with the technology, the underlying health of the animals and the consequential effect on food safety are critical aspects that require investigation to gain regulatory and consumer acceptance. Future improvements in animal cloning will largely arise from a greater understanding of the molecular mechanisms of reprogramming.

Keywords

Cloning – Food safety – Health – Mammal – Nuclear transfer – Reprogramming – Trans-generational effects.

Introduction

Cloned offspring in humans and farm animals are sometimes produced in nature when the early embryo splits in two (or sometimes, more) pieces just a few days after fertilisation, before the cells have become too specialised. However, there are also a number of artificial methods to produce genetically identical mammals. Of these, the nuclear cloning methodology is considered to have the greatest potential application for animal agriculture and medicine. In this review, the current methodology, efficiency and applications of nuclear cloning are described, along with the present limitations of the technology that adversely affect the welfare, including the health, of some cloned animals. To address food safety concerns, data are presented demonstrating that milk from cloned dairy cows is similar in composition to that obtained from conventional cows. Emphasis is placed on the common farmed livestock species, especially cattle and sheep. Relevant data from mouse models, which aid our understanding of the biology and consequences of nuclear cloning, are also discussed.

Nuclear cloning

The production of nuclear clones is a multi-step process that essentially generates an entire organism from the nuclear deoxyribonucleic acid (DNA) of a single donor cell using a technique known as nuclear transfer (NT). The basic methodology was first developed in amphibians in the 1950s and was used to investigate nuclear totipotency in differentiated cell populations. In livestock species, undifferentiated embryonic blastomeres were first used successfully in sheep (82), cattle (46) and pigs (47). In more recent times, embryonic NT has been extended in mice to include the use of other undifferentiated cell types including embryonic stem cells derived from the inner cell mass of blastocysts (68). Conversely, the use of more differentiated cell types obtained from either embryos (6), foetuses or most significantly adult animals, as in the case of 'Dolly' the sheep (83), overturned a dogma in biology concerning nuclear totipotency from adult cells and has opened new opportunities and directions in research. This has been termed somatic cell NT to distinguish it from embryonic NT.

Nuclear cloning methodology

The nuclear cloning process comprises a sequence of five main steps, which are summarised below.

Firstly, oocytes, commonly obtained by aspirating follicles on ovaries collected from commercially slaughtered cows, are matured *in vitro* and then enucleated. This process involves the physical removal of the metaphase chromosomes (which incorporate the oocytes' own nuclear DNA) and the extruded first polar body, using finely controlled micro-surgical instruments. Thus, the nuclear genetic material of the oocyte is removed, resulting in what is termed a cytoplast (a cell containing only cytoplasmic material). The mitochondrial DNA within the cytoplasm of the oocyte remains present.

Secondly, with conventional NT methods, a single donor cell is injected underneath the outer *zona pellucida* and adjacent to the cytoplast membrane. The donor cells can come from a variety of sources representing different degrees of cellular differentiation. For instance, donor cells could be embryonic blastomeres, cell lines such as embryonic stem cells, or primary cultures derived from biopsies obtained from selected adults.

Thirdly, the cytoplast and the donor cell are then fused together utilising a direct current electrical field. Thus, the genetic information contained within the nucleus of the donor cell enters the cytoplast. This is the essence of the term 'nuclear transfer', whereby the genetic information from the oocyte is removed and is replaced with that from the donor cell. Immediately following reconstruction the donor nucleus has the opportunity to be reprogrammed following molecular interactions between factors present in the oocyte cytoplasm and the donor chromatin (see 'Incomplete reprogramming' below).

Fourthly, the reconstructed 1-cell embryos are artificially activated using either specific chemical signals or electrical pulses, in order to initiate embryonic development.

Fifthly, following activation, the reconstructed embryos are cultured *in vitro* in a chemically-defined medium, in the case of cattle, for seven days. After this time, embryos that have developed into blastocysts of suitable quality (that is, embryos comprising around 120 cells) are transferred to the uteri of recipient females that have a synchronised oestrus cycle, where some may develop to term and result in viable cloned animals.

The NT-derived animals are not strictly true clones and possess greater differences than naturally occurring monozygotic twins. Compared to the donor animal, they might for instance possess:

– different mitochondrial DNA derived from the recipient oocyte

– point mutations or other chromosomal rearrangements in the genomic DNA of individual donor cells used for NT

– alternative patterns of X-chromosome inactivation in females

– various other epigenetic alterations arising from *in vitro* culture (of the donor cells and/or reconstructed embryos) or perturbations from the NT process

– differences that occur as a result of environmental influences from the oocyte cytoplasm, the maternal uterus in the surrogate female, or the post-natal environment.

In addition to the heritability of a trait, all these extra variables contribute to potential variations in phenotype (and also genotype in some cases) within a clonal family (a set of nuclear clones derived from the same source of donor cells) and deviations from the original donor animal.

Current efficiency of somatic cell nuclear transfer

Overall, the current efficiency of NT with somatic cells is poor. At AgResearch, the proportion of reconstructed 1-cell cattle embryos that develop to transferable quality blastocysts after seven days of culture (40%) is comparable to that following *in vitro* embryo production (IVP) (i.e. *in vitro* matured, fertilised and cultured) with abattoir-derived

oocytes. However, at present, *in vivo* development is only one-third that following IVP. For example, of 988 somatic cell cloned embryos transferred at AgResearch, only 13% resulted in calves delivered at full term (76). This compares with 30% to 45% embryo survival with IVP (30, 60). Although pregnancy rates in cattle on day 50 of gestation after the transfer of single NT embryos can be as high as 65%, and similar to both IVP embryos and artificial insemination, there is continual loss thereafter with the clones (35). Moreover, peri-natal and post-natal mortality rates with cloned offspring are greater than normally expected, with only 64% of cloned calves surviving to weaning at three months of age (76). Recently, the concerns regarding the long-term health and survival of clones into adulthood have been more fully appreciated (76).

From an animal welfare perspective, in addition to farmer and consumer acceptance of the technology, these losses must be solved before any large-scale cloning opportunities are practicable or tolerated. Ideally, cloning efficiency should have pregnancy rates comparable to those achieved following sexual reproduction, principally artificial insemination or after transfer of *in vivo* produced embryos, which is 55% to 60% (30). It is important to remember, however, that somatic cell NT can be effective in producing what appear to be physiologically normal animals. This provides encouragement for eventually resolving technical issues and elucidating the molecular mechanisms responsible for effecting complete epigenetic reprogramming.

Complete reprogramming

There is evidence that some cloned animals are physiologically normal (33, 76) or at least they may develop a stable metabolism some time after birth (8, 17). It is remarkable that somatic cell NT is successful, for a tremendous amount is asked of a differentiated donor nucleus to re-establish the correct pattern of gene expression to allow normal embryogenesis. Various international studies in a range of species provide evidence that some clones appear to be the same as their non-clone counterparts in the following areas:

– behaviour (63)

– growth rates (84)

– reproduction (33, 67)

– livestock production characteristics (44, 72, 76)

– life spans (43).

Furthermore, the sexually-derived offspring of clones also appear normal (76).

Incomplete reprogramming

There are instances, however, where reprogramming appears to be incomplete. For normal development following NT, it is generally accepted that the epigenetic modifications in the donor nucleus must be reprogrammed to a state comparable to those in a zygote. This is necessary for the correct pattern of gene expression to occur during subsequent embryogenesis. This reprogramming must occur within a short timeframe, in a different cellular context compared with normal development, and is prone to error. There are increasing amounts of data documenting deviations in epigenetic reprogramming, with clones showing inappropriate patterns of:

– DNA methylation (5, 14, 29)

– chromatin modification (56)

– X-chromosome inactivation (86)

– expression of imprinted and non-imprinted genes (28, 39, 53).

The pattern of mortality and clone phenotypes observed presumably reflect the inappropriate expression of various genes, whose harmful effects are exerted at various different stages of development. Aberrations that occurred early in embryonic or foetal development may impair health in adulthood (43). There is a wide spectrum of phenotypic outcomes, ranging from those that are lethal to those that appear neutral. Outcomes which appear neutral do not compromise the health and welfare of the animal, but the epigenetic variations reduce the uniformity in the clonal family, which may be undesirable for some applications (1, 2). The common consequences of incomplete reprogramming following somatic cell NT are:

– higher rates of pregnancy loss

– difficult parturition

– higher rates of post-natal mortality

– some specific clone-associated phenotypes in adulthood.

Placental abnormalities

A failure of the placenta to develop and function correctly is a common feature amongst clones. The majority of early pregnancy failures, before placentome formation, are attributed to an inadequate transition from yolk sac to allantoic-derived nutrition, with poor allantoic vascularisation in sheep (13). Furthermore, there is reported evidence of immunological rejection contributing to early embryonic loss (27). Typically in cattle, 50% to 70% of pregnancies at day 50 are lost throughout the remainder of gestation and up to term (25, 33, 80). This is in stark contrast to only 0% to 5% loss with artificial insemination or natural mating over the same period (15). In extreme cases, placentomes are entirely absent at day 50. Shortly

thereafter, these pregnancies fail. More commonly, cloned placentae only have half the normal number of placentomes, display compensatory overgrowth and are oedematous (25, 35).

Of particular concern are the losses in the second half of gestation; especially the occurrence of hydroallantois, i.e. the excess accumulation of fluid within the allantois. Hydroallantois does occur with other forms of assisted sexual reproduction in cattle, ranging in incidence between 0.07% to 5% for artificial insemination and IVP, respectively (30, 66). With clones, however, typically 25% of those cows pregnant at day 120 of gestation develop clinical hydroallantois (hydrops) (D.N. Wells, unpublished findings). The range is variable (0% to 40%) with the incidence possibly dependent upon the individual cell line. Cases are typically severe enough to render the calves non-viable and on welfare grounds, in order to reduce the risk of mortality to the recipient cow, the standard practice is to electively terminate the hydrops pregnancy. This is an unsatisfactory management procedure and work is underway to identify non-viable pregnancies much earlier in development to lessen the welfare burden. Ideally, a reprogramming marker would enable only viable embryos to be transferred into recipients. Alternatively, abnormal pregnancy development could be determined by measuring specific components present in maternal serum with early detection allowing early elective abortion. The level of pregnancy specific protein b produced by the binucleate cells of the trophoblast was transiently higher at day 35 in those concepti that failed to develop to day 90 (25). Similarly, levels of pregnancy serum protein 60 were elevated over the first four months of gestation in those pregnancies that became pathological (21). Pregnancy monitoring is complemented by detailed ultrasonography (21, 25).

Parturition difficulties

Intervention is often deemed necessary to deliver cloned offspring, as gestation length in NT pregnancies is typically prolonged and the birth weight of cloned calves may be 25% heavier than normal. Newborn cloned calves display functional adrenal glands, so this extended gestation may be due to failure of the placentae to respond to foetal cortisol near term or to a lack of adrenocorticotropic hormone release from the foetus (8). Oversized cloned offspring add to the birth complications. They are larger than IVP, artificially inseminated or naturally-mated controls (52). It has been reported that somatic cloned calves are heavier than embryonic clones (21).

At AgResearch, the occurrence of prolonged gestation and the risk of dystocia initially prompted the delivery of clones by elective caesarean-section, following a brief exposure to exogenous corticosteroids (80). Recognising the welfare issues and the intensive peri-natal veterinary care often required, we have modified our calving management system. The aim is to have a planned vaginal delivery (with manual traction if necessary), using an alternative corticosteroid therapy to aid foetal maturation, especially of the lungs, and to completely induce parturition a week before expected full term (77). This protocol has reduced the incidence of caesarean-section to 5% to 10% and with the majority of cloned calves reared on their recipient dams. Although not completely natural, this approach towards delivering cloned calves in a controlled manner is feasible and acceptable on farm.

Post-natal viability

The viability of cloned offspring at delivery and up to weaning is reduced compared to normal, and this is despite greater than usual veterinary care. Data from our group shows that around 80% of cloned calves delivered at term are alive after 24 h (76). Two-thirds of the mortality within this period is due to a spinal fracture syndrome through the cranial epiphyseal plate of the first lumbar vertebrae or to deaths that occurred either *in utero* or from dystocia. Surviving newborn clones have altered neonatal metabolism and physiology, possibly due to placental abnormalities, and it takes time for these processes to adjust to normal (8, 17).

At AgResearch, typically an additional 15% of calves initially born alive die before weaning (76). In our experience, the most common mortality factors during this period are gastroenteritis and umbilical infections. Other abnormalities noted include defects in the cardiovascular, musculoskeletal and neurological systems, as well as susceptibility to lung infections and digestive disorders (26, 51). Hydronephrosis is particularly common in sheep (79), with correspondingly elevated serum urea levels in some surviving clones (78).

The proportion of cloned calves born that are longer-term survivors ranges between 47% and 80% (22, 33, 44, 76). AgResearch data show that the stage of the donor cell cycle at the time of NT affects subsequent calf viability. The proportion of cloned calves that survive to weaning is significantly greater for those derived from quiescent G0 donor cells (81%) than for those derived from G1 cells (50%) (74). Post-natal survival of cloned sheep is substantially less than that of cattle with both somatic and embryonic cell types (31% and 42%, respectively) (78 and D.N. Wells, unpublished findings).

Clone-specific phenotypes

Whilst there are some studies indicating that clones can be physiologically normal and apparently healthy (33, 76), there are other observations and reports in the literature of

abnormal clone-associated phenotypes that become apparent during the juvenile and adult phases of life. The incidence of these anomalies may vary according to the particular species, genotype or cell type, or according to specific aspects of the NT and culture protocols used. More research is required to determine the following:

– the effects of nuclear-mitochondrial interactions arising from a donor nucleus in a foreign cytoplasmic environment (23)

– the effects of mitochondrial DNA heteroplasmy and possible recombination events (24) on cloning efficiency

– the resulting fitness of the cloned animals.

The cloned offspring syndrome is a continuum, in that lethality or abnormal phenotypes may occur at any phase of development, depending upon the degree of dysregulation of key genes, presumably due to fundamental errors in epigenetic reprogramming. Even apparently normal clones may have abnormal regulation of many genes that are too subtle to result in an obvious phenotype (28).

There has been much debate about Dolly's shortened telomeres (58) and the possibility of premature aging and early onset of disease in clones. Telomeres are regions of DNA at the ends of chromosomes which progressively shorten after each cell division in most somatic cell types. Whilst Dolly may have developed arthritis and was euthanised at a relatively young age because of a virally-induced lung tumour (52), this may have resulted from her largely indoor housing and handling rather than the fact that she was a clone. Other studies have been contradictory with regard to telomere length in clones, with reports of restoration to normal in cattle (3, 64) and mice (69) and even instances of extended telomere lengths (32). The discovery of a telomere length restoration process that occurs during early embryogenesis appears responsible for this (57). Normal telomere lengths have even been reported after repeated recloning in mice (69) and cattle (31) and specifically, in the spermatozoa of somatic cell cloned bulls and subsequent progeny (37). Thus, in cattle and mice at least, it appears that telomere erosion generally does not occur in clones and is therefore unlikely to cause the long-term health and reduced life expectancy concerns raised by many recent reports.

The majority of (male) mice cloned from immature Sertoli cells died after approximately 500 days, which was around 50% of the lifespan in control mice (42). The causes of death were severe pneumonia and hepatic failure. It remains to be determined whether this is a general phenomenon with clones, but it appears to be both cell type and genotype specific, with other cloned mice having apparently normal life spans (43). The mouse model has the advantage of a shorter generation interval and biological life span to screen for these effects. Whilst it is encouraging that some studies report normal health of four year old bovine clones (33), it is too early to detect if phenotypes with shorter life spans will also occur among livestock. Although an important issue, even if cloning were to shorten lifespan, it may be of little significance in agriculture. In commercial beef production, for instance, cattle may be slaughtered at target live weight within two years, or in the dairy industry the average life span of a cow in the herd is only six years. In these examples, the productive life of farmed animals is substantially less than the biological limits for the species. However, studies at AgResearch show that between weaning and four years of age, the annual mortality rate in cattle cloned from somatic cells is at least 8% (76). This is in marked contrast to the negligible mortality experienced with the offspring of clones and the typically accepted mortality of 2% to 3% per annum in conventional pastoral farming. Although the reasons for death amongst the clones are variable, and some potentially preventable, the main mortality factor beyond weaning is euthanasia due to musculoskeletal abnormalities (76). This includes animals with severely contracted flexor tendons and those displaying chronic lameness, particularly in milking cows. This emphasises the point that any underlying frailties in cloned animals may not be fully revealed until the animals are stressed in some manner.

Again in mice, it was initially reported that females cloned from cumulus cells developed an increased body-weight phenotype, commencing eight to ten weeks after birth, (63) that was directly attributable to increased adipose tissue (62). However, this obese phenotype has also been recently observed in cloned mice produced from immature Sertoli cells and appears to occur at a greater frequency in agouti mouse strains (43). At present there is no indication for early onset obesity occurring in livestock.

Evidence of a compromised immune system is a clone phenotype noted in some species. Thymic aplasia has been documented in cloned cattle (50) and lower levels of antibody production in cloned mice (42) and of cytokines in cloned pigs (7) are direct indicators of a reduced immune response. This may increase their susceptibility to infection and disease. The incidence of enteritis, umbilical and respiratory infections are certainly increased in cloned livestock. However, others have reported normal characterisation of peripheral blood lymphocytes and normal responses to periodic infection in cloned cattle (33).

Assessment of animal behaviour and cognitive function provide an indicator of general physiological state and wellbeing. An examination of the behaviour of cumulus cell cloned mice (63) revealed that there was a delay of 0.6 to 2.3 days in the first appearance of three out of ten pre-weaning developmental behaviours and milestones examined. However, subsequent tests on spatial learning,

memory, activity level and motor skills were comparable to controls (63).

Trans-generational effects

It is important to not only monitor the health of the clones but also their subsequent progeny derived following sexual reproduction. Offspring of male and female clones in a range of species have been produced following both natural mating and assisted sexual reproduction, such as artificial insemination, with a non-cloned partner. Conception, pregnancy, parturition and survival are all within normal ranges (33, 67, 75, 78), as is the subsequent fertility of these offspring of clones (D.N. Wells, unpublished findings). More discriminatory, is the mating of cloned females with cloned males. With these matings in sheep (73), cattle (D.N. Wells, unpublished findings) and mice (62) there is no evidence of the placental abnormalities and large birth weights recorded in the clone generation. It has also been claimed that the obese phenotype observed in cumulus cell mouse clones is not heritable following mating with cloned males derived from fibroblasts of the same mouse strain (62). However, it has not been reported whether the males of this strain also have the obese phenotype. If the obesity is truly non-heritable, then another generation of inbreeding would be required to exclude the possibility of a recessive genetic (or epigenetic) trait. The most convincing evidence for the lack of transmission of any obvious deleterious recessive genetic or epigenetic trait has been provided following the mating of cloned male and cloned female mice (derived from XY and XO embryonic stem cells, respectively) obtained from the same cell line (59). The resulting offspring were phenotypically normal; lacking the foetal and placental overgrowth and open-eyelids-at-birth characteristic of their cloned parents.

The observations above, indicating that the clone-associated phenotypes are not transmitted to offspring following sexual reproduction, implies that they are epigenetic in nature and that any errors in the surviving clones appear to be reset or corrected during gametogenesis. This is encouraging for the major application of cloning technology in agriculture; namely, the generation of cloned sires from progeny-tested, genetically elite males. The cloning of elite sires means that their superior genes will be more widely disseminated following either increased semen production for artificial insemination or natural mating. Nonetheless, it is still possible that heritable genetic errors may be present in the clones. Moreover, detailed molecular studies are required to confirm whether the necessary epigenetic modifications in gametes, zygotes and embryos derived from cloned parents are indeed restored to normal. It is critical to investigate this phenomenon more thoroughly, as evidence exists for the germ line transmission of epigenetic states at various endogenous loci (48, 49) and in more artificial situations, following nuclear-cytoplasmic incompatibility (54). Additionally, at a practical level, it remains to be demonstrated that the daughters of cloned dairy sires, for instance, have a similar phenotypic performance to contemporary progeny of the original donor bull.

Livestock production characteristics and food safety

Few scientific reports regarding the production characteristics and food safety of somatic cell clones have been published to date. However, to address safety concerns, information has been provided to national regulatory agencies in a number of countries to demonstrate compositional equivalence of food products derived from cloned livestock (for example: www.fda.gov/cvm/cloning.htm and (Rudento *et al.* [55]).

As expected, given the current state of the technology, subtle epigenetic differences in somatic cell clones appear to increase the variation in animal performance between members of a clonal family (1, 35). This variation in phenotype is anticipated to be greater than that between naturally occurring monozygotic twins. Despite this, the composition of meat (61, 65) and milk (72, 76) from cloned cattle are within the normal ranges for these food products. This is further supported by recent data examining some of the constituents for which milk is an important dietary source. The average levels of minerals, amino acids and vitamins from the milk of nine pastorally-fed cloned cows (with three cows each representing three different clonal families) were comparable to those from five control cows, examined at a similar stage of lactation in springtime (Tables I, II and III; D.N. Wells, unpublished findings).

Overcoming the current limitations of nuclear cloning

If an acceptable and safe nuclear cloning technology that has wide applicability is to be developed, solutions to the cloning abnormalities must be found. It is desirable that the health and wellbeing of cloned animals should be equal to non-clones and that any deficit should be minimised and thoroughly justified in terms of the benefits expected from the application. Achieving this goal will only result from improvements in the efficiency of the cloning process. This is the prime focus of many international groups presently working in this field. Improvements will probably come from modifications to the basic NT manipulation procedure (41, 45), the choice of an appropriate type of donor cell (40, 77) and recipient cytoplast (23), embryo culture media formulations (9) and greater fundamental understanding (53) and control of reprogramming (10). The use of molecular markers to screen new protocols in nuclear

Table I
Mineral composition of bovine whole milk harvested in spring (mean ± standard deviation)

Milk mineral (mg/100g)	Control milk (n = 5)	Clone milk (n = 9)
Calcium	134.4 ± 10.1	133.0 ± 15.7
Iodine	0.0022 ± 0.0009	0.0010 ± 0.0005
Magnesium	10.0 ± 0.0	10.1 ± 1.5
Phosphorus	103.6 ± 5.3	115.2 ± 12.5
Potassium	125.8 ± 15.1	129.9 ± 13.9
Selenium	0.0008 ± 0.0004	0.0005 ± 0.0
Sodium	26.8 ± 5.0	27.0 ± 5.1
Zinc	0.515 ± 0.077	0.495 ± 0.768

Table II
Amino acid composition of bovine skim milk harvested in spring (mean ± standard deviation)

Amino acid (mg/g)	Control milk (n = 5)	Clone milk (n = 9)
Alanine	1.31 ± 0.14	1.31 ± 0.17
Arginine	1.33 ± 0.12	1.32 ± 0.20
Aspartic acid	3.02 ± 0.26	3.07 ± 0.40
Cystine	0.38 ± 0.04	0.36 ± 0.05
Glutamic acid	8.65 ± 0.70	8.78 ± 1.16
Glycine	0.75 ± 0.07	0.74 ± 0.11
Histidine	1.01 ± 0.07	1.02 ± 0.14
Isoleucine	1.76 ± 0.16	1.82 ± 0.28
Leucine	3.75 ± 0.30	3.83 ± 0.51
Lysine	3.16 ± 0.26	3.22 ± 0.45
Methionine	0.88 ± 0.09	0.89 ± 0.12
Phenylalanine	1.83 ± 0.15	1.85 ± 0.26
Proline	3.80 ± 0.33	3.87 ± 0.53
Serine	2.17 ± 0.19	2.19 ± 0.30
Threonine	1.76 ± 0.18	1.78 ± 0.25
Tryptophan	0.48 ± 0.06	0.48 ± 0.08
Tyrosine	1.80 ± 0.17	1.81 ± 0.27
Valine	2.08 ± 0.17	2.15 ± 0.32
Totals	**39.94 ± 3.40**	**40.48 ± 5.57**

Table III
Vitamin composition of bovine whole milk harvested in spring (mean ± standard deviation)

Vitamin	Units	Control milk (n = 5)	Clone milk (n = 9)
A	IU/100 ml	140 ± 29	128 ± 22
B2	mg/100 ml	0.24 ± 0.04	0.27 ± 0.03
B12	µg/100 g	0.29 ± 0.07	0.40 ± 0.09

cloning using either DNA microarrays (28) or candidate gene approaches (85) and to identify viable embryos (4) before transfer to recipient females, will be vital tools for capturing the potential opportunities of this technology.

Applications of nuclear cloning

There are several important issues to be addressed before commercial opportunities for cloning in livestock agriculture can be realised. Firstly, there are significant animal welfare concerns limiting the acceptability and applicability of the technology in its current form. There needs to be confidence in the long-term health status of cloned livestock and in that of subsequent generations. There are issues surrounding the safety of food products derived from clones and their offspring. Regulatory agencies in a number of countries are presently addressing these issues. There needs to be ongoing assessment and modelling of the technology to identify where it is best suited, i.e. to which farming system. Some applications of cloning technology for agriculture and medicine are briefly discussed below. Some will not be realised until well into the future.

Rapid multiplication of desired livestock

Cloning could enable the rapid dissemination of superior genotypes from nucleus breeding flocks and herds, directly to commercial farmers. Genotypes could be provided that are ideally suited for specific product characteristics, disease resistance, or environmental conditions. Cloning could be extremely useful in multiplying outstanding F1 crossbred animals, or composite breeds, to maximise the benefits of both heterosis and potential uniformity within the clonal family. These genetic gains could be achieved through the controlled release of selected lines of elite live animals or cloned embryos. More appropriately, given that cloning is not particularly efficient at present, a niche opportunity exists in the production of small numbers of cloned animals with superior genetics for breeding. These could be clones of performance tested animals, especially sires. This would be particularly relevant in the sheep and beef industries, where cloned sires could be used in widespread natural mating to provide an effective means of disseminating their superior genetics. This could be used as a substitute for artificial insemination, which in these more extensive industries is often expensive and inconvenient.

Animal conservation

Cloning can be used along with other forms of assisted reproduction to help preserve indigenous breeds of livestock, which have production traits and adaptability to local environments that should not be lost from the global

gene pool (81). In some situations, inter-species NT and embryo transfer may be used to aid the conservation of some exotic species (75). At the very least, it is appropriate to consider the cryopreservation of somatic cells from these endangered animals as insurance against further losses in diversity.

Research models

Sets of cloned livestock animals could be effectively used to reduce genetic variability and reduce the numbers of animals needed for some experimental studies. This could be conducted on a larger scale than is currently possible with naturally occurring genetically identical twins (36). Lambs cloned from sheep selected either for resistance or susceptibility to nematode worms will be useful in studies aimed at discovering novel genes and regulatory pathways in immunology (20).

Human cell-based therapies

There are also direct applications of NT technology in human medicine; principally therapeutic cloning (12) as opposed to human reproductive cloning. Patients with particular diseases or disorders in tissues that neither repair nor replace themselves effectively (as occurs, for example, in insulin-dependent diabetes, muscular dystrophy, spinal cord injury, certain cancers and various neurological disorders, including Parkinson's disease) could potentially generate their own immunologically compatible cells for transplantation, which would offer lifelong treatment without tissue rejection. Initially, this approach could employ human NT and embryonic stem cells, the use of which is controversial. In the longer term, however, fundamental understanding of reprogramming will enable one cell type to be directly trans-differentiated into another cell type specifically required for cell-based therapy (10).

Cloning for transgenic applications

A significant application of NT is to clone animals from cells that have been genetically modified in order to produce transgenic livestock. This topic is discussed more comprehensively elsewhere in this issue (Houdebine and Renard; Niemann). Even acknowledging the current problems with NT, the cloning route is more efficient than conventional pronuclear injection of DNA, where typically less than 1% of injected zygotes develop into transgenic animals (70).

Additional advantages of the NT and cell-mediated transgenic approach include the ability to:

– introduce, functionally delete or subtly modify genes of interest

– screen cells for the specific genetic modification before producing the transgenic animal

– introduce a specific transgene into a desired genetic background of the chosen sex (particularly important for agricultural traits)

– produce embryos or offspring that are all transgenic and where none should be mosaic (with a mixture of transgenic and non-transgenic cells in the same organism)

– produce small herds from each cell line in the first generation, rather than individual founder animals that need to be subsequently bred.

Nevertheless, whilst the present NT technology is able to produce a few founder transgenic animals, currently it is desirable to use assisted sexual reproduction thereafter, to further multiply animals and to circumvent potential epigenetic aberrations in the cloned generation (16). The most efficient means of introducing a transgene into the wider livestock population is through artificial insemination. Ideally, the sire should be homozygous for the desired trait so that all progeny receive a copy of the transgene. Animal industries may choose to annually introduce the transgene on a new genetic background using cell lines derived from the most recently selected progeny-tested sires.

Depending upon the particular genes that are manipulated, there are a wide variety of potential uses for genetically modified livestock in both biomedicine and agriculture. Examples include the following:

– human pharmaceutical proteins (harvested from the milk of livestock) (11)

– pig organs for xenotransplantation (34)

– models for human genetic diseases (such as for cystic fibrosis) (19)

– various agricultural applications aimed at improving the quality or quantity of food or fibre products (38), reducing environmental pollution (18) and improving animal disease resistance (71).

For agricultural transgenics, functional genomics will contribute greatly to the understanding of the genes that influence livestock production traits and provide the knowledge to accurately modify the appropriate genes to generate new and desired animal products in the future.

Conclusions

Cloning livestock following somatic cell NT involves numerous steps, each with the potential to perturb the development of the embryo and foetus, and affect health

during adulthood. For these reasons, the technology needs to be thoroughly evaluated to fully appreciate the longer-term consequences on the animals produced. This is especially true from observations at AgResearch of higher than normal annual mortality rates with bovine clones. These effects may be specific to particular species, cell types or the NT and culture methods used. Because nuclear cloning can also result in physiologically normal animals, it is anticipated that the initial commercialisation of this technology will focus on producing small numbers of high-value animals for breeding purposes and transgenic dairy animals producing valuable pharmaceuticals in their milk. In contrast to the cloned generation, the offspring of clones produced following sexual reproduction appear phenotypically normal. An increasing body of international data indicates that the major abnormalities in the clones are probably epigenetic in nature and do not appear to be transmitted to offspring even when male and female clones are mated together. However, to provide greater confidence in large-scale breeding applications of elite cloned livestock there is a need for molecular evidence to determine the possibility of genetic or epigenetic inheritance and the impact on future generations. Despite the present limitations of cloning, the milk and meat from these livestock animals does not appear to be materially different from those of conventionally bred animals. If the acceptability and utility of this emerging technology are to be improved, it is important to understand the biology behind nuclear cloning so as to improve the health and viability of the cloned animals produced and of their surrogate mothers.

■

Clonage animal : problèmes et perspectives

D.N. Wells

Résumé

Une technologie de clonage animal efficace ouvrirait bon nombre de voies nouvelles pour l'élevage, l'agriculture, la médecine humaine et la préservation des espèces animales. Le clonage par transfert de noyau aboutit à la production d'animaux génétiquement identiques aux cellules donneuses utilisées dans une technique appelée transfert nucléaire (TN). Toutefois, cette méthode est pour l'instant inefficace : chez les bovins, environ 6 % seulement des embryons implantés dans l'utérus des vaches receveuses ont produit des clones sains bénéficiant d'une survie à long terme. Le niveau élevé des pertes tout au long de la gestation, pendant la mise-bas et au cours de la période post-natale jusqu'à l'âge adulte est préoccupant. De nombreux avortements sont dus à une anomalie du développement et de la fonction placentaires. Le dysfonctionnement placentaire peut également avoir des conséquences néfastes pour la santé post-natale. Ces anomalies sont probablement dues à une mauvaise reprogrammation d'ordre épigénétique du génome du donneur à la suite d'un TN, ce qui aboutit à des profils inappropriés d'expression des gènes au cours du développement des clones. Si certains tests physiologiques appliqués aux clones survivants donnent des résultats normaux, d'autres rapports font état d'anomalies post-natales liées au clonage. Cette variabilité des résultats peut s'expliquer par des différences de méthodes de clonage et/ou propres à l'espèce. Fait important, à ce jour, il s'avère que ces phénotypes liés au clonage ne sont pas transmis à la descendance obtenue par reproduction sexuée. Cette constatation indique qu'ils représentent des erreurs épigénétiques plutôt que génétiques, qui sont corrigées pendant la gamétogenèse. Bien qu'une confirmation au niveau moléculaire soit requise, c'est un élément qui permet de faire naître la confiance à l'égard de la première application du TN en agriculture. Elle consiste à produire un petit nombre de mâles reproducteurs clonés à partir de taureaux d'élite sur le plan génétique,

destinés à la monte naturelle, dans le but de diffuser efficacement l'amélioration génétique. Outre les questions liées au bien-être animal que pose la technique, la santé des animaux et ses conséquences sur la sécurité sanitaire des aliments représentent des aspects essentiels. Des études doivent être réalisées pour obtenir l'aval des autorités réglementaires et des consommateurs. Les améliorations futures en matière de clonage animal seront en grande partie attribuables à une meilleure compréhension des mécanismes moléculaires de la reprogrammation.

Mots-clés
Clonage – Effet transgénérationnel – Mammifère – Reprogrammation – Santé – Sécurité sanitaire des aliments – Transfert nucléaire.

Problemas y perspectivas de la clonación de animales

D.N. Wells

Resumen
La existencia de una técnica eficaz para clonar animales abriría un gran número de nuevas oportunidades en los terrenos de la ganadería, la medicina humana y la protección de especies animales. La clonación por la llamada transferencia nuclear (TN) supone la obtención de ejemplares genéticamente idénticos al donante. Sin embargo, esta técnica resulta por ahora poco eficaz: en los bovinos, sólo en torno al 6% de los embriones implantados en el tracto reproductor de una vaca receptora dan lugar a clones sanos y longevos. Preocupa el elevado nivel de pérdidas que se registran desde la gestación y el parto hasta el inicio de la edad adulta. Muchos de los abortos guardan relación con problemas en la formación o el funcionamiento de la placenta, alteraciones que también pueden influir negativamente en la salud del recién nacido. Es probable que estas anomalías obedezcan a una deficiente reprogramación epigenética del genoma donante tras la TN, lo que da lugar a procesos incorrectos de expresión génica durante el desarrollo del clon. Aunque en algunos casos las pruebas fisiológicas practicadas a los animales supervivientes arrojan resultados normales, en otros se observan diversas alteraciones postnatales asociadas a los clones. Esta heterogeneidad en los resultados obtenidos podría deberse a diferencias entre las especies o entre los métodos de clonación. Es importante señalar que, por lo que se sabe hasta ahora, esos fenotipos vinculados a la clonación no parecen transmitirse a la progenie engendrada por reproducción sexual, lo que lleva a pensar que no responden a errores genéticos sino epigenéticos, y que éstos quedan subsanados durante la gametogénesis. Este hecho, aunque pendiente de confirmación en el plano molecular, permite en principio ver con optimismo la primera de las aplicaciones de la TN a la producción animal: la obtención de varios clones de un toro dotado de un patrimonio genético excepcional para utilizarlos como sementales y diseminar con eficacia ese acervo genético. Además de las consideraciones relativas al bienestar animal que plantean estas técnicas, otros dos aspectos cruciales son la salud de los animales y los efectos

que de ahí puedan seguirse en cuanto a la inocuidad de los alimentos. Todo ello exige investigaciones para propiciar la aceptación de esa tecnología por parte de la opinión pública y las instancias normativas. En el futuro, las técnicas de clonación de animales irán mejorando a medida que se vayan entendiendo mejor los mecanismos moleculares de la reprogramación.

Palabras clave
Clonación – Efecto transgeneracional – Inocuidad de los alimentos – Mamífero – Reprogramación – Salud – Transferencia nuclear.

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