AJRI 2003; 50: 113–123

American Journal of Reproductive Immunology
ISSN 8755-8920

Cloning Adult Farm Animals: A Review of the Possibilities and Problems Associated with Somatic Cell Nuclear Transfer

Edwards JL, Schrick FN, McCracken MD, van Amstel SR, Hopkins FM, Welborn MG, Davies CJ. Cloning adult farm animals: a review of the possibilites and problems associated with somatic cell nuclear transfer. AJRI 2003; 50:113–123 © Blackwell Munksgaard, 2003

In 1997, Wilmut et al. announced the birth of Dolly, the first ever clone of an adult animal. To date, adult sheep, goats, cattle, mice, pigs, cats and rabbits have been cloned using somatic cell nuclear transfer. The ultimate challenge of cloning procedures is to reprogram the somatic cell nucleus for development of the early embryo. The cell type of choice for reprogramming the somatic nucleus is an enucleated oocyte. Given that somatic cells are easily obtained from adult animals, cultured in the laboratory and then genetically modified, cloning procedures are ideal for introducing specific genetic modifications in farm animals. Genetic modification of farm animals provides a means of studying genes involved in a variety of biological systems and disease processes. Moreover, genetically modified farm animals have created a new form of 'pharming' whereby farm animals serve as bioreactors for production of pharmaceuticals or organ donors. A major limitation of cloning procedures is the extreme inefficiency for producing live offspring. Dolly was the only live offspring produced after 277 attempts. Similar inefficiencies for cloning adult animals of other species have been described by others. Many factors related to cloning procedures and culture environment contribute to the death of clones, both in the embryonic and fetal periods as well as during neonatal life. Extreme inefficiencies of this magnitude, along with the fact that death of the surrogate may occur, continue to raise great concerns with cloning humans.

J. L. Edwards[1], F. N. Schrick[1], M. D. McCracken[2], S. R. van Amstel[3], F. M. Hopkins[3], M. G. Welborn[3], C. J. Davies[4]

[1]Department of Animal Science, Tennessee Agricultural Experiment Station, Institute of Agriculture, The University of Tennessee, Knoxville; [2]Department of Pathobiology, College of Veterinary Medicine, The University of Tennessee, Knoxville; [3]Department of Large Animal Clinical Sciences, College of Veterinary Medicine, The University of Tennessee, Knoxville; [4]Department of Veterinary Microbiology and Pathology, College of Veterinary Medicine, Washington State University, Pullman, WA, USA

Key words: Cloning adult farm animals, animal cloning, Somatic Cell Nuclear transfer

Address reprint requests to J. L. Edwards, 206 Brehm Animal Science, 2505 River Drive, Knoxville, TN 37996, USA. E-mail: jedwards@utk.edu

Revised November 20, 2002;
accepted November 21, 2002.

INTRODUCTION

In 1997, Wilmut and coworkers[1] announced the birth of the first ever clone of an adult animal. Such an announcement was significant because the ability to clone mammals by simple nuclear transfer was thought 'biologically impossible'.[2] Furthermore, the demonstrated totipotency of mammary nuclei inferred that other types of somatic cells could be utilized for cloning adult animals. As somatic cells can be readily obtained and cultured in the laboratory, the procedures of somatic cell nuclear transfer (SCNT) immediately held promise for offering an alternative for introducing targeted additions or deletions to the genome of farm animals.

Dolly was not the result of the union of an oocyte and sperm, but derived from the nuclear genome contained within a quiescent-induced mammary cell and cytoplasm originating from an enucleated oocyte and somatic cell. The concept of cloning an animal by transfer of a nucleus into an enucleated oocyte was first proposed in 1938.[3,4] However, the first success was with amphibians and not reported until 1952.[5] Thirty-one years later, McGrath & Solter announced the first successful cloning of mice.[6] Soon after, the cloning of sheep,[7] cattle,[8] rabbits,[9] and pigs[10] was reported. However, it is important to note that cloning successes before Dolly involved the use of toti- or pluripotent cell types comprising the early embryo. Any and every attempt to clone an adult animal by nuclear transfer

using more differentiated cell types failed.[4] Clearly, Dolly's birth dispelled the dogma that it was 'biologically impossible to clone mammals using nuclear transfer'. To date, adult sheep,[1] cattle,[11] mice,[12] pigs,[13] goats,[14] cats,[15] and rabbits[16] have been cloned using SCNT.

Given the incredible complexity of what must be involved in the ooplasm redirecting functionality of a somatic nucleus, it is remarkable that any cloned embryos develop at all. Very little is known about specific factors or mechanisms involved with the ooplasm reprogramming a somatic nucleus. This current lack of understanding continues to be reflected in the overall inefficiency of the cloning procedures for producing live offspring. Dolly was just one cloned offspring that resulted after 277 attempts (0.3% efficiency).[1] Five years later, similar inefficiencies are still being described for cloning adult animals (cattle,[17,18] goats,[14,19] pigs,[13] rabbits,[16] cats[15]). The focus of this review will be to provide a brief overview of SCNT procedures utilized for cloning adult farm animals, discuss potential applications, problems associated with its use and some of the potential factors contributing to the overall inefficiency of the procedure for producing live offspring.

OVERVIEW OF SOMATIC CELL NUCLEAR TRANSFER UTILIZED FOR CLONING ADULT FARM ANIMALS

The ultimate challenge of cloning using SCNT involves reprogramming a somatic nucleus in a manner conducive for embryonic development. The predominant cell type of choice for reprogramming a somatic nucleus is an oocyte arrested at metaphase II.[20] A matured oocyte is a logical choice as critical components required for initiating and directing early embryo development are contained within the oocyte. Procedures required for cloning adult farm animals using SCNT are in general similar across species and are depicted in Fig. 1. For a detailed review of specific procedural details of nuclear transfer refer to Robl & Stice,[21] Prather & First,[22] First & Prather[23] and Campbell et al.[24]

In general, the first step of the cloning process involves collection of somatic cells from the animal to be cloned. Choice of somatic cell for use in SCNT varies greatly. In cattle, adults have been cloned using cumulus,[11,25,26] fibroblasts,[25,27–32] ovarian/granulosa,[18,33–36] mammary,[27,29] muscle,[37] oviduct,[11,25,38] and uterine[25] cell types. It has also been suggested that some cell types may work better than others for producing live offspring. Wakayama et al. [12] reported cloned embryos originating from Sertoli or neuronal nuclei failed to develop beyond 8 days post-coitum; however, embryos originating from cumulus cells developed to term. After collection, somatic cells may be utilized immediately[12] or after long-term culture.[30] Use of quiescent or quiescent-induced adult cell types may[1] or may not[35,37,39,40] be required for producing cloned offspring of adult animals. However, doing so may improve ability of cloned embryos to establish[35,41] (Table II) and maintain pregnancy to term.[18]

The second and perhaps most labor-intensive part of SCNT requires removal of maternal DNA from an oocyte arrested at metaphase II (MII). Doing so requires the use of microtools beginning approximately 18 hr after oocytes have been placed in maturation medium. To avoid lysis, oocytes may be cultured in the presence of a microfilament inhibitor[22] (cytochalasin B). This induces relaxation of cytoplasm allowing for mechanical removal of <5–15% of egg cytoplasm containing the maternal DNA. Removal of maternal DNA can be verified by Hoechst staining and ultraviolet transillumination. For cloning cattle, oocytes generally originate from a non-descript group of females obtained from commercial sources, abattoirs[18,28–30,32–36] or a descript group of live animals using ultrasound-guided aspiration.[17]

The next step of SCNT is to insert a somatic nucleus into an egg cytoplasm; thereby, constructing the equivalent of a one-cell embryo. In most farm animal species, this is performed by electrical-induced fusion of the somatic cell with an egg cytoplasm.[1,18,28–30,32–36] Initially, a somatic cell is mechanically inserted into the perivitelline space using microtools. Thereafter, the resulting 'couplet' (egg cytoplasm and somatic cell) is aligned between two electrodes and pulsed with an electrical current (for example, use of 2.2 kV/cm for 40 μs will induce greater $>70\%$ of couplets to fuse).[42] Electrofusion is dependent on maintaining contact of the somatic cell with egg cytoplasm such that membranes of each cytoplasm may intermingle after pore formation resulting after electrical current.[23] In mice, however, the preferred method for introducing a somatic nucleus into egg cytoplasm is microinjection.[12] Within minutes of introducing somatic nucleus into egg cytoplasm, the nuclear membrane breaks down and the chromatin condenses.[12,24,42]

In some cases, the electrical pulse utilized for fusion is also sufficient to 'activate' the cloned embryo to begin development,[1] while others choose various chemical combinations.[18,43] Regardless of the method of choice, the ultimate challenge of activation protocols is to mimic actions of a sperm after fertilization. Cloned embryos may be transferred into oviducts of ligated recipients (sheep)[1] or cultured in the incubator

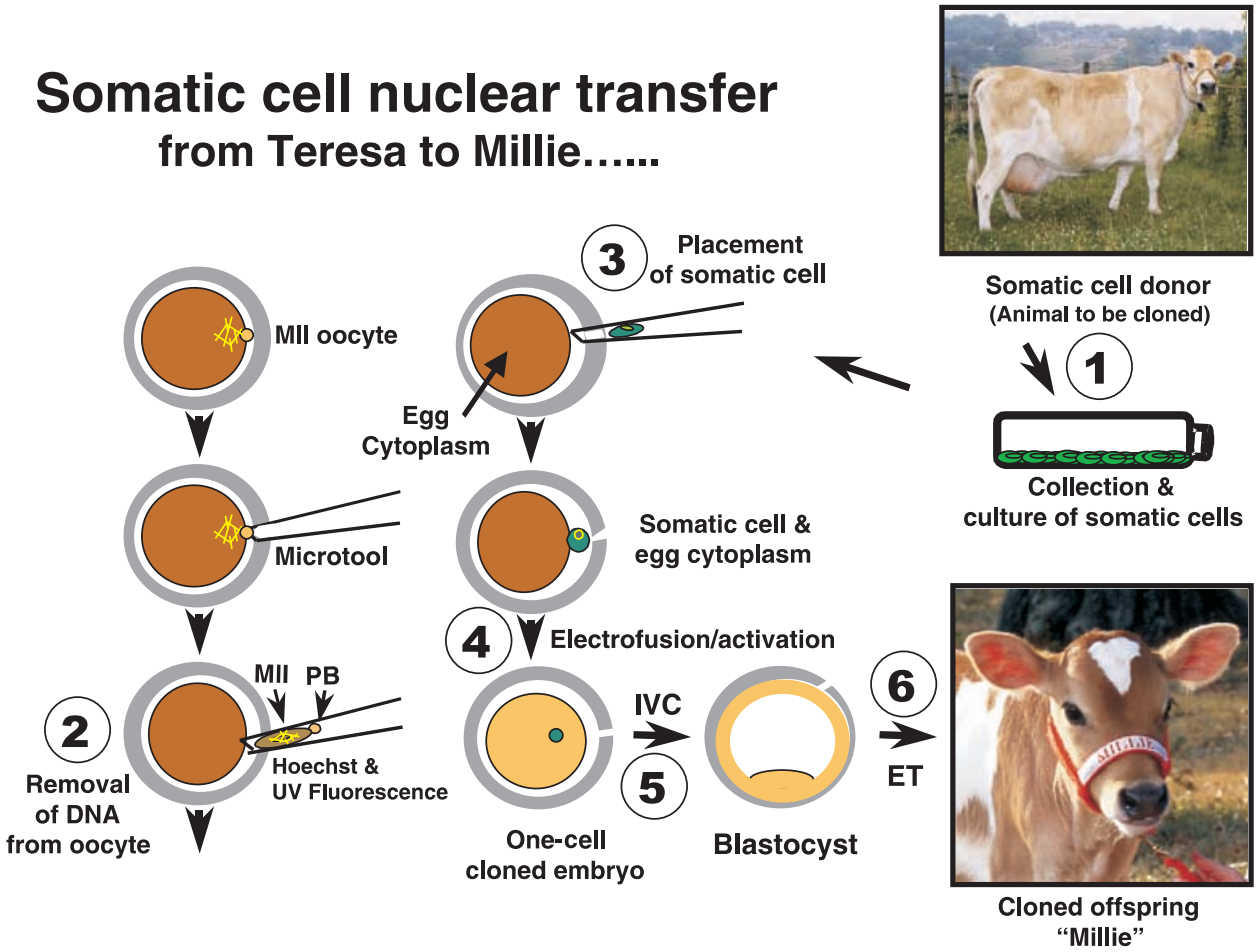

Fig. 1. Schematic depicting procedures of SCNT utilized for cloning adult farm animals. In general, procedures are similar across species. (1) The first step involves collection of somatic cells from the animal to be cloned. (2) Maternal DNA is then removed from an oocyte using microtools. Success in doing so can be confirmed by exposing the portion of cytoplasm removed from the Hoechst-stained oocyte to ultraviolet light. (3) A somatic cell is then placed beside the egg cytoplasm. (4) Exposure of egg cytoplasm and somatic cell to electrical current will induce the majority to fuse, thereby creating the equivalent of a 1-cell cloned embryo. In most cases, electrical-induction of fusion of the egg cytoplasm with somatic cell is sufficient to activate the cloned embryo to begin development. (5) Cloned embryos may be cultured in the laboratory (IVC) for a defined period of time. (6) Thereafter, embryos must be transferred (ET) to surrogate recipients for continued development. Millie was one offspring that resulted out of 95 one-cell cloned embryos made in the laboratory, one of 19 transferred to 17 surrogate recipients, and one of nine that established a pregnancy by 25–29 days.

for a period of time required for development to compact morula or blastocyst.[35] Continued development of cloned embryos is dependent on transfer into surrogate recipients. In cattle, approximately half of the cloned embryos transferred as singles will have established a pregnancy 21 days after transfer as indicated by the presence of an embryo proper with heartbeat, while only a few will progress to term.[35]

APPLICATIONS OF SOMATIC CELL NUCLEAR TRANSFER

A number of applications of SCNT have been described benefiting animal agriculture and human medicine. Success in cloning of adult sheep,[1] cattle,[11] pigs[13] and goats[14] confirms usefulness of SCNT for the purpose of clonal expansion of agriculturally important food-producing animals. There is also increasing interest to utilize SCNT to restore endangered, or even extinct species. Wells et al.[34] reported the first successful use of SCNT for preserving the last surviving cow of the Enderby Island cattle breed. Moreover, Lanza et al.[44] used interspecies SCNT to clone an endangered species (*Bos gaurus*). Finally, given the manipulative aspects of the procedures, SCNT provides one with a novel tool for investigating nuclear and/or cytoplasmic components involved in embryonic development and loss.

Given the ease at which most somatic cells can be clonally expanded and genetically modified, use of

SCNT is rapidly becoming the method of choice for producing transgenic farm animals. Moreover, use of SCNT increases efficiency of producing transgenic farm animals to 100%.[45] Production of transgenic farm animals provides one with a means in agricultural and biomedical disciplines for studying importance of genes involved in a variety of biological systems. And, genetic modification of farm animals has created a new form of 'pharming'; whereby, transgenic animals are used as bioreactors for production of pharmaceuticals or perhaps organ donors for the human population. Announcement of the first ever cloned transgenic sheep[45] and cattle[46] using SCNT immediately obviated the need for producing embryonic stem cells in farm animal species and suggested possible usefulness for targeted additions or deletions to the genome of farm animals. In 2000, McCreath et al.[47] announced the first cloned gene-targeted sheep. Most recently, Lai et al.[48] demonstrated that SCNT could be utilized to produce cloned pigs with the β-1,3-galactosyltransferase gene knocked out. Moreover, Lanza et al. [49] demonstrated that one could use SCNT for generation of histocompatible tissues addressing one of the major challenges in transplantation medicine.

PROBLEMS ASSOCIATED WITH CLONING ADULT ANIMALS USING SOMATIC CELL NUCLEAR TRANSFER

A major limitation of cloning adult farm animals using somatic cell nuclear transfer is the extreme inefficiency of producing live offspring. For example, Dolly was one of 277 cloned embryos that developed to term (0.3% efficiency).[1] 'Millie' was just one offspring that resulted after 95 attempts.[40] Similar inefficiencies regardless of species or somatic cell type continue to be reported.[50] Death of cloned embryos and fetuses occur throughout pregnancy. Moreover, a high proportion of cloned offspring are generally larger than normal (large offspring) and die soon after birth.

In general, there have been at least five periods of loss observed with clones derived from adult animals. The first and perhaps most dramatic occurs during preimplantation development. In cattle[17,18,29,30,34,35,37,51] as well as other species including goats,[14] sheep,[1] and rabbits,[16] >65% of one-cell cloned embryos fail to develop to compact morula or blastocyst (Table I).

Approximately 50% of bovine cloned embryos (compact morula or blastocyst) establish pregnancy after transfer of a single embryo into a surrogate recipient (i.e., presence of an embryo proper with a heart beat between days 29–32; Table II). Development of cloned bovine embryos, at least for the first 29–32 days, parallels that of those embryos produced after in vitro maturation, fertilization and culture (IVMFC).[52,53] However, beginning at approximately 30 days and continuing through day 60 of pregnancy, embryonic death may occur in 50–100% of cloned pregnancies (absence of heartbeat and detachment of placental membranes; Table II). Others[18,28,31,34,51] have reported similar losses. Pregnancy losses in cattle of this magnitude are significantly higher than expected in animals bred by natural service (2–10% loss[54] or developed using IVMFC procedures (16%;).[52,53] Examination of placentae originating from cloned embryos between days 40–50 of gestation reveal placentae that are hypoplastic, partially developed with rudimentary cotyledons, or those that are essentially normal when compared with IVF derived embryos.[51]

TABLE I. Developmental Potential of Clones (Constructed with Adult Somatic Cells Ovarian/Granulosa and Skin Fibroblasts), Parthenotes or *In Vitro* Produced (IVP) Embryos (Edwards et al., unpublished)

Embryos	No. of clones, oocytes or presumptive zygotes	Cleaved (%)	Day 6 & 7 morulae & blastocysts (%)
Clones	686	–	207 (30.2)
Parthenotes	331	–	164 (49.5)
In Vitro produced	863	705 (81.7)	235 (27.2)

The third period of loss that has been noted is associated with an increased incidence of spontaneous abortions during the second trimester of pregnancy.[25,29,30,33,35] Complete macroscopic and histopathologic examinations of aborted fetuses reveal few abnormalities.[55] However, placentae are oftentimes grossly abnormal with a marked reduction in fetal cotyledons (fewer than 20 compared with the expected 70–120).[56] Moreover, fetal membranes are generally thickened and edematous.

The fourth period of loss noted for cloned bovine pregnancies occurs during the third trimester between days 200–265 (280 days = term). Loss during this time period is generally characterized by a marked increase in the incidence of hydrallantois and fetal death (unpublished observations of Edwards et al.).[18,27,32,33,36,57,58] Hydrallantois is accompanied by a marked reduction in placentomes (often fewer than 70, and in some cases <20), marked hypertrophy of many cotyledons, adventitial placentation and severe edema of the intercotyledonary placental membranes (Edwards et al., unpublished; Fig. 2). Fetal anasarca with generalized edema and marked edema of the umbilicus is usually present.

TABLE II. Developmental Potential of Cloned Bovine Embryos Constructed with Quiescent-Induced Versus Proliferating Adult Ovarian/Granulosa Cells After Embryo Transfer[35]

Somatic cells	Reps	Clones	M/B ET	Recipients	Pregnant 29–35 days (%)	Pregnant 60 days (%)	Pregnancy Loss days 30–60* (%)
Proliferating	6	97	27	25	11 (44.0)	7	4 (36.3)
Quiescent-induced	5	91	17	13	9 (69.2)	1	8 (88.8)
Total		188	44	38	20 (52.6)	8	12 (60.0)

M/B ET, Total number of morulae and blastocysts transferred to individual recipient animals.
**Pregnancy loss defined as the absence of embryonic heartbeat followed by detachment of fetal membranes.*

In our experience, death of late term cloned fetuses is primarily the result of inadequate placentation. Thus far, complete gross and microscopic examinations of late term and early neonatal fetuses have not identified any known genetic or inherited abnormalities.[55] The majority of fetal lesions observed can be attributed to changes secondary to inadequate placental development. Amniotic squames and meconium are generally present in the lungs of all late term fetuses indicating some degree of stress in utero before death.

In some cases, a few cloned embryos will develop to term. However, the majority are not born without complications. In particular, cloned calves derived from adult animals are usually larger at birth and have a lower postnatal survival rate when compared to in vitro counterparts (Edwards et al., unpublished).[25,32] Most calves derived from abnormal placentae require intensive monitoring and therapy after birth to treat a whole plethora of complications that may include lung dysmaturity, pulmonary

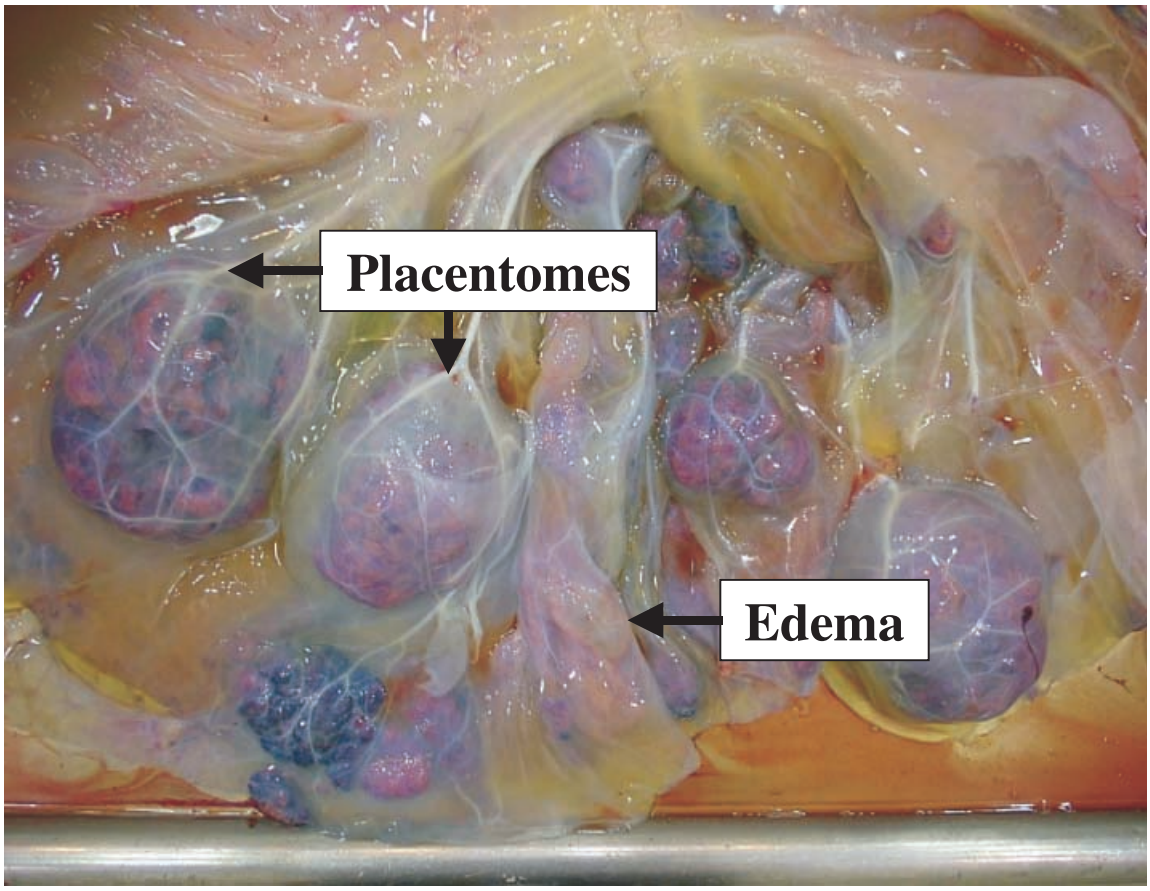

Fig. 2. Abnormal cloned placenta 203 days of pregnancy. Note variations in size of placentomes (50 g–1 kg) and edematous fetal membranes.

hypertension, respiratory distress, hypoxia, hypothermia, hypoglycemia, metabolic acidosis, enlarged umbilical veins and arteries, and/or development of sepsis in either the umbilical structures or lungs (Edwards et al., unpublished observations).[18,33,34,51,57] Severity of complications may not be evident for several months. Gibbons et al.[18] lost one calf at approximately 60 days of age and necropsy revealed adhesions in the lungs consistent with pneumonia, and digestive problems consistent with vagal indigestion. Similar complications have been described for clones derived from fetal[57] and embryo[59] cells.

In spite of the extreme inefficiency of SCNT, there are some clones born "normal and healthy" requiring little if any veterinary care after birth (Edwards et al., unpublished observations).[36,60] Pace et al.[61] reported similar growth rates, reproductive performance and lactational characteristics of clones compared with non-cloned dairy cattle. Moreover, Enright et al.[62] demonstrated that cloned heifers were not different in estrous cycle length, ovulatory follicle diameter, number of follicular waves, or profiles of hormonal changes (leutinizing hormone, follicle stimulating hormone, estradiol, and progesterone). Concentrations of growth hormone, IGF-I and IGFBP3 values recorded for clones derived from a 13-year-old Holstein were all within the range reported for non-cloned calves of similar ages.[63]

However 'normal and healthy' cloned animals may appear, it is possible that undiagnosed pathologies may develop later in life[58] as a result of subtle changes in chromatin structure and/or gene expression. Miyashita et al.[64] noted differences in telomere lengths among cloned cattle derived from different cell types. Moreover, X-chromosome inactivation may (mice)[65] or may not (cattle)[66] be normal. Wrenzycki et al.[67] noted aberrant expression of genes thought to be of importance in stress adaptation, trophoblastic function, and DNA methylation during preimplantation development in cloned bovine embryos. Yet, many mice and other animals have survived to adulthood despite widespread gene dysregulation, indicating that mammalian development may be rather tolerant to epigenetic aberrations of the genome.[68] The ultimate consequences of epigenetic aberrations of the genome in cloned animals remain unclear but may result in an early death.[69]

POTENTIAL FACTORS CONTRIBUTING TO THE EXTREME INEFFICIENCY OF THE CLONING PROCEDURES FOR PRODUCING LIVE OFFSPRING

Given the physically insulting nature of the cloning process and the presumed complexity of factors that must be involved with the ooplasm redirecting the functionality of a somatic nucleus, it is remarkable that any cloned embryos develop at all. Moreover, data are coming forth supporting a role for immunologic rejection in the syndrome of early embryonic loss in cloned bovine pregnancies.[70]

Certainly, many factors related to SCNT procedures and culture environment may be contributing to the embryonic and fetal deaths of cloned embryos. First, construction of cloned embryos requires extensive micromanipulation (refer back to Fig. 1) of oocytes and somatic cells and exposure to otherwise noxious chemicals. Second, enucleation of an oocyte involves the physical removal of 5–15% or more of the ooplasm. Confirmation in doing so oftentimes involves exposure of the ooplasm to ultraviolet light which has been shown to alter membrane integrity, increase methionine uptake, alter protein synthesis and mitochondrial activity.[71] Third, insertion of a somatic cell nucleus within egg cytoplasm generally involves electrical-induced fusion or microinjection. Some degree of lysis occurs after either procedure indicating alterations in membrane integrity of the ooplasm.[72,73] Fourth, a variety of protocols have been described for activating cloned embryos, with the majority involving exposure to otherwise noxious chemicals to initiate embryonic cell cycles.[18,33,46] Fifth, resulting embryos are 'nurtured' in an otherwise hostile culture environment until transfer into surrogate recipients. Doing so may not be without consequence[74] and may further exacerbate problems associated with SCNT. For example, *in vitro* production of embryos is associated with alterations in gene expression in early embryos and fetuses (cattle;[75] sheep[76]), increased abortion (16%),[53] hydrallantois (1%),[52] heavier fetuses,[77] dystocia,[52,78] large offspring,[59,79] and increased mortality (14.9%).[53]

Given the fact that cloned embryos generated after SCNT are not 'true' clones may further complicate matters. Oocyte cytoplasm contains mitochondrial DNA that is transmitted to the cloned embryo.[80] Significance of uniparental inheritance of cytoplasmic organelles (maternal inheritance) remains unclear. In instances whereby nuclear and mitochondrial genes are mismatched, Nagao et al.[81] reported decreased physical performance and growth rates in mice. It is possible that reconstructing cloned embryos with cytoplasts of non-descript origin results in the production of embryos whereby some degree of incompatibility exists between the cytoplasm and donor nucleus.

Clearly, some of the problems associated with SCNT for producing live offspring may in part be due to incomplete reprogramming of the somatic nucleus by egg cytoplasm. As a consequence, expression of

developmentally important genes as required for embryo and fetal development are altered in cloned embryos. Most recently, Yong-Kook et al.[82] found highly aberrant methylation patterns in various genomic regions in bovine cloned embryos. Cloned blastocysts in this study closely resembled somatic cells in their overall genomic methylation status. Similar findings were obtained by Dean et al.[83] and Bourc'his et al.[84] Moreover, analysis of various developmentally important genes in cloned embryos revealed abnormal expression in IL6, FGF4, and FGFr2,[85] G6PD and Xist,[67] and Oct4,[86] to name a few. Furthermore, Wrenzycki et al.[87] reported that alterations in gene expression occurring in cloned embryos may be dependent on the protocol used for SCNT. Finally, ability of the egg cytoplasm to effectively reprogram somatic nuclei may differ according to adult cell type. For instance, nuclei obtained from Sertoli and brain cells following microinjection into MII cytoplasts did not support development to term compared with cumulus cell nuclei (mice).[12] Similar observations have been made in cattle.[25,88]

IMMUNOLOGIC REJECTION OF NUCLEAR TRANSFER FETUSES

The high rate of early embryonic mortality occurring during the first trimester of cloned pregnancies may be due in part to inappropriate expression of trophoblast major histocompatibility complex (MHC) class I antigens.[70] In most species, down regulation of polymorphic MHC class I antigens on the trophoblast cells seems to be required for successful pregnancy. In cattle, trophoblast cells do not express class I antigens early in pregnancy. However, during the third trimester the trophoblast cells in the interplacentomal and arcade regions of the uterine/placental interface commence expression of class I antigens.[89] Using immunohistochemistry, Hill et al.[70] reported abnormal expression of MHC class I antigens by the trophoblast cells of 34–63-day-old cloned fetuses ($n = 8$) when compared with age-matched controls. Moreover, the endometrial stroma of cloned pregnancies contained large numbers of lymphocyte aggregates and had an increased number of diffuse endometrial lymphocytes. Approximately 80% of the lymphocytes in the lymphoid aggregates were CD4+ helper T cells, the remaining cells consisted of about equal numbers of CD8+ cytotoxic T lymphocytes and B lymphocytes plus a very small number of γ/δ-T cells (Davies, unpublished).

In day-34 cloned pregnancies, the uterine stroma was already infiltrated with lymphocytes. Tight attachment and placentome development in cattle begin at approximately day 30 of pregnancy. Consequently, in surrogate recipients the maternal immune response to SCNT fetuses occurred early enough to perhaps inhibit placentome development. Immunologically mediated inhibition of placentome development could explain the types of placental abnormalities observed in SCNT pregnancies.[51,57] It might seem odd that the primary cells responding to inappropriate expression of class I antigens on trophoblast cells are CD4+ helper T cells that recognize peptide antigens presented by MHC class II molecules. There is no information on the expression of B7-1 (CD80) and B7-2 (CD86), the costimulatory molecules required for activation of naive T cells, by bovine trophoblast cells. However, these costimulatory molecules are normally only expressed on professional antigen presenting cells.[90] Furthermore, in other species trophoblast cells express novel members of the B7 family of costimulatory molecules but do not express B7-1 or B7-2.[91–94] It would, therefore, be surprising if trophoblast cells directly activated naive cytotoxic T cells. The primary mode of recognition of fetal class I antigens would be indirect recognition involving processing and presentation of trophoblast cell antigens by maternal antigen presenting cells, macrophages and dendritic cells. In cattle, this would be greatly facilitated by migration of binucleate trophoblast cells across the interface, their fusion with uterine epithelial cells, and ultimately the death of hybrid trinucleate cells within the maternal endometrium.[95,96] If the binucleate cells present at the time of implantation were class I positive, as they appear to be in most SCNT pregnancies, class I antigens would be delivered directly to maternal antigen presenting cells for processing and presentation to the maternal immune system. It is likely that both inflammatory Th1 cells and anti-fetal class I antibodies are produced in response to class I positive trophoblast cells. Hence, effector T cells and/or antibodies could interfere with placental development in SCNT pregnancies.

One strategy for avoiding immunologically mediated rejection of SCNT fetuses is to create MHC class I compatible pregnancies. If a SCNT fetus expresses only class I antigens, also carried by the embryo transfer recipient then the surrogate recipient should be tolerant to the fetal class I antigens. Because of the extreme polymorphism of the MHC, few MHC compatible pregnancies are created by chance. Furthermore, it could be difficult to identify MHC compatible recipients for nuclear donor cell lines with rare MHC genotypes. Recently, a microarray based MHC typing system for cattle has been developed for matching donor cell lines and surrogate recipients (Davies,

unpublished). However, thus far very few MHC compatible SCNT pregnancies have been studied.

An additional strategy for obviating potential problems of trophoblast cell class I expression would be to induce the trophoblast cells of NT fetuses to shut off class I expression. This could involve either addition of a hormone, growth factor or cytokine to the medium used for *in vitro* culture or infusion of a mediator into the uteri of recipient cows. Currently, little is known about the regulation of class I gene expression in bovine trophoblast cells. Consequently, a better understanding of the normal mechanism by which class I expression is turned off will probably be required before headway is made with this approach. If somatic cell cloning is to move forward, gaining an understanding of trophoblast cell class I gene regulation should be a high priority.

SUMMARY AND CONCLUSIONS

Given that the procedures of cloning defy basic principles in developmental biology, it is remarkable any cloned embryos survive to term. Clearly, many, yet unidentified, factors related to the cloning or culture procedures are contributing to the death of cloned embryos, fetuses and offspring. Because of the complexity that must be involved in beginning with just a somatic nucleus and cytoplasm of an oocyte, it is likely that many more years of effort will be required before understanding the specifics of the process. Improvements for increasing survival of cloned embryos, fetuses and offspring are imperative for realizing the ultimate benefits of somatic cell nuclear transfer for animal agriculture and human medicine.

REFERENCES

1. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Viable offspring derived from fetal and adult mammalian cells. Nature 1997; 385:810–813.
2. McGrath J, Solter D. Inability of mouse blastomere nuclei transferred to enucleated zygotes to support development in vitro. Science 1984; 226:1317–1319.
3. Spemann H. Embryonic development and induction. New Haven, Conneticut, Yale University Press, 1938.
4. Di Berardino MA. Animal cloning – the route to new genomics in agriculture and medicine. Differentiation 2001; 68:67–83.
5. Briggs R, King T. Transplantation of living nuclei from blastula cells into enucleated frogs' eggs. Proc Natl Acad Sci USA 1952; 38:455–463.
6. McGrath J, Solter D. Nuclear transplantation in the mouse embryo by microsurgery and cell fusion. Science 1983; 220:1300–1302.
7. Willadsen SM. Nuclear transplantation in sheep embryos. Nature 1986; 320:63–65.
8. Prather RS, Barnes FL, Sims MM, Robl JM, Eyestone WH, First NL. Nuclear transplantation in the bovine embryo: assessment of donor nuclei and recipient oocyte. Biol Reprod 1987; 37:859–866.
9. Stice SL, Robl JM. Nuclear reprogramming in nuclear transplant rabbit embryos. Biol Reprod 1988; 39:657–664.
10. Prather RS, Sims MM, First NL. Nuclear transplantation in early pig embryos. Biol Reprod 1989; 41:414–418.
11. Kato Y, Tani T, Sotomaru Y, Kurokawa K, Kato J, Doguchi H, Yasue H, Tsunoda Y. Eight calves cloned from somatic cells of a single adult. Science 1998; 282:2095–2098.
12. Wakayama T, Perry AC, Zuccotti M, Johnson KR, Yanagimachi R. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 1998; 394:369–374.
13. Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S, Dal Y, Boone J, Walker S, Ayares DL, Coleman A, Campbell KHS. Cloned pigs produced by nuclear transfer from adult somatic cells. Nature 2000; 407:86–90.
14. Zou X, Chen Y, Wang Y, Luo J, Zhang Q, Zhang X, Yang Y, Ju H, Shen Y, Lao W, Xu S, Du M. Production of cloned goats from enucleated oocytes injected with cumulus cell nuclei or fused with cumulus cells. Cloning 2001; 3:31–37.
15. Shin T, Kraemer D, Pryor J, Liu L, Rugila J, Howe L, Buck S, Murphy K, Lyons L, Westhusin M. A cat cloned by nuclear transplantation. Nature 2002; 415:859.
16. Chesne P, Adenot PG, Viglietta C, Baratte M, Boulanger L, Renard JP. Cloned rabbits produced by nuclear transfer from adult somatic cells. Nat Biotechnol 2002; 20:366–369.
17. Bruggerhoff K, Zakhartchenko V, Wenigerkind H, Reichenbach HD, Prelle K, Schernthaner W, Alberio R, Kuchenhoff H, Stojkovic M, Brem G, Hiendleder S, Wolf E. Bovine somatic cell nuclear transfer using recipient oocytes recovered by ovum pick-up: effect of maternal lineage of oocyte donors. Biol Reprod 2002; 66:367–373.
18. Gibbons J, Arat S, Rzucidlo J, Miyoshi K, Waltenburg R, Respess D, Venable A, Stice S. Enhanced survivability of cloned calves derived from roscovitine-treated adult somatic cells. Biol Reprod 2002; 66:895–900.
19. Keefer CL, Keyston R, Lazaris A, Bhatia B, Begin I, Bilodeau AS, Zhou FJ, Kafidi N, Wang B, Bladassarre H, Karatzas CN. Production of cloned goats after nuclear transfer using adult somatic cells. Biol Reprod 2002; 66:199–203.
20. Fulka J Jr., Loi P, Ledda S, Moor RM, Fulka J. Nucleus transfer in mammals: how the oocyte cytoplasm modifies the transferred nucleus. Theriogenology 2001; 55:1373–1380.
21. Robl JM, Stice SL. Prospects for the commercial cloning of animals by nuclear transplantation. Theriogenology 1989; 31:75–86.
22. Prather RS, First NL. Nuclear transfer in mammalian embryos. Int Rev Cytol 1990; 120:169–190.
23. First NL, Prather RS. Production of embryos by oocyte cytoplast–blastomere fusion in domestic animals. J Reprod Fertil Suppl 1991; 43:245–254.

24. Campbell KH, Loi P, Otaegui PJ, Wilmut I. Cell cycle co-ordination in embryo cloning by nuclear transfer. Rev Reprod 1996; 1:40–46.
25. Kato Y, Tani T, Tsunoda Y. Cloning of calves from various somatic cell types of male and female adult, newborn and fetal cows. J Reprod Fertil 2000; 120:231–237.
26. Tani T, Kato Y, Tsunoda Y. Direct exposure of chromosomes to nonactivated ovum cytoplasm is effective for bovine somatic cell nucleus reprogramming. Biol Reprod 2001; 64:324–330.
27. Zakhartchenko V, Alberio R, Stojkovic M, Prelle K, Schernthaner W, Stojkovic P, Wenigerkind H, Wanke R, Duchler M, Steinborn R, Meuller M, Brem G, Wolf E. Adult cloning in cattle: potential of nuclei from a permanent cell line and from primary cultures. Mol Reprod Dev 1999; 54:264–272.
28. Hill JR, Winger QA, Long CR, Looney CR, Thompson JA, Westhusin ME. Development rates of male bovine nuclear transfer embryos derived from adult and fetal cells. Biol Reprod 2000; 62:1135–1140.
29. Kishi M, Itagaki Y, Takakura R, Imamura M, Sudo T, Yoshinari M, Tanimoto M, Yasue H, Kashima N. Nuclear transfer in cattle using colostrum-derived mammary gland epithelial cells and ear–derived fibroblast cells. Theriogenology 2000; 54:675–684.
30. Kubota C, Yamakuchi H, Todoroki J, Mizoshita K, Tabara N, Barber M, Yang X. Six cloned calves produced from adult fibroblast cells after long-term culture. Proc Natl Acad Sci U S A 2000; 97:990–995.
31. Kasinathan P, Knott JG, Wang Z, Jerry DJ, Robl JM. Production of calves from G1 fibroblasts. Nat Biotechnol 2001; 19:1176–1178.
32. Heyman Y, Chavatte-Palmer P, LeBourhis D, Camous S, Vignon X, Renard JP. Frequency and occurrence of late-gestation losses from cattle cloned embryos. Biol Reprod 2002; 66:6–13.
33. Wells DN, Misica PM, Tervit HR. Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. Biol Reprod 1999; 60:996–1005.
34. Wells DN, Misica PM, Tervit HR, Vivanco WH. Adult somatic cell nuclear transfer is used to preserve the last surviving cow of the Enderby Island cattle breed. Reprod Fertil Dev 1998; 10:369–378.
35. Edwards JL, Dorado CM, Wilson TJ, Schrick FN. Development of cloned embryos reconstructed with serum fed or serum starved adult granulosa cells. Theriogenology 2001; 55:265.
36. Piedrahita JA, Wells DN, Miller AL, Oliver JE, Berg MC, Peterson AJ, Tervit HR. Effects of follicular size of cytoplast donor on the efficiency of cloning in cattle. Mol Reprod Dev 2002; 61:317–326.
37. Shiga K, Fujita T, Hirose K, Sasae Y, Nagai T. Production of calves by transfer of nuclei from cultured somatic cells obtained from Japanese black bulls. Theriogenology 1999; 52:527–535.
38. Goto Y, Kaneyama K, Kobayashi S, Imai K, Shin-noh M, Tsujino T, Nakano T, Matsuda S, Nakane S, Kojima T. Birth of cloned calves derived from cultured oviductal epithelial cells of a dairy cow. Anim Sci J 1999; 70:243–245.
39. Wakayama T, Yanagimachi R. Cloning of male mice from adult tail-tip cells. Nat Genet 1999; 22:127–128.
40. Edwards JL, Schrick FN, Dorado CM, Miller J, McCann L, Wilson TJ, Malone M, Hockett M, Towns TM, Blackmon H, Welborn M, Hopkins F. Cloning of an adult Jersey cow using proliferating somatic cells. National Swine Improvement Federation Proceedings; 2000; 25:40.
41. Zakhartchenko V, Durcova-Hills G, Stojkovic M, Schernthaner W, Prelle K, Steinborn R, Muller M, Brem G, Wolf E. Effects of serum starvation and re-cloning on the efficiency of nuclear transfer using bovine fetal fibroblasts. J Reprod Fertil 1999; 115:325–331.
42. Edwards JL, Powell AM, Talbot NC, Garrett WM, Pursel VG. Developmental potential of reconstructed embyos activated immediately or 4 hours after assessment of fusion with fetal fibroblasts. Theriogenology 1999; 51:202.
43. Susko-Parrish JL, Leibfried-Rutledge ML, Northey DL, Schutzkus V, First NL. Inhibition of protein kinases after an induced calcium transient causes transition of bovine oocytes to embryonic cycles without meiotic completion. Dev Biol 1994; 166:729–739.
44. Lanza RP, Cibelli JB, Diaz F, Moraes CT, Farin PW, Farin CE, Hammer CJ, West MD, Damiani P. Cloning of endangered species (*Bos gaurus*) using interspecies nuclear transfer. Cloning 2000; 2:79–90.
45. Schnieke AE, Kind AJ, Ritchie WA, Mycock K, Scott AR, Ritchie M, Wilmut I, Colman A, Campbell KH. Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science 1997; 278:2130–2133.
46. Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce Leon de FA, Robl JM. Cloned transgenic calves produced from nonquiescent fetal fibroblasts. Science 1998; 280:1256–1258.
47. McCreath KJ, Howcroft J, Campbell KH, Colman A, Schnieke AE, Kind AJ. Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. Nature 2000; 405:1066–1069.
48. Lai L, Kolber-Simonds D, Park KW, Cheong HT, Greenstein JL, Im GS, Samuel M, Bonk A, Rieke A, Day BN, Murphy CN, Carter DB, Hawley RJ, Prather RS. Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science 2002; 295:1089–1092.
49. Lanza RP, Chung HY, Yoo JJ, Wettstein PJ, Blackwell C, Borson N, Hofmeister E, Schuch G, Soker S, Moraes CT, West MD, Atala A. Generation of histocompatible tissues using nuclear transplantation. Nat Biotechnol 2002; 20:689–696.
50. Renard JP, Zhou Q, LeBourhis D, Chavatte-Palmer P, Hue I, Heyman Y, Vignon X. Nuclear transfer technologies: between successes and doubts. Theriogenology 2001; 57:203–222.
51. Hill JR, Burghardt RC, Jones K, Long CR, Looney CR, Shin T, Spencer T, Thompson JA, Winger QA, Westhusin M. Evidence for placental abnormality as the major cause of mortality in first-trimester somatic cell cloned bovine fetuses. Biol Reprod 2000; 63:1787–1794.
52. Hasler JF. The current status of oocyte recovery, in vitro embryo production, and embryo transfer in domestic animals with an emphasis on the bovine. J Anim Sci 1998; 76:52–74.

53. Hasler JF, Henderson WB, Hurtgen PJ, Jin ZQ, McCauley AD, Mower SA, Neely B, Shuey LS, Stokes JE, Trimmer SA. Production, freezing and transfer of bovine IVF embryos and subsequent calving results. Theriogenology 1995; 43:141–152.
54. Hawk HW. Infertility in dairy cattle. Allanheld, Osmun and Co. Publishing, Inc. Montclair, 1979.
55. McCracken M, Bochsler P, Schrick F, Hopkins F, Welborn M, Edwards J. Pathologic changes in late term fetuses and neonates cloned from an adult Jersey cow. Vet Pathol 2001; 38:77.
56. Schlafer DH, Fisher PJ, Davies CJ. The bovine placenta before and after birth: placental development and function in health and disease. Anim Reprod Sci 2000; 60–61: 145–160.
57. Hill JR, Roussel AJ, Cibelli JB, Edwards JF, Hooper NL, Miller MW, Thompson JA, Looney CR, Westhusin ME, Robl JM, Stice SL. Clinical and pathologic features of cloned transgenic calves and fetuses (13 case studies). Theriogenology 1999; 51:1451–1465.
58. Chavatte-Palmer P, Heyman Y, Richard C, Monget P, LeBourhis D, Kann G, Chilliard Y, Vignon X, Renard JP. Clinical, hormonal, and hematologic characteristics of bovine calves derived from nuclei from somatic cells. Biol Reprod 2002; 66: 1596–1603.
59. Garry FB, Adams R, McCann JP, Odde KG. Postnatal characteristics of calves produced by nuclear transfer cloning. Theriogenology 1996; 45:141–152.
60. Lanza RP, Cibelli JB, Faber D, Sweeney RW, Henderson B, Nevala W, West MD, Wettstein PJ. Cloned cattle can be healthy and normal. Science 2001; 294: 1893–1894.
61. Pace MM, Augenstein ML, Betthauser JM, Childs LA, Eilertsen KJ, Enos JM, Forsberg EJ, Golueke PJ, Graber DF, Kemper JC, Koppang RW, Lange G, Lesmeiser TL, Mallon KS, Mell GD, Misica PM, Pfister-Genskow M, Strelchenko NS, Voelker GR, Watt SR, Bishop MD. Ontogeny of cloned cattle to lactation. Biol Reprod 2002; 67:334–339.
62. Enright BP, Taneja M, Schreiber D, Riesen J, Tian XC, Fortune JE, Yang X. Reproductive characteristics of cloned heifers derived from adult somatic cells. Biol Reprod 2002; 66:291–296.
63. Govoni KE, Tian XC, Kazmer GW, Taneja M, Enright BP, Rivard AL, Yang X, Zinn SA. Age-related changes of the somatotropic axis in cloned Holstein calves. Biol Reprod 2002; 66:1293–1298.
64. Miyashita N, Shiga K, Yonai M, Kaneyama K, Kobayashi S, Kojima T, Goto Y, Kishi M, Aso H, Suzuki T, Sakaguchi M, Nagai T. Remarkable differences in telomere lengths among cloned cattle derived from different cell types. Biol Reprod 2002; 66:1649–1655.
65. Eggan K, Akutsu H, Hochedlinger K, Rideout W, Yanagimachi R, Jaenisch R. X-Chromosome inactivation in cloned mouse embryos. Science 2000; 290:1578–1581.
66. Xue F, Tian XC, Du F, Kubota C, Taneja M, Dinnyes A, Dai Y, Levine H, Pereira LV, Yang X. Aberrant patterns of X chromosome inactivation in bovine clones. Nat Genet 2002; 31:216–220.
67. Wrenzycki C, Lucas-Hahn A, Herrmann D, Lemme E, Korsawe K, Niemann H. In vitro production and nuclear transfer affect dosage compensation of the X-linked gene transcripts G6PD, PGK, and Xist in preimplantation bovine embryos. Biol Reprod 2002; 66:127–134.
68. Humpherys D, Eggan K, Akutsu H, Hochedlinger K, Rideout WM, Biniszkiewicz D, Yanagimachi R, Jaenisch R. Epigenetic instability in ES cells and cloned mice. Science 2001; 293:95–97.
69. Ogonuki N, Inoue K, Yamamoto Y, Noguchi Y, Tanemura K, Suzuki O, Nakayama H, Doi K, Ohtomo Y, Satoh M, Nishida A, Ogura A. Early death of mice cloned from somatic cells. Nat Genet 2002; 30:253–254.
70. Hill JR, Schlafer DH, Fisher PJ, Davies CJ. Abnormal expression of trophoblast major histocompatibility complex class I antigens in cloned bovine pregnancies is associated with a pronounced endometrial lymphocytic response. Biol Reprod 2002; 67:55–63.
71. Smith LC. Membrane and intracellular effects of ultraviolet irradiation with Hoechst 33342 on bovine secondary oocytes matured in vitro. J Reprod Fertil 1993; 99:39–44.
72. Ritchie WA, Campbell KHS. Intracytoplasmic nuclear injection as an alternative to cell fusion for the production of bovine embryos by nuclear transfer. J Reprod Fertil 1995; 15:175.
73. Van Stekelenburg-Hamers AE, Van Inzen WG, Van Achterberg TA, Kruip TA, de Laat SW, Weima SM. Nuclear transfer and electrofusion in bovine in vitro-matured/in vitro-fertilized embryos: effect of media and electrical fusion parameters. Mol Reprod Dev 1993; 36:307–312.
74. Farin PW, Crosier AE, Farin CE. Influence of in vitro systems on embryo survival and fetal development in cattle. Theriogenology 2001; 55:151–170.
75. Blondin P, Farin PW, Crosier AE, Alexander JE, Farin CE. In vitro production of embryos alters levels of insulin-like growth factor-II messenger ribonucleic acid in bovine fetuses 63 days after transfer. Biol Reprod 2000; 62:384–389.
76. Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C, Broadbent PJ, Robinson JJ, Wilmut I, Sinclair K. Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. Nat Genet 2001; 27:153–154.
77. Farin PW, Farin CE. Transfer of bovine embryos produced in vivo or in vitro: survival and fetal development. Biol Reprod 1995; 52:676–682.
78. Walker SK, Hartwich KM, Seamark RF. The production of unusally large offspring following embryo manipulation: concepts and challenges. Theriogenology 1996; 45:111–120.
79. Young LE, Sinclair KD, Wilmut I. Large offspring syndrome in cattle and sheep. Rev Reprod 1998; 3:155–163.
80. Plante Y, Schmutz SM, Lang KDM. Restriction fragment length polymorphism in the mitochondrial DNA of cloned cattle. Theriogenology 1992; 38:897–904.
81. Nagao Y, Totsuka Y, Atomi Y, Kaneda H, Lindahl KF, Imai H, Yonekawa H. Decreased physical performance of congenic mice with mismatch between the nuclear and the mitochondrial genome. Genes Genet Syst 1998; 73:21–27.
82. Yong-Kook K, Deog-Bon K, Jung-Sun P, Young-Hee C, An-Sik C, Kyung-Kwang L. Aberrant methylation of donor genome in cloned bovine embryos. Nat Genet 2001; 28:173–177.

83. Dean W, Santos F, Stojkovic M, Zakhartchenko V, Walter J, Wolf E, Reik W. Conservation of methylation reprogramming in mammalian development: aberrant reprogramming in cloned embryos. Proc Natl Acad Sci USA 2001; 98(24):13734–13738.
84. Bourc'his D, Le Bourhis D, Patin D, Niveleau A, Comizzoli P, Renard JP, Viegas-Pequignot E. Delayed and incomplete reprogramming of chromosome methylation patterns in bovine cloned embryos. Curr Biol 2001; 11:1542–1546.
85. Daniels R, Hall V, Trounson AO. Analysis of gene transcription in bovine nuclear transfer embryos reconstructed with granulosa cell nuclei. Biol Reprod 2000; 63:1034–1040.
86. Boiani M, Eckardt S, Scholer HR, McLaughlin KJ. Oct4 distribution and level in mouse clones: consequences for pluripotency. Genes Dev 2002; 16:1209–1219.
87. Wrenzycki C, Wells D, Herrmann D, Miller A, Oliver J, Tervit R, Niemann H. Nuclear transfer protocol affects messenger RNA expression patterns in cloned bovine blastocysts. Biol Reprod 2001; 65:309–317.
88. Forsberg EJ, Strelchenko NS, Augenstein ML, Betthauser JM, Childs LA, Eilertsen KJ, Enos JM, Forsythe TM, Golueke PJ, Koppang RW, Lange G, Lesmeister TL, Mallon KS, Mell GD, Misica PM, Pace MM, Pfister-Genskow M, Voelker GR, Watt SR, Bishop MD. Production of cloned cattle from in vitro systems. Biol Reprod 2002; 67:327–333.
89. Davies CJ, Fisher PJ, Schlafer DH. Temporal and regional regulation of major histocompatibility complex class I expression at the bovine uterine/placental interface. Placenta 2000; 21:194–202.
90. Azuma M, Ito D, Yagita H, Okumura K, Phillips JH, Lanier LL, Somoza C. B70 antigen is a second ligand for CTLA-4 and CD28. Nature 1993; 366:76–79.
91. Olivares EG, Montes MJ, Oliver C, Galindo JA, Ruiz C. Cultured human decidual stromal cells express B7-1 (CD80) and B7-2 (CD86) and stimulate allogeneic T cells. Biol Reprod 1997; 57:609–615.
92. Sallinen K, Verajankorva E, Pollanen P. Expression of antigens involved in the presentation of lipid antigens and induction of clonal anergy in the female reproductive tract. J Reprod Immunol 2000; 46:91–101.
93. Chapoval AI, Ni J, Lau JS, Wilcox RA, Flies DB, Liu D, Dong H, Sica GL, Zhu G, Tamada K, Chen L. B7-H3: a costimulatory molecule for T cell activation and IFN-gamma production. Nat Immunol 2001; 2:269–274.
94. Petroff MG, Chen L, Phillips TA, Hunt JS. B7 family molecules: novel immunomodulators at the maternal-fetal interface. Placenta 2002; 23 Suppl A:S95–S101.
95. Wooding FB, Wathes DC. Binucleate cell migration in the bovine placentome. J Reprod Fertil 1980; 59: 425–430.
96. Wooding FB. Current topic: the synepitheliochorial placenta of ruminants: binucleate cell fusions and hormone production. Placenta 1992; 13:101–113.