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Review

Animal cloning for food: epigenetics, health, welfare and food safety aspects

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Cloning *via* somatic cell nucleus transfer (SCNT) is a potential way for using validated genomes in farm animal breeding and to save endangered breeds or species. Although this technique is relatively inefficient and costly, it is envisaged to use it as an assisted reproduction technique. Despite numerous problems observed in the perinatal period, after some time clones appear normal although they may have kept some epigenetic modifications. Meat and milk from cattle and meat from pig clones and their offspring are substantially equivalent to conventional animals with no observed toxicity or allergenicity. Due to limited data, monitoring of clones and their offspring is recommended to detect whether there are unexpected long-term effects of cloning.

Introduction

Cloning is a way to control genetic selection by preventing the random distribution of parent genes into offspring that takes place in sexual reproduction and makes possible the use of genomes from validated genitors. Cloning can be used in breeding to accelerate the introduction of desired traits into herds. Cloning *via* SCNT according to the technique used to generate ''Dolly the sheep'' is laborious and relatively costly but it has been sufficiently improved to make possible its implementation to improve genetic selection of farm animals and particularly of cattle and pigs. Interestingly, cloning can be applied similarly to both sexes making it possible a more intensive use of specific female genotypes with desired phenotypes compared to conventional breeding.

The cloning technique implies a reprogramming of the genetic elements of a somatic cell to transform it into a totipotent embryonic cell. This process is complex and sometimes not fully complete in all instances. The incomplete dysfunctional reprogramming is responsible for the morbidity and mortality of clones during pregnancy and soon after birth. After the juvenile period (up to 6–12 months), cattle clones appear normal although they could keep some epigenetic modifications which seem not to be transmitted to progeny.

Clones are precious genitors primarily intended to be used for breeding and not as human food. On the contrary, offspring of clones are valuable candidates to enter the food chain. The cloning technique in its present state induces a significant reduction of animal health and welfare in a cohort of animals which must be balanced with the advantages cloning may bring for breeders and consumers but also for the animals themselves (e.g. using a cell donor with proven resistance to infection). Cattle and pig clones and their offspring are the two species first likely to be used for human food.

Cloning technique

SCNT is a complex technological procedure, with many steps potentially affecting the reprogramming process, creating different epigenetic patterns and aberrations and thus influencing the foetal development and long-term health of the clone. Due to these processes, the animal health and welfare are influenced by the sum of the effects of the technical steps which consequently may affect the food safety of products derived from the clones. Many of the technology improvements are based on empirical scientific studies, as the exact nature of the interactions is often unclear. Existing information demonstrated major influence by some of the technical components, including the activation methods used, *in vitro* culture conditions and treatment of donor cells or reconstructed embryos with chemicals affecting the methylation processes.

There are numerous variations in the technical steps and applied parameters in SCNT, although all cloning methods

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are fundamentally similar by achieving the replacement of the oocyte's original nucleus with that from a somatic cell. Three protocols can be distinguished based on the presence or absence of the zona pellucida (a coat surrounding the oocyte) during enucleation and reconstruction of the new model embryos: (1) ''conventional'' zona-intact SCNT, (2) zona-free SCNT and (3) zona-free ''hand-made cloning''. The first method has been used for a longer time, and is still the most popular. It can be used to enucleate oocytes in the metaphase II (MII)-arrested developmental stage. In this method the genetic material from the recipient oocyte is removed by aspirating the maternal chromosomes and surrounding cytoplasm in a small plasma membrane envelopes in order to obtain a so-called cytoplast. This SCNT procedure is very labour-, skill- and technology-intensive due to complicated micromanipulation steps (Cibelli *et al*., 1998). The second method is simpler, using zona pellucida-free oocytes which can increase the embryo clone and offspring throughput production and is also easier to establish in a production environment (Oback *et al*., 2003). The third method is using manual sectioning of zona-free oocytes with a microblade and then the chromatin-free portion is fused with another cytoplast to restore the original volume before SCNT (Vajta, Lewis, Hyttel, Thouas, & Trounson, 2001). The different SCNT protocols applied by different users confound the understanding for the biological causes underlying the low efficiency of SCNT and it is practically impossible to standardize all experimental parameters with inherited differences in operator skills, oocyte quality and donor cells (Gao *et al*., 2004).

The core elements of improving the SCNT procedure are the following: choice of donor cells, the recipient cytoplast, and certain modifiers of the reprogramming. Perfecting donor cell culture methods and using low passage number of such cells could help to decrease epigenetic modifications, genetic aberrations and mutations which can accumulate during cell culture (Niemann, Tian, King, & Lee, 2008). The genetic origin of the cells is an important factor according to data mostly generated in mouse. The cell cycle status of the donor cells plays an important, but still controversial role, and several studies reported on the positive effect of serum starvation induced G0 (Wilmut, Schnieke, McWhir, Kind, & Campbell, 1997) or cell culture confluency induced G1 stage (Cibelli *et al*., 1998; Li, Li, Jouneau, Zhou, & Renard, 2003).

Cytoplasts for SCNT are often generated by enucleation of non-activated MII stage oocytes (Cibelli *et al*., 1998; Wilmut *et al*., 2002; Wilmut *et al*., 1997), but Telophase-II stage oocytes (Bordignon & Smith, 1998) and mitotic zygotes are also viable options (Greda, Karasiewicz, & Modlinski, 2006; Schurmann, Wells, & Oback, 2006). *In vitro* approaches to increase SCNT success include treating donor cells with pharmacological agents to modify their epigenetic marks (Enright, Kubota, Yang, & Tian, 2003; Enright, Sung, Chang, Yang, & Tian, 2005) or cell cycle stage (Campbell, Loi, Otaegui, & Wilmut, 1996; Wells *et al*., 2003). Another approach is fusing transiently permeabilized cells containing artificially condensed chromatin (Sullivan, Kasinathan, Kasinathan, Robl, & Collas, 2004). Activating with sperm rather than by artificial methods (Schurmann *et al*., 2006) can have a positive influence on SCNT by providing a better Ca-signaling impulse and contributing some factors (centrosome, mRNAs, microRNAs) to the cytoplast.

Genetic and epigenetic properties of clones

Genetic aspects

Cloning by SCNT generates in principle animals genetically identical to the nucleus donor. In practise, the situation is somewhat more complex. Somatic cells are assumed to harbour all the same genome but mutations of some genes which are silent in somatic cells may have occurred during the life of the animals and, strictly speaking, the genomes used for cloning are not completely known.

Difference between clone and the donor animal may also stems from mitochondrial heterogeneity. In sexual reproduction, male mitochondria are eliminated from the oocyte cytoplasm. In SCNT, embryos may contain mitochondrial DNA from the oocyte cytoplasm only or from both the donor cell and the oocyte cytoplasm (mitochondrial heterogeneity). The vast majority of clones analysed so far contained only a small number, if any, of mitochondria from the donor (Hiendleder *et al*., 2006), although in some exceptional cases a high proportion of donor mitochondria has been detected (Burgstaller, Schinogl, Dinnyes, Muller, & Steinborn, 2007). Yet, it cannot be ruled out that some adult metabolic diseases could result from the presence of donor mitochondria (McConnell, 2006).

Telomeres are short, highly repetitive DNA sequences located at the ends of chromosomes which prevent degradation of chromosomes. An enzyme, telomerase, present in various renewal tissues including germ cells and embryonic cells has the ability to extend, or to hold constant, the length of the telomeres over multiple cell divisions. Telomeres of the first mammalian clone, ''Dolly'', were found to be shorter than those of the age-matched, naturally bred counterparts (Shiels *et al*., 1999). In sheep similar results have been published showing that clones derived from cultured somatic cells have shortened telomere lengths compared to age-matched controls and donor cell cultures beyond 20 population doublings had significantly ($P < 0.05$) shortened telomeres (Alexander *et al*., 2007). The offspring derived from natural mating between clones had normal telomere lengths compared to their age-matched counterparts. However, in later studies, the telomere length in cattle, pig and goat clones were comparable to or even longer than age-matched naturally bred controls, even when senescent donor cells were used for cloning (Betts *et al*., 2005; Jeon *et al*., 2005; Jiang *et al*., 2004; Lanza *et al*., 2000; Schaetzlein & Rudolph, 2005). The telomere length of 30 offspring from the same bull clone was not different from age-matched controls (Ortegon *et al*.,

2007). Overall, the impact of telomere lengths of aging is not fully understood (Niemann *et al*., 2008).

Epigenetic aspects

Mammalian genomes contain about 25,000 genes and only 2000 of them are required for the normal activity of a somatic cell. About 1000 genes are expressed in all cell types and are considered as housekeeping genes. The other 1000 genes are responsible for the differentiated state of somatic cells. Gamete formation implies that the 23,000 silent genes of the somatic gonad cells are reactivated as about 10,000 genes are needed for the early embryo development and the other genes for the different cell function in adults. This reprogramming of the genome in somatic cells to obtain gametes occurs during gamete formation but also during the early steps of embryo development.

Genes are active or not according to the presence or the absence of specific inducers. To be active, a gene must be accessible to its inducers. DNA is associated to proteins and particularly histones. According to the chemical status of histones (e.g. more or less acetylated or methylated), chromatin adopts several conformations considered as open or closed, permitting access or not to specific genes of the inducers and the transcription machinery. Moreover, the cytosine base of the CpG structures in DNA may be methylated or not. The DNA methylation state corresponds to inactive genes. Both the histone and DNA biochemical modifications are essential for the regulation of gene expression and known as epigenetic mechanisms. The biochemical modifications of chromatin proteins are a reversible and dynamic process whereas DNA methylation is much more stable, but also reversible. Somatic cell reprogramming consists to a large extent of DNA demethylation followed by specific remethylation of DNA regions which must remain silent in a given cell type. Epigenetic mechanisms affect the expression of some genes and these modifications may be transmitted to daughter cells (Jablonka & Lamb, 2002).

The dedifferentiation of the somatic donor nucleus after SCNT requires changes in DNA and chromatin which are essentially dependent on components found in the cytoplasm of the recipient oocyte. These changes may partially mimic those taking place after fertilization (Jaenisch & Wilmut, 2001). Consequently, the clone embryos often show aberrant patterns of global DNA methylation at the zygotic stages (Dean *et al*., 2001; Kang *et al*., 2001a, 2001b). Chromosomal disorders after SCNT in bovines have been observed at a relatively high frequency during the preimplantation stages but mainly in morphologically abnormal embryos (Booth *et al*., 2003). Methylation errors evidenced early in the preimplantation period of embryonic development can persist in bovine clone foetuses (Hiendleder *et al*., 2006). Some genes aberrantly expressed in blastocysts are also found aberrantly expressed in the organs of bovine clones that died shortly after birth (Li, Wells, Peterson, & Lee, 2005). Several studies in cattle reveal that significant and relatively normal nuclear reprogramming, in terms of gene expression, can occur by the blastocyst stage after SCNT (Yang, Smith, *et al*., 2007). In the mouse, the pluripotent cells derived *in vitro* from the inner cell mass of cloned blastocysts have been found to be indistinguishable from those obtained from *in vivo* fertilized embryos, both for their transcriptional activities and methylation profile (Brambrink, Hochedlinger, Bell, & Jaenisch, 2006; Kishigami *et al*., 2006). This suggests that the epigenetic status of embryonic cells forming the inner cell mass is relatively well restored after SCNT at the blastocyst stage. On the contrary, the DNA of trophectoderm cells that are the precursors of placenta is excessively methylated (Yang, Smith, *et al*., 2007). This may explain why about 400 genes out of 10,000 examined showed abnormal expression in the placenta of mouse clones and why this organ is often altered in clones. These changes in DNA methylation patterns have also been observed in *in vitro* fertilization and embryo culture (without cloning) and in a protocol- and tissue-specific manner, resulting in foetal overgrowth correlated with endocrine changes (Hiendleder *et al*., 2006).

Not all epigenetic alterations observed in early SCNT embryos result in abnormalities. Hypomethylation of the genes involved in the X-chromosome inactivation process has been observed in various organs of stillborn calves. Although, as no disturbance of sex development has been reported in clones, the implications of the hypomethylation of the X-chromosome observed in dead clones are unclear for healthy clones (Xue *et al*., 2002). More generally, it must be considered that the two copies of a gene have little chance to be simultaneously epigenetically silenced in a clone. The silencing of specific genes by epigenetic mechanisms or inactivation of a pathway may be compatible with a normal life of the clones.

Although global analysis of the methylated status of clones is lacking in domestic species, a study in swine clones included evaluation of methylation in two different regions of the genome (Archer *et al*., 2003). Compared to control pigs, clones demonstrated differences in the methylation status in both transcribed and untranscribed regions of the genome, indicating that the cloning process may alter the pattern of DNA methylation. However, because all of the clones in this study were healthy at the time of study (27 weeks of age) and had no apparent developmental defects, the biological relevance of these differences in DNA methylation is unclear.

Study in mouse indicates that, after cloning, epigenetic abnormalities such as those resulting in an obese phenotype are corrected in the germ cells of clones so that the offspring do not exhibit the obese phenotype (Tamashiro, Wakayama, Blanchard, Blanchard, & Yanagimachi, 2000). Many genes with epi-alleles may exist in the genome but their detection requires a visible effect on the phenotype in both the clone and its progeny (Peaston & Whitelaw, 2006). Recent data indicated that 30 offspring generated

by the same bull clone, lost all the abnormalities observed at birth and postnatally in the genitor (Ortegon *et al*., 2007).

Transgenerational epigenetic inheritance in response to various conditions has been observed in many eukaryotes and may play an important role in mammals (Peaston & Whitelaw, 2006). Environmental influences may induce a number of epigenetic modifications leading to the silencing or activation of specific genes, especially when pregnant females are maintained in conditions resulting in stress in the dam and foetus. The epigenetic modifications observed in the offspring of those pregnancies may then be transmitted to their progeny (Gluckman, Hanson, & Beedle, 2007a, 2007b). Moreover, there is evidence suggesting that RNA can be a determinant of inherited phenotype. In the *Agouti* phenotype, the white tail tip trait in mouse is not transmitted in a Mendelian fashion but by RNAs packaged in sperm (Rassoulzadegan *et al*., 2006). No similar studies or outcomes have been identified in the livestock species. The relevance of these observations to clones and their offspring are not entirely clear. One hypothesis is that clones are epigenetically modified animals, with the cloning process itself functioning as the epigenetic inducer. The healthy clones may then be considered as having epigenetic modifications compatible with a normal life. Epigenetic dysregulation is not a phenomenon unique to cloning and has been observed in all other forms of reproduction, but particularly in ART that have a considerable *in vitro* component. This has been observed in cattle when *in vitro* fertilized embryos and embryos derived *via* SCNT were compared to *in vivo* produced embryos (Camargo, Viana, Sa, Ferreira, & Vale Filho, 2005; Smith *et al*., 2005). It is not known whether these abnormalities are due to the stress of SCNT *per se*, or as the result of the *in vitro* environment that the early embryos are exposed to prior to transfer to the surrogate dam. However, Smith *et al*. (2005) found that the SCNT bovine embryos' gene expression profiles were very different from those of their donor cells and very closely resembled those of naturally fertilized AI embryos, more so than IVF embryos, representing the complexity of this issue.

Health and welfare of clones

Animal health is referred to as a state where an animal can sustain its biological function to sustain its own integrity and animal welfare when its physical and behavioural needs are fulfilled which include the absence of pain, distress and suffering. The evidence for poor health and welfare, or improved health and welfare, may change according to the context of the various phases of the animal life. The current available data with reference to clones have been generated mostly by comparing clones with animals that are not clones. It is important, in regard to the risks associated with the cloning technology, to distinguish clearly between the risks directly related to the technology of cloning itself, and those related to the stage of development of the technology and the degree of the control of the processes which are used.

Qualitative and preferably quantitative data are required to assess welfare indicators directly on the animals concerned. Since animal cloning is a relatively recent technology data are still scarce and it is therefore difficult to draw any conclusions on welfare from the limited behavioural studies available (Archer, Friend, Piedrahita, Nevill, & Walker, 2003a, 2003b; Coulon *et al*., 2007; Savage *et al*., 2003).

The draft EFSA opinion (EFSA, 2008) considers health aspects in relation to the surrogate dams, to clones and their progeny. For surrogate dams, an increased proportion of pregnancy failure has been observed in cattle and pigs and increased frequencies of hydrops and dystocia have been observed especially in cattle (Arnold, Bordignon, Lefebvre, Murphy, & Smith, 2006; Batchelder *et al*., 2005; Lee *et al*., 2004). This and the increased size of the offspring (large offspring syndrome; LOS) make Caesarean sections more frequent in cattle carrying a clone than with conventional pregnancies (Constant *et al*., 2006). These effects have also been observed in surrogate dams carrying pregnancies induced by assisted reproductive technologies (ART) not involving SCNT, albeit at a lower frequency and often with less severity (Farin & Farin, 1995; Walker, Hartwich, & Seamark, 1996). Mortality and morbidity rates in clones are higher than in sexually reproduced animals but most clones that survive the perinatal period in pigs and the juvenile period (up to about 6 months) in cattle are normal and healthy as determined by physiological measurements as well as by behaviour and clinical examinations (Chavatte-Palmer *et al*., 2004; Heyman *et al*., 2007; Panarace *et al*., 2007; Wells, Forsyth, McMillan, & Oback, 2004). There is no evidence indicating adverse outcomes for the sexually reproduced progeny of cattle or pig clones. However, it should be noted that neither clones nor their progeny have yet been studied for their full natural lifespan, and that no studies on the welfare of the progeny of livestock clones have been reported.

The draft EFSA opinion addresses animal welfare and indicates that the cloning procedure itself does in general not affect the welfare of the animals from which the somatic cell nucleus and oocyte are obtained (EFSA, 2008). Reduced welfare of clones is assumed to occur as a consequence of adverse health outcomes. For the surrogate dam carrying calf clone the occurrence of late gestational losses, dystocia and LOS is likely to affect welfare and the frequency of adverse health outcomes is higher in SCNT compared to *in vitro* or *in vivo* reproduction. Due to the low efficiency of the cloning process, also a high number of surrogate dams are required to produce a low number of clones which indirectly may affect the welfare of a cohort of surrogate dams. However, the proportion of adversely affected clones could decrease as a result of good animal management and as the technology improves.

In some experiments but not in others, it has been observed that mouse clones have a lifespan shortened by an average of 10% whereas other studies has not seen signs of premature ageing, but that lifespan was shorted due to increased sensitivity to diseases (Wakayama, 2004). It is not known if clones of farm animals have a shorter lifespan as they are still too young, and because farm animals are normally slaughtered long before the end of their natural life.

The European Group on Ethics (EGE) has adopted an Opinion on the request of the President of the European Commission on the ethical aspects of animal cloning for food supply (EGE, 2008). Considering the current level of suffering and health problems of surrogate dams and animal clones, the EGE has doubts as to whether cloning animals for food supply are ethically justified. Whether this applies also to progeny is open to further scientific research. The draft EFSA opinion as well the EGE opinion state that there is a lack of data on the long-term animal welfare and health implications of clones and their offspring, due to the current limited use of the technology. Therefore it is recommended to perform further studies and analyses on long-term animal welfare and health implications for clones and their offspring, as well as more comparative analyses with other assisted and traditional reproductive technologies in animal farming.

Safety of meat and milk from clones and clone progeny

The safety of food from clones and progeny has been examined in several countries in cows and pigs using various criteria.

Composition of meat and milk

Several parameters have been measured in clones and clone offspring in various studies such as gross carcass characteristics, milk volume, water, fat, proteins, carbohydrates, amino acids, fatty acids, vitamins and minerals (Heyman *et al*., 2007; Norman, Lawlor, Wright, & Powell, 2004; Norman & Walsh, 2004; Shibata *et al*., 2006; Takahashi & Ito, 2004; Tian *et al*., 2005; Tome, Dubarry, & Fromentin, 2004; Walker *et al*., 2007; Walsh, Lucey, Govindasamy-Lucey, Pace, & Bishop, 2003; Yang, Tian, *et al*., 2007). In one study more than 150 parameters in 37 cow clones from 3 independent groups of clones and 38 control animals were examined over a 3-year period (Heyman *et al*., 2007). Only a few very slight differences among the 10,000 individual measurements were observed between the clones and their controls, e.g. in fatty acid composition of milk and muscle of clones and a slight increase of stearoyl-CoA desaturase in milk and muscle. These variations were within the normal range of controls and are not expected to have an impact on food safety.

Meat composition data for 5 pig clones and 15 comparators showed no biologically relevant differences in fatty acid, amino acid, cholesterol, mineral and vitamin values. The composition of 242 pig clone offspring, from four boar clones and 162 controls from the same breed were compared (Walker *et al*., 2007). Among the 58 parameters and the 24,000 individual measurements examined, only 3 individual values of the offspring were different from the normal range of the controls and 2 out of the 3 were within the normal range found in pigs, according to the USDA National Nutrition Database.

Toxicity, allergenicity and genotoxicity of clones

Clones as well as conventional food production animals are unlikely to have genes or biochemical pathways to produce toxins. Toxicity of livestock animals may instead result from indirect effects such as minor changes of protein glycosylation or over accumulation of toxic residues. Evaluating the toxicity of meat or milk is more difficult than for individual compounds as the amount of clone products given to the test animals remains limited by their capacity to eat these products. The evaluation of food products from clones and offspring is therefore based on the concept of substantial equivalence comparing measurements of biochemical parameters and nutritional substances with conventionally reproduced control animals and data found in available databases.

Rats fed for 14 weeks with a diet containing meat and milk derived from embryonic and somatic clones were not affected by the consumption of meat and milk from bovine clones (Yamaguchi, Ito, & Takahashi, 2007). A similar conclusion was drawn with rats fed for 21 days using a diet containing milk and meat of cattle clones (Heyman *et al*., 2007). Rats fed with milk and meat from cattle clones and controls developed, as expected, a weak immune reaction. The antibodies were in both cases IgG, IgA and IgM but not IgE, indicating that the consumption of the cow products induced a classical immune response but no allergenic effect (Takahashi & Ito, 2004). Intraperitoneal injections of meat extracts and milk into mice were performed and no difference in the allergenic potential was observed between samples from clones and comparator control cattle (Takahashi & Ito, 2004). Similarly, Heyman *et al*. did not detect differences in the allergenicity of milk and meat obtained from clones or control animals (Heyman *et al*., 2007). Meat derived from cattle clones did not show any genotoxic potential using the mouse micronucleus assay (Takahashi & Ito, 2004).

In conclusion the available reported studies, and available risk assessments does not indicate that animal clones, their products or offspring from clones would constitute any additional food safety risks compared with their conventional counterparts (EFSA, 2008; FDA, 2008).

Impact on environment and genetic diversity

There is no expectation that cattle and pig clones or their progeny would pose any new or additional environmental risks compared to conventionally bred animals. There is also no information to suggest that such risks may exist.

But it should be noted that there are no studies published on the environmental impact of clones. Cloning does not seem to have a direct effect on genetic diversity but there could be an indirect effect due to overuse of a limited number of breeding animals in breeding programs. An increased homogeneity of a genotype within a population may increase the susceptibility of an animal population to infectious and other risk factors. Cloning offers opportunities to save endangered species or livestock breeds and can be used to restoring populations from infertile or castrated animals (Meirelles *et al*., 2001; NZRBCS, 2002; Oh *et al*., 2008). This implies preservation of cells obtained from animals to produce genitors that could be used in subsequent breeding programs to expand endangered populations.

Conclusions

The substantial epigenetic reprogramming taking place of the genetic material of the donor cell consists of several crucial stages. The process at each stage is subject to possible failures that may constitute an epigenetic dysregulation which consequently may lead to failure of the SCNT cloning process. The health of clones can be adversely affected due to the epigenetic dysregulation, and this health effect can also give rise to decreased welfare of animal clones. Epigenetic modifications have also been seen in healthy clones, and are likely to be a normal process in all animals. There have been no health effects seen in sexually reproduced offspring of clones and conventional animals. Meat and food products (such as milk from cattle) from clones and their offspring are within the normal range observed in conventional products, and it is unlikely to be any food safety issues consuming such products.

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