European Food Safety Authority

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Scientific Opinion of the Scientific Committee

Food Safety, Animal Health and Welfare and Environmental Impact of Animals[1] derived from Cloning by Somatic Cell Nucleus Transfer (SCNT) and their Offspring and Products Obtained from those Animals[2]

(Question No EFSA-Q-2007-092)

Adopted on 15 July 2008

SCIENTIFIC COMMITTEE MEMBERS

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SUMMARY

In 2007 the European Food Safety Authority (EFSA) was asked by the European Commission to provide a scientific opinion on the food safety, animal health, animal welfare and environmental implications of animal clones, obtained through somatic cell nucleus transfer (SCNT) technique, of their progeny and of the products obtained from those animals. In view of the multidisciplinary nature of this subject this task was assigned to the EFSA Scientific Committee. The ethical aspects of cloning are outside the remit of EFSA and the European Commission asked the European Group on Ethics in Science and New Technologies to provide an opinion on the ethical aspects of cloning.[3]

Unlike sexual reproduction, in which the fertilized egg is totipotent (capable of becoming all cells in the resulting organism), in SCNT, the activated embryo containing a differentiated somatic cell first must be "reset" to totipotency, so that it then follows the same path as a fertilized embryo and is able to complete embryonic and foetal development. This process called "reprogramming" changes the biochemical signals that control gene expression. Failure of the epigenetic reprogramming, which may occur to varying degrees, is the source of potential adverse health effects which may affect clones and may result in developmental abnormalities. The production of healthy clones is the main indicator of the successful functioning of epigenetic reprogramming.

Cloning by SCNT has been applied to several animal species. Based on current knowledge and given the data available it was only possible to make a risk assessment on clones of cattle and pigs and their progeny.

[1] The animal species covered in this opinion are cattle and pigs

[2] For citation purposes: Scientific Opinion of the Scientific Committee on a request from the European Commission on Food Safety, Animal Health and Welfare and Environmental Impact of Animals derived from Cloning by Somatic Cell Nucleus Transfer (SCNT) and their Offspring and Products Obtained from those Animals. *The EFSA Journal* (2008) 767, 1-49

[3] http://ec.europa.eu/european_group_ethics/publications/

Uncertainties in the risk assessment arise from the limited number of studies available, the small sample sizes investigated and the absence of a uniform approach to allow all the issues relevant to this opinion to be addressed.

This opinion considers animal health aspects in relation to the surrogate dams, to clones and their progeny. For surrogate dams, an increase in pregnancy failure has been observed in cattle and pigs and increased frequencies of hydrops and dystocia have been observed especially in cattle. This together with the increased size of the offspring (large offspring syndrome) makes Caesarean sections more frequent in cattle carrying a clone than with conventional pregnancies. These effects have also been observed in surrogate dams carrying pregnancies induced by assisted reproductive technologies not involving SCNT, but at much lower frequencies.

A significant proportion of clones, mainly within the juvenile period for bovines and perinatal period for pigs, has been found to be adversely affected, often severely and with fatal outcome. Most clones that survive the perinatal period are normal and healthy, as determined by physiological measurements, demeanour and clinical examinations. There is no indication of adverse effects for the sexually reproduced progeny of cattle or pig clones. However, clones and their progeny have not yet been studied throughout the whole of their natural life span.

The current welfare assessment is extrapolated from mainly animal health data. The welfare of both the surrogate dam and a significant proportion of clones has been found to be affected by the adverse health outcomes observed.

For the evaluation of the safety of bovine milk and meat from cattle and pigs derived from clones or their progeny, the following aspects were considered: compositional and nutritional data, probability of novel constituents to be present, health status of the animal, available data on toxicity and allergenicity. Based on current knowledge, and considering the fact that the primary DNA sequence is unchanged in clones, there is no indication that differences exist in terms of food safety between food products from healthy cattle and pig clones and their progeny, compared with those from healthy conventionally-bred animals.

At present there is no indication that clones or their progeny would pose any new or additional environmental risks compared with conventionally bred animals.

A number of recommendations is given at the end of the opinion.

Key words: Animal Cloning, Animal Health, Animal Welfare, ART, Assisted Reproduction Technology, Bovine, Cattle, Clone, Clones, Environmental Impact, Epigenetic Reprogramming, Food Product, Food Safety, Genetic Diversity, Immunocompetence, Livestock, Offspring, Pig, Progeny, Risk Assessment, SCNT, Somatic Cell Nucleus Transfer, Swine.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

According to experts, animal cloning carried out through somatic cell nucleus transfer (SCNT) is on the verge of widespread commercial use and expected to spread within the global food chain before 2010. Food (e.g. meat and milk) derived, in particular from traditionally produced offspring of clones might therefore become available to consumers in the future.

In the USA, the Food and Drug Administration (FDA) published on 28 December 2006 its comprehensive draft risk assessment, risk management proposal and guidance for industry on animal cloning. The FDA draft risk assessment concluded that edible products from clones and their offspring are as safe as their conventional counterparts. The above mentioned developments will be facilitated if the FDA, as expected, will issue the final version of the Risk Assessment and lift the voluntary moratorium on food from clones and their progeny.[4]

SCNT allows the production of genetic replicas (clones) of adult animals. The EU is already faced with embryos (offspring of a clone) and soon with semen (sperm) from clones offered in a global market for animal germ line products.

Community Interest

The European Commission (DG SANCO) is currently reflecting on the development of its policy in this area, in the framework of legislation on novel foods, zootechnics, animal health and welfare.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

The European Commission requests the EFSA to advise on food safety, animal health, animal welfare and environmental implications of live animal clones, obtained through SCNT technique, their offspring and of the products obtained from those animals.

INTERPRETATION OF TERMS OF REFERENCE

In reply to the request from the European Commission, EFSA, having considered data availability of different species, decided to restrict its opinion to animal health and animal welfare of cattle and pig clones and their offspring, the food safety of products derived from those animals, and the possible implications of SCNT for the environment and genetic diversity. The opinion does not indicate any priority of the assessed areas.

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EFSA would also wish to thank the participants of the drafting group that prepared the final draft of the opinion before its adoption; Ada Knaap, Vittorio Silano, Pierre Le Neindre, John Collins and Jörg Hartung.

[4] The final FDA risk assessment was published on 15 January 2008 and can be found at http://www.fda.gov/cvm/CloneRiskAssessment_Final.htm (last accessed 27 June 2008)

ASSESSMENT

1. Introduction to the opinion

This opinion is based upon published peer reviewed scientific papers, data and other information deemed reliable. EFSA launched through its Advisory Forum and on its website a request for scientific contributions on this subject from third parties; a list of all documents made available to EFSA can be found at the end of the opinion. A draft of the opinion was published on the EFSA web for public consultation between 11 January and 25 February 2008. During the public consultation, on February 7, a technical meeting with the EFSA's Stakeholder Consultative Platform was held. This meeting provided an opportunity to have an exchange of views and feedback from stakeholders, as part of the public consultation. All the public comments received that related to the remit of EFSA were assessed and the opinion has been revised taking relevant comments into consideration. The comments received and a report on the outcome of the public consultation has been published on the EFSA web.

While cloning has been applied to several animal species, only in the case of cattle and pigs has there been sufficient data available to perform a risk assessment. Where appropriate, reference is made also to data concerning other species.

The first farm animal clone was born in 1984, based on the use of embryonic cells as the nucleus source for the cloning procedure. In 1995, the lambs "Megan" and "Morag" were born, for which embryo-derived cells had been cultured *in vitro* for several weeks and then used for cloning. The major breakthrough came with the birth of the lamb "Dolly" in 1996, using adult somatic cell nucleus transfer (SCNT) in the cloning procedure (Wilmut *et al.*, 1997).

Broadly speaking, cloning can be regarded as an assisted reproductive technology (ART) in the sense that it is a method used to achieve pregnancy by artificial means. However, in the context of this opinion, SCNT is not included in the current use of the term ART, as it is unique due to its asexual nature and permits the production of animals from a single animal with a given genotype of a known phenotype. The present opinion takes into account observations on clones in the context of animals produced by ART (such as artificial insemination, *in vitro* fertilization, embryo transfer and embryo splitting), and natural mating as appropriate. It is also acknowledged that ARTs are currently widely used in the zootechnical practice without any underlying formal risk assessment. In Europe artificial insemination (AI) is used in about 48 % of cattle breeding and 49 % of pig breeding; worldwide the figures are 42 % and 28 % respectively (FAO, 2007). The global conception rates following AI average 50-65 % in cattle and 70-80 % in pigs.

In deciding whether significant differences are incurred by SCNT, the choice of appropriate comparators has to be considered as well as the origin of the somatic cells and oocytes used for cloning, since they may have been selected for characteristics whose expression does not reflect those commonly found in a conventional population. For example, an elite animal would have characteristics that may be found at the top of the range compared with the average values of that species or breed line. This therefore might complicate a direct comparison with the normal range.

1.1. Matters not addressed in the opinion

Approaches to cloning other than SCNT, such as embryonic cell nucleus transfer (ECNT) using early embryonic cells (blastomeres) have been carried out, but in comparison with SCNT, relatively few animals have been described in the literature (Yang *et al.*2007b). ECNT as well as genetically modified animals (rDNA animals) that have been propagated by the use of

SCNT are not assessed in the present opinion, nor are the effects of ARTs. Cloning, without involving SCNT is not addressed in this opinion. Moreover, the opinion does not address aspects of established procedures used in animal breeding or aspects related to breeding the progeny of clones.

The ethical aspects of cloning are outside the remit of EFSA and the European Commission has asked the European Group on Ethics in Science and New Technologies to provide an opinion on the ethical aspects of cloning.[5]

1.2. Terms used in the opinion

Some relevant terms are defined below. A glossary of other terms is given at the end of the opinion.

- **Clone**

The word clone is derived from the Greek words *clonos*, "twig" and *clonizo* "to cut twigs". A clone is the animal born as a result of asexual reproduction of animals using SCNT; in the present opinion clones are also referred to as F0.

- **Cloning**

Cloning, as assessed in this opinion, is defined as the technique of somatic cell nucleus transfer (SCNT). Cloning is a process by which animals are reproduced asexually. In the cloning of animals with SCNT, the haploid genetic material of an unfertilized ovum (oocyte) is replaced by the diploid genetic material of a somatic cell derived from foetal or adult tissue. In contrast, genetic modification (which is not assessed in this opinion) alters the characteristics of animals by directly changing the DNA sequence.

- **Progeny (offspring) of clone**

Clone progeny refers to offspring born by sexual reproduction, where at least one of the parents was a clone (F0); in the present opinion clone progeny are also referred to as F1.

2. Animal breeding and reproductive techniques

ARTs have contributed to genetic selection during past decades. These technologies include: artificial insemination from selected sires with its possible extension to sexed semen, oocyte collection from selected dams, embryo selection and transfer from selected genitors, *in vitro* fertilisation, and the long term storage of gametes and embryos.

The genetic diversity of animal species or breeds may, in principle, be managed through the selection of genitors, by generating intra- and inter-hybrids. The advantage of conventional genetic selection is that it creates new genotypes at each generation through the process of meiotic recombination (sexual reproduction) and the segregation of recombined chromosomes into individual gametes. In contrast to sexual reproduction, SCNT, by by-passing the sexual reproduction, is intended to reproduce a particular desired phenotype e.g., disease resistance ability, improved welfare, production or food product quality, with a higher likelihood than sexual reproduction. SCNT allows the replication of the genome of an animal with the intention of producing more animals with a desired trait over a period than might be possible through conventional or assisted breeding. However, as with any other reproductive technique, clones may also develop abnormally and/or possess undesirable traits.

[5] http://ec.europa.eu/european_group_ethics/publications/

2.1. Introduction to Somatic Cell Nucleus Transfer (SCNT)

In SCNT, the nucleus of a differentiated somatic cell (a non-germline cell) is transferred, by cell fusion or direct injection, into an oocyte that has had its nucleus removed. In practice, in livestock cloning the whole somatic cell (including the nucleus) is usually transferred. The reconstructed embryo is artificially activated to start its development before implantation into a surrogate dam where it continues to develop and is delivered, in successful cases, as a healthy newborn clone (F0) (see Figure 1).

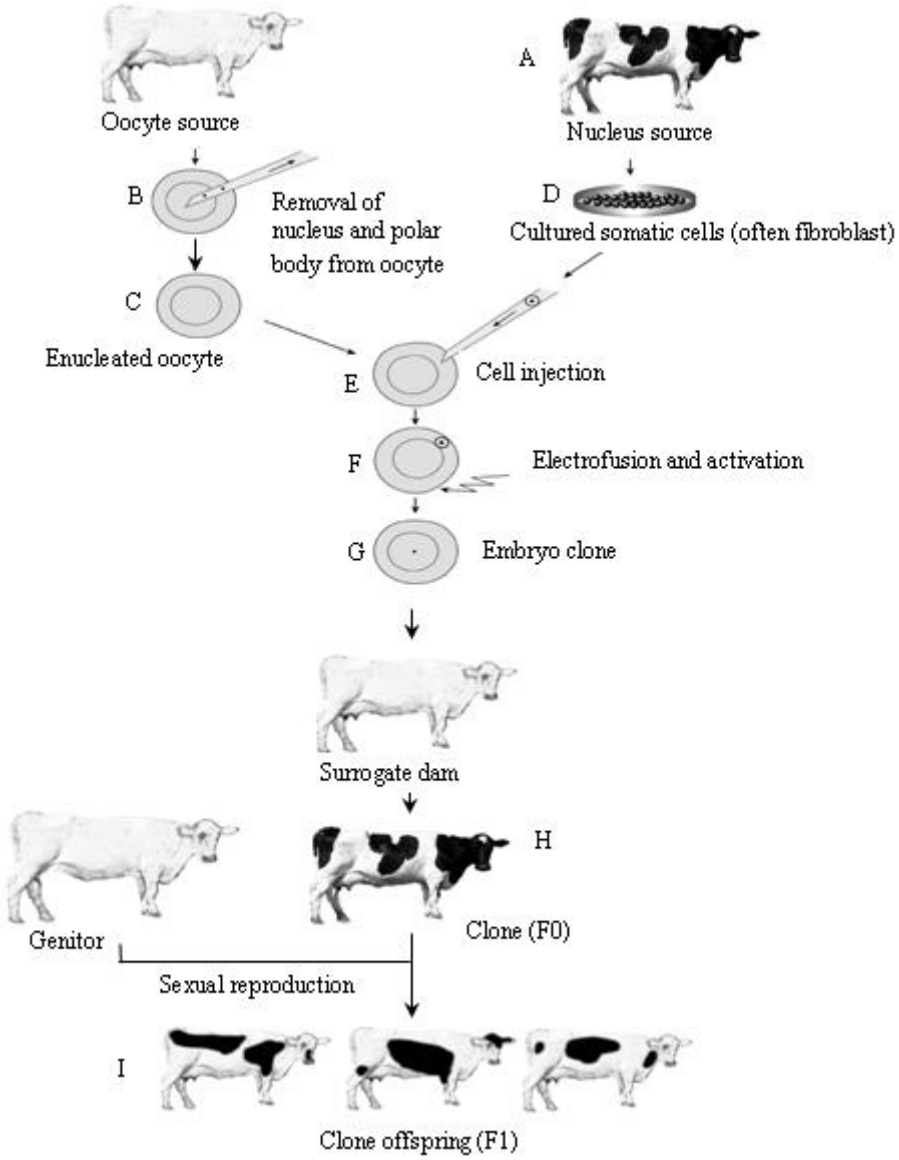

Figure 1. Main steps of somatic cell nucleus transfer (SCNT). (A) nucleus cell source; (B) the nucleus and the polar body are removed from oocyte by aspiration giving an enucleated oocyte (C); (D) culture of somatic cells from the nucleus donor; (E) injection of a somatic cell between the zona pellucida and the membrane of the enucleated oocyte; (F) intermediate association of enucleated oocyte and somatic cell followed by introduction of the somatic cell nucleus (and cytoplasm) into the oocyte cytoplasm by electrofusion of the oocyte and cell membranes; (G) embryo clone formed by an oocyte cytoplasm and a somatic cell nucleus containing two copies of chromosomes; (H) embryo transfer into a surrogate dam generating clone (F0) with coat colour similar to that of the nucleus source (A); (I) clone offspring (F1) generated by the sexual reproduction of the clone (F0) with a normal partner.

Biologically, most steps in the procedure present their own challenges. Examples include how to select and prepare the somatic cell to be used as the nucleus donor; how to prepare the oocyte used as the nucleus recipient; how to combine these two cells, i.e. the fusion process; and how to initiate embryo development after fusion. The opinion does not address the effect of these methodological challenges on the outcome of cloning.

Technical improvements over time are gradually increasing the proportion of clones born (e.g. better *in vitro* culture conditions) and technical innovations in the handling of embryos allow better control of nucleus transfer procedures (Sullivan *et al.*, 2004; Petersen *et al.*, 2007).

2.2. Cloned species and cloning efficiency

Since the birth of the sheep "Dolly" in 1996, SCNT has been applied to livestock and to several other species. Cattle, which are reported to be the animals most frequently used for SCNT, were first cloned in 1998 (Cibelli *et al.*, 1998; Yang *et al.*, 2005), goats in 1998 (Keefer *et al.*, 2002), pigs in 2000 (Onishi *et al.*, 2000), rabbits in 2001 (Chesne *et al., 2002*) and horses in 2003 (Galli *et al.*, 2003).

The overall success rate of the cloning procedure to date is low and differs greatly between species. The overall success rate is often measured as the percentage of embryos transferred and the number of live clones born. Information is often also given on the survival at certain times, such as 24 h, after the perinatal period, weaning or puberty.

Panarace *et al.* report the efficiency of cloning cattle in three countries, Brazil, Argentina and the USA, over five years (Panarace *et al.*, 2007). From the 3374 embryo clones transferred into surrogate dams, 317 (9 %) live calves were born, 24 hours after birth 278 of these clones (8 %) were alive and 225 (7 %) were alive at 150 days or more after birth. The higher overall success rates in cattle are largely attributed to the extensive knowledge of the female (and male) reproductive physiology in that species because of the importance of reproductive management in breeding schemes and in the economy of milk production.

Walker *et al.* described a method for porcine cloning where the overall cloning efficiency was improved from less than 1 % to 5 % and a later study reported an efficiency of up to 17 % (10 live births out of 58 embryos transferred) (Walker *et al.*, 2002; Du *et al.*, 2007).

However, within a given species, success rates can vary extensively, reflecting a lack of full understanding of the role of various factors involved in the cloning process, such as somatic cell and oocyte selection, cell cycle stage, culture conditions, etc. For unknown reasons, about one third of the donor cell lines lead to a success rate, expressed as the percentage of live calves obtained from initiated pregnancy, as high as 40 %, while one quarter of donor cell lines totally failed (Panarace *et al.*, 2007). These differences in the birth rate of live calves occur even when donor cell line cultures, with no evidence of abnormal chromosomal constitution, are run simultaneously within the same experimental programme. Unexpectedly, the different cell lines gave the same high number of blastocysts *in vitro* after nucleus transfer, irrespective of the subsequent success rate of development. This variable efficiency could not be attributed to chromosomal abnormalities in the cell lines resulting in the failure to develop to term (Renard *et al.*, 2007).

2.3. Data on clones and their life span

There is no world-wide register of clones; similarly no register is available in individual countries and therefore the number of living clones is difficult to estimate. From information gathered by EFSA it is estimated that in 2007 in the EU there were about 100 cattle clones and fewer pig clones alive. The estimated number in the USA is about 570 cattle and 10 pig clones.

There are also clones produced elsewhere e.g. Argentina, Australia, China, Japan and New Zealand. EFSA estimates that the total number of clones alive world-wide in 2007 was less than 4000 cattle and 500 pigs.

The relatively small number is considered to be a reflection of both the difficulties of the SCNT techniques and the various voluntary or mandatory moratoria around the world regarding clones and their progeny. The current number of clone progeny (i.e., F1 and subsequent generations) is also limited, largely for commercial reasons. Despite this, however, gametes (primarily semen) from clones have been traded for a number of years.

Although farmed animals may live for decades their production life is relatively short (e.g. beef cattle approximately 1-3 years, dairy cattle approximately 5-7 years, fattening pigs less than 6 months). Consequently, it will be difficult to generate data on the effect of cloning on the natural lifespan on farmed animals. The number of clones reported as reared and living for a considerable time is limited. Only a few reports on cattle clones to date refer to animals of 6-7 years of age (Chavatte-Palmer *et al.*, 2004; Heyman *et al.*, 2004; Panarace *et al.*, 2007) and no data on the full natural life span of livestock clones are yet available.

Several studies indicate that mouse clones may have a lifespan as long as their conventionally bred counterparts, whereas other studies reported a shorter life span (Ogonuki *et al.*, 2002; Tamashiro *et al.*, 2003; Wakayama *et al.*, 2000).

2.4. Possible use of cloning

With SCNT there is the opportunity to clone those animals that have already shown good productivity, a low incidence of disease and ability to cope with the production environment. As a result there may be an even greater chance that clones will propagate 'good' phenotypes as animals can be selected according to their own individual performance criteria. Traditional breeding methods, based on phenotype and population, are more recently complemented with genetic markers that may result in improved, poorer or the same quality of life for the animal and, where often, individual performance testing has not been carried out. However, as with any breeding strategy it is important to carry out longer term risk assessments on critical aspects and at critical ages to address potential genetic risks associated with changes occurring due to the process of genetic reprogramming.

SCNT is starting to be used commercially for purposes of breeding food production animals in a few countries outside the EU. Within the EU, SCNT is used for research purposes as a reproductive technique. The application of SCNT in research allows for the generation of an understanding of fundamental biological mechanisms, with potential benefit in other areas such as medicine.

The Scientific Committee noted that the primary use of clones (F0) commercially is to produce elite animals to be used in breeding, and not to produce animals as a food source.

3. Epigenetic and genetic aspects of SCNT

Successful SCNT requires that the nuclear activities of the differentiated somatic cell used in cloning are reset to those of an undifferentiated embryonic cell and that the new embryo is able to complete foetal development. The somatic cell nucleus has to change its gene expression pattern in relation to changes in its microenvironment in order to be able to replicate all steps of normal development. This process, which is by essence epigenetic, leaves the primary DNA sequence unchanged and is reversible. Epigenetic modifications include biochemically-mediated conformational changes of the proteins surrounding the DNA (i.e. chromatin) and also biochemical modifications of the DNA, particularly methylation. Modification of

chromatin proteins is a reversible and dynamic process. In contrast DNA methylation can be much more stable. Somatic cell reprogramming consists to a large extent of DNA demethylation followed by a specific re-methylation of those DNA regions which must remain silent in a given cell type. Epigenetic mechanisms affect the expression of some genes and such modifications may be transmitted to daughter cells (Jablonka and Lamb, 2002).

The low success rates of SCNT and the underlying physiological abnormalities, frequently observed in clones during embryonic and foetal development and also soon after their birth, appear to be caused mainly by epigenetic dysregulation occurring during inappropriate reprogramming of the genome.

Some considerations about the possibility that SCNT induces genetic alterations are given in 3.2, whereas the epigenetic aspects are discussed in Section 3.1.

3.1. Epigenetic aspects: Reprogramming in clones

Reprogramming of nuclear activities after SCNT is a time-dependent process which involves two main steps: the de-differentiation of the somatic cell nucleus to a totipotent embryonic state, followed by the re-differentiation of embryonic cells to different cell types during later development (Yang *et al.*, 2007a). Only a relatively small proportion of the total genome is active in a somatic cell at any one time. Many of these genes are known as housekeeping genes and are expressed in all cell types; others correspond to the genes that grant specific functions to each cell type. In a somatic cell, therefore, most of the genes available for transcription are actually silent. The reactivation of these genes occurs normally in part during gametogenesis, with the cytoplasm of the oocytes containing the factors allowing reactivation. When genes required for a developmental step are not properly activated, the development of the embryo or foetus is interrupted, usually with fatal consequences. It is this phenomenon that is consistent with the considerable loss of embryo clones during early development and shortly after birth.

The de-differentiation of the somatic nucleus requires changes of the DNA and the 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 and 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; Kang *et al.*, 2001b). A high degree of variability in the epigenetic changes is also observed among individual embryo clones with regard to methylation levels and mRNA expression patterns of genes (Dean *et al.*, 2001; Beaujean *et al.*, 2004; Wrenzycki *et al.*, 2005). Some genes aberrantly expressed in the blastocyst stage are also found aberrantly expressed in the organs of clones that had died shortly after birth (Li *et al.*, 2005). Methylation errors evidenced early in the preimplantation period of embryonic development can persist in bovine clone foetuses (Hiendleder *et al.*, 2004). The extent to which these aberrant methylation patterns are linked to the methylation status of the somatic cell nucleus before its transfer into the oocyte cytoplasm remains largely undetermined. However, several studies in cattle reveal that significant and relatively normal nuclear reprogramming, in terms of gene expression, can occur by the blastocyst stage following SCNT (Yang *et al.*, 2007a).

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* fertilised embryos, both for their transcriptional activities and their methylation profile (Brambrink *et al.*, 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 other hand, the DNA of trophectoderm cells, that are the precursors of the placenta, is excessively methylated (Yang *et al.*, 2007a). 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.

Not all epigenetic alterations observed in early SCNT embryos result in abnormalities. For example, studies of the inactivation of one of the two X chromosomes in female embryos show that the pattern of inactivation in mouse blastocyst clones is apparently normal (Eggan *et al.*, 2000), but that the expression of X-linked genes in the placenta can be deregulated, particularly in mid-to-late gestation (Senda *et al.*, 2004).

In cattle, the expression of X-chromosome related genes has been found to be delayed at early preimplantation stages in embryos of clones compared with *in vivo* derived embryos (Wrenzycki *et al.*, 2002). Hypomethylation of the genes involved in the X-chromosome inactivation process has been observed in various organs of stillborn calves. However, as no disturbance of sex development has been reported in clones, the implications for healthy clones of the hypomethylation of the X-chromosome observed in dead clones are unclear. 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 the inactivation of a pathway may be compatible with a normal life of the clones.

Re-differentiation of the cloned embryo into different somatic cell lineages is initiated after the blastocyst stage when the extra-embryonic lineages, which will contribute to the foetal part of the placenta, differentiate from those embryonic lineages where the patterning events leading to the definition of the first developmental axis become established. In different domestic species including sheep and cattle, several histological and molecular abnormalities thought to be major causes of foetal death have also been identified in the placenta of SCNT embryos (Hill *et al.*, 2000; Heyman *et al.*, 2002; Wilmut *et al.*, 2002; Lee *et al.*, 2004).

A class of genes known as imprinted genes has apparently an important role in the high foetal mortality observed after the transfer of embryo clones into surrogate cattle dams. Imprinted genes are expressed from only one of the two alleles of a gene in a parent-of-origin dependent manner. Many of them are imprinted specifically in the placenta (Coan *et al.*, 2005). In mouse clones an abnormally low expression of several imprinted genes is frequently detected in the placenta but not in foetal tissues (Inoue *et al.*, 2002).

A number of reports have analysed the methylation status of imprinted genes in various tissues of aborted foetal cattle clones (Liu *et al.*, 2007; Long and Cai, 2007; Lucifero *et al.*, 2007). The results suggest a direct link between aberrant methylation profiles and the compromised development after SCNT. A similar conclusion can be drawn from a genome-wide methylation analysis of repeated DNA sequences containing CpG islands (Kremenskoy *et al.*, 2006).

Also, in cattle clones abnormal allelic expression patterns of the imprinted *IGF2R* (Insulin Growth Factor II Receptor) gene have been observed in the placenta but not in calves (Yang *et al.*, 2005). The extent to which abnormal methylation patterns, induced by SCNT and observed in a specific tissue during foetal development, will persist in adult healthy clones remains to be determined. These changes in DNA methylation patterns, which have also been observed in *in vitro* fertilisation and embryo culture (without cloning) and in a protocol- and tissue-specific manner, result in a foetal overgrowth correlated with endocrine changes (Hiendleder *et al.*, 2006).

Several epigenetic changes such as DNA methylation have been observed among different mouse new born clones that look normal in their appearance (Ohgane *et al.*, 2001). A more extensive study concluded that each mouse clone has a different DNA methylation pattern (Shiota and Yanagimachi, 2002). The degree of these variations differed among individual new born clones analysed with an average of two to five aberrantly methylated loci per 1,000 loci in

each tissue. However the few cloned mice that developed to full term seemed to have almost perfectly re-established the genomic DNA methylation patterns necessary for their development (Shiota and Yanagimachi, 2002) The mouse data indicate that animals are obviously not perfect copies of the original animals as far as the methylation status of their genomic DNA is concerned. The extent to which individuals from a set of adult clones remains epigenetically different is however not known. There are some suggestion that abnormalities could disappear with the advancement of animals' aging (Senda *et al.*, 2007).

Although global analysis of the methylated status of clones is lacking in domestic species, one study in swine clones included evaluation of methylation in two different regions of the genome (Archer *et al.*, 2003a). Compared with 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 in swine. 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.

3.1.1. Transgenerational epigenetic inheritance

Limited data are available on whether epigenetic dysregulations occurring during the reprogramming of nuclear activities in clones can be transmitted to their sexually reproduced offspring. 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 and Whitelaw, 2006).

Recent data indicated that 19 female and 11 male offspring generated by the same bull clone lost all the abnormalities observed at birth and postnatally in the bull clone (Ortegon *et al.*, 2007). In cattle a single offspring from mating a cow clone and a bull clone has been produced (Kasai *et al.*, 2007). Various examinations of this offspring at eight months age including growth characteristics, and clinical, serological, haematological, biochemical and telomere length analyses, indicated no abnormalities.

Nine female pig clones mated with a conventional boar produced 14 F1 piglets in which the parameters that were statistically significant between the clones (F0) and the controls were within the normal range in the F1 with exception of two parameters (Mir 2005). The two parameters (Blood Urinary Nitrogen and Alkaline Phosphatase) were not consistently different over time (attributed to an outlier animal).

Similar results have also been seen in mice, where the obesity character of the F0 was not transmitted to offspring produced by natural mating between clones (Tamashiro *et al.*, 2000; Tamashiro *et al.*, 2002).

Overall, these results indicate that aberrant traits exhibited by clones are not necessarily transmitted to their offspring produced by natural mating.

Transgenerational epigenetic inheritance in response to various conditions has been documented in many eukaryotes and may play an important role in mammals. In particular, 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. These phenomena, which are considered as mechanisms of adaptation, have been found to be reversible after three generations in rats (Gluckman *et al.*, 2007a; Gluckman *et al.*, 2007b). Epigenetic inheritance has also been shown to occur occasionally in mouse embryos under *in vitro* experimental conditions (Roemer *et al.*, 1997). Different mouse models are now available to investigate how

epigenetic marks, such as DNA methylation, existing in specific non-imprinted alleles are transmitted as epi-alleles through the paternal and/or maternal germ cell line (Wolff *et al.*, 1998; Cooney *et al.*, 2002). There is now evidence suggesting that RNA can be a determinant of inherited phenotype. In the mouse *Agouti* phenotype, the white tail tip trait is not transmitted in a Mendelian fashion but by RNAs packaged in sperm and down regulating *Kit* gene expression by an RNA interfering mechanism (Rassoulzadegan *et al.*, 2006). No similar studies or outcomes have been identified in the livestock species that are the subject of this scientific opinion. The relevance of these observations to clones and their progeny is not entirely clear. It is also expected that the epigenetic modifications of clones will disappear in future generations as it is the case for those that are naturally induced. Therefore epigenetic effects of adult onset disease and the transgenerational epigenetic inheritance are not specific to cloning and would follow the same mechanisms in conventional animals. Recent data suggest complex epigenetic transgenerational effects on the phenotype of mammals and raise new scientific questions on genotypes bearing epigenetic differences (Han *et al.*, 2008).

3.1.2. Epigenetic telomere modifications

One epigenetic mechanism that has been linked to the ability of donor somatic nuclei to drive the development of SCNT embryos is the length of telomeres of clones. Telomeres are short, highly repetitive DNA sequences located at the ends of chromosomes that prevent those ends from inappropriate fusions and heal them when they are degraded. Telomeres shorten at each round of cell division due to problems associated with DNA replication. Thereby, telomeres have a function in the control of the ageing process. 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 telomere 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). For this reason, clones were first considered to show premature ageing. Subsequently however, the vast majority of studies have reported that telomere length in cattle, pig and goat clones are comparable with or even longer than age-matched naturally bred controls, even when senescent donor cells were used for cloning (Lanza *et al.*, 2000; Tian *et al.*, 2000; Jiang *et al.*, 2004; Betts *et al.*, 2005; Jeon *et al.*, 2005; Schaetzlein and Rudolph, 2005; Kasai *et al.*, 2007). Current data indicate that telomere length restoration is normal in clones derived from fibroblast donor cells (which are the cells predominantly used). The telomere lengths of 30 offspring from the same bull clone were not different from age-matched controls (Ortegon *et al.*, 2007).

3.1.3. Epigenetic dysregulation in perspective

Epigenetic dysregulation is not a phenomenon unique to cloning and has been observed in all other forms of reproduction, but particularly in ARTs 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 with *in vivo* produced embryos (Camargo *et al.*, 2005), as well as in other species (Gardner and Lane, 2005; Wrenzycki *et al.*, 2005). It is not known whether these abnormalities are due to the stresses of SCNT *per se*, or are the result of the *in vitro* environment, that the early embryos are exposed to, prior to transfer to the surrogate dam. Furthermore, as mentioned earlier, it is to be noted that the epigenetic status of any embryo is in part a response to its environment, as is the epigenetic status of any life stage of any organism.

3.2. Genetic aspects

Chromosomal disorders after SCNT are routinely observed at a high frequency during the preimplantation stages but mainly in morphologically abnormal embryos (Booth *et al.*, 2003). In a study of 20 cattle clones two showed high incidences (about 21 %) of abnormal cells with non-diploid chromosomes number. These abnormalities were not transient and indicates that instability in chromosome number can occur in phenotypically normal clones (Hanada *et al.*, 2005). The telomere length and chromosome stability of 30 offspring from the same bull clone showed no abnormalities compared with controls (Ortegon *et al.*, 2007).

Chromosome stability may differ in the mouse between embryonic cells derived *in vitro* from cloned or fertilised embryos but this is probably because of epigenetic rather than genetic causes (Balbach *et al.*, 2007).

3.2.1. Mitochondrial DNA modifications

Genetic and phenotypic differences between clones might derive from mitochondrial DNA. Mitochondria serve mainly as a source of energy for the cell but have other important roles in cellular physiology, notably in steroid synthesis and in programmed cell death, both of which are required for embryonic development. In sexual reproduction, male mitochondria are recognized as foreign and are eliminated in the oocyte cytoplasm in a species-specific manner. Thus the mitochondria show a strict maternal inheritance. After SCNT, embryos can possess mitochondrial DNA from the oocyte cytoplasm only (homoplasmy) or from both the donor cell and the recipient cytoplasm (heteroplasmy) (Steinborn *et al.*, 2000). Adult somatic cells typically contain from a few hundred to several thousand mitochondria. This number is even lower during the specification of the germ line but increases dramatically during oocyte growth and may become as high as 100,000 in the mouse oocyte at the time of fertilisation (Shoubridge and Wai, 2007). It is perhaps not surprising that the vast majority of clones analysed so far have shown little evidence of heteroplasmy but the number of studies is small (Hiendleder *et al.*, 2005). It has been speculated that changes in mitochondrial copy number and function, or the transmission of mitochondrial dysfunction from the recipient oocyte, could be risk factors for adult metabolic diseases with a developmental origin (McConnell, 2006).

3.2.2. Silent mutations

The extent to which SCNT induces silent mutations in the nuclear DNA of clones that could be transmitted to later generations (through sexual reproduction) remains largely undetermined. Such mutations occur spontaneously although at a low frequency in animals born from sexual reproduction and the same is probably true after nuclear transfer. These mutations can lead to aberrant phenotypes at the next generation, depending on the allelic combination of individual offspring, and can be screened for and eliminated in conventional breeding programs.

There are examples in normal breeding showing that mutations occurring spontaneously in the DNA can interfere with the expression but not with the epigenetic status of imprinted genes, resulting in a modification of their contribution to the phenotype of offspring. This is the case in the sheep with the “callipyge phenotype”, an inherited muscular hypertrophy that affects only heterozygous individuals receiving a mutation from their male parent (Charlier *et al.*, 2001). A related situation has also been observed in the pig (Van Laere *et al.*, 2003). There is now evidence to suggest that RNA, and not only DNA can be a determinant of inherited phenotype (Rassoulzadegan *et al.*, 2007).

Since nuclear reprogramming requires a marked reorganisation of the somatic cell nucleus chromatin, SCNT could increase the occurrence of silent mutations in the donor genome which

could further affect the outcome of the breeding schemes used today for genetic selection in livestock.

3.3. Other aspects

The cloning process includes several modifications of the oocyte cytoplasm. Part of the oocyte cytoplasm is removed during the nucleus aspiration and the remaining cytoplasm may become disorganized. This may result in a lack of fully functional cytoplasm required for embryo development. Some protocols, aiming at restoring oocyte cytoplasm, involve the addition of exogenous oocyte cytoplasm or the fusion of several enucleated oocytes. Cytoplasmic modification may also result from the fusion of the enucleated oocyte with the donor cell. This introduces donor cell cytoplasm, including functional mitochondria, into the oocyte. These cytoplasmal disturbances may result in the malfunctioning of the cytoplasm and its organelles which could have an impact on the development of the embryo clone. The implication of cytoplasmic modification has not yet been determined (Tamashiro *et al.*, 2007).

3.4. Conclusions of epigenetic and genetic aspects of SCNT

- Epigenetic dysregulation is the main source of adverse effects that may affect clones and result in developmental abnormalities.
- Epigenetic reprogramming takes place successfully in clinically healthy clones.
- The DNA sequence of a clone is a copy of that of the donor animal, but some differences may exist (e.g. the methylation status of genomic DNA).
- Currently, based on the available limited data, there is no evidence that epigenetic dysregulation induced by SCNT is transmitted to the cattle and pig progeny (F1).
- The extents to which epigenetic and genetic aspects of SCNT are affected are not fully elucidated.

4. Animal health and welfare implications of SCNT

Animal health includes physical and mental fitness, freedom from infectious and non-infectious diseases and the ability to carry out essential life-maintaining tasks. Animal welfare includes the absence of pain, distress and suffering. The evidence for poor health and welfare, or improved health and welfare, is reviewed in the context of the various phases in the life of an animal with reference to clones and to data derived by comparing clones with animals that are not clones.

As the literature on cloning is based on reports of work often carried out in highly monitored populations and environments, the effects observed and recorded may not reflect the conditions of husbandry that exist in everyday production systems. Clones are derived from animals with characteristics deemed valuable, often consisting of production traits that may place them outside of the normal distribution of a population for that particular trait. Therefore, care must be exercised in making comparisons between clone and normal population parameters as well as with animals produced with ARTs.

There are a number of published reports relating to health and welfare of clones where the results are based on observations on mixed populations of clones and transgenic clones. Such studies are of limited relevance to this opinion if there is no direct indication on the possible impact of the transgenic effect.

4.1. Animal health

Animal health is considered in relation to the animals originating the somatic cells and oocytes used in cloning, the surrogate dams, the clones themselves and their progeny.

4.1.1. Health of source animals for somatic cells and oocytes

Cells used as nucleus donors in the SCNT process are usually obtained either from existing cell cultures or from minimally invasive procedures such as ear punches of live animals with desirable phenotypes. The oocyte donor could be any animal of the same species whose oocytes are available after slaughter. In rare circumstances it could be a highly valued and/or monitored animal whose oocytes are collected by ovum pick up *in vivo*. In such cases, the animal health and welfare issues have been extensively addressed. There are reports indicating that the recovery of oocytes from live donor animals using an echography-guided approach is not detrimental to animal welfare provided the operator is licensed (Chastant-Maillard *et al.*, 2003; Petyim *et al.*, 2007). In the remainder of this section, the role of the health of the source animals and the implications of their health for the health of subsequent clones are discussed.

The disease status of the source animals can have an impact on the infection risk for the clone. Some disease causing agents, such as intracellular mycoplasma and viral nucleotide sequences integrated in the genome, can be directly associated with the somatic cell nucleus and oocyte cells (Philpott, 1993). The genomes of all vertebrate species investigated contain endogenous retroviruses. Possible reactivation of bovine endogenous retroviruses (BERV) during SCNT was analysed and compared between sexually reproduced cattle and cattle clones (Heyman *et al.*, 2007a). BERV sequences were not transcribed and no RNA was detected in the blood of clones, donor animals or controls.

At present, voluntary guidelines published by organisations involved with embryo transfer are aimed at reducing the risk of infection in relation to trade. The OIE (World Organisation for Animal Health, www.oie.int) has developed guidelines for embryo transfer in close cooperation with IETS (International Embryo Transfer Society, www.iets.org). Detailed protocols for the biosecure management of source animals and surrogate dam have been developed for animals involved in embryo transfer procedures (*in vivo* derived gametes and embryos). However, not all protocols applied to embryos produced *in vivo* are applicable to *in vitro* derived embryos, or cloned or transgenic embryos (Stringfellow *et al.*, 2004).

4.1.1.1. The somatic cell nucleus source

The source of the somatic cell nucleus is often an animal with the desirable trait that the cloning procedure is designed to propagate, and as such would be subject to health monitoring and surveillance during its lifetime. Selection of the disease susceptibility or resistance of the source animal and its source tissue is important as the clone may be affected by such disease traits.

With SCNT there is the possibility of bringing intracytoplasmic pathogens within the somatic cell into the recipient oocyte. However, this hazard also exists if and when pathogens adhere to sperm or to instruments used during *in vitro* fertilization and intracytoplasmic sperm injection (ICSI). This risk is reduced by sanitary management of source animals (OIE, 2007).

4.1.1.2. The oocyte source

Health risks related to the procedures for oocyte recovery from live animals or from abattoir material and their handling *in vitro* are of equal importance to those encountered in the *in vitro* collection of embryos for transfer. The collection of oocytes from animals at slaughter (as

opposed to surgical interventions) increases the risk of contamination with bacteria and viruses which may be retained by the clones and may affect their viability *in utero* or after birth. These risks have already been carefully identified (Bielanski, 1997) and procedures for their prevention have been proposed by the IETS as licensing guidelines and have been adopted by the OIE. While there are steps in the SCNT technique which differ from the *in vitro* fertilisation procedure, no specific health risks related to oocyte enucleation, the fusion of oocyte with a somatic cell nucleus or the injection of the somatic cell nucleus directly into the cytoplasm of the enucleated oocyte have been reported.

It is not known to what extent the disease resistance of the oocyte source animal will affect the clone, as it does not contribute to the genetics of the clone in the same way as the somatic cell nucleus. The source animal of the enucleated oocyte may, however, contribute through mitochondria-associated inheritance stemming from the oocyte cytoplasm.

4.1.2. Health of surrogate dams

Initial pregnancy rates (at day 50 of gestation after transfer) in cattle serving as surrogate dams were found to be similar between those carrying clones (65 %) and those produced through the use of other artificial methods such as embryo transfer (58 %) and artificial insemination (67 %) (Heyman *et al.*, 2002; Lee *et al.*, 2004). However, there is a continued pregnancy loss throughout the entire gestation period in those surrogate dams carrying clones which is not observed in other ARTs, and embryo survival is only one-third of that following *in vitro* embryo production (Lee *et al.*, 2004; Wells, 2005).

Losses of pregnancy in cattle surrogate dams in the second and third trimester are associated with placental abnormalities, hydrops, enlarged umbilical cords with dilated vessels, and abnormally enlarged and fewer placental cotyledons (Wells *et al.*, 1999; Hill *et al.*, 2000; Chavatte-Palmer *et al.*, 2002; Batchelder *et al.*, 2005).

The high rate of pregnancy failure in the surrogate dam has been linked to the finding of abnormal and/or poorly developed placental formation. Such placental defects have been associated with early embryonic loss, abortions, stillbirths, dystocia and pre- and post-natal deaths (Wakayama and Yanagimachi, 1999; Hill *et al.*, 2001; Tanaka *et al.*, 2001; De Sousa *et al.*, 2002; Hashizume *et al.*, 2002; Humpherys *et al.*, 2002; Suemizu *et al.*, 2003). A detailed histological study of the placenta found that pregnancies of seven cattle clones were associated with abnormalities (Batchelder *et al.*, 2005). Abnormal placental development expressed as a reduction in placentome number and consequences on maternal, foetal exchange is seen as one of the main limiting factors in ruminant SCNT pregnancies (Arnold *et al.*, 2006). This abnormal placental development is present from the early stages after implantation but does not necessarily prevent the development and birth of live clones (Hill *et al.*, 2000; Hoffert *et al.*, 2005; Chavatte-Palmer *et al.*, 2006). An early detection of placental abnormalities offers the possibility to terminate pregnancy without threatening the health of the surrogate dam (Hill and Chavatte-Palmer, 2002).

The incidence of birth by Caesarean section is higher in surrogate dams carrying cattle or pig clone foetuses although there is some difficulty in determining causation since elective Caesarean sections were also often carried out.

After normal breeding, the fertility of cows requiring an elective Caesarean section to assist the delivery of their calf is not altered, whereas fertility is significantly reduced if the Caesarean section is performed because of severe dystocia (Tenhagen *et al.*, 2007), principally due to infection resulting in endometriosis (Gschwind *et al.*, 2003). The future fertility of the surrogate dams is not recorded in the literature on cloning.

4.1.3. Health of clones (F0)

Four different outcomes can be identified concerning the health of clones: (i) clones which present serious abnormalities and where the pregnancy needs to be terminated; (ii) clones which present disorders and die during the postnatal period; (iii) clones which present reversible disorders but which survive after birth; and (iv) clones with no detectable defects.

The most critical time for the health and development of cattle clones occurs during the peri-natal period (Chavatte-Palmer *et al.*, 2004; Wells *et al.*, 2004; Panarace *et al.*, 2007). This can be explained by the fact that most of the observed pathologies are associated with, and are secondary to, placental dysfunctions (Constant *et al.*, 2006).

Further data are required to evaluate whether SCNT has an impact on immune functions and susceptibility of clones to infectious agents. Moreover, it should be noted that, although not specifically related to SCNT, depending on the infectious status of the surrogate dam, transplacental infection from the dam to the clone may occur with some specific viruses (e.g. pestiviruses, herpesviruses). This is not specifically related to SCNT and would also be encountered with those ARTs in which an embryo is introduced into a surrogate dam.

4.1.3.1. Immune function of clones

A limited number of studies have investigated the immune function of clones. A study of 17 cattle clones, aged 2 to 5 years, reflecting the immune function have been reported (Heyman *et al.*, 2007a and b). Lymphocyte populations were represented as normal in apparently healthy clones of all age classes compared to controls. After immune challenge there was no difference between clones and controls in the antibody response but the antigen-specific induced cell proliferation was weaker in clones. This finding may indicate that the bovine clones had a reduced capacity to mount a cellular immune response against a newly encountered antigen. However, when a similar study was performed later on the same animals and also in another set of clones, the immune function appeared normal, suggesting that there was an effect of age.

A study on nine cloned piglets demonstrated that following lipopolysaccharide challenge at 30 days of age, the acute phase response (cortisol, TNF-α, IL-6) was lower in some clones, or the same in other clones compared to controls (Carroll *et al.*, 2005).

In the early lactation stage the proportions of gammadelta and WC1+gammadelta T cells temporarily declined in cow clones, suggesting that cloned cows may fall into an immunosuppressive state in the early lactation stage (Tanaka *et al.*, 2006; Heyman *et al.*, 2007b).

4.1.3.2. Health of clones during gestation and the perinatal period

Large offspring syndrome (LOS), often thought to be a cloning-related phenomenon, was first described in pregnancies derived from the transfer of *in vitro* fertilized embryos in cattle and sheep (Farin and Farin, 1995; Walker, 1996; Kruip and den Daas, 1997; Sinclair *et al.*, 1999). LOS has been observed in clones from cattle and sheep together with changes observed in late gestation that give rise to an increase in perinatal deaths, excess foetal size, abnormal placental development (including an increased incidence of hydrops), enlarged internal organs, increased susceptibility to disease, sudden death, reluctance to suckle and difficulty in breathing and standing (Kato *et al.*, 1998; Galli *et al.*, 1999; Wells *et al.*, 1999; Young and Fairburn, 2000). In another study the incidence of LOS at birth was 13.3 % for somatic cloning based on 15 clones, compared with 8.6 % for 23 embryonic clones, and 9.5 % for a group of 25 IVF calves (Heyman *et al.*, 2002). For somatic cloning the incidence of LOS could be related to the tissue

origin of the somatic cells used and an LOS rate of up to 47 % (9 of 19) has been observed when calf clones were derived from skin, ear or liver cells (Kato *et al.*, 2000).

Placental overgrowth has been shown to induce an increase in the fructose provided to the calf foetus during the neonatal period, resulting in hypoglycaemia and hyperfructosaemia affecting muscle functions including cardiac muscle (Batchelder *et al.*, 2007b). These data may explain why calf clones experience greater difficulty adjusting to life *ex utero*.

Foetuses, placentas and calves resulting from both *in vitro* production and SCNT can differ significantly in morphology, physiology and developmental competence compared with embryos produced *in vivo* (Farin *et al.*, 2006). Mechanisms proposed to explain how *in vitro* conditions may influence subsequent embryo development focus on the modification of epigenetic patterns associated with the DNA, which can affect gene expression without altering the primary DNA sequence.

There are similar findings in sheep, where peri- and post-natal lamb losses were considered to be due to placental abnormalities (Loi *et al.*, 2006). Initially the implanted blastocyst was comparable with that of *in vitro* derived fertilised (IVF) embryos but losses after that time were marked, with only 12 out of 93 clones reaching full-term development, compared with 51 out of 123 lambs born from the IVF control embryos.

A study of eight calf clones delivered by Caesarean section, reported that in the first 48 hours of life the red and white cell counts were reduced in comparison with control calves and their plasma electrolytes were more variable, suggesting that calf clones take longer to reach normal calf levels than the controls (Batchelder *et al.*, 2007a). Calf clones were also reported to have higher total bilirubin levels and fibrinogen levels than normal calves (Batchelder *et al.*, 2007b). However the increases in the level of bilirubin and fibrinogen were not necessarily abnormal since these increases remained within the normal physiological range.

In contrast to the LOS syndrome observed in cattle and sheep clones, some pigs produced by SCNT have an increased incidence of intrauterine growth retardation (piglets weighting less than 1.04 kg). A comparison of 23 SCNT litters (143 individuals of which 41 were transgenic) with 112 artificial insemination (AI) litters (1300 individuals) showed a significant increase (1.8 ± 0.3 for SCNT versus 0.7 ± 0.1 for AI) in the number of intrauterine growth retardations per litter (Estrada *et al.*, 2007). In this study no differences were observed in the parameters studied between the clones and transgenic clones.

A study has reported low birth weights in SCNT piglets, where 27 out of 40 died within the perinatal period from a variety of health problems including diarrhoea, meningitis and heart functional abnormalities. Twelve of the clones survived to adulthood. However, in this study it was not possible to rule out the presence of coexisting infections (Park *et al.*, 2005). A follow-up study by the same group found morphological abnormalities in the placentas of the nonviable clones which may have been caused by apoptosis of placenta cells (Lee *et al.*, 2007). The perinatal mortality rate reported above was not observed by other groups (Du *et al.*, 2007; Estrada *et al.*, 2007).

Gestational lengths of between 114 to 120 days have been reported for a limited number of pregnancies giving birth to pig clones (Walker *et al.*, 2002; Carroll *et al.*, 2005; Park *et al.*, 2005; Williams *et al.*, 2006; Du *et al.*, 2007). Generally, the average gestational length in pigs is about 115 days with a range from 110-120 days.

4.1.3.3. Health of clones after birth up to sexual maturation

One study in cattle reported that a mean of 30 % (21 of 59) of the calf clones died before reaching 6 months of age due to a wide range of pathological causes, including respiratory

failure, abnormal kidney development, and liver steatosis (fatty liver) (Chavatte-Palmer *et al.*, 2004). Heart and liver weights were increased relative to body weight. However after 1 to 2 months the surviving calf clones became indistinguishable from calves born from artificial insemination. Once past the first few months after birth most surviving calf clones develop normally to adulthood (Chavatte-Palmer *et al.*, 2004; Wells *et al.*, 2004; Heyman *et al.*, 2007a).

From 988 bovine embryo clones transferred into recipient cows, 133 calves were born and 89 (67 %) of those survived to weaning at 3 months of age (Wells *et al.*, 2003; Wells *et al.*, 2004). Similar findings were reported by Panarace *et al.,* who summarised 5 years of commercial experience of cloning cattle in 3 countries (Panarace *et al.*, 2007). On average, 42 % of cattle clones died between delivery and 150 days of life. The most common abnormalities were enlarged umbilical cords (37 %), respiratory problems (19 %), depressed or weak calves displayed by prolonged recumbency (20 %) and contracted flexor tendons (21 %).

Cattle clones at about 6 months of age showed no relevant differences from age-matched controls with regard to numerous biochemical blood and urine parameters, immune status, body condition score, growth measures and reproductive parameters. Similarly a large number of physiological parameters (blood profiles) showed no differences between clones and age-matched controls (Laible *et al.*, 2007; Panarace *et al.*, 2007; Walker *et al.*, 2007; Yamaguchi *et al.*, 2007; Heyman *et al.*, 2007a; Watanabe and Nagai, 2008).

EFSA was provided with a raw data set on porcine clones and their progeny by ViaGen Inc. USA, and publications based thereon (FDA, 2008; Williams *et al.*, 2006; Walker *et al.*, 2007). In this dataset, seven pig clones were delivered by Caesarean section whilst comparator controls (produced by AI) were delivered vaginally. The birth weights of the clones were smaller than the comparators (1.12 kg vs 1.73 kg).

A controlled study in a research environment indicates that litter weight and average birth weight, when adjusted for litter size, are significantly ($p<0.05$) higher in AI derived litters compared with SCNT derived litters (Estrada *et al.*, 2007). Additionally, there was a trend towards higher stillbirths and higher postnatal mortality in the SCNT population (Estrada *et al.*, 2007).

Studies on swine clones at 15 and 27 weeks of age showed that they were indistinguishable from their comparators in terms of growth, health, clinical chemistry and immune function (Archer *et al.*, 2003a; Mir *et al.*, 2005; Williams *et al.*, 2006).

4.1.3.4. Health of clones after sexual maturation

In a matched study of heifer clones and controls reared under the same conditions, the heifer clones reached puberty later than the controls. However, after sexual maturation there was no significant variation regarding gestation length and survival of the offspring (Heyman *et al.*, 2007b). Subsequent 305-day lactation curves of clones, as a health parameter, were also comparable for yield, fat and mean cell counts. The mean protein content in milk from the clones was significantly higher but this could be accounted for by the fact that three of the heifer clones were from the same source mother, which had a lower milk production but higher protein content, and by the sample size (12 clones and 12 controls). There were no effects on health and subsequent reproductive data showed no significant differences.

The same study found other significant differences between clones and control cattle although there were no outward signs of health effects. Variations have also been observed in haematological and biochemical parameters, muscle metabolism, fatty acid composition and higher oxidative activity in the muscle biopsies of the semitendinosus muscle at the 8 to 12 month stage (Tian *et al.*, 2005; Yonai *et al.*, 2005).

The growth rates of 11 Friesian heifer clones at 15 months of age was comparable with that seen in non-clones reared in New Zealand (Wells *et al.*, 2004). The same workers report that in 52 cattle clones there had been no sign of obesity. Reproductive ability in cattle clones showed no significant variation from that found within a population derived by normal sexual reproduction, and subsequent foetal maturation and development were normal (Enright *et al.*, 2002; Forsberg *et al.*, 2002; Wells *et al.*, 2004; Shiga *et al.*, 2005; Yonai *et al.*, 2005; Tecirlioglu and Trounson, 2007).

A study of clones derived from an aged infertile bull concluded that although their birth weights were heavier than those of calves produced using artificial insemination, their semen characteristics and fertility were normal (Shiga *et al.*, 2005).

Five gilt clones (of which four were transgenic) were mated to a clone boar and gestation length, litter size proportion of pigs born live and birth weights were comparable with those achieved from controls (Martin *et al.*, 2004). In this study no significant differences were observed in the parameters studied between the clones and the transgenic clones.

Data provided by ViaGen Inc. showed that porcine clones had lower IGF-I (Insulin like Growth Factor 1) than the comparator group after birth and before slaughter, although the levels, with the exception of one pig clone, were within the comparator range (ViaGen Inc. USA). Similarly, estradiol-17β levels were lower in the clones than in the controls, but within the comparator range. As these clones reached market weight within the normal time frame and were able to reproduce successfully, the relevance of the differences in these parameters for alterations in growth rate or reproductive function remains to be seen (Walker *et al.*, 2007).

4.1.3.5. Mortality of adult clones

As SCNT is a developing technology, the number of animals reported as reared and remaining alive for their natural productive lifespan remains limited. Thus the use of the word 'old' in reports often refers to animals only a few years past weaning or birth (Chavatte-Palmer *et al.*, 2004; Heyman *et al.*, 2004; Heyman *et al.*, 2007a). It is unlikely that animals reared for production purposes would ever reach their natural lifespan and, therefore, judgements as to the likelihood of a reduction of lifespan or other aging-related effects are difficult to assess at present.

A study reported that between weaning and 4 years of age the annual mortality rate in cattle clones is at least 8 % (7 out of 59 died in the age period 1-2 years; 3 out of 36 died within the age period 2-3 years and 1 out of 12 died in the age period 3-4 years) and that the main mortality factor is euthanasia due to musculoskeletal abnormalities (Wells *et al.*, 2004). In a study on 21 heifer clones of 4 different genotypes, all but one animal survived the study period of 4 months to 3 years of age (Heyman *et al.*, 2007a). The one animal that did not survive died just after calving during a warm period of 2003.

4.1.4. Health of progeny (F1)

In New Zealand it was found that out of 52 progeny of cattle clones delivered vaginally, 85 % survived after 24 hours and their survival was similar to the calves of control cows (84 %) (Wells *et al.*, 2004). Illness in the progeny of clones was also reported to be of no greater prevalence than in conventionally-bred animals. Similar results have been published from cumulated data on calvings from clones, showing that 21 offspring (F1) were naturally delivered and most calves (20 out of 21 animals) survived after birth (Heyman *et al.*, 2007a). Also a recent review of the data collected on a total of 32 offspring from clones produced in Japan supports these findings (Watanabe and Nagai, 2008). A report on the physiology and genetic status of 19 females and 11 males sired by a single bull clone showed that the offspring

from clones had normal chromosomal stability, growth, physical, haematological and reproductive parameters compared with normal animals at one year of age, although they displayed lower heart rates (P=0.009), respiratory rates (P=0.007) and body temperatures (P=0.03) in their early period of life. Furthermore, they showed moderate stress responses to routine handling (Ortegon *et al.*, 2007). In a study, an aged infertile bull was used as cell donor to produce two bull clones (F0) that exhibited normal fertility both *in vitro* and *in vivo*. Conventional cows were artificially inseminated with the semen from the clones and after normal gestation lengths produced ten F1 with normal birth weights and growth (Shiga *et al.*, 2005).

Semen from four boar clones were used to breed 49 conventional gilts and 293 offspring (F1) survived to weaning (Williams *et al.*, 2006). In a follow-up study, 242 of the pig offspring, raised under commercial conditions, were reported and showed no difference in health status or mortality rates compared to offspring (n=162) of control boars (n=3) (ViaGen Inc. USA; Walker *et al.*, 2007).

Six gilt clones were artificially inseminated with semen from a conventional boar and produced 44 (23 male and 21 female) offspring (Shibata *et al.*, 2006). The birth weight of the F1 were significantly lower compared to the controls and after day 30 the growth rates of the F1 were significantly higher compared to controls. However this difference disappeared at weaning. The mean litter size, the numbers of piglets born alive and surviving to weaning were all similar in the F1 compared to controls.

Nine gilt clones were bred to a conventional boar (Mir *et al.*, 2005). There were no differences in the means of body weight and average litter size between clones and controls, 7.8 ± 2.6 and 7.4 ± 3.0 respectively. At 15 and 27 weeks of age ten blood parameters of 14 and 8 offspring (F1) respectively were reported. Two out of the ten parameters (Blood Urinary Nitrogen and Alkaline Phosphatase) were significantly different between the F1 and controls but they were not consistently different at the two time points (attributed to an outlier animal).

4.1.5. Conclusions on animal health

From the available data, mainly concerning cattle, the conclusions below can be drawn.

The infection status of the somatic cells and oocytes source animals (specifically concerning the tissues from which the cells and the DNA are taken) and of the surrogate dam must be taken into consideration in the choice of the animals for cloning.

In relation to surrogate dams it is concluded that:

- Increased pregnancy failure is observed following the implantation of cloned embryos. Based on information from ARTs this may affect the future fertility of the surrogate dam.
- Increased frequencies of hydrops, dystocia and consequential Caesarean section are observed. These effects may affect the future fertility of the surrogate dam.
- The above-mentioned adverse health effects have all been observed in surrogate dams carrying pregnancies produced by ARTs not involving SCNT, albeit at much lower frequencies.

In relation to clones (F0) it is concluded that:

- Although the data are limited and variable, the mortality rate of clones is considerably higher than in sexually produced animals.

- Increased embryonic and foetal losses occur during pregnancy, mostly observed in cattle rather than in pigs.
- Increased mortality is observed in the perinatal period for pigs and bovine clones and during the juvenile period for bovine clones.
- A small number of studies report an increased mortality in adult clones.

- There is evidence of increased morbidity of clones compared with sexually produced animals.
  - A proportion of bovine clones show several altered physiological effects.
  - During gestation, mainly physiological adverse outcomes, including Large Offspring Syndrome (LOS), are observed in cattle clones at a higher frequency than with other ARTs.
  - In the data available, there is often no clear indication of the causes of morbidity and mortality.
  - The low number of animals and the few assays carried out do not allow precise measurement of the impact of cloning on the immune functions of the cloned animals. Such an impact, if present, could modify the carrier state of the cloned animals with respect to infectious agents of animal and human health concern.
  - The close similarity of adverse effects observed in animals reproduced either by cloning or by ARTs suggests a common genesis although conclusive evidence is still lacking.
  - High levels of husbandry care can enhance the survival and health of clones during early life.

- Bovine clones that survive the juvenile period, and pig clones that survive the perinatal period appear to be normal and healthy as determined by physiological measurements, demeanour and clinical examination.
  - No long-term effects have been observed on the reproductive ability of clones.
  - Most clones have not yet reached the end of their natural life span for their species; therefore it is difficult to draw any conclusions on possible effects of SCNT on their longevity. Further, the production life of animals is shorter than the natural life span.

In relation to progeny (F1) it is concluded that:

- From the data available there is no indication of any abnormal effects in those species examined.

4.2. Animal welfare aspects

Qualitative and preferably quantitative data are required to assess welfare indicators directly on the animals concerned. Since animal cloning is a relatively recent technology the availability of such data is very limited. It is therefore difficult to draw any direct conclusions on the welfare aspect of cloning. The current welfare assessment is largely based on the interpretation of data related to the physical health of the animals as presented in the previous section. The interpretation of affected physical health of animals as an indicator of their mental well-being is hampered by anthropomorphic extrapolations and is of a qualitative and more general nature only. However, the Scientific Committee considered that, in the absence of quantitative direct animal welfare indicators, this somewhat conservative interpretation of animal welfare indicators is the most appropriate approach.

In the context of cloning, the welfare of the source (nucleus donor) animal, the gestation animal (surrogate dam), the clone (F0), and the progeny of the clone (F1) should all be considered.

4.2.1. Welfare of the source animals

The cloning procedure itself does not normally affect the welfare of the animals which are the source of the somatic cell nucleus or oocyte. Ovum pick-up is not detrimental to animal welfare providing the operator is licensed (Chastant-Maillard *et al.* 2003; Petyim *et al.* 2007).

4.2.2. Welfare of the surrogate dam

Due to the effects of SCNT on the placenta and foetal membranes, as well as the large foetuses carried by some of the surrogate dams both during gestation and around parturition, the welfare of the dam is likely to be affected. These adverse effects have been noted primarily in cattle and sheep clone pregnancies; similar effects have not been reported for swine clone pregnancies.

From a welfare viewpoint, dystocia carries the risk of unrelieved "extra" pain during birth due to the large offspring. If the dam has to have a Caesarean section then that itself carries the risk of pain and anxiety due to the procedures involved, including a failure to provide adequate post-operative pain relief. If the Caesarean section is not planned then there is the added burden of the pain of both the dystocia and the Caesarean section. For the neonates Caesarean section may be less stressful.

It has been reported that the occurrence of late gestation losses in surrogate dams carrying embryonic or somatic calf clones was linked to a high level of a specific maternal serum protein (PSP60) (Heyman et al., 2002). Elevated PSP60 levels could be detected as early as day 50 in surrogate dams that later lost their foetus and could be used as a marker for foetal death. Therefore assessing the placental development by day 50 or even day 34 of pregnancy by measuring PSP60, especially when carried out in combination with ultrasonography, could lead to more specific care for the bovine surrogate dam (Heyman et al., 2002; Chavatte-Palmer et al., 2006).

4.2.3. Welfare of clones

The evidence for an impact of SCNT on welfare is reviewed in the context of the various life stages of a clone. Data have been compiled by comparing clones with animals that are not clones, but which have been bred by natural mating, artificial insemination, or some other *in vitro* technique using gametes and embryos.

4.2.3.1. Welfare of clones at the time of birth to weaning

The period immediately after birth is critical for all newborns as the cardiovascular, respiratory and other organ systems adapt to life outside the womb. Offspring delivered naturally show a number of compensatory and regulatory mechanisms to minimize the stress of birth. Hence, even though a neonatal animal can certainly show severe signs of abnormal function e.g. so-called respiratory distress, it does not necessarily mean it is experiencing adverse effects, as adults might do under such conditions. In fact, mild postnatal stressors might instigate beneficial consequences relating to stress coping, fearfulness and learning ability (Casolini *et al.*, 1997).

After birth, the newborn gains a raised awareness due to the increased flow of oxygenated blood in the brain, and may then experience distress. Distress and pain reception have been

shown in neonates in premature human infants and lambs (Slater *et al.*, 2006; Mellor and Gregory, 2003). Distress in newborns could be due to various perinatal resuscitation and survival techniques, e.g. slaps, clearing out the mouth, vigorous rubbing of the skin, forced feeding including gavaging with colostrum.

Clones exhibiting LOS may require additional supportive care at birth. Planned Caesarean sections combined with special postnatal resuscitation measures for the clone neonates may reduce this problem. Calf clones are slower to reach normal levels of various physiological measures than their conventional counterparts (Chavatte-Palmer and Guillomot, 2007; Batchelder *et al.*, 2007b). Endocrine studies of cloned calves have shown lower cortisol concentrations at birth, although according to Batchelder *et al.* these results are difficult to interpret because controls were not born by the same method (Chavatte-Palmer *et al.*, 2002; Matsuzaki and Shiga, 2002; Batchelder *et al.*, 2007b).

In cloning the frequency of placenta dysfunction is increased and, therefore, foetal stress could arise due to altered oxygen exchange or altered placental blood barrier (Kato *et al.*, 1998; Galli *et al.*, 1999; Wells *et al.*, 1999; Young and Fairburn, 2000; Batchelder *et al.*, 2007b). Painful stimuli in late gestation have been shown in other species to cause irreversible effects on later development (Smythe *et al.*, 1994; Grunau *et al.*, 1994a; Grunau *et al.*, 1994b; Lloyd-Thomas and Fitzgerald, 1996; Braastad *et al.*, 1998).

Stress elicited in the dam carrying clone foetuses, such as pain or distress during late gestation and calving due to large foetuses, may also affect the foetus. It is not known whether early pregnancy distress exists in dams carrying clone foetuses but small variations in endogenous steroid hormones have been shown to exert programming effects on the developing brain (Ward and Weisz, 1980; Sikich and Todd, 1988; Grimshaw *et al.*, 1995; Martinez-Cerdeno *et al.*, 2006; Roselli *et al.*, 2007).

In LOS calves and lambs various stressors are likely to be detrimental and cause pain, but in apparently normal clones or clones that can be effectively resuscitated after birth, the pain and stress experienced during birth or postnatally may be no greater than in their sexually reproduced counterparts, whether they are delivered naturally or by Caesarean section.

4.2.3.2. Welfare of clones after weaning to puberty/slaughter/end of natural life

No data on welfare effects have been reported in clones approaching reproductive maturity compared with conventional animals.

There is no evidence that non-genetically based abnormal behaviour traits of the source animal will occur in the clone (F0). A comparison of four F0 clones from one 13-year old Holstein cow with four age-matched control heifers was made to determine whether juvenile clones from an aged adult behave similarly to their age-matched controls and whether clones with identical genetic makeup exhibit any behavioural trends (Savage *et al.*, 2003). A range of behavioural indicators and behaviour challenge tests were performed but no significant differences were observed except that the clones tended to exhibit less play behaviour than the others. Trends were observed indicating that the cattle clones "exhibited higher levels of curiosity, more grooming activities and were more aggressive and dominant than controls". The significance of these observations are, as yet, obscure.

An observation of five clones (from three different origins) and five non-clone Holstein heifers has indicated that social relationships (agonistic and non-agonistic behaviours) were not different between the two groups (Coulon *et al.*, 2007). When exposed to an unfamiliar environment heifer clones showed more exploratory behaviour than controls, however, the

authors concluded that this difference was probably related to the early management of the animals.

Daily activity, reactions to new events, and food preferences have been observed in two genetically identical Duroc clone litters consisting of four and five pigs, respectively, and two non-clone Duroc litters each of four pigs (Archer *et al.*, 2003b; Archer *et al.*, 2003c). The clones were similar to, but more variable than, the non-clone controls. However, according to another paper this study design was not amenable to inferential statistics, in addition to the considerable statistical noise (Shutler, 2005).

From the few publications available, and taking into account the very small sample sizes used, it is difficult to draw any conclusions on possible behavioural differences between clones and their age-matched controls. In addition any observed differences should be considered with caution as social behaviour and reactivity are dependent on the early environment of the animal (Veissier *et al.*, 1994) and on their genetic background (Le Neindre, 1989). In particular calf clones were subjected to more intensive care which could explain the few differences observed. Another explanation is that the few differences observed could be due to the fact that the calf clones had experienced stress during the gestation. One route of transmission of prenatal stress between mother and foetus involves maternal glucocorticoids and this effect is mediated through the transplacental crossing of glucocorticoids from mother to foetus, at least in the last part of gestation. In conventional animals, such stress has been described as changing the post-natal behaviour of male goats (Roussel *et al.*, 2005) and calves (Lay *et al.*, 1997).

4.2.4. Welfare of progeny (F1)

No specific studies on the welfare of the progeny of clones have been reported in livestock species.

4.2.5. Conclusions on animal welfare

- Only limited data are available on welfare implications of SCNT on surrogate dams, clones and progeny.
- The cloning procedure itself does not usually affect the welfare of the animals from which the somatic cell nucleus and oocyte are obtained.
- Reduced welfare of clones can be assumed to occur as a consequence of adverse health outcomes.
- The occurrence of late gestational losses, dystocia and large offspring in SCNT is likely to affect the welfare of the surrogate dams carrying calf clones. The frequency of these adverse health outcomes is higher in SCNT than in conventional reproduction or by using other ART.
- Due to the low efficiency of the cloning process, a high number of surrogate dams suffer pregnancy failure.

5. Safety of meat and milk derived from clones (F0) and their progeny (F1)

5.1. Molecular, biological and chemical aspects considered for safety

In line with the recommended safety assessment strategy, i.e. a case-by case consideration of the molecular, biological and chemical characteristics of the food and the determination of the need for, and scope of, traditional toxicological testing (WHO, 1990), the Scientific Committee considered the following aspects for the evaluation of the safety of milk and meat from cattle

and of meat and meat products from pigs derived from clones and their progeny in comparison with milk and meat from sexually reproduced animals.

5.1.1. Compositional comparison of meat and milk derived from clones and progeny of clones

Compositional data of products derived from animal clones (F0) and their progeny (F1) are compared with the corresponding products obtained from sexually generated animals of the same breed which have a long term history of safe use. Comparisons include details of the nutritional composition. The composition of milk and meat from cows is influenced *inter alia* by the nature of the animals' feed and the environment they live in, leading to large inter-individual variability in foods derived from conventional animals (Palmquist *et al.*, 1993). If subtle changes have occurred that would alter the presence of important nutrients, the most likely dietary risk for humans would be the absence of, or significant decrease in levels of, vitamins and minerals whose daily requirements are in large part met by milk or meat. Therefore, nutrients for which milk or meat make a large contribution to the total daily dietary intake in humans should be examined. Compositional data of meat and milk vary widely in the literature depending on breed, feeding regime, age, stage of lactation. Reference databases, indicating the range variability in the biochemical composition observed in sexually reproduced animals, can be utilized for comparison with the composition of clones and their progeny (Jensen *et al.*, 1995; Caballero, 2003; Belitz, 2004). Therefore it is important to make comparisons only with appropriate comparators, of similar genetic background, managed under similar conditions (Smith, 2005).

Several relevant studies with respect to human nutrition have been conducted on the composition of bovine milk and meat from cattle and meat from pigs derived from clones (F0) and their progeny (F1).

In an extensive study, more than 150 parameters in 37 cow clones (F0) from three independent cloning experiments and 38 control animals were examined over a 3-year period and consisted of more than 10,000 individual measurements (Heyman *et al.*, 2007a). In this study some slight changes were observed in all 3 groups of clones, compared with their controls, e.g. in fatty acid composition of milk and muscle of bovine clones (F0) and a slight increase of stearoyl-CoA desaturase in milk and muscle. However, these variations were still within the normal ranges.

A study on meat composition for five pig clones (F0) and 15 comparator animals showed no biologically relevant differences in fatty acid, amino acid, cholesterol, mineral and vitamin values (ViaGen Inc. USA). In a study of the composition of pig clone offspring, 242 offspring (F1) from four boar clones and 162 control pigs from the same breed were compared (Walker *et al.*, 2007). In this study 58 parameters consisting of more than 24 000 individual measurements (clones and controls) were examined. Only three individual values of the offspring were outside the control range.

Several other studies have analysed a number of parameters including carcass characteristics and meat and milk composition, including water, fat, proteins and carbohydrate content, amounts and distribution of amino acids, fatty acids, vitamins and minerals, and in the case of milk, also volume per lactation. These studies did not identify any differences outside the normal variability (Walsh *et al.*, 2003; Takahashi and Ito, 2004; Tome *et al.*, 2004; Norman and Walsh, 2004a; Norman *et al.*, 2004b; Tian *et al.*, 2005; Shibata *et al.*, 2006; Laible *et al.*, 2007; Walker *et al.*, 2007; Heyman *et al.*, 2007a; Yang *et al.*, 2007b).

In summary, none of the studies mentioned has identified differences outside the normal variability in the composition of meat (cattle and swine) and milk (cattle) between clones or clone progeny, and their comparators. In addition no novel constituents have been detected in

products from clones or their progeny. However, it should be acknowledged that the data base is limited.

5.1.2. Toxicity and allergenicity testing

Conventional toxicity tests are designed for individual chemicals and have major limitations for the testing of whole food. Foodstuffs are bulky, lead to satiation and can only be included in laboratory animal diets at lower multiples of expected human intakes. In addition, a key factor to consider in conducting animal studies on whole foods is the nutritional value and balance of the diets used, to avoid the induction of adverse effects, that are not related directly to the material itself (ACNFP, 1998). The testing of large amounts of milk and meat may be a particular problem in laboratory rodents with respect to departure from their normal diet, which is primarily plant-based.

5.1.2.1. Feeding studies

A subchronic oral feeding study (14 weeks) was conducted in rats to investigate the possible effects of a diet containing meat and milk derived from embryonic and somatic clones. For each product three different concentrations were tested; based on the protein content in raw milk and beef the highest amount administered exceeds the usual daily intake in a human diet. The rats were not affected in any of the groups studied (Yamaguchi *et al.*, 2007). Similar results were obtained in a 21-day feeding test with a diet containing milk and meat from cattle clones (F0) (Tome *et al.*, 2004; Heyman *et al.*, 2007a; Heyman *et al.*, 2007b). A 12-month oral toxicity study in the rat (which included reproduction performance) fed meat and milk from progeny (F1) of cattle clones has recently been published (Yamaguchi *et al.* 2008). The meat was derived from three progeny (F1) of conventional cows inseminated with semen from an SCNT bull. The milk was derived from three progeny (F1) of cow clones (F0) inseminated with semen from a conventionally bred bull. There were no biologically significant differences in the parameters examined (haematology, blood biochemistry, necroscopy, organ weight and histology) between the rats fed meat/milk from F1 compared to those fed conventional meat/milk products.

5.1.2.2. Genotoxicity

Meat derived from cattle clones did not show any genotoxic potential in the mouse micronucleus assay (Takahashi and Ito, 2004).

5.1.2.3. Allergenicity

Rats fed for several weeks with milk and meat from cattle clones and controls developed, as expected, a weak immune reaction. This reaction was qualitatively and quantitatively similar in rats given milk or meat either from clones or controls. The antibodies were in both cases IgG, IgA and IgM but not IgE, indicating that the consumption of the cattle products induced a classical immune response but no allergenic effect (Takahashi and Ito, 2004).

The allergenic potential of several *in-vitro* digested samples of meat and milk from cattle clones (F0) and controls was further assessed by intraperitoneal injection into mice following a classical immunization protocol. No statistically significant difference in the allergenic potential was observed between samples from clones and comparator control cattle (Takahashi and Ito, 2004). Also, Heyman *et al.* did not detect differences in the allergenicity of milk and meat obtained from clones in the rat compared with the same food products derived from non-

cloned animals, age and sex-matched, maintained under the same conditions (Heyman *et al.*, 2007a).

These results are only indicative as the rat and mouse models are not specific for human allergenicity predictive testing (WHO/FAO, 2001). However, changes in the primary protein structure or the presence of novel proteins in the edible products of clones and their progeny are not expected because the nuclear DNA sequence is unchanged.

5.1.3. Probability of novel constituents to be present

Animals commonly used for food production have never developed organs and/or metabolic pathways specialized for producing toxicants to kill prey or avoid predation, as is the case for some wild animal species. Therefore, it is highly unlikely in domesticated animals that genes, coding for "silent" pathways to produce intrinsic toxicants, exist or that their expression is possible even in the case of epigenetic dysregulation. This is in contrast to many food plant families, which do contain genes that code for inherent toxic constituents of the organism, such as glycoalkaloids in potatoes, furocoumarins in celery or nicotine in eggplants. Furthermore, as no new DNA sequences have been introduced into the clones, the generation or the occurrence of new substances, such as toxicants or allergens, are not expected.

5.1.4. Animals and animal products for human consumption

In accordance with EU legislation, animals belonging to species used for meat production are individually inspected *ante-* and *post-mortem* to determine if they meet existing regulatory animal health and food safety requirements. Animals, including clones, which are found to show clinical evidence of ill health at *ante-mortem* inspection, along with their carcasses and offal, would be removed from the food chain either prior to or following slaughter and would, therefore, be excluded from the human food supply. These requirements relate to actions to be taken following the detection of overt signs of disease or injury, either at *ante-* or *post-mortem* inspection. They are also complemented by criteria concerning maximum permissible levels of microbial and chemical contaminants.

Likewise, the production of milk from cattle (and other animals), both conventionally produced and cloned, would be subject to comparable EU legislative controls.

5.1.5. Microbiological aspects

Pathogenic microorganisms are likely to be found in both conventionally produced animals and clones even in the absence of clinical disease. These agents may later be present at slaughter on the carcasses of these animals and in their tissues. Any diminution of immunological competence may lead to clinical disease when the agent is pathogenic for that animal species.

5.1.6. Residue levels

The level of chemical contamination of meat and milk is influenced by feeding, environmental conditions and veterinary medication. As animal clones (F0) generally need more intensive care, especially in the early life stages of growth and development, the use of veterinary medicinal products for treatment may be greater than that in their natural comparators; however, no reliable data are available on comparative levels of veterinary drug residue levels. In all cases, veterinary medicinal products residues in meat and milk have to comply with existing EU regulations.

5.2. Conclusions on food safety

Based on current knowledge, and considering the fact that the primary DNA sequence is unchanged in clones, there is no indication that differences exist in terms of food safety between food products from healthy cattle and pig clones and their progeny, compared with those from healthy conventionally-bred animals.

This conclusion is also based on current evidence that indicates that:

- There are unlikely to be significant differences between healthy clones in the physiological parameters measured from their healthy conventional counterparts (see Chapter 4).
- Differences outside the normal variability are unlikely as regards the composition and nutritional value of meat (cattle and swine) and milk (cattle) between healthy clones or clone progeny and their healthy conventional counterparts.
- Toxicological and allergenic effects related to the consumption of food products from clones and their progeny are unlikely.

However, as information is limited on the immunological competence of clones, it is unclear, in cases where the pathogen is zoonotic in nature, whether or not the prevalence of such infection or infestation (and related public health risk) is the same as that of the conventionally produced animal.

6. Impact on the genetic diversity, biodiversity and environment

6.1. Genetic diversity

Cloning does not appear to have a direct effect on genetic diversity in that no new genetic modifications are introduced, but there could be an indirect effect due to overuse of a limited number of animals in breeding programmes. An increased homogeneity of a genotype within a population may increase the susceptibility of an animal population to infection and other risk factors. This would also be the case in conventional breeding schemes and is not caused by cloning as such. Reduction of genetic diversity in the farm animal populations has happened in the last 100 years when the number of livestock breeds has been significantly reduced because of the rapid spread of intensive livestock production (FAO, 2007).

6.2. Biodiversity

Cloning offers opportunities to save endangered species or livestock breeds and can be used to restore populations from infertile or castrated animals (NZRBCS, 2002). This implies preservation of the DNA in frozen cells. Cryopreserved tissue samples (for example, skin), which are easier to obtain than gametes or embryos, or tissue obtained from infertile animals, can be used to generate reproductively capable animals that could be used in subsequent breeding programs to expand endangered populations.

6.3. Environmental impacts

There is no indication suggesting that clones or their progeny would pose any new or additional environmental risks compared to conventionally bred animals. Cloning does not involve changes in DNA sequences and thus no new genes would be introduced into the environment.

In the event of an overall increased need for the use of veterinary medicinal products in clones there might be an impact on the environment, but no reliable data are available that compare

the extent of veterinary medicinal product use in animals produced by SCNT with those produced by ARTs or with conventional reproduction.

6.4. Conclusions on Impact on the Environment and Genetic diversity

Based on current knowledge:

- If used appropriately SCNT technology is not expected to adversely affect the genetic diversity within domestic species.
- Cloning can offer opportunities to restore endangered animal species.
- There is no expectation that clones or their progeny would pose any new or additional environmental risks compared to conventionally-bred animals. There is also no information available to suggest that such risks may exist.

OVERALL CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

Somatic cell nucleus transfer (SCNT) is a relatively new technology in animal reproduction with limited data available and is increasingly being used in some countries to produce clones. These clones can then be used for further breeding using conventional or other methods.

While cloning has been applied to several animal species, only in the case of cattle and pigs has there been sufficient data available to perform a risk assessment.

Uncertainties in the risk assessment arise due to the limited number of studies available, the small sample sizes investigated and, in general, the absence of a uniform approach that would allow all the issues relevant to this opinion to be more satisfactorily addressed.

The health and welfare of a significant proportion of clones, mainly within the juvenile period for bovines and perinatal period for pigs, have been found to be adversely affected, often severely and with a fatal outcome. Epigenetic dysregulation is considered to be the main source of adverse effects that may affect clones and result in developmental abnormalities. The use of SCNT in cattle and pigs, however, has also produced healthy clones and healthy offspring that are similar to their conventional counterparts based on parameters such as physiological characteristics, demeanour and clinical status. The production of clinically healthy clones provides evidence in those cases that the epigenetic reprogramming has taken place successfully.

In relation to food safety, there is no indication that differences exist for meat and milk of clones and their progeny compared with those from conventionally bred animals. Such a conclusion is based on the assumption that meat from cattle and pigs is derived from healthy animals as assessed by mandatory *ante-mortem* and *post-mortem* examinations, that milk is produced from healthy cows and that in both cases these food products are in compliance with food safety criteria regarding microbiological and chemical contaminants.

No environmental impact is foreseen but there are only limited data available.

RECOMMENDATIONS

General recommendations

- The health and welfare of clones should be monitored during their production life and natural life span.
- As food animals other than cattle and pig have also been produced *via* SCNT, risk assessments should be performed on these species when relevant data become available.
- This opinion should be updated in the light of developments in cloning and/or with new relevant data.

Additional recommendations

In relation to epigenetic and genetic aspects of SCNT it is recommended to determine or further investigate:

- The role of the epigenetic dysregulation as a cause of adverse effects.
- Whether, and if so, to what extent epigenetic dysregulation occurring in clones is transmitted to the progeny (F1).
- Whether, and if so, to what extent SCNT may induce silent DNA mutations.
- The possible consequences of mitochondrial heterogeneity in SCNT.
- The effects of telomere length in clones derived from different cell sources.

In relation to animal health it is recommended to:

- Conduct further research on the possible effects of SCNT on the natural life span of cattle and swine clones.
- Investigate further the causes of pathologies and mortality observed in clones during the gestational and postnatal periods and those observed at a lower frequency in adulthood.
- Further investigate the immunocompetence and the susceptibility of clones and their offspring to diseases and transmissible agents when reared and kept under conventional husbandry conditions.

In relation to animal welfare it is recommended to:

- Perform studies on animal welfare, including behavioural studies, in healthy clones under normal husbandry conditions.
- Monitor the surrogate dams for early markers of abnormal foetal development which could lead to adverse effects on their welfare.

In relation to food safety it is recommended that:

- Should evidence become available of reduced immunocompetence of clones (see animal health recommendations above), it should be investigated whether, and if so, to what extent, consumption of meat and milk derived from clones or their offspring may lead to an increased human exposure to transmissible agents.
- The database on compositional and nutritional characteristics of edible animal products derived from clones and their progeny should be extended.

INFORMATION MADE AVAILABLE TO EFSA

EFSA published a call for data on its website between 27 April and 29 May 2007.
Information was received from the following organisations:

AAVS (American Anti-Vivisection Society), USA

- Comments on the FDA Draft Risk Assessment. 47 pages.

BIO (Biotechnology Industry Organisation), Belgium

- BIO Comments to EFSA, Implications of animal cloning, May 29, 2007. 5 pages

Center for Food Safety, USA

- Report: Not Ready for Prime Time. FDA's Flawed Approach To Assessing The Safety Of Food From Animal Clones. 25 Pages
- Citizen Petition before the United States Food and Drug Administration. Petition seeking regulation of cloned animals. 24 Pages.

CIWF (Compassion in World Farming), United Kingdom

- Report: Farm Animal Cloning from an Animal Welfare Perspective. 10 pages

Danish Centre for Bioethics and Risk Assessment Institute of Food and Resource Economics, Denmark

- Information on current research activities and selected references.

EFFAB (European Forum of Farm Animal Breeders), The Netherlands

- The importance of cloning in bovine selection. 2 pages
- The European Perspective for Livestock Cloning. 19 pages
- Summary. 2 pages
- Possibilities and Concerns – Perspectives of Farm Animal Breeders. 24 pages

Faculty of Agricultural Sciences at Aarhus University, Denmark

- Information on current research activities and selected references.

IETS (International Embryo Transfer Society), USA

- Terms of Reference for Food Safety Subcommittee of the International Embryo Transfer Society (IETS) Health and Safety Advisory Committee (HASAC). 2 pages

- Terms of Reference for Research Subcommittee of the International Embryo Transfer Society (IETS) Health and Safety Advisory Committee (HASAC). 2 Pages

Institut national de la recherche agronomique INRA (Jouy-en-Josas), France

- Information on current research activities and selected references.

I-SiS (Institute of Science in Society), United Kingdom

- Is FDA Promoting or Regulating Cloned Meat and Milk? 7 pages
- Cloned BSE-Free Cows, Not Safe Nor Proper Science. 8 pages

ViaGen Inc, USA

- Letter. 3 pages
- Data (29 files, XL and Word) provided also to US FDA. This data is publicly available in the US FDA 2008 Report. "Animal Cloning: A Risk Assessment", Appendix F, which can be found at:
  http://www.fda.gov/cvm/CloneRiskAssessment_Final.htm
  (Last accessed 27 June 2008)

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GLOSSARY AND ABBREVIATIONS USED IN THE OPINION

To assure a consistent use and understanding throughout this opinion, some words of key importance are defined.

Glossary

Term	Definition used in the opinion
Allele	A gene that occupy a particular chromosomal locus. A diploid organism has two alleles, one on each chromosome.
Blastomere	Any one of the cells formed from the first few cell divisions in animal embryology. The embryo usually divides into two, then four, then eight blastomeres, and so on
Blastocyst	The early stage in the development of mammalian embryos. The blastocysts have an inner cell mass which will become the foetus and an outer cell mass (trophectoderm) that will become part of the placenta.
Caesarean section	Birth by surgical intervention
Chromatin	The complex of DNA and various proteins that makes up the chromosomes
Cloned embryo, embryo clone	Embryo resulting from somatic cell nuclear transfer
CpG	A region of DNA where a Cytosine nucleotide is separated by a phosphate to Guanine nucleotide. A CpG island is a region which has a high concentration of CpG sites.
Cytoplasm	The living content of the cell, except the nucleus, consisting of an aqueous protein matrix or gel, and where vital cellular organelles (e.g. mitochondria) are located
DNA methylation	Biochemical modification to the DNA through the addition of a methyl group
Donor animal	Animal from which the cell is obtained to be used in the cloning procedure
Dystocia	Abnormal or difficult birth giving or labour
Embryo	A multicellular structure of diploid cells formed after fertilization of the oocyte and until all organs have been formed, when it is called a foetus
Embryo, Reconstructed	An embryo that has been reassembled from its component parts by micro manipulations *in vitro*
Epigenetic processes	Alteration of gene expression by biochemical modifications (e.g. methylation) of the DNA or of DNA-binding proteins. The process does not involve changes in the DNA sequence
Epigenetic dysregulation	Abnormal or impaired control of gene expression
Epi-alleles	Alleles that are epigenetically modified
Fibroblast	A cell found mainly in connective tissue, involved in the formation and synthesis of extracellular matrix (e.g. collagen fibres)
Foetus	A developing mammal after the embryo stage and before birth
Gamete	A mature reproductive cell from a male or female containing a haploid number of chromosomes that normally fuses with a another gamete from the opposite sex to form a zygote (diploid) from which a new organism can develop The oocyte and spermatozoon are gametes.
Gametogenesis	The process of the formation of haploid gametes
Genetic diversity	The total number of genetic characteristics in the genetic make up of a species
Genotype	The entire genetic constitution of an individual
Germ line cell	A reproductive cell such as a spermatocyte or an oocyte, or a cell that will develop into a reproductive cell

Heteroplasmy	The presence of more than one type of organelle (e.g. mitochondrial DNA) within a cell
Healthy	Within the range of zootechnical and physiological parameters of mean of any given character from the point of view of food safety and animal health
Heifer	A female bovine that has not yet produced a calf
Hydroallantois	Abnormal fluid accumulation in the allantoic cavity of the placenta
Hydrops fetalis	A condition in the foetus characterized by accumulation of fluid, in at least two compartments (e.g. subcutaneous tissue, pleura, pericardium, abdomen). Hydrops sometimes leads to spontaneous abortion
Imprinting	A genetic phenomenon by which certain genes are expressed in a parent-of-origin specific manner.
Juvenile period	A period referring to young bovine of up to six months of age
LOS	Large Offspring Syndrome. The size of the offspring is greater than mean + 2SD for the species or breed. Symptoms .includes clinical hydrops, placental oedema and asynchronous growth of organs resulting in increased heart and liver size
Natural life span	The typical length of time an individual of a particular species can be expected to live
Oocyte	Unfertilized egg, the female gamete
Oocyte donor	Animal providing the oocyte used in the cloning procedure
Parturition	The act or process of giving birth to offspring
Perinatal period	A species dependent time period around 7 days before and after birth for livestock
Phenotype	The totality of the observable and structural characteristics of an organism as determined by genotype and its interaction with the environment
Placentome number	The number of interfaces between the cotyledons of the foetus and the caruncles of the dam's uterus forming the cotyledonary placenta in ruminants
Pluripotent	The possibility of a stem cell to differentiate into any of the three germ layers. A pluripotent cell can give rise to any foetal or adult cell type but has not the potential of as a totipotent cell.
Postnatal period	Time period (a few days) after birth
Progeny of clone	F1 and subsequent generations of animals born by sexual reproduction where at least one of the ancestors was a clone animals
Sexual reproduction	Normal way of reproduction between male and female, involving fusion between spermatozoon and oocyte
Silent mutation	DNA mutations that do not result in amino acid changes in a protein.
Somatic cell	Any cell of an animal that is not a germ line cell
Surrogate dam	Animal carrying the cloned embryos
Telomere	A region of highly repetitive DNA at the end of a chromosome
Totipotent	The possibility of a single cell to divide into any differentiated cell. See also pluripotent
Transgene	Foreign genetic material inserted, e.g. in a cell, embryo or organism (also: genetically modified)
Trophectoderm	The group of cells in the blastocyst that form the placenta
Zona pellucida	The thick glycoprotein layer surrounding the plasma membrane of an oocyte.
Zygote	The cell that results after fertilization of two haploid cells (usually a spermatozzon and an oocyte)

Abbreviations

Term	Definition used in the opinion
AI	Artificial insemination
ART	Assisted reproductive technology
IVF	*In vitro* fertilization
LOS	Large Offspring Syndrome
mtDNA	mitochondrial DNA
SCNT	Somatic Cell Nucleus Transfer