BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

According to experts, animal cloning carried out thorough 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 be 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.

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 ON 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.

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178 ASSESSMENT

179 ## 1. Introduction to the opinion

180 This opinion is based upon published peer reviewed scientific papers, data and other
181 information deemed reliable. EFSA launched through its Advisory Forum and on its website a
182 request for scientific contributions on this subject from third parties; a list of all documents
183 made available to EFSA can be found at the end of the opinion.

184 Cloning has been applied to several animal species, but only in the case of cattle and pigs were
185 there sufficient data to make possible a scientific assessment for this opinion. Where
186 appropriate, reference is made also to data concerning other species.

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

192 Broadly speaking, cloning can be regarded as an assisted reproductive technology (ART) in the
193 sense that it is a method used to achieve pregnancy by artificial means. However, in the context
194 of this opinion, SCNT is not included in the current use of the term ART, as it is unique due to
195 its asexual nature and permits the production of animals from a single animal with a known
196 phenotype. The present opinion takes into account observations on clones in the context of
197 animals produced by ART (such as *in vitro* fertilization, embryo transfer and embryo splitting)
198 and natural mating as appropriate. It is also acknowledged that current ARTs are widely used in
199 the zootechnical practice without any underlying formal risk assessment. For example, large
200 offspring syndrome, often thought to be a cloning-related phenomenon, was first described in
201 pregnancies derived from the transfer of *in vitro* fertilized embryos in cattle and sheep (Farin
202 and Farin, 1995; Walker, 1996; Kruip and den Daas, 1997; Sinclair *et al.*, 1999).

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

210 ### 1.1. Matters not addressed in the opinion

211 Approaches to cloning other than SCNT, such as embryonic cell nucleus transfer (ECNT) using
212 early embryonic cells (blastomeres) have been carried out, but in comparison with SCNT,
213 relatively few animals have been described in the literature (Yang *et al., 2007b*). ECNT as well
214 as genetically modified animals (rDNA animals) that have been propagated by the use of
215 SCNT are not assessed in the present opinion, nor are the effects of ARTs (e.g. *in vitro*
216 fertilization, embryo transfer and embryo splitting).

217 ### 1.2. Terms used in the opinion

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

- **Cloning**

Cloning, as assessed in this opinion, is defined as the technique of somatic cell nucleus transfer (SCNT). The word clone is derived from the Greek words *clonos*, "twig" and *clonizo* "to cut twigs". 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 genetic DNA sequence.

- **Clone**

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.

- **Progeny (offspring) of clone**

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

2. Animal breeding and reproductive techniques

Assisted reproductive technologies (ARTs) have greatly improved 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.

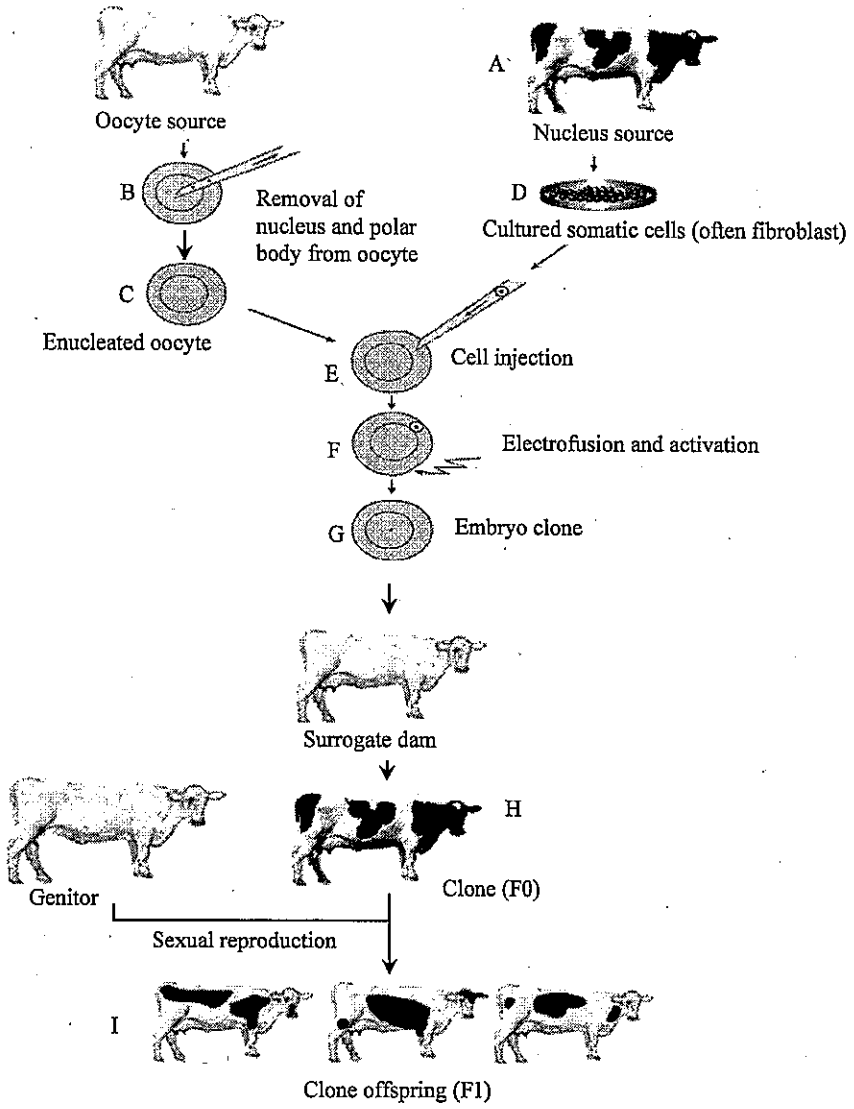
The genetic diversity of animal species or breeds may, in principle, be managed through the selection of genitors, by generating intra- and inter-hybrids or by generating genetically modified animals. The advantage of conventional genetic selection is that it creates new genotypes at each generation 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, will reproduce a particular desired phenotype (such as disease resistance, improved welfare, production or food product quality) with a higher likelihood than sexual reproduction.

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).

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.

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.



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

275

276 ## 2.2. Cloned species and cloning efficiency

277 Since the birth of the sheep "Dolly" in 1996, SCNT has been applied to livestock and to several
278 other species. Cattle, which are reported to be the animals most frequently used for SCNT,
279 were first cloned in 1998 (Cibelli *et al.*, 1998; Yang *et al.*, 2005), goats in 1998 (Keefer *et al.*,
280 2002), pigs in 2000 (Onishi *et al.*, 2000), rabbits in 2001 (Chesne *et al., 2002*) and horses in
281 2003 (Galli *et al.*, 2003).

In livestock species, healthy progeny (F1) have been obtained after the sexual reproduction of a clone. Furthermore, for research purposes, clones have also been produced by using cells taken from clones (i.e. repetitive-cloning) (Cho *et al.*, 2007).

The overall success rate of the cloning procedure is still low and differs greatly between species. The overall success rate, expressed as the percentage of viable offspring born from transferred embryo clones, ranges approximately from 0.5 to 5 %, depending on the species.

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).

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 due 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.

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. Number of clones and data on life span

There is no world-wide register of clones and therefore the number of living clones is difficult to estimate but EFSA has attempted to collect such information. In the EU there are about 100 cattle clones and fewer pig clones. 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, and EFSA estimates that the total number of clones alive world-wide in 2007 is less than 4000 cattle and 1500 pigs. The relatively small number is a reflection of e.g., technical difficulties and the regulatory status, and it can be expected that the number would increase as the efficiency is improved and if cloning is approved for commercial food purposes somewhere in the world. Semen from clones is already available on the market in the USA. However, even if the number of F0 clones remains small, there is potential in the future for a number of F1 and subsequent generation animals that could be produced from F0 clones and enter the food chain.

Similarly, 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 available yet.

2.4. Possible use of cloning

Genetic selection is a method to improve animal production. It is based on the controlled reproduction of animals followed by the identification of individuals with desirable traits, such as high productivity, disease resistance etc. Genetic selection relies on the natural genetic variation and gene redistribution which occurs during sexual reproduction in conventional breeding.

Cloning provides a way in which selected characteristics can be propagated into production herds more rapidly. For example, if an animal with a genetic resistance to a disease has been identified, that animal could be expanded by cloning into several genitors which could then be used to introduce the disease resistance trait via sexual reproduction into the production (or subsequent breeding) herd.

SCNT may also prolong the reproductive life of sires or dams that have already produced high value offspring and are aged beyond their ability to produce gametes effectively or for those whose lives or fertility were shortened by design, accident or misadventure. Cloning may also help to reduce the difference that exists regarding the availability of gametes between male and female genitors. Naturally, females can provide at most a few hundred oocytes whereas males, through their semen can generate thousands of offspring. Cloning, therefore, makes possible a more intensive use of specific female genotypes within a breeding scheme.

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

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. Modifications of chromatin proteins are 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 corresponds 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 fetus is interrupted, usually with fatal consequences. It is this phenomenon that is consistent with the considerable loss of embryo clones at 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 blastocyst stage are also found aberrantly expressed in the organs of clones that 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 after 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 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 successful mouse 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 also differs among individual clones. An average of two to five aberrantly methylated loci per 1,000 loci in each tissue of a clone has been observed in mice. 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. However, these abnormalities can disappear with the advancement of animals' aging, as shown recently from the analysis of kidney cells from new born and adult mouse clones in mid-age or senescence (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.