European Food Safety Authority

The EFSA Journal (2009) RN 319, 1-15

EFSA STATEMENT

Further Advice on the Implications of Animal Cloning (SCNT)[1]

Prepared by the Scientific Committee and Advisory Forum Unit

(Question No EFSA-Q-2009-00449)

Issued on 23 June 2009

SUMMARY

The European Food Safety Authority received in March 2009 a request from the European Commission to expand and further deepen the underlying details related to the recommendations included in the animal cloning opinion of July 2008 (EFSA Journal (2008) 747, 1-49). The request was for EFSA to focus in particular on the health and welfare of animal clones and the recommendations related to investigation of the causes of pathologies and mortality observed in clones during the gestational and postnatal periods and those observed at a lower frequency in adulthood and the health and welfare of clones during their productive life and natural life span. In addition the European Commission requested to know to what extent the current knowledge applies to cloning of sheep, goats and chicken.

A number of scientific publications have been published since the EFSA 2008 opinion indicating that Somatic Cell Nuclear Transfer (SCNT) is an active field both regarding basic and applied research. Most publications have studied embryonic or early development or methodological developments and there are only a few publications and studies on postnatal or adult animals. If the success rate of the epigenetic reprogramming is improved it is likely that the pathologies and mortalities observed in a proportion of clones would decrease.

There is still not sufficient data on species other than cattle and pigs to perform a risk assessment.

This statement confirms that the conclusions and recommendations of the EFSA 2008 opinion are still valid.

Key words: Animal Cloning, SCNT, Somatic Cell Nucleus/Nuclear Transfer

[1] For citation purposes: Statement of EFSA prepared by the Scientific Committee and Advisory Forum Unit on Further Advice on the Implications of Animal Cloning (SCNT). *The EFSA Journal* (2009) RN 319, 1-15

TABLE OF CONTENTS

BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

The College of Commissioners discussed the issue of cloning of animals for food production purposes on 13 January 2009. The College decided to ask Commission services to pursue the contacts and work with the European Food Safety Authority so that open questions can be answered and related scientific work is taken up. Commissioner Vassiliou intends to present to her colleagues an update on the situation before the summer.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

The European Commission requests the European Food Safety Authority to expand and further deepen the underlying details related to the recommendations included in the opinion of July 2008. This should focus in particular on the health and welfare of animal clones as this appears to be the main problem area identified in the EFSA Opinion. The Commission asks the Authority to include information from new scientific evidence which could include information from as yet unpublished data which may be available from experts working in the area.

The areas that should be covered are as following:

- investigation of the causes of pathologies and mortality observed in clones during the gestational and postnatal periods and those observed at a lower frequency in adulthood.
- the health and welfare of clones during their productive life and natural life span.

In addition we [the European Commission] would like to know to what extent the current knowledge applies to cloning of sheep, goats and chicken as due to current knowledge and data available only cattle and pigs were covered by the European Food Safety Authority Opinion of July 2008.

The Commission needs the results of such assessments by June 2009. DG SANCO remains at your disposal for further details and discussion on the work.

ACKNOWLEDGEMENTS

The European Food Safety Authority wishes to thank Andras Dinnyés, Louis-Marie Houdebine, Heiner Niemann, David Morton and Jean-Paul Renard for peer-reviewing the statement.

EVALUATION

1. Introduction to the evaluation

The request of the European Commission was received by EFSA on 6 March 2009. This statement is based on literature searches, information made available to EFSA and discussions with and contributions from experts.

1.1. Scientific Committee Opinion of July 2008

On 24 July 2008 EFSA published a Scientific Opinion of the Scientific Committee on a request from the European Commission regarding 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). In the current statement this opinion is referred to as "EFSA 2008 opinion". The conclusions and recommendations of the EFSA 2008 opinion are found in Annex I.

1.2. Information collection

To collect relevant information in relation to the request EFSA launched a call for data on its website from 11 March 2009 to 30 April 2009. Dedicated dissemination of the call for data was also carried out via the EFSA Advisory Form and its focal points as well as with targeted e-mail to various research groups both within the EU and worldwide. At the closing of the call contributions were received from 12 sources (excluding Member States). A list of the contributions made available to EFSA is found at the end of the statement.

In addition to the call for data a comprehensive literature research was performed. A search strategy was developed based on keywords from the EFSA 2008 opinion and the addition of relevant farm animal species. The search was in general aimed to find publications since the beginning of 2008, as it was assumed that the EFSA 2008 opinion had addressed earlier relevant publications. The search aimed at identifying publications in publicly available databases, mainly Pubmed, ScienceDirect and ISI Web of Knowledge. The literature search included some information presented as abstracts.

The literature search in general excluded several types of studies; transgenic animals, inter-species cloning, studies focusing on methodological developments and improvements and studies involving non-farm animals such as rats and mice. Studies where no live-born animals were reported were in general not considered as the design of many of such studies was not aimed at delivering live animals, but to study *in vitro* development of e.g. blastocysts or embryos. The literature searches in the databases ended on 1 May 2009.

2. Development of recommendations in the EFSA 2008 opinion

The request of the European Commission specifically asked EFSA to expand and further deepen the underlying details related to the recommendations with a focus on the health and welfare of animal clones. Specifically the following recommendations in the EFSA 2008 opinion were to be covered:

- investigation of the causes of pathologies and mortality observed in clones during the gestational and postnatal periods and those observed at a lower frequency in adulthood.
- the health and welfare of clones during their productive life and natural life span.

2.1. Recommendation on causes of pathologies and mortality

This section is related to the EFSA 2008 opinion recommendation related to investigation of the causes of pathologies and mortality observed in clones during the gestational and postnatal periods and those observed at a lower frequency in adulthood.

In the EFSA 2008 opinion, section 3, on the epigenetic and genetic aspects of SCNT it was concluded that it is the epigenetic dysregulation which is the main source of the adverse effects that may affect clones and result in developmental abnormalities. For clinically healthy clones the epigenetic reprogramming takes place successfully. Finally it was concluded that the extents to which epigenetic and genetic aspects of SCNT are affected has not been fully elucidated.

2.1.1. New information

Placental development

Failure of placental development following SCNT is believed to be one of the reasons why cloning has a low success rate (Arnold *et al.*, 2008). Several placental abnormalities have been observed in cattle pregnancies with clones in association with poorly developed placentomes. The placentomes may be abnormal in shape, appear in lower numbers and hypertrophic if pregnancy is maintained. There also seems to be a deregulation of the Vascular Endothelial Growth Factor A (VEGF-A) system in the placenta of SCNT cattle clones which may contribute to the placental alteration and/or malformations reported. Studies on trophoblast development before embryo implantation in the uterus indicate that SCNT trophectoderm does not develop normally which may be one of the causes of pregnancy loss in SCNT bovine embryos. Placental tissues from cloned animals have been shown to proliferate more and have a reduced rate of apoptosis which may be associated with faulty placentation in early pregnancy, placental insufficiency or even a lack of placental and/or foetal maturation towards the end of the pregnancy (Rici *et al.*, 2008).

Disturbances in the embryo-maternal interaction during the peri-implantation period have been observed by investigating mRNA endometrium transcriptome profiles in bovines (Bauersachs *et al.*, 2009; Mansouri-Attia *et al.*, 2009). The mRNA variations in the SCNT embryos are greater compared with IVF (*in vitro* fertilisation) embryos. The placental failure in a proportion of bovine clone pregnancies which manifests at later pregnancy stages may originate from abnormal embryo-maternal communication during the peri-implantation period of early pregnancy. There may be a possibility for evaluating endometrial transcriptome profiles to optimize SCNT embryos for their ability to establish a successful pregnancy, develop a functional placenta and produce viable offspring (Bauersachs *et al.*, 2009; Mansouri-Attia *et al.*, 2009).

Embryo development

The risk of infectious disease transmission in the *in vitro* embryo culturing system has been investigated using Bovine viral diarrhea virus (BVDV) as a model (Gregg *et al.*, 2009). BVDV is a significant bovine pathogen which causes cattle respiratory disease and reproductive disorders, reduced milk production and increased incidence of clinical mastitis. No BVDV was detected in 324 SCNT embryos cultured *in vitro* for 7 days following nucleus transfer to oocytes which were washed to remove cumulus cells. It is essential to screen out BVDV-positive donor cell lines and use only negative cells for SCNT embryo production (Gregg *et al.*, 2009). These results suggest a low risk of BVDV transmission during *in vitro* embryo culture when precautions have been taken (Gregg *et al.*, 2009).

The production of pigs through SCNT presents difficulties probably due to the inefficiencies of *in vitro* oocyte maturation and embryo culture, and the necessity for at least four good-quality embryos to establish a pregnancy (Jiang *et al.*, 2008). Expression of X-linked genes in pig clones surviving to one month of age was largely within the normal range, whereas abnormal expression was prevalent in deceased newborns (Jiang *et al.*, 2008).

DNA mutations and mtDNA heteroplasmy

It has been shown in mouse foetuses that the process of SCNT does not lead to an increase in the frequency of point mutations, and that the clone foetuses accumulated spontaneous mutations at a rate similar to that in foetuses produced by natural conception (Murphey *et al.*, 2009). Moreover, mutations in a marker gene were corrected similarly in early embryos generated by cloning or natural fertilization. It is not yet known if this is also valid for other species.

It has been demonstrated that donor mitochondrial DNA (mtDNA) is transmitted to clone offspring with varying efficiencies (Takeda *et al.*, 2008). Four cows (F1) with mtDNA heteroplasmy showed normal growth, productivity and lactation characteristics and productivity.

Gene expression, imprinting and biomarkers

DNA methylation and histone modifications (acetylation) and specific gene expression may be higher in bovine clones than in controls (Lin *et al.*, 2008). Data in this study also suggest that there are no widespread gene expression abnormalities in deceased clones indicating that the death may be ascribed to abnormal expression of a very limited number of pivotal genes.

Aberrant gene expression patterns in bovine blastocysts, placentomes and cotyledons have been reported (Everts *et al.*, 2008; Aston *et al.*, 2009). Differentially expressed genes (DEG) associated with SCNT are involved in multiple biological pathways including cell cycle, cell death and gene expression.

Abnormal genomic imprinting may lead to developmental abnormalities such as large offspring syndrome (LOS) as well as placental defects (Liu *et al.*, 2008). However, there are also recent data indicating correct reprogramming takes place in the intragenetic differentially methylated region (DMR) of the bovine *IGF2* gene which is an important imprinted locus (Gebert *et al.*, 2009). This result does not support the current view that imprinted genes are the primary suspects for developmental failures.

Gene expression profiling of bovine endometrium indicated that the physiology of the endometrium was affected in SCNT pregnant animals compared with control animals (Mansouri-Attia *et al.*, 2009). The authors suggest that selected endometrial factors could be used as potential biomarkers for discriminating correct from pathological pregnancies.

Oxidative stress from the formation of radical oxygen species (ROS) during in vitro culture may be a cause of apoptosis in SCNT extraembryonic tissue (visceral yolk sac, allantois, umbilical cord and placenta), and the low birth rate of pig clones may be due to abnormal apoptosis in the extraembryonic tissue during early pregnancy (Chae *et al.*, 2008). In addition abnormal expression of translational regulators in extraembryonic tissue in early pregnancy has been found which may also be related to the low birth rate of pig clones (Chae *et al.*, 2009).

Increased levels of certain proteins (e.g. cathelicidin antimicrobial protein (CAMP) and protein inhibitor clade B1)) in the allantoic fluid have been found in bovine SCNT pregnancies which has been suggested to be a direct response to immuno-inflammatory episodes caused by either microbial invasion of the uterine/conceptus space or as a consequence of an inflammatory

event of yet unknown aetiology (Riding *et al.*, 2008). This may contribute to the early foetal losses in SCNT pregnancies.

Results for global gene expression profiling in the pig suggested the maternal responsiveness to SCNT embryos was impaired, leading to abnormal placental and foetal development and increased embryonic loss (Ka *et al.*, 2008).

Parturition

Transforming Growth Factor (TGF)-β_1 is a cell signalling cytokine present in the foetal-maternal interface of the bovine placentome and may be necessary to maintain pregnancy. Delayed parturition (bradytocia) in surrogate dams carrying bovine clones may be associated with persistence of elevated TGF-β_1 expression late in pregnancy (Hwang *et al.*, 2008).

Other information

The epigenetic status of 38 healthy female bovine clones at 1 to 8 years of age was investigated by measuring methylation of cytosine (5mC) as an epigenetic marker. The results demonstrate that the reprogramming of a given donor genotype is compatible with a highly flexible methylation status of its DNA and that genomic copies of adult animals have to be considered as epigenome variants (de Montera *et al.*, 2009).

It has been shown that the mean number of meiotic cross-overs in spermatocytes in SCNT bulls and their offspring was not significantly different from normal control bulls (43 ± 5 for SCNT and offspring, vs 42 ± 4 for normal bulls) (Hart, 2008). These results may indicate that circumventing meiosis in the SCNT process does not influence the meiotic processes in spermatocytes (Hart *et al.*, 2008).

2.1.2. Development of EFSA 2008 opinion recommendation

Several recent publications have studied the causes of pathology and mortality observed in clones during gestation. There are few publications referring to postnatal periods, and studies on adult animals.

Dysregulation of the epigenetic reprogramming is considered to be the main source of adverse effects that may affect a proportion of clones and result in developmental abnormalities leading to morbidity and possible mortality.

Abnormal expression of imprinted genes, X-linked genes, apoptosis related genes and other development related genes have been reported in clones. However it is still not known to what extent these abnormalities cause clone mortality.

The recommendation in the EFSA 2008 opinion is still valid, and additional investigations are warranted, especially aiming to understand epigenetic reprogramming and the role of epigenetic dysregulation as a cause of adverse affects.

2.2. Recommendation on health and welfare of clones during productive life and natural life span

This section is related to the recommendations in the EFSA 2008 opinion on the health and welfare of clones during their productive life and natural life span. In the EFSA 2008 opinion, section 4, it was concluded that most clones have not yet reached the end of their natural life span for their species and therefore it is difficult to draw any conclusions on possible effects of SCNT on longevity.

2.2.1. New information

The immune function of twenty-five heifer clones were compared with age matched controls (Chavatte-Palmer *et al.*, 2009). The humoral and T-cell immune responses of leucocytes to exogenous antigens were studied. The clones presented a normal leukocyte population and their functional immunity was not modified. The authors concluded that this analysis supports earlier data suggesting that cattle clones have normal immunity. The authors further mention "…that most animals used 4 years ago in this study are still alive, regardless of whether they were produced by conventional reproduction or by cloning […]".

A survey in Japan carried out in 2006 reports observations from 482 SCNT cattle (Watanabe and Nagai, 2009). Losses due to stillbirth in calves were: 16.4 % in SCNT, 8.9 % in clone offspring; and 4.6 % in, conventional cattle. Mortality within 24 h of birth was: 14.4 % for SCNT; 0.8 % for clone offspring; and 1.9 % for conventional calves. These results indicate that the mortality due to stillbirth and neonatal mortality (24 h after birth) in clones are higher than those of conventionally bred cattle, but the loss in the offspring are similar to those in conventionally bred cattle. In addition a higher mortality due to disease was observed in clones in the first 30 days of life (24.1 %, 52/216) which reached the same level as that of conventional bred animals at about 200 days of age. In regard to the life span 300-720 days of age (720 days being around the time in Japan when animals would be slaughtered for meat, and dairy cows would calve to produce milk) the incidence of mortality in clones, their progeny and conventional breed cattle was not significantly different. This survey also reports that more than 70 % of the clones were alive and healthy during the observation (Watanabe and Nagai, 2009).

The muscle type of nine heifer clones was studied and compared with conventionally bred cattle heifers (Jurie *et al.*, 2009). Biopsies were taken at regular intervals up to 24 months of age. Young clones had a slower muscle type associated with a more oxidative muscular metabolism but after 12 months of age no significant differences were observed between clones and controls.

Production of third-generation cloned offspring in pigs has been performed without reduction in efficiency or telomere length (Kurome *et al.*, 2008a). The percentage of offspring obtained from the transferred embryos ranged from 1.4 % to 3.3 % over the three generations in this study. One of the first generation clone offspring was followed to 20 months of age, seven of the second generation clone offspring were followed to 18 months of age, and one of a third generation clone offspring was followed for 14 months; there were no observed developmental abnormalities or illnesses in any of the animals. Three of the four second generation clone offspring became pregnant, and blood tests from three other clones conducted at 17 months of age revealed normal blood cell counts and biochemical profiles.

Other information

The OIE (World Organisation for Animal Health) has recommendations on the collection and processing of *in vivo* derived oocytes in their terrestrial animal health code (http://www.oie.int/eng/normes/MCODE/en_chapitre_1.4.7.htm#rubrique_bovins_collecte_in_vivo. The International Embryo Transfer Society has published a recommendation on the health assessment and care for animals involved in the cloning process (IETS, 2008, http://www.iets.org/hasac.htm).

2.2.2. Development of EFSA 2008 opinion recommendation

Since the opinion was published in 2008 it is not expected that the available information referring to natural life span has changed over such short time. There is limited new

information related to the health and welfare of clones during their productive life and natural lifespan and the recommendations in the EFSA 2008 opinion are still valid.

3. SCNT of other species

The request of the European Commission also asked to what extent the current knowledge applies to cloning of other species (e.g. sheep, goat and chickens).

This question is addressed in a pragmatic way. An assessment of additional species would depend on the available data for assessment. In the EFSA 2008 opinion, the food safety aspects were based on comparison of compositional data of products derived from clones or clone offspring and on toxicity and allergenicity testing (See section 5 in the EFSA 2008 opinion). Since the EFSA 2008 opinion no additional publications with such information have been identified referring to sheep, goat, chicken, rabbit, horse, buffalo or fish. The status of the available data has therefore not changed and the EFSA 2008 opinion is still valid.

Based on available publications, fish and chicken have not been cloned by classical SCNT However, it has been reported that genuine diploid clones of medaka fish (*Oryzias latipes*) could be obtained after the transfer of the nucleus from somatic cells into artificially diploidized oocytes (Bubenshchikova *et al.*, 2007). The mechanism leading to the elimination of the oocyte nuclei is still unknown (Bubenshchikova *et al.*, 2008).

4. Additional information

The Japanese Food Safety Commission has released a draft report on "Risk assessment of safety on food products derived from somatic cell cloned cattle and pigs" (Available in Japanese only at http://www.fsc.go.jp/iinkai/i-dai277/dai277kai-siryou3.pdf) whose outcome corresponds to the EFSA 2008 opinion as well as with the final FDA 2008 report on animal cloning (http://www.fda.gov/cvm/CloneRiskAssessment_Final.htm). This draft was published for public consultation until 10 April 2009, and the final assessment is expected shortly.

Information on clone progeny (F1)

Although it was outside the European Commission's request to look at clone progeny, a publication in Japanese reports the outcome of a one year observation of blood parameters of offspring from cattle clones (Matsuda *et al.*, 2009). Several blood parameters analysed in five SCNT cattle offspring were compared with five conventionally bred cows indicating that although some parameters were significantly different almost all values observed were within the normal range. The authors concluded that there were no biologically significant differences in these blood parameters between clone offspring and comparators.

In a Japanese survey, 202 offspring of cattle clones showed the same level of mortality as observed in conventional bred cattle throughout their lifetime (Watanabe and Nagai, 2009).

A group of 39 progeny (F1) from cattle clones have been observed and compared with clones and animals derived with artificially insemination (AI) as controls (Heyman *et al.*, 2009). The mean birth weights of the F1 were not significantly different from AI controls and no Large Offspring Syndrome (LOS) was observed in the F1 group. The postnatal survival of the F1 group at six months of age (94.4 %) was comparable to the control (94.1 %) as well as the body weight (188.25 ± 15.17 kg versus 185.30 ± 19.94 kg) and daily weight gain of 0.820 and 0.803 kg/day respectively. None of the F1 calves presented any of the pathologies observed in clones.

These recent publications confirm the conclusion of the EFSA 2008 opinion that there is no indication of adverse effects for the sexually reproduced progeny (F1) of clones and that there

is no indication that differences exists for meat and milk of F1 compared with those of conventionally bred animals.

CONCLUSIONS

A number of scientific publications have been published since the EFSA 2008 opinion indicating that SCNT is an active field both regarding basic and applied research. Most publications have studied embryonic or early development or methodological developments and there are only a few publications and studies on postnatal or adult animals. If the success rate of the epigenetic reprogramming is improved it is likely that the pathologies and mortalities observed in a proportion of clones would decrease.

There are still not sufficient data on species other than cattle and pigs to perform a risk assessment.

This statement confirms that the conclusions and recommendations (see annex I) of the EFSA 2008 opinion are still valid.

INFORMATION MADE AVAILABLE TO EFSA

EFSA published a call for data on its website between 11 March and 30 April 2009. In addition EU Members States were asked to provide information via the EFSA Focal points. There are a few EU member states where research is carried out using SCNT technology. In addition the following information, some of it unpublished, was received;

Biotechnology Industry Association (BIO)

- BIO Comments to the EFSA call for data. 4 pages.
- Scientific publication.

European Forum of Farm Animal Breeders (EFFAB)

- EFFAB comments to EFSA call for data. 2 pages.
- Sustainable farm animal breeding and reproduction technology platform. 6 pages.
- Fact sheet on cloning. 2 pages.
- Letter with consequences for European innovation and breeding programmes. 7 pages.

Institute National de la Recherche Agronomique (INRA)

- Letter. 1 page.
- 14 scientific publications.

International Embryo Transfer Society (IETS)

- Health Assessment and Care for Animals Involved in the Cloning Process. A consensus document from the International Embryo Transfer Society. 15 May 2008. 15 pages.

Istanbul University, Faculty of Veterinary Medicine, Turkey

- First cloned animals in Turkey. Unpublished information. 8 pages.

National Institute of Livestock and Grassland Science, Japan

- 5 scientific publications were provided, including one *in press.*

ViaGen Inc, USA

- ViaGen comments to the EFSA call for data. 4 pages.
- Compilation of cloning publications 2008-2009 (XL file).
- Scientific publication.

Individual Scientists

- Scientist based in France. Scientific publication.
- Scientist based in Germany. 2 scientific publications.
- Scientist based in Hawaii. 2 scientific publications.
- Scientist based in Italy. Book chapter *under preparation.*
- Scientist based in Japan. 5 scientific publications (1 in press)

REFERENCES

Arnold, D. R., Fortier, A. L., Lefebvre, R., Miglino, M. A., Pfarrer, C. and Smith, L. C. 2008. Placental insufficiencies in cloned animals - a workshop report. Placenta 29 Suppl A: S108-10.

Aston, K. I., Li, G. P., Hicks, B. A., Sessions, B. R., Davis, A. P., Winger, Q. A., Rickords, L. F., Stevens, J. R. and White, K. L. 2009. Global Gene Expression Analysis of Bovine Somatic Cell Nuclear Transfer Blastocysts and Cotyledons. Molecular Reproduction and Development 76 (5): 471-482.

Bauersachs, S., Ulbrich, S. E., Zakhartchenko, V., Minten, M., Reichenbach, M., Reichenbach, H. D., Blum, H., Spencer, T. E. and Wolf, E. 2009. The endometrium responds differently to cloned versus fertilized embryos. Proc Natl Acad Sci U S A 106 (14): 5681-6.

Bubenshchikova, E., Kaftanovskaya, E., Hattori, M., Kinoshita, M., Adachi, T., Hashimoto, H., Ozato, K. and Wakamatsu, Y. 2008. Nuclear transplants from adult somatic cells generated by a novel method using diploidized eggs as recipients in medaka fish (Oryzias latipes). Cloning Stem Cells 10 (4): 443-52.

Bubenshchikova, E., Kaftanovskaya, E., Motosugi, N., Fujimoto, T., Arai, K., Kinoshita, M., Hashimoto, H., Ozato, K. and Wakamatsu, Y. 2007. Diploidized eggs reprogram adult somatic cell nuclei to pluripotency in nuclear transfer in medaka fish (Oryzias latipes). Dev Growth Differ 49 (9): 699-709.

Chae, J. I., Lee, K. S., Kim, D. J., Han, Y. M., Lee, D. S., Lee, K. K. and Koo, D. B. 2009. Abnormal gene expression in extraembryonic tissue from cloned porcine embryos. Theriogenology 71 (2): 323-33.

Chae, J. I., Yu, K., Cho, S. K., Kim, J. H., Koo, D. B., Lee, K. K. and Han, Y. M. 2008. Aberrant expression of developmentally important signaling molecules in cloned porcine extraembryonic tissues. Proteomics 8 (13):

Chavatte-Palmer, P., Heyman, Y., Richard, C., Urien, C., Renard, J. P. and Schwartz-Cornil, I. 2009. The immune status of bovine somatic clones. Cloning and Stem Cells 11 (2): 11.

de Montera, B., El Zeihery, D., Muller, S., Jammes, H., Brem, G., Reichenbach, H. D., Scheipl, F., Chavatte-Palmer, P., Zakhartchenko, V., Schmitz, O. J., Wolf, E., Renard, J. P. and Hiendleder, S. 2009. Highly variable epigenomes in healthy adult bovine clones. Reproduction, Fertility and Development 21 (1): 1.

Everts, R. E., Chavatte-Palmer, P., Razzak, A., Hue, I., Green, C. A., Oliveira, R., Vignon, X., Rodriguez-Zas, S. L., Tian, X. C. and Yang, X. 2008. Aberrant gene expression patterns in placentomes are associated with phenotypically normal and abnormal cattle cloned by somatic cell nuclear transfer. Physiological Genomics 33 (1): 65.

Gebert, C., Wrenzycki, C., Herrmann, D., Gröger, D., Thiel, J., Reinhardt, R., Lehrach, H., Hajkova, P., Lucas-Hahn, A., Carnwath, J. W. and Niemann, H. 2009. DNA methylation in the IGF2 intragenic DMR is re-established in a sex-specific manner in bovine blastocysts after somatic cloning. Genomics In Press, Uncorrected Proof:

Gregg, K., Chen, S. H., Sadeghieh, S., Guerra, T., Xiang, T., Meredith, J. and Polejaeva, I. 2009. Experimental risk assessment of bovine viral diarrhea virus transmission via in vitro embryo production using somatic cell nucleus transfer. Theriogenology:

Hart, E. J., Pinton, A., Powell, A., Wall, R. and King, W. A. 2008. Meiotic recombination in normal and clone bulls and their offspring. Cytogenet Genome Res 120 (1-2): 97-101.

Heyman, Y., Richard, C., Delatouche, L., Renard, J. P. and Chavatte-Palmer, P. 2009. Characteristics of Bovine Clone Offspring (F1): Comparison with Clones. Reproduction Fertility and Development 21 (1): 116-116.

Hwang, S., Chang, Y. M., Go, Y. G., Yang, B. C., Min, K. S., Yoon, J. T., Nho, W. G., Kim, C. K. and Seong, H. H. 2008. Delayed parturition in cloned calves associated with persistently elevated placentomal TGF-beta(1) expression. Anim Reprod Sci:

Jiang, L., Lai, L., Samuel, M., Prather, R. S., Yang, X. and Tian, X. C. 2008. Expression of x-linked genes in deceased neonates and surviving cloned female piglets. Molecular Reproduction and Development 75 (2):

Jurie, C., Picard, B., Heyman, Y., Cassar-Malek, I., Chavatte-Palmer, P., Richard, C. and Hocquette, J. F. 2009. Comparison of cloned and non-cloned Holstein heifers in muscle contractile and metabolic characteristics. Animal 3 (2): 244-250.

Ka, H., Seo, H., Kim, M., Moon, S., Kim, H. and Lee, C. K. 2008. Gene expression profiling of the uterus with embryos cloned by somatic cell nuclear transfer on day 30 of pregnancy. Anim Reprod Sci 108 (1-2): 79-91.

Kurome, M., Hisatomi, H., Matsumoto, S., Tomii, R., Ueno, S., Hiruma, K., Saito, H., Nakamura, K., Okumura, K., Matsumoto, M., Kaji, Y., Endo, F. and Nagashima, H. 2008a. Production efficiency and telomere length of the cloned pigs following serial somatic cell nuclear transfer. J Reprod Dev 54 (4): 254-8.

Lin, L., Li, Q., Zhang, L., Zhao, D., Dai, Y. and Li, N. 2008. Aberrant epigenetic changes and gene expression in cloned cattle dying around birth. BMC Developmental Biology 8 (1): 14.

Liu, J. H., Yin, S., Xiong, B., Hou, Y., Chen, D. Y. and Sun, Q. Y. 2008. Aberrant DNA methylation imprints in aborted bovine clones. Mol Reprod Dev 75 (4): 598-607.

Mansouri-Attia, N., Sandra, O., Aubert, J., Degrelle, S., Everts, R. E., Giraud-Delville, C., Heyman, Y., Galio, L., Hue, I., Yang, X., Tian, X. C., Lewin, H. A. and Renard, J. P. 2009. Endometrium as an early sensor of in vitro embryo manipulation technologies. Proc Natl Acad Sci U S A 106 (14): 5687-92.

Matsuda, H., Yoshioka, H., Oyabu, T., Imai, K. and Yonai, M. 2009. One-year observation aroun d parturition of blood parameters in progeny of holstein cows derived from somatic cell nuclear transfer. Japanese journal of Ebryo transfer 31 (1): 8.

Murphey, P., Yamazaki, Y., McMahan, C. A., Walter, C. A., Yanagimachi, R. and McCarrey, J. R. 2009. Epigenetic regulation of genetic integrity is reprogrammed during cloning. Proceedings of the National Academy of Sciences 106 (12): 4731.

Rici, R. E. G., Facciotti, P. R., Ambrósio, C. E., Maria, D. A., Jr, J. R. K., Bertolini, M. and Miglino, M. A. 2008. Cell cycle and apoptosis in normal and cloned bovine near-term placentae. Animal Reproduction Science In Press, Corrected Proof:

Riding, G. A., Hill, J. R., Jones, A., Holland, M. K., Josh, P. F. and Lehnert, S. A. 2008. Differential proteomic analysis of bovine conceptus fluid proteins in pregnancies generated by assisted reproductive technologies. Proteomics 8 (14): 2967-82.

Takeda, K., Kaneyama, K., Tasai, M., Akagi, S., Takahashi, S., Yonai, M., Kojima, T., Onishi, A., Tagami, T. and Nirasawa, K. 2008. Characterization of a donor mitochondrial DNA transmission bottleneck in nuclear transfer derived cow lineages. Molecular Reproduction and Development 75 (5):

Watanabe, S. and Nagai, T. 2009. Death losses due to stillbirth, neonatal death and diseases in cloned cattle derived from somatic cell nuclear transfer and their progeny: a result of nationwide survey in Japan. Animal Science Journal 80 (3): 6.

ANNEX 1. CONCLUSIONS AND RECOMMENDATIONS FROM THE EFSA 2008 OPINION

Below are the overall conclusions and recommendations (page 32-33) from the 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

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.