European Food Safety Authority

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STATEMENT OF EFSA

Update on the state of play of

Animal Health and Welfare and Environmental Impact of Animals derived from SCNT Cloning and their Offspring, and Food Safety of Products Obtained from those Animals[1]

European Food Safety Authority[2, 3]

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ABSTRACT

The European Food Safety Authority (EFSA) received in December 2011, a request from the European Commission for an update on the possible scientific developments for cloning of farmed animals for food production purposes. The present Statement follows the EFSA 2009 and 2010 Statements and the EFSA 2008 Scientific Opinion, and is based on peer reviewed scientific literature published since the EFSA 2010 Statement, information made available to EFSA following a call for data, and discussions with experts in the field of animal cloning. As reported before, Somatic Cell Nuclear Transfer (SCNT) can produce healthy clones, but a portion of the animal clones suffered from developmental abnormalities likely due to epigenetic dysregulation (incomplete nuclear programming) and died at various stages of development. For some of the live animal clones, in particular calves and piglets, health and welfare were compromised specifically within the perinatal and juvenile period. Also some of the surrogate dams were affected due to abnormal pregnancies. Food products from healthy clones, i.e. meat or milk, did not differ from products from healthy conventionally bred animals. The offspring of clones and their food products showed no differences with conventional offspring or products. Data on clones of farmed species for food production other than cattle and pigs have remained limited and do not allow for the assessment of food safety or animal health and welfare aspects. The cloning efficiency, defined as the number of live offspring as a proportion of the number of transferred embryos, remained about 6-15 % for cattle and about 6 % for pigs. When compared with *in vitro* fertilisation (IVF), for which the background percentage of live offspring per transferred embryo is 45-60%, the efficiency of cattle SCNT relative to IVF is 13-25%. To overcome the relatively low cloning efficiency researchers continue to amend cloning procedures, with limited improvements shown by some researchers.

KEY WORDS

Somatic Cell Nuclear Transfer (SCNT), Embryonic Cell Nuclear Transfer (ECNT), Food Safety, Animal Health and Welfare, Risk Assessment, Efficiency, Cattle, Pig, Cloned Animal.

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SUMMARY

The European Food Safety Authority (EFSA) received in December 2011, a request from the European Commission for an update of the possible scientific developments of cloning of farmed animals for food production purposes.

The present Statement follows the EFSA 2009 and 2010 Statements and the EFSA 2008 Scientific Opinion, and is based on peer reviewed scientific literature published since the EFSA 2010 Statement, information made available to EFSA following a call for data, and discussions with experts in the field of animal cloning.

Cloning by Somatic Cell Nuclear Transfer (SCNT) in cattle and pigs has produced healthy clones and healthy offspring that are similar to their conventional counterparts based on parameters such as physiological characteristics, behaviour and clinical status. With respect to food safety, there were no indication that differences exist for products (i.e. meat and milk) of healthy clones and their offspring compared with those from healthy conventionally bred animals. Data on clones of farmed species for food production other than cattle and pigs have remained limited and do not allow for the assessment of food safety or animal health and welfare aspects.

Health and welfare were compromised in a proportion of clones, mainly observed as increased mortality within the postnatal and juvenile period of calve and piglet clones, as well as in a proportion of the surrogate dams that were affected by abnormal pregnancies. For cattle and pig clones, epigenetic dysregulation remained the main source of anomalies throughout development. The majority of reports published over the last 2 years covers research to try to overcome this dysregulation. So far, no major breakthroughs have been reported on improved overall cloning procedures that would result in an increase of the SCNT cloning efficiency calculated as the number of live offspring as a proportion of the number of transferred embryos. This efficiency is still in the range of 6-15% for cattle and of 6% for pigs, albeit occasionally higher success rates were reported. If the comparator for cattle cloning is *in vitro* fertilisation (IVF), the background (i.e. the percentage of live offspring per transferred embryo from IVF) is 45-60% and the efficiency of SCNT when compared to IVF can be calculated as 13-25%.

No new elements have emerged that would change the previous EFSA Opinion on the possible environmental impact of cloning: i.e. cloning in itself of farmed animals poses no particular threats for genetic diversity or biodiversity; and from the limited data available there are no indications that would suggest new or additional environmental risks from farmed animal clones when compared to conventionally bred farmed animals. There is also no information available to suggest that such risk may exist.

No new information has become available since the EFSA 2009 and 2010 Statements and the EFSA 2008 Scientific Opinion that would lead, at this point in time, to a reconsideration of the conclusions and recommendations related to the food safety, animal health and welfare aspects, and environmental aspects of animal cloning of the 2008 Scientific Opinion and the 2009 and 2010 Statements of EFSA.

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BACKGROUND

As announced in its request to EFSA for the 2010 update on cloning, the European Commission presented a report on somatic cell cloning to the European Parliament and to the Council end 2010, providing details on the market situation of animal clones, their offspring, breeding material, and food products obtained from such animals. The European Commission report reviews science based issues related to the health and welfare of the animals as well as food safety issues as raised by EFSA in 2008, 2009 and 2010 (EFSA, 2008, 2009, 2010).

The European Commission is spear-heading regulatory debates on somatic cell cloning that explore potential measures on animal cloning for food production in the EU. An impact assessment on the possible measures on animal cloning for food production in the EU has begun and is planned to be finalised by the end of 2012. Based on this impact assessment a legislative proposal on animal cloning for food production is envisaged for 2013, with input from stakeholders and a report provided by an external contractor on the possible economic, social and environmental impact of these possible measures.

The main issues reported in the EFSA 2008 Scientific Opinion 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" and the 2009 and 2010 updates were as follows. There were limited numbers of studies reported for clones of farmed animals other than cattle and pigs; the health and welfare of a proportion of clones were found to be adversely affected, often severely and with a fatal outcome at a juvenile stage; there were no indications of any differences in food safety for the meat and milk of clones and their offspring when compared with conventionally bred animals; and no environmental impacts were envisaged, though the limited availability of data was acknowledged. There was also no information available to suggest such risks may exist. The main source of the adverse effects that may affect clones and result in developmental abnormalities was epigenetic dysregulation; the failure of the placental development following cloning was considered to be one of the reasons why the technique has a low success rate; adverse affects, however, varied between species. For example, as for other assisted reproductive technologies, large offspring syndrome (LOS) affects SCNT cattle but not SCNT pigs. This condition could result in difficult birth and health problems for the surrogate dams and cases of stillbirth. Neither of these phenomena appeared to affect the offspring of clones born through conventional breeding protocols. Cloning could contribute to breeding in a more rapid manner of farmed animals that show improved parameters on resistance to the common pathologies (e.g. mastitis, other infectious and parasitic diseases), fertility, welfare and others related to the general robustness of the animal. With respect to the concern that cloning might decrease genetic diversity, it is noted that if used appropriately, in connection with suitable management measures, cloning is not expected to adversely affect the genetic diversity among domestic species.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

Following the failure of the Conciliation procedure in March 2011 of the Novel Food Regulation, the Commission is examining the possibility to introduce a legislative proposal on cloning.
A comprehensive impact assessment on all aspects of cloning is foreseen before adopting the possible legislative proposal on cloning.
The EFSA Statements of 2010 and 2009 confirmed the EFSA Scientific Opinion of January 2008. The Commission would again appreciate an updated assessment of the current situation as regards the scientific developments in this area.
By this letter, it is formally requested to provide an update on the state of play on the possible scientific developments on the issue of cloning of farm animals for food production purposes.
The update would be appreciated by the end of June 2012[4].

[4] The request letter of the European Commission is accessible at http://registerofquestions.efsa.europa.eu/ under the question number EFSA-Q-2011-01270.

ASSESSMENT

1. INTRODUCTION

Cloning of farmed animals aims at producing healthy and fertile offspring through somatic cell nuclear transfer (SCNT) or embryonic cell nuclear transfer (ECNT). For SCNT, a wide variety of nucleus donor cells can be used from adult somatic tissues, while for ECNT, donor nuclei come from an early embryonic stage (and therefore require less reprogramming).

To be successful, a number of steps have to be made correctly during the cloning process. First, the donor nucleus carrying the desired genetic material should be chosen with care so that it is capable to remodelling and subsequent genomic reprogramming. That nucleus is then introduced into an enucleated oocyte cytoplasm capable of facilitating the nuclear remodelling. Further, this reconstructed oocyte needs to be adequately stimulated to initiate pre-implantation development, for which the culture environment must be compatible with the physiological requirements of the particular embryo. As from here, the steps of the cloning process are the same as in other assisted breeding methods: the embryo is transferred into a surrogate dam and the birth may be by natural delivery or by Caesarean section.

Much has been learned about the changes that occur to a nucleus after it is placed in the cytoplasm of an oocyte and about the effects of adapting culture conditions for embryo development. While the understanding of the reorganization that occurs is increasing, and successful SCNT clones continue to be produced around the globe, the process of cloning is still inefficient due to incomplete reprogramming. This may lead to aberrant gene expression profiles during the pre-implantation development, foetal development, and abnormal health conditions of neonates and young growing animals. Below, while reviewing the reported results of mainly SCNT (as there are very few data on ECNT), nuclear remodelling and reprogramming will be discussed, as well as techniques that may improve reprogramming.

Animal cloning is intended to produce virtually identical genetic copies of the donor animal to yield identical phenotypes. The animal clones, however, are very similar to their genetic source, but not completely identical from a genetic point of view: the usual degree of mutations may be present in the chromosomal genomes and the mitochondrial genomes will be different. These animal clones, may also have slightly different phenotypes due to individual variation in their epigenetic status, and due to contributions from the cytoplasm of the recipient oocytes.

Unless otherwise stated, the information in the current Statement refers to intra-species SCNT. Cloners switched from ECNT to SCNT in the early 2000s, because the latter allows to access a larger source of nuclei of the same genotype, to pre-select the gender of the clone and to select from of a larger amount of desirable known phenotypes. Therefore, this Statement does not assess the safety of the ECNT procedure, although limited available data sets have included ECNT clones and are reported herein. Results from inter-species SCNT are not included in this Statement.

The aim of this Statement is to identify recent information pertinent to the efficiency of cloning of farmed animals and its consequences for longevity and animal health and welfare of surrogate dams, clones and their offspring. These last parameters, falling within the remit of EFSA, were identified previously as areas of concern when applying cloning for animal breeding.

Ethical[5], economic and socio-cultural concerns or benefits regarding cloning of farmed animals fall outside EFSA's remit for science-based risk assessment and are not addressed in this Statement.

[5] http://ec.europa.eu/bepa/european-group-ethics/docs/publications/opinion23_en.pdf

2. SPECIES OF FARMED ANIMALS CLONED FOR FOOD PRODUCTION PURPOSES

Previously, EFSA reported (EFSA 2010) that somatic cloning has been successfully used in 22 different species and that most laboratories work on farmed animals, particularly on bovine, porcine, ovine, caprine, and equine species. It needs to be noted however, that clones of other species and their offspring, for instance rabbits, mule, buffalo and deer, could potentially enter the food chain. This Statement focuses on cloning of farmed animals species that are used for food production in Europe. Consequently, cloning of additional species such as elephant and raccoon, as reported recently, is not covered herein.

3. PURPOSES OF CLONING FARMED ANIMALS FOR FOOD PRODUCTION

The main reason for cloning farmed animals is to produce elite livestock for meat and milk production (e.g. Watanabe, 2011). Clones of elite animals give insurance for or facilitate the breeding for the elite quantitative and qualitative traits, such as milk and meat quantity and quality. Other traits that characterise an elite animal might be the resistance to disease (e.g. mastitis, other infectious and parasitic diseases) or improved fertility. Another reason for cloning is to preserve a particular type of wild-life species or domesticated ecotype. Indeed, the capacity of preserving extinct animals and maintaining genetic diversity through cloning has also been successfully applied for domesticated species such as native Anatolian grey cattle (Arat et al., 2010; 2011) and for companion animals. Since this Statement is dedicated to cloning of farmed animals for food production, the cloning of farmed animals for purposes other than for food production is not included herein.

4. CHANGES TO CLONING PROTOCOLS FOR FARMED ANIMALS OVER THE LAST 2 YEARS

As mentioned in the introduction, current cloning protocols are composed of many different steps and inefficiencies are most likely due to incomplete or faulty reprogramming of the genome of the donor cell. Further studies on epigenetic reprogramming of farmed animal clones have been performed and provide some new insights into the fundamental biology of epigenetic reprogramming processes themselves as well as in the way to optimize them. Indeed, there have been contributions of farmed animals to the scientific understanding of animal cloning as epigenetic reprogramming, epigenetic alterations versus (ab)normal phenotype, and the concept of clones as epigenome variants has been further documented in farmed animals.

Many research groups worked on the **handling of somatic nucleus donor cells and recipient enucleated oocytes (ooplasms) at the level of embryo reconstruction and activation conditions**. Under the hypothesis that alterations frequently associated with SCNT stem from the initial remodelling of the somatic nucleus and/or from the early reprogramming of genes that control the first steps of development, several attempts were made to modulate the remodelling and early reprogramming events of the somatic nuclei by modifying their micro-environments when in culture to determine whether it is possible to increase yield of blastocysts and the efficiency of cloning. The duration of exposure of the somatic nucleus to ooplasm factors before induction of the activation process that initiates development, and the activation process itself, are also critical steps of any cloning procedure. These steps were investigated by several research groups; however, so far no marked advances were reported over the last 2 years.

Other research focussed on **the *in vitro* culture conditions of early developing embryos in order to unravel further nuclear remodelling and genomic reprogramming** in relation to epigenetic modification occurring in the genome. Progress in cellular imaging and high-throughput molecular analysis of DNA methylation and histone modifications stimulated an already active field of research including epigenetics, stem cell biology, reproductively biology, and veterinary sciences. In this context, SCNT-derived embryos of farmed animals were often used as models to extend observations made in the mouse. Such mouse experiments have for instance aimed at altering the micro-environments of the embryos to make it compatible with a more complete nuclear reprogramming and ultimately resulting in improvements in SCNT efficiency. However, few papers provide an *in vivo*

validation of observations made *in vitro*, mainly because of the costs of experiments with large farmed mammals.

Also the **risks of disease transmission through cloning** has been addressed. Some data on the potential disease transmission through cloning have become available and show that risks are reduced to negligible when appropriate precautions are adopted in the SCNT embryo production and transfer, in line with published guidance documents (e.g. by the International Embryo Transfer Society IETS).

Yet other studies have analysed **the foetal and placental development** with respect to epigenetics and late emerging effects thereof. Abnormal implantation and placenta formation have been frequently observed after transfer of SCNT embryos into recipient surrogate dams. These contribute to the low efficiency of cloning and are the main origin of most of the welfare problems encountered by recipient surrogate dams around birth. The gene expression profile in the placenta and at foetal stages has been studied as well as the physiological features of clones. Incomplete nuclear reprogramming after SCNT and failure to re-establish appropriate epigenetic marks during development, particularly in regions controlling the expression of imprinted genes, are considered as the major causes of pregnancy failure with SCNT embryos. Some improvements have been made over the past two years to the analysis of pregnancy dysfunctions but these have not yet led to marked improvements in the reduction of pregnancy failures with SCNT embryos in farmed animals.

At this stage, one or more improvements made to cloning procedures cannot be considered as common for all species nor as a major breakthrough during 2010-2012. Suboptimal nuclear reprogramming remains to be considered a main cause of pathologies and mortalities in clones.

Cattle and pigs are the two leading species in terms of numbers of published papers and propositions for improving technical cloning protocols, which differ between the two species. However, several advances in efficiency gains obtained during 2010-2012 but in the mouse academic animal model - where **potentially controllable reprogramming errors have been identified** - may be expected to steadily lead to improvements per species (incl. animals with long production cycles, such as farmed animals).

Furthermore, techniques for **prediction of pregnancy outcomes and the health prognosis of growing foetuses** are in development. Advances in the medical uses of such techniques (e.g. of imaging techniques) are being considered for increasing the prognostic evaluation of clone pregnancy outcome.

An overview of marked studies from the last 2 years in the above-mentioned areas for changing the cloning protocol for farmed animals is given and referenced in Appendix 1.

5. CURRENT EFFICIENCY RANGES OF CLONING IN CATTLE AND PIGS

5.1. Efficiency range calculations

Watanabe and Nagai (2011) evaluated cloning efficiencies as the numbers of (1) pregnant recipient surrogate dams, (2) calves born, (3) calves living at birth, (4) calves living at 24 h, and (5) calves living at 6 months, as a percentage of SCNT embryos transferred into recipient surrogate dams.

Cloning efficiencies can thus reflect different things, such as the implantation rate of the embryos, or the pregnancy rates, or the percentage of live births, or the proportion of live and healthy adult clones.

For cattle, in which 1-2 embryos are implanted per surrogate dam, helpful information can be derived from calculating general cloning efficiency as the number of healthy clones that live past the juvenile stage compared with the number of recipient surrogate dams of transferred SCNT embryos. Wider use of one uniform calculation would allow research results to be more readily compared.

Basing the calculation on the number of healthy clones that live past the juvenile stage informs about the full success of the process, including the element of health and welfare of clones and their surrogate dams. On the other hand, calculations based on live births only are a good indicator of the nuclear reprogramming capacity, meanwhile excluding post-natal deaths. Post-natal deaths can indeed be due to other reasons than the cloning and can be linked to the herd management.

Calculations based on the number of recipient surrogate dams of transferred SCNT embryos and not only on the pregnant recipients, also takes into account all of the surrogate dams used, including those which did not become pregnant.

Calculations based on the number of transferred SCNT embryos, instead of surviving animal clones, would also include the proportion of natural embryonic mortality (abortions as part of natural selection) during the first days after fertilisation. These mortalities occur in any species at rather high frequencies; e.g. 35-50% for cows (Sreenan and Diskin, 1986; Sreenan et al., 1997) and 30-40% for pigs (Bolet, 1986; Lambert et al., 1991).

The above calculation (the number of healthy clones that live past the juvenile stage compared with the number of recipient surrogate dams of transferred SCNT embryos), can also be applied to pig cloning. The domestic pig is a multiparous species with an average litter size of 10-12 and under current cloning practice receives up to 100 or more SCNT embryos per surrogate dam, depending on embryo quality at day of transfer. Therefore, there are two additional parameters to assess cloning efficiency for pigs; the first is the number of pregnant and delivering surrogate dams per total surrogate dams; the second is the average litter size per recipient surrogate dam.

5.2. Variability and comparators for efficiency ranges

Efficiencies of cloning vary not only between species, but even between the type of donor cells, genotypes and culture medium conditions. Variations are important between donor cell lines, as one quarter of donor cells never develop into live offspring (results in cattle reported by Panarace et al. (2007)). No new data that inform about this variability have been reported.

EFSA considers that cloning efficiency should not be compared with natural breeding since the objective of cloning is not to be an alternative for natural breeding but for other ART techniques based on *in vitro* fertilization. However, it should be noted that background success rate of conventional reproduction with AI, as the number of females that did not return to oestrous cycles after insemination, is in the range of 55-60 % for cattle under farm conditions and 45-55% for pigs. Importantly, the factors that cause pregnancy failure here, are equally present for pregnancies of recipient surrogate dams carrying SCNT embryos. For IVF in cattle, the background success rate as the percentage of live offspring per transferred embryo is 45-60% (Chavatte-Palmer et al. (2012), reviewing original data published by Hill et al. (2000) and Heyman et al. (2002)), and again the factors that cause failure for IVF are acting for cloning as well.

Furthermore, if a given % of cloning efficiency would be compared to yield a relative efficiency, it should only be with efficiencies of other techniques using *in vitro* produced embryos of the same species, not *in vivo* embryos; this is to take into account the efficiencies of standard species-specific embryo transfer procedures. For example for cattle, the SCNT percentage of live offspring per transferred embryos is 6-15% (Chavatte-Palmer, 2012), see section below. If the comparator is cattle IVF, then the background percentage of live offspring per transferred embryo is 45-60% (Chavatte-Palmer, 2012) and the efficiency of cattle SCNT relative to IVF can be calculated as 13-25%. If the comparator would be AI, then the background is 55-60% and the efficiency of cattle SCNT relative to AI can be calculated as 11- 25%.

5.3. Efficiency ranges for SCNT in cattle and pigs

So far, no major improvements in cloning efficiency of farmed animals have been published since the EFSA 2010 Statement. Therefore, the efficiency of cloning as previously described for cattle and pigs (EFSA, 2010), and expressed as the number of live offspring as a proportion of the number of embryos transferred, remains approximately 6-15 % for cattle and 6 % for pigs. There are occasional reports from companies offering commercial cloning services indicating that success rates for cattle as high as 40% can be obtained provided the entire chain of the SCNT cloning process, including for example the choice of donor cells and the delivery of the clones, has been optimised. EFSA notes that mere refinements of overall cloning protocols to increase cloning efficiency are unlikely to be published in peer-reviewed journals due to their lack of hypothesis generating issues, and the likelihood that many of these improvements may be the proprietary information of private concerns.

Recent calculations for cattle cloning efficiency in Japan[6] were provided by Watanabe and Nagai (2011) on the basis of clones living from 1998-2007. These calculations were based on higher numbers of SCNT clones (e.g. 301 SCNT calves) and confirmed the previously cited ranges for cattle reported in the EFSA (2010) Statement. While the authors report 8 % as the number of live births as a proportion of the number of transferred embryos, the cloning efficiency calculated as the number of healthy clones that live past the juvenile stage as a proportion of the number of transferred embryos is reduced below 5% due to a considerable amount of post-natal deaths. For additional information on health and welfare observations of live clones in Japan, see Section 6.

For pigs, Schmidt et al. (2010) reported 7.3% ±0.6% as the mean rate of born SCNT piglets as a proportion of transferred embryos into Large White (LW) sows. Liu et al. (2010) using the Guangxi Bama minipig also found that the efficiency of cloning was low: 4 recipients and 328 SCNT embryos resulted in just one male pig clone. Schmidt et al. (2010) achieved greater pregnancy rates and larger litter sizes (6.1 vs 4.2) by increasing the number of transferred embryos further (60-120 embryos compared with less than 60) and when the embryos were placed into both uterine horns rather than into just one (74% vs 44% delivery rate). They took into account that highly-inbred minipigs generally have reduced fertility and litter size.

6. HEALTH AND WELFARE OF ANIMALS INVOLVED IN THE CLONING PROCESS

For cattle, earlier EFSA Statements highlighted concerns over the health and welfare of a proportion of the surrogate dams, and a proportion of the clones at various stages of development (but not for the offspring of clones). The health and welfare of some of the surrogate dams of clones can be adversely affected due to complications in pregnancy from abnormal placentation (e.g. abortions, placental deformities, large foetuses). Abnormalities of some of the clones were found during the perinatal period (joint anklyosis, respiratory disease) with other problems reported up to 6 months of age. It is noted that these types of abnormalities observed are not unique to clones and occur also during conventional breeding albeit at lower frequencies. It has also become clear that once clones have survived the neonatal period and have reached approximately 6 months of age (cattle), further development appears normal. Sexually reproduced offspring of clones appeared normal at all stages of development. The literature from the past 2 years on health and welfare of cattle and pig clones, sexually reproduced offspring of clones, and surrogate dams is summarised below.

6.1. Health and welfare of surrogate dams

Cattle: Foetal overgrowth and loss of allometric growth regulation, collectively known as large offspring syndrome (LOS), is one of the most common SCNT foetal phenotypes, along with placentomegaly (Chavatte-Palmer et al., 2012; Constant et al., 2011; Kohan-Ghadr et al., 2011). Out of 20 SCNT pregnancies reported by Kohan-Ghadr et al. (2011), 12 pregnancies did not proceed to

[6] Some technological peculiarities in the Japanese practice in recent years (cold storage of ovaries for an extended period of time, in order to check the BSE status of the donors) has resulted in a reduction of live births, creating a slight negative bias in the data compared with other countries.

term: (1) therapeutically aborted for humane reasons (N=4), (2) delivered via Caesarean section (N=3), or (3) spontaneously aborted (N=5). Abnormal placentation was observed; the placentomes were larger and longer than normal, especially during the last trimester. Chavatte-Palmer et al. (2012) reported that pre- and post-implantation losses can affect up to 70% of pregnancies. They further observed that steroidogenesis during pregnancy was perturbed and that abnormal oestrogen production and metabolism probably play an important part in the increased gestation length as frequently observed with clones. This may have welfare implications as longer pregnancies can lead to larger calf sizes and a higher chance of dystocia, although this also happens in non-clones. Kohan-Ghadr et al. (2011) found that at day 80 of pregnancy, progesterone concentrations were lower in surrogate dams carrying clones than in controls, whereas oestradiol levels and pregnancy-specific protein B were higher than in conventionally-produced pregnancies (Chavatte-Palmer et al., 2012). Placental glucose transport and fructose synthesis also appeared to be modified in neonatal clones, and gene expression analyses of the bovine SCNT placenta showed that multiple pathways and functions were affected (Chavatte-Palmer et al., 2012). Plasma glucose concentrations were lower in surrogate dams with clones than in the control group at day 257 of pregnancy and at parturition (Hirayama et al., 2011). It was also observed that the plasma glucose concentration at term was lower than normal. As such, this is not considered to create a health problem for the surrogate dam. In view of the fact that the birth weight of the clones was significantly higher than that of the control group, reduced maternal glucose levels could allow a rough prediction of the occurrence of LOS at least after the 4th week pre-parturition and could help to decide on an early Caesarean delivery.

Pigs: Schmidt and co-workers (2011) found an overall pregnancy rate (defined as the number of pregnant sows compared with the number of sows that have received embryos) of 62% in Large White (LW) sows carrying SCNT embryos, with 26% of the pregnancies aborting or resorbing at around day 35 of pregnancy. With LW litters, 59 piglets (+ 3 mummies) were born in total from 15 pregnancies: 47 piglets were born alive, while 12 (+ 3 mummies) were born dead (Schmidt et al., 2010). Of the 47 piglets born alive, 8 were dead in less than 10 days. The authors found several placental abnormalities such as endometrial oedema in 14 sows delivered by Caesarean section.

Watanabe (2011) reviewing pig cloning in Japan (Duroc and Jin-Hua breeds) recorded that 97.8% of the surrogate dams were induced for birth and that 24.4% of the foetuses were stillborn compared with 5.6% of births from conventionally bred pigs. Schmidt et al. (2011) found that stillbirths in spontaneous deliveries by LW and minipigs were higher than in induced deliveries (56% vs 24%) with deaths often being caused by asphyxia (no further details given). The latter observations could be explained by the lack of correct hormonal balance often observed with term SCNT foetuses that do not engage in the delivery as normal. This is considered to be the physiological reason for the prolonged pregnancies and delivery problems with clones. Induced deliveries can reduce the proportion of stillbirths and the proportion of surrogate dams suffering from these abnormal deliveries; however this proportion still remains higher than for the conventionally bred animals.

6.2. Health and welfare of clones

Cattle:
Studies of clones in Japan (Watanabe, 2011) show that those animals surviving for more than 200 days were no different to normal animals in terms of health status, growth rate, fertility and production of meat and milk. However, in the first 30 days of life, losses due to disease in clones (SCNT) were higher than in conventional animals (CV) (see Table 1). For a group of 17 calves, large offspring syndrome (LOS) symptoms were examined: 1 to 3 days after birth 6/17 (35.3%) of these died of respiratory disease and 2/17 (11.8%) died due to deformed hearts. After the 4th day after birth, deaths by pneumonia were observed, but no figures were given.

Table 1: Observations of deaths due to disease in SCNT cattle (Watanabe, 2011). SCNT = Somatic Cell Nuclear Transfer Clones, CV = Conventional animals, CP = Progeny from SCNT Clones crossed with CV, sd = significantly different, nsd = not significantly different, * = sd at p = or < 0.05 from CP and CV.

Death due to disease	Nr of death animals out of the total numbers studied[(a)] (%)			Probability
	SCNT[(b)]	CV	CP	
Overall	94/482 (19.5%)	Figures not given	14/202 (6.9%)	
Stillbirths	79/482 (16.4*%)	26/566 (4.6%)	18/202 (8.9%)	SCNT sd to CP and CV (p <0.05). CV and CP nsd
Neonatal (< 24h)	65/451 (14.4*%)	11/566 (1.9%)	1/124 (0.8%)	SCNT sd to CP and CV (p <0.01). CV and CP nsd
2 - 150 days	72/307 (23.5*%)	55/1289 (4.3%)	5/111 (4.5%)	SCNT sd to CP and CV (p <0.01). CV and CP nsd
150-300 days	5/202 (2.5*%)	6/1207 (0.5%)	0/94 (0%)	SCNT sd to CV (p <0.01).
300-720 days				No significant differences
After 720 days				No significant differences

(a): When examining data in Table 1, it is important to consider that the cattle population groups addressed in this Table are data obtained at one point in time available from the national survey in Japan. Therefore, while the horizontal comparison between groups (i.e. SCNT clones versus conventional animals or clone progeny at the same point in time) is valid, a vertical comparison (over time) is not. Therefore, it is not possible from Table 1 to draw any precise conclusion on how many animals in a specific cattle group died at birth, during the neonatal period, between 2 and 150 days, between 150 and 300 days or later, although general information on lethality observed at different ages among cattle clones, conventional cattle and clone progeny can be derived.

(b): For some of the numbers ECNT clones might be included.

In another report, Watanabe and Nagai (2011) described survival rates of transferred bovine embryos and the percentage of calves derived from SCNT had not improved over a decade (1998–2007). A feature of SCNT embryos was the high incidence of spontaneous abortions, with 8.3– 27.1% of the pregnant recipient surrogate dams losing their foetuses after 100 days of gestation. These survival rates are lower than those for foetuses bred by multiple ovulation and embryo transfer (MOET) or *in vitro* production (IVP). The variation in survival rates for SCNT calves at birth, 24h and 6 months were 4.6–11.0%, 3.4–9.1% and 2.2–7.5%, respectively.
In another pool of 25 neonatal calf clones, 22 suffered from respiratory disease, 9 of which required ventilator support (intra-nasal oxygen); 3 of these later died and one was euthanised (Brisville et al., 2011).

Chavatte-Palmer et al. (2012) observed that neonatal calves had high fructose levels although blood glucose levels were normal. Also a previous report showed that surviving SCNT clones have haematology and clinical chemistry values for plasma glucose within normal ranges (Hirayama et al., 2011).

Ten cow clones obtained from oviduct cells and which had shortened telomeres, had reached the age of 5 years (Miyashita et al., 2011). These clones have delivered healthy offspring with normal telomere length after AI with semen from conventional animals. However, the authors found evidence of poor wound healing, alopecia, rough hair, wrinkled skin and lameness in one of the two groups of clones with shortened telomeres. When the above ten clones with shortened telomeres were 3 years old, they appeared to be normal based on growth rate, time of onset of puberty, age at first conception, calving interval, gestation length; and also volume milk (compared with the cows from which they obtained the donor nucleus). In this experiment, also one clone was generated from uterus cells which

did not have shortened telomeres. The authors also described considerable variation in telomere length in the normal control population and in the clinical signs shown by animals with varying telomere sizes. No direct relation between telomere length and aging of clones could be demonstrated.

The ontogeny of disease observed with certain clones in relation to defective reprogramming of different classes of genes, here imprinted genes, has also been studied:

During the SCNT process itself, the nuclear DNA of the donor adult somatic cell that is transferred into an unfertilised oocyte, needs to be reprogrammed for the development of different tissues. Some of the defects of such reprogramming are not observed until after birth and one of these is for Mash2, an imprinted gene that plays an important role in embryogenesis and embryo development. Imprinted genes are expressed differently based on parental inheritance. Since lungs of deceased bovine clones often exhibit aberrations, the methylation status of Mash2 was checked in such samples. Chen et al. (2010) concluded that Mash2 was abnormally methylated in these animals, which may have contributed to aberrant lung development and the low survival of these clones.

Couldrey and Lee (2010) compared a panel of imprinted, non-imprinted and satellite repeat sequences in mid-gestation SCNT clones. Various tissues were collected from viable and failing mid-gestation SCNT foetuses and compared with similar tissues from gestation-matched normal foetuses generated by AI. Most of the genomic regions examined in tissues from viable and failing SCNT foetuses had DNA methylation patters similar to those in the control tissues. However, specific DNA regions rich in CG nucleotide basepairs (CpG sites) in only some regions of the genome tend to be hypomethylated only in SCNT tissues. Also, DNA methylation pattern in tissues of failing SCNT foetuses were similar to apparently viable SCNT foetuses, although there were individuals showing extreme deviant patterns. Therefore it can be concluded that SCNT foetuses largely undergo reprogramming, but the epigenetic signature at this stage cannot predict whether the foetus will develop to term or not.

Longevity and reproductive physiology of cattle clones
Ten SCNT clones when bred with conventional bulls have given birth to 3 calves each per year (i.e. 30 offspring in total of which 2 calves were stillborn) and they all exhibited normal fertility and productivity parameters (Miyashita et al., 2011).
Male clones (bulls) had semen DNA methylation patterns within the normal range and normal DNA methylation patterns were also reported in their offspring (Couldrey et al., 2011).
Four groups of clones from different breeds (18 clones in total), were artificially inseminated after reaching the breeding age and breeding was repeated until the third, fourth or sixth parity, depending on the breed. Taking into account disease/injury/accident-associated damages, the survival time and mean parity of the clones was found favourable when compared to the control group (Konishi et al., 2011).

Pigs:
In some cases, weights at birth of surviving clones were found to be higher than conventional animals (Martinez-Diaz et al., 2010; Schmidt et al., 2010; Watanabe, 2011) but others have reported the opposite (Estrada et al., 2007; Vajta et al., 2007). However, weight at birth is critically dependent upon litter size; small litters will result in higher individual birth weights, irrespective of the production method. Researchers, having produced >400 transgenic clones of pigs, did not observe oversized piglets in litters with normal size (Niemann et al. (2012), oral communication).
In another study, total perinatal mortality for two pig breeds was 49% although it was lower in LWs (34%) compared with minipigs (56%) but still higher than in conventional pigs (10-15%) (Schmidt et al., 2010). Watanabe (2011) also reported on neonatal deaths, which were seen more frequently in SCNT clones than in their progeny (8.9 vs 1.4%), and post-perinatal losses due to disease were 27.7% for SCNT vs 11.9% for progeny of clones. These figures for clone progeny were very similar to independent data for conventional animals as reported by the National Animal Disease Information Service (NADIS)[7], (2011).

[7] http://www.nadis.org.uk/

The percentage of SCNT piglets born with malformations was higher than in normal animals: 7% for LW clones and 26% for minipig clones. The piglets were killed when they were malformed for example in their legs, heart, diaphragm, tongue or testes, or when they did not function normally and had for example anorexia or no weight gain (Schmidt et al., 2010). At post-mortem examinations the organ weights of the heart, small intestine and kidney were lower than in conventionally bred piglets, whereas the weight of the pancreas was higher, and liver weight was borderline ($p = 0.057$) (Schmidt et al., 2011). In another report, haematology and clinical chemistry of surviving clones appeared normal (Watanabe, 2011).

Longevity and reproductive physiology of pig clones

Schmidt et al. (2010) reporting 39 SCNT surviving clones that have been kept in standard farmed conditions and killed occasionally for experimental purposes, found that all lived and none died naturally (one pig was 3 years old). During their lifetime none of the clones showed abnormalities in growth, behaviour or disease incidence. Nine 1 year-old male clones exhibited normal semen parameters; and when male and female clones were bred with conventional animals, their fertility appeared normal (Schmidt et al., 2010).

Further to the fertility of pig clones, Liu et al. (2010) bred one male clone with conventional females to produce 98 (93 surviving) F1 offspring. They then outbred 3 of these F1 pigs to produce 22 F2 piglets in 3 litters of which 14 piglets survived (one pig gave birth to 8 dead piglets accidently, which is also common in naturally bred groups). None of these breedings was outside normal reproduction parameters (given the small numbers involved).

6.3. Health and welfare of offspring of clones

Adverse effects of cloning due to incomplete reprogramming were shown to result in difficult birth and health problems for a proportion of the surrogate dams and premature mortality of a proportion of the clones; these phenomena were shown to vary between species. However, neither of these phenomena appears to affect the offspring of clones, born through conventional breeding techniques.

Offspring of cattle clones

For cattle this was further supported by the above-mentioned report of Couldrey et al. (2011) who analysed the sperm DNA methylation patterns in ten genomic regions of 3 eighteen-month-old Friesian bull clones. There are very low levels of variation between the DNA methylation patterns from sperm of conventionally bred males even of different genetic background. Equally so, no differences were observed between sperm from the 3 bull clones and respective controls. This provides evidence that the donor cell genome is correctly reprogrammed upon passage through the germ line in males.

Miyashita et al. (2011) found no differences in telomere length in 30 offspring from cattle clones when inseminated with semen from conventional bulls and their zootechnical performance was normal.

Offspring of pig clones

Liu et al. (2010) mated a male Guangxi Bama minipig clone produced from testicular fibroblast cells with 13 conventional sows to produce 93 offspring; all appeared healthy. Farrowing rate, litter size, and piglet survival were no different from controls. To evaluate the fertility of the clones' offspring, the authors also produced 14 F2 normal piglets from three F1 females: three F1 female pigs from the cloned minipig were artificially inseminated with semen from conventional Duroc or Landrace sires, giving rise to 22 piglets, of which in the 3 litters: 13 piglets were born alive, 1 born alive, and in the third litter all 8 were dead; all 14 survivors appeared healthy.

Watanabe (2011) found that stillbirths and neonatal deaths in the offspring of SCNT clones were 5.6% and 1.4% respectively. All of these piglets were delivered by natural parturition and the birth weight from piglets surviving the perinatal period were similar to that from conventionally bred piglets.

As previously reported, there are no indications found for health and welfare concerns of offspring of cattle or pig clones.

Recloning

Recloning is using a somatic cell of an animal clone to make a new clone. Wang et al. (2011) extended the work of Kubota et al. who had already shown in 2004 that second-generation clones (C2), generated from a somatic nucleus of a first-generation donor clone (C1) could be healthy and of normal fertility. Wang et al. (2011) evaluated the cloning efficiency of second-generation cloning of Holstein cow clones derived from ear fibroblasts of a first-generation cow clone, and assessed their health status in terms of physical, growth and reproductive parameters. After embryo transfer, both pregnancy rate to term and calving rate of C2 cloning were approximately half of C1 cloning (5.8% vs. 10.7%; 3.9% vs. 8.6%, $p > 0.05$). Six C2 clones were delivered, and three of them survived. These C2 clones displayed signs of being overweight at birth and tachycardia in the first week after birth. In summary, the results showed that although cloning efficiency of C2 was lower than that of C1, the surviving C2 (3 out of 6 died) clones appeared physically healthy and were fertile.

6.4. Conclusions on health and welfare of surrogate dams, clones and their offspring

Although there have been many attempts to improve cloning techniques, no major changes have been reported that reflect positively on the compromised health and welfare of a proportion of the surrogate dams and of a proportion of the cattle and pig clones themselves. Although guidance[8] (IETS-HASAC, 2008) has been published for veterinary practitioners to check the health and welfare and management of animals involved in cloning, there are few detailed whole animal reports published with a broad range of health measures[9]. Rather the emphasis is on recording gross events such as survival, stillbirths, birth weights, malformations, and clinical chemistry and haematology. The use of more uniform criteria for checking the health status of surrogate dams and clones would be helpful.

Those clones that survive often appear no different to conventional animals, although sample sizes and numbers of animals involved may often not be large enough to make robust conclusions, particularly for less frequent adverse events. Using a uniform and broader range of criteria combined with meta-analyses would increase sample sizes, as was achieved through a nation wide survey of bovine clones in Japan (Watanabe, 2011). This would also be helpful for other species of clones and/or for other regions.
With regard to the health and welfare of the progeny from breeding clones with conventional animals no health problems have been reported.

[8] « Health Assessment and Care for Animals Involved in the Cloning Process. A consensus recommendation from the International Embryo Transfer Society », 15 May 2008, elaborated by the IETS Health And Sanitary Advisory Committee (HASAC) and approved by the Board of Governors of this scientific society (incl. scientists and practitioners).

[9] **Checking the health status** should comprise aspects and measurements as advised by formal or acknowledged experts in the relevant clinical specialities, for example, *inter alia*, (1) Clinical examination and associated laboratory tests; (2) Zootechnical data (e.g. normal development, feed intake, growth rate, fertility, feed conversion rate, productivity indices); (3) Measurements of relevant aspects of physiology, such as immune function (e.g. immunological challenge with antigens producing B-cell or T-cell responses, temperature tolerance); and (4) Health records, for example, evidence of the results of post-mortem and associated laboratory examinations of all animals found sick or dead in order to identify the cause of death; post-mortem examination of all animals slaughtered or euthanised at the end of the experiments; and incidence of body injuries, body malfunctions and disease (infectious and non-infectious), and medicine use, should all be taken into account.
Checking the welfare should address the ability of the animals to carry out normal behaviour, physiological functions and to develop normally. Abnormal behaviours may relate to feeding, locomotion, social behaviour (e.g. aggression), and other responsiveness measures, such as flight distances. The occurrence of stereotypies, the extent to which strongly preferred behaviours can be shown, and the extent of any avoidance behaviours should be recorded (e.g. filming animals). For cattle and pigs, more detailed parameters have been described in Appendix 3 of EFSA 2012a (Scientific Opinion of AHAW Panel: Scientific Opinion on the use of animal-based measures to assess welfare of dairy cows *www.efsa.europa.eu/en/efsajournal/pub/2554.htm*) and in Appendix 2 of EFSA 2012b (Scientific Opinion of AHAW Panel: Scientific Opinion on the use of animal-based measures to assess welfare in pigs *www.efsa.europa.eu/en/efsajournal/pub/2512.htm*), respectively.

7. SAFETY OF MILK AND MEAT DERIVED FROM ANIMAL CLONES OR THEIR PROGENY

The scientific assessment to address the safety of food/feed products from clones requires the availability of a variety of data on food/feed products from animal clones and their offspring including those obtained through analytical and nutritional investigations as well as toxicity testing dealing with many different end points.

7.1. Compositional data

Several studies on the composition of bovine milk and meat and of pig meat, including water, fat, protein and carbohydrate content, amounts and distribution of amino acids, fatty acids, vitamins and minerals, were reviewed by the EFSA's Scientific Committee (EFSA, 2008) without identifying any differences outside the normal (physiological) variability between clones (F0) or clone progeny (F1), and their comparators.

In a study reported by Ito and Watanabe (2011), meat was derived from three progeny (F1) of conventional cows inseminated with semen from an SCNT bull and the milk from three progeny (F1) of cow clones (F0) inseminated with semen from a conventionally produced bull. Both these meat and milk were compared with the corresponding products from conventionally bred cattle. No significant differences in any components, including water content, ash content, cholesterol, calcium, amino acids, fatty acids and vitamins, were observed as depending on the origin of the milk or meat.

In the report by Watanabe (2011) three of each ECNT cows, SCNT cows and conventionally bred cows were employed for producing milk, whereas one ECNT steer, one SCNT steer and three conventionally bred steers were fattened for meat production. Three of each progeny of SCNT cows and conventionally bred cows were employed for producing milk and three of each progeny (heifer) of SCNT cattle and conventionally bred heifers were fattened for producing meat. Normal compositions for a variety of parameters were determined in milk and meat from animal clones and their progeny. Some individual variations were observed, but they were not related to cloning.

Moreover, the use of SCNT cattle and their progeny to evaluate milk and meat productive performance in comparison with conventionally bred cattle was reported by Watanabe (2011). The parameters investigated included, for milk, milk yield, lactation curves and a variety of quality indicators such as total fat, total protein and somatic cell counts, whereas, for meat, they included body weight gain, carcass traits and physiological properties. Only variations within the normal range were detected in this study.

Changes in sensory (taste) traits of longissimus muscle (LM) were investigated using SCNT clones of Japanese black steers slaughtered at different ages (20, 25 and 30 months) and analysed jointly with fat content, soluble collagen content and collagen solubility (Okumura et al., 2012). Although this investigation was not performed comparatively with muscle from conventionally bred animals, the results reported did not point out any major peculiarity possibly associated with cloning.

7.2. Protein digestion rate

Data on protein digestion rates were obtained from rats fed with milk/meat derived from conventionally bred cattle and progeny of SCNT cattle. The digestion rates of milk were 88.8% for that from conventionally bred cattle and 86.8% for that from the progeny of SCNT cattle. With regard to meat, the digestion rates were 90.3% for conventionally bred cattle and 89.5% for the progeny of cattle clones. Therefore, no significant differences in digestibility due to the origin of milk or meat were reported (Ito and Watanabe, 2011). Similar results were also described by Watanabe (2011) for milk/meat derived from the ECNT/ SCNT cattle clones as well as from the progeny of SCNT clones.

7.3. Allergenicity

The 2008 EFSA Scientific Opinion reviewed the studies by Takahashi and Ito (2004) and by Heyman et al. (2007) showing the absence of any statistically significant difference in the allergenic potential

observed in the rat between meat and milk from clones and from comparator control cattle.

The mouse abdominal wall method was also applied more recently by sensitizing test mice through intra-peritoneal injection of the water (saline) soluble fraction of milk/meat powders derived from the ECNT/SCNT cattle or the progeny of cattle clones and from conventionally bred cattle; 14 days after sensitization, the mouse abdominal wall was exposed under anaesthesia and the elicitation solution was injected into the abdominal wall. No significant differences in anaphylactic reactions due to the origin of milk/meat were detected (Ito and Watanabe, 2011; Watanabe, 2011). It is though well known, that these results are only indicative as the rat and mouse models are not specific for human allergenicity predictive testing (WHO/FAO, 2001).

7.4. Genotoxicity

As already reported in the 2008 EFSA Scientific Opinion on animal cloning derived by SCNT, Takahashi and Ito (2004) did not show any genotoxic potential in the meat derived from cattle clones tested using the mammalian erythrocytes micronucleus test.

Data on the genotoxic potential of milk/meat powders derived from the ECNT/SCNT cattle clones or from the progeny of SCNT cattle and conventionally bred cattle were recently reported by Ito and Watanabe (2011) and Watanabe (2011). The micronucleus test was used to assess potential genotoxicity. Mice were fed for 14 days with test diets supplemented with milk powder derived from progeny of cattle clones or from the ECNT/SCNT cattle at 2 or 10% (w/w) and the incidence of micronucleated red blood cells was assessed. No significant differences were observed compared to the control groups. In addition, polychromatic cell rate as a proportion of total erythrocytes was not significantly different from the value observed in the control groups. Moreover, mutagenicity of meat powders derived from progeny and conventionally bred cattle or from the ECNT/SCNT cattle were investigated in mice. In the test diet supplemented with meat powder at 1 or 5% (w/w), the incidence of micronucleated cells was not significantly different compared to the control group. The authors concluded that milk/meat derived from progeny of cattle clones did not show any mutagenic potential.

The results of *in vitro* (bacterial mutation and chromosome aberration) and *in vivo* (micronucleus) genotoxicity studies of SCNT cattle meat were published by Lee et al. (2011). Several concentrations of meat extracts from both conventional cattle and clones, were tested in five strains of bacteria (*Salmonella typhimurium*: TA98, TA100, TA1535, and TA1537; *Escherichia coli*: WP2uvrA) for bacterial mutation and to Chinese hamster lung (CHL/IU) cells for chromosome aberration, respectively. For the micronucleus test, mice were divided into five dietary groups: commercial pellets (control), pellets containing 5% and 10% conventional cattle meat, and pellets containing 5% and 10% meat from cattle clones. No test substance-related genotoxicity was noted in the five bacterial strains, CHL/IU cells, or mouse bone marrow cells.

7.5. Toxicity and reproduction studies

Results of 14-week feeding studies in rats given a diet of milk or meat from ECNT/SCNT cattle in comparison with milk and meat from conventionally bred cattle have been reported by Yamaguchi et al. (2007) and Watanabe (2011) with the conclusions that physiological conditions of the test animals had not been affected by any treatment.

Watanabe (2011) also reported the results of a 12-month feeding study in rats given a diet of milk or meat from progeny of SCNT cattle clones and conventionally bred animals. In this study, in addition to the parameters commonly determined to assess the health status of test and control animals, additional observations focussed on reproduction of dams and the health status of their pups. No biologically relevant indication emerged from this study that milk or meat from cattle clones was different from control products.

Yamaguchi et al. (2008) and Ito and Watanabe (2011) have reported results obtained through 12-month feeding studies with rat groups given a diet supplemented with the milk/meat powder from

progeny of somatic cell nuclear transfer clones or from corresponding products from conventionally bred animals. The rats were subjected to clinical observations of general health conditions. Moreover, sexually mature rats fed the test diets were mated and examined for indices such as the reproductive performances of the dams and health of their pups, without detecting any biologically relevant differences.

Furthermore, two reproduction toxicity studies, one in rats and the other in rabbits fed meat from cattle clones, were previously reviewed in the 2010 EFSA Statement on cloning. No biologically relevant differences associated with cloning were detected for the reproductive parameters.

The effects on the behavioural and reproductive characteristics of F1 rats derived from dams fed during pregnancy a diet supplemented with SCNT cattle meat were investigated by Yang et al. (2011). F1 rats were divided into five diet groups with their dams: commercial pellets (control), pellets containing 5% (N-5) and 10% (N-10) of conventional-cattle meat, and diets containing 5% (C-5) and 10% (C-10) of clone meat. In most cases, the cloned-cattle meat diet did not affect body weight and food consumption in male and female F1 rats during 11 weeks. However, the authors found significantly higher main body weight of males in N-5 (at 3 weeks), males in N-10 (at 3 and 5 weeks), and females in N-10 at 4 weeks. Rates of food consumption of males was significantly higher in the meat-diet groups (conventional- and cloned-cattle meat at 7 to 9 weeks), as compared with the controls, but not for females. No signs of test substance-related toxicities were detected on organ weights and behavioural characteristics (sensory reflex, motor function, and spatial learning and memory tests). Reproductive functions (mating, fertility, and implantation) did not significantly differ among all examined rats.

EFSA notes that not many publications have appeared on long-term toxicity studies of food products from animal clones over the last 2 years. It is understood that researchers might not be inclined to undertake such studies which are unlikely to indicate any significant differences associated with the use of SCNT cloning. Further, not all countries regard whole food feeding studies as providing required information.

7.6. Conclusion of meat and milk of clones and progeny

Since the publication of the EFSA 2010 Statement, several recent research results have become available dealing with genotoxicity and reproduction toxicity of milk and meat from SCNT cattle clones and their progeny. These recently published data, do not affect in any way the conclusion reached in the previous EFSA risk assessments and confirm that there are no indications that differences exist in terms of composition and food safety between food products from healthy cattle and pig clones and their progeny, compared with those from healthy conventionally bred animals. Moreover, data on clones of farmed species for food production other than cattle and pigs have remained limited and do not allow for the assessment of food safety or animal health and welfare aspects.

8. ENVIRONMENTAL IMPACT OF CLONING OF FARMED ANIMALS

No new elements have emerged that would change previous EFSA Scientific Opinions: i.e. cloning in itself of farmed animals poses no particular threats for genetic diversity or biodiversity[10]; and from the limited data available there are no indications that would suggest new or additional environmental risks from cloning farmed animals when compared to conventionally bred farmed animals. There is also no information available to suggest that such risk may exist.

[10] Biodiversity and genetic diversity are important protection goals in the EU : http://ec.europa.eu/environment/nature/biodiversity/comm2006/pdf/2020/1_EN_ACT_part1_v7[1].pdf

OVERALL CONCLUSIONS

The previous EFSA conclusions (EFSA, 2008, 2009, 2010) are confirmed on the basis of the review of the recent scientific literature and other information made available to EFSA.

- With respect to food safety, there are no indications that differences exist between products (i.e. meat and milk) of healthy clones or their offspring when compared with those from healthy conventionally bred animals.

- SCNT can produce healthy clones, but during the SCNT cloning process, animal health and welfare remains a matter of concern mainly due to mortality at all developmental stages (from the perinatal period until the juvenile stage, as well as mortality through abortion), which is higher than for other ARTs with *in vitro* produced embryos. Importantly however, there are no indications found for health and welfare concerns for offspring of clones born through conventional breeding.

- For cloning of farmed animals as such, there is no new scientific information that would suggest risk to genetic diversity, biodiversity or the environment, when compared to conventionally bred farmed animals.

- Data on clones of farmed species for food production other than cattle and pigs have remained limited and do not allow for assessment of food safety or animal health and welfare aspects. Large sample sizes that would allow more robust conclusions are also often not available.

- Continued research with farmed animals for the optimisation of the different steps of SCNT cloning has resulted in increased knowledge on the basic biological processes involved. Limited improvements were shown by some researchers, but so far have not led to a breakthrough for the overall cloning efficiency.

Furthermore it is considered that

- A harmonised framework to report any additional data on cloning efficiency would be helpful to have a normalized set for appropriate comparisons, if and when more data would be generated.

- A harmonised framework and a uniform set of species-specific parameters to report on health and welfare of clones in a more consistent manner would be helpful, if and when more data would be generated in the future.

DOCUMENTATION TO THIS EFSA STATEMENT

In order to answer the Terms of Reference, EFSA collected information as follows.

Literature search

From the background information, the previous EFSA work on cloning and the Terms of Reference, EFSA has identified the issues to be addressed in the present Statement. On 15 March 2012 the databases of ISI Web of Knowledge were searched with the search conditions as shown in Table 2, yielding 548 records. The search protocol was based on the one used for the EFSA 2009 and 2010 Statements; and was updated with the assistance of an information specialist of the EFSA SAS Unit.

Screening

The records were screened by the titles and the abstracts. Those that were considered for this Statement focus on food producing animals (e.g. mainly cattle and pigs), epigenetics, longevity, clones, clone progeny, animal welfare, health, toxicity tests, risk assessment etc. The following records were not considered: papers on molecular cloning of genetic constructs; duplicates; meetings records (unless of particular importance); transgenic animals and their cloning for medical research[11]; inter-species cloning; most reports on cloning of non-food/feed animals (e.g. mouse, rats); and papers from 2010 that were already cited in the EFSA 2010 Statement. The above eligibility criteria are essentially the same as for the 2009 and 2010 EFSA Statements and yielded approximately 90 papers that were studied in further detail. Key papers that are most relevant are reflected in this Statement.

Table 2: Search limitations as used on 15 March 2012 on the ISI Web of Knowledge.

Limitations			
Boolean operator		**AND**	**AND**
Search terms	Topic=(clon*)	Topic=("nucl* transfer")	Topic=(Animal OR embryo OR neonate OR placent* OR endometr* OR donor cell OR blastocyte OR Cow OR Cows OR Cattle OR Bovine OR Calf OR Calves OR Bull OR Bulls OR Bullock$ OR Heifer$ OR Livestock OR Pig$ OR porcine OR piglet$ OR Sow OR Bore OR Sheep OR Ovine OR lamb OR Goat OR Rabbit OR Horse OR foal OR mare OR Fish OR Chicken OR bird$)
Language	= English		
Timespan	= 2010-2012		
Lemmatization	= on		

(* and $): are truncations

Open call for data

As for the 2009 and 2010 EFSA Statements, a call for data was published on the EFSA website for 1 month (i.e. from 17 February to 17 March 2012). This call for data was further disseminated through existing networks such as the EFSA Advisory forum and focal points; the International Food Chemical Safety Liaison group (IFCSLG) that includes other international risk assessment agencies (notably from the US (FDA), UK (FSA), CA, NZ, AU, FR (ANSES) and JP); the European Commission services; and through targeted e-mails to cloning experts from cloning companies, EU research groups and organisations who previously contributed to EFSA's work on cloning.

During the call for data the following information, some unpublished, was received:

1. Individual scientist based in UK. 1 publication.

2. Individual scientist based in Italy. 1 paper accepted for publication.

[11] EFSA notes that most pig clones are transgenic and developed for medicinal research.

3. Individual scientist based in USA. 1 draft paper.

4. Breed society based in UK. E-mail, 1 page; a Policy position paper in relation to registration of the progeny from clones.

5. Individual scientist based in France. Two papers; a list of abstracts published in the proceeding of the International Embryo Transfer Society (IETS) annual meeting 2011; a poster presented at the same annual meeting; a PhD thesis defended in November 2011.

6. FDA liaison officer to EFSA. A list of 57 publications related to animal cloning.

APPENDIX 1:

CHANGES TO CLONING PROTOCOLS FOR FARMED ANIMALS OVER THE LAST 2 YEARS

The state-of-the art technical steps and the best practice in the classical **cloning procedures for bovine or porcine SCNT** were described recently by Niemann et al. (2011).

Aspects that influence the efficiency of cloning, as reported by EFSA in 2008, 2009 and 2010, include the handling of somatic nucleus donor cells and recipient enucleated oocytes (ooplasms) at embryo reconstruction, description of nuclear remodelling, *in vitro* culture conditions of early developing embryos and analysis of genomic reprogramming in relation to epigenetic modification occurring in the genome, gene expression in the placenta and at foetal stages, and physiological features of cloned calves. Substantial numbers of studies on these aspects have been performed and recent records are reviewed below. They reflect the remarkable efforts of the scientific community to try to overcome impeding factors for the efficiency of cloning in farmed animals. A priority is given below to studies based on the *in vivo* development of SCNT-derived embryos at the level of the foetus, neonate or live clone. Reports limited to the *in vitro* development of embryos to the blastocyst stage are also considered but only when of particular relevance for complementary scientific information to the *in vivo* achievements. Whenever possible, improvement in cloning efficiency has been evaluated by comparison with the development of non-cloned embryo obtained by the conventional techniques of *in vitro* fertilization (IVF) or artificial insemination (AI).

The main picture that emerges from the literature published between 2010 and 2012 on farmed animal cloning is that **progress in terms of cloning efficiency remains modest**. Cattle and pig are the two leading species in terms of numbers of published papers and propositions for improving technical protocols, which differ between the two species. However, several advances in efficiency gain obtained during the same period but in the mouse academic animal model - where some potentially controllable reprogramming errors have been identified - may be expected to steadily lead to improvements per species. These **future developments** are briefly presented herein as a conclusion to the improvement of the cloning protocol for farmed animals.

A.1 Update on the contribution of farmed animals to the scientific understanding of cloning

- *Epigenetic reprogramming*

Data published over the past two years have confirmed that demethylation of the DNA of donor cell genome occurs rapidly after SCNT but with differences between species and more importantly between SCNT embryos obtained from the same batch of donor cells. This was clearly shown during *in vitro* culture of cattle embryos by Sawai et al. (2011) in a comparative analysis of DNA methylation in individual blastocysts obtained eight days after SCNT, or *in vitro* fertilisation, or parthenogenetic activation, or embryonic cell nuclear transfer (ECNT), or *in vivo* (artificial insemination). Using the bisulfite method to **measure the methylation level** of cytosines within the satellite 1 region of bovine genome (a DNA repeated sequence with highly conserved motifs amendable to methylation (the CpG motifs)) the authors found that the DNA of SCNT blastocysts although less methylated than the DNA of donor cells remained about twice as more methylated than in non-cloned embryos. Marked individual variations were however observed within a given batch of blastocysts obtained from the same donor cells (40 to 60% of methylated sites). This variability was higher than between the three batches of blastocysts generated from three different donor cells. However, *in vitro* development to the blastocyst stage was similar in SCNT (21.9% from 183 one-cell stage embryos) and *in vitro* fertilised embryos (21.1% from 142 one-cell stage embryos). Moreover, full-term development efficiencies between three different cell donors were not different. Thus, changes in the DNA methylation level of SCNT pre-implantation blastocysts *in vitro*, although they reflect some degree of reprogramming of somatic nuclei, appear to be a poor marker of the further *in vivo* developmental potential in cattle.

The same group provided also evidence that **DNA methylation** of the same target DNA sequence could be in fact **spontaneously corrected *in vivo* during the elongation period that precedes implantation** (Sawai et al., 2010) and after an initial period of *in vitro* development to the blastocyst stage. They evidenced a shift in methylation levels between day 8 (transfer of blastocysts to recipient) and day 16 when the still non-implanted but elongated embryos were recovered. Aberrant methylation level of bovine SCNT embryos at day 8 (hypermethylation) were found to be normal at day 16 when the methylation status of satellite 1 sequence became no more significantly different than in *in vivo* produced embryos. Quantification by real time PCR analysis of the level of DNA (cytosine-5-)-methyltransferase 1 (Dnmt1) expression in SCNT and *in vivo* produced embryos provides evidence that the adjustment of DNA methylation levels was the result of demethylation and retention of methylation processes. The above data illustrate the **advantage of the long gestation period** of farmed mammals (much longer than in rodents) to provide insights into the time-dependent epigenetic modifications (here DNA methylation and Dnmt1 activity) that can contribute to the adjustment of the gene expression levels expressed by a developing reprogrammed genome.

- *Epigenetic alterations and (ab)normal phenotype*

In the pig, tissue-specific differences with respect to both gene expression and DNA methylation were analysed in muscle as well as in liver of two SCNT and two control 6-weeks old male piglets (Gao et al., 2011). While gene expression profiles were generally found to be similar for each tissue between SCNT and controls, the pattern of **DNA methylation was more different than the gene expression pattern**. This was true for the non-repeated sequences as well as for repeated sequences.
On the contrary, Park et al. (2011) found that gene expression patterns of five one-month old pig clones (males and females) of normal phenotypically appearance and generated from foetal fibroblast is altered when compared to controls of the same age (but with different genotypes). The 13K oligonucleotide micro array used by the authors reveals less variability of the global gene expression profiles in the brain, kidney and lung tissues between piglet clones than between controls as would be expected. Several differentially dysregultated genes of clinical relevance were found to be associated with **histological evidences of some altered lung function** (collapsed alevaoli). As for cattle, pig clones were found generally hypermethylated. The above observations confirm that **alterations in DNA methylation are still present in the genome of phenotypically normal clones**. This remains an important issue in farmed animal cloning.

- *Clones as epigenome variants*

In cattle, de Montera et al. (2010) showed by measuring the 5-methylcytosine (5mC) levels in leukocyte DNA of 38 adult female clones of two different breeds (Holstein and Simmenthal, 19 animals for each breed) that healthy SCNT animals must be considered as epigenetic variants. The estimated variance in 5mC level within clone genotypes (2 to 7 animal per genotype for each breed) was higher than between clone genotypes. Quantification of the contribution of SCNT to this unexpected variability was done by comparing clones with monozygotic twins (14 pairs) of similar age and breed. The estimated variability between SCNT animals obtained from the same donor (same genotype) was again significantly higher than between SCNT animals obtained from different donor cells (different genotype). In contrast, twins showed lower variability within genotypes than between genotypes. The **clone-specific variability in DNA methylation** clearly show that healthy adult SCNT clones must be considered as epigenome variants.

This conclusion is also supported by the work of Takeda et al. (2011) who compared **the variations in mitochondrial protein levels** between SCNT derived and AI derived adult cattle and calves. Using the classical 2D gel electrophoresis that allowed the simultaneous detection of 1500 protein spots from one sample, the authors detected several marked difference in the level of expression (up to 5.5-fold up regulation or 3-fold down regulation) of more than 20 protein spots between three adult SCNT females obtained from the same donor cell line and of the same age. No evidence of mitochondrial heteroplasmy (originating from their recipient oocyte at SCNT) could be detected between the genomes of these females. Conversely, the authors could not detect any differences in the pattern of protein synthesis between two adult female clones of the same age (6 years), obtained from the same

batch of donor cells, but with evidence of mitochondrial heteroplasmy. Thus mtDNA heteroplasmy inherited after nuclear transfer is not the cause of **variation in mitochondrial gene expression observed between adult clones**. The authors also found that the liver of all non-viable neonatal SCNT calves overexpressed (by a factor 2) the 78kD glucose regulated protein precursor and the protein disulfite isomerase A3 precursor, both of mitochondrial origin. This result implicates mitochondrial-related gene expression in developmental losses of SCNT embryos.

A.2 Handling of donor cells and ooplasms at embryo reconstruction and activation

Under the hypothesis that alterations frequently associated with SCNT stem from the initial remodelling of the somatic nucleus and/or from the early reprogramming of genes that control the first steps of development, several attempts were made to modulate the remodelling and early reprogramming events of the somatic nuclei by modifying their micro-environments when *in culture* to determine whether it is possible to increase the yield of blastocysts and the efficiency of cloning. The main improvements for the production of transferable SCNT embryos, obtained from modification of the embryo micro-environment and culture conditions, are listed below.

Hand-made cloning (HMC) is a technique initially proposed by Vajta et al. (2003) to facilitate the application of cloning in farmed animals. This technique has the feature of removal of the zona pellucida prior to enucleation and fusion, resulting in a limited (or no) requirement for micromanipulators. The benefits of HMC are low equipment costs, simplification of the manipulations and time reduction of the cloning process. Over the past two years, reports have been published on the birth of live animals obtained after HMC (Buffalo: Panda et al. (2012); Cattle: Akagi et al. (2011); Pig: (Clausen et al., 2011; Schmidt et al., 2010; Schmidt et al., 2011; Staunstrup et al., 2012), and several reports described *in vitro* increase in the rate of blastocysts obtained compared with the traditional SCNT (e.g. for pigs: Zhang et al. (2011)). Further technical improvements, including the possibility of partial or full automation of somatic cell nuclear transfer (Vajta and Callesen, 2012), could contribute to facilitate working with this technique.

The duration of exposure of the somatic nucleus to ooplasm factors before induction of **the activation process** that initiates development, and the activation process itself are critical steps of any cloning procedure. These steps were investigated by several research groups (see paragraphs below), however, no major advances were reported over the last 2 years for improving the overall cloning procedure.

By controlling the usage of **the cell-cycle inhibitor** Butyrolactone I **to allow a time extension for the manipulation of batches of oocytes**, De Bem et al. (2011) could obtain the birth of live calves but with an efficiency of only 7.7%. A similar strategy obtained by preventing the degradation of cyclin B with MG132, an inhibitor of the proteasomal machinery formerly used in rat cloning experiments, was adapted to cattle by Le Bourhis et al. (2010): an exposure of oocytes for 45 to 90 minutes during enucleation and up to electrofusion of the donor somatic cell resulted in a significant increase of cells at the blastocyst stage in comparison to controls (about 25%) and a higher rate of pregnancy at day 35, although no clear conclusion could be drawn in this study because of the reduced number of recipients used (n=4 only). Similar beneficial effect of MG132 exposure of recipient pig oocytes during the long period of maturation required *in vitro* before manipulation (44h) in that species, was reported by You et al. (2012) with an increase in the number of cells at the blastocyst stage of 25%.

Limitation of the cellular stresses induced by fusion and activation conditions was also considered as beneficial to cloning efficiency and studies by several groups but with *in vitro* data only. Useful results were obtained in cattle by Song et al. (2011) who revisited the use of an inactivated viral envelope (Sendai virus, SV) as fusion material for the reconstruction of embryos. This technique had been initially proposed in the mouse in the very first staged of nuclear transfer research (Surani et al., 1984). Sendai virus mediated fusion was shown to reduce the transcription level of several endoplasmic reticulum stress-associated genes stimulated by electrofusion while it improved the number of blastomeres in SV-SCNT blastocysts by 35%; the ratio of inner cell mass cells to the total number of cells tended however to be lower than in control IVF or electrostimulated SCNT embryos.

Aggregation of SCNT embryos (by letting them fuse when locked in a small volume after manipulation) provides an epigenetic compensation between the three reprogrammed genomes (Boiani et al. (2003) in the mouse; Kurosaka et al. (2007) in cattle). Akagi et al. (2011) tested the timing of removal of the zona pellucida of the oocyte and the timing of aggregation of bovine SCNT embryos (i.e. at the 1-cell, at the 8-cell on day 2, and at the 16- to 32 cell stages on day 4). Full term pregnancies were obtained after aggregation of three SCNT embryos at the 8 cell stage (two calves from a total of 11 aggregates, one stillborn, the other healthy) or at the 16- to 32-cell stage (1 calf, but stillborn, generated from a total of 7 aggregates). Given this small number, it is unclear whether aggregation of three SCNT embryos had an influence on perinatal mortality. The overall efficiency expressed in terms of healthy calves/transferred embryo remained low (9%; 1 out of 11).

The **spatial organisation of chromatin** is dramatically affected upon the entry of a somatic nucleus in a recipient cytoplasm. By studying the distribution of the heterochromatin protein CBX1, together with that of the centromeric proteins CENPA and CENPB and trimethylated histone H3, Pichugin et al. (2010) found that two contrasted types of SCNT embryos could be made distinguished in cattle at the 2- and 4-cell stages. They predicted that these two types of SCNT embryos will display different chromosome territory arrangements and proposed to use that nuclear organisation to **screen for the technical conditions that improve epigenetic reprogramming**. A similar suggestion was made by Deshmukh et al. (2012) for SCNT pig embryos which spatial distribution and dynamics of chromatin at the 2- and 4-cell stages display marked variations. They proposed that the lack of a heterochromatin halo around nucleolar precursor reveals altered chromatin remodelling. These promising and innovative approaches are to be confirmed with further results.

In vivo data are considered as scientifically necessary before concluding on any improvement in the technique of nuclear transfer *per se*. This has been convincingly demonstrated by Whitworth et al. (2010) who compared three **activation techniques** for SCNT pig embryos, namely MG132 induced activation, electrofusion only, or exposure to Thimesoral, a sulfhydryl agent that stimulates the release of cytosolic free calcium. None of these techniques could prevent abnormal expression of placental genes at day 30 of gestation.

A.3 *In vitro* culture of developing embryos: nuclear remodelling and reprogramming

Progresses in cellular imaging and high-throughput molecular analysis of DNA methylation and histone modifications stimulate an already active field of research including epigenetics, stem cell biology, reproductively biology, and veterinary sciences. In this context, SCNT-derived embryos of farmed animals are most often used to extend observations made in the mouse with the scope that **altering the microenvironment of the embryos** will lead to a more complete reprogramming and followed by improvements in SCNT efficiency. However, few papers provide an *in vivo* validation of observations made *in vitro*, mainly because of the costs of experiments with large farmed mammals. These papers have been given priority in the below paragraphs.

- *Nuclear remodelling*

Mitosis is essential to make mammalian somatic nuclei prone to reprogramming. Recent results obtained in the mouse (Ganier et al., 2011) have demonstrated that **Xenopus mitotic egg extracts** have a strong reprogramming activity on mouse somatic nuclei (cultured fibroblasts). The experiment reset replication properties of somatic nuclei towards a replication profile characteristic of early development resulting in a four time increase in the rate of blastocyst obtained *in vitro* from 2-cell stage treated SCNT embryos (45%) in comparison with non-treated ones (11%).
This heterologous Xenopus extract was also applied to sheep SCNT as pre-treatment of donor somatic cells. No increase in the frequency of development to blastocyst stage was observed with treated donor cells; however, live births were significantly more after the transfer of treated blastocysts with a 4.7-fold increase in comparison with controls (non-treated embryos). The percentage of lambs surviving to adulthood of blastocysts transferred (5.9%) increased 1.9-fold compared to controls (Rathbone et al., 2010).

In the pig Liu et al. (2012) did observe an increase in the formation of blastocysts with cells exposed to Xenopus extracts and induced to reprogram *in vitro* for 7 to 8 days before being used as a source of nuclei for nuclear transfer. No *in vivo* data have yet been reported in that species. Xenopus egg extracts are known to abruptly erase epigenetic marks at mitosis such as trimethylation of H3K9, H3K4, and H4K20 but their biochemical activity and quality vary from batch to batch. This is a present limitation to a technique that could contribute to characterise the key molecular players of nucleus reprogramming that are well conserved between species.

Inadequate **spatio-temporal reorganization of the donor nucleus** has been described with rabbit SCNT (Bonnet-Garnier et al., 2012). Since nuclear architecture is determinant in cell specialisation (Cremer and Zakhartchenko, 2011) spatio-temporal remodelling should be considered as an additional and more integrated level of SCNT alteration. In the pig Deshmukh et al. (2012) provided clear evidence that whatever the method used for generating embryos *in vitro* (*in vitro* fertilisation, parthenogenetic activation or SCNT), the remodelling of chromatin is altered in comparison with *in vivo* produced embryos. Interestingly early SCNT one-cell stage embryos had high level of DNA methylation similar to somatic nuclei even though the structural remodelling to a pronuclear like structure occurred normally. At later stages SCNT blastocysts displayed high variations in methylation levels but some were similar to *in vivo* produced embryos. The remodelling events that affect higher order structure of chromatin are thus somewhat disconnected from the epigenetic modifications (methylation) that regulate the reprogramming of genome functions.

- *Reprogramming of nuclear functions: DNA methylation and histone acetylation*

DNA methylation and histone acetylation are highly dynamic processes that have a determinant role in the organization of chromatin and the time-dependent control of gene expression which is at the basis of reprogramming.

DNA methylation

DNA methylation plays a major role in the reprogramming of somatic nuclear activities. This highly dynamic process controls the expression of genes critically involved in pluripotency and cell differentiation. During the pre-implantation period of development *de novo* DNA methylation occurs in a species-specific manner and can be affected by *in vitro* culture conditions. Numerous reports had attempted to improve the efficiency of early embryonic development of SCNT-derived embryos by altering the **DNA methylation pattern of reprogrammed nuclei** *in vitro* but with **limited successes in terms of full reprogramming of genomic functions**.
By normalising the DNA methylation levels by the DNA content of pig embryos at different stages of their pre-implantation development, Deshmukh et al. (2011) confirmed **the high level of DNA methylation in SCNT** 1-cell stage embryos relative to *in vivo* produced ones. They found that the methylation level **was even higher in *in vitro* fertilised embryos**. DNA methylation pattern are thus affected by *in vitro* embryo production (and is not specific for SCNT). Interestingly, SCNT embryos displayed a pronounced variation in methylation level at the blastocyst stage but with respectively 50% and 14% of them having similar levels to that of *in vivo* produced embryos at the early (days 5-6) and late (days 6-7) blastocyst stages. These results suggest that measuring the normalised DNA methylation contents of SCNT cultured and *in vivo* produced early and late blastocysts could to some extend allow an empirical mean for a multifactor improvement of culture conditions adapted to SCNT embryos.

Histone acetylation

Modifications to the histone acetylation pattern of donor cells or that of early SCNT embryos by their transient exposure to various drugs (such as **deacetylase inhibitors**) is an active research area in cloning. Many reports examined the proportion of SCNT embryos that develop to the blastocyst stage and consider the allocation of cells between the two first cell lineages that differentiate at that stage (the trophoblast and the inner cell mass cells). Only a few also examine the *in vivo* developmental

potential of such treated embryos. A variety of **deacetylase inhibitors** (HDACi) have been used by different research groups.

Zhao et al. (2010) showed that an exposure of pig embryos to the **histone deacetylases inhibitor Trichostatin A (TSA)** (during 10h after activation) or **Scriptaid** (14-16h after activation) increases the percentage of blastocysts formed in culture at 144h by respectively a factor of 3.2 and 2 without affecting the blastocyst's cell numbers. Exposure to Scriptaid not only improved the histone acetylation on Histone H4 at lysine 8 (AcH4K8) in a pattern similar to that of the *in vitro* fertilized (IVF) embryos but also increased the overall reprogramming efficiency of foetal fibroblasts cells from 0.4% (untreated group, 211 embryos transferred to 2 recipients) to 1.6% (819 treated embryos transferred to 5 recipients). Similar positive effects of Sriptaid were reported in sheep SCNT by Bordignon et al. (2011) with a three time increase in the rate of initiated pregnancy (transfer of 178 1-cell stage embryos to 8 recipients from which 4 became pregnant), in buffalo by Panda et al. (2012) with a 70% significant increase in blastocyst rate (54% versus 38% with controls) and a 100% increase in the number of cells per blastocyst (343 versus 150 for controls).

Exposure of pig embryos to **butyric acid** for 4h immediately after activation (Das et al., 2010) improved the rate of blastocysts formed by a factor of 1.6 (18.3% vs 11.2%) without affecting the total number of cells. Histone acetylation levels became close to those in IVF embryos. There were no effects obtained when donor cells were treated for 24h before (instead of after) SCNT. This is a good indication that nuclear functions have in some manner been improved as a consequence of the action of the inhibitor during the early remodelling of the nucleus after SCNT.
Exposure of cattle embryos to **valproic acid (VPA**) for 24h only marginally improved the development of bovine SCNT embryos at the blastocysts formed after, although it significantly reduced the rate of apoptosis (1.91±0.48% vs. 5.67±0.40%, $p<0.05$) (Xu et al., 2012).

None of the above chromatin modifying agents, nor their combinations (e.g. (Azacytidine + TSA) or (Hydrazaline+VPA)) were found to have a positive effect in cattle when added on donor cell medium before SCNT (Sangalli et al., 2012). When (Hydrazaline+VPA) was added upon SCNT, the acetylation pattern of embryos was increased but without effect on development (both pre- and post-implantation).

- *Removal of animal protein source from the culture media.*

Avoidance of embryo contamination before their transfer *in vivo* into foster recipients is the objective of research that analyses the metabolite uptake and release by the embryo in its microenvironment. Chemically defined medium where Bovine Serum Albumin (BSA) or Foetal Bovine Serum (FBS) were replaced by the **water-soluble polymer polyvinyl alcohol (PVA)** associated with myo-inositola-phosphate was shown by Jang et al. (2011) to lead to a higher rate of live calve clones (20% from 15 blastocysts transferred into 7 recipients) than with the classical synthetic oviductal fluid (SOF) medium developed in the mouse but requiring a supplementation with BSA when used in cattle (11.9% only; 52 blastocysts transferred to 27 recipients). Interestingly, both media provided the same rate of blastocysts with a similar total number of cells. In both cases however cases of dystocia were observed.

- *Mitochondria and oxidative stresses*

Oxydative stresses caused by a high intracellular oxygen concentration induced by *in vitro* culture have detrimental consequences on embryo development (Takahashi, 2012). Exposure of SCNT recipient pig oocytes to the anti-oxidative agent anthocyanin increases the rate of blastocyst formation by a factor of 2 (32.2% vs. 16.1%) while it has no effect during IVF. The quality of the blastocysts in terms of total number of cells is not affected (You et al., 2010). This confirms that **prevention of Reactive Oxigen Species (ROS)** is a positive factor for reprogramming.

Oxydative stresses are linked to an increase in apoptosis that affects the kinetics of cell division in numerous cellular types. Taking this mechanism into account Jeon et al. (2011) propose the mRNA level of survivin, a member of the chromosomal passenger complex (CPC) with a bifunctional function on both the suppression of apoptosis and the regulation of cell cycle, as a new marker for

embryo quality of *in vitro* developed pig embryos. They found that the kinetics of cleavage after the activation of SCNT eggs affects the rate of blastocysts obtained at day 7 with 2-cell embryos at 24h, leading to a significant higher rate of blastocysts when compared to less or more advanced SCNT embryos (18.0%, 376 embryos analysed with 6 replicate experiments). Level of survivin mRNA which parallels that of the protein, were also higher with this classified group suggesting that this class of early SCNT embryo had a higher developmental competence. No complementary *in vivo* data were however produced.

Oxydative stresses provide a convenient, although limited read-out of the alterations that affect cellular metabolism. This implies a highly regulated process involving multiple signalling cascades. It is governed by mitochondrial activity which regulates the level of reactive oxygen species (ROS). To determine whether increasing the level of mitochondrial activity could lead to an improvement in SCNT embryo development, Hua et al. (2012) have used as donor nuclei foetal bovine fibroblasts with different expression levels of Mitofusin1 (Mfn1), the main mediator of mitochondrial homeostasis. Up-regulation or down regulation of the Mfn1 gene were obtained respectively from transgenic, of RNAi -treated donor cells. Results demonstrate that the up-regulation of Mfn1 expression improves the development rate of SCNT embryos to the blastocyst stage (by 40% when compared to control donor cells) while down-regulation of Mfn1 resulted in poor development (only 4.2% of blastocysts). This improvement resulted from an increase in both the ATP level and the mitochondrial membrane potential at the morula stage, while H_2O_2 generation was reduced. This indicated that overexpression of Mfn1 could promote the early development of bovine SCNT embryos by improving oxidative phosphorylation.

That a transient modification of mitochondrial activity at the one-cell stage can exert late emerging developmental effects has been demonstrated recently in the mouse, but with normally fertilized mouse embryos. Indeed, Banrezes et al. (2011) have shown that stimulating or reducing mitochondrial activity by exposure of one-cell stage embryo (to respectively exogenous pyruvate or lactate) resulted in offspring with a permanent smaller or larger size. This result has however not yet been extended to SCNT.

A.4 Risk of disease transmission through cloning

Bovine embryo transfer technology has been widely studied and a **standard operating procedure for embryo production and transfer** has been published by the International Embryo Transfer Society (IETS) (Stringfellow and Seidel, 1998) to ensure safety of this technology. Potential disease transfer was also taken into account in the guidance on Health Assessment and Care for Animals Involved in the Cloning Process (consensus recommendation from the International Embryo Transfer Society, 15 May 2008, http://www.iets.org/comm_hasac.asp). Nonetheless, since oocytes used for somatic cell nuclear transfer embryo production are predominantly from abbatoirs and the nuclear transfer procedure produces small openings on the zona pellucida, the concern regarding potential disease transfer was further raised by Gregg et al. (2010a). An experimental risk assessment of **bovine viral diarrhoea virus (BVDV)** transmission via *in vitro* embryo production by SCNT published in 2009 (Gregg et al.) focused on critical steps of the embryo production process for its risk of BVDV disease transmission (i.e. the donor somatic cell, the oocyte collected from commercial abbatoirs and the *in vitro* SCNT embryo culture system). This study made it possible to identify some key risk factors and to develop a SCNT standard operating procedure aiming at minimizing the risk for BVDV transmission through pre-nuclear transfer donor cell testing, oocyte decontamination and virus-free cell and embryos culture conditions.

The large scale *in vivo* risk of transmitting viruses responsible for infectious diseases through SCNT-derived embryos was further investigated for the BVDV by Gregg et al. (2009; 2010a; 2010b) and the porcine reproductive and respiratory syndrome virus (PRRSV) by Gregg et al. (2011) with the main conclusion that such a risk is reduced to very low (negligible) levels when appropriate precautions are adopted in the SCNT embryo production and transfer.

Similar conclusions have been reached through an assessment of a considerable amount of experimental data on the transmission, via SCNT embryos, of porcine reproductive and respiratory syndrome virus by Gregg et al. (2011) and of equine infectious anaemia virus by Gregg and Polejaeva (2009) and by Asseged et al. (2012).

One source of alteration to reprogramming with potential consequence on disease affecting SCNT offspring remains still poorly documented. It deals with sequences of retroviral origin that occupy approximately 10% of mammalian genomes. Endogenous retroviruses are inactivated by mutations and/or deletions but endogenous terminal repeat (LTR) **retrotransposons are markedly activated during zygotic genome activation**. This suggest a general requirement for retrotransposon transcription for progression through cleavage as shown recently by genome-scale, base-resolution analysis of DNA methylation at the cleaving stage of embryonic development in the mouse (Smith et al., 2012). Since SCNT involves nuclear remodelling and genome re-activation, this might influence or be influenced by the activation of retrotransposons.

A.5 Transfer into *in vivo* environment

In a large scale retrospective analysis of the transfer conditions of SCNT embryos in the pig, involving 100 to 300 recipients per variable considered, Koo et al. (2010) showed that the environment of the recipients and **the technique of SCNT embryo transfer** *per se*, are important determinants of cloning efficiency. The transfer of day-2 SCNT embryos in the oviduct of pre-ovulatory instead of post-ovulatory recipients resulted in a significant increase in their pregnancy rate at day 25 (36.2% instead of 22.7%) and a marked 4.4 time increase in the rate of full-term pregnancies (9.4% and 2.1% respectively). Season also appeared to influence the *in vivo* development of porcine embryos (52% pregnancy rate after the transfer of SCNT embryos in autumn and only 18% in winter) and delivery rates (12.7% in spring but 4.3% in autumn); other parameters (embryo transfer (ET) method, prevention of cold shock to embryos before transfer prove to be also of importance.

A.6 Analysis of foetal and placental development: epigenetics and late emerging effects

Abnormal **implantation and placenta formation** are frequently observed after the transfer of SCNT embryos into recipients. They contribute to the low efficiency of cloning and are the main origin of most of the health and welfare problems encountered by recipients at or before birth. Incomplete nuclear re-programming after SCNT and failure to re-establish appropriate epigenetic marks during development, particularly in regions controlling the expression of imprinted genes, are considered as the major causes of pregnancy failure with SCNT embryos. Some improvements have been made over the past two years to the analysis of pregnancy dysfunctions but these have not yet led to marked improvements in the reduction of pregnancy failures with SCNT embryos in farmed animals.

- *Pre-implantation period up to placenta formation*

In cattle about 40% of transferred SCNT cattle blastocysts fail to develop through the elongation stage which initiates at day 14 (after the one-cell stage) and leads to implantation around day 18. Numerous factors, including recipient type, number of embryos transferred per recipient and season, have previously been shown to affect the *in vivo* development of SCNT embryos.

Smith et al. (2010) found that **gene expression profiles** during the second week of embryogenesis do not differ between SCNT and IVF embryos. For that, the authors have transferred into the same recipient groups of 10 SCNT blastocysts obtained from a donor cell line harbouring a reporter gene (GFP) together with 5 (grade and stage matched, day 7) IVF blastocysts. Doing so, they provided the same *in vivo* environment to all the embryos while being able to screen for SCNT versus IVF embryos at recovery after 2, 4, 7 and 10 days. Gene expression profiles of nine trophoblast and two epiblast relevant marker genes were found to be similar between SCNT and IVF embryos of the same trophoblast length. Thus the gene expression program that regulates the critical phase of embryo elongation is spatially and temporally correctly reprogrammed in the SCNT blastocysts up to the

initiation of implantation. Consequently the reduced birth rates with SCNT embryos compared to IVF embryos must be predominantly due to reprogramming-specific errors manifesting their effect after implantation. In other words the SCNT related defects observed at later developmental stages have to be considered as predominantly of epigenetic origin.

A further step was taken by Degrelle et al. (2012), who proposed **that gene expression pattern in the extra-embryonic tissues** at implantation (day 18) do not reflect the pregnancy outcome while that of the embryo itself does. For this conclusion, the authors made a general analysis of the genes expressed from the extra-embryonic part of bovine SCNT-derived embryos at day 18 using a dedicated array of 10214 unique sequences. These were obtained from the cDNAs of extra-embryonic tissues of bovine embryos collected at different developmental stage between day 14 and day 24 as well as cDNAs from young foetuses (day 36 - day 64) and from term placenta. Expression patterns were compared between SCNT, *in vitro* fertilized and AI derived (*in vivo* fertilized) embryos. The **general patterning (gastrulation) of the embryonic part** of each embryos was also analysed. Finally and in order to provide an insight into the interactions between the expression profile of embryonic versus extra-embryonic tissues within the same embryo they use day 18 SCNT embryos obtained from 3 different donor cell lines (10 embryos per line) already known from their ability to provide similar rates of blastocysts (rates higher than 30% from reconstructed 1-cell stage embryos) but highly different calving rates (respectively 2.3%, 6.1% and 20%). From this multifactorial analysis they discovered that the embryonic part of SCNT embryos exhibited 20 to 60% of abnormalities that mirrored the different pregnancy rates and recalled some defective specification of the primitive streak whereas the extra embryonic part was still functionally normal since only a low proportion of genes (72 out of the 10 000 unique sequences analysed) were surprisingly found to be differentially expressed between the groups of embryos including *in vivo* produced ones. From those genes, only 7 were classified as of importance to nuclear reprogramming and cell differentiation. Thus an uncoupling between the extra-embryonic functions, mostly normal at day 18 and the embryonic functions, already altered at gastrulation, is an ontological mark of developmental failures after SCNT.

An additional observation of the pattern of expression of a small set of **genes expressed at later stages of pregnancy** (day 26 and day 63) **in differentiated extra-embryonic tissues** (yolk sac and chorion) suggested that a reduced expression of key regulatory genes could be at the origin of the dysfunctions of SCNT clones. A similar situation is observed also in the pig. In their analysis of the effect of activation procedure on SCNT embryo development, Whitworth et al. (2010) studied several functional activities of the extra-embryonic membranes of SCNT and control day-30 conceptus. Up-regulated pathways in SCNT extra-embryonic membrane included blood circulation and gas exchange, cell surface receptor-mediated signal transduction, G-protein and ligand mediated signalling. Major histocompatibility class 1 genes were both up- and down-regulated suggesting that there are major differences in reproductive tract immunity between conventional and clone pregnancies in pig. Interestingly, PAG2, a trophoblast-specific transcript was found to be ectopically expressed in the luminal epithelium of the maternal side with SCNT conceptus but not with *in vivo* produced embryos. This is potentially an important observation since PAG2 is a member of a large family of pregnancy associated glycoproteins.

Further insights into the he causal origin of gestational death of pig SCNT conceptus was provided by Kim et al. (2011). These authors analysed 12 SCNT foetuses obtained from 5 recipients at day 30 post transfer. All SCNT foetuses were alive and of normal size and shape but their foetal membranes displayed avascularisation and hypovascularisation. Several placenta related proteins were barely detectable at the basement membrane of the SCNT cytotrophoblast cells whereas they were markedly expressed in the cytotrophoblasts of developing controls. Genes were also down regulated with evidence of hypermethylated pattern of their promoter region (ex MMP9) and poor immunohistochemical staining. Some genes however (exHand-1) were over expressed while others were expressed similarly to controls. Evidence of a marked apoptosis in the developing endometrial gland of the recipient transferred with SCNT embryos was also provided. The authors conclude that death of pig SCNT conceptus is caused, at least in part, by disruption of the developing endometrial gland due to impaired trophoblast migration and invasiveness.

In summary, the above results lead to the view that early gestational death of SCNT conceptus (cattle and pig) stems from an initial uncoupling of developmental functions between the embryonic and extra-embryonic compartments of the implanting embryo. All the functions are not altered but the first perturbations influence tissue growth and differentiation within each compartment.

- *Early placenta development*

Abnormal placentation associated with hydrallantois and foetal overgrowth, known as the large offspring syndrome (LOS) account for most of the foetal losses observed in cattle in the mid to late gestation period after SCNT. During the period of the **initiation of placentome formation** (day 35-50), SCNT embryos can show impaired cotyledon formation but also advanced cotyledon development and vascularisation. Many of the cotyledon - caruncle interactions fail to successfully form placentomes, resulting in far fewer placentomes. Overall, placentome numbers and morphology are far more variable in SCNT pregnancies compared to artificially inseminated (AI) controls, suggesting a deregulation of placental development. References are Guillomot et al. (2010) on Phlda2 gene with data from day 32 to day 200 and Kohan-Ghadr et al. (2012) on E-cadherin and b catenin at the attachment period.

- *Late placenta development*

Foetal losses continue to occur sporadically throughout the second and third trimesters in bovine SCNT pregnancies, affecting 25 to 75% of pregnancies from Day 90 onwards. Whether the numbers of placentomes are decreased or are normal, there is increased total placental mass and the enlarged placentomes are much larger and thicker in these pregnancies and develop hydrallantois (a rapid and uncontrolled increase in allantoic fluid volume) and oedema of the foetal membranes and placental tissues. Hydrallantois accounts for most of the foetal mortality in the second half of gestation in bovine SCNT compared to <1% in normal bovine pregnancies. These placental abnormalities are additionally associated with other foetal lesions, such as enlarged umbilical cord and ascites (excessive fluid accumulation in the abdominal cavity), cardiac enlargement, liver steatosis and renal lesions, such as hydronephrosis, all of which compromise foetal viability. References for cattle are Yan et al. (2010) on mitochondrial compatibility at day 90 of gestation and Takeda et al. (2011) on liver mitochondrial status in new born calves. A reference for pigs is Wei et al. (2010) on Methylation status of placenta tissues at birth in the pig.

A.7 Future developments and expectations for additional research

The efforts that are being made in the scientific community to overcome the identified processes that work sub-optimally during the cloning of farmed mammals, have lead over the past two years to some improvement in the development of early SCNT embryos and a more comprehensive description of the failures affecting placental function compromising foetal development. It is also confirmed that species differences in terms of the kinetics of pre-implantation development, *in vitro* culture requirement, and timing of implantation and duration of gestation have to be taken into account to steadily lead to improvements in cloning efficiency.

Recent publications in the mouse species offer promising prospects also for mammalian SCNT cloning. They provide convincing evidence that marked improvement in terms of live offspring obtained from transferred SCNT embryos is becoming an achievable goal at least in mouse. Since these reports also offer a conceptual framework for the unravelling of the complex events linked to the remodelling and reprogramming of somatic nuclei, they support basic research with the more demanding (in terms of replication time and consequently research costs) farmed animals.

First, the group of Ogura et al. (Inoue et al., 2010; Matoba et al., 2011) identified an RNA which appears to act as a general key regulator of the level l of expression of many genes during early development and leading to impaired development of mouse clones. By injecting oocytes just before SCNT or at the pronuclear stage with **a siRNA against Xist,** a gene responsible for X chromosome inactivation (XCI), they obtained evidence of a seven- to nine-fold increase in overall cloning

efficiency compared to non-treated controls. From 304 2-cell stage embryos injected in different experiments performed by different operators 45 live pups were delivered (overall efficiency 14.8%) whereas the transfer of 293 control embryos resulted in only 6 live pups (overall efficiency 2%). A synergetic treatment of siRNA injected embryos with TSA to overcome some of the non-random errors in genome reprogramming resulted in an overall efficiency of 20% (14 live pups from 69 treated embryos versus only 4 pups from 68 control embryos). Moreover, only limited gene dysregulations were observed in the gene expression profiles of livers in neonate clones supporting the view that correction of Xist expression in pre-implantation embryos might have long-term beneficial effects during postnatal life. It has been shown recently that different mammals have very different strategies for initiating X-chromosome inactivation, for example eutherian mammals including rabbit (and human) have very different strategies for initiating X chromosome inactivation (Okamoto et al., 2011).

Second, Balbach et al. (2012) provided a comprehensive study of the cell cycle during early phases of reprogramming after SCNT. Using bright field and live fluorescence imaging of histone H2b-GFP expressing mouse embryos to quantitavely analyse cleavage kinetics up to the blastocyst stage, they showed that **SCNT embryos presented similar rates of errors in M phase than *in vitro* (ICSI) fertilized controls, but were considerably less tolerant to subsequent mitotic errors.** While this does not affect pre-implantation development, it has detrimental consequences at post-implantation stages. **Different needs** of SCNT and control embryos **in terms of amino acid supplementation (arginine)** was also outlined in the Balbach's paper.

From these reports above (Ogura et al. and Balbach et al.), the view emerges that contrarily to the current assumption, **the fate of mouse clone embryos is largely if not almost exclusively determined before implantation**. Bright field microscopy and live imaging of chromatin remodelling of embryos during the first mitosis are useful new tools for screening of SCNT embryos with a high developmental potential, before their transfer *in vivo*.

This has important consequences for research with farmed animals where the late emerging effects of SCNT markedly affect both surrogate dam and clone health and welfare. Under the hypothesis that the basic mechanisms underlying reprogramming in mammals are well conserved between species (a reasonable assumption) the above assumption indicates that a research on the early cellular and molecular events of SCNT could lead to more rapid improvement in terms of health and welfare.

- *Prediction of pregnancy outcome*

Not only for the particular field of cloning, research is evolving to predict pregnancy outcome and full-term development of foetuses, for instance with 3D imaging. Also the developmental potential of SCNT embryos into live offspring remains today largely unpredictable. While most gestational losses occur during the pre-implantation period, the prevalence and severity of abnormal placentation and late foetal losses is a matter of concern for applications of cloning. Attempts to predict pregnancy outcome after SCNT embryo transfer remains an important objective. Few improvements have however been obtained over the past two years.

At late foetal stages, the mean umbilical vessel diameter is consistently larger in SCNT compared to control AI pregnancies. Le Cleac'h (2011) used the non-invasive ultrasound imaging to provide evidence that not only the diameter of umbilical vessels but also rib sizes and interval between two consecutive ribs were higher in clones from the 5th month of pregnancy onwards. For that he used 2D Doppler technique to monitor the pregnancies established with clones (n= 7) or after insemination (n=11). Evaluation of placentome morphology and size, echogenicity of allantoic fluid, rib sizes and interval between two consecutive ribs, diameter of umbilical vessels, were recorded from a mean of 4.8 sessions per animal and 2 placentome analysed per session. Only the last two parameters were found to be higher in clones already at 5 month and up to term.

This approach has been extended to a quantitative evaluation of **blood flow within the placentome** unit the sheep by Morel et al. (2010). Although these authors did not have examined yet pregnancies established from SCNT embryos, their work provides a first demonstration that several Power Doppler

Angiography (PDA) indices can be used *in vivo* to monitor placental functions. Advances in the medical use of imaging techniques should prove to be helpful for increasing the prognostic evaluation of pregnancy outcome in clones.

Earlier prediction of pregnancy outcome with clones has been proposed by Constant et al. (2011) who analysed **the plasma levels of pregnancy associated glycoproteins (PAG)** produced by the placental trophoblast in ruminants. These glycoproteins are known to migrate to the uterine luminal epithelium. These authors found that with abnormal SCNT pregnancies, maternal PAG concentrations are higher compared to conventional pregnancies established after AI or IVF from the end of the second month of pregnancy (day 62) onwards. This was shown using three different immunoassay quantifications measuring the level of PAG-1 (67kDa) in the peripheral blood. They also provide some evidence that this increase is not the result of an increase in protein expression of PAG-1 and propose that altered secretion (clearance) of the glycoprotein rather explains this result. This approach could constitute the detection of abnormal SCNT pregnancies in cattle already from the end of the second trimester.

Attempt for an even earlier prediction of pregnancy outcome already at implantation has been proposed by Degrelle et al. (2011) in cattle. These authors examined whether **a molecular signature originating from extra-embryonic tissues** could relate to the developmental stage of the embryo proper and be used to predict its full term development. To this end, these authors analysed the expression profile of day 18 extra-embryonic tissues which can be sampled from *in vivo* developing conceptus. They had previously identified a set of seven extra-embryonic genes which were discriminative to distinguish between two different consecutive development stages of embryonic patterning (at gastrulation and early neurulation). Abnormal patterning at implantation is a major bottleneck to full reprogramming of SCNT embryos. To determine if the expression profile of the extra-embryonic compartment could be used to predict the status of the embryonic compartment they examined 15 day-18 SCNT embryos collected *in vivo*. They found that the normality of the patterning process could be accurately predicted for 14 out of the 15 embryos (accuracy of 93%) with a panel of only 6 extra-embryonic expressed genes.

In the pig, **methylation status of early embryos** has also been proposed by Niemann et al. (2010) to evaluate the quality of SCNT blastocysts. A comprehensive study on epigenetic reprogramming that occurs in ARTs was conducted by comparing 25 developmentally important genes of *in vitro* (IVF and SCNT) and *in vivo* (AI) bovine embryos. The study revealed a subset of amplicons that distinguished between the three groups. This subset of amplicons can be used to evaluate blastocyst quality and reprogramming following SCNT, and can also be employed for the localization of the epigenetic control regions within individual genes.

- *Health prognosis of growing foetuses*

Further to the prediction of pregnancy term as mentioned above, prediction methods are also used for making health prognosis of growing foetuses, for proposing improvements to the conditions in the uterus and for adaptation of foetuses to their uterine environment. This can be achieved for instance by real-time echography to **monitor the development of the foetus in association with the management of the surrogate dam**, adjusted to the developmental needs of the growing foetus. By modifying the diet of the surrogate dams, meeting the nutritional requirements of the surrogate dam during the pregnancy, and altering the management of the mothers, the growth of the foetal clones could be influenced. Applying these methods during cloning, aims to overcome some of the animal health and welfare challenges of cloning, such as LOS in cattle, and eventually aim at more live SCNT clone births.

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ABBREVIATIONS

AI: Artificial Insemination
ART: Assisted Reproductive Technology
BVDV: Bovine Viral Diarrhoea Virus
CHL/IU: Chinese Hamster Lung Cells
CP: Cloned Progeny
CPC: Chromosomal Passenger complex
CV: Conventional animals
C1: First generation Clone
C2: Second generation Clone, cloned from a first generation clone
F1: First generation
F2: Second generation
ECNT: Embryonic Cell Nuclear Transfer
h: hour
IVF: *In vitro* Fertilization
IVP: *In vitro* Production
LM: Longissimus Muscle
LOS: Large Offspring Syndrome
LW: Large White
MOET: Multiple Ovulation Embryo Transfer
N: Number
mtDNA: Mitochondrial DNA
ROS: Reactive Oxigen Species
SCNT: Somatic Cell Nuclear Transfer
vs: versus

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.
SCNT-derived embryo, SCNT 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 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 a mixture of more than one type of an organellar genome (e.g. mitochondrial DNA (mtDNA)) 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.
Hydronephrosis	Abnormal fluid accumulation in the kidney.
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.
Large Offspring Syndrome (LOS)	The size of the offspring is greater than mean + 2SD for the species or breed. Symptoms include 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. Progeny is used interchangeably herein with Offspring.
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 SCNT-derived 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 spermatozoon and an oocyte).