

# **The Science and Technology of Farm Animal Cloning**

A review of the state of the art of the science, the technology,  
the problems and the possibilities



**Report from the project Cloning in Public  
A specific support action within the 6th framework  
programme, priority 5: Food quality and safety**

**Coordinator: Danish Centre for Bioethics and Risk Assessment (CeBRA)**



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# 1: Introduction

This report is the first deliverable from the project “CLONING IN PUBLIC; a specific support action within the sixth framework programme, Priority 5, Food quality and safety” (Contract no. 514059).

The main objectives of CLONING IN PUBLIC are: (a) to develop recommendations on the preparation of European regulation of, and guidelines covering, research on farm animal cloning and its subsequent applications (e.g. in genetically modified animals for bio-reactors); and (b) to stimulate informed public debate across Europe on these issues involving key stakeholders, university students and members of the public. These two aims are of equal importance. They are also interrelated, because if regulations and guidelines are to serve their purpose, they must take public concerns into account. In addition, stimulating, informing and reporting public debate is part of the more general, long-term aim of improving communication between science, civil society and European authorities at different levels, and hence facilitating discussion of European public affairs connected with science and technology.

The report reviews the current state of the art of farm animal cloning from a scientific and technological perspective. It does not pretend to give an exhaustive review of the all the literature available; instead it pinpoints issues and events pivotal to the development of current farm animal cloning practices and their possible applications. In the report, the history of cloning technology is set out along with an analysis of scientific and technological developments since the cloning of the sheep Dolly was announced by Wilmut et al. in 1997 (chapter 2). The report also outlines the challenges, barriers and problems that the technology faces today (chapter 3) and describes the most important potential applications of farm animal cloning (chapter 4).

This report will be supplemented by a report based on qualitative interviews with cloning researchers. The latter, which will deal mainly with the expectations and concerns of researchers to the technology in question, is intended to develop our understanding of the scientists’ objectives and way of reasoning. Reports on the legal and ethical aspects of farm animal cloning will also be made available by CLONING IN PUBLIC. A list of deliverables and dates of their publication is available at the project website: <http://www.bioethics.kvl.dk/cloninginpublic.htm>.

CLONING IN PUBLIC concentrates on the cloning of farm animals and the possible applications of this technology. The term “cloning” refers here to reproductive biology in the sense of *asexual reproduction*, or more precisely the production of *individuals with (virtually (NRC, 2002 p. 18, Poland & Bishop, 2002)) identical genetic material by asexual reproduction*. In recent debates, interest has centred on cloning by means of nuclear transfer. Less attention has been paid to monozygotic twinning (on which see 2.2. below). Details of the first mammal born after nuclear transfer cloning were published by Steen Willadsen in 1986 (Willadsen, 1986). However, in spite of its enormous scientific significance, this discovery failed to trigger much public concern, possibly because the donor cells were derived from pre-implantation stage embryos. The major breakthrough, in terms of public recognition, happened when Ian Wilmut and colleagues in 1997 published a paper describing

the successful use of exactly the same method, using the nuclei of somatic cells from an *adult* mammal, to create Dolly the sheep (Wilmut et al. 1997). Now it was theoretically possible to create an unlimited number of genetic replica animals from an adult or post-implantation foetus.

The thematic scope of the project extends to both biomedical and agricultural applications of farm animal cloning. In biomedical research, a wide range of potential applications of farm animal cloning are now being explored. These applications include the production of genetically identical animals for research purposes, and the use of cell nuclear transfer as method of producing genetically modified animals. Research into the importance of other factors' influence on the development of the phenotype (epigenesis) will also be facilitated by the technology. Apart from their use for research purposes, such animals may be used as so-called 'bio-reactors' to produce valuable proteins. In the agricultural sector, cloning can be used as a tool within farm animal breeding.

The work of the project is confined to animal biotechnologies in which the use of cell nuclear transfer is an essential part and applications of these technologies to farm animal species. Thus neither triploid fish nor genetically modified mice for use in research are examined.

A first draft of the report was presented at a collaborative workshop in Seville involving the Institute of Prospective Technological Studies (DG JRC-IPTS, Sustainability in Agriculture, Food and Health unit) and the Danish Centre for Bioethics and Risk Assessment (CeBRA)/DG RTD (Biotechnology, Agriculture and Food directorate), attended by a panel of around 35 experts on the science of animal cloning. Where appropriate, information presented at this workshop will be used to qualify the information from the literature presented in this report.

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The front page picture has been downloaded from the Roslin Institute Image Library: <http://www.roslin.ac.uk/imagelibrary/>

## **2: Different kinds of cloning**

This chapter outlines various kinds of cloning. It describes in detail the methods currently used to clone farm animals, and the latest developments in this technology.

### **2.1: Cloning in nature**

Cloning as asexual reproduction is a very common form of multiplication in *plants*. All plant organs can be sources of asexual reproduction, but stems are the most common. Above-ground stems (stolons) of strawberry plants produce new plants. Underground stems include rhizomes, bulbs, corms and tubers. Leaves in irises and roots of aspen are also sources of new plants (Kimball's Biology Pages, 2005).

In *animals* the reproductive process is also diversified to the point that almost any mechanism we can imagine has already been implemented. The various forms of asexual reproduction coexist with hermaphroditism and bisexual external and internal copulation (Benagiano and Primero, 2002). Asexual reproduction includes budding (jellyfish, corals and tapeworms), fragmentation (worms), and parthenogenesis (some fishes, insects, frogs and lizards). However, most of the animals that are able to reproduce asexually reproduce through parthenogenesis only at certain times. Aphids use parthenogenesis in the spring when they find themselves with ample food. Parthenogenesis is more rapid than sexual reproduction and permits quick exploitation of available resources. In honeybees, fertilised eggs become females, while haploid unfertilised (parthenogenetic) eggs become males (Kimball's Biology Pages, 2005). However, it should be noted that asexual reproduction of mammals is not a naturally occurring phenomenon although in mammalian reproduction genetically identical individuals, known as monozygotic twins, do occur. These can however not be considered clones in this respect because they a: are not the result of asexual reproduction and b: they share all their genetic material where artificially produced clones only share their core DNA, whereas the mitochondrial DNA differs.

Active induction of asexual plant reproduction (using grafting and rooting) has been a common practice in agriculture since early human history. It has been used to breed and retain particularly desirable traits such as growth, flavour and resistance. In this case, it can be said that humans have used the naturally occurring process of reproduction of the desired plant species. But when it comes to farm animals, asexual reproduction in nature is as good as non-existent. They all reproduce by combining the genes of two individual organisms. Again cloning is a new phenomenon in the history of human development of farm animal species.

### **2.2: Embryo splitting**

The first cloning experiments on animals date from the nineteenth century. In 1891 Hans Driesch separated the blastomeres (cells formed in the first stages of embryonic development) of a two-cell embryo of sea urchin mechanically by shaking them in sea water. The cells started to grow independently and formed two whole sea urchins (Driesch, 1891). Eleven years later the same experiment, with similar results, was performed by Hans Spemann in a

vertebrate (salamander) using a hair from his baby boy to separate the cells (Spemann, 1902). However, the unavailability of an efficient handling system and, more importantly, lack of recognition that mammalian oocytes and preimplantation embryos require strictly controlled temperature for development (Bavister, 2002) hampered the application of the procedure to mammals for almost 80 years. Eventually the first successful embryo splitting was performed in domestic animals with the purpose of rapid multiplication of valuable individuals (Willadsen, 1979; Ozil et al., 1982). However, following initial enthusiasm, the procedure failed to become as efficient as expected: technical difficulties, compromised pregnancy rates and the simple mathematical limits of the procedure had not been fully appreciated. Even if the technical difficulties had been resolved and the pregnancy rates improved, it would have remained the case that an embryo can only be split 1-2 times and thus can create at most 2-4 genetically identical siblings through artificial splitting.

More recently individual blastomeres from cattle embryos at the 2-, 4-, and 8-cell stage have been isolated and grown into blastocysts. These were then transferred into surrogate zonae pellucidae and used to produce genetically identical quadruplets (Johnson et al., 1995).

### ***2.3: Embryonic cell cloning***

A completely different approach to asexual reproduction was discovered by accident by Jacques Loeb in 1894. In attempt to induce parthenogenesis in sea urchin embryos using different salt concentrations, he observed the formation of a large bleb in some of the early embryos. While the rest of the embryo started to develop, this bleb remained unchanged. However, in certain cases one cell nucleus entered the bleb, and this part of the embryo started to develop as well. Moreover, when it was separated from the original embryo, the new part continued to develop alone (Loeb, 1894). What Loeb had discovered was that embryos could be created by moving the cell nucleus between cells. In a more consistent model, Hans Spemann (1914) again used the hair-loop method to bring about transient yet complete separation in salamander embryos. Retrospectively, both models were ingenious and extremely innovative and both of them were by-products of other experimental models for which these authors have become famous.

The first success in mammalian nuclear transfer used embryonic cells as donors and was reported by Illmensee and Hoppe (1981). Illmensee and Hoppe described the birth of three mice following the transplantation of early embryo cells into enucleated zygotes (one-cell embryos). However, nobody (including the authors) could repeat this experiment. Many scientists supposed that the report was fraudulent, and after frustrating, unsuccessful attempts to use the procedure, leading experimental embryologists declared that the cloning of mammals by simple nuclear transfer was biologically impossible (McGrath and Solter, 1984).

However, not all scientists accepted this verdict. At Cambridge University, Steen Willadsen, a domestic animal embryologist, made a fresh attempt to repeat the experiments of Illmensee and Hoppe in sheep. After improving the technology in many details, but obtaining no significant success, he decided to use oocytes (egg cells) instead of zygotes as recipients. This idea was unsupported at the time, but it resulted in the vital breakthrough: the first cloned mammals (Willadsen, 1986). Since Willadsen's pioneering work, the same principles have

resulted in births of embryonic cell-cloned offspring in other domestic species, including cattle (Prather et al., 1987) and pigs (Prather et al., 1989).

Somewhat surprisingly, this scientific breakthrough created only a minor reaction in the scientific community and went almost entirely unnoticed by the public. Some animal breeding companies regarded it as a potentially useful way of rapidly multiplying animals with valuable genetic traits, but the efficiency of the technique remained stubbornly low. (The theoretical maximum is 32 transferable embryos from a single 32-cell donor embryo. This was never reached.) As it turned out, income generated by the advance did not cover the costs of the laboratory and embryo transfer work. Eventually, support for research in this field dramatically decreased in the early 1990s, and except for a few laboratories embryologists throughout the world have focused on other subjects.

## **2.4: Somatic Cell Nuclear Transfer (SCNT)**

All the experiments described above used embryonic cells as sources for donor nuclei. However, the possibility of reversing the process of cell differentiation and, hence, of using developmentally later cells for nuclear transfer had been described in 1938 by Hans Spemann. He suggested that one could transfer nuclei of morula stage embryos (approximately 4 days old) or "older nuclei of various cells" into enucleated eggs. He called the idea "somewhat fantastic" because of the foreseeable technical difficulties (Spemann, 1938).

Strangely, the two scientists who eventually carried out Spemann's fantastic experiment had not even heard about his proposal at the time. Briggs and King (1952) were the first to perform nuclear transfer (as here defined). Using frogs, they removed the nuclei of recipient eggs and inserted a donor nucleus. Initially, the donor nuclei were obtained from morula stage embryos. Later they came from tadpoles. This work was continued by many scientists, including John B. Gurdon (1962). Although some reviews state otherwise, the full developmental cycle was never completed: donor cells from tadpoles resulted in fully developed frogs, and donor cells from fully developed frogs resulted in tadpoles, but in spite of extensive efforts no adult frogs were cloned from adult frog cells (Di Berardino, 2001).

In retrospect, it may appear peculiar that scientists did not attempt to use adult somatic cells as nucleic donors in the mammalian experiments performed between 1986 and 1997<sup>1</sup>. The technical problems of the approach were negligible, and the move to adult somatic nucleic donation, and the overcoming of the problems to which it gave rise, were the only real innovations of the team (led by Ian Wilmut at the Roslin Institute) that eventually succeeded in cloning the sheep Dolly: serum starvation of somatic cells before transfer was later proved insignificant, although it was initially seen as the key to success. So even though some earlier publications had indicated that cultured cells of embryos at an advanced stage of development could be used as donors for nuclear transfer (Sims and First, 1993; Campbell et al., 1996), it

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<sup>1</sup> Part of the explanation for this could be the shockwaves sent through the scientific community and the associated funding system by the Karl Ilmensee affair in 1984-85. Professor Karl Ilmensee, an embryologist at the University of Geneva, was accused of falsifying his nuclear manipulation experiments in mice. Prof. Ilmensee lost his NIH grant and later resigned from his post at the University. It is tempting to speculate that applications involving nuclear cloning would have been regarded as neither scientifically nor politically correct for a number of years after that.

was only after the birth of Dolly (Wilmut et al., 1997) that it became accepted that it was possible to clone a grown animal by removing the nucleus of a somatic cell from an adult and inserting it into an enucleated egg. It was a discovery that stunned most researchers, made it necessary to rewrite all the books on developmental biology, and faced the world with new scientific and commercial — as well as ethical — challenges.

### **2.4.1: The traditional way of SCNT**

Although considerable variation exists, the basic features of the somatic cell nuclear transfer procedure currently used in farm animals can be summarised as follows.

Recipient oocytes are usually obtained from ovaries sourced from abattoirs. They are matured *in vitro*. This abundant and inexpensive supply compensates for the fact that the oocytes are slightly lower in quality than those derived directly from adult females after *in vivo* maturation induced by hormonal treatment.

Donor somatic cells are now derived from various tissues and from animals of various ages (foetuses, newborn, young and elderly animals, and even from deceased animals a relatively short period after death). Despite extensive efforts, live offspring have been obtained from only a dozen of the approximately 200 types of adult differentiated tissue that exist in mammals. The reasons for this are unknown. Some tissues, including those present in the female genital tract (granulosa cells, oviductal epithelial cells), seem to be more suitable for generating offspring than others. One might sensibly suppose that the tissue of younger animals and tissue with less differentiated cells would be the best sources of donor nuclei, but no conclusive evidence supports this hypothesis. And various pre-treatments of the donor cells have resulted in limited success so far (Vajta, 2004).

The standard SCNT procedure includes three steps: the enucleation of oocytes, the insertion of the donor cells (or nuclei) and the activation of the reconstructed embryo. Subsequently, the cloned embryos are cultured *in vitro* for a period, and when they reach the optimal stage for embryo transfer they are transplanted into a “mother” animal.

In standard SCNT the zona pellucida is preserved. This flexible, transparent acellular shell of the egg is regarded as important in supporting further development until day 6-8 (depending on species), when embryos normally outgrow, and “hatch” from, the zona. To make the necessary delicate manipulations inside the zona pellucida, when the original nucleus is removed and the new one transferred, special and expensive instruments such as micromanipulators are required. The handling of these micromanipulators, as well as the preparation of micropipettes used during micromanipulation, are difficult tasks and require a specially trained, highly skilled workforce and considerable investment.

Enucleation is usually performed mechanically by fixing the oocytes in the appropriate position with the polished end of a holding pipette and a slight vacuum, and by aspirating the chromatin-containing part of the oocyte into a sharp enucleation pipette that has been passed through the zona pellucida. There are several strategies for finding the chromatin-containing part of the cytoplasm. None is completely reliable and harmless, but careful use of a strategy may result in a good overall success rate and minor damage.

The most common way of inserting somatic cells is by injecting them under the zona pellucida and then introducing an electric impulse to induce cell membrane fusion between the enucleated oocyte and the somatic cell. Alternative ways include injection of the cytoplasm-free donor nucleus or the whole somatic cell into the cytoplasm. Earlier attempts to induce fusion with chemical and viral agents have been less successful and would now be rarely used.

When the embryo has been reconstructed, it must be activated. In normal fertilisation, the level of maturation promoting factor (MPF) decreases as a result of the repeated rise of calcium ion level in the cytoplasm induced by the sperm's penetration. Just about any mechanism inducing increased cytoplasmic calcium levels may be used for activation, including various mechanical, electrical and chemical agents. The subsequent drop in MPF level may be maintained by protein synthesis inhibitors for several hours until the reconstructed embryo escapes from developmental arrest. The usual triggers of activation are an electric impulse or short exposure to a chemical agent followed by an extended block on protein synthesis.

After reconstruction and activation, the embryos start to cleave. Under appropriate culture conditions they develop to the stage at which they are optimally ready to be transferred. This stage depends on the species. In pigs, embryos are usually transferred at one-cell stage, as surgical procedure is unavoidable, anyway, and the developmental rates under in vitro culture conditions are rather compromised. On the other hand, in cattle non-surgical transfer can be performed at day seven after activation, and the in vitro culture is relatively efficient. So here, extended in vitro growth of embryos is common. To date, standard SCNT (with some modifications) has resulted in the birth of live offspring in 11 species, including cattle, pigs, sheep and goats.

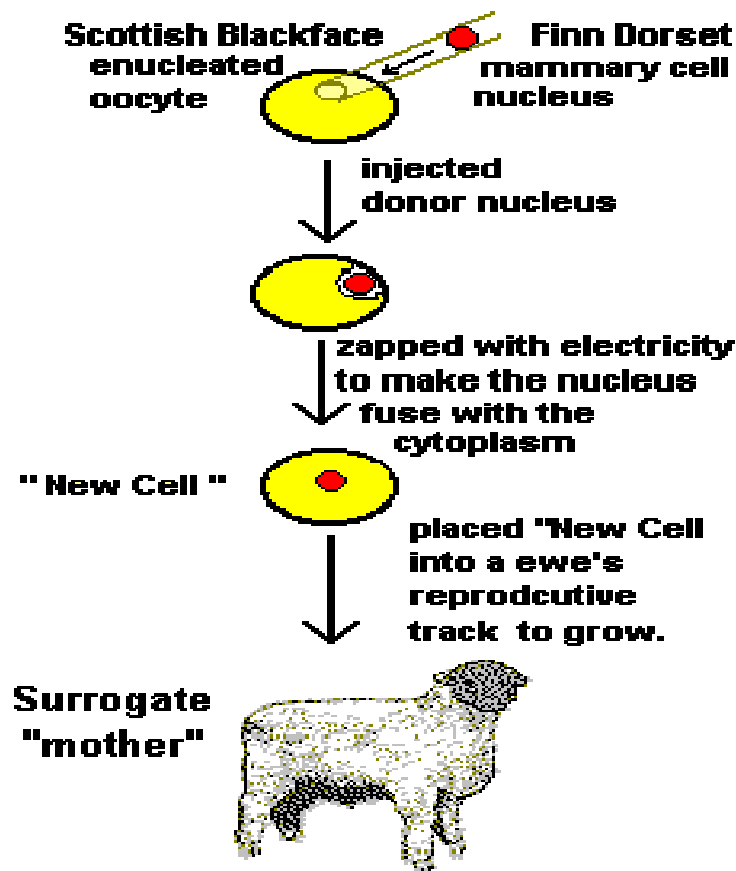


Figure 1: How Dolly was made ([www.synapses.co.uk/science/clone.html](http://www.synapses.co.uk/science/clone.html))

#### 2.4.2: Handmade Cloning (HMC)

A relatively new approach to nuclear transfer is the so-called Handmade Cloning Technique (HMC). This was initially used for embryonic cell nuclear transfer (Peura et al., 1998) and then modified for SCNT (Vajta et al., 2001; 2003). In this method, the zona pellucida is removed after maturation and before enucleation.

The advantage of this approach is that micromanipulators are not needed for enucleation and fusion. This reduces the costs associated with investment in laboratory equipment and the employment of highly skilled workforce to operate it. With some modifications, the procedure delivers highly efficient enucleation, fusion and activation results. The *in vitro* culturing of embryos without zonae pellucida might seem hazardous, but with modifications to the culture system, appropriate developmental rates can be achieved. Additionally, the procedure has the potential to be standardised and automated (Vajta 2004).

HMC and similar zona-free techniques have resulted in the birth of approximately 20-25 healthy calves in Australia, New Zealand and South Africa, and ongoing experiments, now at an advanced stage, suggest possible porcine pregnancies in the foreseeable future. It is still too early to tell if HMC will be able to replace the traditional way of performing SCNT, but the technology seems to have considerable potential.

### **3: Challenges, barriers and problems**

The major technical obstacle to widespread application of SCNT is the inefficiency of the method. This inefficiency varies from species to species, but it generally involves all three phases of the procedure: the laboratory part, the in utero part (i.e. collectively before birth), and the postnatal development part. It is estimated that today about 4% of cloned embryos result in viable offspring (Paterson et al., 2003).

#### **3.1: *In vitro* phase**

Considering the delicate handling of genetic material during the normal process of fertilisation and natural gamete development, it is simply a miracle that a drastic intrusion such as somatic cell nuclear transfer ever results in embryos, foetuses and viable offspring. It seems that nature is more flexible than science once supposed and can — to a degree — compensate for the enormous damage caused by the primitive manipulations of the scientist. The most promising overall approach today is therefore to study the naturally occurring processes, and then to perform the manipulations accordingly. This should minimise injury and also support the natural mechanisms of repair.

Various deviations from the normal developmental pattern have been reported. Short-term developmental abnormalities include decreased embryonic growth, fragility and chromosome abnormalities. In addition, there can be an inappropriate distribution of cells between the inner cell mass (ICM) and the cells that differentiate into the outermost layer of cells of the blastocyst (trophectoderm). In contrast with short-term abnormalities, which are either eliminated during the first weeks of development or cause the early death of embryos, the more stable epigenetic changes may arise early, i.e. during *in vitro* culture, but reveal themselves much later in foetal or even adult life. Thus, the diagnosis of epigenetic changes involves studies of DNA and histone modifications rather than the detection of changes in gene expression.

On present knowledge, anomalies related to nuclear transfer can be caused by the following factors (Vajta 2004):

- Inappropriate donor cell or/and recipient oocyte
- Inappropriate synchrony between the cell cycle phase of donor nucleus and recipient cytoplasm
- Inappropriate reprogramming of the donor genome
- Inappropriate handling of oocytes, somatic cells and embryos during maturation, various manipulations and cultural techniques causing mechanical, osmotic, electrical, toxic, thermal and other types of damage

Some of these factors are self-evident; others, including the problems occurring during reprogramming of the nucleus, need further explanation. As a general rule, all cells in the body contain a full set of genetic information, and changes in gene activity reflect a more or less open configuration of the genes. The inaccessible gene regions are often modified by methylations from the DNA base cytosine and the associated histone proteins. These

methyations are passed on to daughter cells, but they may be modified by cytoplasmatic factors such as those occurring in the oocyte's cytoplasm. The success of SCNT makes a strong argument that sufficient deprogramming activity is present in the ooplasm to completely change the programming of adult cells, but the nature of the factors has not been precisely characterised. Observations on changes in the global level of methylation in embryos are rather contradictory; the mechanisms may differ from species to species. Attention has therefore turned to include the functional aspect of methylation change, and its role on the imprinted genes. Imprinted genes are particularly interesting since they affect the balance of foetal growth and foetal membrane growth. Imprinted genes show a parentage-specific, stable silencing of one of the two alleles, and silencing is often related to differential methylation of the two alleles. Since disturbances in placental as well as foetal growth have been repeatedly associated with in vitro culture and SCNT, these genes are key candidates for study in work on epigenetic alterations due to SCNT.

### **3.2: *In vivo phase***

Developmental anomalies produced by SCNT after embryo transfer include low pregnancy rates, an unacceptably high level of losses during early and late pregnancy, stillbirths, early postnatal deaths, short lifespan, obesity and malformations. So far, these phenomena are poorly understood — a fact that is reflected in the lack of consensus in nomenclature and classification.

The term often used to describe some of these developmental anomalies is “large offspring syndrome” (LOS). Essentially, LOS refers to increased birth weight. However, LOS is not confined to SCNT. It was first described after in vitro embryo production in ruminants (Walker et al., 1998). It was also observed after the vitrification of bovine oocytes (Jacobsen et al., 2000). In sheep, in vitro maturation and fertilisation may result in developmental abnormalities irrespective of the subsequent in vivo or in vitro culture (Holm et al., 1996). Embryo culture involving a high level of serum and co-culture with somatic cells have also been reported to cause large offspring in sheep (Sinclair et al., 1997; Jacobsen et al., 2000). It is interesting to note that LOS sheep had significantly altered levels of the foetal growth factor IGF2R and simultaneous alteration in the methylation's status as the regulatory part of this gene (Young et al., 2001). IGF2R is an imprinted gene. Hence changes in its foetal expression could be due to alteration of its methylation programme induced by the high serum level during in vitro development.

LOS is now used to describe a number of malformations and diseases. Increased birth weight is just one of these manifestations. Depending on the author, some, most or all of the phenomena listed below are regarded as elements of LOS. The common feature is that all of these phenomena have been found in animals in which embryos were in some way manipulated in vitro — in the majority of cases exclusively by nuclear transfer. In sheep, cows and mice the following problems were detected (Vajta, 2004):

- Placental abnormalities
- Foetal overgrowth, prolonged gestation
- Stillbirth, hypoxia, respiratory failure and circulatory problems, lack of post-natal vigour

- Increased body temperature at birth
- Malformations in the urogenital tract (hydronephrosis, testicular hypoplasia)
- Malformations in liver and brain,
- Immune dysfunction, lymphoid hypoplasia, anaemia, thymic atrophy
- Bacterial and viral infections

Although they are suspected by many, neither a common origin nor a common mechanism behind these phenomena has been proved so far. Eggan et al. (2001) found no correlation between placental and embryonic overgrowth and neonatal survival in cloned mice. However, in cattle a reduction in viability in utero has been shown to be associated with abnormal placental development (Hill et al., 2000) while neonatal deaths are often associated with cardiopulmonary maladies (Hill et al., 1999; Jones, 2001).

According to Chavatte-Palmer et al. (2003) there are two categories of loss after birth: those in the early neonatal period (within one week) and those in the following phase lasting up to several months. Early death is caused by the failure of the cardiovascular system and the renal system, sometimes because of internal haemorrhages, skeletal deformations, gastrointestinal problems and multiple organ failure. Hydrocephalus and infections were also reported in this period. It seems that initial problems are related to events that took place during pregnancy or defects at placentation. Later, the main cause is thymic aplasia, the increased size of pituitary. Some animals die without any real pathological finding, others with diseases that cannot be directly connected with the death, including, for example, liver steatosis. During this period some laboratory parameters may be abnormal, but these return to normal later. After six months, cloned cattle do not display major differences from control animals.

It should be emphasised again that not only the increased birth weight, but almost all elements of the LOS, can be detected after processes other than nuclear transfer (Young et al., 1998). A good example here is the interspecies embryo transfer between *Bos gaurus* and *Bos taurus*, where severe placental abnormalities and respiratory problems are both consistently found and similar to those occurring (sporadically) in in vitro produced and (more frequently) cloned pregnancies (Hammer et al., 2000). Similar findings have been reported in interspecies crosses in mice and used to identify candidate genes for the LOS phenotype (Singh et al., 2004).

On the other hand, the occurrence of LOS seems to be species-specific: it is quite frequent in cattle, sheep and mice, and some elements have also been described in humans after assisted reproductive procedures (Ménézo et al., 2000). However, almost no malformations were detected in pigs and goats (Betthausen et al., 2000; Reggio et al., 2001, respectively), although these results — especially regarding goats — require confirmation. Some publications question the sharp difference between species. A recent paper by Carter et al. (2002) has shown decreased survival, infections and cardiac problems in transgenic cloned piglets. Also, Phelps et al. (2003) have found large tongue and kidneys in piglets produced with galactosyltransferase deficient somatic cells, and have attributed this phenomenon to nuclear transfer, not the transgenic cell line.

As should be clear by now, the reasons for, and implications of, LOS remain unclear. But whether or not LOS is a specific problem within the cloning technology or more broadly within reproduction technology, and whether or not it is species-specific, it is clear that a

better understanding of the scientific background will increase not just the *efficiency* of the cloning technology but the *welfare* of the cloned animals.

A final problem that should be discussed here concerns the age of cloned animals. At the end of the chromosomes there are stabilisers known as telomeres. They consist of the same chemical bases as the rest of the genome. What is especially interesting about telomeres is that they shorten a little every time the cell replicates itself. This has led many to view telomeres as a kind of biological clock in the cells showing the age of the cells (Xu and Yang, 2003). The most famous example of this is the cloned sheep Dolly. When examined, her telomeres were shown to be shorter than would be expected given her biological age, but comparable to that of the 6-year-old sheep that donated the nucleus from which she developed (Shiels et al., 1999).

Other studies, however, have shown that animals cloned by SCNT did not have shortened telomeres; in some cases, indeed, the animals had longer telomeres than the donor animals. So far the evidence is inconclusive, but it seems to suggest that the length of the telomeres depends on not just the age of the donor animal but also a range of parameters, including cell type, cell culture, nuclear transfer procedure, sampling and the measuring protocol (Xu and Yang, 2003).

At present we possess very limited data on the life-span of clones. Dolly's longevity may not be typical. The first cloned mouse, Cumulina, died at the age of 2 years and 7 months, which is slightly longer than the average lifespan of a mouse. The importance of telomere length to the survival and health of cloned animals, require (like many other factors in cloning) further research.

### **3.3: Experience and technical choices profoundly influence the outcome**

It has been stated that the cumulative damage following each step of nuclear transfer (NT) may reduce the developmental potential of NT embryos and could ultimately cause embryonic or foetal death at any point in development (Dominko et al., 1999). Among the factors that can influence the outcome of a cloning programme, the activation process has probably been investigated most thoroughly.

Numerous experiments have shown that even small changes in the way that the reconstructed egg is activated can affect the number of transferable embryos. The types of chemical used, the parameters of the induced electric impulse, the in vitro system, and also the donor cell source and the culture medium that the cell is reconstructed in, all play an important role (Vajta, 2004).

It should be noted, however, that relatively little information has been published on the long-term effects of enucleation and reconstruction, although many would agree with Yanagimachi (2002), who states that the technical skill here greatly contributes to the success rate of cloning. According to Wakayama (2003), the extent to which the outcome of NT reflects the technical details of the methodologies used rather than properties inherent in the biological material is unknown. Seemingly, many of the differences observed are due to relative

disparities in operator skills. One factor may be speed of manipulation. Workers performing experiments side by side with shared samples can produce irreconcilable data (Perry and Wakayama, 2002). These differences might reflect technical influences.

This suggests that technical considerations should be strongly taken into account when data are being interpreted. This is especially true when a slightly modified method has been employed. One publication provides surprisingly strong evidence that small changes in the nuclear transfer protocol may result in profound changes in the outcome of NT (Walker et al., 2002). The simplified technique used here, with rational use of media and a micromanipulator, resulted in 5% overall efficiency, i.e. healthy piglet, per oocyte (ibid.). This result could imply a breakthrough in porcine NT and may encourage a new approach to research in other species.

At any rate, it is becoming clear that an element of craftsmanship (experience, technical skills, intuitive understanding and luck) influences the outcome of the cloning procedure. This influence cannot be eradicated, although some think that it can be reduced through more elaborate protocols.

### ***3.4: The first and the second generation***

It is a fact that only a few clones are long-term survivors, and that even these animals may have altered pattern of gene expression (Yanagimachi, 2002). Even apparently healthy cloned animals may still have epigenetic defects affecting the expression of genes activated later in development or adulthood (Chavatte-Palmer et al., 2002).

On the other hand according to observations of various groups, the offspring of cloned offspring are almost always healthy (Yanagimachi, 2002; Wells et al., 2004), as are the offspring of two cloned parents (Wells et al., 2004). Bulls cloned from a top bull have the same performance in respect of semen quality, IVF and in vitro fertilising ability, pregnancy and calving outcome. Clinical parameters of the offspring here were normal (Galli et al., 2003). Even obese phenotypes give birth to normal mice (Tamashiro et al., 2002). Reproductive parameters of cloned females here do not differ from those in the control group, and the offspring are alive and normal (Heyman et al., 2004). The pre-weaning performance and health of piglets born from cloned boars and cloned females (including number of piglets, birth weight, perinatal and pre-weaning mortality) did not differ from that of the control group (Martin et al., 2004). This parallels the relatively intact chromosome constitution of the cloned embryos, but is in contrast with the extent of the various problems related to gene function. As would be expected, abnormalities connected with epigenetic alterations seem to be erased when cell nuclei go through the germ line, i.e. corrected during gametogenesis (Yanagimachi, 2002). This last observation is very important from the point of view both of basic research and (even more so) practical application. It needs, however, to be confirmed at the molecular level, i.e. by investigations of germ cells from both male and female SCNT animals.

The cloning of farm animals involves young technology and so, around it, there are many unanswered questions. We have not mentioned all of them here. We have merely highlighted some of the more important ones. The latter must be answered if the theoretical applications

that will be discussed in the next chapter are to become reality. Whether these questions are solvable, present long-term challenges, or introduce insurmountable barriers, remains to be seen.

## **4: Applications of farm animal cloning**

Theoretically, the applications of cloning in research, industry and agriculture are almost limitless. At present, low levels of efficiency are the only factor hampering most applications from a technical point of view. Considerable efforts are therefore being made either to increase efficiency or identify situations where even the present levels of efficiency would result in competitive outcomes.

Recently the current as well as potential applications of farm animal cloning have been reviewed (Colman, 1999; Faber et al., 2003; Denning and Priddle, 2003; Paterson et al., 2003), and it has been suggested that these applications can be divided into two general areas of applications: *biomedical* and *agricultural*. An application that falls outside this scheme is basic research. Here the cloning of farm animals could have a potentially huge impact on our understanding of the factors that determine what genes are expressed in the phenotype (Reick & Dean, 2002). There is a growing awareness within the scientific community that epigenetic factors might play a large role in determining the traits of cloned animals. These factors may limit the project of creating identical individuals through cloning.

It should be noted that many applications are only theoretically possible at present owing to the inefficiency of current technology. These applications will only become useful if many of the problems mentioned in section 3 of this report are solved or at least substantially better understood.

### **4.1: Biomedical applications**

The area in which cloning of farm animals has the greatest potential so far is the biomedical area. This is because the technology, combined with the possibility to introduce genetic changes into the animals, opens up new opportunities in both research and drug development, and because here the inefficiency of the technology, from a strictly economic perspective, is a minor problem since the animals that are created will have a relatively high value (as compared with livestock).

The use of SCNT on farm animals is a relatively new technology (eight years old). This explains to some degree the difficulties, from a technological and scientific perspective, in producing high efficiency rates. But it also means that there is as yet no clear picture of the application possibilities. (Perhaps it is fairer to say that insufficient experience has been built up as yet to rule out some of the possibilities.) In the literature there seems to be no set boundaries to the areas where the technology will be able to improve biotechnology within the biomedical sector.

Since the 1980s it has been possible to genetically modify mammals by injecting copies of desired genes into one of the two pronuclei in the zygote. This method is, however, very inefficient. Most of the injected embryos do not develop, and less than 1% of the individuals born have the desired genetic change. Furthermore, the method can only introduce new genes

into the genome and these often cause problems, because the integration site is random (Paterson et al., 2003).

Cloning offers an opportunity to use genetically modified cells for the nuclear transfer. By introducing the genetic changes into cells, and then choosing the ones with the desired changes for the cloning procedure, other and more precise genetic modifications can be introduced into the genomes of farm animal species.

Another possibility is to clone individual transgenic animals that are known to carry desired changes (Paterson et al., 2003). Hereby, new possibilities are opened up involving transgenic and cloned farm animals. In this report we have chosen to describe the two applications that seem the most realistic within the next 3-5 years, namely: the creation of *disease* models and *bioreactors*. Furthermore we will briefly describe a series of applications in agriculture. These are often mentioned in the literature and have in common that they by most scientists are seen as possibilities that lie 10 years, or more, ahead and are highly dependent on the technology becoming more efficient. Finally, we will describe possibilities within *xenotransplantation* — not because it is a realistic opportunity within the next few years, but because it is often mentioned when the advantages of cloning of farm animals are discussed, and because it is one of the most controversial applications from a societal and ethical point of view. In the report on the ethical aspects of the cloning technology that will be written as part of the CLONING IN PUBLIC project, we will return to these aspects of the technology.

#### **4.1.1: Disease models**

Disease models are animals that have been designed to express, either at the genotypic or phenotypic level, a certain human disease. They can be used both to further understanding of the disease and to do initial tests on possible treatments. Since the 1980s, a large number of mouse models for human diseases have been developed. These models, however, have limitations owing to the physiological differences between mice and men. The combination of transgenesis and cloning is seen as a solution to this problem, since it will enable scientists to design and create larger and physiologically more congruent animals, such as sheep that express human diseases. An example of this is mentioned in Paterson et al. (2003), where the creation of a sheep that expresses cystic fibrosis is envisaged. Sheep are ideal animals because of the similarities, in physiology and size, of their lungs and those of humans. From a biomedical point of view, the creation of such a sheep would lead to an improvement in research into cystic fibrosis as well as a more suitable model to test new therapies on.

Our scientific understanding of the impact of introducing genetic changes into farm animals, and of cloning them, is still developing, and the technologies needed to create disease models consequently remain inefficient. But given the huge biomedical potential and the considerable economical value of such disease models, this will undoubtedly be an early application of cloning technology.

### **4.1.2: Bioreactors**

Bioreactors are transgenic animals that have had genes that produce human proteins inserted into their genome. These proteins can subsequently be harvested from the animal and used within the biomedical sector as medicine. Most of the research in this field has been designed to get the transgenic animals to express the desired proteins in their milk. Goats, cows, sheep and pigs have been genetically modified and cloned in attempts to create such proteins. Potentially, proteins for the treatment of a range of human diseases can be produced this way in the future. Proteins such as human coagulation factor IX, Human anti-thrombin and Alfa-1-antitrypsin have all been harvested in an experimental setting from transgenic and cloned animals today.

However, the inefficiency of the technologies and tight regulation of medicine in general are obstacles to the deployment of bioreactor technologies. Especially the risk of introducing new diseases (zoonoses) into humans by using animals as medicine factories generates a serious need for these biological compounds to be thoroughly tested before commercialisation. High demands on the safety of the products and low levels of efficiency combine to slow down their development and marketing. However, the production of, for example, transgenic cows that secrete commercial quantities of valuable human proteins will face less stringent efficiency requirements owing to the potential value of the end product. Still, the total production costs, including the gene construction, transgenic animal production, and extraction and purification of the product, must be balanced against income opportunities to gauge commercial viability (Lewis et al., 2004). Regulatory requirements to cope with these new production methods are still evolving.

### **4.1.3: Xenotransplantation**

Xenotransplantation (the transplantation of organs between distinct species) is considered by some as the most promising solution to the growing gap between demand and supply of organs suitable for humans (Niemann et al., 2003). For several reasons, the pig appears to be the most suitable donor animal: it has organs of a similar size to those found in humans; porcine anatomy and physiology are not too different from their human counterparts; pigs have short reproductive cycles and large litters; pigs have rapid growth; practical maintenance is relatively cheap; and pigs are a domesticated species (Pinkert, 1994). The use of non-human primates as donors has largely been ruled out owing to problems with raising them, welfare problems affecting the animals (e.g. isolation of infants) and a suspected higher risk of transferring diseases from donor to human recipient (Paterson et al., 2003). Finally, it should be mentioned that many scientists believe that it will be more acceptable to the public to use pigs as donors, because, at least in most of the Western world, we are used to viewing the pig as a production animal (Videnskabsministeriet, 2003)

The use of pigs as organ donors is one of the most widely discussed uses of transgenesis and cloning, although it seems to be an application that will not be viable for a long time. Not only does it raise much public concern and many ethical questions (which we will address in a subsequent report on the ethical issues of farm animal cloning), but, from a scientific and

technological point of view, additional challenges arise. Three problems have to be solved before the technology can even be considered as a practical option.

A: The prevention of transmission of zoonoses from donor animal to human recipient. It has been shown that endogenous retroviruses can be produced by porcine cell lines and infect human cell lines (Patience et al., 1997). However, to date no infection has been reported in patients who had received various living porcine tissues for periods up to 12 years (Paradis et al., 1999). By employing high quality hygienic management, it has been shown that transgenic animals can be kept free from all known bacteria and viruses. Recent studies have shown that the risk of virus transmission is very low (Dinsmore et al., 2000). Furthermore, a certain inbred strain of miniature swine has been identified that failed to produce known human-tropic replication competent porcine endogenous retroviruses (PERVs). It should be noted, however, that these pigs may still contain PERV loci that are not detectable by current methods (Oldmixon et al., 2002). To date three retroviruses (PERV A, B and C) have been discovered. It is believed that these can be controlled, although not eradicated, by selective breeding. Another virus that could cause cross-species infections is porcine cytomegalovirus. It has been shown that it can be excluded from a herd by early weaning of piglets. Finally a porcine lymphotropic herpesvirus has been identified. The risks associated with this virus and possible ways of excluding it from potential donor animals is still unidentified (Fishman & Patience 2004).

In a review article Fishman & Patience (2004: 1390) conclude that current data suggest consideration now can be given to research “into other barriers to clinical xenotransplantation (immune and metabolic) as a prelude to clinical trials. The conclusion – based on an interpretation of the reviewed literature – is that “the risks of human infection resulting from xenotransplantation are manageable”. This conclusion may seem a bit rash considering the reservations taken in their review stemming from the lack of knowledge about the pig genome, possible viral transmitters and so on.

One issue should be briefly mentioned here though. Fishman and Patience suggest that to manage the risks associated with xenotransplantation a routine monitoring programme of patients that have received xeno-organs and their intimate contacts must be put in place. At a first glance this may seem unproblematic, but at the very least the discrepancy between developing a scientifically based monitoring programme that would work if people behave as they are supposed to and the way that people actually behave in a social context, demands more attention before such a programme could be said to provide any kind of safety against a possible infection being spread to the general populace.

Finally it must be acknowledged that our understanding of the pig genome and the possible viruses that could be biologically inactive and therefore go undetected will have to improve dramatically before it can be said with any certainty that the procedure is safe. One of the problems is that even keeping the pigs in high quality hygienic conditions will not prevent the transfer of PERVs (Paterson et al., 2003).

B: Compatibility of the donor organs in anatomy and physiology with the human organ system. Not much is known in this area. Porcine organs transplanted into non-human primates were able to maintain a number of biochemical parameters within the physiological range (Vajta, 2004), but no firm conclusions can be drawn on this basis. Only future research into

this area will show whether the physiological, anatomical, hormonal and chemical differences between pigs and humans can be overcome.

C: Overcoming the immunological rejection of the transplanted organs. Differences between pigs and humans mean that a normal pig organ, transplanted into a human, would be rejected by the body within a few minutes. This hyperacute rejection (HAR) response occurs because antibodies react with antigenic structures on the surface of the porcine organ and induce HAR by activating the complement cascade. Many reports indicate that the enzyme  $\alpha$ 1,3 galactosyltransferase (present on cell surfaces in almost all mammals with the exception of humans, apes and Old World monkeys) is involved in the HAR response (Phelps et al., 2003). Pigs have been produced that have disrupted genes for the enzyme thus being unable to express  $\alpha$ 1,3-gal sugars on the cell surfaces (so called knock-out pigs) (Lai et al., 2002, Fishman & Patience, 2004). The hope is that an absence of the enzyme in the donor organ will prevent HAR.

A similar problem is acute vascular rejection (AVR), which occurs within a few days of transplantation and is thought to be induced by xenoreactive antibodies. Its pathological symptoms are similar to those of HAR. It is not yet known whether AVR is also provoked by the presence of the  $\alpha$ 1,3 galactosyltransferase, or whether something else triggers rejection of the donor organ (Videnskabsministeriet, 2003).

A cellular rejection process occurs within a few days of transplantation. During this process, the blood vessels of the new organ are damaged by T-cells which invade the intercellular spaces and destroy the organ. This rejection also occurs after allotransplantation (human-to-human transplantation) and is normally countered by immunosuppressive drugs. These have to be taken by the recipients of transplanted organs for the rest of their lives (Vajta, 2004).

Recent research in which organs from alpha1,3-galactosyltransferase gene-knockout donors have been transplanted to baboons has shown promising results. The organs functioned in their new host organisms for up till 83 days (Yamada et al., 2005).

A chronic rejection process is the final challenge in this area. It is a complex immunological process that may result in the rejection of the transplanted organ after several years. It is slow, progressive, and its aetiology is largely unknown. The only therapeutic possibility is another transplantation (Vajta, 2004).

## **4.2: Agricultural applications**

In general, uses of cloning technology in agriculture are seen as a possibility that lies further in the future than biomedical applications. Although the technical and scientific problems remain similar, it is obvious that, from an economic point of view, agricultural applications need to be especially productive to be cost-effective. None the less, several suggestions as to how the technology can be used to improve livestock have been suggested in the literature.

Cloning can be used to create copies of animals with highly valued traits, such as a dairy cow with high milk production or bulls with especially good meat (Paterson et al., 2003). Alternatively, it can be used to create copies of animals whose traits are especially in demand

in breeding programmes, thereby bypassing the need for artificial insemination. One could, for example, clone a bull that has desirable traits and use it for breeding thus enlarging the number of possible offspring (Vajta, 2004); or one could clone a large number of animals to improve the general genetic quality of a given herd (Paterson et al., 2003). Again, however, the low level of efficiency of the technology means it is unable to compete with traditional breeding programmes and other technologies. For the foreseeable future, cloning will therefore play at most a very limited role in agricultural breeding. The advantages of the technology do not measure up to the necessary investments.

None the less a number of studies examining the biological and biochemical properties of products from cloned animals in relation to risks to human health have already been published. Takahashi and Ito (2004) examine the differences in meat samples taken from embryonic cloned, somatic cloned and non-cloned cattle and find no significant biological differences. Tomé et al. (2004) review examinations of the nutritional value of milk and meat products derived from cloned cattle and find no significant differences in products from cloned and non-cloned animals. Although the amount of research in this field is still limited it seems that products from cloned animals do not pose any risks to human health, although both the limited amount of research and the methodological limitations in the research should be taken into account.

In the US the FDA is currently working on a report expected to confirm these findings and so permit products from cloned animals or their off-spring to enter the food production. It is believed that the report will conclude that there are no health risks relating to such products, and that cloning poses no animal welfare problems but has not been given the go-ahead yet “due to the complexity of the issue” (FDA, 2005). This work is based on suggestions as to how to evaluate the safety of products from cloned animals and their offspring from the National Research Council (NRC, 2004 pp. 217-233).

Cloning combined with genetic modification, as described in 4.1, stands a better chance of competing with traditional breeding schemes, since traits that cannot otherwise be introduced into the animals can thereby be disseminated throughout a population. So, for example, the overall inefficiency of the production of the cloned animal might be counterbalanced by rapid dissemination of the improved genetics of the modified bulls through existing artificial insemination systems. Candidate breeding goals here include increased resistance to disease (such as mastitis), transgenic bulls that produce only female or only male offspring (Faber et al., 2003), and dairy cattle with altered  $\beta$ -casein and  $\kappa$ -casein ratio and thus improved milk quality (a possibility noted by Brophy et al., 2003).

Another possibility is the manufacturing of animals that can reduce negative agricultural effects on the environment. The most known of these today is the Enviropig<sup>TM</sup>: a pig that has the capability to digest plant phytate, leading to less phosphate in the manure from the animal and thus less environmental pollution. This animal is currently under development in Canada, and is often mentioned in the literature as an example of an environmentally friendly use of biotechnology (GM and cloning) being close to the market (Kues & Niemann, 2004). However, on the company’s web page (<http://www.uoguelph.ca/enviropig/>) it is stated that: “We cannot reliably predict when the Enviropigs<sup>TM</sup> will reach the consumers”.

Finally, it must be mentioned that some authors see a possibility in using both cloning and GM technology to enhance animal welfare. Here we define enhanced animal welfare as a reduction in physical and/or mental suffering compared with the present situation, well knowing that the concept of animal welfare can be defined in other and broader ways (Dahl et al., 2003). We have already mentioned the possibility of manufacturing cattle with increased resistance to mastitis. This would not only increase milk production but also improve the welfare of the cow. But also resistance to e.g. BSE and scrapie and various viral and bacterial infections is seen as a, albeit distant, possibility (Paterson, 2003)

In this area, it seems indeed to be only the imagination of the author that limits the range of new traits that might be introduced into farm animals. There is no doubt that, as the genomes of farm animals are better understood and the technology to make genetic modifications to them is developed, an even larger number of possibilities will emerge. If, and when, these possibilities will become reality depends, from a technological point of view, only on the development of the technology to a stage at which it is able to compete in economic terms with existing technologies. One thing that must be taken into account is that economic viability will not be decided merely by the level of the technology but also, to a large degree, by public acceptance of cloning technology in agriculture (Denning et al., 2003). And it seems fair to say that this observation goes for all applications of cloning technology involving farm animals.

Finally, it should be remembered that the basic science and the technology of cloning do not possess all the answers. Running through much of the literature reviewed in this report is a river of optimism that tends to reduce the unanswered questions within farm animal cloning to mere technological problems that will soon be fixed (Kues & Niemann, 2004). This official optimism is, in our experience, often modified at conferences and in private talks with scientists, for in these situations there is room to air the scientific uncertainty that inevitably follows in the wake of a technology as new as cloning.

Thus at a CLONING IN PUBLIC workshop on the scientific aspects of animal cloning held jointly by the Institute of Prospective Technological Studies (DG JRC-IPTS, Sustainability in Agriculture, Food and Health unit) and the Danish Centre for Bioethics and Risk Assessment (CeBRA)/DG RTD (Biotechnology, Agriculture and Food directorate) and attended by a panel of around 35 scientific experts, it became very apparent that there are highly divergent expectations about the future applications of cloning within agriculture.

Scott Davis, from Viagen, presented the company's results on cloning of farm animals – on cattle and pigs. Most notable, were the results on pigs. These far surpassed any previously published success rates, and Davis even stated that the animal welfare problems connected with cloning (LOS) has been solved. The data on methodology has not been published in any scientific journal. According to Davis, Viagen is able to produce around 15 piglets for every 25 blastocysts inserted. And at the workshop, Davis confirmed that Viagen was waiting for the above-mentioned FDA report before putting cloned embryos on the market.

It is impossible to validate the claims made by Davis, since there has been no scientific publication of the relevant results. However, there is no reason to doubt that Viagen will market their cloned piglets if the US FDA deems that they pose no risks to human health.

What is not clear is whether this marketing will be economically feasible. At the workshop a series of barriers to the economic success of the project were mentioned.

Some of these barriers are connected with the fact that clones are not exact copies of an already existing animal. The mitochondrial DNA will differ since this comes from the donor egg. Furthermore, epigenetic effects (see chapters 3.1 and 3.2) influence phenotypic similarities between the original animal and the clone. Doubts were therefore raised both from a scientific and breeder perspective as to whether the technology could produce copies of animals with desired traits for the breeding industry.

Finally, it was made very clear by Margareta Håård, chair of the European Forum of Farm Animal Breeders (EFFAB), that any implementation of the technology into breeding programmes in Europe depends not only on the genetic merits of the cloned animals, but also public acceptance of the technology.

The next technical report from CLONING IN PUBLIC will attend to these themes and will in this sense function as a supplement to this report. It will contain an in-depth analysis of the expectations of cloning technology within the scientific community; and it will seek to qualify the present discussion of the feasibility of the different applications. It is noteworthy that discussions of this kind are seldom found in the literature, where it tends to be assumed that the problems are merely technological.

The question of if, when and how products from cloned animals or their offspring should be allowed to enter food production is closely connected with the issue of labelling. If cloned products are allowed, should they be labelled, and how exactly should the labelling system be designed and administered? This question will be discussed in the first CLONING IN PUBLIC report, which covers legal aspect of farm animal cloning. In that report, both the state of the existing legislative framework and the actual practice of animal biotechnology regulation, within and outside the EU, are reviewed.

Finally, it is evident that realisation of the possibilities mentioned in chapter 4 will depend on further research. In this connection it is of some interest to get a picture of the countries and regions in which research is currently active. This information is not easy to obtain, but using an ongoing study, from which some initial conclusions were available at the workshop in Seville June 2005, Bruce Whitelaw, from the Roslin Institute, presented a map showing where articles on cloning science since 1991 have originated from. This showed that about 26 % of the articles were published from European research institutions, 30 % from America, 8 % Australia and New Zealand and 31% originated in the Far East (Japan, Korea and China). Although the study is still in its initial stages, it seems that research is moving from Europe to the Far East and the US. What impact this might have on future research strategies and goals is, as of yet, unclear.

## 5: Conclusions

This report seeks to evaluate the state of the art of farm animal cloning. It is based on a literature review together with additional information gathered at a joint workshop, held under the auspices of CLONING IN PUBLIC in June 2005, involving the Institute of Prospective Technological Studies (DG JRC-IPTS, Sustainability in Agriculture, Food and Health unit) and the Danish Centre for Bioethics and Risk Assessment (CeBRA)/DG RTD (Biotechnology, Agriculture and Food directorate).

The main findings are:

Since the birth of the cloned Dorset Ewe, Dolly, several other mammalian species have been cloned using the SCNT technology that produced Dolly and variations of this technology. The success rates remain low (less than 5%) regardless of methodology. However, the so-called Handmade Cloning approach, in which nuclear transfer can be performed at lower cost (both in equipment and trained personnel), seems to have some advantages — mainly, that it is inexpensive and can be performed at low-tech laboratories.

The reasons for the low success rates are not yet clear, but it seems that both methodological choices (donor cell lines, cell nutrition, maturation period of egg cell, level of technical skill etc) and so-called epigenetic factors affect the way the cloned embryo reacts to reprogramming and the in vitro phase.

Problems associated with animal cloning emerge in the first stages of the development of the cloned egg and following birth. The more frequently occurring difficulties are:

- Placental abnormalities
- Foetal overgrowth, prolonged gestation
- Stillbirth, hypoxia, respiratory failure and circulatory problems, lack of post- natal vigour
- Increased body temperature at birth
- Malformations in the urogenital tract (hydronephrosis, testicular hypoplasia)
- Malformations in liver and brain
- Immune dysfunction, lymphoid hypoplasia, anaemia, thymic atrophy
- Bacterial and viral infections

Low success rates are the most important technical factor hampering the economic feasibility of a range of applications of farm animal cloning suggested in the literature. These include:

- *Basic research.* Using the cloning technology to gain a deeper insight into the fertilization process and the development of the early foetus
- *Disease models.* Together with genetic modification, cloning can produce animals that mimic human diseases and thus help to provide a better understanding of these; it can also generate tailored animals on which new drugs can be tested
- *Bioreactors.* Together with genetic modification, cloning can produce animals that function as ‘biological factories’ which produce human proteins for medicine

- *Agricultural applications.* Alone, or together with genetic modification, cloning may in future be used to produce animals that will increase productivity, animal welfare or the quality of food products.

- *Pets, endangered species, etc.* Cloning can be used for a range of more exotic purposes, including the replication of favourite pets and the artificial reproduction of individuals of endangered species so as to increase breeding populations.

It is debatable whether the technology can actually be used for all these purposes. Putting aside the low success rates, which may yet be improved on, cloning does not produce genetically identical individuals, and a pair of almost genetically identical individuals might differ greatly at the phenotypic level. This is especially relevant in agricultural applications, where high success rates, public acceptance and the production of individual animals with specific desirable traits are required if cloning is to be economically feasible.

The future of animal cloning is therefore unclear. It is obviously a technology with many potential applications, but given the state of the technology's development today, it must be expected that in the near future, at least, cloning will mostly be confined to basic research and biomedicine where the economic value of each individual animal is very high. Whether the technology will become interesting from an agricultural point of view remains to be seen.

## 6: Literature

- Bavister BD (2002): Early history of in vitro fertilization. *Reproduction* 124:181-196.
- Benagiano G, Primiero FM (2002): Human reproductive cloning. *Internatl. J. Gynecol. Obstetr.* 79:265-268.
- Bethhauser J, Forsberg Eg, Augenstein M, Childs L, Eilertsen K, Enos J, Forsythe T, Goluenke P, Jurgella G, Koppang R, Lesmeister T, Mallon K, Mell G, Misica P, Pace M, Pfister-Genskow M, Strelchenko N, Voelker G, Wat S, Thompson s, Bishop M (2000) Production of cloned pigs from in vitro systems. *Nature Biotechnology* 18:1055-1059.
- Briggs R, King TJ (1952): Nuclear transplantation studies on the early gastrula (*Rana Pipiens*). *Develop. Biol.* 2: 252-270.
- Brophy B, Smolenski G, Wheeler T, Wells D, L'Huillier P, Laible G (2003): Cloned transgenic cattle produce milk with higher levels of  $\beta$ -casein and  $\kappa$ -casein. *Nature Biotechnology* 21: 157-162.
- Campbell KH, McWhir J, Ritchie WA, Wilmut I (1996). Sheep cloned by nuclear transfer from a cultured cell line. *Nature* 380: 64-66.
- Carter DB, Lai L, Park KW, Samuel M, Lattimer JC, Jordan KR, Estes DM, Besch-Williford C, Prather RS (2002): Phenotyping of transgenic cloned piglets. *Cloning and Stem Cells* 4:131-145.
- Chavatte-Palmer P, Heyman Y, Richard C, Monget P, LeBourhis D, Kann G, Chilliard Y, Vignon X, Renard JP (2002): Clinical, hormonal, and haematologic characteristics of bovine calves derived from nuclei from somatic cells. *Biol. Reprod.* 66: 1596-1603.
- Chavatte-Palmer P, Rémy D, Mialot JP (2003): Health status of cloned animals at different ages. *Cloning and Stem Cells* 6: 94-100
- Colman A (1999): Somatic cell nuclear transfer in mammals: progress and applications. *Cloning* 1:185-200.
- Dahl K, Sandøe P, Johnsen PF, Lassen J and Kornerup Hansen A (2003): Outline of a risk assessment: the welfare of future xeno-donor pigs. *Animal Welfare* 12: 219-237.
- Denning C & Priddle A (2003): New frontiers in gene targeting and cloning: success, applications and challenges in domestic animals and human embryonic stem cells. *Reproduction* 126: 1-11.
- Di Berardino MA (2001): Animal cloning — the route to new genomics in agriculture and medicine. *Differentiation* 68: 67-83.

- Dinsmore JH, Manhart C, Raineri R, et al (2000): No evidence for infection of human cells with porcine endogenous retrovirus (PERV) after exposure to porcine fetal neuronal cells. *Transplantation* 70(9): 1382-9
- Dominko T, Ramalho-Santos J, Chan A, Moreno RD, Luetjens CM, Simerly C, Hewitson L, Takahashi D, Martinovich C, White JM, Schatten G (1999): Optimization strategies for production of mammalian embryos by nuclear transfer. *Cloning* 1:143-152
- Eggan K, Akutsu, H, Loring J, Jackson-Grusby L, Klemm M, Rideout 3rd WM, Yanagimachi R, Jaenisch R (2001): Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. *PNAS* 98:6209-6214.
- Faber DC, Molina JA, Ohlrichs CL, Van der Zwaag DF, Ferne LB (2003): Commercialization of animal biotechnology. *Theriogenology* 59: 125-138.
- Fishman, JA & Patience, C (2004): Xenotransplantation: Infectious Risk Revisited. *American Journal of Transplantation* 4: 1383-1390.
- Food and Drug Administration (2005): Statement from FDA on the Safety of Animal Cloning in the Food Supply. <http://www.fda.gov/bbs/topics/NEWS/2005/NEW01188.html>
- Gurdon JB (1962): Adult frogs derived from nuclei of single somatic cells. *Devel. Biol.* 4: 256-273.
- Hammer CJ, Tyler HD, Loskutoff NM, Armstrong DL, Funk DJ, Lindsey BR, Simmons LG (2000): Compromised development of calves (*Bos gaurus*) derive from in vitro-generated embryos and transferred interspecifically into domestic cattle (*Bos taurus*). *Theriogenology* 55:1447-1455.
- Heyman, Y, Richard, C, Rodriguez-Martinez, H, Lazzari, G, Chavatte-Palmer, P, Vignon, X & Galli, C (2004): Zootechnical performance of cloned bulls and their offspring. *Cloning and Stem Cells* 6: 111-120
- Hill JR, Roussel AJ, Cibelli JB, Edwards JF, Hooper NL, Miller MW, Thompson JA, Looney CR, Westhusin ME, Robl JM, Stice SL (1999) Clinical and pathological features of cloned transgenic calves and fetuses (13 case studies). *Theriogenology* 51:1451-1465.
- Hill JR, Burghardt RC, Jones K, Long CR, Looney CR, Shin T, Spencer TE Thompson JA, Winger QA, Westhusin ME (2000) Evidence for placental abnormality as the major cause of mortality in first-trimester somatic cell cloned bovine fetuses. *Biol. Reprod.* 63: 1787-1794.
- Holm P, Walker SK, Seamark RF (1996): Embryo viability, duration of gestation and birth weight in sheep after transfer of in vitro matured and in vitro fertilized zygotes cultured in vitro or in vivo. *J. Reprod. Fertil.* 107:175-181.
- Illmensee K, Hoppe PC (1981): Nuclear transplantation in *Mus musculus*: developmental potential of nuclei from preimplantation embryos. *Cell* 23: 9-18.

Jacobsen H, Schmidt M, Holm P, Sangild PT, Vajta G, Greve T, Callesen H (2000): Body dimensions and birth and organ weights of calves derived from in vitro produced embryos cultured with or without serum and oviduct epithelial cells. *Theriogenology* 53: 1761-1769.

Johnsen WH, Loskutoff NM, Plante Y, Betteridge KJ: Production of four identical calves by the separation of blastomeres from an in vitro derived four-cell embryo. *Vet Rec.* 1;137(1):15-6.

Jones KL, Hill J, Shin TY, Lui L, Westhusin M (2001): DNA hypomethylation of karyoplasts for bovine nuclear transplantation. *Mol. Reprod. Dev.* 60: 208-213.

Kimball's Biology Pages (2005): <http://users.rcn.com/jkimball.ma.ultranet/BiologyPages>

Kues, Wilfried A. & Niemann, Heiner (2004): The contribution of farm animals to human health. *Trends in Biotechnology* 22: 286-294

Lai L, Kolber-Simonds D, Park KW, Cheong HT, Greenstein JL, Im GS, Samuel M, Bonk A, Rieke A, Day BN, Murphy CN, Carter DB, Hawley RJ, Prather RS. (2002): Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. *Science.* 295:1089-92.

Lewis IM, French AJ, Tecirlioglu RT, Vajta G, McClintock AE, Nicholas KR, Zuelke KA, Holland MK and Trounson AO (2004): Commercial aspects of cloning and genetic modification in cattle. *Australian Journal of Experimental Agriculture* 44(11) 1105—1111

Loeb J (1894): Über eine einfache methode, zwei oder mehr zusammengesachsene Embryonen aus einem Ei hervorzubringen. *Pflügers Arch.* 55: 525-530.

Martin M, Adams C, Wiseman B (2004): Pre-weaning performance and health of pigs born to cloned (fetal cell derived) swine versus non-cloned swine. *Theriogenology* (epub).

McGrath J, Solter D (1984): Completion of mouse embryogenesis requires both the maternal és paternal genomes. *Cell* 37: 179-183.

Ménézo YJR, Veiga A, Dale B (2000): Assisted reproductive technology (ART) in humans: facts and uncertainties. *Theriogenology* 53: 5999-610.

National Research Council of the National Academies (2002): *Animal Biotechnology. Science-Based Concerns.* National Academy of Science, USA.

National Research Council and Institute of Medicine of the National Academies (2004): *Safety of genetically engineered foods. Approaches to assessing unintended health effects.* National Academy of Science, USA.

Niemann H, Rath D, Wrenzycki C (2003): Advances in biotechnology: new tools in future pig production for agriculture and biomedicine. *Reprod. Dom. Anim.* 38: 82-89.

- Ozil JP, Hayman Y, Renard JP (1982): Production of monozygotic twins by micromanipulation and cervical transfer in the cow. *Vet Rec* 110:126-127.
- Oldmixon BA, Wood JC, Ericsson TA, Wilson CA, White-Scharf ME, Andersson G et al. (2002): Porcine endogenous retrovirus transmission characteristics of an inbred herd of miniature swine. *J Virology* 76: 3045-3048
- Paradis K, Langford G, Long Z, Heneine W, Sandstrom P, Switzer WM, Chapman LE, Lockey C, Onions D, Otto E. (1999): Search for cross-species transmission of porcine endogenous retrovirus in patients treated with living pig tissue. *Science* 285: 1236-1241.
- Paterson L, DeSousa P, Ritchie W, King T, Wilmut I (2003): Application of reproductive biotechnology in animals: implications and potentials. Applications of reproductive cloning. *Animal Reproduction Science* 79: 137-143
- Perry ACF, Wakayama T (2002): Untimely ends and new beginnings in mouse cloning. *Nature Genetics* 30: 243-244
- Peura TT, Lewis IM, Trounson AO (1998): The effect of recipient oocyte volume on nuclear transfer in cattle. *Mol. Reprod. Dev.* 50:185-91.
- Phelps CJ, Koike C, Vaught TD, Boone J, Wells KD, Chen SH, Ball S, Specht SM, Polejaeva IA, Monahan JA, Jobst PM, Sharma SB, Lamborn AE, Garst AS, Moore M, Demetris AJ, Rudert WA, Bottino R, Bertera S, Trucco M, Starzl TE, Dai Y, Ayares DL (2003): Production of  $\alpha 1,3$ - galactosyltransferase deficient pigs. *Science* 299: 411-414.
- Pinkert CA (1994): Transgenic pig models for xenotransplantation. *Xeno* 2: 10-15.
- Poland & Bishop (2002): 'Bioethics and Cloning, Part I', *Kennedy Institute of Ethics Journal* 12: 304-324
- Prather RS, Barnes FL, Sims MM, Robl JM, Eyestone WH, First NL (1987): Nuclear transplantation in the bovine embryo: assesment of donor nuclei és recipient oocyte. *Biol. Reprod.* 37: 859-866.
- Prather RS, Sims MM, First NL (1989): Nuclear transplantation in early pig embryos. *Biol. Reprod.* 41: 414-418.
- Reggio BC, James AN, Green HL, Gavin WG, Behboodi E, Echelard Y, Godke RA (2001): Cloned transgenic offspring resulting from somatic cell nuclear transfer in the goat: oocytes derived from both follicle-stimulated and nonstimulated abattoir-derived ovaries. *Biol. Reprod.* 65:1528-1533.
- Rieck W & Dean W (2002): Epigenetic reprogramming: Back to the beginning. *Nature* 420:127
- Shiels PG, Kind AJ, Campbell KH, Waddington D, Wilmut I, Colman A, et al. (1999): Analysis of telomere lengths in cloned sheep. *Nature* 399: 316-317.

- Sims M, First NL (1993): Production of calves by transfer of nuclei from cultured inner cell mass cells. *Proc. Natl. Acad. Sci. USA*. 91: 6143-6147.
- Sinclair KD, Maxfield EK, Robinson JJ, Maltin CA, McEvoy TG, Dunne LD, Young LE, Broadbent PJ (1997): Culture of sheep zygotes can alter fetal growth and development. *Theriogenology* 47:380.
- Singh U, Fohn LE, Wakayama T, Ohgane J, Steinhoff C, Lipkowitz B, Schulz R, Orth A, Ropers HH, Behringer RR, Tanaka S, Shiota K, Yanagimachi R, Nuber UA, Fundele R (2004): Different molecular mechanisms underlie placental overgrowth phenotypes caused by interspecies hybridization, cloning, and Esx1 mutation. *Dev Dyn*. 230(1):149-64.
- Spemann H (1902): Entwicklungsphysiologische Studien am Tritonei II. *Arch. f. Entw. Mech.*, 15: 448-534.
- Spemann H (1914): Über verzögerte Kernversorgung von Keimteilen. *Verh. Deutsch. Zool. Ges. Leipzig (Freiburg)* 24:216-221.
- Spemann H (1938): *Embryonic development és induction*. Yale University Press, New Haven, Connecticut, p. 211.
- Tomé D, Dubarry M and Fromentin G (2004): Nutritional value of milk and meat products derived from cloning. *Cloning and Stem Cells*. 6: 172-177
- Takahashi S & Yoshihio I (2004): Evaluation of meat products from cloned cattle: biological and biochemical properties. *Cloning and Stem Cells* 6: 165-171.
- Tamashiro KL, Wakayama T, Akutsu H, Yamazaki Y, Lachey JL, Wortman MD, Seeley RJ, D'Alessio DA, Woods SC, Yanagimachi R, Sakai RR. (2002): Cloned mice have an obese phenotype not transmitted to their offspring. *Nat. Med.* 8: 262-267.
- Vajta G, Lewis IM, Hyttel P, Thouas GA, Trounson AO (2001): Somatic cell cloning without micromanipulators. *Cloning* 3: 89-95.
- Vajta G, Lewis IM, Trounson AO, Purup S, Maddox-Hyttel P, Schmidt M, Pedersen HG, Greve T, Callesen H (2003): Handmade somatic cell cloning in cattle: Analysis of factors contributing to high efficiency in vitro. *Biol. Reprod.* 68: 571-578
- Vajta G (2004): *Handmade Cloning — Summary*. Unpublished.
- Videnskabsministeriet (2003): *Genmodificerede og klonede dyr*. Ministeriet for Videnskab, Teknologi og Udvikling, København.
- Wakayama S, Cibelli JB, Wakayama T (2003): Effect of timing of the removal of oocyte chromosomes before or after injection of somatic cell nucleus on development of NT embryos. *Cloning and Stem Cells* 5: 181-189.

Walker SK, Hartwich KM, Robinson JS, Seamark RF (1998): Influence of in vitro culture of embryos on the normality of development. In: Lauria A, Gandolfi F, Enne G, Gianaroli L, (eds.): *Gemetes: Development and Function*. Serono Symposia, Milan, Italy, pp. 457-484.

Wells DN, Forsyth JT, McMillan V, Oback B (2004): The Health of Somatic Cell Cloned Cattle and Their Offspring. *Cloning and Stem Cells* 6: 101-110

Willadsen SM (1979): A method for culture of micromanipulated sheep embryos and its use to produce monozygotic twins. *Nature* 277:298-300.

Willadsen SM (1986): Nuclear transplantation in sheep embryos. *Nature* 320: 63-5.

Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH (1997): Viable offspring derived from fetal és adult mammalian cells. *Nature* 385: 810-3

Yamada K, Yazawa K, Shimizu A, Iwanaga T, Hisashi Y, Nuhn M, O'Malley P, Nobori S, Vagefi PA, Patience C, Fishman J, Cooper DK, Hawley RJ, Greenstein J, Schuurman HJ, Awwad M, Sykes M, Sachs DH (2005): Marked prolongation of porcine renal xenograft survival in baboons through the use of alpha1,3-galactosyltransferase gene-knockout donors and the cotransplantation of vascularized thymic tissue. *Nat Med*. 11(1):32-4.

Yanagimachi R (2002): Cloning: experience from the mouse and other animals. *Mol. Cell. Endocrin.* 187: 241-248.

Young LE, Sinclair KD, Wilmut I (1998): Large offspring syndrome in cattle and sheep. *Rev. Reprod.* 3:155-163.

Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C, Broadbent PJ, Robinson JJ, Wilmut I, Sinclair KD (2001): Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nat Genet.* 27(2):153-4.

Xu J & Yang X (2003): Will cloned animals suffer premature aging — The story at the end of clone's chromosomes. *Reproductive Biology and Endocrinology* 1:105.