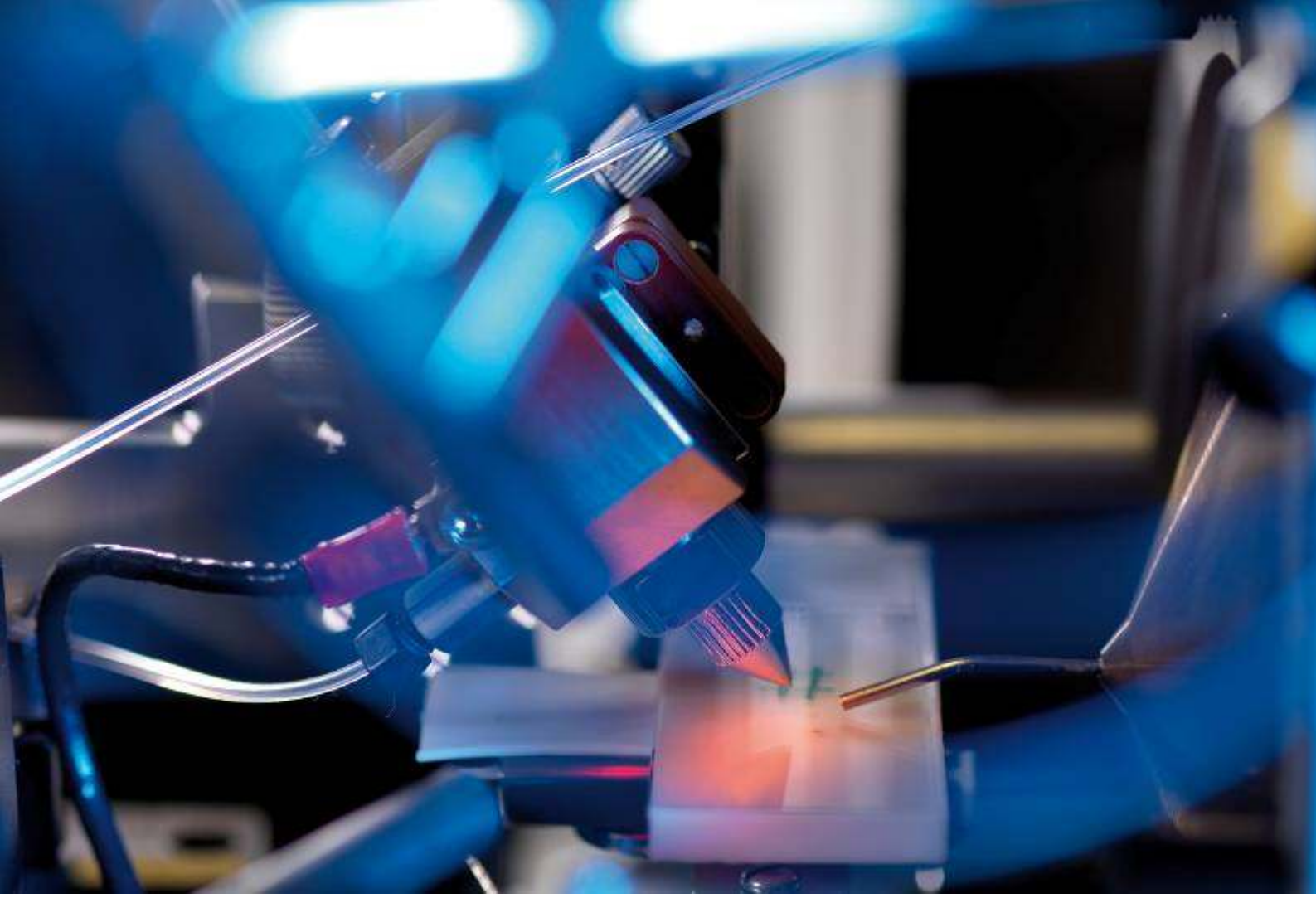

A Review of Potential Analytical Approaches for Detecting Cloned Animals and their Offspring in the Food Chain

Government Chemist Programme 1 May 2011 – 31 May 2012
Project RF1/3.1

Report number: LGC/P/2012/130

Susan Pang

26 June 2012

Executive summary

This report provides an overview of the process of animal cloning and reviews the current scientific knowledge pertaining to genetic anomalies and biomarkers that may be characteristic of cloned animals. Such biomarkers, if sufficiently robust and distinct from those present in naturally conceived animals, may be applied for detecting food products derived from cloned animals and their offspring in the food chain. In summary, four different types of biomarker (telomerase length/activity, epigenetic modifications, gene expression and protein expression) have been studied to varying degrees in cloned animals. However, to date such studies have been limited in size and scope and have often resulted in conflicting findings. Therefore, at this point in time reproducible traits discriminatory for healthy adult cloned animals cannot be defined. Further scientific research on larger numbers of cloned animals will be required before consensus among the scientific community as to the validity of potential biomarkers can be reached.

Introduction

The cloning of animals has always been a contentious issue as it is commonly viewed as tampering with nature. Animal welfare campaigners believe that cloned animals suffer far more than standard animals, and many consumers acknowledge the ethical dilemma of the practice of animal cloning. Somatic cell nuclear transfer (SCNT) is a process used to create genetically identical animals by transferring the nucleus (a cell structure that is termed as an organelle which contains most of the cell's genetic material) from a donor adult cell (somatic cell) of the animal to be cloned, into an egg

devoid of a nucleus. If the egg begins to divide normally it is transferred into the uterus of the surrogate mother. Although the nuclear DNA is transferred from the donor animal, the resultant clones are not strictly identical to the donor adult as the DNA in the mitochondria will be distinct from the donor adult. Mitochondria are organelles that provide energy for all cellular processes. Mitochondrial DNA of the clone will be inherited solely from the mother, which in this case is the enucleated donor egg rather than the surrogate birth mother. In spite of the risk of low level mitochondrial DNA carry-over during the process of SCNT, there are only reports of mitochondrial DNA inheritance from the egg donor.

Dolly the sheep was the first animal cloned from an adult cell using SCNT by scientists at the Roslin Institute in 1996 (1). The news was released in February 1997 sparking a global media frenzy as the moral, social, medical and legal ramifications of this work were brought into question (2); (3). Numerous species have been cloned since Dolly, including pig (4); (5), cattle (6); (7), dog (8), cat (9); (10); (11), mouse (12); (13); (14), rat (15), rabbit (16), horse (17), mule (18), camel (19), goat (20), deer (21) and fish (22).

The process of SCNT is highly inefficient and expensive, with a 0.1-3 % success rate of a clone surviving birth as a healthy animal (23). This has led to investigations into the cause of the high failure rate and ways to improve on the success rate of cloning (24); (25); (26). However, the technique may offer the potential benefit of producing ‘copies’ of desirable animals to allow more farmers to rear animals of the highest genetic merit (e.g. product yield, food quality, and disease resistance) more quickly (27). Nonetheless, the process is accompanied by a high mortality rate during gestation, abnormal placentas and increased incidence for spontaneous abortions

during the second trimester (6); (28); (29). For animals that survive birth, there have also been reports of aberrantly high birth weight, respiratory and metabolic abnormalities as well as a high incidence of sudden death syndrome of the adults (30); (31); (32); (33).

Given the high reported incidence of abnormalities with cloned animals, there are public concerns regarding animal welfare and food safety associated with the consumption of meat and diary products derived from cloned animals. Admissions by farmers complicit with the sale of meat or milk from cloned animals or the offspring, as well as by official bodies such as the Foods Standards Agency (FSA) investigating such claims, have fuelled the debate. Under European Union (EU) laws, the milk and meat from cloned animals are categorised as “novel foods” which require the supplier to obtain special authorisation prior to their sale. The EU rules state that novel foods cannot be legally sold without a scientific assessment of safety, and a breach of this rule may lead to a fine of up to £5K.

In 2008, the European Food Safety Authority stated there was "no clear evidence" to suggest differences between food products from clones or their offspring compared to products from conventionally bred animals on the grounds of food safety. The United States Food and Drug Administration (FDA) had previously arrived at the same conclusion in December 2006, and published a risk assessment report approving the human consumption of meat and other products from cloned animals without special labelling being granted (34). Since the FDA approval, food products derived from cloned animals have been regularly consumed in the US. However the consumption of such food products has caused much public concern in the UK.

In the case reported in August 2010, relating to meat sold from a Highland farm by farmer Steven Innes, the FSA traced two bulls born in the UK from embryos harvested from a cloned cow in USA. The investigation revealed that one of the cloned animal offspring was slaughtered in July 2009 and its meat subsequently entered the food chain. The FSA reported that the farmer and butchers were in breach of the rules as the so-called "novel food" licence had not been obtained. In July 2010, the second bull was slaughtered, but its meat was stopped from entering the food chain. Claims regarding the retail of milk from the offspring of a cloned cow by an anonymous UK dairy farmer also emerged in August 2010, and that the same farmer was reputed to be selling embryos from the same cow to breeders in Canada.

The public debate led a review of scientific investigation by the FSA. In March 2011, the FSA concluded that there was no scientific evidence to suggest that cloned meat or milk could not be eaten safely, but stipulated that a licence for the sale of such products was necessary. Currently there is no existing test for the detection of characteristics of a cloned animal or its offspring. To avoid reliance on the paper trail in investigations of suspect food, there is a need for the development of a robust food-based test. Hence, it is necessary to establish the robustness of putative indicators of cloning in animals should food products derived from the cloned animal or possibly their offspring enter the food chain. In this paper, we evaluate the scientific research conducted to characterise anomalies with reputed links to the cloning of animals. The comparability and robustness of these studies will be assessed to determine the validity of any reported detectable traits of a cloned animal or its offspring to form the basis of food testing.

Much scientific research has been untaken to evaluate genetic changes in cloned animals. Four main areas have been reviewed: (1) telomere length and activity, (2) epigenetic aberrations, (3) gene expression and (4) protein expression.

(1) Telomeric length and activity

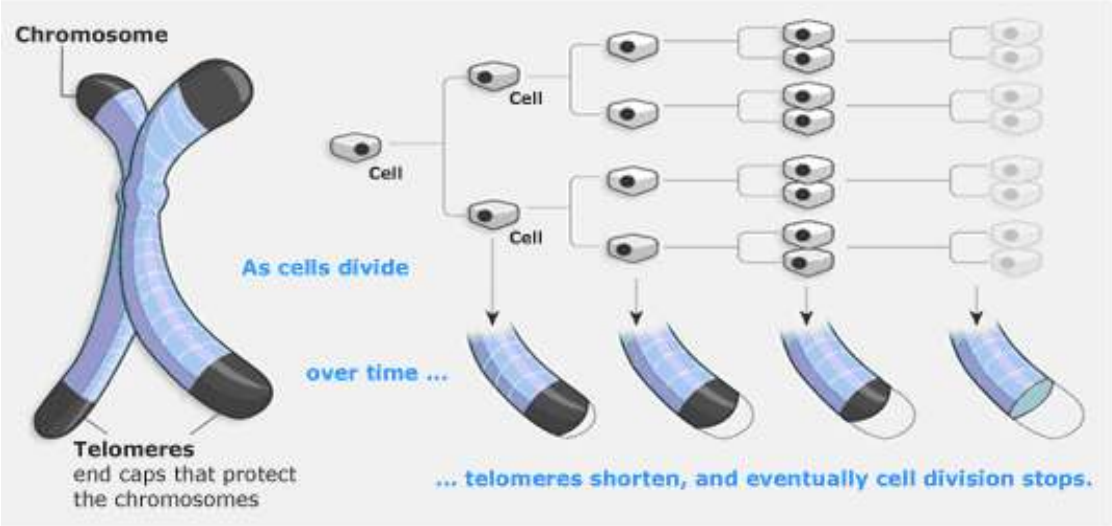

Figure 1: An illustration of telomere shortening during the process of cell division. The image is obtained from:

http://telomeres.net/wpcontent/uploads/2011/10/telemeres-science21.jpg

A telomere (Figure 1) is a region of repetitive sequences of nucleotides (the building blocks of DNA) at the end of a chromosome, which is a highly ordered structure comprising of a single piece of coiled DNA and protein that is found in cells. The telomere cap protects the end of the chromosome from deterioration or from fusion with neighboring chromosomes. During cell division, enzymes that duplicate DNA cannot continue their duplication completely to the end of chromosomes. Without telomeres acting as buffers at the ends of the chromosomes, genetic information

within the ends of the chromosomes will be lost. The telomeres are consumed during cell division, and are replenished by an enzyme, telomerase reverse transcriptase. The link between telomere shortening and cellular ageing has been much documented, and can shorten between 50-200 base pairs in somatic cells of most mammals (35); (36); (37); (38). There have been numerous investigations evaluating the telomerase activity and telomere length within cloned animals and their normal counterparts given concerns that cloned animals suffer from premature ageing as the clone may inherit its age from its cell donor (39); (3). Telomerase activity is measured by telomere repeat amplification protocol and the mean telomere length is determined by terminal restriction fragment (TRF) analysis using a variety of commercial kits.

The length of telomeres in Dolly and other cloned sheep appeared shorter than in normal age-matched sheep (40); (41), albeit signs of premature senescence were not observed in such cloned animals. In 1999, Shiels *et al*. reported that the telomeres shorten by approximately 172 base pairs (bp) in sheep during each cell division event. In this study, there were three cloned sheep; one was Dolly who had received nuclear DNA transferred from mammary epithelial cells from a sheep (aged six years), the second clone had nuclear DNA from embryos (aged nine days), and the third had DNA derived from fibroblasts from a fetus (aged 25 days). Although the telomere length of all three clones (19.14, 20.37 and 21.19 kilobases (kb) for clones 1 (Dolly), 2 and 3, respectively) were shorter than the mean length in the age-matched controls (23.9 ± 0.18 kb), the telomere length of the third clone derived fibroblasts was not statistically significantly different at 95% confidence intervals (40). A more recent study in 2007 supports the observation that sheep clones derived from cultured somatic cells have shorter telomeres compared with their age-matched normal counterparts (41). Of the four adult sheep clones incorporated in this study, the mean

telomere length was shorter than the mean derived from the 35 age-matched controls. The length of the telomeres of the four clones ranged from 11.3-14.45 kb. However the telomere length for one of the clones was not statistically significantly shorter at 95% confidence limits.

Overall, the investigations with sheep telomere length may indicate that telomere length in the clones may not be fully restored. However this conclusion is drawn from a total population of only seven cloned sheep (from two studies) including two clones not exhibiting a statistically significant difference in telomeric length from the control animals. Hence, this may not be a robust indicator given the low level of replication.

Only a single investigation of telomere length of cloned goats has been conducted. Betts *et al.* reported significantly shorter telomeres in six goat clones derived from cultured cumulus-granulosa cells (13.28 ± 0.41 kb) compared with age-matched controls (14.96 ± 0.25 kb) (42). The same study showed that this phenomenon is likely to be dependent on the donor cell type as four goat clones derived from fetal fibroblast donor cells appeared shorter than the controls (12.60 ± 0.51 kb compared with 14.03 ± 0.44 kb, respectively) but this difference was not statistically significant at 95% confidence limits.

Three investigations of telomere length in cloned pigs have been performed. The first investigation by Jiang *et al*. in 2004 revealed that there was no significant difference between the telomere length of skin samples derived from six cloned pigs and their age-matched controls (43). These findings are consistent with the later (and most recent) study on the subject by Kurome *et al*. in 2008 who evaluated the average telomere lengths of serially cloned pigs up to three generations. The mean TRF length of the first, second and third generation clones were 16.3, 18.1 and 20.5 kb,

respectively, and these values were comparable to those in age-matched controls (17, 18.4, and 22.1 kb, respectively) (4). However, these two investigations contrast the findings of the third study which chronologically predates the study by Kurome *et al* in 2008. In 2005, Jeon *et al*. documented the elongation of the mean TRF length in cloned pigs (24.8 ± 0.5 kb) compared with age-matched control piglets (21.8 ± 0.5 kb), albeit without any difference in the telomerase activity between the two groups (44). Overall, the investigations in cloned pig telomere length are very limited, and a single report of elongated telomeres in cloned pigs by Jeon *et al*. must be further substantiated.

More studies of telomere length have been performed with cattle than with other animals. However these investigations have generated greater variability in findings Miyashita *et al*. have also reported shorter telomere length in cloned cattle when donor cells were sourced from the oviductal and mammary epithelial cells of an adult cow, or oviductal epithelial cells from younger cattle (45).

However, normal telomere lengths in cloned cattle has been documented in numerous independent investigations (46); (47); (48); (49); (7); as well as normal levels of telomerase activity (44). Tian *et al.* reported that the telomere length from four live clones (15.38 ± 0.62 kb) was not statistically different from the mean TRF length from the four age-matched controls (15.38 ± 0.62 kb). Furthermore, the same study also established that the telomere length of cloned animals that died shortly after birth (15.87 ± 0.40 kb derived from 6 clones) was not statistically different from the four live clones (46). Hence aberrant telomere length is not the inherent cause of lethality. In addition to evaluating the length of telomeres in the first generation of cloned cattle (where a mean TRF length of 15.4 ±0.5 kb from 4 clones was derived), Kubota *et al*.

also reported normal length telomeres in second generation cloned cattle (16.1 ± 0.5 kb from 2 clones) with the analysis of fibroblasts from the animals (7). TRF analysis of the same animals using blood leukocytes also generated the same findings (7). The telomere length of cloned calves was reported by Betts *et al.* as approximately 20 kb, and was not significantly different from the age-matched controls at 95% confidence limits, when the cloned animals were derived from fibroblasts and cumulus cells (49). These studies may suggest the reversal of inherited genomic modifications acquired from the donor cell.

However, in contrast to the above findings, there have been reports of cloned cattle exhibiting longer telomeres than the control normal calves (50); (45). The Lanza *et al.* study have reported that fetal calf clones exhibit longer telomeres than their aged-matched control animals (50). Other investigators have documented that embryonic cell-cloned cattle (n=6) have significantly longer telomeres than their control counterparts at 99% confidence limits (45). Additionally, the telomere length of the offspring of one of these female clones was determined to be somewhere between the length of the embryonic cell-cloned cattle and their controls. These two reports suggest that fetal donor cells may cause telomerase elongation in cattle. However in practice, the cloning of adult farm animals is more realistic given the desire to retain particular commercially advantageous traits of cattle as aforementioned. Hence this phenomenon may not be of relevance for detecting evidence of cloning if the farm animal has been cloned from an adult donor cell which is the more realistic scenario. Table 1 summarises the telomere lengths reported in cloned animals.

Donor Cell				TRF (kb)			Clone vs. Control	Tissue assayed	Telomerase	Ref
Species	**Origin**	**Cell type**	**Note**	**Donor**	**Control**	**Clone**				
Sheep	Adult Female	Mammary gland			23.9	19.14	Shorter	Blood		(40)
Sheep	Fetal Female	Embryonic	Very short culture time		23.9	21.19	Similar	Blood		(40)
Cattle	Fetal Female	Fibroblast	Extremely long culture time	15.2	18.3	20.1	Longer	Blood cells	High in blastocyst	(50)
Cattle	Adult Female	Fibroblast cumulus		12.4	14.7	15.4	Similar	Fibroblast	High in blastocyst	(46)
Cattle	Adult Female	Fibroblast		13.68	20.9 (fetal)	17.95 (fetal)	Similar	Fibroblast	High in blastocyst	(49)
					20.5 (calf)	15.3 (calf)	Similar			(49)
Cattle	Adult Male	Fibroblast		17.85	20.9 (fetal)	22.74 (fetal)	Similar		High in blastocyst	(49)
					20.5 (calf)	21.33 (calf)	Similar			(49)
Cattle	Adult Male	Ear					Longer			(48)
Cattle	Adult Male	Ear					Similar			(48)
Cattle	Adult Female	Epithelial					Shorter			(45)
Cattle	Adult Female	Oviduct					Shorter			(45)
Cattle	Adult Male	Muscle					Similar			(45)
Cattle	Adult Male	Skin fibroblast					Similar			(45)
Cattle	Fetal	Embryonic cells					Longer			(45)
Cattle	Adult male			14.7 ± 0.4	G1:	15.4 ± 0.5 (1st Gen)	Similar	Fibroblasts	Similar in clone & control	(7)
					G2:	16.2 ± 0.7 (2nd Gen)	Similar		Similar in clone & control	(7)
				15.7 ± 0.8	G1:	15.3 ± 0.8 (1st Gen)	Similar	Blood leukocytes	Similar in clone & control	(7)
					G2:	15.7 ± 0.8 (2nd Gen)	Similar		Similar in clone & control	(7)
Cattle	Adult Female				18.5	18.0 ± 0.5			Similar in clone & control	(44)
Pig	Fetal	Fibroblast			21.8 ± 0.5	24.8 ± 0.5	Longer		Similar in clone & control	(44)
Pig		Fibroblast cell line			18.0 ±1.4	18.7 ± 2.2	Similar			(43)
Pig				G1:	17.1 ± 0.1	18.7 ± 2.2	Similar			(5)
				G2:	18.4 ± 0.2	18.7 ± 2.2	Similar			(5)
				G3:	22.1 ± 0.4	18.7 ± 2.2	Similar			(5)
Goat	Fetal	Fibroblast			14.03 ± 0.44	12.60 ± 0.51	Shorter (but not significant at 95%CL)			(42)
Goat		Cultured cumulus-granulosa cells			14.96 ± 0.25	13.28 ± 0.41	Shorter			(42)

Table 1: A summary of the telomere lengths reported in cloned animal (reproduced from Xu and Yang 2003, and supplemented with further updates since 2003). G1, G2, G3 denote 1st, 2nd and 3rd generation animals.

The discrepancy between the observation of shortened telomeres in "Dolly" the sheep and normal length or even elongated telomeres within cattle may be attributed to a number of reasons. The studies involving the use of multiple types of donor cells indicate that differences in telomere lengths among cloned cattle may be attributed to the donor cell types and age. The Lanza *et al.* investigation that reported longer telomeres in clones used fetal donor cells (50), whereas the study by Tian *et al.* utilised adult donor cells rather than fetal. Hence, the use of different sources of donor cells cultured in distinct culturing conditions may be a factor for the differing conclusions between the two studies. There is also speculation that the duration of the cell culturing period may account for contrasting reports on the telomere length of cloned sheep (51). The length of telomeres could perhaps be dependent upon the species and the source of DNA used in the analyses by terminal restriction fragment to ascertain the telomere length. It is also possible that the observation of short telomeres within sheep was atypical, as this conclusion was based on only five out of a total of seven cloned sheep from two studies. The Tian *et al.* study which showed that adult-derived cloned cows have normal length telomeres was based on ten cloned animals (46). Hence, the low level of efficiency in producing sufficiently healthy cloned animals for these studies is a real limitation.

Overall, there no robust conclusion may be drawn regarding the correlation in the length of the telomeres in cloned animals without taking into consideration the species cloned, the source of the donor cell and its age, in addition to period of the cell culturing. Given that this information would not be known when screening meat at the point of entry into the food chain, there is no possibility of utilising telomere length as an indicator of a cloned animal. The assessment of telomere length would serve no purpose for detecting the entry of offspring of adult cell cloned animals into the food

chain as all reports indicate normal length telomeres. The telomere length among the offspring of cloned sheep derived by natural mating are reported to be of normal length (41), as are serially cloned bulls (up to the second generation) (7), and serially cloned pigs (up to the third generation) (4).

(2) Epigenetic aberrations

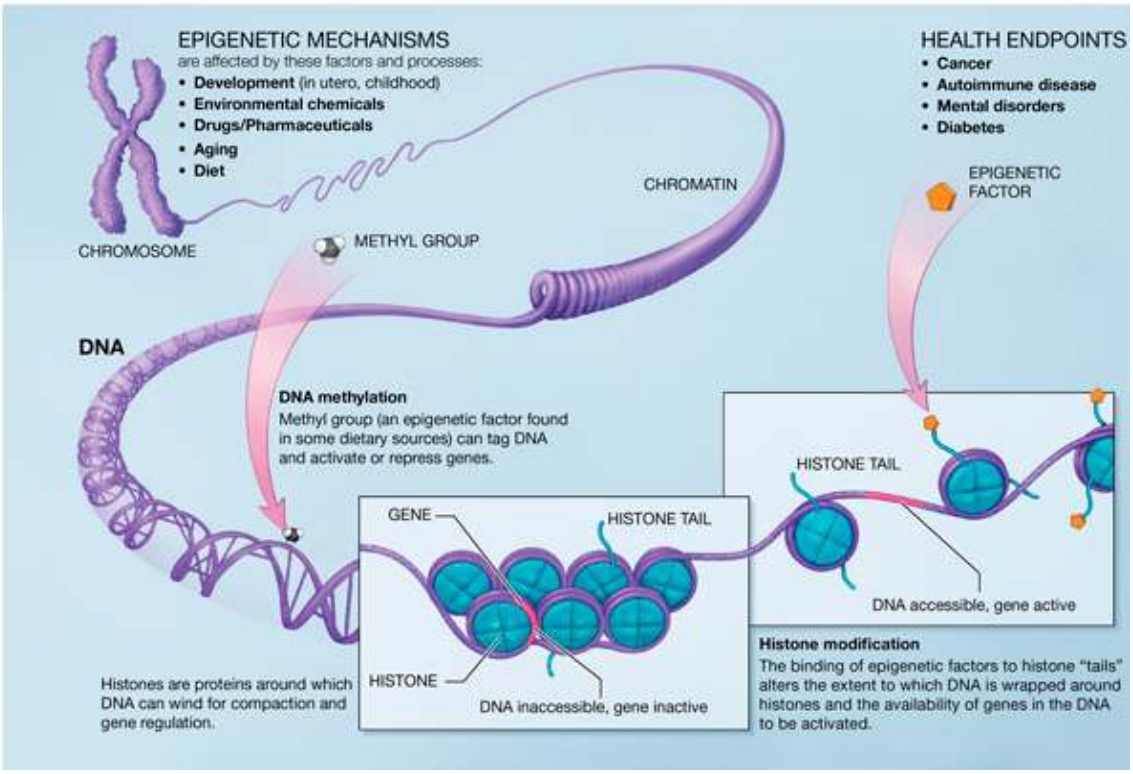

Figure 2: An illustration of epigenetic mechanism sourced from:

http://en.wikipedia.org/wiki/File:Epigenetic_mechanisms.jpg

Epigenetics is the study of heritable changes in gene expression or cellular phenotypes as a result of mechanisms (Figure 2) that do not alter the underlying DNA sequence. DNA methylation and histone modifications are the primary epigenetic modification

implicated in gene regulation. The histone modifications can take the form of methylation (addition or removal of methyl groups) or acetylation (the addition or removal of acetyl groups) (Figure 3) and these cause the chromatin (a structure with a combination of DNA and proteins that make up the contents of the nucleus of a cell) to open or close which regulates gene transcription, the first step leading to gene expression.

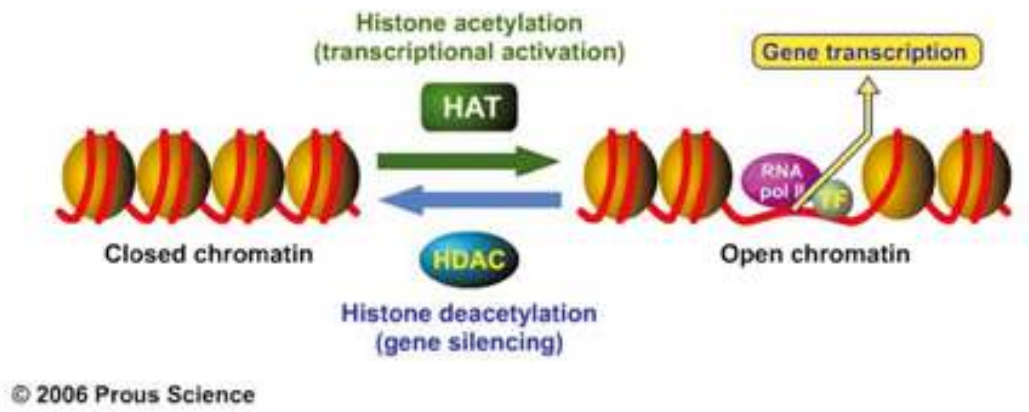

Figure 3. An illustration of the effects of histone acetylation and deacetylation on chromatin structure obtained from http://journals.prous.com/journals/dof/20073201/html/df320045/images/image01.jpg

The different levels of chromatin organisation are shown in Figure 4. The DNA wraps around eight histone proteins forming nucleosomes. Euchromatin is the light packaged form of chromatin where the DNA is loosely wound around the histones in the "beads on a string" structure. Multiple histones may then wrap into a 30 nm fibre consisting of nucleosome arrays in a tightly packed form known as heterochromatin. Higher-level DNA packaging of the fibre into the chromosome then occurs during cell division.

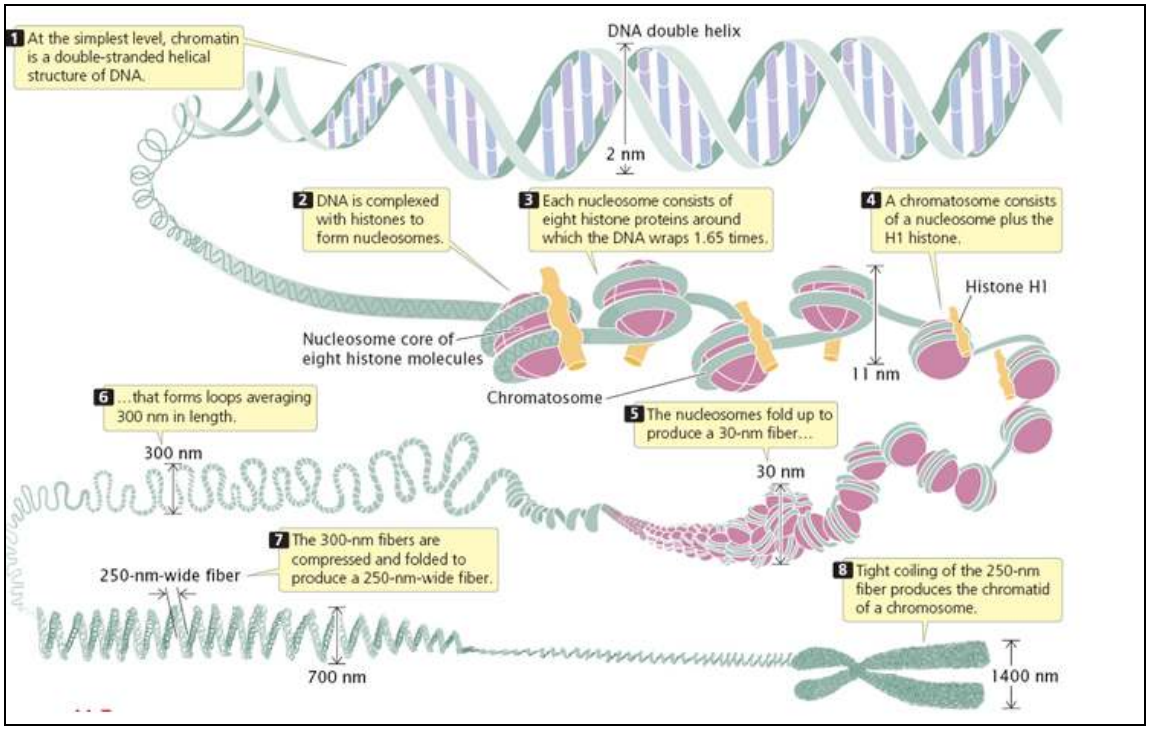

Figure 4: A schematic of the levels of organisation of chromatin, obtained from http://www.nature.com/scitable/content/18847/pierce_11_5_large_2.jpg

Heavy DNA methylation of heterochromatin has been observed in cloned bovine embryos by immunofluorescent staining, contrasting the occurrence of low methylation in normally fertilised embryo chromatin prior to implantation (52). Dean *et al.* have documented aberrant time dependent methylation during the early development of some cloned embryos (53). This aberrant epigenetic reprogramming may result in the high incidence of physiological anomalies occurring during the progression of the pregnancy or after the birth of the cloned animal. Abnormal DNA methyltransferase 1 (DNMT1) expression, a key enzyme that catalyses the transfer of methyl groups to DNA, has also been reported in cloned bovine embryos (54).

In spite of reports of global aberrant methylation patterns within various genomic regions in bovine cloned embryos (53); (55), there is no statistical difference in the

methylation patterns of centromeric satellite DNA (the DNA in the middle of the chromosome) of cloned pig embryos and their normal counterparts (56). This may suggest there are species-specific differences in epigenetic reprogramming of a cloned donor genome. Tissue-specific DNA methylation has also been documented with cloned embryos, such as the DNA hypermethylation at the *Spalt*-like gene 3 locus in the placenta of mice carrying the clones (57).

DNA methylation is the primary mechanism for repressing the expression of imprinted genes (the genes whose expression is determined by the parent). The degree of DNA methylation at imprinted genes is influenced by the donor cell type (55). The loss of DNA methylation at imprinted genes may be less pronounced in some species (e.g. sheep (58)) than others (e.g. mouse (59)).

Hypomethylation of the imprinted *Igf2* and *H19* genes has been detected in liver and placenta of cloned bovine calves that either died immediately after birth or required sacrifice shortly after birth due to complications linked to large offspring syndrome (60). However, as this study did not incorporate apparently healthy live clones, there is no suggestion that this hypomethylation is characteristic of cloned cattle that survive birth without severe lethal aberrations necessitating immediate termination. Indeed, a recent study by Sukuzi *et al*. also suggests that embryos with widespread demethylation of *H19* are unable to implant (61). Hence, the detectable epigenetic aberrations at the embryonic stage may require significant reprogramming to give rise to viable animals, with tolerance of minor but variable epigenetics aberrations that cannot be pinpointed as a characteristic of all cloned animals. Cezar *et al*. reported reduced genome-wide methylation in bovine cloned fetuses, but not in adult clones (62). This is consistent with the investigation by Yang *et al*. reporting that the

expression of *Igf2*, *Igf2r* and *H19* genes are relatively normal in surviving adult clones (63). Hence aberrant hypomethylation of *Igf2* and *H19* genes is more likely to be indicative of clonal lethality.

Abnormal histone modifications (acetylation and methylation) accompany changes in DNA methylation. Aberrant hyperacetylation of histone H3 lysine 9 (AcH3K9) has been documented in cloned preimplantation bovine embryos created by SCNT, in addition to abnormal DNA methylation (55). At the eight-cell stage of development in cloned bovine embryos, the level of acetylated histone H4 lysine 5 (AcH4K5) is significantly lower than in control embryos (64). More recently, high levels of trimethylation of histone H3 lysine 27 (i.e H3K27me3) have been reported in the cloned mice at the blastocyst stage (structures formed during early embryogenesis which have the potential to develop into cloned animals) (65). Incomplete nuclear programming may generate abnormal epigenetic marks on the X chromosomes of cloned cattle which may be attributable to a number of aberrant histone modifications (i.e. the presence of H3K9me2 and H3K27me3 amidst the absence of H3K4me3) (66). However a major limitation of this particular study was the analysis of bovine somatic cell lines derived from ear skin cells from the animals rather than use of tissues from live animals. Aberrant DNA methylation and histone H4 acetylation have been reported in the tissues extracted from clones that died prior to or during birth (67). However, these findings may possibly be irrelevant to live clones.

Numerous investigations have been undertaken to identify possible epigenetic aberrations that may be characteristic of a cloned animal. However, none of the investigations have incorporated the analysis of tissues directly from live cloned animals that survive birth. The cloning of bovine embryos using bovine fetal

fibroblast nuclei as donors typically results in a 30-50% chance of development to the blastocyst stage and only 2-5% of the embryos will result in live births (68). Given the low success rate of SCNT, epigenetic aberrations characterised in embryos or blastocysts may not necessarily yield live animals, as the abnormalities may prove to be lethal. Any further downstream genetic reprogramming during later stages of embryonic development is also not taken into account. Given the desire to screen for the entry of healthy cloned animals and their offspring into food chain, any epigenetic markers need to be linked to these populations specifically. Currently there are no robust epigenetic markers indicative of the healthy cloned animals or their offspring.

(3) Gene expression

There have been a number of reports of abnormal gene expression linked to the cloning of animals. Differential gene expression (351 out of 13,610 genes) has been established with uterine tissues with SCNT pig embryos by microarray analysis (69). Global changes the gene expression profile of placental tissue of the cows carrying SCNT cloned embryos have also been documented, with 291 genes exhibiting more than a two-fold elevation, and 77 genes with expression reduced by at least 50% (70).

Abnormal expression of developmental genes characterised in cloned embryos include *Oct4* (71); (72), *IL6*, *FGF4*, and *FgFr2* (73), *G6PD* and *Xist* (74) to name but a few. Rideout *et al*. reported that the aberrant expression of imprinted genes may cause abnormal overgrowth in cloned animals that survive beyond birth, although it cannot be linked to just a single imprinted gene (75). Irregular expression of imprinted genes (e.g. *H19*, *Igf2*, *Peg1/Mest* and *Meg 1/Grb10*) in cloned mice have been

reported to result in the abnormal development of the fetus and placenta (76); (77); (78). The abnormal expression profiles of two imprinted genes *Xist* and *Ndn* have been observed during various stages of early development of cloned bovine embryos (64). A more recent study by Guillomot *et al.* identified the reduced expression of the imprinted gene *Phlda2* in cloned bovine placenta (79). However, this is a marker inherent to the placenta of the surrogate mother carrying the bovine clone, rather than the cloned animal.

A study by Xue *et al*., revealed that the pattern of expression of X-linked monoamine oxidase type A (*MAOA*) and *Xist* and eight other X-linked genes from skin and blood samples taken from four live cloned cattle (produced by SCNT using ovarian donor cells) were indistinguishable from those of their control counterparts, in spite of aberrant expression patterns exhibited by the cloned animals that died within 24 hours of birth (80). This investigation suggests that the success of nuclear transfer in producing viable, healthy cloned animals is dependent on epigenetic modifications required to reprogramme gene expression in the genome of the differentiated cell. X-chromosome inactivation (a mechanism for dosage compensation in mammals that is achieved by the silencing of one X-chromosome in female somatic cells) involving *Xist* expression was reported to be normal within cloned mice (81); (82). Hence there is concordance between different investigators that X-chromosome inactivation is normal within healthy full-term cloned animals. Thus the conflicting reports of aberrant *Xist* expression from investigations using cloned blastocysts or embryos may not be of relevance for healthy cloned animals (81); (82).

Aberrant expression of *Acrogranin*, *Cdx2*, and *ERR2* have been also documented in cloned bovine blastocysts (83). In a more recent study, abnormal expression of

selected genes in defined tissues (i.e. reduced beta actin in lung, and elevated spleen VEGF , lung Oct4, liver TERT, spleen H19, heart and kidney Igf2) was established in cloned cattle (67). However, as the tissues were derived from clones that died prior to or during birth the findings from this study may not be of relevance to live clones. Kremenskoy *et al.* have reported on differential expression of *leptin* and *POU5F1* genes in bovine fetal clones at 59 of the pregnancy compared with the gene expression in fetuses produced by artificial insemination 48 days into the pregnancy (84).

Aberrant expression of wide variety of target genes has been characterised as potential traits of cloned animals. However, the lack of independent verification of findings for some of the targets is a limitation, as is the use of embryonic, fetal, or uterine cells or placental tissue for the studies as opposed to tissues from live healthy cloned animals. For food analysis purposes, reports of aberrant gene expression must be linked to clones that survive birth, which is not the case with many of the reported investigations to date. Only Xue *et al*. investigated aberrant expression of X-linked genes using non-invasive samples from healthy adult cell-derived cloned animals, and no abnormalities were reported for the subset of genes evaluated (80); this was concordant with reports of normal *Xist* expression in mice.

(4) Protein expression

Aberrant protein expression has been documented in placenta from cloned mice (29)**,** cat (85), pig (86) and cattle (87); (88). A recent study by Bang *et al*. identified a set of

differentially expressed proteins that are linked to oxidative damage, senescence and apoptosis within the placentas of cloned cat embryos (85).

Kim *et al.* reported abnormal expression of key developmental proteins (tissue inhibitor of metalloproteinase-2 (TIMP-2), superoxide dismutase (SOD), vimentin and plasminogen activator inhibitor-1 (PAI)) in the placentae of SCNT cloned Korean native cattle that died immediately after birth (88). The upregulation of TIMP-2 and SOD expression in SCNT placenta, and accompanying downregulation of vimentin and PAI may have resulted in clonal mortality. In pigs carrying clones, the placenta exhibits increased osteopontin, and decreased expression of retinol-binding protein and fibroblast growth factor 7 (FGF7) (86). All of these markers are linked to the placenta from the birth of an unhealthy cloned animal, and therefore are unlikely to serve as biomarkers in healthy cloned animals.

The major limitation of these reports of aberrant protein expression is the analyses of the placenta of the surrogate mother of the clones rather than tissues from live healthy cloned animals. Abnormal protein expression inherent to live cloned animals have not been characterised *per se*. Hence there is no suggestion that these reported abnormalities will be detected in an adult cloned animal that may enter the food chain.

Conclusions

In summary, robust biomarkers that reliably distinguish naturally conceived animals from live cloned animals or their offspring have not been identified following a review of current literature. The limitation with many of the investigations is the use of embryos or even derived cell lines for the analyses of anomalies rather than tissues

from full term healthy cloned animals. The studies utilising tissues from full term clones have not necessarily included sufficient numbers of healthy cloned animals that do not necessitate immediate termination. The majority of the scientific investigations focus on the lethal aberrations linked with cloning. The main obstacle to these studies is the low efficiency of SCNT for the production of viable cloned cattle. The occurrence of conflicting reports may be attributable to the variability of the SCNT procedure itself, as well as the age and type of donor cell, and species of the clone. One robust study citing the use of blood and skin extracted from healthy live cloned cattle (n=4) reports normal X chromosome inactivation that is indistinguishable from the four control animals (80). Potential genetic or protein markers identified from the analyses of real samples from the live healthy clones would be more robust than cell line derived from somatic cells of a live clone, given the cell culturing is a process which may introduce variability. There is also concordance among independent investigators that the offspring of the cloned cattle do not exhibit any detectable difference in telomerase length compared with control animals (41); (7); (4). These robust studies may suggest that the healthy cloned animals and offspring are indistinguishable from animals without any legacy of SCNT. Hence further scientific research is required to identify if there are markers that may ultimately form the basis of a food test for cloned animals and their offspring.

Acknowledgements

This work was funded by the UK National Measurement System as part of the Government Chemist programme 2011-2014.

References

1. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Viable offspring derived from fetal and adult mammalian cells. Nature 1997;385:810-3.

2. Turner L. A sheep named Dolly. CMAJ 1997;156:1149-50.

3. Edwards JL, Schrick FN, McCracken MD, van Amstel SR, Hopkins FM, Welborn MG, Davies CJ. Cloning adult farm animals: a review of the possibilities and problems associated with somatic cell nuclear transfer. Am J Reprod Immunol 2003;50:113-23.

4. Kurome M, Hisatomi H, Matsumoto S, Tomii R, Ueno S, Hiruma K et al. Production efficiency and telomere length of the cloned pigs following serial somatic cell nuclear transfer. J Reprod Dev 2008;54:254-8.

5. Kurome M, Zakhartchenko V, Kessler B, Gungor T, Richter A, Klymiuk N et al. 23 developmental potential of cloned transgenic porcine embryos produced by serial nuclear transfer can be improved by treatment with histone deacetylase inhibitors. Reprod Fertil Dev 2011;24:123-4.

6. Forsberg EJ, Strelchenko NS, Augenstein ML, Betthauser JM, Childs LA, Eilertsen KJ et al. Production of cloned cattle from in vitro systems. Biol Reprod 2002;67:327-33.

7. Kubota C, Tian XC, Yang X. Serial bull cloning by somatic cell nuclear transfer. Nat Biotechnol 2004;22:693-4.

8. Jang G, Hong SG, Oh HJ, Kim MK, Park JE, Kim HJ et al. A cloned toy poodle produced from somatic cells derived from an aged female dog. Theriogenology 2008;69:556-63.

9. Yin XJ, Lee HS, Lee YH, Seo YI, Jeon SJ, Choi EG et al. Cats cloned from fetal and adult somatic cells by nuclear transfer. Reproduction 2005;129:245-9.

10. Gomez MC, Pope CE, Dresser BL. Nuclear transfer in cats and its application. Theriogenology 2006;66:72-81.

11. Yin XJ, Lee HS, Yu XF, Kim LH, Shin HD, Cho SJ et al. Production of second-generation cloned cats by somatic cell nuclear transfer. Theriogenology 2008;69:1001-6.

12. Wakayama T, Shinkai Y, Tamashiro KL, Niida H, Blanchard DC, Blanchard RJ et al. Cloning of mice to six generations. Nature 2000;407:318-9.

13. Tamashiro KL, Wakayama T, Blanchard RJ, Blanchard DC, Yanagimachi R. Postnatal growth and behavioral development of mice cloned from adult cumulus cells. Biol Reprod 2000;63:328-34.

14. Suzuki T, Kondo S, Wakayama T, Cizdziel PE, Hayashizaki Y. Genome-wide analysis of abnormal H3K9 acetylation in cloned mice. PLoS One 2008;3:e1905.

15. Zhou Q, Renard JP, Le Friec G, Brochard V, Beaujean N, Cherifi Y et al. Generation of fertile cloned rats by regulating oocyte activation. Science 2003;302:1179.

16. Chesne P, Adenot PG, Viglietta C, Baratte M, Boulanger L, Renard JP. Cloned rabbits produced by nuclear transfer from adult somatic cells. Nat Biotechnol 2002;20:366-9.

17. Galli C, Lagutina I, Duchi R, Colleoni S, Lazzari G. Somatic cell nuclear transfer in horses. Reprod Domest Anim 2008;43 Suppl 2:331-7.

18. Holden C. Genetics. First cloned mule races to finish line. Science 2003;300:1354.

19. Zhou H, Guo Z. Heterogeneous nuclear-transferred-embryos reconstructed with camel (Camelus bactrianus) skin fibroblasts and enucleated ovine oocytes and their development H-M. Anim Reprod Sci 2006;95:324-30.

20. Keefer CL, Keyston R, Lazaris A, Bhatia B, Begin I, Bilodeau AS et al. Production of cloned goats after nuclear transfer using adult somatic cells. Biol Reprod 2002;66:199-203.

21. Berg DK, Li C, Asher G, Wells DN, Oback B. Red deer cloned from antler stem cells and their differentiated progeny. Biol Reprod 2007;77:384-94.

22. Naruse K, Ijiri K, Shima A, Egami N. The production of cloned fish in the medaka (Oryzias latipes). J Exp Zool 1985;236:335-41.

23. Wakayama T, Yanagimachi R. Cloning the laboratory mouse. Semin Cell Dev Biol 1999;10:253-8.

24. Ju S, Rui R, Lu Q, Lin P, Guo H. Analysis of apoptosis and methyltransferase mRNA expression in porcine cloned embryos cultured in vitro. J Assist Reprod Genet 2010;27:49-59.

25. Thuan NV, Kishigami S, Wakayama T. How to improve the success rate of mouse cloning technology. J Reprod Dev 2010;56:20-30.

26. Mizutani E, Yamagata K, Ono T, Akagi S, Geshi M, Wakayama T. Abnormal chromosome segregation at early cleavage is a major cause of the full-term developmental failure of mouse clones. Dev Biol 2012;364:56-65.

27. Smith LC, Bordignon V, Babkine M, Fecteau G, Keefer C. Benefits and problems with cloning animals. Can Vet J 2000;41:919-24.

28. Wei Y, Zhu J, Huan Y, Liu Z, Yang C, Zhang X et al. Aberrant expression and methylation status of putatively imprinted genes in placenta of cloned piglets. Cell Reprogram 2010;12:213-22.

29. Kim HR, Han RX, Wakayama T, Park CS, Jin DI. Aberrant protein expression in the placenta of cloned mouse derived from embryonic stem cell. Placenta 2010;31:853-9.

30. Park MR, Cho SK, Lee SY, Choi YJ, Park JY, Kwon DN et al. A rare and often unrecognized cerebromeningitis and hemodynamic disorder: a major cause of sudden death in somatic cell cloned piglets. Proteomics 2005;5:1928-39.

31. Young LE, Sinclair KD, Wilmut I. Large offspring syndrome in cattle and sheep. Rev Reprod 1998;3:155-63.

32. Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C et al. Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. Nat Genet 2001;27:153-4.

33. Brisville AC, Fecteau G, Boysen S, Dorval P, Buczinski S, Blondin P, Smith LC. Respiratory disease in neonatal cloned calves. J Vet Intern Med 2011;25:373-9.

34. Animal Cloning. A Draft Risk Assessment. 2006.
Available at:
http://www.fda.gov/cvm/Documents/Cloning_Risk_Assessment.pdf

35. Greider CW, Blackburn EH. Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. Cell 1985;43:405-13.

36. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. Nature 1990;345:458-60.

37. Jiang H, Ju Z, Rudolph KL. Telomere shortening and ageing. Z Gerontol Geriatr 2007;40:314-24.

38. Savela S, Saijonmaa O, Strandberg TE, Koistinen P, Strandberg AY, Tilvis RS et al. Physical activity in midlife and telomere length measured in old age. Exp Gerontol 2012.

39. Xu J, Yang X. Will cloned animals suffer premature aging--the story at the end of clones' chromosomes. Reprod Biol Endocrinol 2003;1:105.

40. Shiels PG, Kind AJ, Campbell KH, Wilmut I, Waddington D, Colman A, Schnieke AE. Analysis of telomere length in Dolly, a sheep derived by nuclear transfer. Cloning 1999;1:119-25.

41. Alexander B, Coppola G, Perrault SD, Peura TT, Betts DH, King WA. Telomere length status of somatic cell sheep clones and their offspring. Mol Reprod Dev 2007;74:1525-37.

42. Betts DH, Perrault SD, Petrik J, Lin L, Favetta LA, Keefer CL, King WA. Telomere length analysis in goat clones and their offspring. Mol Reprod Dev 2005;72:461-70.

43. Jiang L, Carter DB, Xu J, Yang X, Prather RS, Tian XC. Telomere lengths in cloned transgenic pigs. Biol Reprod 2004;70:1589-93.

44. Jeon HY, Hyun SH, Lee GS, Kim HS, Kim S, Jeong YW et al. The analysis of telomere length and telomerase activity in cloned pigs and cows. Mol Reprod Dev 2005;71:315-20.

45. Miyashita N, Shiga K, Yonai M, Kaneyama K, Kobayashi S, Kojima T et al. Remarkable differences in telomere lengths among cloned cattle derived from different cell types. Biol Reprod 2002;66:1649-55.

46. Tian XC, Xu J, Yang X. Normal telomere lengths found in cloned cattle. Nat Genet 2000;26:272-3.

47. Vogel G. In contrast to Dolly, cloning resets telomere clock in cattle. Science 2000;288:586-7.

48. Kato Y, Tani T, Tsunoda Y. Cloning of calves from various somatic cell types of male and female adult, newborn and fetal cows. J Reprod Fertil 2000;120:231-7.

49. Betts D, Bordignon V, Hill J, Winger Q, Westhusin M, Smith L, King W. Reprogramming of telomerase activity and rebuilding of telomere length in cloned cattle. Proc Natl Acad Sci U S A 2001;98:1077-82.

50. Lanza RP, Cibelli JB, Blackwell C, Cristofalo VJ, Francis MK, Baerlocher GM et al. Extension of cell life-span and telomere length in animals cloned from senescent somatic cells. Science 2000;288:665-9.

51. Xu J, Yang X. Will cloned animals suffer premature aging--the story at the end of clones' chromosomes. Reprod Biol Endocrinol 2003;1:105.

52. Bourc'his D, Le Bourhis D, Patin D, Niveleau A, Comizzoli P, Renard JP, Viegas-Pequignot E. Delayed and incomplete reprogramming of chromosome methylation patterns in bovine cloned embryos. Curr Biol 2001;11:1542-6.

53. Dean W, Santos F, Stojkovic M, Zakhartchenko V, Walter J, Wolf E, Reik W. Conservation of methylation reprogramming in mammalian development: aberrant reprogramming in cloned embryos. Proc Natl Acad Sci U S A 2001;98:13734-8.

54. Giraldo AM, Hylan DA, Ballard CB, Purpera MN, Vaught TD, Lynn JW et al. Effect of epigenetic modifications of donor somatic cells on the subsequent chromatin remodeling of cloned bovine embryos. Biol Reprod 2008;78:832-40.

55. Santos F, Zakhartchenko V, Stojkovic M, Peters A, Jenuwein T, Wolf E et al. Epigenetic marking correlates with developmental potential in cloned bovine preimplantation embryos. Curr Biol 2003;13:1116-21.

56. Kang YK, Koo DB, Park JS, Choi YH, Kim HN, Chang WK et al. Typical demethylation events in cloned pig embryos. Clues on species-specific differences in epigenetic reprogramming of a cloned donor genome. J Biol Chem 2001;276:39980-4.

57. Ohgane J, Wakayama T, Senda S, Yamazaki Y, Inoue K, Ogura A et al. The Sall3 locus is an epigenetic hotspot of aberrant DNA methylation associated with placentomegaly of cloned mice. Genes Cells 2004;9:253-60.

58. Young LE, Schnieke AE, McCreath KJ, Wieckowski S, Konfortova G, Fernandes K et al. Conservation of IGF2-H19 and IGF2R imprinting in sheep: effects of somatic cell nuclear transfer. Mech Dev 2003;120:1433-42.

59. Mann MR, Chung YG, Nolen LD, Verona RI, Latham KE, Bartolomei MS. Disruption of imprinted gene methylation and expression in cloned preimplantation stage mouse embryos. Biol Reprod 2003;69:902-14.

60. Curchoe CL, Zhang S, Yang L, Page R, Tian XC. Hypomethylation trends in the intergenic region of the imprinted IGF2 and H19 genes in cloned cattle. Anim Reprod Sci 2009;116:213-25.

61. Suzuki J, Jr., Therrien J, Filion F, Lefebvre R, Goff AK, Perecin F et al. Loss of methylation at H19 DMD is associated with biallelic expression and reduced development in cattle derived by somatic cell nuclear transfer. Biol Reprod 2011;84:947-56.

62. Cezar GG, Bartolomei MS, Forsberg EJ, First NL, Bishop MD, Eilertsen KJ. Genome-wide epigenetic alterations in cloned bovine fetuses. Biol Reprod 2003;68:1009-14.

63. Yang L, Chavatte-Palmer P, Kubota C, O'Neill M, Hoagland T, Renard JP et al. Expression of imprinted genes is aberrant in deceased newborn cloned calves and relatively normal in surviving adult clones. Mol Reprod Dev 2005;71:431-8.

64. Wee G, Koo DB, Song BS, Kim JS, Kang MJ, Moon SJ et al. Inheritable histone H4 acetylation of somatic chromatins in cloned embryos. J Biol Chem 2006;281:6048-57.

65. Zhang M, Wang F, Kou Z, Zhang Y, Gao S. Defective chromatin structure in somatic cell cloned mouse embryos. J Biol Chem 2009;284:24981-7.

66. Geng-Sheng C, Yu G, Kun W, Fang-Rong D, Ning L. Repressive but not activating epigenetic modifications are aberrant on the inactive X chromosome in live cloned cattle. Dev Growth Differ 2009;51:585-94.

67. Lin L, Li Q, Zhang L, Zhao D, Dai Y, Li N. Aberrant epigenetic changes and gene expression in cloned cattle dying around birth. BMC Dev Biol 2008;8:14.

68. Zakhartchenko V, Durcova-Hills G, Stojkovic M, Schernthaner W, Prelle K, Steinborn R et al. Effects of serum starvation and re-cloning on the efficiency of nuclear transfer using bovine fetal fibroblasts. J Reprod Fertil 1999;115:325-31.

69. Ka H, Seo H, Kim M, Moon S, Kim H, Lee CK. Gene expression profiling of the uterus with embryos cloned by somatic cell nuclear transfer on day 30 of pregnancy. Anim Reprod Sci 2008;108:79-91.

70. Oishi M, Gohma H, Hashizume K, Taniguchi Y, Yasue H, Takahashi S et al. Early embryonic death-associated changes in genome-wide gene expression

profiles in the fetal placenta of the cow carrying somatic nuclear-derived cloned embryo. Mol Reprod Dev 2006;73:404-9.

71. Boiani M, Eckardt S, Scholer HR, McLaughlin KJ. Oct4 distribution and level in mouse clones: consequences for pluripotency. Genes Dev 2002;16:1209-19.

72. Beyhan Z, Forsberg EJ, Eilertsen KJ, Kent-First M, First NL. Gene expression in bovine nuclear transfer embryos in relation to donor cell efficiency in producing live offspring. Mol Reprod Dev 2007;74:18-27.

73. Daniels R, Hall V, Trounson AO. Analysis of gene transcription in bovine nuclear transfer embryos reconstructed with granulosa cell nuclei. Biol Reprod 2000;63:1034-40.

74. Wrenzycki C, Lucas-Hahn A, Herrmann D, Lemme E, Korsawe K, Niemann H. In vitro production and nuclear transfer affect dosage compensation of the X-linked gene transcripts G6PD, PGK, and Xist in preimplantation bovine embryos. Biol Reprod 2002;66:127-34.

75. Rideout WM, III, Eggan K, Jaenisch R. Nuclear cloning and epigenetic reprogramming of the genome. Science 2001;293:1093-8.

76. Humpherys D, Eggan K, Akutsu H, Hochedlinger K, Rideout WM, III, Biniszkiewicz D et al. Epigenetic instability in ES cells and cloned mice. Science 2001;293:95-7.

77. Inoue K, Kohda T, Lee J, Ogonuki N, Mochida K, Noguchi Y et al. Faithful expression of imprinted genes in cloned mice. Science 2002;295:297.

78. Ogawa H, Ono Y, Shimozawa N, Sotomaru Y, Katsuzawa Y, Hiura H et al. Disruption of imprinting in cloned mouse fetuses from embryonic stem cells. Reproduction 2003;126:549-57.

79. Guillomot M, Taghouti G, Constant F, Degrelle S, Hue I, Chavatte-Palmer P, Jammes H. Abnormal expression of the imprinted gene Phlda2 in cloned bovine placenta. Placenta 2010;31:482-90.

80. Xue F, Tian XC, Du F, Kubota C, Taneja M, Dinnyes A et al. Aberrant patterns of X chromosome inactivation in bovine clones. Nat Genet 2002;31:216-20.

81. Eggan K, Akutsu H, Hochedlinger K, Rideout W, III, Yanagimachi R, Jaenisch R. X-Chromosome inactivation in cloned mouse embryos. Science 2000;290:1578-81.

82. Wrenzycki C, Wells D, Herrmann D, Miller A, Oliver J, Tervit R, Niemann H. Nuclear transfer protocol affects messenger RNA expression patterns in cloned bovine blastocysts. Biol Reprod 2001;65:309-17.

83. Hall VJ, Ruddock NT, French AJ. Expression profiling of genes crucial for placental and preimplantation development in bovine in vivo, in vitro, and nuclear transfer blastocysts. Mol Reprod Dev 2005;72:16-24.

84. Kremenskoy M, Kremenska Y, Suzuki M, Imai K, Takahashi S, Hashizume K et al. Epigenetic characterization of the CpG islands of bovine Leptin and POU5F1 genes in cloned bovine fetuses. J Reprod Dev 2006;52:277-85.

85. Bang JI, Bae DW, Lee HS, Deb GK, Kim MO, Sohn SH et al. Proteomic analysis of placentas from cloned cat embryos identifies a set of differentially

expressed proteins related to oxidative damage, senescence and apoptosis. Proteomics 2011;11:4454-67.

86. Kim M, Seo H, Choi Y, Hwang W, Lee CK, Ka H. Aberrant expression of retinol-binding protein, osteopontin and fibroblast growth factor 7 in the porcine uterine endometrium of pregnant recipients carrying embryos produced by somatic cell nuclear transfer. Anim Reprod Sci 2009;112:172-81.

87. Kim HR, Kang JK, Yoon JT, Seong HH, Jung JK, Lee HM et al. Protein profiles of bovine placenta derived from somatic cell nuclear transfer. Proteomics 2005;5:4264-73.

88. Kim HR, Naruse K, Lee HR, Han RX, Park CS, Jin DI. Abnormal expression of TIMP-2, SOD, vimentin and PAI proteins in cloned bovine placentae. Reprod Domest Anim 2009;44:714-7.