REPRODUCTION

REVIEW

Focus on Mammalian Embryogenomics

Epigenetic reprogramming in embryonic and foetal development upon somatic cell nuclear transfer cloning

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Abstract

The birth of 'Dolly', the first mammal cloned from an adult donor cell, has sparked a flurry of research activities to improve cloning technology and to understand the underlying mechanism of epigenetic reprogramming of the transferred somatic cell nucleus. Especially in ruminants, somatic cell nuclear transfer (SCNT) is frequently associated with pathological changes in the foetal and placental phenotype and has significant consequences for development both before and after birth. The most critical factor is epigenetic reprogramming of the transferred somatic cell nucleus from its differentiated status into the totipotent state of the early embryo. This involves an erasure of the gene expression program of the respective donor cell and the establishment of the well-orchestrated sequence of expression of an estimated number of 10 000–12 000 genes regulating embryonic and foetal development. The following article reviews the present knowledge on the epigenetic reprogramming of the transferred somatic cell nucleus, with emphasis on DNA methylation, imprinting, X-chromosome inactivation and telomere length restoration in bovine development. Additionally, we briefly discuss other approaches towards epigenetic nuclear reprogramming, including the fusion of somatic and embryonic stem cells and the overexpression of genes crucial in the formation and maintenance of the pluripotent status. Improvements in our understanding of this dramatic epigenetic reprogramming event will be instrumental in realising the great potential of SCNT for basic biological research and for various agricultural and biomedical applications.

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Introduction

The most dramatic epigenetic reprogramming occurs in somatic cell nuclear transfer (SCNT) cloning when the expression profile of a differentiated cell is abolished and the new embryo-specific expression profile is established to drive embryonic and foetal development. It is estimated that this includes an abrogation of the expression of 8000–10 000 genes of the somatic cell program and the initiation of the embryonic program with ~10 000 genes. Pre-zygotic reprogramming includes the erasure of somatic cell epigenetic modifications and is followed by a post-zygotic establishment of embryonic modifications. Other post-zygotic reprogrammings include X-chromosome inactivation and adjustment of telomere lengths (Hochedlinger & Jaenisch 2003). Here, we review some aspects of epigenetic reprogramming in pre- and post-implantation development with emphasis on imprinting, X-chromosome inactivation and telomere elongation in cattle. As it is difficult to discriminate between effects of *in vitro* culture and the cloning process itself on epigenetic marks, we refer to important information related to the effects of *in vitro* culture during early development. In addition to SCNT, alternative approaches to reprogram the genome of a somatic nucleus have been developed and we briefly refer to recent exciting developments in this field because the underlying basic epigenetic mechanisms should be similar to SCNT.

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Epigenetic reprogramming in early development

Normal development depends on a precise sequence of changes in the configuration of the chromatin, which are primarily related to the acetylation and methylation status of histones and the methylation of genomic DNA. These epigenetic modifications control the precise tissue-specific expression of genes. It is estimated that the mammalian genome with its ~25 000 genes contains at least 30 000–40 000 CpG islands, i.e. areas of between 200 and 2000 nucleotides where the C+G content is >50% and the observed:expected numbers of CpG dinucleotides is >0.6. These CpG islands are predominantly found in the promoters of housekeeping genes, but are also observed in tissue-specific genes (Antequera 2003). The correct pattern of cytosine methylation in CpG dinucleotides is required for normal mammalian development (Li *et al.* 1993, Li 2002). DNA methylation is also thought to play a crucial role in suppressing the activities of parasitic promoters and is thus part of the gene silencing system in eukaryotic cells (Jones 1999). Previously, methylation was thought to be associated with silencing of a given gene, but an increasing number of genes is now found to be activated by methylation marks, specifically tumour suppressor genes and differentiation-associated genes (Bestor & Tycko 1996, Jones 1999, Li 2002). Epigenetic regulation is critical to achieve the biological complexity of multicellular organisms and the complexity of epigenetic regulation increases with genomic size (Mager & Bartholomei 2005).

During early mammalian development, reprogramming of genomic DNA modifications is observed shortly before and after the formation of the zygote. The paternal DNA is actively and rapidly demethylated after fertilisation, whilst the maternal DNA undergoes passive demethylation, as shown in bovine, murine, porcine, rat and human zygotes (Mayer *et al.* 2000, Oswald *et al.* 2000, Dean *et al.* 2001, Santos *et al.* 2002, Beaujean *et al.* 2004, Xu *et al.* 2005). The embryonic DNA is increasingly remethylated between the two-cell and the blastocyst stages (Dean *et al.* 2001) in waves, which correlate with the species-specific onset of transcription from the embryonic genome (Telford *et al.* 1990, Reik *et al.* 2001). These mechanisms ensure that critical steps during early development, such as the timing of first cell division, compaction, blastocyst formation, expansion and hatching are regulated by a well-orchestrated expression of genes. The application of assisted reproductive technologies, such as *in vitro* fertilisation and culture, is frequently associated with aberrant mRNA expression patterns in the resulting embryos, greater epigenetic disturbances and a higher risk of aberrant phenotypes (DeRycke *et al.* 2002, Niemann *et al.* 2002, Powell 2003).

DNA methylation critically depends on the activity of a class of enzymes, the DNA-methyltransferases (Dnmts). DNA-methytransferase 1 (Dnmt1) is a maintenance enzyme that is responsible for copying the methylation pattern of CpG dinucleotides to the newly synthesised strand in hemi-methylated DNA after replication (Bestor 1992). The oocyte-specific isoform, Dnmt1o, maintains maternal imprints. Dmnt3a and Dmnt3b catalyse *de novo* methylation and are thus critical for establishing DNA methylation during development (Hsieh 1999, Okano *et al.* 1999). Dmnt3L co-localises with Dnmt3a and -b and presumably is involved in establishing specific methylation imprints in the female germline (Bourc'his *et al.* 2001*b*).

Overview on epigenetic reprogramming approaches

A long-held dogma in developmental biology is that mammalian somatic cell differentiation is irreversible as illustrated in the landscape model of cell differentiation (Waddington 1940, Keeton & Gould 1984). The process of differentiation is depicted as a ball rolling down a hill with many valleys. When the ball is on top of the hill, it can roll down through any valley below, representing a totipotent cell that can differentiate into any tissue of the body. However, when the ball reaches the bottom of the hill, it cannot move to another valley or back to the top of the hill, illustrating that once a cell is terminally differentiated, it can no longer transdifferentiate into another cell type or become totipotent again (Fig. 1). The successful cloning of mammals using differentiated somatic cells by SCNT, however, challenged this dogma (Wilmut *et al.* 1997) and provided first convincing evidence for the enormous plasticity of the mammalian epigenome. The process of returning a differentiated somatic nucleus to a totipotent state is termed nuclear reprogramming. Over the past decade, alternative reprogramming approaches have been developed,

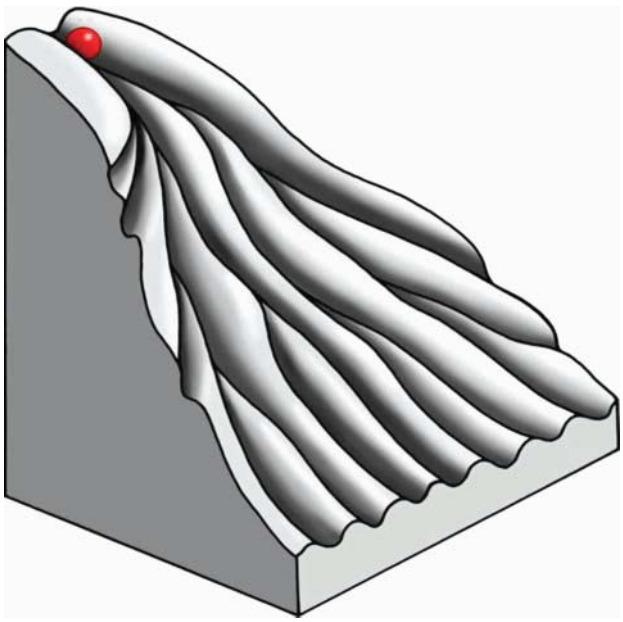

Figure 1 The landscape model of mammalian differentiation (modified from Keeton & Gould 1984).

mainly with the goal to make the entire process less error prone and to avoid the use of oocytes.

Cell-fusion or cell extract-based reprogramming

Various attempts have been made to directly de-differentiate somatic cells by fusing them with pluripotent stem cells or by the use of extracts from these cells. While these approaches can de-differentiate somatic cells to a certain degree and are good research models for proof-of-concept, each of them has intrinsic problems that are hard to overcome. Extracts from embryonic stem cells (ESC), pluripotent cancerous cells (Taranger *et al*. 2005) or differentiated somatic cells like cardiomyocytes (Gaustad *et al*. 2004), have been found to reprogram, to some extent, terminally differentiated cells. However, ESC themselves or other cell types are needed first to generate ESC or the desired cell types. Additionally, the number of cells needed for extract preparation is several magnitudes greater than the number of cells that can be reprogrammed. It would be difficult, if at all possible, to obtain such enormous number of cells from human sources for reprogramming purposes. Cancerous cells may be relatively easier to obtain but it is unlikely that they will be approved for human clinical use for fear of reprogramming cells to a cancerous state. For the cell fusion approach, both mouse (Tada *et al*. 1994) and human (Cowan *et al*. 2005) somatic cells have been fused with ESC of the respective species. However, extensive genetic modification of the somatic cells is necessary for the incorporation of markers to select cells that fused correctly, and the complete removal of the ESC chromosomes from the fused cells is no small task.

The use of Xenopus *egg extract for reprogramming*

Xenopus laevis eggs are 1–1.3 mm in diameter, and are a thousand times larger than mammalian oocytes. They are easily collected in large quantities; as many as 300–1000 eggs are spawned upon hormonal stimulation. Somatic cells injected into the nuclei of *Xenopus* eggs have been induced to express *OCT4* (Byrne *et al*. 2003) and *BRG1* was shown to be essential in this process (Hansis *et al*. 2004). These observations demonstrated that the reprogramming factors in the nuclei of *Xenopus* eggs are capable of de-differentiating mammalian somatic cells. Because *Xenopus* eggs are ovulated at the metaphase II stage of meiosis (MII), the nuclear contents are released into the cytoplasm, and methods of preparation for these nuclear components are well established. The use of *Xenopus* egg cytoplasm extracts to reprogram somatic cells has not been researched extensively although some progress has been made (Alberio *et al*. 2005, Miyamoto *et al*. 2007).

Transfection of pluripotent factors into somatic cells

Recently, a revolutionary breakthrough in the direct nuclear reprogramming of mouse somatic cells has been reported (Takahashi & Yamanaka 2006, Okita *et al*. 2007, Wernig *et al*. 2007). Cells transfected with *Oct4*, *Sox2*, *c-Myc* and *Klf4*, carried in lentiviral vectors, were turned into a totipotent state and are indistinguishable from ESC generated from fertilised embryos with regard to differentiation potential and morphology. ESC derived from the somatic cells were even able to populate the germ line upon injection into blastocysts and after transfer into recipient mice, clearly indicating complete reprogramming (Maherali *et al*. 2007, Okita *et al*. 2007, Wernig *et al*. 2007). The same genes have already been found to be effective in reprogramming human fibroblasts and other human somatic cells into cells with true pluripotent properties (Takahashi *et al*. 2007). While these findings are extremely exciting, the introduction of *c-Myc* can potentially induce cancer formation. Further refinement of this technology is necessary to avoid the use of retroviral vectors and potentially carcinogenic molecules.

Epigenetic reprogramming by SCNT

During somatic cloning, a differentiated somatic cell is fused with an enucleated oocyte to generate a reconstructed embryo. Common somatic cloning protocols involve the following major technical steps: (1) enucleation of the recipient oocyte, (2) preparation and subzonal transfer of the donor cells, (3) fusion of the two components, (4) activation of the reconstructed complex, (5) temporary culture of the reconstructed embryo and finally (6) transfer to a foster mother or storage in liquid nitrogen. Because the resulting embryos contain totipotent cells, SCNT thus indirectly converts the differentiated somatic cell into a totipotent state.

Typically, the success rate (live births) of mammalian somatic nuclear transfer is low and usually only $<2\%$ of embryos transferred result in live births. Cattle seem to be an exception to this rule as levels of 15–20% can be reached (Kues & Niemann 2004). Pre- and post-natal development is often compromised and a variable proportion of the offspring show aberrant developmental patterns and increased pre- and perinatal mortality. These abnormalities include a wide range of symptoms, such as extended gestation length, oversized offspring, aberrant placental development, cardiovasculatory and respiratory problems, immunological deficiencies, problems with tendons, adult obesity, kidney and hepatic malfunctions, behavioural changes, and a higher susceptibility to neonatal diseases. These are all commonly described as the 'large offspring syndrome' (LOS; Renard *et al*. 1999, Tamashiro *et al*. 2000, Ogonuki *et al*. 2002, Perry & Wakayama 2002, Rhind *et al*. 2003). These pathologies have most often reported in SCNT cloning of cattle, sheep and mice.

The occurrence is stochastic and has not been correlated with the aberrant expression of particular genes or specific pathophysiology.

Despite these limitations and the development of new transfection methods (transfection of pluripotent factors into somatic cells), reprogramming by SCNT remains a very attractive approach for the generation of ESC. A key question is whether ESC generated by SCNT are normal because reproductive cloning by SCNT has been associated with low birth rates and high incidence of developmental abnormalities. This question is partially answered by several lines of existing evidence accumulated in different species. First, the development rate of bovine SCNT embryos to the blastocyst stage is similar to that of *in vitro* fertilised bovine embryos and the global gene expression profile of bovine SCNT embryos is similar to that of embryos produced *in vivo*, suggesting that by the blastocyst stage, the SCNT embryos have undergone a relatively complete and correct nuclear reprogramming at the molecular level (Smith *et al*. 2005). Secondly, ESC derived from SCNT mouse embryos show indistinguishable expression profiles from ESC derived from embryos produced by natural fertilisation (Brambrink *et al*. 2006, Wakayama *et al*. 2006). Thirdly, ESC lines can be derived from SCNT mouse and bovine embryos with a high efficiency, suggesting that NT embryos are reprogrammed and contain totipotent cells. Taken together, these studies indicate that the cytoplasm of oocytes is capable of completely reprogramming somatic cells to true stable totipotency.

Aberrant DNA methylation in cloned bovine embryos and foetuses

Global and gene-specific changes in DNA methylation have been demonstrated in bovine embryos derived from *in vitro* fertilisation or SCNT (Dean *et al*. 2001, Kang *et al*. 2001). Studies comparing the changes in DNA methylation patterns in cell nuclei using antibodies to methylcytosine showed that the pattern of nuclear staining was different in the cells of normal embryos when compared with SCNT embryos (Bourc'his *et al*. 2001*a*, Dean *et al*. 2001). The staining pattern in the SCNT embryos better resembled that of the somatic donor cells used for cloning, leading to the conclusion that nuclear reprogramming was incomplete and the epigenetic modifications of differentiated donor cells could not be erased and returned to the totipotent state of early zygotic cells (Bourc'his *et al*. 2001*a*, Dean *et al*. 2001). Although most of these SCNT embryos do not result in live offspring, a high percentage do establish pregnancies, form foetuses and develop placentas, which the original somatic donor cell could never do. Thus, the DNA of the donor nuclei must have been reprogrammed to a large extent. Those regions that are heavily methylated in the somatic donor cells may be regions of DNA that do not encode genes and hence, need not be demethylated for the nuclear DNA to drive embryonic development. Gene expression profiling of the donor cells and SCNT blastocysts generated from these cells show that significant reprogramming had occurred (Smith *et al*. 2005), as the difference in appearance between the cells and the embryos should suggest. One study examining the change in methylation status of the galanin gene, showed that the CpG sites that were methylated in the donor cell DNA were demethylated after nuclear transfer and underwent re-methylation at exactly the same positions in a manner that recapitulated the events in normal bovine IVF development (Kang *et al*. 2003). Studies of the methylation in promoters of specific genes, such as the epidermal cytokeratin gene, the differentially methylated region (DMR) of X-chromosome inactive-specific transcript (*XIST*) and in the satellite I repeat DNA revealed no difference in methylation between SCNT and control tissues for the DMR of *XIST*, despite apparent loss of imprinting in the chorion from some bovine SCNT samples (Dindot *et al*. 2004). The methylation levels of cytokeratin promoter and the satellite I DNA were not different between SCNT and the control livers, but a small difference was detected in the chorion, suggesting that there may be a tendency for dysregulation of methylation in the chorion. Alterations in methylation levels, either global or at specific sequences, have been observed in abnormal or dead bovine SCNT foetuses or calves, compared with either normal controls or apparently normal clones. Some studies reported hypermethylation (Hiendleder *et al*. 2004) and others, hypomethylation (Cezar *et al*. 2003). Such conflicting data emphasise the importance of comparing the same DNA sites in equivalent tissues and developmental stages between studies and using comparable methodologies. The significance of these observations is unclear as the effect of altered methylation levels on gene expression is generally unknown. It is not possible to conclude if the altered methylation levels caused the abnormalities and developmental failure or if abnormal development has lead subsequently to improper DNA methylation.

It is important to appreciate that DNA methylation patterns are dynamic and not static during development and can change in response to environmental influence. The aberrant methylation patterns or levels seen in those SCNT animals that die *in utero* or shortly after birth may bear no resemblance with methylation levels and patterns seen earlier in development. Simply, we do not know if the genome was incorrectly methylated to start with or if a stochastic event perturbed the environment at a critical time in development, altering gene expression and subsequently the DNA methylation status later on. Such alterations may set the embryo or foetus on a developmental path that is incompatible with a live offspring outcome.

We have developed the hypothesis that deviations from the normal pattern of mRNA expression due to epigenetic alterations, which are observed in the early preimplantation embryo, persist throughout foetal development up to birth and that the many effects of this period of culture only become manifest later in development (Niemann & Wrenzycki 2000). Consistent with this hypothesis, genes aberrantly expressed in blastocysts were also aberrantly expressed in the organs of clones that died shortly after birth (Li *et al*. 2005). This is particularly true for Xist and heat shock protein for which aberrant expression patterns had been found in cloned blastocysts (Wrenzycki *et al*. 2001, 2002).

X-chromosome-linked development and gene expression

X-chromosome inactivation in mammals

Mammals have evolved a sex determination mechanism that is chromosome based with the primary sex determinant for females being the presence of two X chromosomes, and the presence of an X and a Y chromosome for males. The X chromosome consists of ~160 Mb DNA (3–5% of the genome) that codes for over a thousand genes with a diverse range of functions, while the Y chromosome is comparatively gene-depleted, consisting of a variable amount of DNA that codes for less than 100 genes mainly involved in sex determination and fertility. One in particular, sex determining region on the Y chromosome (SRY), is considered to be the initiator of a cascade of events that leads to the development of the testis. The absence of SRY results in a different cascade of gene expression culminating in ovarian development. Restriction of the Y chromosome in males is a strategy for ensuring that key genes for testis induction and function such as SRY are kept separate from females. However, a consequence of this strategy is that genes located on the X chromosome are present in two copies in females and one in males. To overcome the potential unequal expression of genes resulting from unequal copy number of chromosomes, female mammals have developed an epigenetically regulated process of dosage compensation known as X- chromosome inactivation. In general terms, transcription of X-linked genes is restricted to a single, active X chromosome (Xa) and in females inhibited on the other inactive X chromosome (Xi).

The X-inactivation process is initiated early in embryogenesis by transcription of *XIST* from one of the two X chromosomes that is set to be inactivated (Xi) and subsequent coating of the same X chromosome by the untranslated *XIST* RNA (Brockdorff 2002, Heard 2004, Chang *et al*. 2006). The choice of which X chromosome becomes inactive appears to be under an imprinted control, where random X-inactivation occurs in the inner cell mass derivatives and preferential inactivation of the paternal X occurs in the trophoblast derivatives (Chang *et al*. 2006). Immediately after *XIST* RNA coating begins, the Xi undergoes various chromatin modifications such as loss of methylation on H3 lysine 4 (H3-K4), methylation of histone H3 lysine 9 (H3-K9) and methylation on H3 lysine 27 (H3-K27) and these changes lead to transcriptional silencing (Peters *et al*. 2002, Plath *et al*. 2003, Chadwick & Willard 2004) and late-replication of the Xi (Keohane *et al*. 1999). However, histone modification such as H3-K27 methylation is not sufficient for silencing of the X chromosome (Plath *et al*. 2003). Hence, the inactive state is synergistically maintained through other chromatin modifications such as hypoacetylation at histone H4, macroH2A recruitment and DNA methylation (Csankovszki *et al*. 2001). Nonetheless, the functional links between methylated DNA and histones on the X chromosome are extremely stable and are maintained throughout all subsequent cell divisions and life (Avner & Heard 2001).

Effects of in vitro culture on X-chromosome-linked mRNA expression

The chronology of initiation of X-inactivation has been investigated from several perspectives. Mouse, human and cattle embryos express *XIST* at the two-, four- and eight-cell stages, respectively. The shift from synchronous replication to asynchronous (late) replication of the X chromosomes in female bovine embryos and other species has been shown to occur after the initial expression of XIST (De La Fuente *et al*. 1999, Chang *et al*. 2006). By the morula stage, female bovine embryos exhibited a late replicating X chromosome in a proportion of their cells, which progressively increased as the embryo developed to the hatched blastocyst stage and beyond (De La Fuente *et al*. 1999). Determination of the relative expression levels of a small panel of X-linked genes in bovine male and female morulae and blastocysts showed that the process of dosage compensation or equalisation of expression of genes such as *PGK, G6PD* and *HPRT* was initiated or completed in the blastocyst stage embryos (Peippo *et al*. 2002, Wrenzycki *et al*. 2002). Thus, it would appear that X-inactivation is initiated and established as the embryo develops from early cleavage stages through to the blastocyst stage.

There is a growing body of evidence that suggests substantial developmental differences between embryos produced *in vivo* and those produced and cultured *in vitro* (IVP; Niemann & Wrenzycki 2000, Wrenzycki *et al*. 2005). One of the first observations on IVP embryos was that the rate of development to the blastocyst stage differed between males and females, with males developing more rapidly (Yadav *et al*. 1993) with the resulting sex ratios of embryos and offspring skewed in favour of males (Bousquet *et al*. 1999). Other studies

showed that the composition of the culture media affects the rate of development and the overall sex ratio of IVP embryos. Medium that is rich in glucose tended to yield a preponderance of male blastocysts on Day 7 after *in vitro* fertilisation when compared with media deficient in glucose (Bredbacka & Bredbacka 1996). Culture media supplemented with serum and factors from co-cultured somatic cells tended to have a preponderance of female embryos arrested at the morula stage (Gutierrez-Adan *et al*. 2000). It was suggested that there is a greater survival of male embryos in media supplemented with serum and glucose, which were both detrimental to female embryos (Gutierrez-Adan *et al*. 2001*a*,*b*). Furthermore, the exposure of IVP embryos to elevated temperature has been shown to cause a greater loss of female embryos (Edwards *et al*. 2001).

A possible cause for this developmental dimorphism is that *in vitro* techniques and/or culture conditions influence the expression genes on the X chromosome and the X-inactivation process. A recent study reported 384 genes that differed in expression levels between bovine embryos produced *in vivo* and *in vitro* when examined by microarray analysis (Corcoran *et al*. 2006). Overall, about 85% of these differentially expressed genes were downregulated in IVP embryos. Studies conducted on a selected subset of X-linked genes in bovine *in vivo* and IVP embryos revealed differences in the level of expression (Wrenzycki *et al*. 2002, Nino-Soto *et al*. 2007). However, it appears that with few exceptions, and in contrast to genes located on autosomes, the mRNA of X-linked genes was upregulated in IVP embryos compared with *in vivo* controls. When comparisons were made between male and female IVP embryos, the levels of expression of these X-linked genes were generally similar between the two sexes. However, no consistent trend was observed, rather the levels of gene expression varied among culture conditions and individual developmental stages (Nino-Soto *et al*. 2007). Thus, the cause of the sexual dimorphism in early embryonic development remains unexplained.

Effects of SCNT on X-chromosome-linked mRNA expression

It has been suggested that the faulty reprogramming and re-establishment of epigenetic regulators of development may explain, in part, the low survival rates of embryos and high abnormality rates among SCNT offspring. One of the epigenetically regulated developmental mechanisms potentially affected by faulty reprogramming is X-inactivation. Indeed, some SCNT embryos and offspring have been shown to exhibit aberrations in the X-chromosome inactivation. Although the inactive X of the donor cells can be successfully reactivated by the recipient cytoplast, there was heterogeneity within SCNT mouse blastocysts for X-inactivation, with cells showing zero, one or two inactive X chromosome(s) (Nolen *et al*. 2005). The kinetics of preferential paternal X inactivation differed significantly between normal and cloned mouse embryos, the latter showing an abnormal Xist expression pattern (Bao *et al*. 2005).

In bovine embryos, Xist RNA, the initiator of X-inactivation, was found in samples taken from pools of male SCNT blastocysts, but not male IVP or *in vivo* blastocysts (Nino-Soto *et al*. 2005). The pattern of X-inactivation in aborted bovine SCNT foetuses and dead newborn calves was found to be altered (Xue *et al*. 2002). Placental samples exhibited random X-inactivation as opposed to the non-random preferential paternal X-inactivation seen in normal controls and healthy SCNT calves. In addition, nine out of ten X-linked genes examined in various organs and tissues of the dead cloned calves were aberrantly expressed. The use of a donor cell line containing an X chromosome which causes preferential inactivation of the normal X chromosome resulted in aberrant inactivation patterns in the organs and tissues of stillborn and dead new-born calves which included random inactivation of the normal and abnormal X, and inactivation of both X chromosomes (King WA, Joudrey EM & Nino-Soto MI; unpublished observations). These combined data provide empirical evidence to suggest that faulty removal of existing epigenetic marks and subsequent reprogramming of the donor nucleus during SCNT leads to abnormal patterns of X-chromosome inactivation that is deleterious for development of female SCNT embryos. To remedy the faulty reprogramming of the X chromosomes after SCNT, DNA demethylating agents such as 5-aza-2′-deoxycytidine and *S*-adenosyl homocysteine have been used on donor cells to chemically reactivate the Xi prior to nuclear transfer. The treatments partially reactivated the Xi, as evidenced by the re-expression of silenced alleles and earlier chromosome replication (Haaf *et al*. 1988, Sasaki *et al*. 1992, Lee *et al*. 2004*b*). Both approaches have resulted in improvement in the rate of development of SCNT to the blastocyst stage (Enright *et al*. 2003, 2005, Shi *et al*. 2003, Jeon *et al*. 2006). Recently, healthy calves were born from cloned embryos derived from trichostatin (TSA)-treated donor cells (Tian unpublished observation) demonstrating the compatibility of the chemical treatment with normal full term development.

Telomere length and somatic cloning

Telomeres are the natural ends of linear chromosomes and play a crucial role in maintaining the integrity of the chromosomal DNA by preventing loss of terminal coding DNA sequences and preventing end-to-end chromosome fusion. Telomeres are composed of repetitive DNA elements and specific DNA proteins, which together form a nucleoprotein complex at the ends of eukaryotic chromosomes (Blackburn 2001). Although

the sequence of these terminal DNA structures varies between organisms, telomeres are generally composed of concatamers of short sequences of the form 5′-TTAGGG-3′. A specialised RNA-dependent DNA polymerase, the telomerase, is then required to maintain the natural length of telomeric DNA. This ribonucleoprotein enzyme is composed of two essential subunits: the telomerase RNA component and the telomerase reverse transcriptase component (Nakayama *et al.* 1998). Telomerase is critically involved in maintaining normal telomere length and is active in haematopoietic, cancer and germ cells and in early embryos at the blastocyst stage (Blasco *et al.* 1999). Changes in the telomere length are closely related to ageing and cancer (de Lange 2002). As a general rule, some loss of telomere length occurs with each cell division as a result of the incomplete replication of the lagging strand.

Telomeres of Dolly, the cloned sheep, which was derived from an adult mammary epithelial cell, were shorter when compared with age-matched, naturally bred counterparts and correlated with telomere length of the donor cells (Shiels *et al.* 1999). Subsequently, however, the vast majority of cloning studies reported that telomere length in cloned cattle, pigs, goats and mice are comparable with age-matched, naturally bred controls even when senescent donor cells were used for cloning (Jiang *et al.* 2004, Betts *et al.* 2005, Jeon *et al.* 2005, Schaetzlein & Rudolph 2005). The regulation of telomere length is to some extent related to the type of donor cells employed for cloning. The telomere length in cattle cloned from fibroblasts or muscle cells was similar to that of age-matched controls, while clones derived from epithelial cells did not have telomeres restored to normal length (Miyashita *et al.* 2002). A check point for the elongation of telomeres to their species determined length has been discovered at the morula to blastocyst transition in bovine and mouse embryos (Schaetzlein *et al.* 2004). The morula/blastocyst transition is a critical step in the preimplantation development leading to first differentiation into two cell lineages: the inner cell mass and the trophoblast, which coincides with dramatic changes in morphology and gene expression. Telomeres were at the level of the donor cells in SCNT morulae (Fig. 2), whereas at the blastocyst stage telomeres had been restored to normal length (Fig. 3). The telomere elongation process at this particular stage of embryogenesis is telomerase dependent since it was abrogated in telomerase deficient mice (Schaetzlein *et al.* 2004).

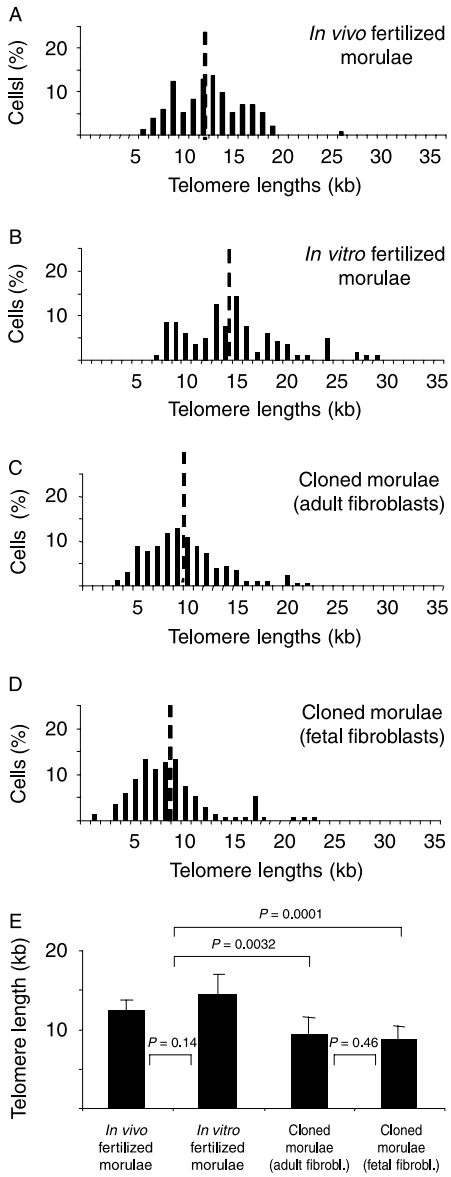

Figure 2 Telomere length of cloned and fertilised bovine morulae by Q-FISH. (A–D) Distribution of mean telomere length over all analysed nuclei of morulae (Day 6 of embryonic development) derived from (A) *in vivo* fertilisation ($n=234$), (B) *in vitro* fertilisation ($n=118$), (C) cloning from adult bovine fibroblasts ($n=203$), (D) cloning from foetal bovine fibroblasts ($n=134$). (E) Mean value of the mean telomere lengths determined for individual morulae derived by cloning from foetal fibroblasts (8.71 ± 1.81 kb, $n=8$), adult fibroblasts (9.47 ± 2.07 kb, $n=6$), from *in vivo* fertilisation (12.42 ± 1.37 kb, $n=7$) or *in vitro* fertilisation (14.36 ± 2.67 kb, $n=4$). The difference in telomere length between morulae derived from *in vivo* and *in vitro* fertilisation was not significant (from Schaetzlein *et al.* 2004).

Expression of imprinted genes in bovine conceptus development

The imprinted genes are a subset of genes in mammals whose expression is dependent on the parental inheritance of the allele. Imprints established during gametogenesis determine whether the imprinted gene expressed solely or predominantly from the maternal or paternal allele. Usually, imprinting is achieved through DNA methylation of imprinting control regions (ICRs) and then the appropriate allele is silenced throughout development and the entire life by covalent methylation of CpG dinucleotides by the *de novo* methyltransferase, Dnmt 3a

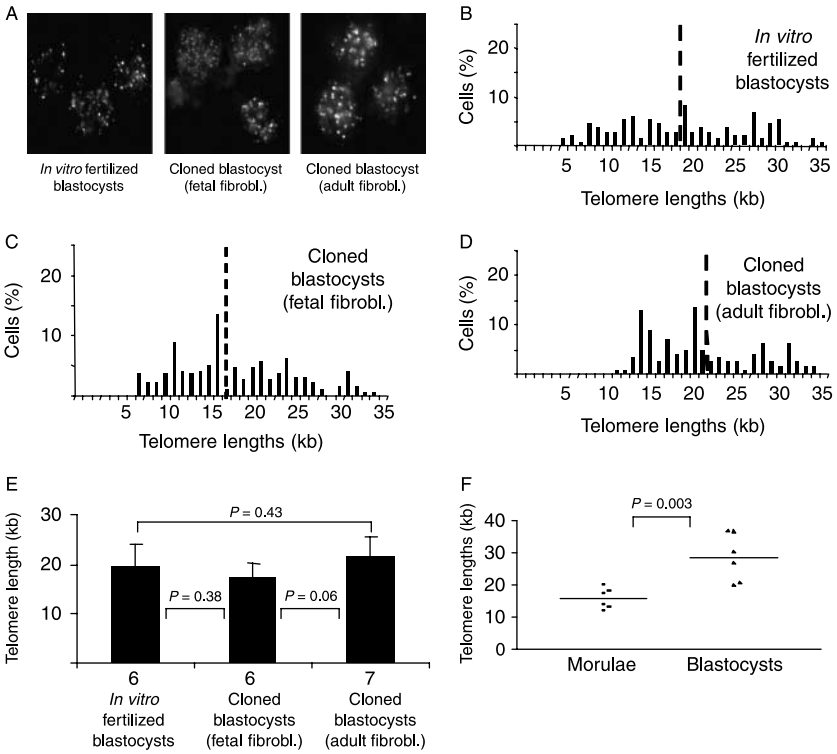

Figure 3 Telomere lengths of cloned and fertilised bovine blastocysts by Q-FISH. (A) Representative photographs of telomere spots in nuclei of blastocysts (Day 8 of development) derived from cloning of adult and foetal fibroblasts and *in vitro* fertilisation. Magnification bar: 20 μm. (B–D) Distribution of mean telomere length over all analysed nuclei of blastocysts derived from (B) *in vitro* fertilisation ($n=129$), (C) cloning from foetal bovine fibroblasts ($n=192$) and (D) cloning from adult bovine fibroblasts ($n=126$). (E) Mean value of the mean telomere lengths determined for individual blastocysts derived from *in vitro* fertilisation (21.67±3.92 kb, $n=5$) and from cloning using foetal fibroblasts (17.3±2.66 kb, $n=6$) and adult fibroblasts (19.53±4.6 kb, $n=6$). Note that telomeres were elongated in all groups when compared with the morula stage and that in contrast to the differences in telomere length at the morula stage there was no significant difference between the different groups of blastocysts. (F) A sequential analysis of the telomere length in embryos derived from a single *in vitro* fertilisation experiment showed telomere elongation at the morula–blastocyst transition with an average of 15.8±3.1 kb for morula stages ($n=6$) and 28.7±7.4 kb for blastocyst stages ($n=6$; from Schaetzlein *et al.* 2004).

(Constancia *et al.* 2004). A typical feature of imprinted genes is that they are found in clusters on the chromosomes and the ICRs exert a regional control of expression of genes from that cluster (Reik & Walter 2001). In the mouse no more than 50, in humans no more than 80 and in cattle no more than 10 imprinted genes have been identified (Dean *et al.* 2003, Constancia *et al.* 2004).

The role of imprinting in ruminant placenta formation and function

It is postulated that imprinting has evolved in placental mammals as a genetic mechanism to regulate the demand and provision of resources to the offspring during foetal and neonatal development. Usually, genes expressed from the paternally inherited allele tend to favour resource transfer from the mother to the foetus, whereas maternally expressed genes tend to restrict resource transfer to safeguard the mother's long-term well being (Constancia *et al.* 2004). Based on this parental conflict hypothesis (Wilkins & Haig 2003), genomic imprinting in the placenta is postulated to play a critical role in regulating growth of the foetus through nutrient partitioning between the mother and the foetus. The placenta is an organ that exists solely to secure maternal resources for foetal development.

The stage at which the placenta forms and the type of placentation is species-specific and is significantly different in ruminants compared with the mouse and human. Placentation in ruminants occurs at a much later stage in development than in the mouse or human and requires the extensive development and differentiation of the extra-embryonic membranes. At the initiation of placental formation, with the attachment of the conceptus to the endometrium, early foetal organogenesis has occurred and a beating heart and circulatory network has formed. Unlike rodents and the human, the allantois plays a pivotal role in the formation of the ruminant chorioallantoic placenta. The allantois contributes the blood vessels which eventually perfuse both the placenta and the foetus. These developmental differences may explain why the temporal and spatial expression of imprinted genes differs between ruminants and rodents or human. In sheep and cattle, some imprinted genes are expressed in the extra-embryonic membranes at a far earlier time point than others. *PEG1/Mest* and *Grb10* are both expressed from the elongation stage (Lee *et al.* unpublished observation), whereas significant levels of insulin-like growth factor 2 (*IGF2*) and *H19* are not detected until a few days later, at allantoic emergence and initial of trophoblast attachment to the endometrium (Lee *et al.* 2002). Although *IGF2*, *H19* and *PEG1/Mest* are all expressed in mesodermally derived cells, which are present at the elongation stage, the timing of the onset of expression for each gene is different. For *IGF2* and *H19*, it appears that the timing of 'implantation' rather than the stage of embryonic development determines the onset of enhancer-driven expression of these two genes in the extra-embryonic membranes (Lee *et al.* 2002). As *IGF2* and *H19* are reciprocally imprinted and have opposite effects on growth, the onset of expression coinciding with the time of implantation supports the parental conflict hypothesis

since the demand of the embryo on maternal resources is insignificant up to this stage. On the other hand, IGF2 receptor (*IGF2R*) is expressed from the pre-elongation stage, hence the *IGF2* receptor may be regulating the availability of maternally derived *IGF2* to the early embryo well before the conceptus produces its own *IGF2*. The timing of when each of these genes is expressed during peri-implantation development may depend on when the epigenetic modifications are established after early zygotic demethylation and on the temporal and spatial expression of transcription factors or those factors that interact with enhancers or regulatory elements of these imprinted genes.

Despite the key roles imprinted genes play in placental development across species, there is a difference in the conservation of imprinting in the placenta. Whether the species are monotocus or polytocus may determine the evolutionary conservation of placental gene imprinting, even when the type of placentation and the stage of implantation are similar, as with the human and the mouse (Monk *et al*. 2006). An example is the *KCNQ1* domain, where in the human placenta, many of the genes are expressed biallelically, from first trimester to term (Monk *et al*. 2006), whereas the majority are expressed exclusively from the maternal allele in the mouse placenta. The lack of imprinting of some of these genes in human placenta is independent of the methylation status of the CpG islands in the promoter and correlates more with allelic histone modifications.

The lack of conservation of imprinting extends to the *IGF2R* gene, which is an important regulator of both placental and foetal growth. It is expressed from the maternal allele in the mouse, cattle and sheep (Killian *et al*. 2001). In the human, this gene is polymorphically imprinted in the placenta, with only a minority of term placenta samples showing monoallelic maternal expression (Monk *et al*. 2006). Polymorphic imprinting was also reported for *SLC22A3*, another gene in the same region as the *IGF2R* gene. Thus, even within the same species, imprinted expression can vary in a temporal manner, depending on the tissues, and even between individuals. At present, there is not much known about genomic imprinting in the ruminant placenta.

Expression of IGF family of genes after SCNT

The many aberrant phenotypes described in SCNT cloned foetuses or offspring bear strong similarities to abnormalities associated with either deletion or mutations in imprinted genes or aberrant expression of these genes. The 'LOS' frequently associated with somatic cloning, is sometimes also observed, although in a less severe form, in offspring derived from IVP embryos cultured under certain conditions (Farin *et al*. 2006). Placental abnormalities are frequently encountered in SCNT pregnancies and even IVP pregnancies without obvious foetal overgrowth show greater variability in placental morphometry (Lee *et al*. 2004*a*). Thus, the exposure of the pre-implantation embryo to unfavourable conditions may precipitate changes in the expression of those imprinted genes that drive proliferation and growth and placental development appears to be particularly vulnerable to these perturbations. Overexpression of IGF2 in the mouse results in foetal and placental overgrowth and oversize of certain organs (Eggenschwiler *et al*. 1997), similar to those that are often reported to be affected in cloned foetuses. This puts *IGF2* as one of the prime candidate genes responsible for foetal overgrowth in bovine and ovine SCNT; however, increased expression of *IGF2* itself in ruminants has not been consistently correlated with foetal overgrowth after SCNT or IVP. The recent identification of an intragenic DMR within exon 10 of the bovine *IGF2* gene provides a useful tool to evaluate the methylation patterns of embryos derived *in vivo*, *in vitro* or by SCNT (Gebert *et al*. 2006). Changes in the expression of *Igf2R* have been reported for large offspring sheep (Young *et al*. 2001). In the mouse, the absence of *Igf2r* expression results in excessive foetal growth, failure of the placenta to cease growing in late gestation and perinatal lethality chiefly due to cardiac failure (Lau *et al*. 1994). Placental overgrowth and cardiovascular abnormalities are frequently encountered in abnormal SCNT foetuses but an association with altered IGF-II receptor levels has not been found. *IGFBP2* is another gene whose expression was affected in large ovine foetuses resulting from IVF and exposure of embryos to culture media containing serum (Young *et al*. 1999). Although not an imprinted gene, the protein binds IGF-II and therefore regulates its availability in a tissue-specific manner. Aberrant expression of other IGFBPs has also been reported to be associated with SCNT (Ravelich *et al*. 2004, Li *et al*. 2007*a*, 2007*b*). Together, present data demonstrate the importance of *IGF2* in controlling proliferation and differentiation during foetal development and the need to tightly regulate its availability.

Altered expression of *IGF2R* in oversized lambs was associated with a decrease in DNA methylation in the ICR (Young *et al*. 2001). In cattle, the equivalent of the ICR2 was found upstream of exon 3 and showed a high level of homology with ICR2 in sheep (Long & Cai 2007). This region was differentially methylated between sperm and blood cells and may be involved in imprinting of *IGF2R*. A comparison of the extent of methylation in 347 bp of the >2 kb ICR2 region between one control animal and five SCNT foetuses from two different cell lines suggested that the methylation levels tended to be lower in the SCNT samples (Long & Cai 2007). The effect of these methylation differences on the level of *IGF2R* expression is unknown although the data of Young *et al*. (2001) implied that there was an association with large offspring sheep. It will be difficult to prove whether these methylation changes are responsible in some way for the phenotypic abnormalities bovine SCNT foetuses.

Although it is widely hypothesised that the abnormalities associated with cloning by SCNT may be due to faulty expression of imprinted genes or loss of imprinting, this has not been proven conclusively. The expression of several candidate-imprinted genes in days 40 and 72 foetal and placental tissues was from the appropriate parental alleles, indicating that the allele-specific imprints of the donor cells were maintained after nuclear reprogramming (Dindot *et al*. 2004). This is perhaps not surprising, given that these SCNT embryos have successfully developed to the stage where placentation had been established and they are morphologically not that different from normal foetuses. As 'survivors' to this stage in development, they would be expected to be closer to 'normal' than the ones that failed much earlier on.

Conclusions and perspectives

It is evident from available literature that the effect of the SCNT cloning procedure or even extended *in vitro* culture of embryos on gene expression (imprinted or non-imprinted) and on DNA methylation in the resultant foetuses and offspring is highly variable. Given the variability in the phenotypes observed in clones with the same nuclear genetics, it is not surprising that a unifying molecular explanation for why SCNT cloning goes wrong has remained elusive.

Since the birth of Dolly, the first mammal cloned from an adult somatic cell, significant progress has been made in increasing the efficiency of somatic cloning. Up to now, 12 animal species have been cloned. While the majority of offspring derived from SCNT cloning are outwardly normal, cloning may be still associated with pathological side-effects summarised as LOS, which appear to be due to incomplete and/or faulty reprogramming of the donor nucleus genome by the oocyte's cytoplasm. Epigenetic reprogramming involving the appropriate sequential establishment of DNA methylation and histone modifications that ensure a well-orchestrated pattern of gene expression is essential for successful cloning. X-chromosome inactivation (for females) and telomere length restoration are post-additional zygotic epigenetic tasks that need to be properly performed for successful cloning. Identification of the specific factors present in the ooplasm, which are necessary for epigenetic reprogramming, will provide a better understanding of the underlying mechanisms and would improve cloning efficiency. Progress in the understanding of epigenetic gene regulation in development has been aided by the advent of SCNT, which has a promising application potential and is a valuable tool in basic research. SCNT has become an important technique for producing transgenic animals, re-establishment of endangered species and in human medicine for producing patient-specific ESC for use in autologous tissue replacement without immune rejection. The application of this technology in human medicine, however, requires the creation and destruction of a cloned human embryo, which is ethically controversial and unacceptable to people of various religious beliefs. This ethical controversy can be avoided by directly reprogramming somatic cells using the reprogramming properties of the oocyte cytoplasm to de-differentiate somatic cells in culture dishes without conducting SCNT. The direct reprogramming of somatic cells has the potential of generating a large number of patient-specific totipotent cells for therapeutic tissue replacement without the need to create and destroy human embryos.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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