Published in final edited form as:
Cytogenet Genome Res. 2004 ; 105(2-4): 285–291.

Nuclear reprogramming in mammalian somatic cell nuclear cloning

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Abstract

Nuclear cloning is still a developing technique used to create genetically identical animals by somatic cell nuclear transfer into unfertilized eggs. Despite an intensive effort in a number of laboratories, the success rate of obtaining viable offspring from this technique remains less than 5%. In the past few years many investigators reported the reprogramming of specific nuclear activities in cloned animals, such as genome-wide gene expression patterns, DNA methylation, genetic imprinting, histone modifications and telomere length regulation. The results highlight the tremendous difficulty the clones face to reprogram the original differentiation status of the donor nuclei. Nevertheless, nuclei prepared from terminally differentiated lymphocytes can overcome this barrier and produce apparently normal mice. Study of this striking nuclear reprogramming activity should significantly contribute to our understanding of cell differentiation in more physiological settings.

Introduction

Given the enormous complexity of the gene regulatory pathways, it is remarkable that the entire cell differentiation program can be completely erased and properly re-established in somatic cell nuclear cloning (Hochedlinger and Jaenisch, 2002b; Oback and Wells, 2002). Nuclei taken from terminally differentiated B cells can produce the entire body of embryos with apparently normal functions as long as the extra-embryonic tissues are supplied externally by the tetraploid blastocysts (Hochedlinger and Jaenisch, 2002a). Simple nuclear injection was insufficient for the B cell nuclei to acquire pluripotency and the clones had to pass through ES cells to produce live pups in this experiment; nevertheless, this remarkable finding indicates that differentiated nuclei can be de-differentiated in the oocyte and embryonic environment. Nuclear cloning is arguably one of the most powerful experimental systems to study the reprogramming of cell differentiation. In *Xenopus* cloning, all active genes in the donor nuclei are shut off soon after nuclear transfer is completed. Several hours after nuclear transfer, embryonic nuclei start to express development specific genes that follow the normal time course of the zygotic gene activation (Chan and Gurdon, 1996; Byrne et al., 2002). One can argue that this genetic reprogramming is simply due to dilution of the donor nuclear components by the proteins and RNA stored in the large frog eggs. However, that argument is not convincing in the case of mouse cloning, where reprogrammed gene expression initiates as early as the 2-cell stage in tiny embryos. Although the success rate of animal cloning is still extremely low (Table 1), extensive reprogramming of differentiation at the cellular level is accomplished in aborted embryos that contain well differentiated tissues. Since such a drastic and rapid nuclear reprogramming is rare in living cells, nuclear cloning will provide a unique window to dissect the cell differentiation mechanisms.

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The cloning of Dolly the sheep in 1997 triggered a wide interest in mammalian cloning (Wilmut et al., 1997) followed by a number of mammalian clones created from adult cell nuclei as shown in Table 1. The recent progress in mammalian cloning needs to be interpreted in the context of the long cloning history initiated by Briggs and King, who created swimming frog tadpoles by injecting blastomere nuclei into unfertilized eggs in 1952 (Briggs and King, 1952). In the pre-Dolly era, key concepts, such as the importance of cell cycle compatibility between the donor nuclei and host eggs, and the progressive decrease of cloning efficiency related to the donor differentiation stage, were already established (Gurdon, 1986; Sun and Moor, 1995). One factor that has accelerated the cloning research is the successful mouse cloning by Wakayama and colleagues using adult cumulus cell nuclei (Wakayama et al., 1998). Until then, the blastocyst was believed to be the last stage compatible as a nuclear cloning donor (Solter, 2000). Supported by a wealth of background information, mouse cloning enabled us to investigate reprogramming of genetic imprinting, reactivation of the inactive X chromosome, and potential problems of ES cells as the source of the donor nuclei, none of which were possible with other species (see below for references).

In addition to low birth rate, live cloned animals demonstrate a variety of pathological conditions such as respiratory failure, placental dysfunctions and large offspring syndrome (Young et al., 1998; Rhind et al., 2003). It is usually difficult to trace the origin of these ailments to a few responsible genes. Probably, they reflect the cumulative effects of many faulty gene expressions. Because these abnormalities were not passed on to the offspring of the cloned mice, these phenotypes represent aberrant gene expression by deficient epigenetic reprogramming rather than genetic changes in cloned animals (Tamashiro et al., 2002). Even though nuclear reprogramming is a complicated process, by focusing on a certain aspect of the nuclear events, it is possible to dissect and understand the basic science behind the reprogramming as demonstrated by our recent finding of the nucleolar disassembly in egg cytoplasm (Gonda et al., 2003).

In this review, we will discuss the reprogramming of genome-wide gene expression, DNA methylation, histone modifications and telomere length regulation that occur during nuclear cloning. Reflecting the recent wide attention to the cloning field, numerous insightful reviews are available on various aspects of nuclear cloning. The readers are recommended to refer to the following papers: Campell (1999), Gurdon (1999), Wakayama and Yanagimachi (1999), Kikyo and Wolffe (2000), Hochedlinger and Jaenisch (2002b), Oback and Wells (2002), Mullins et al. (2003) for a more general account of cloning, McLaren (2000), Solter (2000), Gurdon and Byrne (2003) for the historical background, Jaenisch et al. (2002), Dean et al. (2003) for epigenetic reprogramming and Wade and Kikyo (2002) for biochemistry of the nuclear reprogramming.

Abnormal gene expression in cloned animals

Several groups have compared gene expression patterns in clones and control animals as summarized in Table 2. By analyzing expression of eight developmentally important genes in cloned blastocysts, Wrenzycki and colleagues reported that several genes were properly activated in the blastocysts, but with a marked difference in the gene expression levels (Wrenzycki et al., 2001). These differences were found to be dependent on parameters in the nuclear transfer procedure, including the activation protocol, the cell cycle of the donor cells and the passage number of the donor cells. Daniels and colleagues also reported a similar finding based on the study of a different set of genes specific to early embryonic development (Daniels et al., 2000,2001).

To understand the genome-wide difference in the gene expression patterns between cloned mice and fertilization-derived controls, a DNA microarray was employed using RNA isolated

from placentas and livers of these mice (Humpherys et al., 2002). The result showed that less than 3% of over 12,000 genes were expressed abnormally in the clone's placentas. Placentas tend to overgrow in clones, but there was no clear relationship between the additional growth and the aberrantly expressed genes. The livers of the clones showed a less conspicuous abnormality in gene expression than placentas, which may occur as liver is a more homogeneous tissue with smaller number of differentiated cell types than placenta. It is impressive that more than 97% of the genes could be properly silenced or activated in the cloned embryos in this comprehensive genome-wide analysis. However, it is important to note that this study examined RNA isolated from a whole tissue; and by doing so, an irregularity of the gene expression in each cell may have been averaged (see below for improper spatial distribution of Oct4 as an example).

The transcription factor Oct4 is essential to maintain pluripotency of early mouse blastomeres (Pesce and Scholer, 2001). *Oct4* is exclusively expressed in germ cells and early embryonic cells; therefore, it must be reactivated soon after nuclear transfer in the somatic cell clones. Indeed, more than 80% of the cumulus cell clones reactivated *Oct4* at the correct stage, but 54.7% of the clones showed aberrantly high level of the *Oct4* transcript in the trophectoderm at the blastocyst stage when *Oct4* expression is normally limited to the inner cell mass (Boiani et al., 2002). Recently, it was reported that *Oct4* expression could be specifically reactivated in mouse thymocyte nuclei and human lymphocyte nuclei injected into *Xenopus* oocytes, suggesting that the regulatory mechanisms for this pluripotency-specific gene are probably evolutionarily conserved (Byrne et al., 2003). Another pluripotency gene *Nanog* is a newly discovered homeoprotein specifically expressed in morulae, inner cell mass and ES cells (Chambers et al., 2003; Mitsui et al., 2003). Nanog is required to maintain these cells pluripotent, independently of the LIF/Stat3 pathway used by the Oct4 signaling system. It remains to be examined if *Nanog* demonstrates correct spatial and temporal profiles of reactivation in cloned embryos.

Reprogramming of DNA methylation and imprinting

DNA methylation of cytosine at the CpG dinucleotides plays vital roles in the regulation of gene expression in mammalian development (Bird, 2002; Li, 2002). DNA methylation suppresses gene expression by recruiting methyl-CpG binding proteins, such as MeCP2, MBD1, MBD2 and MBD3, as well as associated histone deacetylases, co-repressor proteins and chromatin remodeling machineries to the promoter of specific genes. At least three DNA methyltransferases are involved in the methylation of new CpG sites and maintenance of the already methylated CpG during DNA replication. Ubiquitously expressed DNMT1 functions primarily as a maintenance methylase that methylates CpG sites on the newly synthesized DNA strand copying the existing methylation pattern on the template DNA strand. Developmentally regulated DNMT3a and DNMT3b are responsible for methylation of new CpG sites to establish de novo CpG methylation patterns, especially in early development and germ cell development.

The DNA methylation pattern shows global changes during early mouse development (Dean et al., 2003). Upon fertilization a majority of the sperm-derived genomic DNA is rapidly demethylated before the onset of DNA replication by an uncharacterized active mechanism (Mayer et al., 2000; Santos et al., 2002). In contrast, oocyte-derived DNA is passively demethylated only after DNA replication initiates, by the nuclear exclusion of DNMT1. The global level of DNA methylation remains at the lowest level in the morula and blastocyst stages until implantation, when sudden genome-wide de novo methylation occurs by DNMT3a and DNMT3b. The genome-wide demethylation and remethylation in early embryos seems to be conserved across species as observed in cow, rat and pig, although their timing with respect to developmental stages is slightly different (Dean et al., 2001). Successfully cloned embryos

have to follow these methylation dynamics to erase the tissue-specific DNA methylation pattern and establish a new embryo-specific DNA methylation pattern on numerous genes.

A majority of the cloned bovine embryos show a gross abnormality in the genome-wide DNA methylation level and DNA methylation patterns on various repetitive sequences when compared with fertilization-derived controls (Table 3). The DNA methylation level in clones can be higher or lower than that in the control embryos depending on the donor cell types, target DNA sequences, examined embryonic stages and detection methods. The abnormality of the DNA methylation level is also substantially variable among individual clones (Kang et al., 2001a) and extremely abnormal embryos may have died before the analysis was done. Indeed, DNA methylation was undetectable in six out of nine spontaneously aborted bovine clones, but the methylation level was normal in the clones that survived to adulthood (Cezar et al., 2003).

Bovine somatic nuclei are resistant to the erasure of DNA methylation in early embryogenesis described above and the clones have a tendency to preserve the DNA methylation patterns inherited from the donor cells (Bourc'his et al., 2001; Dean et al., 2001). Re-establishment of DNA methylation was also potentially deregulated by precocious de novo methylation in clones (Dean et al., 2001). This abnormal methylation transition in cloned embryos could be due to the specific features of the somatic chromatin structure and/or defective regulation of DNMTs. For example, cloned mouse embryos expressed the somatic form of DNMT1 at abnormally high level and showed defective nucleo-cytoplasmic translocation of the oocyte form of DNMT1 (Chung et al., 2003). Culture conditions of the cloned embryos are also known to affect DNA methylation as shown by loss of methylation in the regulatory CpG site of the *H19* gene depending on the culture medium of the embryos (Doherty et al., 2000).

DNA methylation of imprinted genes is established during germ cell development and is protected from the genome-wide demethylation and re-methylation in early development by an unknown mechanism (Li, 2002). It is intriguing to understand whether methylation imprinting in the donor somatic nuclei is protected from the global changes of DNA methylation in the early embryos as effectively as that in the fertilized nuclei. While Inoue and colleagues found normal allele-specific expression of seven imprinted genes in mouse embryos obtained from Sertoli cells (Inoue et al., 2002), two other groups reported grossly disrupted imprinting in cumulus cell clones (Humpherys et al., 2002;Mann et al., 2003). This abnormality in the imprinting status may suggest susceptibility of the methylation imprinting in the somatic nuclei to the global methylation changes during early embryogenesis. Epigenetic markers for the inactive X chromosome can also be erased and re-established on either X chromosome in cloning (Eggan et al., 2000) with the exception of some X-linked genes (Xue et al., 2002). ES cell-derived mouse clones show a striking variation in the DNA methylation pattern and imprinted gene expression, perhaps reflecting the instability of DNA methylation during the ES cell culture. In spite of this, some ES cell-derived clones developed to term implying that the epigenetic noise paused by aberrant DNA methylation and imprinting can be compensated by other mechanisms (Humpherys et al., 2001;Jaenisch et al., 2002). This notion is consistent with the routine success in producing ES cell chimeras in transgenic experiments.

Histone modifications in cloned animals

Global release and uptake of linker histone H1 is another challenge for the donor nuclei during the nuclear reprogramming. The histone H1 exists at a very low level in mature mouse oocytes and gradually becomes abundant around the 4-cell stage (Clarke et al., 1997; Adenot et al., 2000). Following this temporal profile, blastomere nuclei lose histone H1 upon injection into oocytes and reacquire histone H1 during the subsequent development (Bordignon et al., 2001). This DNA replication-independent transition of the histone H1 level was also observed

in bovine clones (Bordignon et al., 1999). In *Xenopus* somatic nuclei incubated in egg extract, the molecular chaperone nucleoplasmin is responsible for the exchange of the somatic linker histone with the egg type linker histone B4 (Dimitrov and Wolffe, 1996). It is likely that mammalian nucleoplasmin (Burns et al., 2003) is involved in the loss of histone H1 from the donor nuclei, although its physiological meaning is unknown.

Alteration of histone modifications is also an important aspect of chromatin remodeling in cloning. Histones receive a number of covalent modifications including acetylation, methylation, phosphorylation, ubiquitination and ADP-ribosylation at the amino termini protruding from the chromatin core. A specific combination of these histone modifications on a given gene provides a recognition site for interacting molecules and thus contributes to regulating the gene activity (histone code hypothesis) (Strahl and Allis, 2000; Jenuwein and Allis, 2001). Bovine oocytes and early embryos express several histone acetylases and deacetylases with some variability in the transcript levels depending on the developmental stages (McGraw et al., 2003). In mouse oocytes, histone H3 and H4 are globally deacetylated on several lysines at the metaphase II of the second meiosis, which was reproduced in somatic nuclei transferred into the same stage of oocytes (Kim et al., 2003). This genome-wide decrease of histone acetylation may contribute to the erasure of the previous gene expression patterns specific to the donor cell differentiation.

Methylation on histone H3 lysine 9 (H3-K9) is usually associated with gene inactivation and acetylation on H3-K9 is linked with gene activation (Fischle et al., 2003). Fertilized control mouse embryos become hypoacetylated on H3-K9 at the 4-cell stage and are gradually hyperacetylated after the 8-cell stage (Santos et al., 2003). In contrast, cloned embryos retain hyperacetylation on H3-K9 throughout these stages. At the blastocyst stage, the cloned embryos show hypermethylation on H3-K9 in the trophectoderm compared with the controls. The detailed enzymology responsible for these transitions of histone acetylation and methylation in early embryos is not yet available, but these aberrant histone modifications should almost certainly affect expression of a number of genes.

Telomere restoration in clones

Telomeres are DNA-protein complexes at the ends of eukaryotic chromosomes essential for chromosomal integrity and normal cell growth (McEachern et al., 2000; Blasco, 2002). Vertebrate telomere DNA is composed of tandem repeats of the sequence TTAGGG and a 3′-overhang that forms a t-loop with the double-stranded DNA protecting the 3′ end of telomeres. Because conventional DNA polymerases cannot replicate the lagging strand at the 5′ end, telomeric DNA is progressively lost with each round of cell division, 50–150 base pairs per cell division in human cells, unless the cells express the ribonucleoprotein complex telomerase. The enzymatic core of telomerase consists of the reverse transcriptase TERT (telomerase reverse transcriptase) and its template RNA TR (telomerase RNA). While TR is ubiquitously expressed, TERT expression is limited to germ cells and stem cells in the normal human body. When telomeres become shorter than the critical threshold in somatic cells due to a lack of TERT, p53- and Rb-regulated DNA damage responses trigger growth arrest (replicative senescence) (Maser and DePinho, 2002). If TP53 and RB are inactivated, these cells can bypass this growth arrest, but the cells will eventually die because of massive chromosome end fusions triggered by the cumulative telomere loss (crisis).

The telomerase activity is subjected to multiple levels of regulatory mechanisms (Blasco, 2002; Kyo and Inoue, 2002). Transcriptional regulation of TERT by c-Myc, Max and Sp1 is one of the most critical control mechanisms. Alternative splicing of TERT also regulates the telomerase activity by producing more than six forms of transcripts including truncated forms and dominant-negative forms of TERT. Subcellular localization of TERT adds another layer

of regulation to the telomerase activity. For instance, telomerase activity in activated T cells is not dependent on the total TERT protein level, rather the activity is defined by nuclear translocation of TERT accompanied by phosphorylation (Liu et al., 2001). While transfected TERT is localized in the nucleoli in normal cells and released into nucleoplasm only in the S phase, it is always widely distributed in the cancer cell nucleoplasm (Wong et al., 2002). In addition to these direct modifications of TERT, a number of telomere binding proteins play essential roles in modulating the telomere length (Blasco, 2002). For example, TRF1 and TRF2 bind to the TTAGGG repeats and negatively regulate the telomere length through interactions with other proteins on telomeric DNA (de Lange, 2002).

Since telomere length is tightly linked with cellular senescence, it is intriguing to examine whether shortened telomeres in somatic nuclei can be restored in nuclear cloning as one aspect of re-juvenilization at the cellular level. A comparison of telomere length among donors, clones and age-matched controls was reported by several groups (Table 4). These results indicate that shortened adult cell telomeres can be restored during early development of cloned animals but the degree of telomere elongation is quite variable. Even in a single study, there was significant variation in telomere length among individual clones and among different tissues isolated from a single clone (Betts et al., 2001;Miyashita et al., 2002), underscoring the complexity and difficulty of telomere length control in clones. If aborted embryos, which potentially harbor telomeres with extremely abnormal length, are included, the efficiency of telomere restoration may be even less than what is being reported. It is not known whether the telomere elongation in clones is a secondary effect of non-specifically activated telomerase or a regulated telomere restoration reflecting the cell's effort to compensate for defective telomeres.

To understand the functional consequences of telomere restoration, two groups examined whether the replicative lifespan of senescent cells could be elongated by nuclear cloning. Lanza and colleagues found that clone-derived bovine fibroblast cells, which contained fully restored telomeres comparable to the age-matched controls, showed longer proliferative lifespan than the senescent donor fibroblast cells (Lanza et al., 2000). However, when Clark and colleagues tested cloned sheep fibroblast cells that harboured partially restored telomeres, the proliferative lifespan of the cells was not extended (Clark et al., 2003). Thus, it remains to be examined to what extent the restored telomeres can influence the proliferative lifespan of these cells. It is also still unclear whether the resetting of proliferative lifespan of isolated cells has something to do with the lifespan of cloned animals. When whole embryonic extract was tested, the temporal profile of the telomerase activity during development was similar in cloned bovine embryos and fertilized embryos (Xu and Yang, 2001). Because a number of factors contribute to define the telomerase activity and telomere length in vivo as described above, the next step will be to examine individual accessory factors of the telomerase complex and subnuclear localization of TERT in cloned animals.

Conclusion

To be successful, clones have to erase the previous differentiation memory and establish embryo-specific gene expression profiles within a short period of time. This is accomplished through large-scale reorganization of chromatin structure and functions, as exemplified by genome-wide DNA methylation and re-methylation, adjustment of expression level for imprinted genes, reactivation of inactive X chromosome genes, global changes of histone modifications and exchange of linker histones. They also have to repair shortened telomeres as an essential step to restore replicative competence. It is almost inconceivable that differentiated cell nuclei can achieve this daunting task, albeit with extremely low efficiency. Some of the successful clones may have been derived from nuclei of stem cells embedded within somatic tissues, requiring less extensive nuclear reprogramming. The rare occurrence of stem cells in these tissues may explain the low efficiency of cloning and this possibility

needs to be carefully examined using strict criteria for the donor cell differentiation. If creation of perfectly normal animals is the goal of cloning, it may be quite difficult, if not impossible (Rhind et al., 2003), but if the goal of the cloning study is to understand how the embryonic environment is trying to reprogram the differentiated nuclei, then the study will be a precious source of insight into the normal cell differentiation mechanisms.

Acknowledgements

We thank Justin Wudel for his critical comments on the manuscript.

This work is supported by the NIH (GM068027), the American Cancer Society (IRG58-001-43-IRG40), the Minnesota Medical Foundation, and the Graduate School of the University of Minnesota.

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Table 1

List of cloned mammals

Reference	Species	Donor Cells	Cloning Efficiency (Live birth/Manipulated oocytes) (%)
Wilmut et al., 1997	Sheep	Mammary epithelial cells	1/277 (0.4)
Wakayama et al., 1998	Mouse	Cumulus cells	41/2468 (1.7)
Kato et al., 1998	Bovine	Cumulus cells	5/99 (5.0)
		Oviductal cells	3/150 (2.0)
Cibelli et al., 1998	Bovine	Fetal fibroblasts	4/276 (1.4)
Baguisi et al., 1999	Goat	Fetal fibroblasts	3/285 (1.1)
Onishi et al., 2000	Pig	Fetal fibroblasts	1/210 (0.5)
Polejaeva et al., 2000	Pig	Adult granulosa cells	5/183 (2.7)
Betthauser et al., 2000	Pig	Fetal cells	Not available
Chesne et al., 2002	Rabbit	Cumulus cells	6/1852 (0.3)
Shin et al., 2002	Cat	Cumulus cells	Not available
Woods et al., 2003	Mule	Fetal fiborblasts	1/334 (0.3)
Galli et al., 2003	Horse	Fibroblasts	1/841 (0.1)
Roh et al., 2003	Rat	2-cell stage embryos	6/139 (4.3)
Zhou et al., 2003	Rat	Fibroblasts	Not available

Table 2

Aberrant gene expression patterns in cloned mammals

Reference	Donor	Cells	Number of the tissue	Aberrantly expressed genes[a]/total number of genes examined	Detection method
Daniels et al., 2000	Bovine	Granulosa cells	2-cell embryo to blastocyst (4 −10)	3/7 (42.9 %)	RT-PCR
Daniels et al., 2001	Bovine	Fetal epithelial cells	Blastocyst (62)	1/4 (25 %)	RT-PCR
Wrenzycki et al., 2001	Bovine	Follicular cell line	Blastocyst	1/8 (12.5%) to 3/8 (37.5%)	RT-PCR
Humpherys et al., 2002	Mouse	ES cells	Placenta (12) Liver (13)	221/12,654 (1.7%) 26/12,654 (0.2%)	Microarray
		Cumulus cells	Placenta (14)	286/12,654 (2.3%)	
Suemizu et al., 2003	Mouse	ES cells	Placenta (2)	Clone 1: 1,807/15,247 (11.9%) Clone 2: 1,964/15,247 (12.9%)	Microarray

[a]Gene expression is defined as aberrant in the microassay analysis when the gene expression level in the cloned mice is 2-fold higher or lower than in the controls derived from fertilization.

Table 3

DNA methylation status in cloned mammals

Reference	Species	Donor cells	DNA sequence	Degree of DNA methylation[a]	Detection method
Kang et al., 2001b	Pig	Fetal fibroblasts	Centromeric satellite PRE-1 (euchromatic repeat)	Embryos: C=A Embryos C<A	Bisulfite
Dean et al., 2001	Bovine	Fetal fibroblasts	Whole genome	Embryos: C>A	Immunofluorescence[b]
Bourc'his et al., 2001	Bovine	Adult fibroblasts	Whole genome	Embryos: C>A	Immunofluorescence[b]
Kang et al., 2001a	Bovine	Fetal fibroblasts	Satellite I, Satellite II, SINE and 18S rRNA	In all sequences, Embryos D=C>A	Bisulfite
Kang et al., 2002	Bovine	Fetal fibroblasts	Tissue specific promoters	Embryos: C=A	Bisulfite
Cezar et al., 2003	Bovine	Genital ridge cells Fetal skin cells Adult skin cells Fetal and adult cumulus cells	Whole genome	Summary of 4 donor cell types Aborted fetuses: C<<A Live fetuses: C<A Adults: C=A	Reverse phase HPLC, restriction enzyme

[a] A: age-matched controls derived from fertilization, C: cloned animals and D: the donor animals.

[b] Immunofluorescence with anti-5-methylcytosine antibody.

Table 4

Telomere length in cloned mammals

Reference	Species	Donor cells	Number of cloned animals	Telomere length in clones[a]	Telomere activity in clones
Shiels et al., 1999	Sheep	Mammary epithelial cells	3	D=C<A	Not tested
Wakayama et al., 2000	Mouse	Cumulus cells	35	D<C	Not tested
Tian et al., 2000	Bovine	Adult fibroblasts and cumulus cells	10	D<C=A	Detected
Kato et al., 1998	Bovine	Adult ear cells	3	Ear cells: D<C White blood cells: D>C=A	
Lanza et al., 2000	Bovine	Senescent fibroblasts	6	D<C>A	Detected
Betts et al., 2001	Bovine	Adult fibroblasts Fetal fibroblasts Granulosa cells	Total 21	D<C=A	Detected
Miyashita et al., 2002	Bovine	Oviductal epithelial cells	9	D>C<A	Not tested
		Mammary epithelial cells	1	D>C<A	Not tested
		Muscle cells	2	D=C<A	Not tested
		Skin fibroblasts	2	D=C<A	Not tested
		Blastomere cells	6	C>A	Not tested
Clark et al., 2003	Sheep	Fetal fibroblasts	2	D<C<A	Not tested

[a] A: age-matched control animals derived from fertilization, C: cloned animals and D: donor animals.