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# Will cloned animals suffer premature aging – The story at the end of clones' chromosomes

Jie Xu*[1] and Xiangzhong Yang[2]

Address: [1]Evergen Biotechnologies, Inc. 1392 Storrs Road, Unit 4213, University of Connecticut Incubator Program, Storrs, CT 06269, USA and [2]Department of Animal Sciences and the Center for Regenerative Biology, 1392 Storrs Road, University of Connecticut, Storrs, CT 06269, USA

Email: Jie Xu* - jxu@evergen.com; Xiangzhong Yang - jyang@canr.uconn.edu

* Corresponding author

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## Introduction

Dolly the sheep, the 1st cloned mammal in the world [1], was put down on February 14th of this year. She was suffering from a virus that caused a tumor in the lung [2]. This has triggered a new round of debate on cloning, particularly on the aging problems of cloned animals. Scientists have long worried that cloned animals might inherit its age from its cell donor, thus being born old and die early. In this review we focus on – telomeres – the units which are at the end of all chromosomes and discuss how telomeres are related to the ageing problems of clones.

## The telomeres

The ends of eukaryotic chromosomes are capped with copies of a hexamer repeat sequence (5'TTAGGG3' in human) and associated proteins [3]. These structures are known as telomeres. Telomeres stabilize the ends of chromosomes during replication [4]. Conventional DNA polymerases replicate DNA only in the 5' to 3' direction and cannot initiate de novo synthesis of a DNA chain [5]. The DNA polymerases that replicate eukaryotic chromosomes use an 8- to 12-base stretch of RNA to prime DNA synthesis. As a consequence, after DNA replication, one end of a linear chromosome will be replicated to the very end, whereas the other end will have a short 8- to 12-base gap generated by removal of the RNA primer. Because a conventional DNA polymerase can not fill in this 5' gap, in every subsequent cell division a given DNA end will be incompletely replicated. In yeast, the end of a linear chromosome will shorten by an average of 4 to 6 bases per cell division unless telomeres act as substrates for an alternative replication mechanism (for example, telomerase is able to add telomeric repeats onto the ends of chromosomes) [3]. In human fibroblast cell culture, telomere length decreases at a rate of 48 ± 21 base pairs per population doubling [6,7]. In vivo, Hastie et al [8] studied human lymphocytes and found that the rate of telomere loss is about 33 base pairs per year. Many scientists propose the telomere as a "mitotic clock" [8-10], whose length correlates with the number of cell divisions and indicates the molecular age of the cell (Fig. 1).

## The telomere length of the clones' chromosomes

In the nuclear transfer process, a somatic cell, with shortened telomere length, is transferred into an enucleated oocyte and activated to start embryo development (Fig. 1.). A question immediately comes to mind, are the shortened telomeres of donor cells restored to full length in animals produced by nuclear transfer? It was a concern that if indeed shortened telomeres were inherited by cloned animals, that these animals may inherit the shortened life span of the adult donor rather than that of their age-matched controls produced through traditional reproduction. Dolly's death, if natural and premature, might be the first indication that these concerns were valid.

As early as 1999, Shiels et al. published their report on the telomere lengths of Dolly and two other clones [11]. At two years of age, the clones were phenotypically healthy and similar to control animals [11]. But inside the cells, researchers found Dolly's telomeres shorter than those of control animals of her age (19 kb vs. 23 kb). They discovered the length of her telomeres was actually comparable to that found in the mammary tissues of the 6-year-old

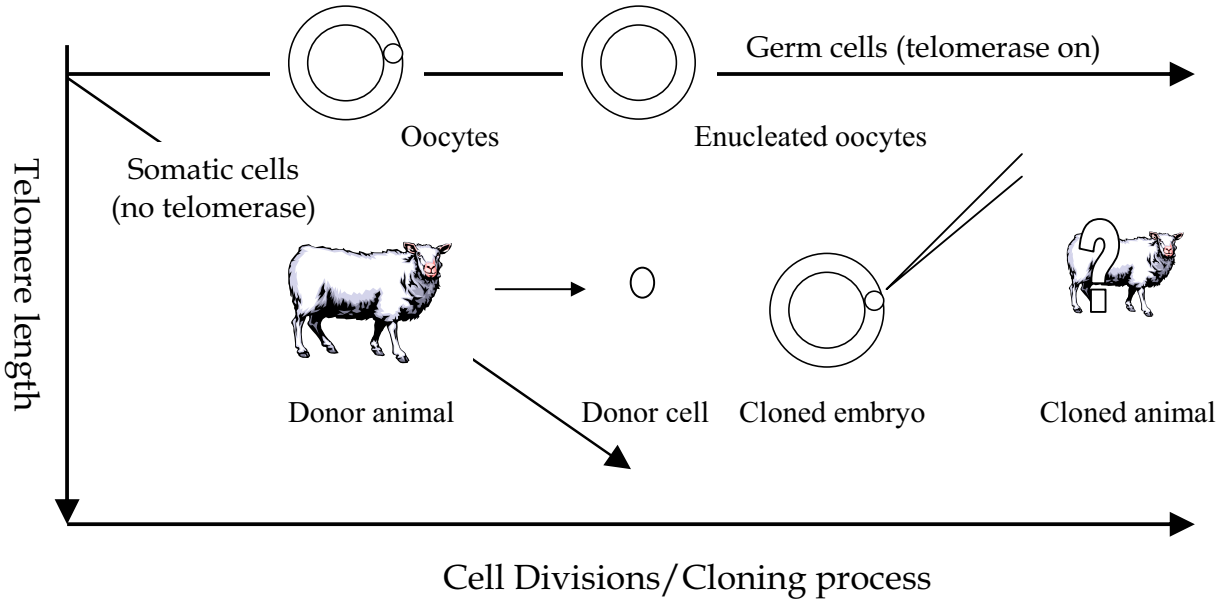


**Figure 1**
Telomeres shorten in somatic cells with each cell division.

donor animal. Another clone that was produced using a donor cell from a 9 day old embryo showed shortened telomere length (20 kb vs. 23 kb) as well. Only the third clone, which was produced by using fetal tissue to produce a donor cell, appeared to have telomeres non-distinguishable in length from those of controls. The authors attributed this exception to the minimal culture duration of these cells [11], rather than the cell type difference. They believed that full restoration of telomere length did not occur in these clones largely because they were produced without germ line involvement. Only in germ line cells, or gametes, but not in most somatic cells, is telomerase activity high and telomere length maintained [3]. In this way telomere length is fully transmitted from generation to generation (for example, a child is born with telomeres the similar length as those his father or grand father were born with, although a father's would be considerably shorter than his son's) while most somatic cells, body cells not involved in reproduction, erode the ends of their chromosomes with each cell division. The results corresponded to the reasoning quite well: the somatic donor cell does not have telomerase activity – telomere length can not be restored – clones should be born with telomere length similar to that of their donor cells.

Although results equivalent to Dolly's were found by Kato et. al. [12], when they observed many characteristics of ageing in male clones derived from a 10 year old bull (numerous wrinkles in the skin, thick bone structure and rough hairs) as well as shortened telomeres, many other research teams soon discovered that telomere restoration can take place in cloned animals. In other words, the gametes – oocyte/sperm are sufficient, but not necessary, for telomeres of normal length in newborns because germ line cells are not the only cells that possess telomerase activity or other factors with the ability to restore telomeres

Researchers at Advanced Cell Technology, in Massachusetts, cultured the cells to extreme: donor cells were in vitro cultured for prolonged periods of time, until 95% of their life span was completed [13]. Six healthy cloned calves were produced from these senescent donor somatic cells, and amazingly, these clones had longer telomeres (20.1 kb) than control animals (18.3 kb); the senescent cells that were used as DNA donors had an average of 15.2 kb [13]. The fact that cloning could extend telomere length drew media attention and swung the pendulum of popular, and scientific, opinion from pessimism to optimism: if indeed, telomere length is the indicator of an

**Table 1: Telomere lengths 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 | | [11] |
| Sheep | Fetal Female | Embryonic | Very short culture | | 23.9 | 21.19 | Similar | Blood | | [11] |
| Cattle | Fetal Female | fibroblast | Extreme Long culture | 15.2 | 18.3 | 20.1 | Longer | Blood cells | High in Blastocyst | [13] |
| Cattle | Adult Female | Fibroblast cumulus | | 12.4 | 14.7 | 15.4 | Similar | Fibroblast | High in blastocyst | [15] |
| Cattle | Adult Female | fibroblast | | 13.68 | 20.9 (fetal)<br>20.5 (calf) | 17.95 (fetal)<br>15.3 (calf) | Similar<br>Similar | Fibroblast | High in blastocyst | [16] |
| | Adult Male | | | 17.85 | 20.9 (fetal)<br>20.5 (calf) | 22.74 (fetal)<br>21.33 (calf) | Similar | Fibroblast | High in blastocyst | [16] |
| Cattle | Adult Male | Ear | | | | | Longer | Ear | | [12] |
| | Adult Male | Ear | | | | | Similar | White blood cells | | [12] |
| Cattle | Adult Female | Epithelial | | 15–16 | 20.43 | 12.5–14.3 | Shorter | White blood cells | | [21] |
| | Adult Female | Oviduct | | 16.9 | 20.43 | 14.9–16.0 | Shorter | White blood cells | | [21] |
| | Adult Male | Muscle | | 20.1 | 20.43 | 19.6–19.9 | Similar | White blood cells | | [21] |
| | Adult Male | Skin fibroblast | | 18.2 | 20.43 | 20.0–20.4 | Similar | White blood cells | | [21] |
| | Fetal | Embryonic cells | | | 20.43 | 21.7–26.2 | Longer | White blood cells | | [21] |
| Mouse | Adult | Cumulus | | 48 | 48 | 48 | Similar | | | [17] |

individual's life span, and our understanding of the elongation mechanism is or will be good enough to make the process controllable, the nuclear transfer technique is offering us the possibility to rejuvenate cells thus making the dream of immortality closer to a reality. It is noteworthy to mention here that telomere length does not necessarily correlate with an animal's lifespan, nor do we know whether the actual physiological age of cloned animals is accurately reflected by their telomere length. The telomere model is very popular among the many mechanisms scientists have proposed for ageing at the cellular level. Other explanations include oxidative damage, accumulation of genomic changes, Mitochondrial DNA mutations, etc. (for review, see [14]).

In a further deviation from the above mentioned results, Tian et. al. [15] and Betts et. al. [16] found that telomere lengths of their clones' were not different from those of control animals; they were neither longer, nor shorter. In the smaller-sized mice, which have longer-telomere-length, Wakayama et al. [17] found the telomeres remained about the same size (48 kb) in peripheral blood lymphocytes throughout six generations of clones/reclones they tested. In fact, the trend was for the telomeres to lengthen with each generation, but this could not be shown statistically significant [17]. Successive generations of clones G1-G6 showed no signs of premature aging as judged by gross behavior parameters. These results, together with that from Lanza et al. [13], Tian et al. [15] and Betts et al. [16] verified that telomere shortening is not a necessary outcome of the cloning process. It appears that the embryo has inherited ability to determine the extent to which the telomeres should go. Although the mechanisms remain unclear, telomerase is no doubt an indispensable key element in telomere reprogramming events and many cattle cloning groups have reported telomerase activity in early stage reconstructed embryos [13,15,16].

The dynamics of telomerase activity during early embryo development was extensively studied in cattle. Telomerase activity is found up-regulated at the blastocyst stage, in *in vitro* fertilization (IVF) systems [18], parthnogenetic activation (PA) systems [19], and nuclear transfer systems [19]. This is consistent with the findings, in human and mice early IVF embryos [20], suggesting a conserved telomerase reprogramming pattern during early mammalian embryo development. Cloned bovine embryos displayed telomerase dynamics, similar to those of IVF and PA embryos [18,19]. The up regulating of telomerase activity at the blastocyst stage may contribute to telomere length restoration in embryo development after nuclear transfer and could explain the normal telomere length found in some cloned animals, including cattle and mice [13,15-17]. No reports of telomerase activity in cloned embryos of other species (including sheep) are available to date.

The discrepancy of reported telomere restoration (shorter, similar, longer) after nuclear transfer in different reports (Table 1.) may come from various factors, which include: species, donor cell type, donor cell culture time, nuclear transfer procedures, sampling and measuring protocols.

Donor cell effect on telomere length is well studied in the recent Miyashita [21] paper. In this study, they produced 14 cloned cattle using nuclei of donor cells derived from muscle, oviduct, mammary and ear skin. All three types (normal, shorter, longer) of telomeres were found when different donor cell types were compared. Clones from muscle or ear skin cell origin, had longer telomeres than the donor animal, but were within the normal range of same age controls. Clones from oviduct and mammary epithelia cell origin had telomeres that were shorter than those of controls. While clones derived from embryonic cell origins have telomeres longer than those of their age-matched controls. This concurs with the work by Lanza et. al [13], in which elongated telomeres were found when fetal fibroblast cells were used as donor cells. In Dolly's study, the animal produced from fetal tissue appeared to have telomere lengths non-distinguishable from normal controls, while the other two, including Dolly, which originated from adult cells, were found to have shorter telomeres. It will be an interesting study to compare the extent of telomere restoration using cells with different degrees of pluro-potency and different levels of telomerase activity. The availability of telomere reprogramming machinery in donor cells will at least not inhibit the process from happening, or it may trigger the restoration.

The time cells spend in culture is a complicated factor. Shorter culture time resulted in similar telomere length to controls in Shields et al.'s paper [11]. Extremely long culture resulted in extended telomere length in Lanza et al.'s paper [13]. One explanation might be: short time culture allowed the donor cell nucleus to be transferred with relatively long telomeres that is, they would start out long and remain long. On the other hand, extremely long culture time erodes the telomeres so short that the repair mechanisms are triggered and result in the over-elongation of telomeres. While the authors are writing this review, Clark et. al. published their results on telomere lengths in cloned sheep [22], which supports this hypothesis. They have shown in their study that NT does extend the telomeres from donor nuclei derived from late-passage cells that are near-senescent, however, telomeres of cultures established from fetuses generated by NT from early passage donor cells are not significantly different in length to those in the donor cell [22].

After many groups have demonstrated the various possibilities of cloning, it is at the point of investigating the molecular basis of telomere/telomerase (and other epigenetic) programming/reprogramming events in embryo development (and through nuclear transfer) and only this can provide a conclusive answer to questions such as 'how old was Dolly'. Up to now, our understanding is limited to observations of the telomere lengths of the animals, the telomerase activity of the cells and embryos, and the correlation between many factors (culture time, donor cell type, etc.) with telomere lengths. We remain limited by insufficient numbers of animals for research. Keep in mind, although eight other species: cow [23-25], mouse [26,27], pig [28-30], goat [31], rabbit [32], cat [33], mule [34] and horse [35] have been cloned since Dolly, nuclear transfer is still a very inefficient process (2%) [36] and very small numbers of clones are available for research [2]. However, telomere length appears to have been exonerated for the inefficiency: Tian et. al. [15] produced 4 live clones and 6 dead clones (died soon after birth). Both the live (15.38 kb) and dead (15.87 kb) clones had telomeres of the same lengths as controls (14.73 kb). The dead clones were found to have abnormal X chromosomes inactivation in a later study [37], indicating that the high fatality rate of clones is related to abnormal gene expression during embryo development.

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