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Aging of Cloned Animals: A Mini-Review

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Abstract
The number of species for which somatic cell nuclear transfer (SCNT) protocols are established is still increasing. Due to the high number of cloned farm, companion, and sport animals, the topic of animal cloning never ceases to be of public interest. Numerous studies cover the health status of SCNT-derived animals, but very few cover the effects of SCNT on aging. However, only cloned animals that reach the full extent of the species-specific lifespan, doing so with only the normal age-related afflictions and diseases, would prove that SCNT can produce completely healthy offspring. Here, we review the available literature and own data to answer the question whether the aging process of cloned animals is qualitatively different from normal animals. We focus on 4 main factors that were proposed to influence aging in these animals: epigenetic (dys)regulation, accumulation of damaged macromolecules, shortened telomeres, and (nuclear donor-derived) age-related DNA damage. We find that at least some cloned animals can reach the species-specific maximum age with a performance that matches that of normal animals. However, for most species, only anecdotal evidence of cloned animals reaching high age is available. We therefore encourage reports on the aging of cloned animals to make further analysis on the performance of SCNT possible.

Introduction

It is a basic, yet still quite mysterious fact that at fertilization the aging clock in metazoans is "reset to zero." While every individual "ages" over time, and consequently dies at some point, the cells in the germline seem completely resistant to age-related changes – otherwise a species would age as quickly as the individual itself [1]. While individual germ cells do age along with its organism, various control and selection mechanisms assure that the next generation starts relatively "unchanged" and healthy [2, 3]. It is, for example, now known that both nuclear and mitochondrial genomes are likely to acquire a small number of mutations between parents and offspring [4]. We regard this minimal change that occurs during natural reproduction, within the physiological reproductive lifespan of the parents, as the ideal 'reset to zero' of the aging clock, against which the aging of cloned animals has to be compared.

Jörg Patrick Burgstaller

In somatic cell nuclear transfer (SCNT), the nucleus of an adult cell is transferred to an enucleated oocyte, and is thought to not only regain pluripotency, but is also "rejuvenated" by factors in the ooplasm. Starting with works based on frogs [5], SCNT fully took off with the birth of Dolly the sheep [6]. Since then, SCNT has been applied successfully in numerous species (mouse, cattle, goat, pig, mouflon, domestic cat, rabbit, horse, mule, rat, African wildcat, dog, ferret, wolf, red deer, buffalo, camel, and coyote) (see online suppl. Table S1; for all online suppl. material, see www.karger.com/doi/10.1159/000452444 for details). Efficiency of SCNT is still rather low, with success rates of 0.3–1.7% per reconstructed oocyte and 3.4–13% per transferred SCNT embryo (as reviewed in [7] for farm animals). There are relatively high losses of individuals derived from SCNT during their perinatal and early postnatal development, but they are thought to be indistinguishable from controls once they reach higher age. In fact, they are reported to have comparable performance on traits like beef and milk production [8]. While there are clearly factors that limit the efficiency of cloning, at least some nuclei seem to be completely reprogrammed and rejuvenated to result in a completely "normal" adult individual. However, is it possible with a nucleus derived from a somatic cell, to completely start at time point zero, like gametes after a conventional fertilization?

Factors That Influence Aging of SCNT-Derived Animals

The effects of aging are quite complex, and cellular biomarkers of aging remain somewhat elusive [1]. Nevertheless, 4 main factors were proposed to underlie (possible) changes in the aging characteristics in cloned animals. They are (a) epigenetic (dys)regulation, (b) accumulation of damaged macromolecules, (c) shortened telomeres, and (d) age-related DNA damage.

Epigenetic Reprogramming

Epigenetic regulation is a process that tells genetically identical cells which role to adopt. Epigenetic regulation happens on multiple levels. "Cis-epigenetics" refers to direct methylation and demethylation of DNA bases, as well as to chromatin modifications. "Trans-epigenetics" covers proteins and RNAs. Both cis-and trans-epigenetics lead to the expression and repression of genes. In SCNT, the "cis-acting" nucleus of the differentiated donor cell gets exposed to the "trans-acting" factors of the recipient. In consequence, a sufficient number of genes to allow SCNT of the donor cells are reprogrammed to resemble that of the recipient cell. However, other genes remain in the expression pattern of the donor cell. This incomplete "reprogramming" is thought to be responsible for most of the SCNT-associated problems like fetal and placental anomalies (reviewed, e.g., in [9, 10]). Our understanding of these reprogramming effects greatly increased with the introduction of induced pluripotent stem cells (iPSCs) [11]. Terminally differentiated adult cells, initially from mouse and human and more recently from other species, can be converted to pluripotent stem cells by the introduction of a small number of transcription factors such as Oct4, Sox2, and Klf4 (reviewed in [12]). Like in SCNT, the reprogramming efficiency is still very low. Importantly, it has recently been shown that this low efficiency is not based on few 'elite' cells in a tissue that respond to reprogramming. Virtually every cell can be reprogrammed, but stochastic effects mean that only a small number of the cells finish the process [12]. It has been speculated that SCNT protocols lead to a selection process, as only a rather small portion of cells can support the development of a healthy animal [13]. Therefore, further optimization of SCNT and iPSC protocols will very likely be able to increase the efficiency of both approaches. In fact, the application of a histone deacetylase inhibitor, trichostatin A, improved the success rate of mouse cloning up to 5-fold. Moreover, with this protocol, the mice could be serially re-cloned for 25 generations, a feat that had not been previously achieved [13] (see below). It will be very interesting to see whether this success can be repeated in other, larger (domestic) species.

What are the consequences of incomplete reprogramming on aging? The relatively high losses of cloned animals during their perinatal and early postnatal development are thought to be mainly caused by failed epigenetic reprogramming [9]. Later in life, a certain dilution effect is expected to act upon epigenetic markings: if the enzymes that modify DNA and histones fail to reinforce the modifications during replications and cell division, both cis- and trans-epigenetics of the cells can be altered [1]. This might lead to a completely normal phenotype later in life. Moreover, successful clones are presumably derived from nuclei that, by chance, fulfilled all or most of the steps that lead to sufficient/complete reprogramming [12].

It is of course difficult to distinguish the overlapping areas of disease and aging. It is noteworthy, however, that perfectly reprogrammed cloned animals seem to be possible by optimizing the cloning protocols. Diseases of cloned animals are beyond the scope of this review (for a

Table 1. Telomere length of cloned animals (see also Table S2 for details)

Species	Relative telomere length compared to control animals	Studies, n	Cloned animals, n	Cloned animals with normal telomeres, %	Reference
Cattle	Normal/longer	5	42	64.6	[27–31]
	Shorter	3	23		[8, 30, 32]
Pig	Normal/longer	3	32	69.6	[26, 33, 34]
	Shorter	2	14		[26, 35]
Sheep	Normal/longer	3	6	37.5	[22, 36, 37]
	Shorter	3	10		[22, 36, 37]
Goat	Normal/longer	2	8	36.4	[38, 39]
	Shorter	3	12		[38–40]
Mouse	Normal/longer	2	535	100.0	[13, 41]
	Shorter	No	No		No
Wolf	Normal/longer	No	No	0.0	No
	Shorter	2	5		[42, 43]
Dog	Normal/longer	2	2	100.0	[44]
	Shorter	No	No		No

detailed review on this topic see, e.g., [10]); therefore, we focus on differences between cloned and control animals towards the end of the natural lifespan of the respective species (see below).

Accumulation of Damaged Macromolecules

Another cellular mediator of aging is accumulation of damaged macromolecules, including proteins and lipids, and highly stable aggregates of those molecules [14]. Like epigenetic regulation, damaged macromolecules are theoretically reversible by dilution, i.e. by cell division and new synthesis of macromolecules. So, this mechanism has very likely limited influence on the rejuvenation effect on SCNT; at least at a higher age damaged macromolecules derived from the nuclear donor cell should be diluted sufficiently to not cause any harm. If cloned animals produced a higher amount of damaged macromolecules and/or were less adept to handle them during their aging process, this mechanism could nevertheless play an indirect role in the aging process. Aggregation-associated degenerative disorders are well studied in humans (and animal models) as they cause severe, age-related degenerative diseases [15]. It has yet to be determined whether similar mechanisms could influence the efficiency and long-term outcome of SCNT.

Shortened Telomeres

Mammalian telomeres are repeated guanine-rich sequences that cap the end of chromosomes to preserve genome stability [16, 17]. The telomere length is different among individuals and species [18]. At each cell division, the telomere length is shortened in normal cells, leading to irreversible growth arrest (varying in a tissue-specific way) when reaching a certain threshold [18]. In active germ cells and during early embryogenesis, the enzyme telomerase is active, guaranteeing restoration of telomere length for the next generation. Consequently, it could be expected that when using an aged somatic cell with shortened telomeres for cloning, the offspring might start with a diminished replicating capability of its cells and consequently age, or at least reach senescence, faster. Moreover, such offspring might also suffer from telomere dysfunction-induced diseases such as cancer or dyskeratosis congenita [19]. While such diseases seem not to be abundant at least earlier in life, for which time-sufficient data are available [20], premature aging was a serious concern from the beginning. In fact, the telomeres of the first adult clone [21] Dolly were 20% shorter when compared with age-matched controls [22], and she died (of a viral illness, but also suffering from arthritis that was speculated to be SCNT-derived [23]) at the age of 6 years, while the life expectancy of her breed would have been 12 years [1]. However, further work on several species showed that at least in some clones telomere length was normal or even elongated when compared to age-matched controls. It was found that telomere length can be restored in the embryo during SCNT [13, 24, 25]. It is still unclear why this does not happen in all cases. We have summarized the results of various studies with regard to telomere length in Table 1 (see also online suppl. Table S2 for further de-

tails). In one-third to half of the studies (and cloned animals), the telomere length is reduced, either compared to the cell line used for SCNT or (more significant) to age-matched control animals. In cattle, several studies report normal or even elongated telomere length, while others find shortened telomeres. Also in pig, several studies report normal telomere length, while other studies find shortened telomeres. Curiously, in sheep almost all reported cloned animals show shorter telomeres than control animals (in absolute numbers, if not significantly. See online suppl. Table S2). In goats, 3 reports show both normal and shortened telomeres. Telomeres of 2 cloned dogs were normal, while in 5 cloned wolves, telomere length was reduced. In mouse (a species with notably long telomeres) telomere length was found normal, even after recloning of 25 generations (as described below).

Elongation of telomeres during SCNT takes place between the morula and blastocyst stage [24, 25]. However, this clearly does not work perfectly and might be a critical issue for the long-term outcome of SCNT. The reasons for these ambiguous results are currently unclear. Possible factors are species differences, donor cell origin and of course the NT protocol itself. Incidentally, the degree of telomere lengthening was found to be associated with nuclear reprogramming [16]. Currently, the application of trichostatin A, as already mentioned, seems not only to improve the success rate of cloning but also to favourably influence the telomere length [26].

Genomic Changes in Nuclear and Mitochondrial DNA

The primordial germ cells in the embryo are separated from the somatic cells at a very early stage. Moreover, at least in the ovary there seems to be a selection mechanism that ensures the "fitness" of a developing oocyte, resulting in the degradation of numerous follicles [2]. Also for mitochondrial DNA (mtDNA), a purifying selection exists at the oocyte level (and possibly during gestation) [3]. Therefore, DNA in the germ line is preserved at a very high level, and harmful mutations are likely to get sorted out ensuring the genetic fitness of the offspring. In contrast, somatic cells accumulate a high number of mutations both in nuclear and mtDNA. While in postmitotic tissues these mutations get fixed, in dividing tissues cells that are dysfunctional are thought to be replaced by others that have no or less detrimental mutations, counteracting loss of tissue function [3]. But how high is the danger of selecting a somatic cell with detrimental mutations?

The mutation rate of mtDNA is believed to be at least 100-fold higher than that of the nuclear genome [3]. In a mouse model, somatic mutations of mtDNA were shown to potentially aggravate aging [45]. Also iPSCs of adult individuals were recently found to harbour age-related mutations [46]. While these mutations are potentially harmful to the somatic cell, in SCNT the mtDNA of the somatic cell is largely replaced or outnumbered by the vast majority of mtDNAs derived from the donor oocyte [47, 48]. However, the mtDNA of the recipient oocyte is foreign to the donor cell nucleus. This could theoretically lead to nucleo-mitochondrial incompatibility, i.e. errors in the normally fine-tuned orchestration of gene expression and replication between the nuclear and mitochondrial genome that guaranties optimal energy supply. The extent of this incompatibility and its physiological influence is currently debated [49]. Even if present in small amounts, the mtDNA of the nuclear donor cell could theoretically have a "replicative advantage," thereby dominating the cells after some time [50]. First evidence of such a mechanism was found in cloned sheep [47]. Also in mice ([50] and references therein) and cattle [51] that harbour 2 types of mtDNA, biased segregation towards 1 of the 2 mtDNA types in the cell (heteroplasmy) was observed. However, no extensive analysis of this phenomenon has been conducted to date on cloned animals.

Nuclear DNA, especially that of dividing cells, is also very likely to accumulate mutations over time [52] in a tissue- and species-specific way [18]. These mutations can definitely not be reversed by SCNT, and very likely represent the only irreversible differences between an aged and a juvenile (donor) cell [1]. For example, a cell line with aberrant genetic material led to accelerated aging in 3 cloned pigs [35]. On the other hand, whole-genome comparison of a cloned dog and its respective nuclear donor showed less de novo differences than between 2 human monozygotic twins [4], showing that in SCNT-derived animals the DNA can be conserved to a very high level.

Can Cloned Animals Reach a Life Expectancy Similar to That of Control Animals?

The ultimate outcome of aging is death, and therefore life expectancy is perhaps the most easily measurable parameter of aging (the question of aging can of course not be reduced to life expectancy alone). The time since several species were first cloned outdates, or is at least close to, the life expectancy of the respective species by now: goat, cattle, dog, sheep, mouse, cat, and pig. Therefore, we should be able to finally answer the question of whether

Table 2. Reported maximum lifespans of cloned animals

Species	Breed	Typical life expectancy of species/breed, years	Reported maximum lifespan of cloned animals, years	Reference
Goat	Dairy goats	15	>15	[Gavin, pers. commun.; 54]
Cattle	Jersey Simmental Fleckvieh	15	11.8 oldest dairy SCNT cow, 2011 14.4 "Lara 8" (euthanized due to project end)	[55] [Brem, unpubl.]
Dog	Afghan hound	10–12	>10	[44]
Sheep	Finn Dorset	<10	9	[53]
Mouse	C57/BL6, DBA/2, 129/Sv	2–3	3	[13]
Cat		15	10 (in 2011)	[56]
Pig	Large, white, Göttingen, Yucatan	15–17	6	[57]

We report here the typical life expectancy as reported (often compared to control animals) in the respective reference; or in [58] (cattle, cat) and [59] (pig). For maximum lifespans, see [18] and references therein.

at least some cloned animals can reach a life expectancy similar to that of the control animals.

Table 2 shows the reported (maximum) lifespans of cloned animals compared to the respective typical lifespan of the species. In several species, cloned animals reach indeed the expected lifespan. Cloned dogs seem to reach a high age. Snappy, an Afghan hound and the first cloned dog, was 10 in 2015; and cloned female dogs of the same breed were 9. Also 3 cloned dairy goats lived to a normal age of 15 years, and Yang Yang, China's first cloned goat turned 15 in 2015. Also for cloned mice, several studies report a normal lifespan, most outstanding in serial cloning (see below). While Dolly, the first cloned sheep, only reached 6 years, very recently, important further work on the aging of cloned sheep was published by the lab of the late Keith Campbell. Thirteen aged (7–9 years old) cloned sheep, with 4 of them derived from the cell line that gave rise to Dolly, were analyzed. Detailed measurements of blood pressure and metabolism, as well as musculoskeletal tests showed no significant differences from age matched controls. Notably, these cloned sheep are already close to their typical natural lifespan (<10 years) [53]. The oldest reported dairy cattle reached 11.8 years in 2011. A cloned Simmental Fleckvieh cow reached healthy 14.4 years and was euthanized only due the project end [Brem, unpubl. data]. Copycat, the first cloned cat turned 10 in 2011, which is at least respectable for a cat, if still several years from the maximum lifespan. Pigs were first cloned in 2000, but the highest age reported to the best of our knowledge was 6 years.

While the question which age cloned animals can reach is asked very often, it is surprising that actual data in the scientific literature are scarce, even about the "celebrated" first cloned animals of several species. Therefore, we had to resort to own data, personal communication and even newsletters to finalize Table 2.

Nevertheless, including the very recent report about the aging of cloned sheep [53], it is now possible to say that at least for those species where the question of longevity of cloned animals was addressed (mouse, goat, sheep), a normal lifespan is possible. Also cats and dogs seem to reach a high age, as well as cloned cattle that reach a respectable age at least for dairy cows. For the other cloned species, the time is either too short to reach maximum lifespan, or we were unable to find reliable data. It would be interesting to find out what proportion of cloned animals indeed reaches old age, but with the current amount data it is impossible to do so.

Serial Cloning: Summing up the Years?

One of the biggest concerns regarding aging of cloned animals is the age of the nuclear donor cell. It was argued that if this cell is old, and consequently has shortened telomeres, the clone would already start at the age of the donor cell. In further consequence, serial cloning, i.e. us-

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