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Assessment of telomere length during post-natal period in offspring produced by a bull and its fibroblast derived clone

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ABSTRACT

Objective: To investigate the telomere length in bovine offspring produced by a cloned and control bull, and the telomerase activity in embryos produced with the same technology. **Methods:** Five daughters of a control and five daughters of a bull cloned using a fibroblast of the control were produced by IVF using sperm of the two bulls. Blood samples of the offspring were collected at 2, 6, and 12 months of age and the relative telomere length (RTL) was assessed by flow cytometry. At same time the body growth, hematological profile, and clinical biochemistry of the same progeny was extensively surveyed, and results have been reported in a previous work. Thereafter, the telomerase activity was assessed using a real time PCR quantitative assay in groups of embryos produced with the same technology. **Results:** The offspring of the clone exhibited a modest, but significant ($P<0.05$), shortening of the telomeres (21.36%, 20.56% and 20.56%) compared to that of the control (23.78%, 23.53% and 22.43%) as mean values determined at 2, 6 and 12 months, respectively. Shortening of telomeres in respect to the age was not significant. No statistical difference was reported between telomerase activity assessed in 144 cloned ($3.4^{-03} \pm 2.4^{-03}$ amoles/ μ L) and 80 control ($2.1^{-03} \pm 1.8^{-03}$ amoles/ μ L) embryos. **Conclusions:** The results have revealed a moderate shortening of telomeres in the offspring of the clone with respect to control. However, this study did not evidence differences in the two progenies that suggest welfare problems during the first year of life.

1. Introduction

Telomeres are specific DNA complexes at the terminal part of the chromosomes in the eukaryotic cells. Their function is essential for chromosomal stability keeping the chromosome ends intact, avoiding their fusion and recombination, and they are implicated in regulating the replication and senescence of cells. In the vertebrates these specialized structures are composed by a sequence of six nucleotides (TTAGGG) repeated from a few hundred to several thousand times. Mammalian telomeric complex is a lasso-like structure called "*t*-loop" [1], and the maintenance of its correct structure is important for their appropriate function. However, during somatic cell division the telomere length is progressively reduced due to the inability of the DNA polymerase to replicate the latest copy of the filament bases at 3′. This results after each cell division in a loss of terminal nucleotides and t-loop structure with a permanent blockade in G1 phase [2].

Some specialized cells, such as stem cells, germ cells and fetal cells, are able to maintain unvaried telomeric

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length and capacity to divide by the action of the enzyme telomerase. Telomerase is a ribonucleoprotein enzyme, a specialized RNA dependent DNA polymerase that synthesizes telomeric DNA in the chromosomal ends using a fragment of its RNA component as a template. The telomerase is active during embryogenesis, remains active in germ cells and is suppressed postnatally in most somatic tissues. Studies in mammalian species have shown a telomere elongation program at the embryonic transition from morula to blastocyst that seems to be required for ensuring sufficient telomere reserves [3, 4].

Somatic cell nuclear transfer (SCNT) as a new frontier of reproductive technology is now a reality. Not later than in the 2008, the USDA organization estimated that approximately 600 cloned animals in the U.S. were primarily used for breeding, and although it can likely be assumed that these cloned cattle produced thousands of progeny, there have been no reports of reproductive problems with the cloned animals or their progeny. The improvement of somatic cloning procedures in cattle has brought about widespread interest in its commercialization, which is mainly practiced with the distribution of semen of the cloned animals, or less frequently of cloned embryos. On the other hand, animals cloned from SCNT exhibit a variable shortening in their telomere length, which mostly depends on the source and the age of the nuclear donor cell [5]. This poses interesting questions on the consequences that shorter telomeres in cloned animals could have on their lifespan and on the potential hereditability of this alteration by the progeny.

The aim of this study was to investigate the telomere length in the offspring obtained using frozen/thawed sperm of a bull and its clone. To this aim, flow cytometry FISH technique was employed for estimating the telomeric length in white blood cells of progeny. Offspring produced for this study was also monitored from birth to 60 wk of life to assess body growth, hematological profile, clinical biochemistry, and to assess age at puberty and results have been provided [6]. As a further investigation on cellular aging in relation to cloning, the activity of telomerase was assessed using a real time PCR quantitative TRAP assay in groups of embryos produced with the same SCNT technology and control IVF embryos.

2. Materials and methods

2.1. Telomere length in clone offspring

2.1.1. Animals

Female offspring was produced as briefly described as follows: an Italian Holstein Friesian bull (control) used for commercial semen production known for its high genetic merit, and a bull (CLONE) cloned by SCNT using a fibroblast deriving from a cultured ear biopsy taken at an age of 6 years of the control, with procedures described below, were used as sires. Offspring was obtained after IVF and embryo transfer using frozen/thawed semen of the CLONE and control bulls and oocytes retrieved by ovum pick up from a group of six donor Holstein cows, equally distributed between the two sires.

2.1.2. Telomere length measurement

Blood samples of offspring were collected at 2, 6, and 12 months of age in sodium-heparin vacutainers and frozen at -80 ℃, after washing and resuspension to a final concentration of 8 to 10 x 10^6 cells/mL in RPMI culture medium, containing 10% DMSO and 25% fetal calf serum (FCS) (Sigma-Aldrich, Milan, Italy), in ice. A human commercial kit (telomere PNA kit/FITC for flow cytometry-DAKO Cytomation) was set up for telomere mean length detection. The assay is based on the use of a fluorescein-conjugated peptide nucleic acid (PNA) probe, which is a synthetic DNA/RNA analogue that links DNA/RNA. PNA probes are conjugated with FITC, a fluorescein that emits green fluorescence (515 nm) when excited by a light source at 488 nm. PNA probes are very sensitive and specific; therefore they are optimal for estimation of telomere length in flow cytometry as the fluorescence intensity of the cells is directly correlated with telomere length [7]. Thus, the relative telomere length (RTL) of sample cells is determined compared to telomere length of control cells.

A human tetraploid sub-line of the EBV-genome negative T-cell leukemia line (1301 cell line), which is characterized by very long telomeres (> 30 Kilo-bases), was used as a control. As the correct telomere length must be evaluated on leukocytes in the $G_{0/1}$ phase, samples were dyed for discriminating the cells in this phase. To this aim, some modifications at the original manufacturer's protocol were performed: the dye Propidium Iodide (PI) was replaced with 7-Amino-Actinomicyn D (7-AAD) Viability Dye (Beckman Coulter), which possesses spectral properties particularly suitable for flow cytometry analysis [8]. Moreover, the determination of the RTL requires DNA-index calculation of the two populations of control and sample cells. DNA-index is represented by the ratio between medium fluorescence value of cellular aneuploid population (not 2n) in $G_{0/1}$ phase, and medium fluorescence value of diploid cellular population (2n) in the same phase. Therefore, a suitable kit (Coulter DNA-Prep. Reagent kit, Beckman Coulter) for cellular cycle evaluation was used. Minor modifications were required to adapt this kit to bovine cells. Briefly, blood sample cells were hemolyzed with NH_4Cl 0.87% and washed in PBS, while control 1301 cells were harvested

and simply washed in PBS. Control and sample cells were then mixed at same concentration of 2×10^6/mL and used for telomere length determination. Mixed cells suspension was divided into two aliquots that were processed with and without the PNA probe, respectively. The two aliquots were denatured at 82 ℃ for 10 minutes and then maintained at room temperature overnight in the dark. Samples were then washed using the specific Wash Solution and incubated at 40 ℃ for 10 minutes. Finally, the samples were stained with 7-AAD and analyzed at flow cytometer. Cells were detected for cellular cycle using the Kit Coulter DNA-Prep.

Analyses were performed using a flow cytometer Facs-Vantage SE (Beckton Dickinson), equipped by an Argon laser Innova 300 emitting at 488 nm, with an absorbance filter of 675/20 nm long-pass for deep red fluorescence, and absorbance filter of 530/30 nm long-pass for green fluorescence. At least duplicate measurements were performed for each cell sample. The analysis of telomere fluorescence was conducted using Cell Quest (Becton Dickinson) acquiring 10 000 events, at a rate of about 150 cells per sec. Cells hybridized with PNA probes emit a signal in FL1 (photomultiplier that acquires green fluorescence) higher than autofluorescence emitted by the same cells hybridized without probe. The RTL was calculated as follows:

$$RTL = \frac{(\text{mean FL1 sample cells with probe} - \text{mean FL1 sample cells without probe}) \times \text{DNA index of control cells}}{(\text{mean FL1 control cells with probe} - \text{mean FL1 control cells without probe}) \times \text{DNA index of sample cells}} \times 100$$

2.2. Telomerase activity in cloned embryos

2.2.1. Production of cloned embryos by SCNT

Oocytes aspirated from ovaries of slaughtered Holstein Friesian cows/heifers were matured for 20 to 21 hours in TCM199 (Sigma) supplemented with 10% (v:v) FBS (Sigma), 1 μL/mL ITS Media Supplement (cat. no. I1884, Sigma), 1 mM sodium pyruvate, 0.5 mM L-cystein, 10 mM glycine, 100 μM β-mercaptoethanol, and gonadotropins (0.05 IU/mL FSH and 0.05 IU/mL LH; Pergovet 75, Serono, Rome, Italy) at 38.5 ℃ in 5% CO_2 in humidified air. Fibroblasts derived from an ear skin biopsy were cultured in TCM199:DMEM (1:1) supplemented with 10% FCS were used as nuclear donor cells. Two days before nuclear transfer, the donor cells were passaged, and the day after were serum starved (0.5% FBS in the growth medium) for 24 hours. Before use as nuclear donors, the cells used in this study were passaged from 6 to 15 times.

The oocytes were denuded of granulosa cells by vortexing in the presence of hyaluronidase. Zona-free method of NT-embryo construction was used [9]. Zona pellucida of oocytes with extruded polar bodies was digested with 0.5% pronase in PBS. Zona-free oocytes were stained with Hoechst 33342 in the presence of cytochalasin B (5 μg/mL) and enucleated under UV light by enucleation pipette with perpendicular break. All manipulations were performed in SOF-Hepes with 10% FBS. Zona-free cytoplasts were individually washed for several seconds in 300 μg/mL phytohemagglutinin P in PBS and then quickly dropped over a single donor cell [10] that settled to the bottom of the drop of SOF-Hepes. Formed cell couplets were washed in 0.3 M mannitol (100 mM Mg) solution and fused by double DC-pulse of 1.2 Kv/cm applied for 30 μsec. NT embryos (2 to 3 hours after fusion) were activated at 27 to 28 hours after the onset of maturation by treatment with 5 μM ionomycin in SOF-Hepes for 4 minutes, followed by 4 hours culture in 2 mM 6-DMAP in medium SOF, supplemented with MEM essential and non-essential amino acids and 4 mg/mL BSA (m-SOFaa). Embryos were cultured individually in wells in 20 μL drops [11] of m-SOFaa with 4 mg/mL BSA in 5% CO_2 and 5% O_2 in humidified air at 38.5 ℃ under mineral oil. Half of the medium was renewed on day 3 with fresh m-SOFaa and on day 6 with TCM199 supplemented with 16 mg/mL BSA (day 0 was the day of fusion and activation). Cloned embryos were produced using five female cell lines (4 to 6 years animals) as source of nucleus, while control IVF embryos were produced using frozen/thawed semen of five bulls after culturing of embryos under the same conditions as for the cloned embryos.

2.2.2. Pools creation

Embryos at 8 days of culture were washed twice in Ca^{2+} and Mg^{2+} free PBS with 0.1% BSA, and transferred in microvials in 1 μL volume of the same PBS solution. Single embryos were then incubated in 1 × lyses buffer (Allied Biotech, Inc. USA) for 1 hour at 4 ℃, at a ratio of one embryo per 5 μL of buffer [12]. After incubation, the suspension was briefly centrifuged at 12 000 × g for 30 sec and the supernatant was quickly frozen and stored at -80 ℃ until use, to preserve telomerase activity. For each female nuclear donor, pools were formed by combining the extracts of eight embryos (2 μL of protein extract for each embryo).

2.2.3. Telomerase activity assessment

Telomerase activity was determined using the Quantitative Telomerase Detection Kit (QTD kit) (Allied Biotech), according to the manufacturer's protocol. The QTD kit, a real

time quantitative TRAP assay, is based on the fluorescent dye SYBR Green which is capable of binding the double-stranded amplicons and generating fluorescence signals in a PCR reaction. For each pool, a reaction mixture was prepared in a total volume of 25 μL, containing 12.5 μL QTD premix, 11.5 μL distilled water and 1 μL protein extracts.

The reaction mixture was first incubated at 25 ℃ for 20 minutes to allow the active telomerase to elongate the telomerase substrate oligonucleotide (TSR) primer (5′-AATCCGTCGAGCAGAGTT-3′) by adding a TTAGGG-repeat sequence. Thereafter, PCR was performed at 95 ℃ for 10 minutes followed by 40 cycles of amplification at 95 ℃ for 30 sec, 60 ℃ for 33 sec and 72 ℃ for 30 sec, on an ABI PRISM 7500 Real Time PCR System (Applied Biosystems). The increase in fluorescence was collected and analyzed, in the late extension step of each cycle, with Sequence Detector Software (SDS). Real time PCR was conducted under conditions in which primer dimer formation is negligible and the extent of repeat amplification is directly proportional to telomerase activity.

Heat inactivated template and a negative control consisting of a reaction mixture containing only 1 × lyses buffer, were also assayed for quality control purposes. Because the telomerase is a heat sensitive enzyme, the inactivated template was obtained by incubating 4 μL of extract of each pool at 85 ℃ for 10 minutes. Telomerase activity was expressed relative to the telomere short repeat TSR control template. Thus, a calibration standard curve was generated from serial dilutions of the TSR (0.5, 0.1, 0.02, 0.004, 0.008, 0.00016 amoles/μL), according to the manufacturer's instructions. Melting curve analysis was performed on each run to verify specificity and identity of the PCR products.

2.3. Statistical analysis

For telomere length evaluation, the following variables were defined: AGE, FATHER and RTL, where:

AGE = age of the offspring (1 = 2 months; 2 = 6 months; 3 = 12 months)

FATHER = bull used for insemination (1 = Clone; 2 = control)

RTL = relative telomere length

Statistical analysis was conducted using the following linear model:

$RTL_{ijk} = \mu + FATHER_i + AGE_{j(i)} + e_{ijk}$

where: RTL_{ijk} = dependent variable RTL

μ = general mean

$FATHER_i$ = effect of i-th level of the fixed factor FATHER (i = 1, 2)

$AGE_{j(i)}$ = effect of i-th level of the fixed factor AGE nested in FATHER (j = 1, 2, 3)

e_{ijk} = random error

For evaluation of telomerase activity, a dataset was implemented using the following variables: ORIGIN, CT, TELO, where: ORIGIN = pool of embryos (1 = cloned; 2 = control);

CT = cycle of real time PCR; TELO = quantity of telomerase measured at a specific CT;

Data were normalized to obtain a normal distribution, and the variable LogTELO was used for statistical evaluation of telomerase activity. The analysis of variance was performed using the following linear model: $Y_{ij} = m + ORIGIN_i + b(CT) + e_{ij}$

where: Y_{ij} = dependent variable LogTELO; m = general mean; $ORIGIN_i$ = effect of the i^{th} fixed factor ORIGIN (i = 1, 2); b(CT) = effect of covariate CT; e_{ij} = common random error N(0, s); Statistical tests were conducted using the software R [13].

3. Results

3.1. Telomere length in clone offspring

A total of five daughters of CLONE and four daughters of control bulls were followed to 12 months of age for the determination of telomere length. One daughter of the controldied at eight months of age, therefore for this subject only blood samples collected at two and six months could be analyzed. The values of RTL in the offspring of the CLONE bull were (21.36 ± 2.05) %, (20.56 ± 2.72) % and (20.56 ± 1.49) % as mean values determined at 2, 6 and 12 months, while the corresponding values for the control offspring were (23.78 ± 4.37) %, (23.53 ± 3.98) % and (22.43 ± 4.65) %. At statistical analysis by linear model, a significant effect of the FATHER ($P<0.05$) was reported in relation to RTL, while the effect of AGE was not significant. Figure 1 presents the histogram of RTLs showing a tendency toward a telomere shortening in the offspring of the CLONE with respect to that of the control bull.

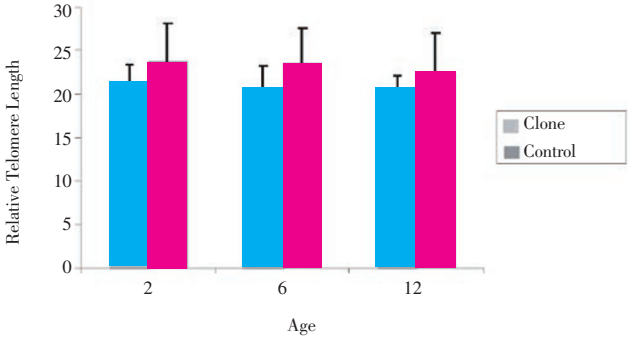

Figure 1. Relative telomere length, expressed as percentage, measured at 2, 6 and 12 months of age in five daughters of a clone and four daughters of a control bull, showing a tendency ($P<0.05$) toward a telomere shortening in the clone respect to control offspring.

3.2. Telomerase activity in cloned embryos

A total of 18 pooled protein extracts from 144 cloned embryos and 10 pools from 80 control embryos were subjected to PCR, and results were presented in Table 1. The Ct values increased linearly with the decrease in the amount of protein extract. Because of the formation of primer dimer artifacts, a fluorescence signal was also detected in the negative control. However, these signals in negative controls were only detected at late stages of amplification, higher than 34 cycles.

Table 1
Ct values and corresponding telomerase activity evaluated by a real time PCR quantitative TRAP assay in protein extracts of 144 cloned, 80 control IVF embryos and the relative heat inactivated templates.

Sample	Ct	Telomerase activity (amoles/ μ L)
Cloned embryos	28.94 ± 1.16	$3.4^{-03} \pm 2.4^{-03}$
Control IVF embryos	29.60 ± 1.17	$2.1^{-03} \pm 1.8^{-03}$
Heat inactivated	35.48 ± 1.19	$2.7^{-05} \pm 1.9^{-05}$

Data were expressed as Mean ± SD.

The data relative to quantification of the telomerase activity were then changed into logarithmic values to generate the LogTELO variable, and a normal distribution was obtained (W=0.9482, P=0.1789, Shapiro-Wilk test). Thus, the mean values relative to cloned and control embryos were respectively 1.41 ± 0.34 and 1.20 ± 0.36. The box-plot of the variables LogTELO is presented in Figure 2. At Barlett test, the variances were homogeneous (K^2 = 0.8856, df = 1, P=0.3467), and at analysis by linear model the effect of the ORIGIN of embryos (cloned and control) was not significant (P=0.472).

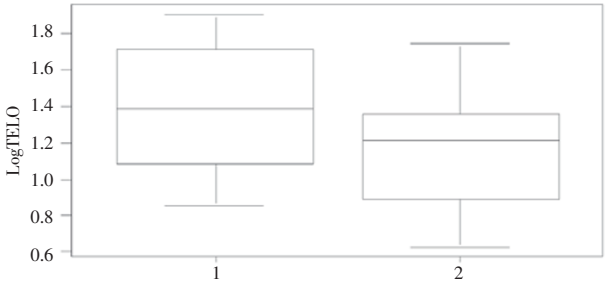

Figure 2. Box-plot of the variable LogTELO derived from the quantification of telomerase activity in pools of cloned (1) and control IVF (2) embryos. At analysis by linear model the effect of the origin of embryos (cloned and control) was not significant (P=0.472).

4. Discussion

Animal cloning as reproductive technology has raised moral and ethical objections mostly addressed toward health risk, wellbeing and life expectancy of the animals. Because animals cloned from SCNT exhibit a variable shortening in their telomere length, the potential hereditability of this alteration by the progeny has been frequently put under debate.

In the present work a reduction of telomere length in daughters of clone was detected with respect to the daughters of control father, while the tendency toward the reduction of RTL in relation to the age did not reach a statistical level of significance within the first year of life. In the literature, it has been reported that telomere length in cloned animals largely depends on the type and the age of the cell used as nucleus donor. Mammals cloned from fibroblasts have shown a broad pattern of elongation of their telomere length relative to age-matched controls, as measured in nucleated blood cells [14] and fibroblasts [15]. Furthermore, it has been reported that telomere lengths in leukocytes of cloned cattle derived from muscle cells of an old bull were longer than those of the donor animal [5]. On the contrary, SCNT from oviductal and mammary epithelial cells of an equally old cow resulted in clones with telomere length shorter than age-matches controls, so as also reported for the first cloned mammal, the sheep Dolly. As regard to the age of the nucleus donor cell, cloning from senescent cells that were passaged until greater than 95% of their life-span was completed has shown an increase in the replicative life in the cells of cloned compared to senescent cells progenitors, and a telomere length even greater compared to the age-matched controls [14]. Similarly, it has been shown that senescence of donor cells was associated with a greater length of telomeres after the reiterative cloning of mice to four and six generations in two independent lines [16].

In a previous study we have evaluated the telomeric length variability in peripheral blood leukocytes of different age Friesian cattle, evidencing an effective telomere length reduction in relation to age [17]. The magnitude of this reduction became statistically significant at the age of 2 years, with respect to the group of newborns. In the present work the negative trend toward a reduction of RTL in relation to the age, in accordance to what previously reported, did not reach a statistical level of significance within the first year of life.

With respect to life expectancy, although fetal development is the most subject to a drastic selection, the perinatal period has been frequently reported to be critical for the fate of the animals generated by SCNT. On the other hand, adult cattle clones appear to have a health risk similar to their normal counterparts. In the work of Abeni *et al.* [6], a wide range of parameters have been examined during the perinatal period of the same progeny that we have used in the present work, to explore any potential difference as

regards the immune system, its differentiation and activity of hematopoiesis. Other than the hematological parameters described in the cited work, in our present study at birth and at two months of age blood samples were collected for the determination of the serum lysozyme, serum bactericidal activity, hemolytic complement, haptoglobin, A/G ratio and total protein (data not shown). In this respect, the serical lysozyme characterizes the activity of the macrophagic monocyte system, which also indicates inflammatory processes; the serical bactericidal characterizes the ability of serum to inhibit the growth of the main environmental germs (Enterobacteriaceae); the hemolytic complement identifies the amount of free complement in the blood, and the haptoglobin is a protein marker of the acute inflammatory phase. These parameters represent an important first line of defense against infection, known as natural immunity. This response is activated within a few minutes or hours after contact of the organism with the pathogen and is able to keep under control, and in some cases to eradicate the infection, before immunity response is activated. In the present work no differences between offspring of clone and control bull have been evidenced as regards to non-specific immune parameters, so that it is realistic to assign to these progenies the same health status at 60 days.

As a further investigation on the mechanisms involved in the restoration of telomeres related to cloning, in the present work we have assessed the telomerase activity in embryos produced by SCNT and we have found only a moderate tendency toward an increase of activity in the embryos cloned from adult fibroblasts with respect to control IVF embryos. This data confirms previous findings, whereas bovine blastocysts cloned from fibroblasts of an age similar to that of our nuclear donors [12], and blastocysts reconstructed with the use of fetal fibroblasts and granulosa cells [18] exhibited telomerase activity similar to their in vitro fertilized counterparts. In the work of Lanza *et al*. [14], the authors found that animals cloned from senescent cells had telomeres longer then those of the age-matched controls. Although high levels of telomerase activity have been detected in the reconstructed embryos, no comparisons were made between cloned and control IVF embryos to establish if an over expression of telomerase activity was the responsible of this unexpected increased telomere length. In this regard, many reports have suggested that telomerase activity during early embryo development is not the only mechanism that comes into play in the remodeling of telomeres after SCNT. For instance, high levels of activity of this enzyme have been detected in fibroblasts, kidney and liver tissues of bovine cloned fetuses [18], but more in general it could be detected in a variety of tissues of a 16-week human fetus [19]. Intriguingly, Bermejo-Álvarez *et al*. [20] have identified differences in telomere length between bovine male and female embryos, being the telomere length shorter in male blastocysts with respect to females. Supported by similar findings in humans [21], it has been hypothesized that such differences between sexes would be due to some regulating mechanisms, partially identified in a locus on the distal X chromosome [22], and other methyltransferases indicated by the authors as negative regulators of telomere length that were differentially expressed between male and female embryos. This hypothesized mechanism could partially explain the tendency toward the increase of telomerase activity that we have found in the cloned with respect to control IVF embryos, when considering that in the present study cloned embryos were obtained from female cell lines and IVF embryos were of a mixed sex. This difference, however, was not significant as reported in the cited works in which embryos of fully opposite sex were studied.

It has been demonstrated that the telomeres in the spermatozoa of human, porcine and bovines are elongated by 69%, 24% and 14%, respectively, in comparison with somatic tissues [23]. Thus, the elongation of telomeric DNA during mammalian spermatogenesis appears as part of a wider process of telomere remodeling, whose major role is to preserve and restore telomere reserve through generations. Allsopp *et al*. [24] found that telomere length in human sperm was increased with respect to the age of donors, and Unryn *et al*. [25] confirmed a positive association between age of fathers at conception and telomere length of their offspring. The results presented in our work, which show that telomerase activity in cloned embryos is only moderately increased during nuclear reprogramming, remarks how the elongation of telomeric DNA during embryogenesis appears as part of a wider process of telomere remodeling, which comprises the elongation of telomeric DNA during spermatogenesis which may promote telomere extension, and possibly telomere length restoration, in cloned offspring via germ line cells.

In conclusion, this study has shown that the female progenies produced by a bull and its fibroblast derived clone were substantially similar as regards to skeletal development, clinical biochemistry and hematological profile [6], to which we have also added the results characterizing the status of natural immunity. In the present work, a reduction of telomere length in daughters of clone was detected with respect to the daughters of control father, as the only difference among the features analyzed. However, this condition did not evidence potential source of concern during the peri and postnatal period until to the achievement of puberty. In agreement with other studies which have also shown some moderate effects of cloning on telomere length, the possible consequences of such a shortening at later

stages of life is still a theme which requires further study.

Conflict of interest statement

We declare that we have no conflict of interest statement.

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