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Telomere Dynamics during Bovine Preimplantation Development

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Nothing in Biology makes sense except in the light of evolution

Theodosius Dobzhansky, 1972

Gracias a todos los que hicieron posible la realización de este documento.

A mis directores de tesis por abrir las puertas de sus laboratorios y guiarme durante este proceso.
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CONTENTS

SUMMARY

Telomeres are dynamic structures that protect the ends of linear eukaryotic chromosomes from aberrant fusions or from being misidentified as DNA breaks, playing an important role in development, differentiation, senescence and health. Although linear DNA shortens at each cell division due to incomplete replication of the 5'-end, telomeres have two mechanisms to maintain their length: a specialized enzyme called telomerase and the Alternative Lengthening of Telomeres (ALT) mechanism, based on homologous recombination. In complex organisms like mammals, both mechanisms are turned off in somatic cells after the preimplantation development, leading to a programmed cellular senescence, whereas immortal cells such as stem cells and most cancer cells possess mechanisms to maintain long telomeres. Short telomere length (TL) in newborns has been related with the appearance of certain disorders such as Dyskeratosis congenital, and telomere length has been proposed to be reset during preimplantation development. Telomere lengthening dynamics and mechanisms during preimplantation development have been widely studied in the laboratory mouse. However, laboratory mice contain longer telomeres than other mammals, including wild mouse, bovine and humans, and thus there may be also differences in telomere lengthening dynamics during preimplantation development. The objectives of this Master Thesis have been to determine the dynamics of telomere length during bovine preimplantation development and to test the effect of the injection of a plasmid expressing mouse *Tert* in bovine zygotes on the telomere length of the blastocyst. Telomere length was analyzed by qPCR at different stages (oocytes, zygotes, 2-cell, morula and blastocyst) observing that a significant increase occurred after embryonic genome activation, doubling its length at the morula stage and ending at the blastocyst stage with a telomeres 10 times longer than those of the oocyte. However, the increase in telomere length observed at the 2-cell stage in mouse embryos was not noted in bovine embryos, suggesting species-specific differences in the mechanisms involved in telomere lengthening at these developmental stages. Telomere length of the blastocysts derived from bovine zygotes injected with a plasmid encoding for mouse *Tert* did not differ from that of blastocysts obtained after injection with a control plasmid expressing EGFP. This result suggest that mouse *Tert* was not able to further elongate telomeres at the blastocyst stage because either it is not be able to form the telomerase complex with bovine components, or telomere length is tightly regulated during preimplantation development to reach a maximum length that cannot be exceeded by exogenous expression of telomerase components.

RESUMEN

Los telómeros son estructuras dinámicas que protegen los extremos de los cromosomas lineales eucariontes de fusionarse entre sí o de ser confundidos como roturas en el ADN, jugando un papel importante en el desarrollo, diferenciación, senescencia y en la salud. A pesar de que el DNA lineal sufre acortamiento en cada división celular debido a la replicación incompleta del extremo 5', los telómeros tienen dos mecanismos de elongación: una enzima especializada llamada telomerasa, y el mecanismo de Elongación Alternativa de los Telómeros (EAT), basado en recombinación homóloga. En organismos complejos como los mamíferos, ambos mecanismos no son funcionales en células somáticas después del desarrollo preimplantacional, derivando en una senescencia celular programada, mientras que las células inmortales como las células troncales y la mayoría de las células cancerígenas constan de mecanismos para mantener telómeros largos. Se han relacionado longitudes teloméricas cortas en recién nacidos con la aparición de ciertas enfermedades como la disqueratosis congénita, y se ha propuesto que la longitud telomérica se establece durante el desarrollo preimplantacional. La dinámica de elongación telomérica durante el desarrollo preimplantacional se ha estudiado ampliamente en el ratón de laboratorio. Sin embargo el ratón de laboratorio contiene telómeros más largos que otros mamíferos, incluyendo el ratón salvaje, bovinos y humanos, y por ello también puede haber diferencias en la dinámica de elongación telomérica durante el desarrollo preimplantacional. Los objetivos de esta Tesis de Máster han sido caracterizar la dinámica de la longitud telomérica durante el desarrollo preimplantacional bovino y determinar el efecto de la microinyección de un plásmido de expresión para la telomerasa transcriptasa inversa (*Tert*) de ratón en cigotos bovinos sobre la longitud telomérica en el blastocisto. La LT se analizó por qPCR en distintas etapas (ovocitos, cigotos, embriones de 2 células, mórulas y blastocistos), observando un aumento significativo posterior a la activación del genoma embrionario, doblando la LT en el estadio de mórula y acabando en el estadio de blastocisto con telómeros 10 veces más largos que los del ovocito. Sin embargo, no se observó un incremento en la longitud telomérica en el estadio de dos células descrito en ratones, sugiriendo que existen diferencias entre especies en los mecanismos implicados en la elongación telomérica durante estas etapas de desarrollo. La longitud telomérica de blastocistos derivados de cigotos bovinos inyectados con un plásmido codificante para *Tert* de ratón fue similar a la obtenida en blastocistos derivados de cigotos bovinos inyectados con un plásmido control que expresa EGFP. Este resultado sugiere que *Tert* de ratón no es capaz de aumentar la longitud telomérica en el estadio de blastocisto porque o no puede formar el complejo de la telomerasa con los componentes bovinos o porque la longitud telomérica está estrechamente controlada durante el desarrollo preimplantacional para alcanzar un máximo que no puede superarse mediante la expresión exógena de componentes de la telomerasa.

INTRODUCTION

Telomeres are the natural ends of eukaryotic chromosomes, where they evolve to solve two major problems: 1) a protection problem, avoiding chromosome ends to be recognized as double-strand breaks (DSB) and 2) a replication problem, avoiding the progressive shortening of chromosomes due to the end-replication problem.

Telomeres Role in Protecting the Chromosome Ends, an Evolutionary Perspective

While prokaryotes have circular DNA, the later developed eukaryotes have linear chromosomes, a feature that faced some constrains in the prokaryotic environment. In an environment where circular DNA was ubiquitous, the first problem that linear chromosomes faced is being misidentified as DNA-damage and alert the DNA-repair machinery. In most organisms, double strand breaks (DSB) activate the ATM-kinase pathway. When this happens, cell cycle may be arrested until the DSB is repaired, either by Non-Homologous End Joining (NHEJ) or by homology-directed repair (HDR), leading to end-to-end fusions or triggering cell dead. In order to avoid this problem, eukaryotes developed telomeres, specialized structures located at their chromosome ends that avoid them to be recognized as DSB, thereby allowing a stable linearization of the chromosome and their effective transmission through generations.

The word “telomere” (from the Greek τελος “end” and μερος “part”) was coined by Hermann Muller to describe the ends of eukaryotic chromosomes, that appeared to have distinctive properties not present in the rest of the chromosome. Barbara McClintock also observed that chromosome ends have special features that prevent them from fusing together. In particular, she used X-rays and mechanical forces to induce chromosomal breaks and noted that broken ends tended to fuse one another, 2-by-2 or to form circular chromosomes, whereas telomeres were never involved in those fusions (McClintock 1938; McClintock 1941). Subsequent experiments carried out by Jack Szostack and Liz Blackburn showed that telomeres was a highly conserved system across eukaryotes. These researchers linearized yeast circular plasmids and inserted telomeres from the ribosomal DNA (rDNA) of Tetrahymena pyriformis, a ciliated protozoan, in the broken ends. The transferred telomeres augmented, suggesting that both organisms share a common telomere maintenance system that included a lengthening mechanism (Szostak & Blackburn 1982).

The stability of linear chromosomes is given by the most basic component of telomeres, the tandem array of repetitive elements. It has been proposed that during eukaryotic evolution, the archaeal genome could have experienced a massive invasion of Group II introns, coming from the endosymbiotic phagocytosis of α-proteobacterial cells (Martin & Koonin 2006). After the accumulation of enough short sequences in a circular chromosome, a DBS in one of those repeats is more likely to occur. In that situation, the DNA repair machinery would either ligate the broken ends by NHEJ or recruit the HDR

system. In the second case, the use of other Group II intron from the same chromosome would lead to a strand invasion, forming a terminal loop that no longer can be recognized as a DSB, as shown in Figure 1. This structure, known as a telomeric loop (t-Loop), could have variable size and sequence composition, but allowed a linear chromosome to be stable (De Lange 2015).

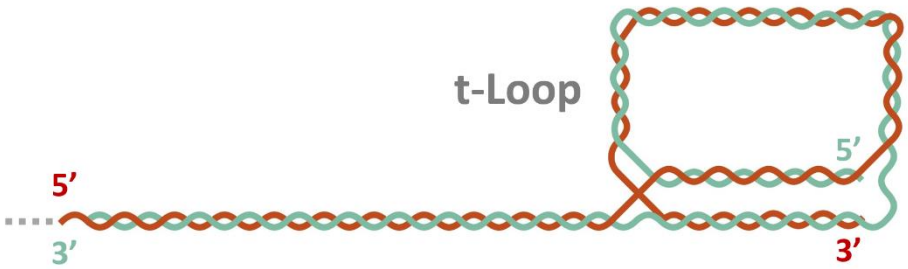

Fig. 1. Linear chromosome stabilized by a t-Loop, mediated by HDR factors after a DBS in a circular chromosome. Strand-invasion between homologous repetitions hides chromosomal ends from NHEJ or DNA-damage machinery.

As seen by electron microscopy, the complementarity of the telomeric sequence not only allows to the formation of secondary structures like t-Loops but also other configurations like cruciform formation that can hide chromosomal ends from being misidentified as DSB (Karrer et al. 1976).

The end replication problem and cellular senescence

Being recognized as DSB is not the only problem a linear DNA faced in the prokaryotic environment. The second constraint that linear DNA confronted was the incomplete replication by DNA-polymerases as these enzymes, evolved in the prokaryotic environment to replicate circular DNA, were unable to replicate the very end of the linear chromosomes. Russian biologist Alexey Olovnikov was the first to note that a shortening would occur in linear DNA if only known replication mechanisms were involved. DNA-Polymerases evolved in the context of circular DNA, using a short RNA primer with a free 3'-end to initiate replication. After finishing the linear copy, the removal of the terminal primer at the 5'-end leaves a gap that cannot be filled in. If left unfixed, this gap leads to a shortening of the molecule at their termini. In chromosomes, shortening would happen at a predictable constant rate per cell cycle, depending on the size of the missing template (Olovnikov 1973; Olovnikov 1996). This shortening provided a molecular explanation to cellular senescence, the so called Hayflick limit, which was discovered a decade earlier.

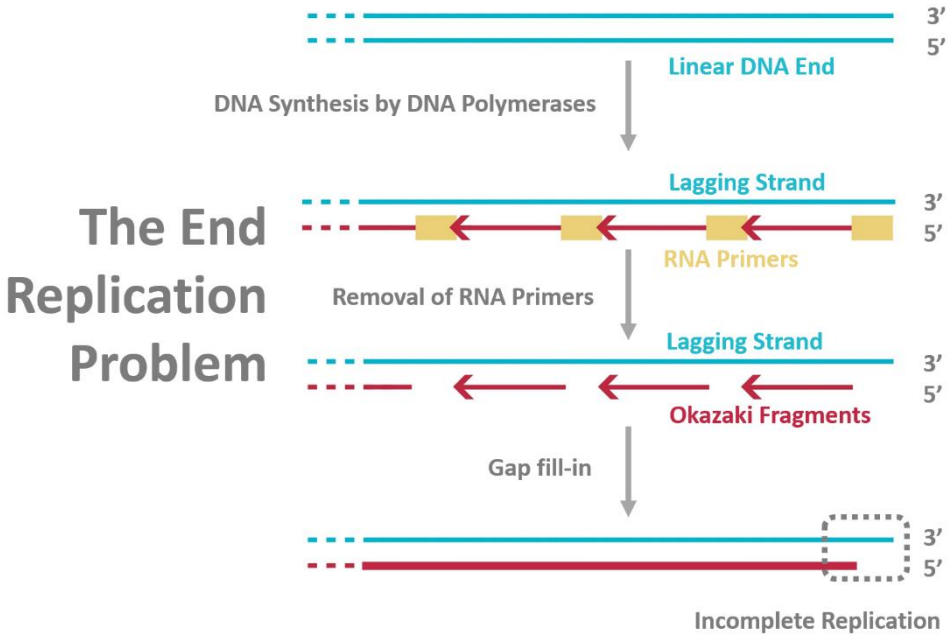

Figure 2. The end replication problem. Incomplete replication at the 5'-end of a linear DNA molecule after RNA primers removal.

Cellular senescence was initially confirmed by the discovery of the Hayflick limit. Before this discovery, several experiments claimed that cell cultures could be maintained indefinitely *in vitro*, never stopping doubling their population, regardless of their nature. In this context, when a cell culture was no longer viable, the common explanation used to be the lack of proper manipulation and knowledge on cell biology during *in vitro* cultures, and thereby the general consensus was that senescence was an attribute of the body as a whole rather than a cellular phenomenon. This paradigm was shifted by Leonard Hayflick who was testing whether human cancers have a viral etiology. In order to do so, he established two human cell lines, one derived from tumorous tissue and other from fetus, observing that cancer cells survived indefinite population doublings, whereas normal fetal cells were unable to sustain a culture indefinitely. Normal cells appeared to have an internal replication countdown that avoided them to replicate beyond passage ~ 50. This countdown, which Hayflick called the replicometer, stopped if the culture was frozen-stored and continued after thawing and even let him predict the eventual dead of specific cultures (Hayflick & Moorhead 1961; Rattan 2000).

Hayflick limit was partially explained by the end replication problem, where telomere length could be the "replicometer", i.e. the indicator of the number of division a cell could sustain before senescence. In agreement, telomere length decreased with cumulative population doublings of fibroblast *in vitro*, as predicted by Olovnikov (Harley et al. 1990). In this perspective, the fact that cancer cells could divide indefinitely suggested the existence of a mechanism for maintenance or de-novo synthesis of telomeric DNA, present in cancer cells but not in somatic cells (Olovnikov 1973). On the same premises, such mechanism should be present in the germ line or during embryogenesis in order to avoid telomere attrition between generations.

Mechanisms to prevent telomere shortening

Telomeres can maintain or increase their length through two mechanisms: 1) telomerase, the main responsible for telomere lengthening in mammalian cells and 2) alternative lengthening of telomeres (ALT).

It has been proposed that the first mechanism appearing in evolution to compensated telomere shortening the t-Loop, which, as already explained, also plays a crucial role in preventing the telomeres to be recognized as a DSB. The strand invasion can act as a replication fork and use the replicative machinery already found in prokaryotes. The t-Loop mechanism is based in homologous recombination, and constitutes the simplest version of alternative lengthening of telomeres (ALT). The chromosomes with t-Loops can survive and hide from the DNA-repair machinery Furthermore, they can use the DNA-replication machinery to increase their length (De Lange 2015)(De Lange 2015). In this sense, more ALT mechanisms have been found across species (Lundblad & Blackburn 1993), with the common characteristic of being mediated by homologous recombination.

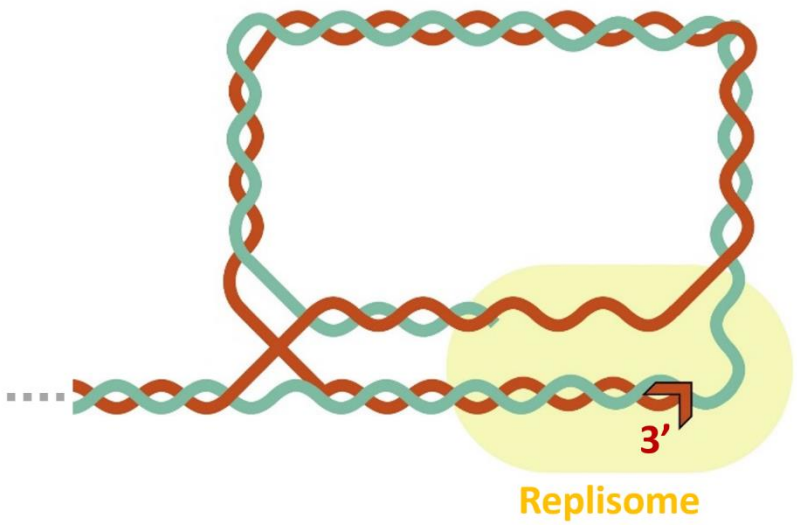

Figure 3. The end replication problem can be solved by the strand-invasion of the 3' overhang in the t-loop, providing a template for telomere lengthening.

In contrast to ALT mechanism, which can be considered as an adaptation of homologous recombination mechanisms, telomerase is a highly specific mechanism specifically developed to maintain or increase telomere length. The existence of such mechanism was predicted based on the evidence that telomeres were formed by the same hexamer in a high number of tandem repeats in the ciliated protozoan Tetrahymena Pyriformis (Karrer et al. 1976), other protozoans (Yao et al. 1981) as well as other eukaryotes. Later, Carol Greider and Liz Blackburn found an enzyme specialized on *de novo* synthesis of DNA at the end of linear chromosomes, the telomerase (Greider & Blackburn 1985).

Telomerase is a ribonucleoprotein functionally composed mainly by two subunits, the telomerase Reverse Transcriptase (TERT) and the Telomerase RNA Component (TERC). TERT and TERC form a tight

complex that is sufficient for *in vitro* synthesis of telomeric repeats (Bryan et al. 2003; Weinrich et al. 1997). TERT is closely related the reverse transcriptases of non-long-terminal-repeat (non-LTR) elements, which date back to the origin of eukaryotes and now can be found in many retrotransposons, including retroviruses and procaryotes (Nakamura & Cech 1998). Because TERC is used as a template for every hexamer synthesis, the use of telomerase promotes the homogeneity of telomeric sequences, allowing the use of a common set of sequence-specific proteins, known as the shelterin complex. Together, telomerase and the shelterin complex are able to finely regulate telomere dynamics and maintain telomere length.

TERT is the catalytic subunit of telomerase and is evolutionary more conserved than TERC (Sandin & Rhodes 2014). The protein contains three domains: 1) RNA binding domain, interacting with the region CR4/CR5 of TERC, 2) the catalytic reverse transcriptase (RT) domain, and 3) the C-terminal domain (CTE) which is the less conserved of the three. The role of TERT in telomere lengthening has been confirmed by different transgenic approaches. Mice homozygous for a null allele of TERT (TERT-/-) are viable, normal in size and do not display any gross physical or behavioral abnormalities, but do not exhibit any telomerase activity and exhibit telomere shortening (Yuan et al. 1999; Liu et al. 2000; Chiang, Hemann, et al. 2004). Homozygous intercrossing for multiple generations shortens their telomere to a point where telomere instability appears and infertility arises together with degenerative phenotypes in highly proliferative organs such as testes, intestine and bone marrow (Chiang, Kim, et al. 2004). In contrast the overexpression of TERT also in mice increases telomere length showing an improvement in the fitness of certain tissues, particularly epithelial barriers with a high cellular proliferation rate, but also increased risk of cancer appearance which shortens their average life expectancy (Artandi 2002; González-Suárez et al. 2001; González-Suárez et al. 2005). These findings show the dual role of telomeres in health: on one side, lengthy telomeres prevent from the premature loss of cellular proliferative capacity which would lead to premature senescence, but on the other hand high telomerase activity may predispose to cancer. To reconcile both sides of the same coin, a mouse model was generated in which the overexpression of TERT was combined with the overexpression of three tumoral suppressors (Sp53/Sp16/SArf) obtaining the highest increase (40 %) in the median life span reported to date (Tomás-Loba et al. 2008).

TERC is a non-coding RNA which not only acts as the template for telomere synthesis, but also is involved in the catalytic reaction of nucleotide incorporation, mediates the translocation process (Cristofari et al. 2007; Qiao & Cech 2008; Theimer et al. 2007), is involved in the assembly of telomerase complex (Robart & Collins 2010) and regulates telomerase activity (Cairney & Keith 2008). In contrast to TERT, TERC is poorly conserved across evolution, although its secondary structure is similar, containing three clearly identified regions: 1) the template region required for the synthesis of telomere repeats, 2) the adjacent element called pseudoknot, also required for the catalytic activity (Sandin & Rhodes 2014), and 3) a distal loop element (stem terminus element or STE) also called CR4-CR5, essential for TERT recognition (Mitchell et al. 1999). TERC role on telomere maintenance and lengthening was evidenced by the mouse deficient in TERC (TERC-/-), which was the first evidence of the role of telomerase in aging (Blasco et al. 1997). TERC-/- mice were normal for the first three generations, but, similar to TERT KO, telomeres shortened along successive mouse generations (Blasco et al. 1997). After telomeres were sufficiently short, mice suffered accelerated telomere shortening linked to premature ageing, decreasing both median and maximum longevity (Blasco et al. 1997). In particular, phenotype arose in tissues with high

turnover rates such as testicle, hematopoietic system, gastrointestinal tract and skin (Lee et al. 1998; Herrera et al. 2000; Hemann, Rudolph, et al. 2001).

Although TERT and TERC are sufficient for elongating telomeres *in vitro*, other factors are required for telomere lengthening in vivo. These factors are involved in the holoenzyme assembly, activity and cellular translocation (Smekalova et al. 2012). Among the other factors required, dyskerin is a RNA binding proteins recognizing the Hinge-hairpin-ACA (H/ACA) motif. Dyskerin (DKC1) is a protein conserved across evolution and is the third telomerase component more studied behind TERT and TERC (Cohen et al. 2007). Several other components that have been suggested to be involved in the active telomerase complex are indeed dyskerin associated proteins such as NOP10, GAR1 and NHP2 (Dragon et al. 2000; Kiss et al. 2006; Pogačić et al. 2000). The role of dyskerin in telomere homeostasis in humans is evidenced by the syndrome known as dyskeratosis congenita (DC), which can be caused by mutations in DKC1 gene itself or in genes encoding for proteins associated to dyskerin in the active telomerase complex such as NOP10, NHP2(Savage & Bertuch 2010) and TCAB1 (Dokal 2011). Dyskeratosis congenita is a rare disorder characterized by short telomeres and manifested by a highly variable phenotype that resemble premature aging, including premature bone marrow failure, defective stem cells reservoirs and increased tumor susceptibility (Heiss et al. 1998).

Role of telomeres in aging and disease

Although most somatic cells shorten their telomeres on each cell division in the absence of telomerase, immortal cell types such as stem cells, required for tissue regeneration in both physiological and pathological conditions, and cancer cells do express telomerase or, in some types of cancer, activate ALT mechanisms in order to maintain telomere length. Actually, telomere shortening in somatic cells has been proposed as a tumor suppression pathway that prevents chromosomal instability and tumor outgrow by establishing a replicative limit (de Lange 2004). On the other hand, as documented in the case of the TERT KO already mentioned, proper telomere length maintenance is critical for keeping the fitness of the tissues, especially those with a high proliferative length.

The link between abnormal telomere length and disease is difficult to establish due to the complex relations between telomere length and other molecular pathways. However, a clear linkage has been found for both short and long telomeres and a broad spectrum of diseases (Stanley & Armanios 2015). Patients exhibiting a critically short telomere often manifest developmental abnormalities, bone marrow failure syndrome, aplastic anemia, idiopathic lung fibrosis and liver cirrhosis, with the onset and severity depending on the extent of telomere shortening (Armanios 2009). Meanwhile, long telomeres have been linked to increased cancer risk, particularly melanomas and gliomas, highlighting the importance of senescence checkpoints for tumor suppression (Stanley & Armanios 2015).

Telomere maintenance across generations

The progressive shortening of telomeres along lifespan would imply a progressive shortening of telomeres across generations if the telomeres of the germline and embryonic cells shorten at each cell division as somatic cells do. In order to avoid intergenerational variations in telomere length, telomerase maintain telomere length in the germline and during embryogenesis.

Telomerase is active in male and female germline. Germ cells are the precursors of the gametes that will form the individual and therefore, appropriate telomere length segregation must be ensured to avoid telomere attrition across generations. Both sexes present a high telomerase activity in their germline experiencing a reduction in telomerase activity during gamete maturation (Achi et al. 2000; Eisenhauer et al. 1997; Wright et al. 1996). The differences in telomere length between male and female gametes have been a matter of controversy, but it seems generally accepted that spermatozoa chromosomes contain longer telomeres than those in oocytes (De Frutos et al. 2016), probably due to the differences in male and female gametogenesis (Siderakis & Tarsounas 2007), in the sense that male gametogenesis is a continuous process not detained at birth and thereby telomerase is continuously expressed in the testis.

Differences in gametes telomere length have been observed and it has been proposed that they may exert an influence on the telomere length of the offspring. This influence would be not driven by genetic loci and is in agreement to the non-Mendelian inheritance observed for telomere length. Thereby, telomere length inheritance would be governed by genetic components and by an epigenetic inheritance mediated by the different telomere length in the gametes.

The genetic component of telomere length inheritance is manifested by the association of several polymorphisms (SNPs) in TERT and TERC genes with telomere length. However, telomere length show non-mendelian inheritance: a preferentially paternal inheritance has been consistently observed by several post-hoc studies in humans (Atzmon et al. 2010; De Meyer et al. 2007). Furthermore, paternal age show a strong positive correlation with offspring telomere length (De Meyer et al. 2007; Eisenberg et al. 2012; Unryn et al. 2005) a finding that cannot be explained by the presence of different genetic variants. In this case, the effect of paternal age seems to be mediated by spermatozoa telomere length, which is longer in old men compared to young men (Allsopp et al. 1992; Baird et al. 2006; M. Kimura et al. 2008). Supporting the role of spermatozoa telomere length on telomere length inheritance, a recent study carried out in two mouse models differing in their spermatozoa telomere lengths (old vs young Mus musculus mouse and Mus musculus vs Mus spretus) observed that spermatozoa telomere length determined that of the offspring (De Frutos et al. 2016). The differences in telomere length between embryos produced with spermatozoa carrying long telomeres, short telomeres or produced by parthenogenetic activation (i.e. without any spermatozoa) appeared as early as the zygote stage, in the absence of telomerase and thereby likely involving ALT mechanisms (De Frutos et al. 2016).

Telomere lengthening during preimplantation development

After fertilization and syngamia, telomere lengthening mechanisms are required in order to avoid the progressive loss caused by the intense cell proliferation taking place during development. Studies in the mouse model suggest that both two mechanisms involved in telomere lengthening (i.e. telomerase and ALT) act during preimplantation development. Telomerase levels are low or absent in the mature oocyte and spermatozoon and thereby the increase in its activity can only be caused by the translation of newly synthesized RNA from the embryonic genome. Embryonic genome is transcriptionally inactive following syngamia and must be activated in a process named Embryonic Genome Activation (EGA). The transition from maternally inherited mRNAs to embryo-derived mRNA depends on finely tuned mechanisms of gene regulation including local modifications of DNA, changes on the chromatin structure and topological rearrangements of chromosomes along the nucleus (Mason et al. 2012). EGA timing varies depending of the species starting at the 2-cell stage in mouse embryos and at the 4-8 cell stage in humans or bovine (Braude et al. 1988; Crosby et al. 1988; Frei et al. 1989; Jarrell et al. 1991; Latham et al. 1991). Following EGA, all mammalian species studied show an increase in the expression of both main telomerase components (TERT and TERC) that correlates with an increase in their telomere length (Liu et al. 2007; Schaetzlein et al. 2004; Wright et al. 2001).

However, before the onset of telomerase expression, a sharp increase in telomere length occurs in the mouse model at the first embryonic cleavage, between zygote to two-cell stages. In the absence of telomerase, this increase occurs by ALT likely mediated by telomere sister-chromatid exchange (T-SCE) and associated to co-localization of the DNA recombination proteins Rad50 and TRF1 in early cleavage embryos (Liu, Bailey et al. 2007). This mechanism may explain why spermatozoa telomere length exert an effect on offspring telomere length (De Frutos et al. 2016), as they can act as a template or a guide to lengthen the shorter oocyte´s telomeres. An elegant study observed that tagged telomeres are inherited in a Mendelian way, therefore suggesting that telomeres are not copied from one chromosome to the other (Neumann et al. 2013). On that basis, the spermatozoa telomere should act as a guide rather than a template for lengthen oocyte telomeres. However, whether this mechanism exists in other mammalian species remains unclear.

After the first increase in telomere length mediated by ALT, a second telomere lengthening mediated by telomerase occurs at the morula-to-blastocyst stage, coinciding with the onset of telomerase activity (Ozturk et al. 2014). This second lengthening sets telomeres at a defined length that has been suggested to constitute the telomere reserve for the new-born, as after implantation the primary culture of cells derived from mouse embryos do not show a significant increase in the telomere length (Schaetzlein et al. 2004). Given the crucial role of this developmental timeframe on telomere length of the new born, which in turn has been involved in adult health, the study of the mechanisms involved in telomere lengthening dynamics during preimplantation development is important to understand the individual differences in telomere length, its inheritance patterns and the possible negative effects of Artificial Reproductive Techniques mediated by long term effects in the offspring.

Rationale of the study

Telomere lengthening program during preimplantation development has been documented on different species, but the results obtained seem to be species-specific and in many cases, there is no consensus within the same species. The laboratory mouse is by far the most studied mammalian model and it has been reported that MII oocytes starts with a mean telomere length around 16 kilobase (kb) pair, growing to 26 kb at the end of the one to two cell transition and ending with 37 kb at the blastocyst stage (Liu et al., 2007). The ALT mediated increase at the zygote stage has not been observed in a study in humans, where the average telomere length in MI oocytes (11 kb) decreases at the cleavage stage (8 kb) and significantly increases reaching blastocyst (13 kb), showing an U-shaped trend without significant differences in telomere length between GV oocytes and blastocysts (Turner et al. 2010). Bovine embryos, in contrast, did show a significant increase in the morula to blastocyst transition similar to that observed in mouse (Schaetzlein et al. 2004). In contrast, another recent study in bovine found no statistically significant difference between telomere lengths of 8-cell, morula and blastocysts (Gilchrist et al. 2015), in striking disagreement with the reference model (the mouse) and with the dynamics of telomerase expression in the same species.

Telomerase activity is only present in the morula to blastocyst transition, after the onset of the transcription of TERT and TERC in the mouse model (Liu et al. 2007), suggesting that the oocyte contain very little or no telomerase activity and that telomere lengthening occurring at the first embryonic division is mediated by ALT. In agreement, in mouse, telomerase null and wild type embryos did not differ in their telomere length before the blastocysts stage (Schaetzlein et al. 2004). Similar findings were obtained in human embryos, where despite a high telomerase activity was detected in fetal ovaries, three different unfertilized eggs showed no or very little telomerase activity in contrast to the blastocyst, showing a high telomerase activity (D. L. Wright et al., 2001). In bovine, it was observed that telomerase activity decreased during oocyte maturation and subsequent development through the 8-cell stage, experiencing a 40-fold increase in the morula-to blastocyst transition (Betts & King, 1999), which is compatible with the increase in telomere length observed on that stage by (Schaetzlein et al. 2004) but not to the lack of significant differences observed by (Gilchrist et al. 2015). However, this later study also found an increased telomerase activity in blastocyst compared to oocytes, cleavage stages or morulae (Gilchrist et al. 2015).

The discrepancies among studies may be caused by methodological pitfalls or to the wide species-specific differences. Regarding to the methodologies used to determine telomere length and telomerase activity, both determinations face the problem of the scarce biological material present in embryonic samples. The limited material challenges the detection limit of many techniques, such as the TRAP assay used in the mentioned studies to detect telomerase activity and the golden standard to determine telomere length, telomere restriction fragment (TRF), extremely difficult to apply to embryo samples. For this reason two techniques have been used to determine telomere length: quantitative fluorescence hybridization (qFISH) and real time PCR (qPCR).

qFISH relies on fluorescence signal emitted by a telomere-specific hybridization of a fluorescent probe and can be applied to single cells (Poon et al.. However, it is a semiquantitative technique that greatly depends on the tridimensional conformation of the chromosomes, which in the case of embryos is highly

variable due to the dynamic changes in epigenetic conformations occurring during preimplantation development. qPCR depends on the amplification of the telomeric sequence by primers specifically designed to produce a single PCR product out of the repetitive telomeric sequence (Cawthon 2002). In contrast to qFISH, the technique is not influenced by the changing epigenetic states occurring during preimplantation development and its high sensitivity allows the accurate determination of relative telomere length in embryonic samples. For these reasons and based on the experience of the laboratory with telomere length determination in embryos (Bermejo-Alvarez et al. 2008; De Frutos et al. 2016) we have chosen this technique to perform our analysis. In order to complement the study with an indirect measurement of telomerase activity we have used real time PCR coupled with retrotranscription to determine the dynamics of transcription of the main components of telomerase complex, using protocols previously validated for embryonic samples (Bermejo-Alvarez et al. 2010).

Finally, regarding to the species-specific differences, telomere length varies greatly between the laboratory mice, which exhibited extraordinarily long telomeres ranging 30 to 150 kb (Kipling & Cooke 1990; Starling et al. 1990; Hemann & Greider 2000) and other mammals such as humans (10-15 kb) (de Lange et al. 1990), bovines (~13 kb) (Garrels et al. 2012; Iqbal et al. 2011) and wild mice (8-10 kb) (Coviello-McLaughlin & Prowse 1997; Hemann & Greider 2000). These differences may have erased due to the intensive breeding in isolated colonies (Hemann & Greider 2000) and it may confer certain protection against telomerase deficiency. As already mentioned, the telomeres of the telomerase KO laboratory mouse (strains 129/C57BL/6J (Lee et al. 1998) and C57BL/6J (Hemann, Strong, et al. 2001)) are not critically short until the third or fourth generation being viable for at least 6 generation (Vidal-Cardenas & Greider 2009). In contrast, telomerase deficiency in mice on the wild CAST/EiJ genetic background, which exhibits telomere lengths comparable to humans, result in evident defects in tissue renewal appearing in the first generation of both TERC (Hao et al. 2005) of TERT (Strong et al. 2011) homozygous KO, with heterozygous KO (TERC+/- or TERT+/-) showing a progressive phenotype alterations across generations. The striking differences between laboratory mice and other mammals in telomere length may arise from different telomere dynamics during preimplantation development, so although the laboratory mouse has been the most studied model for mammalian telomere biology, it may not be the most suitable one to understand human telomere biology. Bovines exhibit a similar telomere length than humans and a preimplantation development closer to humans on timing and dynamics of epigenetic events than that of the mouse. For these reasons, and also because of being a relevant farm animal where artificial reproductive techniques involving embryo manipulation are routinely applied, we have chosen the bovine model for our study.

Objectives

The main objective of this thesis has been to determine telomere dynamics during preimplantation bovine development by pursuing the following specific objectives:

1) To determine by qPCR the dynamics of telomere lengthening during preimplantation development at the mature oocyte, zygote, 2-cell, 8-cell, morula and blastocyst stages.
2) Analyze the effects of TERT overexpression by microinjection of an expression plasmid at the zygote stage on the telomere length of bovine blastocysts.

MATERIALS AND METHODS

In vitro Production (IVP) of Bovine Embryos

The bovine *in vitro* production protocol consists in 3 differentiated steps: 1) oocyte recovery and *in vitro* maturation, 2) *in vitro* fertilization and 3) *in vitro* culture.

OOCYTE RECOVERY AND *IN VITRO* MATURATION (IVM)

Bovine ovaries are collected from heifers and cows at a local slaughterhouse, immediately after removal of the internal organs, in the abattoir chain. The ovaries are transported inside a plastic bag containing 0.9% sterile saline solution supplemented with 1% gentamycin, inside a thermos bottle with water at temperatures ranging 34 ºC to 38 ºC. Once in the lab, ovaries are washed once in water at 37 ºC and twice in saline at 37 ºC and kept in a flask containing saline in a water bath at 37-38ºC, while waiting to be processed. Cumulus-oocyte complexes (COCs) recovery is performed by aspiration of 2-8 mm follicles with an 18 gauge sterile needle attached to a 5 ml syringe, keeping a constant negative pressure. Follicular liquid presumptively containing the COCs is gently poured to a 50 ml sterile flask that is kept in a water bath at 37-38 ºC until all the ovaries of the batch are processed. Ten minutes after pouring the last content into the flask, the supernatant is removed using a sterile disposable Pasteur pipette inside a laminar-flow cabinet. The precipitate, containing the COCs and follicular debris and a minimum follicular liquid, is resuspended with PBS at 39 ºC and poured in sterile petri dishes. Under a stereoscope, COCs are recovered and selected based on the following criteria:

Type	Characteristics	*In vitro* production suitability
1	Compact multilayered cumulus investment; homogeneous ooplasm; light and transparent	Yes
2	Compact, but less than 4 cumulus cells layer. Darker zone at the oocyte periphery	Yes
3	Denuded oocyte	No
4	Expanded cumulus investment, degenerated pyknotic cytoplasm	No

Table 1. Morphological criteria for bovine cumulus-oocyte complexes (COCs) selection for *in vitro* production (IVP)

Only grade 1 and 2 COCs are washed twice in PBS and once in *in vitro* maturation media before being placed in groups of ~50 per well in 4-well NUNC® dishes, each well containing 500 µl of *in vitro* maturation media. COCs are allowed to mature for 24 hours at 39 ºC in a humidity saturated 5% CO_2 atmosphere.

IN VITRO FERTILIZATION (IVF)

After 24 hours of IVM, matured oocytes are washed twice in tempered PBS and once with the *in vitro* fertilization (IVF) medium, and transferred to a sterile 4 well NUNC plate with 250 µl IVF medium. Frozen semen from a proven high fertility bull is processed with BoviPure®, according to the protocol provided by Nidacon, in order to discard dead spermatozoa. Briefly, a two layer gradient of BoviPure® is made by adding a 40% concentration layer over an 80 % concentration layer in a 15 ml sterile plastic tube. A semen straw, stored in liquid nitrogen is thawed in a 37 ºC water bath, poured gently at the very top of the gradient and centrifuged for 10 minutes at 1000 g. Carefully and keeping the pellet intact, the supernatant is extracted and the remaining pellet is washed with 1 ml BoviWash® and centrifuged for 5 more minutes. Carefully, the supernatant is removed until 300 µl are left in the tube. The pellet is homogenized and 5 µl are transferred to an Eppendorf tube containing 95 µl distilled water to determine spermatozoa concentration. In order to have a final concentration of spermatozoa of 1 million spz/ml, the purified spermatozoa are diluted with *in vitro* fertilization medium to 2 million spz/ml and 250 µl are added to the well containing the matured COCs. Fertilization takes place at 39 ºC in a water saturated atmosphere of 5 % CO_2.

IN VITRO CULTURE (IVC)

Presumptive zygotes are washed once in PBS and once in IVC medium (HSOF + 5 % Fetal Calf Serum (FCS)) after cumulus cells removal by vortex in PBS. Groups of 25 presumptive zygotes are transferred into 25 µl culture medium micro droplets under a 3 ml layer of mineral oil (NidOil®) in 35 mm cell culture dishes (Corning), and kept in incubator at 39 ºC, 5% 02, 5% CO2 and maximum humidity for 8 days, with development evaluation at 2, 6, 7 and 8 days post-fertilization.

Average Relative Telomere Length Quantification

For the first objective, bovine embryos were produced *in vitro* as previously described and telomere length was analyzed in 20 samples per stage: matured oocytes (8/sample, collected after IVM), zygotes (collected at 18 hours post insemination –hpi-, 8/sample), 2-cell embryos (collected at 32-34 hpi, 4/sample), morulae (collected at 125 hpi, 1/sample) and blastocysts (collected at 200 hpi, 1/sample). Zona pellucida was removed by incubating the embryos in a 0.5 % pronase solution in PBS in order to improve subsequent embryo digestion and to eliminate spermatozoa bound to the zona pellucida. Immediately after zona pellucida removal, embryos were stored in PCR tubes and frozen at -80 ºC until sample analysis. Samples were digested in 8 µl of a 100 µl/ml proteinase K buffered solution for 1 h at 65 ºC and proteinase K was inactivated by incubating at 95 ºC for 10 min. Average relative telomere

length was measured by quantitative real-time PCR, according to the protocol reported in (Cawthon, 2003) with some minor modifications (Bermejo-Alvarez et al. 2008). Briefly, TL is determined by contrasting the amplification of the telomeric sequence to the *Rn18S* genomic sequence, which served as an internal control relative to the amplification of the telomeres to the total DNA present in the lysate. The specific sequence and the product length of the primers used for amplification is detailed in Table 2. The qPCR was performed in a Rotorgene 6000 Real Time Cycler (Corbett Research), incubating for 3 min at 94 ºC, followed by 40 cycles of 10 sec at 94 ºC, 30 sec at 60 ºC and 30 sec at 72 ºC. Quantification was performed by the comparative Ct Method (Schmittgen & Livak 2008): Fluorescence was acquired in each cycle to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background for each sample. Within this region of the amplification curve, a difference of one cycle is equivalent to doubling of the amplified PCR product. According to the comparative CT method, the ΔCt value was determined by subtracting the *Rn18S* Ct value to the *Telomere* CT value for each sample. For representation purposes, ΔΔCt was calculated by normalizing each Ct value to the highest Ct observed value (i.e. the lowest relative telomere length). Fold changes in the relative telomere length were determined using the formula $2^{-\Delta\Delta Ct}$.

Gene	Primer sequence (5´-3´)	Fragment Size, bp	Gene Bank Accession No.
Telomere	F:CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT R:GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT	79	NT_039202.7
Rn18s	F:AGAAACGGCTACCACATCCAA R:CCTGTATTGTTATTTTTCGTCACTACCT	91	NR_003278.1
Mouse Tert	F: GGATTGCCACTGGCTCCG R: TGCCTGACCTCCTCTTGTGAC	279	NM_009354.1

Table 2: Details of primers used for telomere length determination and plasmid injection verification

TERT Overexpression

GENERATION OF PLASMIDS FOR MOUSE *TERT* AND *EGFP* EXPRESSION IN MAMMALIAN CELLS

For the second objective, a plasmid for exogenous TERT expression was generated by cloning the complete mouse *Tert* cDNA sequence into the *Eco*RI site of the pCAGGs vector (Hitoshi et al. 1991). The plasmid contains the CAG promoter, a strong synthetic promoter widely used in mammals. The CAG promoter contains the cytomegalovirus (CMV) early enhancer element (C), the promoter, first exon and first intron of the chicken β-actin gene (A) and the rabbit β-globin (G) splice acceptor. As a microinjection control, a commercial plasmid (pEGFP, Clontech) that codes for the enhanced green fluorescent protein (EGFP) was also used. Plasmids were multiplied by transforming DH5α competent bacteria. Briefly, ~50 ng of plasmid-containing solution is added an aliquot of competent *E. coli* previously thawed in ice for five minutes. After 30 min incubation in ice, a 30 seconds 42 ºC heat-shock follows. Bacteria is allowed to recover in ice for 5 min and 500 µl SOC media is added before 1 hour 37 ºC shaking incubation. The aliquot is centrifuged at 800 g for 7 min, bacteria are plated into selective (ampicillin) LB agar plates and incubated overnight at 37 ºC. In order to recover the plasmids, a MiniPrep was performed using the FavorPrep™ Plasmid Extraction Mini Kit, accordingly to the manufacturer's instructions (FAVORGEN). Plasmids were purified by phenol-chloroform extraction followed by isopropanol precipitation.

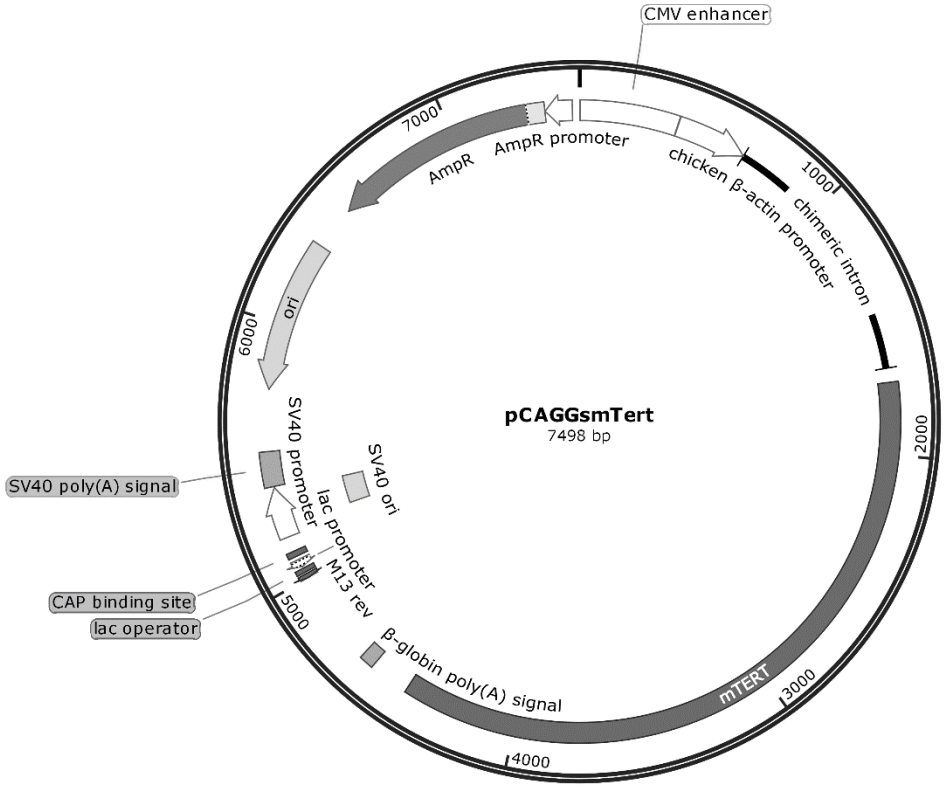

Figure 5. Schematic representation of pCAGGsmTert.

MICROINJECTION

In order to achieve a TERT overexpression before embryonic genome activation, we microinjected the CAG TERT plasmid in bovine zygotes. As a control, other group was microinjected with pEGFP, a commercial plasmid that codes for the enhanced green fluorescent protein (EGFP). Prior to microinjection, the holding pipette and microinjection pipettes need to be crafted. The holding pipettes were made from TW100-6, thin-wall capillaries, 0.6”” 1.0 mm (World Precision Instruments) in a P-97 Pipette Puller (Sutter Instrument CO.) under a program with the following settings: Heat 800, Pull 75, Velocity 130, Time 100. The pipette was broken by heat and fire-polished in a microforge (*Microforge de Fontbrune BEAUDOIN 5262*) to obtain an internal diameter of around 50 µm. The microinjection pipettes were made from TW100F-4 Glass thin 1.0 mm 4 inches with an internal filament (World Precision Instruments) in the same Pipette Puller, but with different settings (Heat 775, Pull 150, Velocity 90, and Time 120). The working drop was made by placing 20 µl of PBS at the center of a microscope slide and covering it by enough mineral oil.

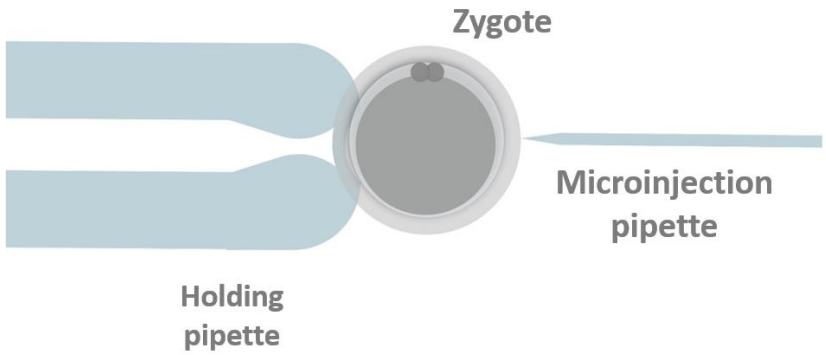

Figure 6. Representative image of the microinjection setting for cytoplasmic injection.

Following microinjection, embryos were washed in IVC medium and transferred into IVC dishes for culture at 39 ºC, 5% 02, 5% CO2 and maximum humidity. Blastocysts were recovered at day 7 and processed to analyze relative telomere length as previously described for objective 1. Proper delivery of the plasmid was analyzed in twelve blastocyst obtained after zygote injection of pEGFP and 9 blastocysts obtained after injection of pCAGGsmTert. Embryos injected with EGFP were tested positive by detecting EGFP by epifluorescence microscope. Embryo injected with pCAGGsmTert were tested by a PCR specific to the mTert sequence following the digestion protocol previously described for telomere length analysis. Telomere length was analyzed as previously described.

Statistical analysis

Data were analyzed using the SigmaStat (Jandel Scientific, San Rafael, CA, USA) software package. One-way analysis of variance (ANOVA) was performed to analyze differences between groups.

RESULTS AND DISCUSSION

Objective 1:
Telomere length dynamics during bovine preimplantation development

Relative telomere length was analyzed before fertilization (oocytes), between fertilization and embryonic genome activation –EGA- (zygotes and 2-cell embryos) and after EGA (morulae and blastocysts), Fig. 6. Oocytes contained the shortest telomeres of all the stages analyzed (1±0.15, mean±standard error of the mean). After fertilization telomeres seem to be longer than in oocytes (1.4±0.17, meaning ~1.4 times longer than those of the oocytes based on $2^{-\Delta\Delta Ct}$), but this increase was not significantly different. Two cell embryos contained similar telomeres to those of oocytes or zygotes (1.13±0.1). After EGA, a statistically significant increase in telomere length was observed at the morula stage (2.3±0.33) followed by a sharper increase in the morula to blastocyst transcription (10.37±1.37).

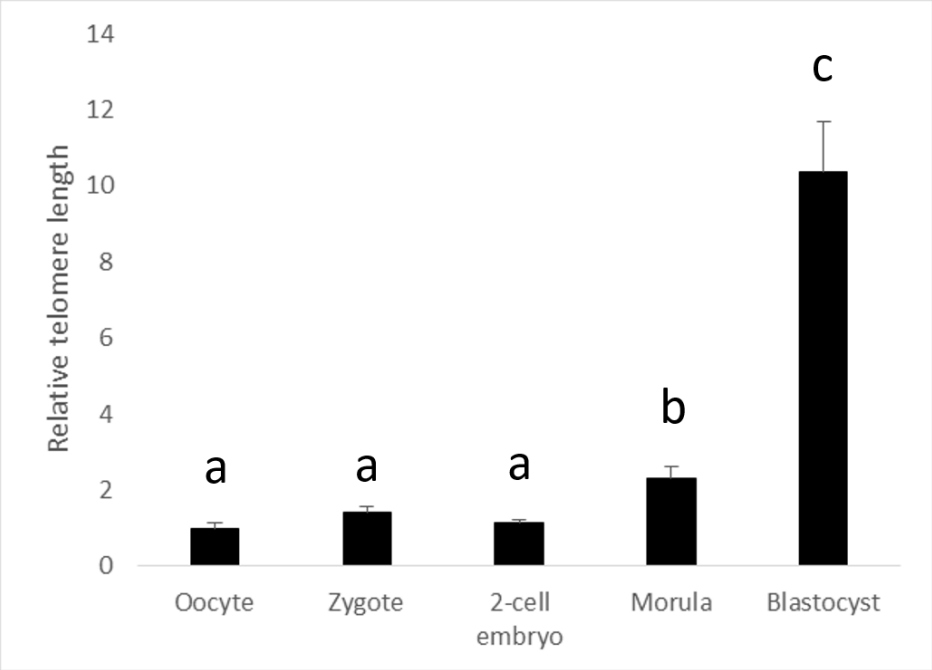

Figure 7. Relative telomere length before fertilization (oocytes), between fertilization and EGA (zygotes and 2-cell embryos) and after EGA (morulae and blastocysts). Different letters indicate statistical differences based on ANOVA ($p<0.05$).

These results suggest that, in contrast to the mouse model (Liu et al. 2007), there is not a significant increase in telomere length before EGA. Right after fertilization, a small increase in telomere length was noticed, but was not statistically significant. This small numeric increase may be the consequence of the

spermatozoa telomeres being longer than those of the oocytes, as reported for the mouse model (De Frutos et al. 2016). However, in the absence of statistical significance and without conducting a study to determine the differences between gametes we cannot test that hypothesis. The absence of an increase in telomere length in the transition from zygote to the 2-cell embryo suggests that, in contrast to the mouse model (De Frutos et al. 2016; Liu et al. 2007) ALT mechanisms do not elongate telomeres before EGA. After EGA, a significant increase was noted in the morula stage concluding with a sharp telomere lengthening occurring in the morula-to-blastocyst transition, resulting in telomeres ~10 times longer than those at the oocyte stage. These findings are in agreement with previous findings that observed a sharp increase in telomerase activity at the blastocyst stage in different mammalian species, including bovine (Liu et al. 2007; Schaetzlein et al. 2004; Wright et al. 2001). In agreement with our results, telomere length detected by qFISH has been observed to significantly increase from the morula to the blastocyst stage (Schaetzlein et al. 2004) although the lengthening was less evident than in our study (~14 kb in morula vs ~20 kb in blastocyst). The numeric differences between studies may be caused by the different techniques used to determine telomere length. In particular, qFISH relies on the intensity of a fluorescent probe against the telomeric sequence and that intensity does not only depend on telomere length, as the conformation of telomeres and subtelomeric regions do affect probe accessibility and thereby fluorescence intensity. In this perspective, qFISH tend to underestimate telomere length differences, as an example, human spermatozoa telomere length quantified by qFISH (Turner & Hartshorne 2013) are half of those obtained using telomere restriction fragment (TRF) analysis (Baird et al. 2006; K. Kimura et al. 2008; Kozik et al. 1998; Pickett et al. 2011), and in mouse embryos telomere length analyzed by qFISH yielded inconsistent results depending on the strain, in contrast to qPCR (Liu et al. 2007).

A recent study using qPCR is the only article published so far analyzing telomere lengthening in bovine embryos from the oocyte to the blastocyst stage (Gilchrist et al. 2015). In contrast to our study, they observed no significant differences between oocytes, zygotes, 2-cell, 4-cell, 8-cell, morula and blastocyst stages, even though they pointed a numeric increase from ~0.9 arbitrary units in the zygote to ~2.4 in the blastocysts. Although the tendency was similar to our study, the lack of significant differences between stages and the higher standard errors obtained by these authors may be explained by the reference sequence used to contrast the CT obtained for the telomeric sequence to the amount of DNA. In particular, these authors used a single copy gene (ZAR1) whose CT levels are very far from those of the Telomere sequence, which exponentially enhance the calculation errors following $2^{-\Delta\Delta Ct}$. Furthermore, a single copy gene contains too few copies in the samples analyzed containing pools of maximum 15 single zygotes (i.e. 30 copies in the total lysate) for a reliable PCR amplification. This approach can be used when a significant number of genomes is present in the sample (such as in blastocysts (Bermejo-Alvarez et al. 2008)), but a multicopy sequence should be used for earlier stages to avoid these problems (De Frutos et al. 2016).

Objective 2.
Analysis of telomere length in embryos injected with a plasmid expressing *mTert*

Microinjection of bovine zygotes resulted in successful delivery of the plasmids. Embryos injected with pEGFP showed green fluorescence that was maintained until the blastocyst stage (Fig. 8A), as expected, no embryo injected with pCAGGsmTert showed green fluorescence. The presence of mTert sequence was detected by PCR in all 9 blastocyst derived from zygotes injected with pCAGGsmTert, whereas no mTert sequence was detected in the pEGFP injected group. However, telomere length was similar between both groups (Fig. 7B). This result suggests that the exogenous expression of mouse *Tert* does not elongate telomeres during bovine preimplantation development.

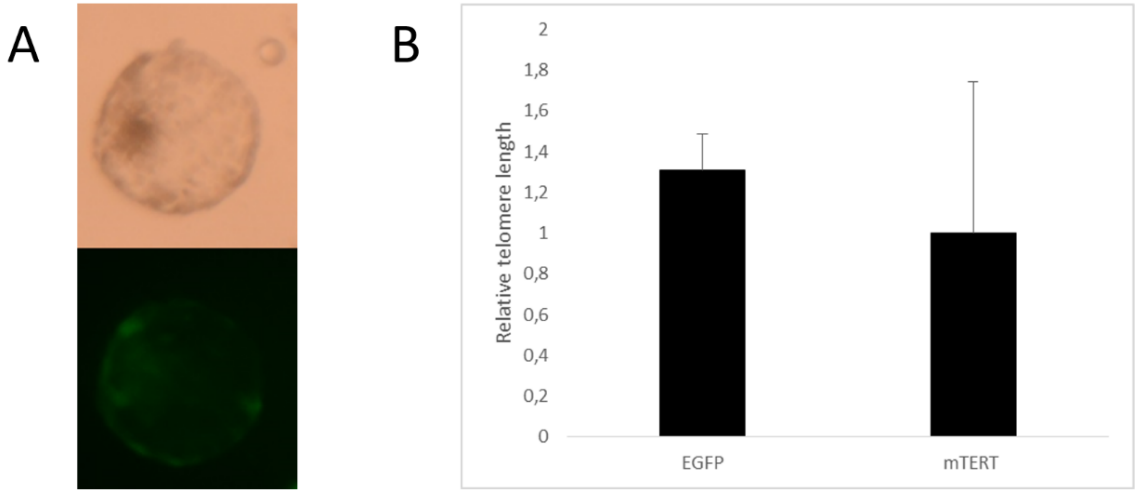

Figure 8. Effects of microinjection of the mTERT and pEGFP expression plasmids on blastocyst at 200 hpi. (**A**) Photographs of a bovine blastocyst microinjected with pEGFP in an epifluorescence microscope under white light (above) and epifluorescence (below). (**B**) Relative telomere length determined by qPCR in blastocysts derived from zygotes injected with either pEGFP or CAGGsmTERT (1 ± 0.741). The differences between groups were not significant based on ANOVA ($p>0.05$).

Transfection of *TERT* expression constructs in telomerase-negative cells elongates telomeres avoiding senescence (Bodnar et al. 1998; Yang et al. 1999). In contrast, TERC is ubiquitously and constitutively (i.e. nonregulated) expressed in most somatic cells which are indeed telomerase negative (Artandi 2002; Fu et al. 2003) strongly suggesting that *TERC* is not a limiting factor for telomerase activity. However, in our study, the exogenous expression of mouse *Tert* in bovine embryos did not increase telomere length. Several reasons may explain the absence of an effect on telomere lengthening. First, it is possible that mouse *Tert* do not recognize the endogenous bovine *Terc* or other proteins involved in the active telomerase complex. However, TERT is evolutionary conserved across species (Sandin & Rhodes 2014)

and the exogenous expression of human *TERT* has been effective to elongate telomeres in rabbits (Xiang et al. 2000) and bovine (Iqbal et al. 2011). In particular, it has been reported that the injection of a plasmid encoding for human *TERT* in bovine zygotes resulted in increased telomerase activity and telomere length at the blastocyst stage (Iqbal et al. 2011). The same group also observed that the exogenous expression of human *TERC* alone was able to increase telomere length, although they failed to observe a significant increase in telomerase activity (Garrels et al. 2012). The later finding is surprising as TERT is supposed to be the limiting factor for telomerase activity, being TERC ubiquitously expressed (Chiang, Hemann, et al. 2004). TERT protein sequence and motifs are very similar between mouse, human and bovine, but slight changes may have impeded to form an active telomerase complex with bovine proteins. Another possibility that may explain the absence of differences is that telomere lengthening mechanisms during preimplantation development may be tightly regulated to a reach a maximum length which cannot be modified by the exogenous expression of telomerase components.

CONCLUSIONS

1) During bovine preimplantation development telomeres elongates after embryonic genome activation, doubling its length at the morula stage and ending at the blastocyst stage with telomeres 10 times longer than those of the oocyte.
2) The zygote injection of a plasmid encoding for mouse *Tert* does not increase telomere length in bovine blastocysts.

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