Species-Specific Telomere Length Differences Between Blastocyst Cell Compartments and Ectopic Telomere Extension in Early Bovine Embryos by Human Telomerase Reverse Transcriptase

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ABSTRACT

The enzyme telomerase is active in germ cells and is critically involved in maintenance of telomere length in successive generations. In preimplantation mammalian embryos, telomerase activity is present from the morula stage onward and is associated with an increase in telomere length in blastocysts. Herein, we show that telomere length regulation in murine and bovine blastocysts differed between trophectodermal and inner cell mass cells in a species-specific manner. Ectopic expression of human telomerase reverse transcriptase (TERT) in bovine embryos increased telomerase activity and in turn increased telomere length. Transient expression of human TERT could be targeted to the 4-cell to morula stages and to the morula to blastocyst stages using unmodified and cytosine-methylated expression plasmids, respectively. Introduction of human TERT constructs in bovine embryos resulted in functional telomerase expression and effective telomere elongation, allowing us to study the effects on embryonic development. Ultimately, these studies may lead to a large-animal model for telomere regulation and aging.

cytoplasmic injection, inner cell mass, qFISH, telomerase, trophectoderm, zygote

INTRODUCTION

The ends of mammalian chromosomes are insulated by telomeric DNA, which is a highly repetitive sequence of hexameric TTAGGG repeats [1, 2]. Telomere length is restored by a reverse transcriptase called telomerase, which was first discovered in Tetrahymena [2, 3]. In humans, it consists of a 127-kDa catalytic protein component known as telomerase reverse transcriptase (TERT) and an RNA subunit (TERC) that acts as a template for telomere extension [4, 5]. The DNA and protein sequences of TERT and TERC are highly conserved, and telomerase from all mammals shares striking sequence homology [6].

The telomeres of somatic cells progressively shorten during successive mitotic cell divisions due to incomplete end replication [7, 8], and cultured cells reach senescence after a certain number of divisions. Telomere shortening limits the regenerative capacity of cells and is correlated with onset of cancer, aging, and chronic diseases [9–11]. Telomerase expression is reactivated in most human cancerous cells [11]. Within nontumorigenic cells, telomerase activity is usually found in the germ line in regenerative tissues with proliferative capacity [12–14] and preimplantation embryos [15–17]. In early embryos, the first telomere elongation is telomerase independent and occurs at the first cleavage by recombination events [18], followed by telomerase-dependent telomere elongation at the morula to blastocyst transition [19].

Introduction of the human TERT protein catalytic subunit into telomerase-negative rodent and human cells reconstitutes telomerase activity, bypasses senescence, and immortalizes cells, accompanied by telomere length elongation [20–24]. Because TERC is ubiquitously expressed in most cells, ectopic expression of TERT results in a functional telomerase holoenzyme [4, 5]. The effects of ectopic expression of human TERT have been studied in the laboratory mouse model [25–28], which has significantly longer telomeres (50–150 kilobase [kb]) than humans (10–15 kb) [29, 30]. The excessively long murine telomeres maintain viability of the animals after Tert gene knockout for at least six generations, when gross abnormalities first become apparent [31, 32]. In contrast to laboratory mice, telomere length in bovine cells (12–23 kb) is largely similar to that of human cells, rendering Bos taurus an alternative model for studying telomere regulation in human aging, regeneration, and carcinogenesis. In addition, bovine preimplantation development, physiology, life span, pathology, and genomic structure closely resemble those of humans, making B. taurus an important model organism [33–36]. Previously, it was shown that ectopic expression of human TERT in bovine lens epithelial and microvascular endothelial cells reconstitutes functional telomerase activity [37, 38]. Herein, we introduced human TERT in early bovine zygotes and investigated the effects of human telomerase expression on telomere length and preimplantation embryonic development as a first step toward the production of cattle with modulated telomere length.

MATERIALS AND METHODS

Collection and In Vitro Culture of Bovine and Murine Embryos

Bovine zygotes were produced as previously described [39]. In brief, bovine ovaries were collected at a local abattoir and cumulus-oocyte complexes...
(COCs) were released by slicing. In vitratummaturation, groups of 15 to 20 COCs were cultured in 100 µl of tissue culture medium (TCM) 199 supplemented with 10 IU of equine chorionic gonadotropin (eCG) and 5 IU of human chorionic gonadotropin (hCG) (Intervet) and 0.1% bovine serum albumin (BSA) (Sigma-Aldrich) under silicone oil in a humidified atmosphere composed of 5% CO₂ in air at 39°C for 24 h. Matured COCs were fertilized in vitro using semen from one bull of proven fertility. Following fertilization, cumulus cells were removed by vortexing.

Female NMRI mice were superovulated with an injection of 10 IU of eCG, followed by an injection of 10 IU of hCG 46 h later and were allowed to mate with males of the same strain. Blastocysts were collected on Day 3.5 after fertilization by flushing the excised uterus with PBS supplemented with 0.3%

### Amplification of Expression Plasmids and In Vitro Methylation

Ultrapure plasmids for microinjection were isolated from XL-10 bacteria grown in Luria-Bertani medium supplemented with antibiotics by ion-exchange columns. The pcDNSec-human TERT and pEGFP plasmids encoded human TERT and EGFP, respectively, under transcriptional control of the cytomegalovirus (CMV) immediate-early promoter (Supplemental Fig. S1, available at www.biolreprod.org). The construct pChrng-EGFP encoding EGFP under control of a muscle-specific promoter of acetylcholine receptor was used for mock injections. The DNA was incubated with CpG methylase (SssI) in the presence of 5'-adenosyl methionine to induce DNA methylation at cytosine residues of CpG dinucleotides. Completeness of CpG methylation was verified by treatment with the methylation-sensitive restriction enzyme MspI and the nonsensitive isoschizomer Msp2.

### Cytoplasmic Microinjection of Expression Plasmids

Cumulus-denuded presumptive zygotes were injected with purified plasmids. The plasmid mixture (10 fg/pI) was prepared in injection buffer (10 mM Tris-HCl and 0.25 mM edetic acid [pH 8.0]) and backfilled in glass injection pipettes [40]. Zygotes were microinjected in TCM-air medium containing Hepes. Individual zygotes were gently sucked to the holding pipette, and approximately 10 pl of plasmid solution was injected into the cytoplast using a transjector (5246; Eppendorf) at 320× magnification. In approximately 20% of the microinjected zygotes, signs of lysis were observed, and these zygotes were discarded. Bovine embryos were kept in culture for 8 days. As controls, either untreated embryos (culture control) or embryos injected with pEGFP (CMV promoter-EGFP), pChrng promoter-EGFP (nonexpressing construct in embryos), or buffer (sham controls) were kept under identical conditions.

### Monitoring Fluorescence

A microscope (Axioskop 35M; Zeiss) equipped with fluorescence optics for EGFP (band-pass filter, 460–490 nm; dichroic mirror, 505 nm; and barrier filter, 515 nm) was used for fluorescence microscopy of intact embryos. Images were recorded on film (Ektachrome 320T; Kodak) with a camera (167MT; Contax). EGFP fluorescence signals were normalized by a fixed exposure time of 10 sec. Quantitative fluorescence in situ hybridization (qFISH) fluorescence signals were detected with UPlanFl 100× oil objectives on a fluorescence microscope (BX60F-3; Olympus) equipped with a filter block containing an excitation filter of 530 to 550 nm, a DM570 dichroic mirror, and a barrier filter of 590 nm. Images were recorded with a camera (DP71; Olympus) and processed with commercially available software (Cell®P; Olympus).

### Reverse Transcription-Polymerase Chain Reaction

Poly (A) RNA was prepared from pools of eight embryos using a kit (Dynabeads mRNA DIRECT Kit, 610.11; Dynal-Invitrogen) as previously described [41]. Reverse transcription was carried out in a total volume of 20 µl. As negative controls, the reaction was performed without reverse transcriptase. For discrimination between human and bovine TERT, species-specific intron-spanning PCR primers were designed from the coding regions of each gene sequence using a software program Oligo (http://www.olygo.net/). Sequences of the primers, annealing temperatures, and fragment sizes are summarized in Table 1.

### Separation of Inner Cell Mass and Trophodermal Cells

A differential cell release by lysis solution (0.01 N HCl-0.1% Tween 20) was used to isolate inner cell mass (ICM) and trophodermal (TE) cells in blastocysts. The TE cells were separated from the blastocyst under microscopic control, and the ICM aggregate was transferred into a new microdrop on the same glass slide. Purity of the resulting cell fractions was verified using blastocysts stained by the double-dye method [42].

### Quantitative Fluorescence In Situ Hybridization

Quantitative FISH analysis was performed as previously described [19, 43]. For quantification of the telomere length, 20 to 30 nuclei of each embryo were evaluated. The telomere spot fluorescence intensities from each nucleus were measured and analyzed. The digital images were recorded using a camera (DP71; Olympus) on a fluorescent microscope (BX60; Olympus) equipped with a multiple filter wheel; slides were excited with Cy-3 filter. Microscope control and image acquisition were performed using a software package (Cell®P; Olympus). Images were acquired using a 100× oil immersion objective (UPlanFl, NA 1.30; Olympus). Fluorescence images were taken at 100 milliseconds (ms) for mouse telomeres and at 500 ms for bovine telomeres. This precludes a direct comparison of murine and bovine data in Figure 1. All other camera settings remained identical. Quantification of the telomere fluorescence intensities was performed using a software package (version 2.0; TFL-TELO) [44]. The software-mediated quantification of interphase qFISH spots by TFL-TELO is standardized and automatically eliminates out-of-focus signals and questionable signals. The expected number of chromosome ends in bovine cells is 120 (60 × 2). A reduced number of chromosome ends can be found in interphase qFISH nuclei, which is attributed to out-of-focus telomere ends that are excluded from analysis. We usually recorded several qFISH interphase nuclei per embryo. Typically, more than 50,000 data points were measured and calculated per sample.

To allow consistent images for day-to-day comparison, a batch of slides with spreads of human 293 cells was prepared, and one slide with human 293 cells was processed on each experimental day in parallel with the embryonic samples and used for normalization.

### Verification of qFISH by Real-Time PCR

For verification of qFISH data, four cell lines were selected (bovine tumor cell lines GM7373 and MDBK, adult bovine fibroblasts in passage 3, and adult bovine fibroblasts in passage 11) for simultaneous telomere length measurement by real-time PCR and in q-FISH. GM7373 is a bovine tumor cell line of endothelial origin, and MDBK cells originate from adult bovine kidney. Both tumor lines were obtained from the Veterinary Cell Bank, Friedrich-Loeffler-Institut. For q-FISH, two slides per group and 20 nuclei each were measured (as already described). Data were logarithmically transformed (log T) to improve approximation to a gaussian curve, and the mean telomere length was determined. Real-time PCR was performed on a commercially available system (ABI 7500 Fast Real-Time System; Applied Biosystems) in 96-well plates.
using genomic DNA. The real-time PCR protocol was based on previous publications [45, 46], with minor modifications. Each 20-μl reaction included 10 μl of master mix (2× Power SYBR Green Master Mix; Applied Biosystems), 300 nM each of the two primers, and 8 ng of genomic DNA, with three replicates for each sample. Primer sequences, annealing temperature, and fragment size used in real-time verification experiments are given in Table 2. Denaturation and activation of Taq polymerase were achieved for 10 min at 95°C, followed by 35 cycles of 95°C for 15 sec, followed by annealing, elongation, and data acquisition at 60°C (for telomere PCR) or 60°C (for single-copy gene PCR) for 1 min. For standard curve calculation, genomic DNA from the MDBK cells was serially diluted from 40 to 0.064 ng per well. Standard curves were generated using commercially available software (Sequence Detection Software 1.4; Applied Biosystems) and were transferred to a spreadsheet (Excel; Microsoft, Redmond, WA) for analysis.

The mean telomere length ratio was calculated by quantifying telomeric DNA, and dividing the amount by the quantity of the bovine-specific single-copy gene [45, 46]. The calculation was performed according to the relative standard curve protocol.

**Determination of Telomerase Activity Using Real-Time Quantitative Telomeric Repeat Amplification Protocol**

Bovine embryos and human 293 cells were lysed and then analyzed by real-time quantitative telomeric repeat amplification protocol (qTRAP) assay. An immortalized human embryonic kidney cell line (human 293 cells) served as a telomerase-positive cell sample. The human 293 cells were cultured in Dulbecco modified Eagle medium with 10% fetal calf serum, trypsinized, counted, and used to prepare six dilutions in 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS) lysis buffer. In total, 10° cells were sedimented, and the cell pellet was resuspended in 200 μl of CHAPS lysis buffer, incubated on ice for 30 min, and then centrifuged for 20 min at 4°C at 16,000 × g. One batch of cells was prepared, and aliquots of the supernatant were stored at −80°C.

Pools of 10 blastocysts were resuspended in 10 μl of CHAPS lysis buffer and were incubated for 30 min on ice. After centrifugation at 16,000 × g at 4°C for 20 min, the supernatant was transferred to a new tube. Two microliters of each sample was transferred to a separate tube for heat inactivation (85°C for 2 min) as a negative control. The master mix for the PCR reaction was prepared, and 24 μl was added to each well sample of a 96-well PCR plate. Each sample was analyzed in triplicate. A 1-μl sample was added to each well to bring the total volume to 25 μl per well. The 96-well plate was incubated at 25°C in the dark for 25 min. The PCR was performed on a commercially available machine (ABI 7500 Fast; Applied Biosystems) using the standard protocol (i.e., 95°C for 10 min, 40 cycles at 95°C for 15 sec, and 60°C for 60 sec) and the TS primer (100 ng/μl) 5′AAT CCG TCG AGC AGA GTT and the ACX primer (100 ng/μl) 5′GCG CGG CTT ACC C8 8 bp GAGCAATGGGAAAGGCTTC −ATGCACTCGGAAAGGCAAG with three replicates for each sample. Primer sequences, annealing temperature, and fragment size used in real-time verification experiments are given in Table 2. Denaturation and activation of Taq polymerase were achieved for 10 min at 95°C, followed by 35 cycles of 95°C for 15 sec, followed by annealing, elongation, and data acquisition at 60°C (for telomere PCR) or 60°C (for single-copy gene PCR) for 1 min. For standard curve calculation, genomic DNA from the MDBK cells was serially diluted from 40 to 0.064 ng per well. Standard curves were generated using commercially available software (Sequence Detection Software 1.4; Applied Biosystems) and were transferred to a spreadsheet (Excel; Microsoft, Redmond, WA) for analysis.

The mean telomere length ratio was calculated by quantifying telomeric DNA, and dividing the amount by the quantity of the bovine-specific single-copy gene [45, 46]. The calculation was performed according to the relative standard curve protocol.

**Statistical Analysis**

Individual telomere spot intensities showed a skewed distribution. To compensate for this, the data were logarithmically transformed (log_{10}) to improve approximation to a gaussian curve. To identify differences between the experimental groups, one-way ANOVA was applied, followed by multiple comparisons of means (Tukey honestly significant difference). In experiments with only two groups, Student t-test was used. A spreadsheet (Excel) and software program (JMP, version 7.0.1; SAS Institute Inc.) were used to calculate statistical significance, SDs, SEMs, and medians. The obtained SEM and SD values cannot be directly related to the median values resulting from logarithmic transformation, as shown in the figures. P ≤ 0.05 was considered significant.

**RESULTS**

**Verification of qFISH for Telomere Length Measurement**

Telomere length in bovine GM7373 cells (643 telomere fluorescence units [tu]) and MDBK cells (887 tu) was significantly shorter (P < 0.05) compared with two bovine fibroblast cell cultures (ABF3 [1260 tu] and ABF11 [1171 tu]). No significant difference was observed in telomere length between the two bovine fibroblast cell lines. Results shown in Figure 1 demonstrate that both methods have high reproducibility and deliver similar results on telomere length. Quantitative FISH was used in all subsequent experiments.

**Telomere Lengths in ICM and TE Cells of Murine and Bovine Blastocysts**

The blastocyst stage contains the following two distinct cell lineages: 1) the TE, which covers the fluid-filled blastocoel,

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**TABLE 2. Primers used for telo-PCR.**

| Amplicon        | Primer sequence                        | Annealing temp. | Fragment size |
|-----------------|----------------------------------------|-----------------|---------------|
| Telomere        | 5'-CCGTTGTTGGTTGGTTGGTTGGTTGGTTGGTT   | 56°C            | 79 bp         |
|                 | 5'-GCGTTGTTGGTTGGTTGGTTGGTTGGTTGGTT   |                 |               |
| GAPDH*          | 5'-TGGTTAATCTTCCGCGCAGCTT             | 60°C            | 99 bp         |
|                 | 5'-AGACTGGGAGAGATGAACT                |                 |               |
| SLC2A3*         | 5'-ATGACTCGGAAAAGGACAG                | 60°C            | 88 bp         |
|                 | 5'-GGAGAAATGCGAGAGGCT                 |                 |               |

* GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SLC2A3 (GLUT3), glucose transporter, member 3.
and 2) the ICM. The 2-cell compartments differ in their life span due to their different physiological functions. The TE interacts with the uterine wall during implantation and ultimately forms primary parts of the placenta, whereas the ICM forms the embryo proper and ultimately the adult organism. We were curious whether the telomere elongation discovered in bovine and murine blastocysts [19] is regulated in a cell lineage-specific manner. The TE and ICM cells from murine and bovine blastocysts were isolated by sequential detachment of TE and ICM cells, and telomere length in each compartment was analyzed by qFISH (Fig. 2). The specificity of the labeled peptide nucleic acid (PNA) probe for both bovine and murine telomeric regions was confirmed by hybridization to metaphase spreads of embryos. In both species, the hybridization spots were exclusively found at the ends of chromosome arms (Fig. 3A). The mean telomere fluorescent intensity in murine ICM (1353 tfu) was significantly higher than that in murine TE (1129 tfu) (Fig. 2). In
contrast, the mean fluorescence intensity in bovine blastocysts in ICM (973 tfu) was significantly lower than that in TE (1153 tfu) (Fig. 2, D and E).

Endogenous Bovine TERT Expression in Preimplantation Bovine Embryos

The endogenous levels of telomerase mRNA of in vitro-cultured bovine embryos were determined by RT-PCR. The highest mRNA levels were found at the blastocyst stage (Fig. 3C), corresponding to the known time course of telomerase activity [47]. However, a transient increase in bovine TERT activity [47] was observed at the 4-cell stage. Overall, 61% of the cultured embryos showed EGFP fluorescence (Table 3). Expression of EGFP was found to be homogeneous in approximately 70% of the embryos, and the rest showed a variegated EGFP fluorescence profile (Fig. 3D). A total of 132 blastocysts developed, and 45 blastocysts (34%) displayed EGFP fluorescence (Table 3). Both plasmids use a CMV immediate early promoter to drive gene expression in eukaryotic cells. Onset of EGFP fluorescence was detected at 30 to 35 h after injection, corresponding to the 4-cell stage in bovine embryo development (Fig. 3D). Overall, 768 bovine zygotes were injected with EGF-human TERT plasmids, and 61% of the cultured embryos showed EGFP fluorescence (Table 3). Expression of EGFP was found to be homogeneous in approximately 70% of the embryos, and the rest showed a variegated EGFP expression profile (Fig. 3D). A total of 132 blastocysts developed, and 45 blastocysts (34%) displayed EGFP fluorescence (Table 3).

The EGFP-positive embryos were selected at various stages of development for RT-PCR analysis to determine onset of ectopic human TERT mRNA expression. The presence of human TERT mRNA was first detected at the 4-cell stage (Table 3). Indication that human TERT is expressed before onset of bovine endogenous TERT transcription. Maximum expression of human TERT was found at the morula stage, and expression had virtually disappeared in blastocysts and expanded blastocysts (Fig. 4A).

TABLE 3. Development of plasmid-injected bovine embryos.

| Plasmids                        | Number of injected zygotes (n) | Cleavage rate (%) | Number of fluor. embryos (%) | Number of blastocysts (%) | Number of GFP positive blastocysts (%) |
|---------------------------------|--------------------------------|-------------------|------------------------------|--------------------------|---------------------------------------|
| Human TERT/GFP                  | 10                             | 768               | 576 (75%)                    | 468 (61%)                | 132 (17%)                             | 45 (34%)               |
| Culture control                 | 179                            | 143 (79%)         | 0 (0%)                       | 60 (34%)                 | 0 (0%)                                | 34 (50%)               |
| 5 μg human TERT/GFP microinjected embryo | 526                           | 207 (74%)         | 174 (63%)                    | 69 (25%)                 | 34 (50%)                              | 34 (50%)               |
| Culture control                 | 148                            | 115 (78%)         | 0 (0%)                       | 48 (32%)                 | 0 (0%)                                | 34 (50%)               |
| GFP plasmid                     | 225                            | 168 (75%)         | 142 (63%)                    | 55 (24%)                 | 24 (44%)                              | 24 (44%)               |
| Injection buffer                | 211                            | 151 (70.8%)       | 0 (0%)                       | 53 (24.8%)               | 0 (0%)                                | 0 (0%)                 |
| (y-AChR)-GFP                    | 57                             | nd                | 0 (0%)                       | 15 (26.3%)               | 0 (0%)                                | 0 (0%)                 |

*nd, Not determined.
The effects of human TERT injections on telomere length were determined by qFISH in morulae and blastocysts. At the morula stage, telomere length in human TERT-microinjected embryos was significantly longer compared with that in noninjected control embryos. The mean fluorescence intensities were 764 tfu in injected morulae vs. 575 tfu in noninjected control morulae. The mean fluorescence intensities were 706 tfu in injected blastocysts vs. 657 tfu in noninjected blastocysts (Fig. 5). The difference between human TERT-injected and noninjected groups with regard to telomere fluorescence intensities at the morula stage was statistically significant (P < 0.0001). At the blastocyst stage, a statistically significant difference between the two groups was not detected.

Expression of Methylated Human TERT Plasmids in Bovine Preimplantation Embryos

Recent data from our laboratory suggest that DNA methylation affects expression of marker gene plasmids in embryos [40]. Herein, the CMV-human TERT construct was methylated by CpG methyltransferase incubation; DNA methylation was verified by methylation-sensitive restriction analysis. Coinjections of 5mCpG-human TERT and EGFP plasmids were performed in 276 zygotes. The EGFP-positive embryos were collected for RT-PCR and qFISH analysis at various developmental stages. Human TERT mRNA expression was found in morulae and blastocysts (Fig. 4, B and C) but not in premorula stages. Injection of 5mCpG-human TERT resulted in higher yields of viable embryos than injection of nonmethylated constructs (Table 3). The mean telomere fluorescence intensities were 985 tfu in morulae injected with 5mCpG-human TERT at the zygote stage and 912 tfu in noninjected morulae (P > 0.05). The mean telomere fluorescence intensities were 1044 tfu in blastocysts produced from 5mCpG-human TERT-injected zygotes vs. 690 tfu in noninjected blastocysts (P < 0.0001) (Fig. 6). This observation is in line with the finding that ectopic 5mCpG-human TERT expression starts at the early morula stage and remains stable until the blastocyst stage.

To determine whether human TERT transcript levels and extended telomere ends correlate with telomerase activity in treated embryos, blastocysts were analyzed by qTRAP. Sensitivity and the linear dynamic range of the qTRAP assay were verified in a dilution series with human 293 cells (Fig. 4F). Telomerase activity was assessed in seven pools of 10 blastocysts, and the ratio of telomerase activity in treated vs. controls was calculated. Blastocysts derived from injection of unmodified human TERT plasmids (Fig. 4, G and H) showed no increased telomerase activity (40%–108% [three independent replicates]), whereas blastocysts derived from 5mCpG-TERT-injected zygotes contained 150% to 250% more telomerase activity compared with the controls (four independent replicates). To exclude the possibility that the plasmid injection per se or the presence of high copy numbers of plasmids affected telomere length homeostasis, a plasmid coding for EGFP

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**FIG. 4.** Stage-targeted expression of human TERT transcripts in bovine embryos. A and B Targeted expression of human TERT in 4-cell stage to morulae by injection of unmodified plasmids into zygotes (A) and in morulae and blastocysts by injection of cytosine-methylated human TERT (B). C The mRNA transcript levels of ectopic human TERT, endogenous bovine TERT, and Poly (A) transcripts in zygotes, morulae, and blastocysts after injection of unmodified plasmids. D Ectopic expression of human TERT transcripts in blastocysts after injection of methylated plasmids. E Ectopic human TERT expression in degenerated embryos of different stages arrested during in vitro culture. F Total telomerase activity in dilution series of human 293 cells as assessed by qTRAP. G and H Ratio of telomerase activity in blastocysts derived from TERT (three replicates) (G) and from 5mCpG-TERT-injected zygotes (four replicates) (H). The relative ratios of telomerase activity in the treatment groups to control nontreated blastocysts are calculated from qTRAP measurements.
driven by a muscle-specific promoter that is inactive in early embryos [48, 49] was microinjected, and telomere length was assessed. The mean telomere fluorescence in this group was similar to that in noninjected controls and was significantly lower than that in the 5mCpG-human TERT-injected group (P < 0.0001) (Fig. 6I).

Embryo Development In Vitro in Human TERT-Injected and Control Groups

Embryonic development in vitro was monitored until the expanded blastocyst stage 7 to 8 days after fertilization in injected and noninjected groups to study effects of human TERT expression. A significant difference was observed in the developmental rates between the human TERT-microinjected and noninjected groups (P < 0.05). The blastocyst rate was 17% in the unmodified human TERT plasmid-injected group, which was significantly lower than the 32% rate in noninjected controls (P < 0.05). Control injections with CMV-EGFP and injection buffer yielded a blastocyst rate of 24%. The proportion of fluorescent blastocysts among the total blastocysts was higher when CMV-EGFP and 5mCpG-human TERT-EGFP was injected (Table 3). This suggests that human TERT may have detrimental effects on embryonic development when expressed prematurely at the 4-cell to 8-cell early stage.

DISCUSSION

The laboratory mouse model has been widely used for studying effects of ectopic TERT expression [25–28]. Inbred mice strains have excessively long and highly variable telomeres up to 150 kb. This is in apparent contrast to human and bovine cells, which have telomeres of 10 to 15 kb and 15 to 23 kb, respectively [19, 29, 50]. Herein, we have shown that telomere length is differentially regulated in ICM and TE cells of murine and bovine blastocysts, suggesting a species-specific regulation of telomere length at this time point of development. The prominent telomere elongation in murine ICM compared with TE may contribute to the excessively long telomeres found in this species. To assess reliable telomere fluorescence data from murine blastocysts, the murine qFISH data were recorded with reduced exposure time (100 vs. 500 ms.
This precludes a direct comparison between the murine and bovine data in Figure 2. The simultaneous analysis of telomere length by qFISH and real-time PCR revealed distinct differences in telomere length among the four different cell lines irrespective of the method. This shows that our qFISH method yields highly reproducible data and could be used as the method of choice for all subsequent experiments.

For the first time to date, ectopic human TERT was successfully expressed in mammalian embryos. Human TERT was expressed in a stage-specific manner after injection of plasmids into bovine zygotes and resulted in increased telomerase activity and in turn in significantly elongated telomeres in bovine preimplantation embryos. Early bovine embryos are a unique in vitro model, in which telomerase activity is dynamically regulated over a short period compared with somatic cells [16, 51].

Recent publications from our laboratory [35] and others [52] have shown that bovine preimplantation embryos are a superior model to investigate human preimplantation development compared with traditional rodent models (laboratory mouse, rat, and rabbit). In addition, physiology, life span, pathology, and genomic structure are surprisingly similar between B. taurus and Homo sapiens [34, 36]. In a previous study [19] from our laboratory, we explored telomere dynamics in bovine-cloned in vivo- or in vitro-derived bovine morulae and blastocysts and discovered a telomere length regulatory mechanism specifically at the morula to blastocyst transition.
that presumably operates in most mammalian embryos. The bovine and human TERT genes, as well as the RNA gene TERC, are highly conserved. By BLAST alignment, higher conservation was shown between human and bovine sequences than with murine sequences, highlighting the usefulness of the bovine model. This promising background prompted us to embark on studies to establish the bovine preimplantation embryo as a model system for telomere modulation. Moreover, the data we have generated herein are promising for developing B. taurus as a alternative model in aging research.

High telomerase activity has been shown to confer a significant proliferative advantage in vitro [17, 53, 54]. Overexpression of telomerase confers a selective proliferation advantage and increases resistance to stress in murine embryonic stem cells [54]. The enhanced telomerase activity in early bovine embryos and the increase in telomere length by ectopic expression of human TERT could be a promising approach to isolate bovine embryonic cells with high self-renewal and proliferating capacity.

Knowledge of the regulatory mechanisms of telomere length in germ cells is fragmentary [55]. Recent data suggest that epigenetic factors may have a role in the regulation of telomere length. Shortening of telomeres is associated with modification of telomeric and subtelomeric chromatin [56]. Telomere shortening is associated with deregulation of the mammalian transcriptome and thereby changes the gene expression toward an aging profile, affects different pathways, and relaxes X-chromosome inactivation by modifying the histone methylation profile [57].

Injection of the human TERT construct triggered human telomerase expression as early as 30 to 40 h after microinjection, in addition to expression of the endogenous bovine telomerase. Potential reasons for the failure to express human TERT from the nonmodified plasmid in blastocysts could be that plasmids were degraded or silenced, albeit this is not likely in view of our recent results [40]. Obviously, the human TERT injection provided early embryos with additional telomerase activity, and expression of human TERT in the first cleavage stages may have interfered with cell cycle progression and resulted in developmental arrest [54, 58]. The control injections with marker plasmid and with buffer also resulted in reduced developmental rates, indicating a deteriorating effect from the microinjection technique itself. Besides telomere length regulation, TERT expression or overexpression can interfere with other physiological functions and has been shown to impair cell viability, primarily by translocation of active telomerase into mitochondria [59, 60]. The high mitochondrial load [61] could render early embryos specifically vulnerable to these adverse effects of human TERT expression.

In an alternative approach, the human TERT plasmid was methylated (5mCpG-human TERT) before injection into bovine zygotes. The 5mCpG methylation delayed expression of marker genes in plasmid-injected embryos [40]. With the aid of methylated human TERT, we targeted human TERT expression to morulae and blastocysts. Hence, targeted expression of human TERT in different stages of preimplantation development can be obtained using methylated and unmethylated human TERT. Our data show that neither the presence of high copy numbers of foreign DNA nor the microinjection procedure per se has measurable effects on telomere length homeostasis in early embryos.

Human embryos with critically short telomeres could not implant [62]. It is tempting to transfer these human TERT-expressing bovine embryos to the reproductive tract of synchronized recipients to study telomere length homeostasis in vivo. Mice normally do not exhibit age-related meiotic dysfunction, as their telomeres are longer than telomeres in humans and, as shown herein, their ICM cells display dramatically increased telomere elongation. In vivo development of bovine embryos with prolonged or shortened telomeres would allow investigation of the effect of telomeres on the high proportion of embryonic and fetal losses in this species. However, ectopic expression of TERT in fetuses and offspring may affect other important physiological systems. Expression of TERT and TERC is critical for maintenance and elongation of mammalian telomeres. Specifically, the number of gene copies and modulation at the transcriptional level of TERC are limiting factors for telomerase activity [63]. However, one has to take into account that, in addition to telomerase activity, expression of the telomere-associated proteins TERF1 (TRF1) and TERF2 is crucial for telomere regulation. Overexpression of TERF2 is associated with progressive shortening of the telomeres [64].

Absence of telomerase activity is compatible with normal development in various species even over several generations. In humans, reduced telomerase activity is associated with bone marrow diseases, lung fibrosis, and various forms of cancer [65]. Patients with Werner syndrome are characterized by premature aging, increased genetic instability, and elevated risk of tumor formation, mainly attributed to shortened telomeres [66]. Patients with X chromosome-linked dyskeratosis congenita show reduced TERC levels, which in turn leads to reduced telomerase activity and shortened telomere length [67].

In conclusion, human telomerase can be functionally reconstituted in early bovine embryos by injection of human TERT expression plasmids into zygotes. The present study describes a targeted expression profile of human telomerase and telomere elongation in early bovine embryos. Further determination of the physiological effects of human TERT expression during in vivo development in the reproductive tract may reveal effects on aging, regeneration, and carcinogenesis.

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