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# An Essential Role for Functional Telomeres in Mouse Germ Cells during Fertilization and Early Development

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**Late generations of telomerase-null ($TR^{-/-}$) mice exhibit progressive defects in highly proliferative tissues and organs and decreased fertility, ultimately leading to sterility. To determine effects of telomerase deficiency on germ cells, we investigated the cleavage and preimplantation development of embryos derived from both *in vivo* and *in vitro* fertilization of $TR^{-/-}$ or wild-type ($TR^{+/+}$) sperm with either $TR^{-/-}$ or $TR^{+/+}$ oocytes. Consistently, fertilization of $TR^{-/-}$ oocytes with either $TR^{+/+}$ or $TR^{-/-}$ sperm, and $TR^{-/-}$ sperm with $TR^{+/+}$ oocytes, resulted in aberrant cleavage and development, in contrast to the normal cleavage and development of $TR^{+/+}$ oocytes fertilized by $TR^{+/+}$ sperm. Many (>50%) of the fertilized $TR^{-/-}$ eggs developed only one pronucleus, coincident with increased incidence of cytofragmentation, in contrast to the normal formation of two pronuclei and equal cleavage of wild-type embryos. These results suggest that both $TR^{-/-}$ sperm and oocytes contribute to defective fertilization and cleavage. We further found that a subset (7–9%) of telomeres was undetectable at the ends of some metaphase I chromosomes from $TR^{-/-}$ spermatocytes and oocytes, indicating that meiotic germ cells lacking telomerase ultimately resulted in telomere shortening and loss. Dysfunction of meiotic telomeres may contribute to aberrant fertilization of gametes and lead to abnormal cleavage of embryos, implying an important role of functional telomeres for germ cells undergoing fertilization and early cleavage development.** 

***Key Words:*** **telomerase-null mice; telomere; fertilization; embryo; apoptosis.**

## INTRODUCTION

Vertebrate telomeres consist of tandem repeats of the TTAGGG sequence that cap the ends of chromosomes, protecting them from degradation and fusion. The length of telomere repeats is primarily maintained by active telomerase, which is composed of telomerase RNA (TR) and a catalytic subunit telomerase reverse transcriptase (TERT) (Blackburn, 2001). Extensive evidence has shown that telomere shortening and erosion lead to chromosome end-to-end fusions and genomic instability, causing replicative senescence in human cultured cells (Blackburn, 2000). Maintenance of telomere length is proposed to be essential for bypassing senescence and crisis checkpoints in cancer cells (Greider, 1990; Greider and Blackburn, 1996). Reversal of telomere shortening by the forced expression of telomerase rescues cells from senescence and extends cell life span (Bodnar *et al.*, 1998; Vaziri and Benchimol, 1998).

Laboratory mice have long telomeres compared with humans, limiting use of most mouse strains for studies on telomere-associated diseases and aging. Nonetheless, telomerase-null ($TR^{-/-}$) mice show progressive telomere shortening and chromosome instability with increasing generations and also exhibit defects mostly in highly proliferative tissues, affecting development, growth, immune function, and carcinogenesis. Late generation $TR^{-/-}$ mice (Blasco *et al.*, 1997; Herrera *et al.*, 2000, 1999a,b; Lee *et al.*, 1998; Rudolph *et al.*, 1999) display defects similar to phenotypes that recently have been found associated with human diseases, resulting from mutations in human telomerase and telomere shortening (Marciniak *et al.*, 2000;

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Vulliamy *et al.*, 2001). Thus, $TR^{-/-}$ mice provide an instructive model for studying telomerase and telomere dysfunction in humans.

Female human fertility declines with increased maternal age. Many factors might contribute to aging-associated infertility in women. However, extensive evidence demonstrates that oocyte defects are a major cause of age-related decline in female fertility (Abdalla *et al.*, 1993; Janny and Menezo, 1996; Navot *et al.*, 1991, 1994). The frequency of chromosome abnormalities in human oocytes, especially aneuploidy, increases with maternal age (Plachot *et al.*, 1988), which impairs embryo development (Kornafel and Sauer, 1994). In particular, an increased incidence of nondisjunction was observed after meiotic resumption and during meiotic division in oocytes from older women (Angell, 1997).

In contrast to male germ cells, female germ cells arrest at the prophase I stage for weeks in mouse and years in human until puberty. Telomere-mediated nuclear reorganization is proposed to be a prerequisite step for the pairing and recombination of homologous chromosomes before the meiotic arrest, although this has not been formally demonstrated in mammalian cells (Bass *et al.*, 2000; de Lange, 1998; Scherthan *et al.*, 1996, 2000; Tease and Fisher, 1998). Conceivably, even minor damage inflicted on telomeres prior to and during this long resting period could impair resumption of meiosis and meiotic division, resulting in aneuploidy and abnormal embryo development. Indeed, $TR^{-/-}$ female mice exhibit decreased fertility and reduced litter size with increasing generations, eventually resulting in sterility (Herrera *et al.*, 1999a,b; Lee *et al.*, 1998). $TR^{-/-}$ males showed increased apoptosis in germ cells (Hemann *et al.*, 2001a; Lee *et al.*, 1998). During normal development, telomerase activity is relatively low in mature oocytes and remains low in cleavage-stage embryos, until morula and blastocyst stage, when telomerase is reactivated (Betts and King, 1999; Wright *et al.*, 1996; Xu and Yang, 2000).

While telomerase-null mice gradually develop infertility, it is not clear how telomerase deficiency disrupts reproductive function. In this study, we evaluate fertilization and early embryo development and apoptosis in telomerase-null mice. We observed aberrant fertilization and first mitotic divisions in telomerase-null mice, suggesting that germ cells with dysfunctional telomeres lead to impaired fertilization and defects in early cleavage.

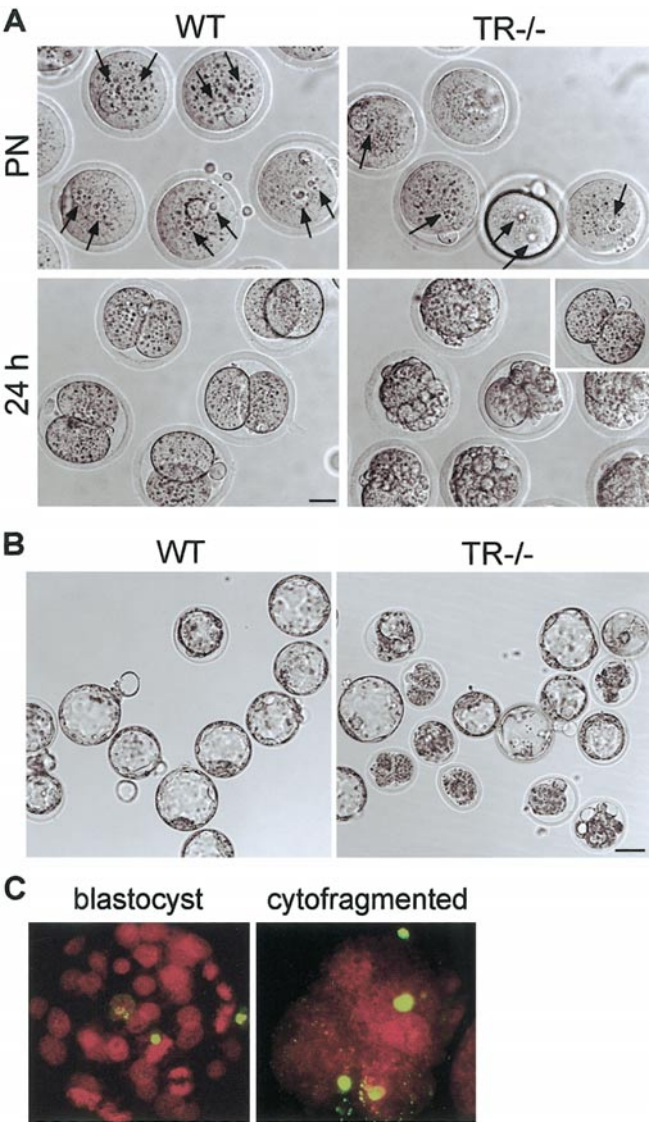
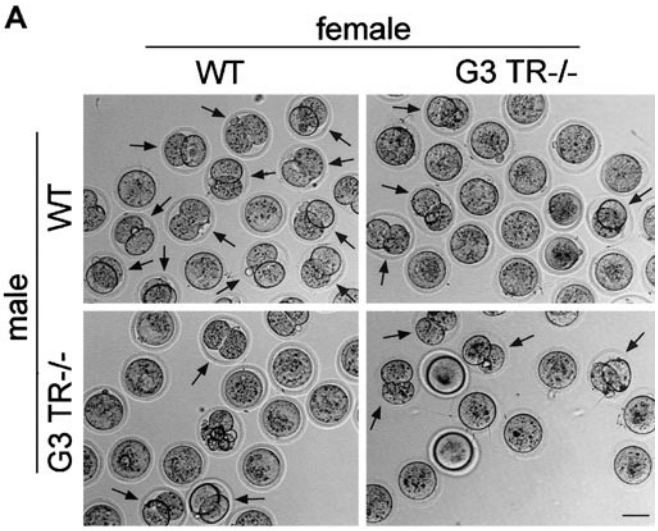


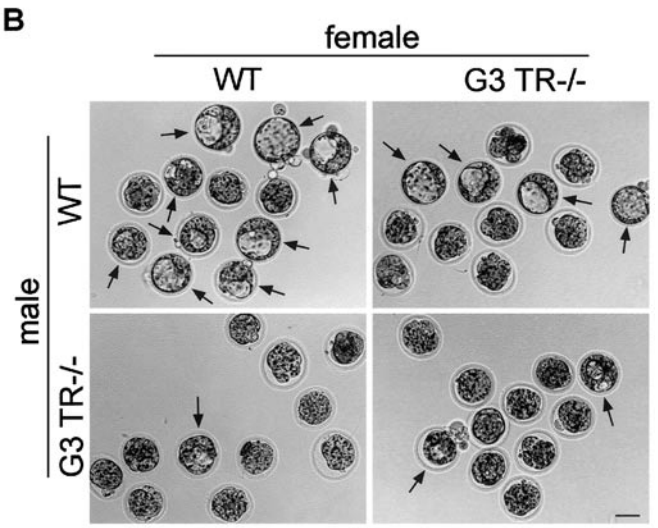

**FIG. 1.** Morphology of cleavage and blastocyst stage of embryos and apoptosis of *in vivo*-fertilized eggs from G2 or G3 $TR^{-/-}$ female mice mated with G2 or G3 $TR^{-/-}$ males. (A) Pronucleus (PN, indicated by arrows) and cleavage of wild-type (WT) and G2 or G3 $TR^{-/-}$ eggs, showing normal cleavage to two-cells at 24 h in culture of WT eggs but cytofragmentation from G2 or G3 $TR^{-/-}$ females. (Inset) Normal two-cell cleaved from an egg with two pronuclei. Bar, 20 $\mu$m. (B) Most WT eggs developed to blastocysts 96 h in culture, in contrast to many arrested embryos from G2 $TR^{-/-}$ eggs. Bar, 50 $\mu$m. (C) TUNEL assay for nuclear DNA fragmentation in blastocysts, and fragmented arrested embryos of G3 $TR^{-/-}$ mice 72 h after cleavage. Red indicates nuclei. TUNEL-positive stain appeared green by FITC filter and yellowish in merged images.

## MATERIALS AND METHODS

### *Animals, Oocytes, and in Vitro Culture of Embryos*

Adult telomerase-null G2 and G3 mice and age-matched wild-type controls (Blasco *et al.*, 1997; Lee *et al.*, 1998) were superovulated with 5 IU pregnant mare's serum gonadotrophin (PMSG) (Calbiochem, La Jolla, CA) followed by 5 IU human chorionic gonadotrophin (hCG) 46–48 h later. Oocytes enclosed in cumulus masses were collected from oviduct ampullae at 14 h post-hCG injection. Fertilized eggs were obtained from mice 19–20 h after injection of hCG and successful mating, judged by presence mating plugs, with males. Cumulus cells were removed by pipetting after brief incubation in 0.03% hyaluronidase prepared in Hepes-buffered KSOM (Lawitts and Biggers, 1993), containing 14 mM




Embryos with 16- or more cells

| male \ female | WT | $TR^{-/-}$ |
|---|---|---|
| WT | 78% (n=23) | 28% (n=18) |
| $TR^{-/-}$ | 10% (n=10) | 18% (n=11) |

n = No. embryos examined

**FIG. 2.** Cleavage and development to morula and blastocyst 72 h after *in vitro* fertilization of WT and G3 $TR^{-/-}$ germ cells. (A) Normal cleavage to two-cells at 24 h, indicated by arrows. (B) At 72 h, morula and blastocyst (arrows). Bar, 50 $\mu$m.

Hepes and 4 mM sodium bicarbonate (HKSOM) (Liu and Keefe, 2000), washed, and incubated in 50-$\mu$l droplets of preequilibrated KSOM (Ho *et al.*, 1995; Lawitts and Biggers, 1993), supplemented with nonessential amino acids and 2.5 mM Hepes, covered with mineral oil at 37°C in a humidified atmosphere of 7% $CO_2$ in air. Successful fertilization was evaluated by the equal cleavage to two-cells at 24 h in culture. All manipulations were carried out at 36–37°C on heated stages, chambers, or in incubators. Unless specified, all reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

### *In Vitro Fertilization*

Sperm were expelled from the cauda epididymis of male mice into 400 $\mu$l TYH medium containing 0.4% BSA and incubated under mineral oil for 1–2 h at 37°C to capacitate (Wakayama and Yanagimachi, 1998). Sperm suspension at the final concentration of $6–7 \times 10^5$ sperm/ml was used to inseminate oocytes in a 400-$\mu$l drop of TYH medium supplemented with 0.4% BSA, under mineral oil at 37°C in a humidified atmosphere of 7% $CO_2$ in air. After coincubation with sperm for 6 h, the inseminated oocytes were washed and cultured in 50-$\mu$l droplets of the KSOM, covered with mineral oil at 37°C in a humidified atmosphere of 7% $CO_2$ in air. Cleavage and embryo development were examined every 24 h. Successful fertilization also was evaluated by the equal cleavage to two-cells at 24 h in culture.

### *Detection of Apoptosis by TdT-Mediated dUTP Nick-End Labeling (TUNEL) Assay*

The TUNEL assay for detection of nuclear DNA fragmentation has been commonly used for detecting apoptosis in preimplantation embryos (Brison and Schultz, 1997; Exley *et al.*, 1999; Liu *et al.*, 2002b; Moley *et al.*, 1998). Embryos were fixed in 3.7% paraformaldehyde prepared in Dulbecco's phosphate-buffered saline supplemented with 0.1% polyvinylpyrrolidone. Nuclear DNA fragmentation in embryos was detected by the TUNEL method using an In Situ Cell Death Detection Kit (Roche Diagnostics Corporation, Indianapolis, IN) according to the manufacturer's instruction, and the total cell nuclei were counterstained with 50 $\mu$g/ml propidium iodide. Embryos were washed, then mounted onto a slide under a coverslip in the Vectashield mounting medium, and sealed with nail polish. The number of total nuclei and the nuclear morphology were evaluated with a rhodamine filter and the DNA fragmentation was assessed with a fluorescein filter by using an inverted Zeiss microscope with epifluorescent optics. The total number of cells reported includes the mitotic cells. The percentage of apoptotic cells per embryo is expressed as apoptotic nuclei/total number of nuclei $\times$ 100%.

### *Immunofluorescence Microscopy*

Egg were fixed and extracted for 30 min at 37°C in a microtubule-stabilizing buffer (Allworth and Albertini, 1993), then washed extensively and blocked overnight at 4°C in wash medium (PBS, supplemented with 0.02% $NaN_3$, 0.01% Triton X-100, 0.2% nonfat dry milk, 2% goat serum, 2% BSA, and 0.1 M glycine). Afterwards, eggs were incubated with $\beta$-tubulin mouse monoclonal antibody (1:150; Sigma), washed, and then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (1:200; Molecular Probes, Eugene, OR) at 37°C for 2 h, washed again and mounted onto a slide under a coverslip in the Vectashield mounting medium (Vector Laboratories, Burlingame, CA), added with 0.5 $\mu$g/ml Hoechst 33342 for DNA stain. The samples were observed by using a Zeiss fluorescence microscope (Axioplan 2 imaging) and images were captured by an AxioCam using AxioVision 3.0 software.

**TABLE 1**

Cleavage, Cytofragmentation, and Development following *in Vivo* Fertilization of Eggs from G2 $TR^{-/-}$ Female Mice Mated with G2 $TR^{-/-}$ Males

| | | 24 h | | 96 h | | |
|---|---|---|---|---|---|---|
| Eggs | No. cultured | No. cleaved (%) | No. frag (%) | No. blastocyst | (%/cultured) | (%/cleaved) |
| WT | 68 | 38 (56)[a] | 14 (21)[d] | 31 | (46)[a] | (82)[c] |
| $TR^{-/-}$ | 99 | 15 (15)[b] | 55 (56)[c] | 8 | (8)[b] | (53)[f] |

*Note.* The different superscripts within the same column mean significant differences (a vs b, c vs d, $P < 0.001$; e vs f, $P < 0.05$). frag, cytofragmentation.

### *Analysis of Telomeric Function Using Quantitative Fluorescence in Situ Hybridization (Q-FISH) with Telomere Probe*

Q-FISH has become the method of choice for examination of both telomere length and loss in single cells (Zijlmans *et al.*, 1997). Chromosome spreads were prepared by a hypotonic treatment of oocytes or spermatocytes with 1% sodium citrate for 20 min, followed by fixation in methanol:acetic acid (3:1), and air dried. FISH with FITC-labeled $(CCCTAA)_3$ peptide nucleic acid (PNA) probe (Applied Biosystems, Framingham, MA) was performed according to the manufacturer's protocol. Chromosomes were counterstained with 0.2 $\mu$g/ml Hoechst 33342. Samples were mounted onto a glass slide in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Telomeres were detected with a FITC filter by using a Zeiss fluorescence microscope (Axioplan 2), and images were captured by an AxioCam using AxioVision 3.0 software. Integrated fluorescence intensity of individual telomeres in chromosome spreads indicates relative length of telomeres (Romanov *et al.*, 2001).

### *Statistical Analysis*

Each experiment was repeated at least three times. Comparison of group means was carried out by ANOVA and Fisher's protected least-significant difference using StatView software (SAS Institute Inc., Cary, NC). Percentages were compared using $\chi^2$ analysis.

## RESULTS

### *Germ Cells of $TR^{-/-}$ Mice Exhibited Impaired Fertilization and First Mitotic Divisions*

In the first series of experiments, cleavage and preimplantation development were compared between eggs of the second generation (G2) homozygotic telomerase knockout ($TR^{-/-}$) and wild-type ($TR^{+/+}$) mice controls. G3 $TR^{-/-}$ eggs fertilized *in vivo* were collected from G2 $TR^{-/-}$ female mice after successful mating as determined by the presence of plugs, with G2 $TR^{-/-}$ male mice, while $TR^{+/+}$ fertilized eggs were obtained from age-matched female $TR^{+/+}$ mice successfully mated with $TR^{+/+}$ males. Morphologically normal eggs were cultured *in vitro* for 96 h. As shown in Table 1, cleavage to two-cell stage was significantly ($P < 0.01$) reduced with G3 $TR^{-/-}$ eggs (15%), compared with that of wild-type eggs (56%) after 24 h culture. The incidence of cytofragmentation, a morphological sign of apoptosis, was significantly increased in G3 $TR^{-/-}$ eggs (56%), compared with a lower rate (21%) of cytofragmentation observed in wild-type eggs (Fig. 1A). While the majority of $TR^{+/+}$ embryos developed to blastocysts by 72 h in culture, most G3 $TR^{-/-}$ embryos remained at morulae stage (data not shown). After 96 h in culture, the rate of development to blastocyst of G3 $TR^{-/-}$ eggs, based on both cultured eggs and cleaved eggs, was significantly ($P < 0.001$) lower than that of wild-type embryos (Table 1; Fig. 1B).

In the second series of experiments, both G3 $TR^{-/-}$ female mice and age-matched wild-type $TR^{+/+}$ female controls were mated with wild-type $TR^{+/+}$ males, to control for effects of telomere function in males. The resulting G4 $TR^{-/+}$ and $TR^{+/+}$ fertilized eggs were cultured *in vitro* for 96 h and compared for cleavage and development. Wild-type eggs exhibited a significantly higher rate (79%) of cleavage at 24 h of culture than G4 $TR^{-/+}$ eggs (31%; $P < 0.001$) (Table 2). Also, the incidence of cytofragmentation was significantly increased in G4 $TR^{-/+}$ eggs (59%), compared with wild-type eggs (10%). Fragmented embryos in general exhibited one to five apoptotic nuclei when examined after 72 h in culture (Fig. 1C). After 96 h in culture, the rate of development to blastocyst of G4 $TR^{-/+}$ eggs (based on cultured eggs) was significantly ($P < 0.001$) lower, but was only marginally lower ($P < 0.05$), if based on cleaved embryos, compared with wild-type embryos. There was no statistically significant ($p > 0.05$) difference in total cell number and apoptotic cell number (expressed as %) between wild-type (43 $\pm$ 15, and 9 $\pm$ 7%) and G4 $TR^{-/+}$ blastocyst-stage embryos (38 $\pm$ 12, and 13 $\pm$ 12%), suggesting that the developed blastocysts were comparable between $TR^{-/+}$ and $TR^{+/+}$ embryos. On the other hand, rates of fertilization, cleavage, and preimplantation development were decreased in telomerase heterozygous eggs. Many $TR^{-/+}$ embryos that failed to cleave normally underwent apoptosis, suggesting that G4 $TR^{-/+}$ fertilized eggs exhibited impaired developmental potential. Homozygous G4 $TR^{-/-}$ eggs, fertilized *in vivo* by crosses between G3 $TR^{-/-}$ males and females, manifested cleavage and cytofragmen-

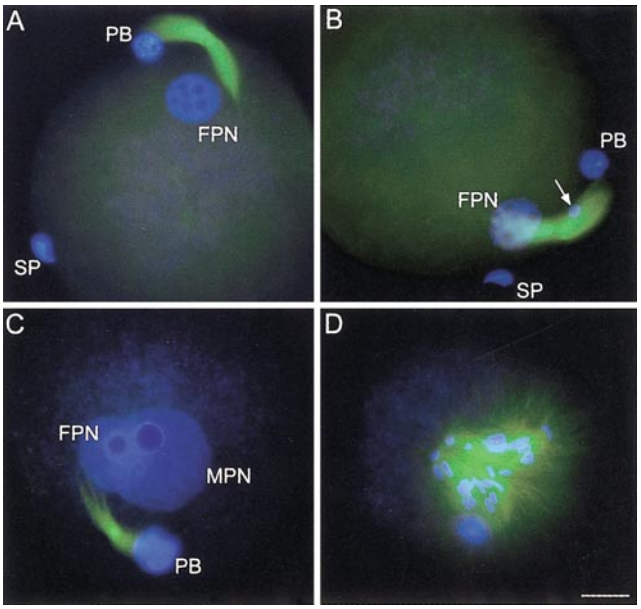



**FIG. 3.** Immunofluorescence imaging of $TR^{-/-}$ eggs collected from G3 $TR^{-/-}$ female mice after mating with G3 $TR^{-/-}$ males. (A) A fertilized egg with one female pronucleus (FPN) and one polar body (PB), with telophase spindle (green) left between them. SP, sperm head. (B) A fertilized egg with one FPN and one PB, and missegregation of chromosomes over the residue of telophase spindles (arrow). (C) A fertilized egg with two pronuclei and one polar body. (D) An egg with spindle disruption and chromosome dispersal. Bar, 10 $\mu$m.

tation at 24 h, and blastocyst formation in 96 of culture, at rates very similar to those of heterozygous G4 $TR^{-/+}$ eggs (Table 2).

To rule out possible effects of mating variations on the observed reproductive defects, we also performed *in vitro* fertilization by fertilizing G3 $TR^{-/-}$ and $TR^{+/+}$ oocytes with the same source of sperm collected from either G3 $TR^{-/-}$ or $TR^{+/+}$ males. To ensure that the observed effects were derived specifically from gametes rather than from host animal variations, morphologically normal eggs were fertilized *in vitro* and cultured for 72 h, and the cell number and apoptosis in the developing embryos were counted. Consistently, fertilization of G3 $TR^{-/-}$ oocytes with either $TR^{+/+}$ or G3 $TR^{-/-}$ sperm, and G3 $TR^{-/-}$ sperm with $TR^{+/+}$ oocytes, resulted in significantly ($P < 0.001$) lower rates of cleavage, compared with those of $TR^{+/+}$ oocytes fertilized with $TR^{+/+}$ sperm (Table 3; Fig. 2A). Further, rates of development to blastocyst and morula stages observed in G3 $TR^{-/-}$ oocytes fertilized with either $TR^{+/+}$ or G3 $TR^{-/-}$ sperm, as well as rates of development of $TR^{+/+}$ oocytes with G3 $TR^{-/-}$ sperm, were significantly lower than those of fertilization between $TR^{+/+}$ oocytes and $TR^{+/+}$ sperm, based on total cultured eggs. However, the rate of development to morula and blastocysts, based on cleaved eggs, did not differ among the four combination groups of fertilization. Wild-type fertilization did produce more blastocysts than G3 $TR^{-/-}$ gamete fertilizations, which also was confirmed by the number of cells in the developed embryos (Fig. 2B). The percentage (6–9%) of apoptotic cells in the developed embryos at 72 h in culture was not significantly different among four groups. These results demonstrate that both male and female germ cells of telomerase-deficient mice can contribute to compromised fertilization and cleavage and blastocyst formation of embryos. We observed that embryos from wild-type mice of this strain formed blastocysts with 16 or more cells, compared with about 32 cells for blastocysts from many other mouse strains. Fertilization and cleavage of wild-type mice of this strain also produced relatively lower rates compared with those (>90%) of hybrid B6C3F1 (C57BL × C3H) mice.

*In vivo*-fertilized wild-type eggs exhibited two pronuclei, whereas most G3 $TR^{-/-}$ eggs only developed one pronucleus, although they did extrude a polar body (Fig. 1A), suggesting that those eggs had been properly activated during fertilization. Most (>50%) fertilized G3 $TR^{-/-}$ eggs that developed only one pronucleus underwent cytofragmentation (Fig. 1A, 24 h). We further examined nuclear morphology and microtubules in more detail by employing immunostaining and fluorescence microscopy. In 15 eggs

**TABLE 2**

Cleavage, Cytofragmentation, and Development following *in Vivo* Fertilization of Eggs from G3 $TR^{-/-}$ Female Mice Mated with Wild-Type Males or G3 $TR^{-/-}$ Males

| | | | 24 h | | 96 h | | |
|---|---|---|---|---|---|---|---|
| Sperm | Eggs | No. cultured | No. cleaved (%) | No. frag (%) | No. blastocyst | (%/cultured) | (%/cleaved) |
| WT | WT | 72 | 57 (79)[a] | 7 (10)[d] | 50 | (69)[a] | (88)[c] |
| WT | $TR^{-/-}$ | 113 | 35 (31)[b] | 67 (59)[c] | 26 | (23)[b] | (74)[f] |
| $TR^{-/-}$ | $TR^{-/-}$ | 92 | 24 (26)[b] | 51 (55)[c] | 17 | (18)[b] | (71)[f] |

*Note.* The different superscripts within the same column mean significant differences (a vs b, c vs d, $P < 0.001$; e vs f, $P < 0.05$). frag, cytofragmentation.

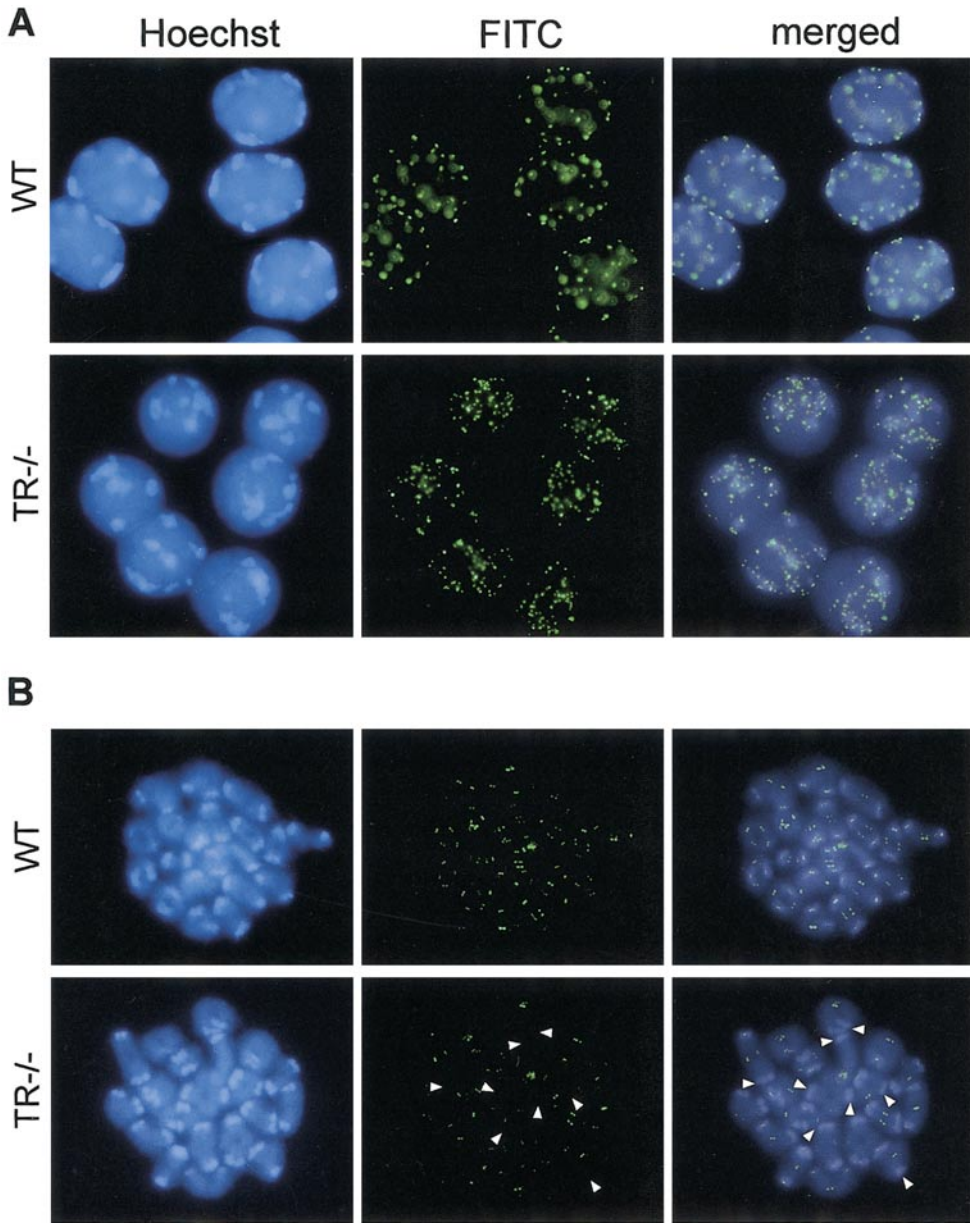



**FIG. 4.** Telomere distribution in mouse spermatocyte I and metaphase I. Telomeres are displayed by FITC immunofluorescence (green). Hoechst staining of nuclear DNA is given as blue. Hoechst-bright heterochromatin is seen as whitish clusters. (A) Late pachytene or diplotene nuclei showed prominent peripheral heterochromatin clusters and peripheral telomere signals in wild-type mouse but both peripheral and dispersed telomere signals in G2 $TR^{-/-}$ mouse spermatocytes. (B) Telomere signals detected at the chromosome ends of metaphase I spermatocytes. Arrowhead, no obvious telomere fluorescence, indicative of possible telomere erosion.

analyzed, 11 eggs showed 1 pronucleus and 1 polar body, with telophase spindle residue left between them, and sperm head attachment was seen around the egg membrane (Fig. 3A), demonstrating obvious defects in fertilization process. Three of the eggs with 1 pronucleus failed in segregation of 1 or more chromosomes that were still congregated along the residue of telophase spindles (arrow, Fig. 3B). Two eggs developed 2 pronuclei and 1 polar body (Fig. 3C), and the other 2 eggs displayed spindle disruption and chromosome dispersal (Fig. 3D).

### *Germ Cells of $TR^{-/-}$ Mice Exhibited Short Telomeres*

The rates of blastocyst formation and cell number in blastocysts were decreased in G3 $TR^{-/-}$ eggs, suggesting

**TABLE 3**
Cleavage and Development following *in Vitro* Fertilization of Eggs and Sperm from G3 $TR^{-/-}$ Mice

| Sperm | Oocyte | No. cultured | 24 h | 72 h | | |
|---|---|---|---|---|---|---|
| | | | No. cleaved (%) | No. bl & m | %/cultured | %/cleaved |
| WT | WT | 190 | 118 (62)[a] | 115 | 61[a] | 97[a] |
| WT | $TR^{-/-}$ | 112 | 33 (29)[b] | 30 | 27[b] | 91[a] |
| $TR^{-/-}$ | WT | 167 | 54 (32)[b] | 48 | 29[b] | 89[a] |
| $TR^{-/-}$ | $TR^{-/-}$ | 98 | 33 (34)[b] | 30 | 31[b] | 91[a] |

*Note.* The different superscripts within the same column mean significant differences ($P < 0.001$). bl, blastocyst; m, morulae.

that telomerase deficiency and telomere dysfunction also prevented many cleaved embryos from developing to morula and blastocyst stages. Both *in vivo* and *in vitro* fertilization experiments suggest that both G3 $TR^{-/-}$ sperm and oocytes may contribute to defective fertilization and cleavage.

These results prompted us to determine whether the disrupted fertilization and embryo development could be attributable to shortened telomeres during meiosis. We labeled telomeres by Q-FISH in germ cells collected from both male and female gonads. Late pachytene or diplotene nuclei at prophase I of spermatocytes exhibited prominent peripheral heterochromatin clusters and perinuclear distribution of telomeres in wild-type mice, but both peripheral and dispersed telomere signals in G2 $TR^{-/-}$ mice (Fig. 4A). Further, telomere signals were found at the ends of chromosomes of metaphase I spermatocyte nuclei of wild-type mice, with only 0.5% of telomere signals (4/800) undetectable. In contrast, 9% (72/800) of telomere signals were undetectable from five chromosome spreads at G2 $TR^{-/-}$ spermatocyte I cells (arrowheads, Fig. 4B). Consistent with a recent report (Hemann *et al.*, 2001a), we did not see chromosome fusion during meiosis, despite telomere erosion at chromosome ends.

The number ($20-40 \times 10^6$) of sperm obtained from cauda epididymis of G3 $TR^{-/-}$ mice was reduced two- to threefold compared with that of wild-type mice. This result is not surprising because extensive apoptosis has been observed in the testis of late generations of $TR^{-/-}$ mice (Hemann *et al.*, 2001a; Lee *et al.*, 1998). The motility of sperm from G3 $TR^{-/-}$ males was extremely low, in contrast to wild-type sperm. By the end of a 6-h coincubation with oocytes, wild-type sperm were still motile, swirling around and attached to oocytes, while $TR^{-/-}$ sperm were no longer motile. We could obtain only spermatids, but no viable motile mature sperm from the very small testes of G4 $TR^{-/-}$ mice. Female G4 $TR^{-/-}$ mice also showed growth retardance and ovarian atrophy. However, the sizes of G2 and G3 $TR^{-/-}$ mouse ovaries and body size were comparable to those of wild-type controls.

We also collected GV oocytes from wild-type and G3 $TR^{-/-}$ mouse ovaries and analyzed telomere signals. Wild-type GV oocytes ($n = 17$) exhibited many telomere signals, whereas five of nine G3 $TR^{-/-}$ GV oocytes showed relatively fewer telomere signals (Fig. 5). Due to the uneven focal planes of the large GV, the telomeres of GV oocytes could not be quantified reliably. When we performed *in vitro* maturation of GV oocytes in MEM supplemented with 10% FBS to obtain metaphase chromosome spreads at the GVBD-MI stage (Liu *et al.*, 2002a), telomere signals of chromosomes spreads were observed in wild-type oocytes ($n = 9$), with only one chromosome end (0.4%) of 280 chromosomes showing no clear telomere signal. By contrast, 7.3% (16/220) of chromosome ends from G3 $TR^{-/-}$ oocytes ($n = 6$) manifested loss of at least one telomere signal (arrows, Fig. 5). The average of relative FITC fluorescence intensity, indicative of relative telomere length, was significantly lower in G3 $TR^{-/-}$ oocytes compared with wild-type oocytes ($1474 \pm 1306$, $n = 649$; and $2166 \pm 1494$, $n = 761$, respectively; $P < 0.0001$, Wilcoxon signed rank test).

## DISCUSSION

From both *in vivo* and *in vitro* fertilization experiments, it appears that the absence of telomerase leads to telomere dysfunction, which in turn results in aberrant fertilization and cleavage of $TR^{-/-}$ gametes.

We found that fertilization of $TR^{-/-}$ eggs with either wild-type or $TR^{-/-}$ sperm and fertilization of $TR^{-/-}$ sperm with wild-type eggs all manifested similar low rates of cleavage and development to morula and blastocysts, indicating that only a small proportion of gametes from telomerase-null mice are capable of fertilization and preimplantation development, regardless of whether they were hetero-or homozygous for the telomerase deletions. These results suggest that reintroduction of telomerase, by fertilizing $TR^{-/-}$ eggs with wild-type sperm (one copy of TR) or vice versa, does not immediately reverse aberrant fertilization, cleavage, and early development. That homozygous $TR^{-/-}$ embryos could develop to morula and blastocysts suggests that telomerase is dispensable for the early cleavage stage of embryo development.

We further observed that blastocysts developed from some normally fertilized and cleaved $TR^{-/-}$, or $TR^{+/-}$ eggs

showed apparently normal chromosome ploidy, with appropriate telomere signals, and no chromosome fusions (unpublished observations). Presumably, these embryos most likely emerged from that subset of the original population of oocytes with the longest telomeres. During normal development, telomerase activity is relatively low in mature oocytes and spermatozoa, as well as in embryos at cleavage stages, and its activity increases only at the morulae and blastocyst stages (Betts and King, 1999; Eisenhauer *et al.*, 1997; Prowse and Greider, 1995; Wright *et al.*, 1996; Xu and Yang, 2000). Thus, the fertilization and early cleavage stages of embryo development, characterized by low telomerase activity, may provide a bottleneck, which allows development only of eggs and embryos with sufficient telomere length.

It is telomere dysfunction rather than telomerase deficiency that causes defects in fertilization, cleavage, and development, since G1 $TR^{-/-}$ mice showed no abnormalities in reproductive function and also produced normal litter sizes. In contrast, late generation $TR^{-/-}$ mice exhibited profound abnormalities in fertilization, cleavage, and development. Indeed, the level of telomerase activity in biopsied blastomeres was not predictive of embryonic growth potential during preimplantation development (Wright *et al.*, 2001). Without reactivation of telomerase at the blastocyst stage, shortened telomeres may trigger a pathway that decreases survival during subsequent embryonic development, as reported previously (Herrera *et al.*, 1999a). By contrast, reintroduction of telomerase restores telomere function and rescues chromosomal instability and premature aging in telomerase-deficient mice with short telomeres (Hemann *et al.*, 2001b; Samper *et al.*, 2001).

Oocyte dysfunction of telomerase-null mice could be attributable to immaturity of oocytes, aneuploidy, and/or oocyte apoptosis. Short telomeres resulting from telomerase deficiency could perturb oocyte function by acting on cumulus cells that communicate with and nourish oocytes. The ovarian atrophy observed in late generations of $TR^{-/-}$ mice, and the failure to ovulate, regardless of exogenous hormone stimulation, probably indicate defects in both germ cells and surrounding somatic cells, which presumably also have undergone extensive apoptosis as their telomeres shorten to critical length in the absence of telomerase.

Fertilized eggs from $TR^{-/-}$ mice exhibited a high incidence of apoptosis, as evidenced by both cytofragmentation and nuclear DNA fragmentation. Cytofragmentation also coincided with development of only one pronucleus. The aberration in fertilization and normal cleavage appears to result from defects in both oocytes and sperm, since $TR^{-/-}$ eggs manifested only female pronuclear formation, regardless of fertilization by either wild-type or $TR^{-/-}$ mouse sperm (Figs. 3A and 3B). Consistently, $TR^{-/-}$ eggs underwent cytofragmentation after fertilization *in vivo* by either wild-type or $TR^{-/-}$ mouse sperm (Table 2). Some $TR^{-/-}$ eggs showed missegregation of chromosomes during polar body extrusion. Cytofragmentation might be attributable to telomere dysfunction that results in meiotic defects (Liu *et al.*, 2002a). The missegregation of chromosomes observed in G3 $TR^{-/-}$ eggs after fertilization probably indicates that misalignment of metaphase chromosomes occurred in meiosis. The incidence of cytofragmentation was high at the two-cell cleavage stage of embryos and was one major factor associated with poor cleavage and development in $TR^{-/-}$ mice. Blastomere fragmentation is common during early human embryo development, when approximately 40% of embryos exhibit fragmented cells before developmental arrest (Antczak and Van Blerkom, 1999; Van Blerkom *et al.*, 2001). It has been proposed that blastomere fragmentation in mouse and human preimplantation embryos is indicative of apoptosis (Jurisicova *et al.*, 1996, 1998a,b). Mouse embryos from crosses involving certain parental genotypes also have been shown to exhibit an increased incidence of blastomere fragmentation at the two-cell stage (Hawes *et al.*, 2001).

Mammalian female germ cells are produced by a special type of cell division, called meiosis, characterized by pairing and genetic recombination of homologous chromosomes at the leptotene/zygotene stages of early prophase I. Telomere dysfunction might already have compromised chromosome array during the early prophase I stage. Defects in meiotic division resulting from telomere dysfunction, as evidenced by chromosome misalignment and spindle disruption (Liu *et al.*, 2002a), could lead to aneuploidy and compromise subsequent normal cleavage and embryo development, such as decreased rates of blastocyst development (Lee *et al.*, 1998).

The heterogeneity of both mouse and human telomere length observed in somatic cells (Londono-Vallejo *et al.*, 2001; Zijlmans *et al.*, 1997) is also seen in mouse oocytes, as evidenced by large variation of telomere relative fluorescence intensity in the present study. The heterogeneity of individual telomere lengths may facilitate chromosomal organization within the nucleus, and thus proper pairing of homologous chromosomes (de Lange, 1998). Although the significance of telomere length heterogeneity in replicative senescence is not fully understood, telomere shortening-associated cellular senescence has not been attributed to a specific telomere. Interestingly, telomere loss preferentially occurred at the p-arm, which is close to centromeric regions. The accumulation of several short telomeres seems to signal senescence in cell culture (Martens *et al.*, 2000) and may signal senescence in germ cells as well. Cells in rapidly dividing tissues, with progenitors that usually express telomerase, are more strongly affected (Marciniak and Guarente, 2001; Vulliamy *et al.*, 2001). Germ cells are rapidly dividing prior to the arrest of prophase I stage, and fertilization and early development are severely impaired by erosion of telomeres.

Obvious defects in reproductive function were observed by the G2 generation of telomerase-null mice, compared with previous reports of reproductive defects starting from the G4 generation (Blasco *et al.*, 1997; Lee *et al.*, 1998). This difference in the timing of onset of reproductive dysfunc-

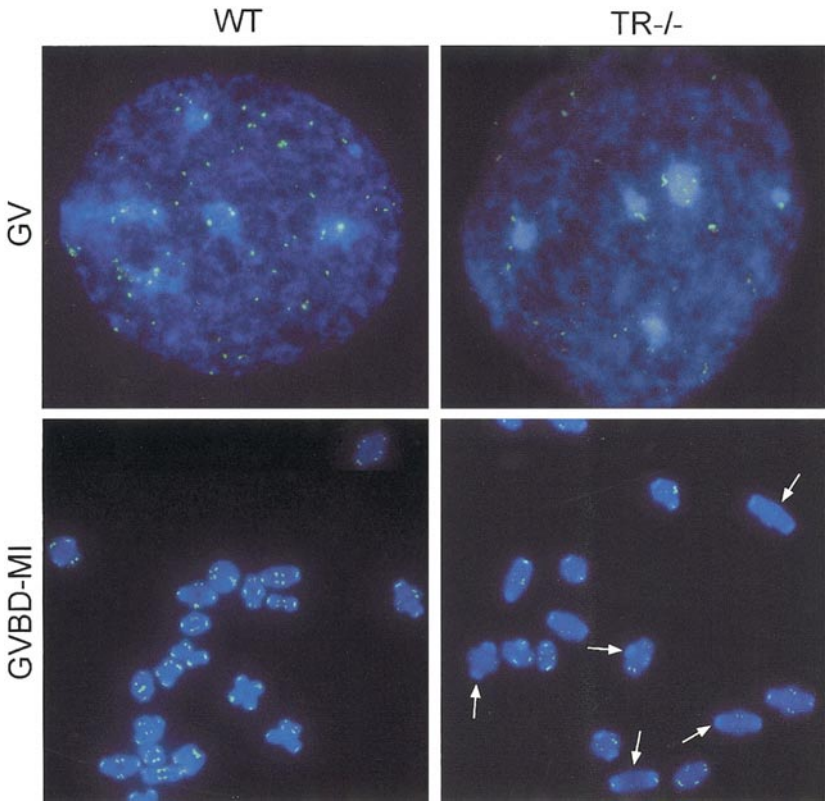



**FIG. 5.** Telomere detection of wild-type and G3 $TR^{-/-}$ mouse oocytes by FISH. (A) Many telomere fluorescence spots in the germinal vesicle (GV) of wild-type oocytes but less spots in the $TR^{-/-}$ GV oocytes. (B) Telomere fluorescence spots observed at the end of chromosomes of wild-type oocytes at germinal vesicle breakdown–Metaphase I stage (GVBD-MI), but undetectable (indicated by arrows) at the ends of some chromosomes in $TR^{-/-}$ mouse oocytes. Blue, DNA stained by Hoechst 33342; green, telomere fluorescence.

tion could be attributable to two factors. Our mice were bred from many generations of intercrosses between heterozygous $TR^{+/-}$ mice, so gradual telomere shortening may have occurred across generations. Indeed, a wild-type telomere length distribution is not restored in $mTR^{+/-}$ mice (Hemann *et al.*, 2001b; Samper *et al.*, 2001). As shown in this study, heterozygous gametes exhibited aberration in both fertilization and cleavage and early development (Tables 2 and 3). Moreover, growth retardation and ovarian atrophy were obvious in G4 $TR^{-/-}$ females. Coincidentally, we have been unable to obtain pregnancies from G4 $TR^{-/-}$ females after natural mating. We also observed a significant reduction in litter size of G3 $TR^{-/-}$ intercrosses (unpublished observations). A second possible reason for the observed pathology in G2 mice is that we employed superovulation by administration of low doses of exogenous gonadotropins, instead of natural ovulations, to obtain more eggs for study, and to mimic human clinical settings, where older women undergo superovulation for treatment of age-related infertility. Superovulations have been routinely utilized in studies on embryology and reproduction in mammals, including mouse and human. We did not observe obvious unfavorable effects of gonadotropins on fertilization and preimplantation development of wild-type and hybrid B6C3F1 mice in control experiments with our colonies. However, superovulation possibly could recruit immature oocytes and follicles with dysfunctional telomeres that would otherwise have been committed to apoptosis.

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## REFERENCES


Abdalla, H. I., Burton, G., Kirkland, A., Johnson, M. R., Leonard, T., Brooks, A. A., and Studd, J. W. (1993). Age, pregnancy and miscarriage: Uterine versus ovarian factors. *Hum. Reprod.* **8,** 1512–1517.

Allworth, A. E., and Albertini, D. F. (1993). Meiotic maturation in cultured bovine oocytes is accompanied by remodeling of the cumulus cell cytoskeleton. *Dev. Biol.* **158,** 101–112.

Angell, R. (1997). First-meiotic-division nondisjunction in human oocytes. *Am. J. Hum. Genet.* **61,** 23–32.

Antczak, M., and Van Blerkom, J. (1999). Temporal and spatial aspects of fragmentation in early human embryos: Possible effects on developmental competence and association with the differential elimination of regulatory proteins from polarized domains. *Hum. Reprod.* **14,** 429–447.

Bass, H. W., Riera-Lizarazu, O., Ananiev, E. V., Bordoli, S. J., Rines, H. W., Phillips, R. L., Sedat, J. W., Agard, D. A., and Cande, W. Z. (2000). Evidence for the coincident initiation of homolog pairing and synapsis during the telomere-clustering (bouquet) stage of meiotic prophase. *J. Cell Sci.* **113,** 1033–1042.

Betts, D. H., and King, W. A. (1999). Telomerase activity and telomere detection during early bovine development. *Dev. Genet.* **25,** 397–403.

Blackburn, E. H. (2000). Telomere states and cell fates. *Nature* **408,** 53–56.

Blackburn, E. H. (2001). Switching and signaling at the telomere. *Cell* **106,** 661–673.

Blasco, M. A., Lee, H. W., Hande, M. P., Samper, E., Lansdorp, P. M., DePinho, R. A., and Greider, C. W. (1997). Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. *Cell* **91,** 25–34.

Bodnar, A. G., Ouellette, M., Frolkis, M., Holt, S. E., Chiu, C. P., Morin, G. B., Harley, C. B., Shay, J. W., Lichtsteiner, S., and Wright, W. E. (1998). Extension of life-span by introduction of telomerase into normal human cells. *Science* **279,** 349–352.

Brison, D. R., and Schultz, R. M. (1997). Apoptosis during mouse blastocyst formation: Evidence for a role for survival factors including transforming growth factor alpha. *Biol. Reprod.* **56,** 1088–1096.

de Lange, T. (1998). Ending up with the right partner. *Nature* **392,** 753–754.

Eisenhauer, K. M., Gerstein, R. M., Chiu, C. P., Conti, M., and Hsueh, A. J. (1997). Telomerase activity in female and male rat germ cells undergoing meiosis and in early embryos. *Biol. Reprod.* **56,** 1120–1125.

Exley, G. E., Tang, C., McElhinny, A. S., and Warner, C. M. (1999). Expression of caspase and BCL-2 apoptotic family members in mouse preimplantation embryos. *Biol. Reprod.* **61,** 231–239.

Greider, C. W. (1990). Telomeres, telomerase and senescence. *Bioessays* **12,** 363–369.

Greider, C. W., and Blackburn, E. H. (1996). Telomeres, telomerase and cancer. *Sci. Am.* **274,** 92–97.

Hawes, S. M., Gie Chung, Y., and Latham, K. E. (2001). Genetic and epigenetic factors affecting blastomere fragmentation in two-cell stage mouse embryos. *Biol. Reprod.* **65,** 1050–1056.

Hemann, M. T., Rudolph, K. L., Strong, M. A., DePinho, R. A., Chin, L., and Greider, C. W. (2001a). Telomere dysfunction triggers developmentally regulated germ cell apoptosis. *Mol. Biol. Cell* **12,** 2023–2030.

Hemann, M. T., Strong, M. A., Hao, L. Y., and Greider, C. W. (2001b). The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. *Cell* **107,** 67–77.

Herrera, E., Martinez, A. C., and Blasco, M. A. (2000). Impaired germinal center reaction in mice with short telomeres. *EMBO J.* **19,** 472–481.

Herrera, E., Samper, E., and Blasco, M. A. (1999a). Telomere shortening in mTR−/− embryos is associated with failure to close the neural tube. *EMBO J.* **18,** 1172–1181.

Herrera, E., Samper, E., Martin-Caballero, J., Flores, J. M., Lee, H. W., and Blasco, M. A. (1999b). Disease states associated with telomerase deficiency appear earlier in mice with short telomeres. *EMBO J.* **18,** 2950–2960.

Ho, Y., Wigglesworth, K., Eppig, J. J., and Schultz, R. M. (1995). Preimplantation development of mouse embryos in KSOM: Augmentation by amino acids and analysis of gene expression. *Mol. Reprod. Dev.* **41,** 232–238.

Janny, L., and Menezo, Y. J. (1996). Maternal age effect on early human embryonic development and blastocyst formation. *Mol. Reprod. Dev.* **45,** 31–37.

Jurisicova, A., Latham, K. E., Casper, R. F., and Varmuza, S. L. (1998a). Expression and regulation of genes associated with cell death during murine preimplantation embryo development. *Mol. Reprod. Dev.* **51,** 243–253.

Jurisicova, A., Rogers, I., Fasciani, A., Casper, R. F., and Varmuza, S. (1998b). Effect of maternal age and conditions of fertilization on programmed cell death during murine preimplantation embryo development. *Mol. Hum. Reprod.* **4,** 139–145.

Jurisicova, A., Varmuza, S., and Casper, R. F. (1996). Programmed cell death and human embryo fragmentation. *Mol. Hum. Reprod.* **2,** 93–98.

Kornafel, K. L., and Sauer, M. V. (1994). Increased rates of aneuploidy in older women. Increased rates of aneuploidy do not occur in gestations of older embryo recipients. *Hum. Reprod.* **9,** 1981–1982.

Lawitts, J. A., and Biggers, J. D. (1993). Culture of preimplantation embryos. *Methods Enzymol.* **225,** 153–164.

Lee, H. W., Blasco, M. A., Gottlieb, G. J., Horner, J. W., 2nd, Greider, C. W., and DePinho, R. A. (1998). Essential role of mouse telomerase in highly proliferative organs. *Nature* **392,** 569–574.

Liu, L., Blasco, M. A., and Keefe, D. L. (2002a). Requirement of functional telomeres for metaphase chromosome alignments and integrity of meiotic spindles. *EMBO Rep.* **3,** 230–234.

Liu, L., and Keefe, D. L. (2000). Cytoplasm mediates both development and oxidation-induced apoptotic cell death in mouse zygotes. *Biol. Reprod.* **62,** 1828–1834.

Liu, L., Trimarchi, J. R., and Keefe, D. L. (2002b). Haploidy but not parthenogenetic activation leads to increased incidence of apoptosis in mouse embryos. *Biol. Reprod.* **64,** 204–210.

Londono-Vallejo, J. A., DerSarkissian, H., Cazes, L., and Thomas, G. (2001). Differences in telomere length between homologous chromosomes in humans. *Nucleic Acids Res.* **29,** 3164–3171.

Marciniak, R., and Guarente, L. (2001). Human genetics. Testing telomerase. *Nature* **413,** 370–373.

Marciniak, R. A., Johnson, F. B., and Guarente, L. (2000). Dyskeratosis congenita, telomeres and human ageing. *Trends Genet.* **16,** 193–195.

Martens, U. M., Chavez, E. A., Poon, S. S., Schmoor, C., and Lansdorp, P. M. (2000). Accumulation of short telomeres in human fibroblasts prior to replicative senescence. *Exp. Cell Res.* **256,** 291–299.

Moley, K. H., Chi, M. M., Knudson, C. M., Korsmeyer, S. J., and Mueckler, M. M. (1998). Hyperglycemia induces apoptosis in pre-implantation embryos through cell death effector pathways. *Nat. Med.* **4,** 1421–1424.

Navot, D., Bergh, P. A., Williams, M. A., Garrisi, G. J., Guzman, I., Sandler, B., and Grunfeld, L. (1991). Poor oocyte quality rather than implantation failure as a cause of age-related decline in female fertility. *Lancet* **337,** 1375–1377.

Navot, D., Drews, M. R., Bergh, P. A., Guzman, I., Karstaedt, A., Scott, R. T., Jr., Garrisi, G. J., and Hofmann, G. E. (1994). Age-related decline in female fertility is not due to diminished capacity of the uterus to sustain embryo implantation. *Fertil. Steril.* **61,** 97–101.

Plachot, M., Veiga, A., Montagut, J., de Grouchy, J., Calderon, G., Lepretre, S., Junca, A. M., Santalo, J., Carles, E., Mandelbaum, J., *et al.* (1988). Are clinical and biological IVF parameters correlated with chromosomal disorders in early life: A multicentric study. *Hum. Reprod.* **3,** 627–635.

Prowse, K. R., and Greider, C. W. (1995). Developmental and tissue-specific regulation of mouse telomerase and telomere length. *Proc. Natl. Acad. Sci. USA* **92,** 4818–4822.

Romanov, S. R., Kozakiewicz, B. K., Holst, C. R., Stampfer, M. R., Haupt, L. M., and Tisty, T. D. (2001). Normal human mammary epithelial cells spontaneously escape senescence and acquire genomic changes. *Nature* **409,** 633–637.

Rudolph, K. L., Chang, S., Lee, H. W., Blasco, M., Gottlieb, G. J., Greider, C., and DePinho, R. A. (1999). Longevity, stress response, and cancer in aging telomerase-deficient mice. *Cell* **96,** 701–712.

Samper, E., Flores, J. M., and Blasco, M. A. (2001). Restoration of telomerase activity rescues chromosomal instability and premature aging in Terc−/− mice with short telomeres. *EMBO Rep.* **2,** 800–807.

Scherthan, H., Jerratsch, M., Li, B., Smith, S., Hulten, M., Lock, T., and de Lange, T. (2000). Mammalian meiotic telomeres: Protein composition and redistribution in relation to nuclear pores. *Mol. Biol. Cell* **11,** 4189–4203.

Scherthan, H., Weich, S., Schwegler, H., Heyting, C., Harle, M., and Cremer, T. (1996). Centromere and telomere movements during early meiotic prophase of mouse and man are associated with the onset of chromosome pairing. *J. Cell Biol.* **134,** 1109–1125.

Tease, C., and Fisher, G. (1998). Analysis of meiotic chromosome pairing in the female mouse using a novel minichromosome. *Chromosome Res.* **6,** 269–276.

Van Blerkom, J., Davis, P., and Alexander, S. (2001). A microscopic and biochemical study of fragmentation phenotypes in stage-appropriate human embryos. *Hum. Reprod.* **16,** 719–729.

Vaziri, H., and Benchimol, S. (1998). Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. *Curr. Biol.* **8,** 279–282.

Vulliamy, T., Marrone, A., Goldman, F., Dearlove, A., Bessler, M., Mason, P. J., and Dokal, I. (2001). The RNA component of telomerase is mutated in autosomal dominant dyskeratosis congenita. *Nature* **413,** 432–435.

Wakayama, T., and Yanagimachi, R. (1998). Fertilisability and developmental ability of mouse oocytes with reduced amounts of cytoplasm. *Zygote* **6,** 341–346.

Wright, D. L., Jones, E. L., Mayer, J. F., Oehninger, S., Gibbons, W. E., and Lanzendorf, S. E. (2001). Characterization of telomerase activity in the human oocyte and preimplantation embryo. *Mol. Hum. Reprod.* **7,** 947–955.

Wright, W. E., Piatyszek, M. A., Rainey, W. E., Byrd, W., and Shay, J. W. (1996). Telomerase activity in human germline and embryonic tissues and cells. *Dev. Genet.* **18,** 173–179.

Xu, J., and Yang, X. (2000). Telomerase activity in bovine embryos during early development. *Biol. Reprod.* **63,** 1124–1128.

Zijlmans, J. M., Martens, U. M., Poon, S. S., Raap, A. K., Tanke, H. J., Ward, R. K., and Lansdorp, P. M. (1997). Telomeres in the mouse have large inter-chromosomal variations in the number of T2AG3 repeats. *Proc. Natl. Acad. Sci. USA* **94,** 7423–7428.

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