Molecular Human Reproduction Vol.7, No.10 pp. 947–955, 2001

Characterization of telomerase activity in the human oocyte and preimplantation embryo*

Diane L.Wright[1,3], Estella L.Jones[2], Jacob F.Mayer[2], Sergio Oehninger[2], William E.Gibbons[2] and Susan E.Lanzendorf[1]

[1]Technology Development Center and [2]Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, The Jones Institute for Reproductive Medicine, Eastern Virginia Medical School, Norfolk, Virginia, USA

[3]To whom correspondence should be addressed at: Vincent Memorial Obstetrics & Gynecology Service, In Vitro Fertilization Unit, Massachusetts General Hospital, 55 Fruit Street – VBK 225, Boston, MA 02114, USA

Telomerase, a ribonucleoprotein, has been described as an essential component of highly proliferative cells as it stabilizes the telomeres and avoids cellular senescence. The objective of this study was to modify the polymerase chain reaction-based telomeric repeat amplification protocol to detect telomerase activity in the single cell and to characterize the activity expressed in the human oocyte through to the blastocyst stage embryo. A comparative evaluation of telomerase activity and developmental stage was conducted using discarded or donated human oocytes and embryos. Telomerase activity was detected in all developmental stages evaluated from immature oocytes through to blastocyst stage embryos. Immature oocytes and blastocysts had similar levels of telomerase activity; however, both groups had significantly ($P < 0.05$) higher activity than zygote through to pre-morula stage embryos. Seventy-five thawed zygotes were cultured to day 3, biopsied by removing 1–2 cells, and the biopsied embryos were cultured to blastocyst stage. There was no difference ($P < 0.05$) in telomerase activity between cells biopsied from embryos that reached the blastocyst stage and cells from those that arrested in growth. This study has shown that human oocytes through to blastocyst stage embryos express telomerase activity, but that the level of telomerase activity in biopsied blastomeres, of the day 3 cleavage stage embryo, is not predictive of embryonic growth potential.

Key words: embryo/human/oocyte/telomerase/TRAP

Introduction

Telomeres are located at the terminal ends of chromosomes and have a unique structure of 6 bp repetitive sequences (Blackburn, 1991). In humans and most mammals, the sequence is TTAGGG (Counter *et al*., 1992). Depending on the cell types evaluated, human telomeres vary in length from 3 to 14 kb (de Lange *et al*., 1990). Telomeres prevent non-specific chromosomal recombination, allow the complete replication of chromosomal DNA without the inherent loss due to the DNA polymerase machinery, and help the chromosome bind to the nuclear matrix (Counter *et al*., 1992, 1994; de Lange, 1992). Telomeres shorten with each division of a somatic cell, due to the inability of DNA polymerase to completely replicate the end of chromosomes (Watson, 1972). Counter *et al*. (1992) established that the DNA of mortal cells is shortened by ~65 bp per generation. This shortening phenomenon has led to the theory that telomeres serve as a 'mitotic clock' for the cell (Allsopp *et al*., 1992).

In some cell types, the inherent loss of telomeric DNA during cellular replication is stabilized through addition of the 6 bp repeats (TTAGGG) to the chromosome ends by telomerase (Morin, 1989). Germline cells (Allsopp *et al*., 1992; Kim *et al*., 1994; Wright *et al*., 1996), embryonic tissues (Kim *et al*., 1994; Sharma *et al*., 1995; Wright *et al*., 1996), embryonic stem cell lines (Thomson *et al*., 1998), most tumour cells (Counter *et al*., 1994; Kim *et al*., 1994; Sharma *et al*., 1995), and some haematopoietic cells (Chiu *et al*., 1996; Engelhardt *et al*., 1997; Ogoshi *et al*., 1997) maintain telomerase activity to allow infinite divisions without disruption of the genome. Telomerase activity involves the concerted efforts of the RNA template component (telomerase RNA; TR) and the catalytic subunit (telomerase reverse transcriptase; TERT). The critical role of telomerase has been evaluated using a mouse strain lacking the telomerase RNA (*mTR*) gene (Blasco *et al*., 1997; Lee *et al*., 1998). Blasco and co-workers (1997) created mice that lacked the RNA component of telomerase ($mTR^{-/-}$). These mice were not capable of producing offspring by generation 6 (Lee *et al*., 1998) and experienced decreased longevity, delayed wound healing and increased incidence of malignancy (Rudolph *et al*., 1999).

*Presented at the meeting of the American Society for Reproductive Medicine, San Diego, California, USA, 2000.

Kim *et al*. first described the use of the telomeric repeat amplification protocol (TRAP), to evaluate telomerase activity, in various human tissues, immortal cell lines, and cancer cells (Kim *et al*., 1994). Of 100 immortal human cell types, 98 were found positive for telomerase activity while the 22 mortal cell types contained no detectable telomerase activity. Normal ovaries and testis were also positive for telomerase activity. Developmental studies in human fetal and adult somatic tissue have demonstrated a loss of telomerase expression with increasing gestational age and cellular differentiation. Wright *et al*. found fetal lung, adrenal, muscle, skin, and liver at 16 weeks of gestation to be positive for telomerase activity, while brain, kidney and bone were negative (Wright *et al*., 1996). Ulaner and Giudice demonstrated similar results for positive telomerase activity in the same fetal tissues through to 21 weeks gestation, except in the brain and kidney tissues where activity dropped off after 16 weeks (Ulaner and Giudice, 1997). These researchers also studied the fetal heart tissue and detected a loss of telomerase activity after 12 weeks gestation. Both studies showed the fetal and adult gonads exhibiting positive telomerase activity. Placental tissue and cultured fetal amniocytes were devoid of telomerase activity. All tissues were also evaluated in the 2 month neonate and telomerase activity was no longer detectable except in the gonad.

Embryos could be considered to be the ultimate stem cell due to the embryo's totipotent nature and research has begun to explore the role of telomerase in early embryonic development. A study in the rat provided information on relative levels of telomerase activity in pooled oocytes, 4-cell embryos and various maturity stages of male germ cells (Eisenhauer *et al*., 1997). Telomerase activity was high in the pre-ovulatory rat oocytes and decreased considerably in the mature oocytes and 4-cell embryos. Similar results were reported with the male germ cells. Immature spermatocytes and spermatids exhibited telomerase activity that was absent from mature spermatozoa. Two recent studies in the bovine established similar trends in relative telomerase activity for pooled oocytes and followed embryonic patterns of telomerase activity through to the blastocyst stage (Betts and King, 1999; Xu and Yang, 2000). These researchers detected telomerase activity in all stages of oocyte and embryo development. As oocytes matured, they noted a decrease in activity that persisted through to the 8-cell stage of embryo development. A considerable increase in telomerase activity was reported from the 8-cell to the morula and blastocyst stage embryo.

Very few researchers have studied human gametes and embryos for telomerase activity. Wright *et al*. analysed single mature oocytes retrieved from ovarian tissue of patients undergoing hysterectomy and adult sperm samples and reported that both mature gametes lacked telomerase activity (Wright *et al*., 1996). However, fertilized human embryos were thawed and cultured to the blastocyst stage, analysed individually, and found to express very high levels of telomerase activity. Alternatively spliced forms of the telomerase catalytic subunit have also been described in the human oocyte and embryo, and the results suggest a relationship with embryo quality (Brenner *et al*., 1999). The objectives of this study were to establish a reliable fluorescent method to measure telomerase activity in the single cell, evaluate the telomerase activity of individual human oocytes through to blastocyst stage embryos, and determine the reliability of using telomerase activity measurements of biopsied blastomeres to predict future embryo development.

Materials and methods

Human oocytes and embryos

Discarded human oocytes and embryos were used for this study, following approval by the Institutional Review Board (IRB) of Eastern Virginia Medical School. In addition, some cryopreserved embryos were donated by infertility patients, also following IRB approval. Zygotes were thawed using protocols that corresponded with the protocol under which they were frozen (Testart *et al*., 1986; Freedman *et al*., 1988; Veeck *et al*., 1993).

Human embryos were cultured in sequential style medias, following instructions provided by the manufacturers (Enhance Day 1/Day 3 from Conception Technologies, San Diego, CA, USA; P1/Blastocyst Medium from Irvine Scientific, Santa Ana, CA, USA; G1.2/G2.2 from IVF Science, Scandinavia). Briefly, pronuclear stage (day 1) embryos were placed at 37°C under oil in 500 µl of CO_2 equilibrated media, which was designated for day 1–2 stage embryos. After 48 h in the first stage media, embryos were moved to media designated for day 3 to blastocyst stage embryos. Embryos were placed in fresh medium every 48 h until the final growth stage was reached or until growth had arrested for 24 h. Zonae pellucidae were removed from embryos, when appropriate, by exposure to acidified Tyrode's solution as previously described (DeScisciolo *et al*., 2000).

Cell lines

All cell lines, except mouse cells, were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cell lines were cultured according to instructions defined by the ATCC. The three cell lines from ATCC used in this study are as follows. DU145: carcinoma, prostate cancer metastasis to brain, human, epithelial-like; Hs27: newborn foreskin, male, human, fibroblast; Detroit 551: female, human, skin, embryonic. The mouse cell line (PMEF: primary mouse embryonic fibroblasts) was created by the homogenization of fetuses at 13 days gestation (Abbondanzo *et al*., 1993). These pregnancies were the result of a cross between B6CBAF1/J females (Jackson Laboratories, Bar Harbor, MA, USA) and CD1 males (Charles River Laboratories, Wilmington, MA, USA).

Cell lines were cultured in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, MD, USA) supplemented with 15% fetal bovine serum (Hyclone, Logan, UT, USA). The culture medium was replaced every 48 h with pre-equilibrated 37°C medium. The passage of cell cultures was conducted following ATCC guidelines. Cell suspensions were then counted and aliquoted for subsequent passage or lysis.

Embryo biopsy

Embryo biopsy was performed in 100 µl drops of equilibrated Earl's balanced salts solution with 15% synthetic serum substitute (Irvine Scientific) with a mineral oil overlay. The embryo biopsy technique was performed as previously described (Gibbons *et al*., 1995) on day 3 cleavage stage embryos that reached the 4–9-cell stage. Briefly, the embryo was held in place using a holding pipette, while a hole was placed in the zona pellucida using acidified Tyrode's solution. After the hole was made next to a blastomere exhibiting a visible nucleus, gentle suction was applied with the biopsy pipette until it had separated from the embryo. Once the biopsy procedure was complete, blastomeres were individually

removed from the micromanipulation dish in a 1 µl column of media and added to a micro-centrifuge tube containing 9 µl of CHAPS lysis buffer [10 mmol/l Tris–HCl (pH 7.5), 1 mmol/l $MgCl_2$, 1 mmol/l EGTA, 0.1 mmol/l benzamidine, 5 mmol/l β-mercaptoethanol, 0.5% CHAPS, 10% glycerol]. These sample tubes were snap-frozen in liquid nitrogen and stored at –80°C.

Immunosurgery of blastocysts

Immunosurgery was conducted on embryos that reached the blastocyst stage. The trophectoderm was removed from the blastocysts as previously described (Lanzendorf *et al*., 2001). Blastocysts that had not hatched free of their zona pellucida were treated with acidified Tyrode's solution and through gentle pipetting the zona pellucida was removed. Whole blastocysts were exposed to rabbit anti-BeWo serum followed by exposure to guinea-pig complement. The trophectoderm cells lysed during the complement step and were removed from the inner cell mass (ICM) by repeated pipetting through a finely drawn Pasteur pipette. The longest length of each ICM was measured under the ×20 objective using an ocular micrometer. Isolated ICM in 1 µl of media were added to micro-centrifuge tubes containing 9 µl of CHAPS lysis buffer and snap-frozen in liquid nitrogen with storage at –80°C.

Telomeric repeat amplification protocol (TRAP)

The TRAPeze™ telomerase detection kit (Intergen, Inc., Purchase, NY, USA) was used to evaluate telomerase activity. Modifications were made in lysate preparation for the single cell (Holt *et al*., 1996) and for the use of fluorescent DNA analysis (Hisatomi *et al*., 1997). Cell lysates were prepared as described by the manufacturer using the CHAPS lysing buffer provided in the kit. The number of cells ranged from 1×10^5 to 1×10^6 cells used in each lysing reaction. Lysates were stored at –80°C. Individual cells from cell line suspensions were isolated in Dulbecco's phosphate-buffered saline (D-PBS; Gibco, Gaithersburg, MD, USA) + 1% BSA (Sigma, St Louis, MO, USA). Single cells within 5 µl of D-PBS were added to a micro-centrifuge tube containing 5 µl of CHAPS and incubated, on ice, for 30 min. The cellular extracts were snap-frozen in liquid nitrogen and stored at –80°C. After removal of the zona pellucida with acidified Tyrode's solution, as previously described (DeSciscIolo *et al*., 2000), whole embryos were placed in a micro-centrifuge tube with 10 µl of CHAPS and snap-frozen in liquid nitrogen, with subsequent storage at –80°C. For thawing, the whole embryo tubes were incubated on ice for 30 min. All oocytes, embryos and the individual or biopsied cells were analysed using the entire 10 µl lysate as the sample.

The assays were conducted in 25 µl reactions with 5 pmol of the TS primer (5′-AATCCGTCGAGCAGAGTT-3′) in 20 mmol/l Tris–HCl (pH 8.3), 1.5 mmol/l $MgCl_2$, 63 mmol/l KCl, 0.05% Tween 20, 1 mmol/l EGTA, 0.05 mmol/l each dNTP, 1 U Taq DNA polymerase, and the primer mix (which includes the reverse 'RP' primer and the K1 primer and TSK1 template for the internal standard). All of the reaction components were provided in the TRAPeze kit. A specifically labelled TS Primer was purchased from LI-COR (Lincoln, NE, USA) to obtain the IRD800 infrared label necessary for use with the LI-COR IR^2 DNA Sequencer. Reaction tubes were incubated at 30°C for 30 min for all cellular lysate samples, except the single cell and whole embryo samples which were incubated for 60 min. A two-step polymerase chain reaction (PCR) was conducted for 38 cycles at 94°C for 30 s and 59°C for 30 s, after an initial hold at 94°C for 3 min. Products were diluted 5:1 with IR^2 Stop Solution (LI-COR) to prevent DNA strands from re-annealing and to facilitate loading of the samples into the gel. The diluted products were heated to 93°C for 3 min and placed directly on ice. Each product was loaded (1 µl/lane) onto a 7% Long Ranger denaturing gel (FMC BioProducts, Rockland, ME, USA) with 7 mol/l urea, fitted to the IR^2 infrared DNA sequencer (LI-COR) and electrophoresed at 45°C and 1500 V.

The fluorescent data were analysed using the Gene Imager software (LI-COR). Each reaction and assay includes various controls to provide the most accurate data, which are also provided with the TRAPeze kit. Every assay included two negative reactions (CHAPS lysis buffer and Hs27 cell lysate) in addition to the positive control cell lysate. All reactions contained an internal PCR amplification control that was 36 bp in size. Finally, a quantification control template, TSR8, was run with each assay. Each sample was quantified in terms of peak band density and peak area (integrated optical density) of the fluorescence. Using the following formulas, values were obtained for total product generated (TPG) and relative telomerase activity (RTA):

$$\text{TPG (units)} = \frac{\left(\dfrac{\text{Measured total area of telomerase activity (50 bp, 56, 62, etc.)}}{\text{Measured area of internal 36 bp control}}\right)}{\left(\dfrac{\text{Measured total area of telomerase activity (50 bp, 56, 62, etc.) in TSR8}}{\text{Measured area of internal 36 bp control of TSR8}}\right)} \times 100$$

$$\text{RTA (units)} = \frac{\left(\dfrac{\text{Measured total area of telomerase activity (50 bp, 56, 62, etc.)}}{\text{Measured area of internal 36 bp control}}\right)}{\left(\dfrac{\text{Measured total area of telomerase activity in positive control cells}}{\text{Measured area of internal 36 bp control positive control cells}}\right)}$$

Statistical analysis

Levene's *F*-test was used to test the assumption of homogeneity of variance, resulting in statistical significance ($P < 0.05$). We concluded that there was no homogeneity of variance; therefore, Dunnett's T3 post-hoc test was used for multiple comparisons to determine significant differences at $P < 0.05$ between the mean TPG and RTA activities of each growth stage. Variance component analysis was conducted on the disaggregated embryo data. For the biopsy experiment, Levene's *F*-test statistic was used for testing of homogeneity of variance and analysis of variance was used for comparison of the two groups with a significance level of 0.05.

Results

Telomerase assay verification

The first series of testing explored the ability of the IR^2 assay system to detect telomerase activity. The diluted lysates from four cell types were tested, including three human (DU-145, Hs27 and Detroit 551) and one mouse (PMEF) cell lines. The positive control cell pellet lysate was also evaluated with each assay. A comparative evaluation was initially made of human and mouse cells to establish the cross-species abilities of the assay; this showed that both cell types (DU-145 and PMEF) had measurable telomerase activity (data not shown).

A subsequent experiment compared telomerase activity in cells from cell lines known to express telomerase activity and those known to lack telomerase activity (Figure 1). As expected, telomerase activity was found in the DU-145 and PMEF cells; however, there was no measurable activity in the Hs27 or the Detroit 551 cell lines.

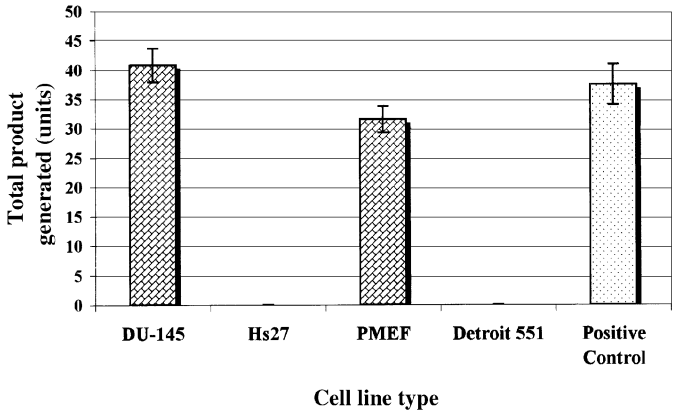

Figure 1. Telomerase activity in cell lysates of positive and negative cell lines. Cell lysates from DU-145, Hs27, PMEF, Detroit 551 and the positive control cells were diluted to 100 cell equivalents. Data are the means ± SD of five samples from one assay.

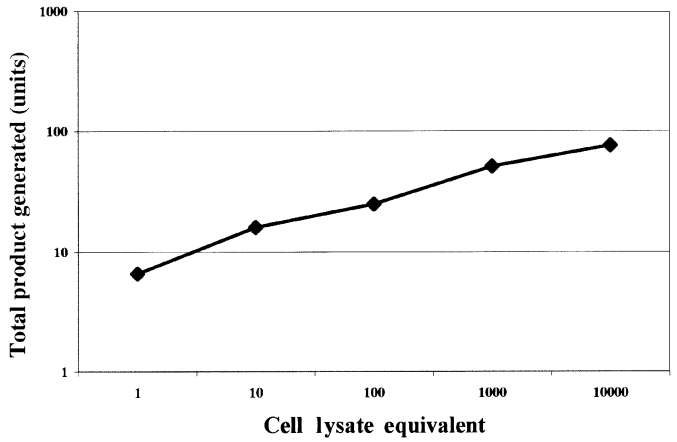

Figure 2. Telomerase activity measured in 10-fold dilutions of the positive control cell lysate. Data represent the mean of single samples loaded twice in one telomeric repeat amplification protocol assay.

A dilution series of the positive control cell lysate was evaluated from a 1-cell equivalent dilution through to 10 000 cells equivalent, increasing by factors of 10. A logarithmic increasing trend was noted in the measured telomerase activity of the lysate dilutions (Figure 2) from the 1 cell equivalent to the 10 000 cells equivalent (Figure 2).

To evaluate the ability of the assay to detect telomerase activity in single cell samples, 60 individual DU-145 cells were isolated and assayed. All cells exhibited measurable telomerase activity, with a mean TPG of 20.1 ± 12.2 units per sample (data not shown).

Telomerase activity in whole oocytes, embryos, and ICM

A total of 167 discarded human oocytes and embryos were evaluated for telomerase activity. Of this study group, 22 were oocytes, 30 were zygotes, and the remaining 115 were cleaving embryos. These embryos were received as fresh (83.7%) discarded embryos and as thawed, donated (16.3%) embryos that arrested in their development. Each oocyte or embryo was analysed individually. Telomerase activity was detected in 97.6% of samples tested. Two methods were used to calculate telomerase activity in these samples, RTA and TPG. When both methods were evaluated statistically, no significant difference was detected in the results.

Oocytes were studied at the immature germinal vesicle (GV) stage through to the mature metaphase II (MII) stage (Figure 3). The immature oocyte study group included 10 with a visible germinal vesicle (GV), and six oocytes with no GV and no polar body and considered to be metaphase I (MI) stage oocytes. Sixteen immature oocytes were tested and all had measurable telomerase activity. One of the six MII oocytes tested had no detectable telomerase activity. The average telomerase activity measured in the immature oocytes was higher than the level observed in the mature oocytes, zygotes and early cleavage stage embryos.

The data collected on embryos ranged from the zygote through to the blastocyst stage (Figure 3). The pronuclear status varied with these discarded embryos. A single pronucleus developed in nine (6.7%) of the samples, 62 (45.9%) exhibited the normal complement of 2 pronuclei, and 64 (47.4%) of the embryos were multipronuclear (⩾3 pronuclei). Embryos were grouped by cleavage stage for statistical analysis. Telomerase activity was detected in all developmental stages. There was no statistical difference between the mature and immature oocytes, but telomerase activity in the immature oocytes was significantly greater than the zygote through 8–16 cell cleavage stage embryos ($P < 0.05$). Telomerase activity in the blastocyst group was significantly higher ($P < 0.05$) than in the zygote through to 8–16-cell stage embryos, but was not different from the oocytes or morula sample means.

Thirty-one blastocysts derived from normal pronuclear embryos were treated with immunosurgery. Of those blastocysts treated, six (19.4%) did not have an identifiable ICM. Nine ICM were analysed individually using the telomerase assay. The mean telomerase activity of the ICM was compared to that of oocytes and numerous growth stages of embryos (Figure 3). Although telomerase activity was detected in all ICM evaluated, there was no statistical difference ($P < 0.05$) measured between ICM and the other comparison groups.

The same set of whole gamete and embryo data was also analysed on a per cell basis by dividing the embryo telomerase value by the number of cells in the embryo (Figure 4). Morula cell number was estimated at 15, and ICM and whole blastocyst cell number were estimated based on a publication (Devreker *et al*., 1998) at 20.7 and 64.5, respectively. The telomerase activity was significantly higher in the immature oocyte group than all other groups ($P < 0.05$), excluding the mature oocyte group, which was not different from any of the comparison groups (Figure 4). The zygotes were statistically different from all other groups excluding the mature oocytes ($P < 0.05$). The blastocyst telomerase activity was significantly lower than all zygote and early cleavage stage groupings ($P < 0.05$).

Analysis of each blastomere of entire embryos

Blastomeres of cleavage stage embryos were separated and assayed individually to provide information on the variability of telomerase activity from one cell to the next within an entire embryo. Twenty-three donated or discarded embryos were evaluated from 11 patients. The embryos ranged from

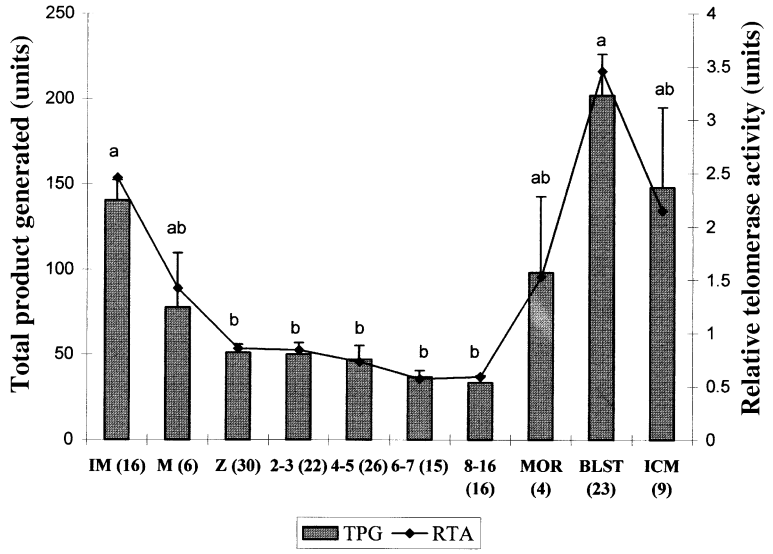

Figure 3. Telomerase activity in human oocytes and embryos. The samples are grouped by developmental stage and calculated as total product generated (TPG) and relative telomerase activity (RTA) for immature oocytes (IM), mature oocytes (M), zygotes (Z), 2–3-cell, 4–5-cell, 8–16-cell and morula (MOR) stage embryos, blastocysts (BLST) and inner cell mass (ICM). Numbers in parentheses represent the number of oocytes or embryos analysed to achieve the mean represented. Y-error bars represent SEM TPG values. Different superscripts represent significant differences ($P < 0.05$).

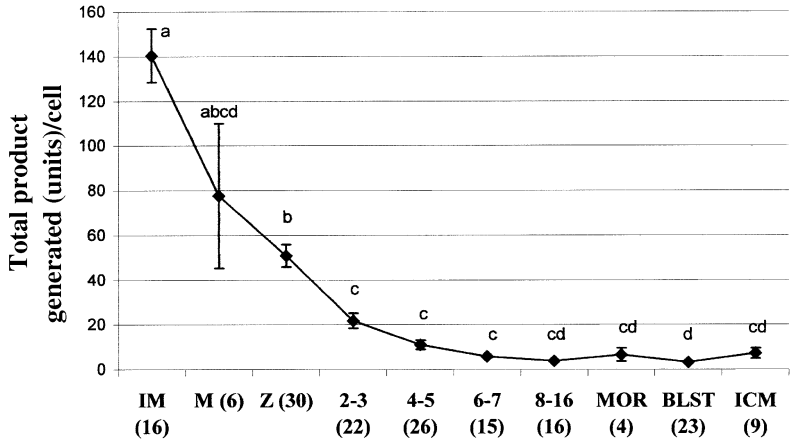

Figure 4. Telomerase activity in each developmental group presented on a per cell basis. Cleavage stage embryos were divided by their respective cell number. Y-error bars represent SEM. Different superscripts represent significant differences ($P < 0.05$).

2-cell to 14-cell with two (8.7%) single pronuclear, seven (30.4%) normal pronuclear, and 14 (60.9%) multi-pronuclear in development; 143 blastomeres were isolated and evaluated from these 23 embryos. During isolation, 28 (19.7%) of the individual blastomeres lysed. Of the 143 blastomeres evaluated, 24 had no measurable telomerase activity and one sample was lost due to technical difficulty. Twelve (52.2%) of the 23 embryos had at least one cell that did not express telomerase activity. Variance analysis of the blastomere TPG levels found that 18% of the variance was the result of differences between embryos and 82% of the variance was due to differences between blastomeres.

Telomerase activity as predictor of blastocyst development

Cryopreserved zygotes were donated by 33 IVF couples. In all, 162 embryos were thawed. Of those 162 thawed, 142 (88%) survived and 123 (79%) cleaved following 24 h incubation. Seventy-five embryos were biopsied. One or two cells were removed from each biopsied embryo and the cells were assayed individually, resulting in 127 blastomeres biopsied from 75 embryos. The blastocyst development rate for embryos that were biopsied was 33 blastocysts of 75 embryos biopsied, or 44%. Nuclear status was evaluated in each blastomere and 23% had no visible nucleus, 73% had one nucleus and 4% had two nuclei. Lysis or partial lysis of the blastomere biopsied occurred in 18% of blastomeres biopsied.

The average cell number at biopsy for the arrested group was 5.78 ± 1.2 compared to the blastocyst group with 7.13 ± 0.92 cells. Telomerase activity was compared between embryos biopsied that subsequently arrested and those that subsequently progressed to blastocyst (Table I). There were two methods used to provide the TPG value for an embryo, when there were two cells biopsied. The first method was to average the two values and the second method was to choose

Table I. Comparison of embryo growth potential with telomerase activity measured in biopsied blastomeres

	Embryos	Blastomere TPG units	
		Average	Highest
Arrested	42	11.4 ± 4	12.8 ± 4
Blastocyst	33	10.3 ± 5	12.6 ± 6

the higher value of the two to represent that embryos activity. No statistical differences were noted between the two groups of biopsied embryos when either the averaging or higher value method was analysed.

Discussion

With the development of the PCR-based TRAP by Kim *et al.* (1994), researchers have initiated a broader understanding of the mechanisms involved in extension of the chromosomal telomeric ends. This original protocol has been modified in numerous ways to improve the speed and sensitivity. This laboratory is utilizing a modified version of TRAP, which is available in a kit form (TRAPeze), decreasing the time needed to prepare and test reagents. A comparison study was conducted to evaluate the difference between the original TRAP assay and the TRAPeze kit (Holt *et al.*, 1996). The investigators found that the kit version was faster due to a decrease from a three-step to a two-step PCR method. The kit was determined to be superior to the original method in sensitivity and quality control additions. The purpose of this study was to modify the current TRAP method to analyse telomerase activity of individual human oocytes and embryos, and to use this method to study the trends in expression of telomerase activity in whole oocytes through development to the blastocyst stage.

This laboratory incorporated infrared labelling into the TRAPeze system to increase the quantifying sensitivity. The substrate primer (TS) was labelled with an infrared fluorochrome, necessary for using the IR^2 DNA sequencing system (LI-COR). This modification allowed for the use of fluorescence instead of radioactivity or staining to quantify telomerase activity. Hisatomi *et al.* first described the combination of TRAP with fluorescent labelled products (F-TRAP) in the analysis of human liver tissue (Hisatomi *et al.*, 1997). Subsequent to this original study, F-TRAP has been utilized to evaluate hepatocellular carcinoma (Wada *et al.*, 1998; Nagao *et al.*, 1999), pheochromocytoma (Kubota *et al.*, 1998), urothelial neoplasia (Ohyashiki *et al.*, 1998), and indomethacin treatment of murine colon adenocarcinoma (Ogino *et al.*, 1999). Finally, most of the investigations of telomerase activity have involved the use of large quantities of cells. A few researchers have described the capability of the assay to measure telomerase activity in single cells with minor modifications. Some adjustments to the assay to allow for measurement of telomerase activity in the single cell have been described in human cancer cells (Holt *et al.*, 1996), in human oocytes and blastocysts (Wright *et al.*, 1996), and in rat oocytes and embryos (Eisenhauer *et al.*, 1997). An extended initial incubation along with increased PCR cycles and the use of whole lysates were employed to enhance the sensitivity of the assay in our laboratory.

Since the TRAPeze kit was modified in use from the manual provided, some preliminary testing was necessary to verify accuracy of results. Evaluation of two cell lines from two different species was conducted to measure telomerase activity which was detected in the human (DU-145) and the mouse (PMEF) cell types using the TRAP assay that was modified in this laboratory. Two other human cell types, Hs27 and Detroit 551, were analysed for telomerase activity and none was detected. Hs27, newborn foreskin, was previously shown to lack telomerase activity (Bodnar *et al.*, 1998). The fact that the Detroit 551 cell line, female embryonic skin, did not express telomerase activity was not unexpected due to the cell type having a finite number of subcultures, suggesting that the cells are terminally differentiated. To establish the range of the assay after the modifications were incorporated, an initial study was conducted using dilutions of the positive control cell lysate. The lysate was diluted from 10 000 to 1-cell equivalents and a correlating trend was noted in telomerase activity, being lowest in the 1-cell equivalent and highest in the 10 000-cell equivalent. Finally, 60 individual cells were isolated from the human carcinoma cell line, DU-145, and each cell was analysed individually. This experiment would more closely replicate the use of the assay for evaluating biopsied cells of embryos. Telomerase activity was detected in each cell analysed. This entire series of preliminary experiments established the initiation of a successful TRAP assay system for evaluating telomerase activity in human oocytes and preimplantation embryos.

Very few data, previous to this study, on telomerase activity in human oocytes and embryos have been published. Human oocytes and embryos (Brenner *et al.*, 1999) have been evaluated for alternative splice variants of the telomerase catalytic subunit (hTERT). A relationship was found between poor quality embryos and an increase in alternative splicing variants of hTERT. Wright *et al.* studied a few samples of mature oocytes, spermatozoa, and blastocysts that were discarded material from IVF cycles (Wright *et al.*, 1996). No telomerase activity was detected in the mature spermatozoa or oocytes, but very high levels were measured in the blastocysts evaluated. In the study conducted in our laboratory, whole oocytes and embryos were tested for telomerase activity after removing the zona pellucida. Each oocyte or embryo was analysed individually and 97.6% of the samples evaluated had detectable levels of telomerase. The fact that telomerase activity was detected in mature oocytes evaluated in this study and was not found in the oocytes analysed by Wright *et al.* (1996) may be due to an increased sensitivity obtained with the assay we employed. Some studies in other species, bovine (Betts and King, 1999; Xu and Yang, 2000) and rat (Eisenhauer *et al.*, 1997), have found measurable, albeit low, levels of telomerase activity in the mature oocyte. In this study, immature oocytes and blastocysts expressed the highest levels, greater than zygotes or any of the cleavage stage groupings. Interestingly there was no difference between the levels of telomerase activity detected in the immature oocyte and in the blastocyst with ~65 cells (Devreker *et al.*, 1998). This high level of telomerase activity

in the single cell, immature oocyte suggests a possible role for telomerase in meiosis (Betts and King, 1999). A high level of expression of telomerase in the immature oocyte was also seen in the bovine species (Betts and King, 1999) and in the rat (Eisenhauer *et al*., 1997). Although similar trends were seen in the data from this study, both the bovine and rat studies utilized pooled samples of their oocytes and embryos for TRAP analysis, differing from this laboratory's method of individual analysis. The authors of the bovine and rat studies also used different methods to calculate telomerase activity. The bovine studies employed the relative telomerase activity (RTA) calculation using a positive control cell line (Betts and King, 1999) or background standard (Xu and Yang, 2000) as methods to normalize data. The Eisenhauer *et al*. (1997) rat study used a similar calculation method, but expressed the value on a per cell basis as the percentage relative to a positive cell (293 cells) assayed in parallel. However, the same trends were seen in expression of telomerase activity in the oocyte and embryo.

This is the first study to evaluate telomerase activity of ICM isolated from the trophoblast cells of the whole blastocyst. Nineteen per cent of blastocysts evaluated did not contain an ICM. Yet, all ICM analysed had detectable levels of telomerase activity that were not significantly different from the whole blastocyst. Although the test group was small, these data could suggest that a significant level of telomerase activity of the whole blastocyst is originating from the ICM, although the ICM only constitutes ~30% of the cell number associated with the blastocyst (Devreker *et al*., 1998).

When the data for whole oocytes and embryos were evaluated on a per cell basis, telomerase activity declined from the immature oocyte through maturation and fertilization and levelled off to a similar value per cell through subsequent cleavages. The same decline on a per cell basis was also noted in the bovine (Xu and Yang, 2000). The immature oocyte has a much larger cytoplasmic volume than cells from subsequent divisions and this could certainly be part of the reason for the significantly higher levels of telomerase activity. However, the mature oocyte has a similar cytoplasmic volume to the immature oocyte, with an apparent decline in telomerase activity and this trend continues through the 1-cell zygote. Perhaps this decline in telomerase activity is due to depletion of the maternal stores of proteins known to exist in the oocyte or a shift in alternative splicing variants of hTERT (Brenner *et al*., 1999). As the transition from oocyte to embryonic genome takes places at approximately the 4-cell stage (Braude *et al*., 1988) there is an up-regulation of critical proteins. Analysis of the data on a per cell basis does not show a change in expression of telomerase activity at this stage.

Since a large percentage of the embryos analysed in this study was polypronuclear, we also compared the normal embryos to polypronuclear embryos within each growth stage for differences in telomerase activity. No differences were noted with the exception of zygote stage embryos. Telomerase activity was significantly higher in normal pronuclear zygotes compared to that in polypronuclear zygotes. This reduction in telomerase activity within polypronuclear embryos may be related to the alternative splice mechanism of hTERT (Ulaner *et al*., 1998; Brenner *et al*., 1999). Ulaner *et al*. (1998) found that fetal kidney only expresses the full-length transcript of hTERT through the time period of actual telomerase activity. Once the activity in the fetal kidney had ended, splice variants were the only hTERT forms remaining. Three alternative splice forms have been described in human oocytes and embryos, but telomerase activity was not compared between these oocytes and embryos (Brenner *et al*., 1999). The polypronuclear zygote may contain more splice variants than the normal zygote resulting in a loss of telomerase activity.

Results from the blastomeres of disaggregated embryos showed a significant level of variability from one cell to the next within an embryo. Certainly, it has been well established that cells within an embryo may be different. Several authors have published data regarding the high incidence of mosaicism of normal and abnormal embryos (Munné *et al*., 1993; DeScisciolo *et al*., 2000). Results here show that, in this population of embryos evaluated, 18% of the variance of blastomere TPG units is the result of different embryos and 82% of the variance is due to differences between the blastomeres.

Human embryo biopsy has become a standard procedure for certain couples trying to avoid genetic disease in their offspring. Research has shown that there is no detrimental effect on the embryo by removing one or two cells from the day 3 embryo (Hardy *et al*., 1990). In the present study, embryos were cryopreserved at the zygote stage, thawed, and cultured to the appropriate stage for biopsy. There are no publications that refer to the use of thawed, cultured pronuclear embryos for biopsy or the evaluation of telomerase activity within these blastomeres. A 44% blastocyst rate was achieved with these thawed, cultured, and biopsied embryos. This study's blastocyst formation rate was lower than the average rate of 71% from biopsied embryos (Hardy *et al*., 1990). Hardy *et al*. (1990) used fresh embryos and only embryos that reached the 8-cell stage. Our laboratory biopsied embryos when they reached the 4–9-cell stage. Telomerase levels were not significantly different for biopsied blastomeres evaluated from the embryos that arrested in development and those that progressed to blastocyst. Variability of measurable telomerase activity undoubtedly played a role in the inability to distinguish differences between the two groups. The source of variability in telomerase activity of these blastomeres can be partly explained by the critical timing at which samples were taken. Results from this study show a decline in telomerase activity through all stages in the cleaving embryo. This decline in telomerase activity coincides with the shift to embryonic genome activation. Biopsies were conducted on 4–9-cell embryos, but less variability might have been observed if biopsies had been conducted only at one specific cell stage. This study found no correlation with blastomere telomerase activity of the 4–9-cell biopsy stage embryo and blastocyst development potential.

Culture systems for the human preimplantation embryo are constantly evolving. Research in the mouse (O'Neill, 1997, 1998) has found a beneficial effect of growing groups of mouse embryos within microdrops. These mouse embryos appeared to require certain autocrine signals to survive through

the 2-cell stage and progress in growth. Various researchers have studied haematopoietic cells and noted an ability to stimulate telomerase activity within these cells by the addition of specific cytokines and stimulation of cell receptors (Chiu *et al*., 1996; Engelhardt *et al*., 1997; Ogoshi *et al*., 1997). Future studies, in the human, should address the relationship of autocrine factors and embryo development with respect to telomerase activity. Studies could be designed to address whether various culture systems currently employed inhibit or enhance the embryonic genome's ability to express telomerase activity.

In summary, this is the first study that has evaluated the level of telomerase activity in discarded embryonic material from whole oocytes through to blastocysts, as well as in the ICM of blastocysts, and finding that all stages expressed telomerase activity. Immature oocytes and blastocysts had the highest levels of telomerase activity and showed significantly greater levels than zygotes through to pre-morula stage embryos. The isolated ICM was found to express telomerase activity similar to the whole blastocyst. Variability within cells of human embryos was found to be very high. Telomerase activity was also measured in one or two biopsied cells, from day 3 cleaving embryos. This study found no statistical relationship between telomerase activity of biopsied blastomeres and developmental potential of the biopsied embryos.

References

Abbondanzo, S.J., Gadi, I. and Stewart, C.L. (1993) Derivation of embryonic cell lines. *Methods Enzymol*., **225**, 803–823.

Allsopp, R.C., Vaziri, H., Patterson, C. *et al*. (1992) Telomere length predicts replicative capacity of human fibroblasts. *Proc. Natl. Acad. Sci. USA*, **89**, 10114–10118.

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. (1991) Structure and function of telomeres. *Nature*, **350**, 569–573.

Blasco, M.A., Lee, H.W., Hande, M.P. *et al*. (1997) Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. *Cell*, **91**, 25–34.

Bodnar, A.G., Quellette, M., Frolkis, M. *et al*. (1998) Extension of life-span by introduction of telomerase into normal human cells. *Science*, **279**, 349–352.

Braude, P., Bolton, V. and Moore, S. (1988) Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature*, **332**, 459–461.

Brenner, C.A., Wolny, Y.M., Adler, R.A. *et al*. (1999) Alternative splicing of the telomerase catalytic subunit in human oocytes and embryos. *Mol. Hum. Reprod*., **5**, 845–850.

Chiu, C.P., Dragowska, W., Kim, N.W. *et al*. (1996) Differential expression of telomerase activity in hematopoietic progenitors from adult human bone marrow. *Stem Cells*, **14**, 239–248.

Counter, C.M., Avilion, A.A., LeFeuvre, C.E. *et al*. (1992) Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *EMBO J*., **11**, 1921–1929.

Counter, C.M., Hirte, H.W., Bacchetti, S. *et al*. (1994) Telomerase activity in human ovarian carcinoma. *Proc. Natl. Acad. Sci. USA*, **91**, 2900–2904.

De Lange, T. (1992) Human telomeres are attached to the nuclear matrix. *EMBO J*., **11**, 717–724.

De Lange, T., Shiue, L., Myers, R.M. *et al*. (1990) Structure and variability of human chromosome ends. *Mol. Cell. Biol*., **10**, 518–527.

DeScisciolo, C., Wright, D.L., Mayer, J.F. *et al*. (2000) Human embryos derived from *in vitro* and *in vivo* matured oocytes: analysis for chromosomal abnormalities and nuclear morphology. *J. Assist. Reprod. Genet*., **17**, 284–292.

Devreker, F., Winston, R.M.L. and Hardy, K. (1998) Glutamine improves preimplantation development *in vitro*. *Fertil. Steril*., **69**, 293–299.

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

Engelhardt, M., Kumar, R., Albanell, J. *et al*. (1997) Telomerase regulation, cell cycle, and telomere stability in primitive hematopoietic cells. *Blood*, **90**, 182–193.

Freedman, M., Farber, M., Farmer, L. *et al*. (1988) Pregnancy resulting from cryopreserved human embryos using a one-step *in situ* dilution procedure. *Obstet. Gynecol*., **72**, 502–505.

Gibbons, W.E., Gitlin, S.A., Lanzendorf, S.E. *et al*. (1995) Preimplantation genetic diagnosis for Tay–Sachs disease: successful pregnancy after pre-embryo biopsy and gene amplification by polymerase chain reaction. *Fertil. Steril*., **63**, 723–728.

Hardy, K., Martin, K.L., Leese, H.J. *et al*. (1990) Human preimplantation development *in vitro* is not adversely affected by biopsy at the 8-cell stage. *Hum. Reprod*., **5**, 708–714.

Hisatomi, H., Nagao, K. and Komatsu, H. (1997) Quantification of telomerase activity in human liver tissues by fluorescence-based TRAP analysis. *Hepatol. Res*., **7**, 35–42.

Holt, S.E., Norton, J.C., Wright, W.E. *et al*. (1996) Comparison of the telomeric repeat amplification protocol (TRAP) to the new TRAP-eze telomerase detection kit. *Meth. Cell. Sci*., **18**, 237–248.

Kim, N.W., Piatyszek, M.A., Prowse, K.R. *et al*. (1994) Specific association of human telomerase activity with immortal cells and cancer. *Science*, **266**, 2011–2015.

Kubota, Y., Nakada, T., Sasagawa, I. *et al*. (1998) Elevated levels of telomerase activity in malignant pheochromocytoma. *Cancer*, **82**, 176–179.

Lanzendorf, S.E., Boyd, C.A., Wright, D.L. *et al*. (2001) The use of human gametes obtained from anonymous donors for the production of human embryonic stem cell (ESC) lines. *Fertil. Steril*., **76**, 132–137.

Lee, H.W., Blasco, M.A., Gottlieb, G.J. *et al*. (1998) Essential role of mouse telomerase in highly proliferative organs. *Nature*, **392**, 569–574.

Morin, G.B. (1989) The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. *Cell*, **59**, 521–529.

Munné, S., Lee, A., Rosenwaks, Z. *et al*. (1993) Diagnosis of major chromosome aneuploidies in human preimplantation embryos. *Hum. Reprod*., **8**, 2185–2191.

Nagao, K., Tomimatsu, M., Endo, H. *et al*. (1999) Telomerase reverse transcriptase mRNA expression and telomerase activity in hepatocellular carcinoma. *J. Gastroenterol*., **34**, 83–87.

Ogino, M., Hisatomi, H. and Hanazono, M. (1999) Effectiveness of indomethacin as an antitumor agent in colon 26-bearing conventional and nude mice, and telomerase activity in the tumors. *Exp. Anim*., **48**, 15–21.

Ogoshi, M., Takashima, A. and Taylor, R.S. (1997) Mechanisms regulating telomerase activity in murine T cells. *J. Immunol*., **158**, 622–628.

Ohyashiki, K., Yahata, N., Ohyashiki, J.H. *et al*. (1998) A combination of semiquantitative telomerase assay and in-cell telomerase activity measurement using exfoliated urothelial cells for the detection of urothelial neoplasia. *Cancer*, **83**, 2554–2560.

O'Neill, C. (1997) Evidence for the requirement of autocrine growth factors for development of mouse preimplantation embryos *in vitro*. *Biol. Reprod*., **56**, 229–237.

O'Neill, C. (1998) Autocrine mediators are required to act on the embryo by the 2-cell stage to promote normal development and survival of mouse preimplantation embryos *in vitro*. *Biol. Reprod*., **58**, 1303–1309.

Rudolph, K.L., Chang, S. and Lee, H.W. (1999) Longevity, stress response, and cancer in aging telomerase-deficient mice. *Cell*, **96**, 701–712.

Sharma, H.W., Sokoloski, J.A., Perez, J.R. *et al*. (1995) Differentiation of immortal cells inhibits telomerase activity. *Proc. Natl. Acad. Sci. USA*, **92**, 12343–12346.

Testart, J., Lassalle, B., Belaisch-Allart, J. *et al*. (1986) High pregnancy rate after early human embryo freezing. *Fertil. Steril*., **46**, 268–272.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S. *et al*. (1998) Embryonic stem cell lines derived from human blastocysts. *Science*, **282**, 1145–1147.

Ulaner, G.A. and Giudice, L.C. (1997) Developmental regulation of telomerase activity in human fetal tissues during gestation. *Mol. Hum. Reprod*., **3**, 769–773.

Ulaner, G.A., Hu, J.F., Vu, T.H. *et al*. (1998) Telomerase activity in human development is regulated by human telomerase reverse transcriptase (hTERT) transcription and by alternate splicing of hTERT transcripts. *Cancer Res*., **58**, 4168–4172.

Veeck, L.L., Amundson, C.H., Brothman, L.J. *et al*. (1993) Significantly enhanced pregnancy rates per cycle through cryopreservation and thaw of pronuclear stage oocytes. *Fertil. Steril*., **59**, 1201–1207.

Wada, E., Hisatomi, H., Moritoyo, T. *et al*. (1998). Genetic diagnostic test of hepatocellular carcinoma by telomerase catalytic subunit mRNA. *Oncol. Reports*, **5**, 1407–1412.

Watson, J.D. (1972) Origin of concatemeric T7 DNA. *Nat. New Biol*., **239**, 197–201.

Wright, E., Piatyszek, M.A., Rainey, W.E. *et al*. (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.

Received on January 5, 2001; accepted on July 11, 2001