Telomerase Activity in Human Preimplantation Embryos

Diane Lynne Wright
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TELOMERASE ACTIVITY IN 
HUMAN PREIMPLANTATION EMBRYOS

by

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A Dissertation Submitted to the Faculties of
Old Dominion University and
Eastern Virginia Medical School
in Partial Fulfillment of the
Requirement for the Degree of
DOCTOR OF PHILOSOPHY
BIOMEDICAL SCIENCES
OLD DOMINION UNIVERSITY and
EASTERN VIRGINIA MEDICAL SCHOOL
December 2000

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ABSTRACT

TELOMerase Activity in Human Preimplantation Embryos

Diane Lynne Wright
Old Dominion University and
Eastern Virginia Medical School, 2000
Director: Dr. Susan E. Lanzendorf

Telomerase, a ribonucleoprotein, has been described as an essential component of highly proliferative cells, which stabilizes the telomeres and avoids cellular senescence. Telomerase has been identified in various embryonic cell stages, hematopoietic cells, and in >85% of tumor tissue biopsies analyzed. The ability to measure the potential to proliferate successfully could provide an objective measure of an embryo’s quality. The objectives of this study were to modify the telomeric repeat amplification protocol (TRAP) assay system for increased sensitivity to allow detection of telomerase activity in the single cell of an oocyte and embryo, obtain telomerase activity levels for the oocyte through blastocyst, and finally, evaluate the use of measuring telomerase activity within biopsied blastomeres to predict blastocyst development. Telomerase positive (DU-145 and PMEF) and negative (Hs27 and Detroit 551) cell lines were used to evaluate the assay system followed by the use of discard and donated oocytes and embryos for the comparative evaluation of telomerase activity and maturation level. Immature oocytes, mature oocytes, zygotes, 2-3 cell, 4-5 cell, 6-7 cell, 8-16 cell embryos, morulae, and blastocysts were evaluated individually for telomerase activity. Thawed zygotes cultured to day three were biopsied, by removing 1-2 cells and the biopsied embryos followed through culture to blastocyst. Telomerase activity was detected in positive cell lines and none measured in negative cell lines. Analysis of 60 single DU-145 cells showed...
detectable levels of telomerase activity in all cells. Of discard oocytes and embryos analyzed, 97.6% had measurable levels of telomerase activity. Telomerase activity was detected in all developmental stages. Immature oocytes and blastocysts had similar levels of telomerase activity, however both groups had significantly higher activity than the zygote through pre-morula stage embryo. There was no difference in telomerase activity of cells biopsied from embryos that reached the blastocyst stage or those that arrested in growth. Human oocytes through blastocyst stage embryos express telomerase activity, however the level of telomerase activity in a single blastomere of the day 3 cleavage stage embryo was not able to predict the growth potential of an embryo.
ACKNOWLEDGMENTS

When I initiated the pursuit of a Ph.D., I naively envisioned a process where I would be essentially alone throughout the navigation of the degree. I realize, with great humility, that I would have been unable to complete, or even begin, this huge undertaking without the assistance and support of numerous outstanding individuals.

First, I would like to thank Dr. Susan Lanzendorf for taking me under her wing and teaching me what it is to be a person with the letters Ph.D. following their name. Thank you, also, to each of my Research Committee members, Dr. Mary Mahony, Dr. Sergio Oehninger, and Dr. William Gibbons. You each provided a unique and important aspect to my graduate development.

To conduct any research project requires and, in fact, benefits from the cooperative efforts of various laboratories and researchers. I would like to thank Catherine Boyd, the Jones Institute for Reproductive Medicine’s IVF Lab, Susan Wilson, Susan Gitlin, and Shelly Billeter.

In addition to the professional help I received, there was a large group of people that provided me with much needed emotional support. Thank you, Valerie Clark, for your endless support and for putting up with the roller coaster ride of emotions over the past few years. I also appreciate the support of many good friends and family, Bill Wright, Estella Jones, Knox Garvin, Joan Earnest, Lana Hollingsworth, Elaine Wile, Kathy Santikos, Melynda Cloud, Carin and Larry Cowell. It is important to recognize two other dear friends and a wonderful grandfather, although gone not forgotten, Jim Otto, Ray Wright, and Claron Waggoner.
Thank you to my parents, Barbara and Jerry Wright, for the many years of support through more degrees than I am sure you ever imagined. A special thank you goes out to my grandmother, Helen Waggoner, for I believe it is her undeniable strength that serves as an inspiration to the entire family. I also appreciate the love and support of my sister, Stephanie Pipkin, and her family.
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CHAPTER I

INTRODUCTION

Methods to evaluate accurately the quality of an embryo remain elusive. Morphological measures have allowed some improvement in the ability to identify good embryos (potential to produce viable offspring), nevertheless, transfer of excellent quality embryos does not always result in a pregnancy. Preimplantation genetic evaluations have allowed for the deselection of genetically unsuitable embryos. Unfortunately, none of these techniques offer a complete picture of the embryo’s potential viability. Distinguishing a good quality embryo from bad, before transferring, remains more guesswork than science. The ability to measure the zygote’s potential to proliferate successfully could provide one more piece of the information. Telomerase, a ribonucleoprotein, has been described as an essential component of highly proliferative cells, which stabilizes the telomeres and avoids cellular senescence. The stability of an embryo’s chromosomes is essential for successful proliferation and differentiation into the various tissues during development. Measuring the level of telomerase activity could provide information on the proliferative capability of an embryo.

Telomeres

Telomeres are located at the terminal ends of chromosomes and have a unique structure of 6 bp repetitive sequences (Blackburn, 1991). In humans, the sequence is TTAGGG (Counter et al., 1992). Telomeres prevent nonspecific chromosomal recombination, allow complete replication of the chromosomal DNA without the inherent loss due to the DNA polymerase machinery, and help the chromosome bind to the

The model for this dissertation is Molecular Reproduction and Development

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nuclear matrix (Counter et al., 1992; Counter et al., 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 mortal cells shortened by approximately 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). Human fibroblasts were found to be capable of only a limited number of population doublings before they exited the cell cycle, thus limiting the replicating potential of somatic cells. The researchers also established a direct correlation in the length of the telomere at the start of culture and the doubling capacity.

Telomerase

In some cell types, telomerase is active and the telomeres are stabilized during divisions with no telomeric shortening (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 tumor cells (Kim et al., 1994; Counter et al., 1994; Sharma et al., 1995), and some hematopoietic 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. The critical role of telomerase was evaluated using a mouse strain lacking the telomerase RNA (mTR) gene (Blasco et al., 1997; Lee et al., 1998). Blasco and coworkers (1997) created mice that lacked the RNA component of telomerase (mTR-). When primary mouse embryonic fibroblasts (PMEF) were analyzed from generation 2 (G2), G4, and G6 by FISH analysis of telomeric ends, a decreasing trend was noted in the level of fluorescence signals with increasing generations. In the later generations, the authors
also noted an increase in aneuploid cells and end-to-end fusions of chromosomes, which included Robertsonian fusions. These mTR−/− mice were capable of producing offspring through G5, but not in the sixth (Lee et al., 1998). A significant decline in fertility was noted at G4 and a significant decrease in germ cells was identified in G5. The seminiferous tubules were almost completely devoid of spermatogenesis in G6 males. In G6 females, almost all levels of reproduction were affected from decreased number of oocytes ovulated to compromised uterine structure and function. A FISH probe for telomere repeats found a significant increase in the lack of 4 end signals of chromosomes in the G6 mice (−58.75%) verses the wild type (−7.5%). Developing fetuses showed an increasing trend in neural tube defects (Herrera et al., 1999). By the fifth generation, 30% of developing embryos experienced a failure of the neural tube to close. When the cells from the embryos with neural tube defects were compared to cells from embryos with closed neural tubes, significantly shorter telomeres were discovered. A subsequent publication reported an expected decrease in life span, wound healing, and response to hematopoietic insult, and an increase in the incidence of spontaneous malignancies in the latter generations of this telomerase deficient mouse (Rudolph et al., 1999). The fact that abnormalities in these mTR−/− mice did not become apparent, until later generations, may be explained by the ultra-long telomere length in mice (Kipling and Cooke, 1990). In humans the length varies, but averages approximately 10 kb in blood cells and in mice the length is highly variable from strain to strain reaching up to 150 kb (Kipling and Cooke, 1990). Since humans are a longer-living species with a significantly shorter telomeric length suggests that telomerase activity, or the lack of, may play a more critical role in cellular immortality and senescence than in the mouse. Some researchers propose
that the lack of telomerase in somatic cells serves as a way of avoiding cancerous growth (Greider and Blackburn, 1996).

As researchers began evaluating telomerase activity, somatic cell types were found to generally be lacking in telomerase activity, but "immortal" cell types presented activity (Counter et al., 1992). A follow-up study by Counter and coworkers (1994) provided the evidence that tumor cells (ovarian carcinoma) exhibited a shorter telomere length on average, but no loss of length through subsequent divisions was observed as compared to non-tumor cells. An upregulation of telomerase activity was noted in these ovarian carcinoma cells with shortened yet stable telomeres. A significant advance was made in telomerase evaluations by the development of the PCR-based telomeric repeat amplification protocol (TRAP) by Kim et al. (1994). Of 100 immortal human cell types, 98 were found positive for telomerase activity and the 22 mortal cell types contained no detectable telomerase activity. Normal ovaries and testis were also positive for telomerase activity. Sharma et al. (1995) documented the loss of telomerase activity when immortal cell types were induced to differentiate. Murine embryonic stem cells (mES) and teratocarcinoma cells (F9) were induced to differentiate by the withdrawal of leukemia inhibitory factor (LIF) or addition of retinoic acid (RA), respectively. The mES cell line showed no change in telomerase activity after 6 days, but by 12 days post LIF withdrawal telomerase activity was absent as the cells reached a differentiated state. A similar result was established with the F9 cell line. This relationship of telomerase activity to highly proliferative cells was also demonstrated in a novel study using transfection. Telomerase-negative human somatic cells (retinal pigment epithelial cells - 340 and BJ foreskin fibroblasts) were transfected with vectors that coded for the catalytic
subunit of telomerase, producing telomerase activity and extension of life span in cells that would otherwise senesce after a finite number of population doublings (Bodnar et al., 1998).

Developmental studies in human fetal and adult somatic tissue have demonstrated a loss of telomerase expression with increased gestational age. Wright et al. (1996) found fetal lung, adrenal, muscle, skin, and liver at 16 weeks gestation positive for telomerase activity, while brain, kidney and bone were negative. Ulaner and Giudice (1997) demonstrated similar results for positive telomerase activity in the same fetal tissues through 21 weeks gestation, except in the brain and kidney tissues where activity dropped off after 16 weeks. 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, excluding the gonad.

Progeria and X-linked dyskeratosis congenita (DKC) are the first two diseases to be associated with a failure of telomerase activity and subsequent decreased longevity. DKC is passed on to sons of carrier mothers. Patients experience problems with prolifically dividing tissues like skin and bone marrow. Researchers have established that DKC is the result of a mutation in the gene encoding dyskerin (Mitchell et al., 1999). These investigators demonstrated a relationship between dyskerin and telomerase, through a co-immunoprecipitating study, showing that dyskerin can interact with the RNA component of telomerase. Primary fibroblasts and lymphoblasts from affected males were found to express lower levels of telomerase activity and shorter telomeres.
than the controls. Progeria is another disease that results in a shortened life span of the affected individuals. Telomeres from Hutchinson-Gilford progeria patients were discovered to be shorter than those of matched controls, which was expected due the dramatically shortened life span experienced by sufferers (Allsopp et al., 1992).

All blood cellular components originate from hematopoietic progenitor cells that lie within the bone marrow. Various subpopulations of hematopoietic cells were evaluated for their level of telomerase activity (Chiu et al., 1996). The primitive, quiescent, hematopoietic subset exhibited low-level expression of telomerase activity that could be stimulated to increase transiently when a mixture of cytokines was added. Conversely, the early progenitor subset had high levels of telomerase activity and the cytokine mixture downregulated the telomerase activity. More mature hematopoietic cells remained unchanged in their low telomerase activity in the presence of cytokines. Engelhardt et al. (1997) experienced similar results with primitive human hematopoietic cells. When the primitive hematopoietic cells were cultured in the presence of a combination of cytokines for 48-72 hours, telomerase activity was upregulated, peaked at 1 week of culture, and declined after 3-4 weeks. The treatment of these cells with a single cytokine did not produce the same level of upregulation. Another study in the mouse demonstrated the same cytokine receptor associated telomerase upregulation using T cells isolated from the spleen (Ogoshi et al., 1997). T cells with little or no telomerase activity were induced to upregulate telomerase activity by exposure to the mitogens Concanavalin A or immobilized anti-CD3 monoclonal antibody. When these mitogens were added at a suboptimal level no change was noted in telomerase activity, however, when the cytokine IL-2 was later added telomerase activity returned. This information
indicates not only does stimulation of the T-cell receptor (TCR) convey an increase in telomerase activity, but also binding of cytokine receptors can produce the same upregulation of telomerase activity. Present research suggests that telomerase activity is present in any cell type that has a role as a stem cell for renewing the population of terminally differentiated cell types.

**Gametes and Embryos**

Embryos could be considered the ultimate stem cell due to an 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), using an immortalized human embryonic kidney cell line (293) as the comparative control. The relative percentage values for telomerase activity, in the various oocyte stages on a per cell basis, were as follows: early antral was 83%, preovulatory was 159%, ovulated was 2%, and 4 cell embryo was 7%. Similar results were seen with the male germ cells. Immature spermatocytes and spermatids exhibited telomerase activity that was absent from mature spermatozoa. A recent study in the bovine established similar trends in relative telomerase activity for pooled oocytes and followed embryonic patterns of telomerase activity through the blastocyst stage (Betts and King, 1999). Relative telomerase activity was based on a comparison of telomerase activity of the oocytes or embryos and a positive control cell lysate (293 cells). The researchers detected telomerase activity in all stages of oocyte and embryo development. As oocytes matured, they noted a decrease in activity that lasted through the 8-cell stage of embryo development. A dramatic 40-fold 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. (1996) analyzed single mature oocytes from hysterectomy tissue and adult sperm samples finding that both mature gametes lacked telomerase activity. However, fertilized human embryos were thawed and cultured to the blastocyst stage, analyzed individually, and found to express very high levels of telomerase activity.

Not only does the oocyte express telomerase activity during maturation, Lavranos and coworkers (1999) detected telomerase activity in the granulosa cell layer of developing bovine ovarian follicles. Telomerase activity exhibited an inverse relationship to follicle size. Granulosa cells from the small preantral follicle had the highest level of activity and a decline was seen through increasing follicle size to 6-8 mm follicles. The researchers also stated that the telomerase activity in the positive control cell line and follicles was not related to the amount of telomerase, but to the log of the amount of telomerase.

Subunits

Telomerase activity involves the concerted efforts of the RNA template component (TR), a catalytic subunit (TERT/TRT/EST2/TCS1/TCS), and additional related proteins important for the regulation of telomerase activity (TLP1/TP1, hnRNP A1, TRF1, TRF2). A clone of the human TR (hTR) subunit was reported in 1995 by Feng et al., opening the door to understanding the levels of regulation available to cellular expression of telomerase activity. Cloning of the RNA component defined an approximately 460 base length transcript with an 11 nucleotide (5' -CUAACCCUAAC) template region complementary to the human telomerase sequence (TTAGGG)n. These
researchers also found that germline tissues and tumor cell lines expressed greater levels of hTR than somatic tissues that lack telomerase activity. Transfecting HeLa cells with antisense hTR resulted in death of the cells after approximately 25 population doublings with telomeric DNA shortening. The results suggested an essential role for hTR in telomerase activity. Related work from the same laboratory (Blasco et al., 1995) characterized the difference between mouse and human hTR. The mouse telomerase RNA (mTR) subunit was found to share a 65 percent sequence homology with the human TR. Differentiated mouse tissues were evaluated for levels of mTR and expression followed predicted levels of differentiation or immortalization. As primary Mus spretus fibroblasts transformed to an immortal cell line, the level of mTR was 18 times more in the immortal cells than the parental cells. Detailed analysis of various prenatal, newborn and adult tissues revealed a down regulation of mTR in the mouse brain, kidney, and lung; however, testis, intestine, liver and spleen maintained mTR expression into adulthood. The telomerase RNA subunit expression varies in somatic tissues of the human more than in the mouse. Yashima et al. (1998) exhibited a defined decline in hTR expression as cells differentiated into somatic cell types, excluding testicular and adrenal tissue by 10 weeks postnatal development. The highest level of expression of hTR was undifferentiated embryonic neuroepithelium, but hTR expression was absent in the nervous system by the tenth week of postnatal development. Although the trend is for hTR expression to decrease with level of differentiation, there are examples where hTR transcripts are present in cells of late differentiation or lacking detectable telomerase activity. Yashima et al. (1998) described cells that had differentiated and were postmitotic still expressing hTR. A northern blot analysis study also found cell lines and
tissue lacking telomerase activity that still expressed hTR (Avilion et al., 1996), suggesting that the RNA component is not a good predictor of telomerase activity.

The most current studies suggest that the catalytic subunit (TERT) is more directly predictive of telomerase activity than the RNA component (Martin-Rivera et al., 1998; Ulaner et al., 1998). A study evaluating mouse TERT (mTERT) found a high correlation between mTERT expression and telomerase activity, but mTERT expression was independent of mTR expression. This study employed the novel use of a TR-/- strain of mouse. When the hTR gene was transfected into the mTR-/- strain, functional telomerase activity resumed. This was not true for the combination of mTR and hTERT subunit (Beattie et al., 1998). A final discovery from this group (Martin-Rivera et al., 1998) was the location of mTERT and hTERT in the cell nucleus.

Regulation

The controversy over the RNA and catalytic subunits and their relationship to actual telomerase activity appears to be related to various regulatory mechanisms utilized by various tissue types. Ulaner et al. (1998) found the human fetal heart and liver to express hTERT and telomerase activity in a direct relationship, but the fetal kidney suppressed telomerase activity after the fifteenth week of gestation and continued to express hTERT through the twenty-first week. In addition, multiple alternative splice forms of hTERT were identified in these tissues consisting of the full-length version as well as spliced messages containing vital deletions. With regard to the alternative splice forms, the fetal kidney only expresses the full-length transcript of hTERT through the time period of actual telomerase activity. Once the telomerase activity is gone in the fetal kidney, splice variants were found to be the only forms existing of hTERT in the tissue.
These authors suggest at least two mechanisms available within cells to regulate telomerase activity: at the DNA level of transcription of hTERT gene and manipulation at the RNA level of the hTERT transcript by alternative splicing. Human oocytes and embryos (Brenner et al., 1999) have also been evaluated for alternate splice variants of the human telomerase catalytic subunit. Oocytes and embryos were found to express hTERT, but there was a huge variation between various embryos and cells. The alternate splice forms were 430 bp, 400 bp, and 250 bp in size. Embryos tended to express all sizes and oocytes predominately expressed the 430 bp size.

Chromosome 3 has been shown to contain telomerase related genes. Soder et al. (1997) mapped the hTR gene to 3q26.3 and found increased copy number of this locus in 97% of tumors evaluated. A tumor repressor gene has also been located on chromosome 3 of the human. The gene is located on the p arm of the chromosome in the region 3p14.2-p21.1 (Tanaka et al., 1999). Other researchers had shown a narrow region on the p arm of chromosome 3 (3p21.3-p22 and 3p12-21.1) could be introduced through normal chromosome 3 microcell transfer, into breast cancer cells and cause repression of tumor cell growth (Cuthbert et al., 1999). At least one gene from this repressor region of chromosome 3 exhibits its activity by causing a reduction in the catalytic subunit of telomerase (Horikawa et al., 1998).

The proto-oncogene Myc has been identified as a transcriptional regulator of telomerase activity through the activity of c-Myc, the encoded protein which is known to be involved in the control of cell proliferation and differentiation (Wang et al., 1998; Wu et al., 1998; Greenberg et al., 1999). The transduction of two normal cell lines, human mammary epithelial cells (HMEC) and human diploid fibroblasts (IMR-90 and WI-38),
with HPV-16 E6 protein, which induces c-Myc expression, produced a measurable increase in telomerase activity within 1 passage (Wang et al., 1998). The researchers also identified the catalytic component of telomerase as a target of c-Myc. However, in the rat embryo fibroblast transformation assay used, TERT alone was not able to replace the transforming activities of c-Myc in the cells, suggesting that Myc does more within the cell that just its influence on TERT levels (Greenberg et al., 1999).

Some newly discovered proteins also support a fine level of telomerase regulation. In the human, telomeric repeat binding factor 1 (TRF1) has been shown to provide a negative feedback on telomeric length (van Steensel and de Lange, 1997). TRF1 did not have a detectable influence on telomerase expression, but overexpression of TRF1 in a telomerase positive tumor cell line resulted in a gradual shortening of telomeres. A similar protein has been elucidated in fission yeast called Tazlp (Cooper et al., 1997), exhibiting the suppressive role on telomere length. In the mouse, a protein with abilities to modulate telomeric length, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1, A1), has been described (Le Branche et al., 1998). An A1 deficient mouse strain did not exhibit a significant difference in telomerase activity to the wild type mouse and A1 had a positive influence on telomeric lengths. Another telomeric protein, TRF2, is implicated in the recent discovery of “t loop” formation at the 3’-end of telomeric repeats (Greider, 1999; Griffith et al., 1999). The results suggest that TRF2 induces the single stranded 3’-end of the telomere to loop back on itself and actually bind to the double stranded telomere sequence. Telomerase appears to have numerous levels at which its activity is regulated, suggesting an important purpose for the ribonucleoprotein within the cell.
Telomeric Repeat Amplification Protocol

Current methods to evaluate and measure telomerase activity originate from a PCR-based assay termed telomeric repeat amplification protocol (TRAP). Kim et al. (1994) first described the use of this assay to evaluate various human tissues, immortal cell lines, and cancer cells. The assay involves two major steps beginning with telomerase from the lysed cells adding the 6 bp repeats to the substrate primer. The second step utilizes PCR to amplify these telomeric additions to a level necessary to measure. Shortly after this publication, a kit became available commercially (TRAPeze®) that provided increased sensitivity and additional controls. A study comparing the original method to the commercial kit found that the kit decreased sample processing time due to a decrease from a three-step PCR cycle to a two-step cycle (Holt et al., 1996). The researchers concluded the TRAPeze® was superior to the original assay because of the improved sensitivity, quality control additions, and decreased time needed to run the assay.

Most of the TRAP assays to this point relied on the use of radioisotope labeled reaction products to evaluated banding patterns on the product gels. Hisatomi et al. (1997) described the combination of TRAPeze® with fluorescent labeled products (F-TRAP) and the alleviation of radioactivity in the analysis of human liver tissue. The substrate (TS) primer was labeled with Cy-5 and an automated DNA sequencer (ALF red™: Pharmacia Biotech) was utilized to evaluate the product run on a denaturing gel. 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).

**Gene Regulation in the Early Embryo**

The human oocyte, during growth and maturation, builds up stores of essential organelles, mRNA, and proteins that will be necessary for the mature oocyte and early fertilized embryo to use until the embryonic genome can take over the synthesis of these vital elements (Schultz, 1999). In the human, this shift to embryonic control takes place at the four to eight-cell stage (Braude et al., 1988). The study showed that various qualitative changes occur in polypeptide levels during the four-cell to eight-cell stage and are dependent on transcription. A related study also demonstrated this shift to embryonic driven transcription at the four-cell stage. Using RT-PCR, paternal transcripts for the X-linked glucose-6-phosphate dehydrogenase and the Y-linked ZFY gene were first detected at the four-cell stage (Taylor et al., 1997). Heikinheimo et al. (1995) measured human cyclin B1 and beta-actin in oocytes and embryos, using RT-PCR, and demonstrated a dramatic increase in embryo-derived expression of these genes at the 6-cell stage.

**Biopsy of Human Embryos**

Since the publication of a viable technique for biopsy of human embryos (Handyside et al., 1989) and first pregnancies (Handyside et al., 1990), preimplantation genetic diagnosis (PGD) has become a common technology, offered to couples with genetic disorders, in over 50 assisted reproduction programs worldwide. The challenge of this technique is not only the ability to remove one to two cells from an embryo without harm, but to also correctly amplify and evaluate the DNA of a single cell. Numerous studies have established the ability to remove cells from an embryo, in the
mouse (Wilton et al., 1989) and in the human (Handyside et al., 1990; Grifo et al., 1992), yet the embryo experiences no deficit in its ability to grow and implant within the uterus. Since the original publications that evaluated the sex of the embryos to avoid sex-linked diseases, the repertoire of diseases that programs can test these embryos for has grown immensely (ESHRE PGD Consortium Steering Committee, 1999). Biopsy of the human embryo has moved from a research technique to an established method for preventing patients from terminations, or the consideration of terminations, of ongoing pregnancies.

The study of telomerase activity in gametes and embryos is in its infancy. Although there is a wealth of information in the literature regarding telomeres, telomerase and cancer, little is known about the role of telomerase in the preimplantation embryo. Already we know that: 1) telomerase is important for normal, and abnormal, highly proliferative cell types, 2) telomerase activity is regulated on numerous cellular levels to achieve optimal telomeric lengths, and 3) cells deemed terminally differentiated can become immortal by restoring telomerase activity through transfection. Certainly, the information already published in the aging and cancer fields will speed the pursuit of research in reproductive biology. Human developmental studies have discovered the presence of telomerase activity in various tissue types at numerous fetal growth stages. Research has established that in the bovine, rat, and in the human blastocyst stage embryo, telomerase activity is present. Baseline information on telomerase activity in human gametes and embryos will be necessary, before research can move into the areas of regulation and differentiation. As the baseline information is compiled we will begin to understand the role of telomerase in another highly proliferative cell type, the embryo, and have an additional way to define embryonic potential for a viable pregnancy.
CHAPTER II

STATEMENT OF PURPOSE

Telomerase is a ribonucleoprotein that provides a mechanism for the stabilization of telomeres on the ends of chromosomes during cellular replication. DNA Polymerase is unable to replicate these ends and telomerase provides the template for the 6 bp repeating sequence. Without telomerase, a cell has a limited number of divisions possible as the telomeres shorten to a critical point at which the cell enters senescence. Telomerase has been identified in various embryonic cell stages, hematopoietic cells, and in >85% of tumor tissue biopsies analyzed. These cell types have in common the need to elude cellular senescence and appear to use telomerase as the mechanism to stabilize the telomeres and attain ‘immortality’. Human blastocysts have been evaluated for the presence of telomerase and found to express the enzyme, where the mature human oocyte did not show measurable levels of telomerase activity.

The purpose of this study was to evaluate the quantitative level of telomerase from the gamete to blastocyst stage. The hypothesis to be tested was that telomerase is a gene transcribed from the initial startup of the embryonic genome and that individual embryos differ in their successful expression of telomerase activity. Although an embryo has the appearance of normal morphology, it might be lacking in telomerase gene expression, which would not be apparent until latter divisions as the telomeres shorten. Results that include a comparative value for the levels will provide information on the embryonic genome and further studies could use this parameter to evaluate the mechanisms involved in signaling the transcription of telomerase. Long-term application of this method could be to evaluate biopsied embryos for telomerase activity and provide
more information on the viability potential of the embryo.

**Specific Objectives**

1) Demonstrate that telomerase levels are low in mature, ovulated oocytes and increase with continued division of the fertilized embryo due to the transcription of telomerase-associated genes in the embryonic genome.

2) Establish that embryos vary in their upregulation of telomerase activity, providing a predictive measure of embryonic viability, through the comparative evaluation of biopsied embryos.

**Study Design**

**Preliminary Cell Line Experiments**

A group of cell lines were used to modify the TRAP assay for single cell evaluation, from the TRAPeze® kit's defined lower-end sensitivity of approximately 80 cells. Four cell lines (primary mouse embryonic fibroblasts, human prostate carcinoma, human foreskin, human embryonic skin) and the positive control cell line, supplied with the kit, were used for these experiments. An initial cross-species assay was conducted using the mouse fibroblast and human cancer cell types, followed by an evaluation of the assay’s ability to detect known positive and negative cell lines. Next, a series, of ten-fold dilutions of the positive control cell lysate (10,000 to 1 cell equivalents), was evaluated for correlation with telomerase activity detected. Finally, 60 cells, from the known telomerase positive human carcinoma cell line, were tested individually for the assay’s ability to detect telomerase activity in the single cell.

**Experiment Series One - Individual Oocytes and Embryos**

Whole oocytes and embryos were assayed individually to establish the pattern of
telomerase activity expressed from the oocyte through various embryonic cell stages. The following cell stages were evaluated: immature oocyte, mature oocyte, zygote, 2–3 cell, 4–5 cell, 6–7 cell, 8–16 cell, morula, and blastocyst.

Experiment Series Two – Blastomeres

Part A - Individual Blastomeres of Entire Embryos

A selected group of cleaving embryos was completely disaggregated and each blastomere tested for telomerase activity. This section of data served to distinguish the variances between embryos and blastomeres in their expression of telomerase activity.

Part B - Biopsy and Comparative Growth Potential

Day 3 cleavage stage embryos were biopsied and 1-2 cells removed, allowing the remainder of the embryo to continue developing. Telomerase activity of the blastomeres removed was compared with the final growth stage of the biopsied embryo, to allow for the comparison of cleavage potential and telomerase activity levels of the biopsied blastomere. A statistical comparison was made between the values obtained for telomerase activity in the biopsied blastomeres of embryos that successfully developed to the blastocyst stage and those that arrested in growth prematurely.

Those embryos that reached the blastocyst stage were treated with immunosurgery to isolate the inner cell mass (ICM) and telomerase activity was measured in the ICM for comparison with telomerase activity of the previously biopsied blastomeres.
CHAPTER III
MATERIALS AND METHODS

Materials

The sequential culture medias used came from three sources: 1) Enhance Day 1/D3 from Conception Technologies (San Diego, CA), 2) P1/ Blast Medium from Irvine Scientific (Santa Ana, CA), and 3) G1.2/ G2.2 from IVF Science (Scandinavia). Earls Balanced Salt Solution and Synthetic Serum Substitute were obtained from Irvine Scientific (Santa Ana, CA). The thaw media components, acidified Tyrode’s components, Bovine Serum Albumin (BSA) and Dulbecco’s Phosphate Buffered Saline, were obtained from Sigma Chemical Company (St. Louis, MO). Other media additives, which were purchased from Sigma Chemical Company, included 0.25% trypsin and hyaluronidase. The TRAPeze® kit was purchased from the Intergen Company (Purchase, NY). The Long Ranger gel solution was obtained from FMC BioProducts (Rockland, ME). All tissue culture dishes, aerosol barrier pipet tips, borosilicate glass Pasteur pipets and Urea came from Fisher Scientific (Pittsburgh, PA). PCR tubes were obtained from USA Scientific (Ocala, FL). Taq Polymerase and Dulbecco’s Modified Eagle Medium (DMEM) came from Gibco / Life Technologies (Gaithersburg, MD). Fetal bovine serum was purchased from Hyclone (Logan, UT). Molecular weight markers (50-350 bp) were obtained from LI-COR (Lincoln, NE). Borosilicate glass (B100-75-15) for micromanipulation pipets was obtained from Sutter Instrument Company (Novato, CA). Rabbit anti BeWo serum was prepared by Atlantic Antibodies (Wyndham, ME). Standard guinea pig complement was purchased from Cedarlane Laboratories Limited (Hornby, Ontario).
Equipment

Embryos requiring a programmed thaw cycle were thawed using the Planer KRYO 10 Series II from TS Scientific (Perkasie, PA). Polymerase Chain Reactions were conducted using a Thermolyne Series 669 Thermocycler (Barnstead/Thermolyne Corporation). Electrophoresis and densitometric analysis were conducted using the LI-COR 4200 DNA Sequencer (Lincoln, NE) and fluorescent banding analysis was performed using the Gene ImagIR Software (Version 3.55, LI-COR). Blastomere isolation and deposit in reaction tubes was conducted under a Nikon SMZ-10 dissecting microscope. Biopsy of human embryos was performed using a Nikon Diaphot inverted microscope (Nikon Inc., Melville, NY), equipped with 3 sets of Narishige MO-202 micromanipulators and Narishige MN-2 course manipulators. A PLI-188 picoinjector (Medical Systems, Inc., Melville, NY) was used for control of fluid in hatching and biopsy pipets for biopsy of blastomeres. The pipet system used to hold the embryo in place was managed by the use of a Narishige IM-6 microinjector. Micromanipulation pipets were created using a P87 Flaming/Brown Micropipette puller (Sutter Instrument Company, Novato, CA) and a Narashige MF-9 microforge.

Human Oocytes and Embryos

Some of the human material used in this study was obtained from the In Vitro Fertilization Laboratory at the Jones Institute for Reproductive Medicine. The use of patient material for research was approved by the Institutional Review Board (IRB) of Eastern Virginia Medical School and included the following protocols:
1) #09-05-96-0243 - allows for the use of abnormally fertilized human embryos, which cannot be used by the patients, to be used in preclinical research studies.

2) #07-08-93-0052 - allows for the use of mature and immature oocytes that the patients do not require for their treatment to be used for preclinical research studies.

3) #07-08-93-0043 - allows the use of fertilized embryos for preclinical research studies.

In addition cryopreserved zygotes were received, for the purpose of research, from five centers for assisted reproductive medicine; Cooper Institute for IVF (Marlton, NJ), Beach Center for Infertility (Virginia Beach, VA), Fertility Center of Maryland (Baltimore, MD), Christ Hospital (Cincinnati, OH), New Hope Center for Reproduction (Virginia Beach, VA). These donated cryopreserved embryos were also used under the IRB protocol #07-08-93-0043. All couples signed and consented to the disposition of their embryos.

**Cell Lines**

All cell lines, except mouse cells, were obtained from the American Type Culture Collection (ATCC - Rockville, MD). 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, 1993). These pregnancies were the result of a cross between B6CBAF1/J females (Jackson Laboratories, Bar Harbor, MA) and CD1 males (Charles River Laboratories, Wilmington, MA).

Methods

Embryo and Cell Line Culture

Human Embryo Culture

Human embryos were cultured in sequential style media, following instructions provided by the manufacturers. Briefly, pronuclear stage (Day 1) embryos were placed in 500 μl of CO₂ equilibrated media under oil, which was designated for day 1-2 stage embryos. After 48 hours 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 hours until the final growth stage was reached or until growth had arrested for 24 hours.

Cell Line Culture

Cell lines were cultured in DMEM supplemented with 15% fetal bovine serum. The culture medium was replaced every 48 hours with pre-equilibrated medium. Passage of cell cultures was initiated by the removal of culture media and addition of trypsin for 1 min followed by 3 min incubation at 37°C with trypsin removed. As cells begin to lose connection with culture vessel, culture media was added by pipetting over the cells. Cell suspensions were then counted and aliquoted for subsequent passage or lysis.

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Thaw protocols

Zygotes were thawed according to established protocols obtained from the corresponding donating programs. Those zygotes requiring a programmed slow thaw were thawed as described by Veeck et al. (1993). Another group of embryos were frozen with a similar protocol, but in straws, which does not require a programmed thaw (Testart, 1986). Zygotes that required a one-step thaw protocol were thawed according to the methods described by Freedman et al. (1988).

Embryo Biopsy

Embryo biopsy was performed in 100 µl drops of equilibrated Earl’s Balanced Salts Solution with 15% Synthetic Serum Substitute with a mineral oil overlay. Two drops were placed on each micromanipulation dish. Only one embryo at a time was in the micromanipulation dish. This embryo was out of the culture system for a maximum of 15 min. A second embryo would be biopsied in the other drop with the same time constraint after the first biopsy was complete. Once two embryos were biopsied, a new micromanipulation dish was prepared.

Embryo biopsy consists of 2 parts: 1) a hole is placed in the zona pellucida directly in front of the cell designated to be removed, by the use of Acidified Tyrode’s solution and 2) one or 2 blastomeres are removed from the embryo through the hole in the zona pellucida. Embryos were biopsied when the majority of the embryos had reached the 6-8 cell stage. If an embryo was a 4 cell at biopsy, only 1 cell was removed, otherwise 2 blastomeres were removed for analysis.

The embryo biopsy technique was performed as described by Gibbons et al. (1995). The embryo is held in place using a holding pipet (120 o.d., 40 i.d.), while a hole
is placed in the zona pellucida using a hatching pipet (10 o.d.) loaded with Acidified Tyrodes' solution. After the hole is made next to a blastomere exhibiting a visible nucleus, the biopsy pipet (40-45 o.d.) is aligned with the hole in the zona pellucida. This biopsy pipet is connected to the picoinjector to allow fine movement of fluid within the pipet. The biopsy pipet is preloaded with a small column of mineral oil followed by an equal size column of the Earle's medium. Gentle suction was performed on the blastomere until it had separated from the embryo. The blastomere was then expelled on the bottom of the micromanipulation dish a safe distance from the holding pipet and embryo. Blastomeres were individually removed from the micromanipulation dish in a 1 μl column of media, once the biopsy procedure was complete, and added to a microcentrifuge tube containing 9 μl of CHAPS lysis buffer. 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 trophoderm was removed from the blastocysts as previously described in the mouse (Solter and Knowles, 1975), rhesus monkey (Thomson et al., 1995), and human (Thomson et al., 1998). Blastocysts that had not hatched free of their zona pellucida were treated with acidified Tyrode's solution and through gentle pipeting the zona pellucida was removed. Whole blastocysts were exposed to rabbit anti-BeWo serum followed by exposure to guinea pig complement. The trophoderm cells lyse during the complement step and are removed from the inner cell mass by repeated pipeting through a finely drawn Pasteur pipet. The longest length of each ICM was measured under the 20 X objective using an ocular micrometer.
Telomerase Assay

The original description and use of TRAP was published by Kim et al. (1994). This polymerase chain reaction (PCR)-based assay allows the telomerase of the cells being tested to add telomeric (TTAGGGn) repeats to the 3' end of the substrate oligonucleotide (TS) (Step 1) and these extended products are then amplified through PCR to allow detection (Step 2). The TRAP assay has since been modified for sensitivity and accuracy and a kit is commercially available (TRAPeze distributed by Intergen, Inc.) for easier and more reliable testing. The kit also provides a 36 bp internal amplification standard, a quantitation control template - TSR8, and a pellet of telomerase positive cells for controls. A comparison study of the original TRAP protocol and the TRAPeze kit found the kit to be a superior system (Holt, 1996).

Preparation of Cell Lysates

The lysing buffer was provided in the kit, CHAPS, (10 mM Tris-HCL [pH 7.5], 1 mM MgCl₂, 1 mM EGTA, 0.1 mM Benzamidine, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% Glycerol). The procedure for lysis of cell lines, to liberate telomerase components, is described in the TRAPeze kit manual. The number of cells ranged from 1 x 10⁵ to 1 x 10⁶ cells used in each lysing reaction. Cells were centrifuged to a pellet in micro centrifuge tubes for 6 minutes at 6,000 X G. The supernatant was removed and the cells snap frozen in liquid nitrogen and stored at -80°C. At thaw, 200 µl of CHAPS was added to the pellet, tubes vortexed, and incubated on ice for 30 minutes. After the lysing period, samples were centrifuged at 12,000 X G for 20 minutes at 4°C. From the cell lysates, 160 µl of the top supernatant was removed and flash frozen in liquid nitrogen. The aliquots were then stored at -80°C.
Individual cells from cell line suspensions were isolated in DPBS + 1% BSA. A single cell, plus 5 µl of media, were added to a micro centrifuge tube containing 5 µl of CHAPS and incubated, on ice, for 30 minutes. The cellular extract was then snap frozen in liquid nitrogen and stored at -80°C. After removal of the zona pellucida with Acidified Tyrode's solution, whole embryos were placed in a micro centrifuge tube with 10 µl of media and snap frozen in liquid nitrogen, with subsequent storage at -80°C. At thaw, these tubes were incubated on ice for 30 minutes to allow lysis of the cells. Both the embryos and the individual cells were analyzed using the entire 10 µl lysate as the sample.

**PCR Amplification**

The assay was conducted in a 25 µl reaction with 5 pmol of the TS primer (5'-AATCCGTCGAGCAGAGTT -3') in 20 mM Tris-HCl [pH8.3], 1.5 mM MgCl₂, 63 mM KCl, 0.05% Tween 20, 1 mM EGTA, .05 mM 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. The TS primer from the kit was not used. A specifically labeled TS Primer was purchased from LI-COR to obtain the IRD800 label necessary for use with the LI-COR system.

The first step of the assay was incubation at 30°C to allow any telomerase within the cellular lysate to add telomeric repeats to the TS primer. This step was conducted for 30 minutes in all cellular lysate samples, except the single cell and whole embryo samples. The single cell and whole embryo assays were incubated for 60 minutes to allow the lower level of telomerase to be detected.
After the preliminary incubation, the actual PCR step was conducted. An initial hold at 94°C for 3 minutes preceded amplification, to stop the telomerase reaction. The PCR was conducted for 38 cycles of 94°C for 30 seconds and 59°C for 30 seconds.

**Analysis of Telomerase product**

Products were diluted 5 to 1 with IR² Stop Solution (LI-COR), heated to 93°C for 3 minutes and placed directly on ice. Each product was then loaded (1 µl / lane) onto a 7% Long Ranger denaturing gel (FMC BioProducts, Rockland, ME) with 7 M urea, fitted to an automated, infrared, DNA sequencer (IR2 from LI-COR, Inc., Lincoln, NE) and electrophoresed at 45°C and 1500 V.

The fluorescent data was analyzed 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 using CHAPS lysis buffer as the sample to evaluate primer-dimer PCR artifacts and contamination and the Hs27 cell lysate was run to show a negative cell reaction. A dilution of the lysate from the positive control cell line, provided in the kit, was run in every assay. All reactions contained the template (TSK1) and reverse primer (K1) for an internal PCR amplification control that is 36 bp in size. Finally, a quantitation control template was run with each assay, TSR8. This control provided a standard to estimate the amount of TS primers that were extended by telomerase in each extract. Each sample was quantified in terms of peak band density of the fluorescence and peak area (integrated optical density). Using the following formulas values were obtained for total product generated (TPG) and relative telomerase activity (RTA):

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Measured total area of telomerase activity (50 bp, 56, 62, etc)

Measured area of internal 36 bp control

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

Measured total area of telomerase activity (50 bp, 56, 62, etc) in Positive Control Cells

Measured area of internal 36 bp control Positive Control Cells

RTA (units) = \frac{\text{Measured total area of telomerase activity (50 bp, 56, 62, etc)}}{\text{Measured area of internal 36 bp control}}

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 not homogeneity of variance, therefore, Dunnett T3 post hoc test was used for multiple comparisons and determine significant differences at P < 0.05 between mean TPG and RTA activities of each growth stage in the first experiment. Variance component analysis was conducted with the embryo data that had all blastomeres of the embryo tested for telomerase activity. For the final biopsy experiment, Levene's F-test statistic was used for testing of homogeneity of variance and ANOVA was used for comparison of the 2 groups. A two-tailed test for Pearson correlation was used to evaluate the relationship between ICM size and telomerase activity in the blastomeres biopsied.
CHAPTER IV

RESULTS

Telomerase Assay Verification

The first series of testing explored the ability of the assay system to pick up telomerase activity, when cells contained telomerase, and the ability to find no activity in cells that are known to lack telomerase activity. The diluted lysates from four cell types were tested, which included three human and one mouse. The cell types were DU-145 (human, prostate carcinoma), Hs27 (human, newborn foreskin), Detroit 551 (human, female embryonic skin) and PMEF (primary mouse embryonic fibroblasts). 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 set of data established that each cell type had measurable telomerase activity (Fig. 1).

![Graph showing telomerase activity](image)

**Fig. 1.** Telomerase activity in cell lysates of human and mouse cell lines. Cell lysates from DU-145 (human), PMEF (mouse) and the positive control cells were diluted to 500 cell equivalents. Data represents the mean of single samples loaded in duplicate.
A subsequent experiment compared telomerase activity in cells from cell lines known to express telomerase activity and those known to lack telomerase activity (Fig. 2). In Fig. 2, not only was the presence of telomerase activity duplicated in the DU-145 and PMEF cells, however there was no measurable activity in the Hs27 or the Detroit 551 cell lines.

![Bar graph showing telomerase activity in cell lysates of positive and negative cell lines.](image)

**Cell Line**

**Type**

DU-145  | Hs27  | PMEF  | Detroit 551 | Positive Control

**Fig. 2.** 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 ± 1 SD of 5 samples from one assay.

A dilution series of the positive control cell lysate was evaluated from a 1 cell equivalent dilution through 10,000 cells equivalent, increasing by factors of 10. A logarithmic increasing trend was noted in the measured telomerase activity of the lysate dilutions (Fig. 3) from the 1 cell equivalent to the 10,000 cells equivalent. To evaluate repeatability of the gel loading and analysis system, the identical product was loaded into
a gel 23 times and the product was measured in all 23 lanes (Fig. 4). This comparison was made using the portion of the formula (see methods) integrated optical density of TRAP product ladder bands / integrated optical density of short internal control band. The mean value was 0.7729 with a standard deviation of 0.06299 for these 23 lanes. One standard deviation represented an 8% deviation from the mean value.

![Graph](image)

**Fig. 3.** Telomerase activity measured in ten-fold dilutions of the positive control cell lysate. Data represents the mean of single samples loaded twice in one TRAP assay.

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.
Fig. 4. Twenty-three identical loadings of a sample of positive control cell lysate diluted to 100 cell equivalents.

Telomerase Activity in Whole Oocytes and Embryos

A total of 167 deselect 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%) deselects and from thawed, donated (16.3%) embryos that arrested in their development. Each oocyte or embryo was analyzed individually. Telomerase activity was detected in 97.6% of samples tested. Two methods were used to calculate telomerase activity in these samples, relative telomerase activity (RTA) and total product generated (TPG). When both methods were evaluated statistically no difference was detected in the results.

Oocytes were studied at the immature germinal vesicle (GV) stage through the mature metaphase II (MII) stage. The immature oocyte study group included 10 with a visible GV, and 6 oocytes with no GV and no polar body that are considered metaphase I
(MII) 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 these 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 blastocyst stage. The pronuclear status varied with these deselecteds embryos. A single pronucleus developed in 9 (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). Embryos were grouped by cleavage stage for statistical analysis. Additionally, nine embryos that reached the blastocyst stage in the final experiment of this study were subjected to immunosurgery and the inner cell masses (ICM) of the blastocysts were also evaluated for telomerase activity (Fig. 5).

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 statistically greater than the zygote through 8-16 cell cleavage stage embryo (p < 0.05). Telomerase activity in the blastocyst group was statistically higher (p < 0.05) than the zygote through 8-16 cell stage embryos as well, but was not different from the oocytes, morula, or ICM sample means.
Fig. 5. Telomerase activity in human oocytes and embryos. The samples are grouped by developmental stage and calculated as TPG and RTA for immature (IM) oocytes, mature (M) oocytes, zygotes (Z), 2-3 cell, 4-5 cell, 8-16 cell, morula (MOR), blastocyst (BLST), and, inner cell mass (ICM). Numbers in parentheses represent the number of oocytes or embryos analyzed to achieve the mean represented. Y-error bars represent standard error of mean TPG values only.

The same set of whole gamete and embryo data was also analyzed on a per cell basis by dividing the embryo telomerase value by the cell stage (Fig. 6). Morula cell number was estimated at 15, inner cell mass and whole blastocyst cell number were estimated based on a publication by Devreker et al. (1998) at 20.7 and 64.5, respectively.
Fig. 6. Telomerase activity in each developmental group presented on a per cell basis. Cleavage stage embryos were divided by their respective cell number and Y-error bar represents standard error of the mean.

The telomerase activity was significantly higher in the immature oocyte (Fig. 6) group than all other groups ($p < 0.05$), excluding the mature oocyte group, which was not different from any of the comparison groups. The zygotes were statistically different from all other groups excluding the mature oocytes. The blastocyst telomerase activity was significantly lower than all cleavage stage groupings.

**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 deselect embryos were evaluated from 11 patients. The embryos ranged from 2-cell to 14-cell with 2 single (8.7%), 7 normal (30.4%), and 14 (60.9%) multi-pronuclear in development. One hundred forty-three
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 and RTA levels showed no difference in the method to evaluate telomerase activity, but did find that 18% of the variance was the result of different embryos and 82% of the variability is the result of differences in blastomeres.

**Telomerase Activity as Predictor of Blastocyst Development**

This experiment utilized frozen 2-pronuclear embryos donated by 33 IVF couples. One hundred sixty-two embryos were thawed. Of those 162 thawed, 142 (88%) survived and 123 (79%) cleaved following 24 hours incubation. Eighty-eight reached the appropriate stage for biopsy. One to 2 cells were removed from each biopsied embryo and the cells were assayed individually, resulting in 133 blastomeres biopsied from 88 embryos. Four blastomeres were lost during the biopsy procedure, resulting in the loss of information on two embryo biopsied. In addition to the 88 embryos biopsied, 5 embryos were unable to be biopsied due to premature morulation. Of the 162 embryos thawed for this study, 43 (26.5%) progressed to the blastocyst stage. The blastocyst rate related to embryos that were biopsied was 39 blastocysts of 88 embryos biopsied or 44.3%.

Nuclear status was evaluated in each blastomere and 23% had no visible nucleus, 73% had one nucleus and 4% had 2 nuclei. Lysis or partial lysis of the blastomere biopsied occurred in 18% of blastomeres biopsied. Technical difficulties with the TRAP assay
resulted in the loss of blastomere data from 11 biopsied embryos, which resulted in a remaining 75 embryos that could be evaluated. The primer used with these samples appeared to be lacking the fluorescent label necessary to evaluate the bands on the gel.

Both methods were used to calculate telomerase activity and the results were not different using TPG or RTA (Table 1). 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 arrested and embryos biopsied that progressed to blastocyst. There were two methods used to provide the TPG or RTA value for an embryo, when there were 2 cells biopsied. The first method was to average the two values and the second method was to choose 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 analyzed.

<table>
<thead>
<tr>
<th>TABLE 1. Telomerase activity in blastomeres biopsied from 75 day 3 embryos and evaluated for blastocyst growth</th>
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<tbody>
<tr>
<td><strong>TPG Units</strong></td>
</tr>
<tr>
<td>Embryos</td>
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<td>Arrested</td>
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<td>Blastocyst</td>
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The final segment of this study involved the isolation and analysis of telomerase activity in the inner cell masses of the blastocysts that developed. Forty blastocysts were
treated to immunosurgery, but technical difficulties with the procedure resulted in the inability to treat 9 blastocysts. Six (19.4%) of the blastocysts treated did not have an identifiable ICM. The ICM sized ranged from 20 to 150 μm. Each ICM was analyzed in whole for the telomerase assay. During the TRAP procedure an additional 18 ICM experienced technical difficulties related to the primer problem discussed in the earlier experiment. Nevertheless, a comparison of ICM size verses TPG or RTA value for the biopsied blastomere(s) associated with 13 blastocysts was conducted. No statistical relationship could be identified between ICM size and blastomere TPG or RTA. Nine ICM did undergo the TRAP assay successfully and the data is presented in Fig. 5 and Fig. 6. The telomerase activity detected in these ICM did not statistically differ from that of the whole blastocyst.
CHAPTER V
DISCUSSION

With development of the polymerase chain reaction (PCR)-based Telomeric Repeat Amplification Protocol (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®, Intergen Inc.), decreasing the time needed to prepare and test reagents. Another modification was incorporated into the TRAPeze® system to increase the quantifying sensitivity in this laboratory. The substrate primer (TS) was labeled with an infrared fluorochrome, necessary for using the IR²DNA sequencing system (LI-COR).

The purpose of this study was to 1) modify the current TRAP method to analyze telomerase activity for the whole embryo and the single cell of an embryo, 2) use this method to study the trends in expression of telomerase activity in whole oocytes through blastocyst development and finally, 3) establish if the telomerase activity measured in a blastomere of a day 3 embryo can be used to predict blastocyst development.

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 the first assay conducted to measure telomerase activity and 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 analyzed for telomerase activity and none was detected. Hs27,
newborn foreskin, was previously shown to lack telomerase activity by 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 the cells are terminally differentiated. To establish the range of the assay after the modifications 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 analyzed 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 analyzed. 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 little data, previous to this study, on human oocytes and embryos had been published. Wright et al. (1996) studied a few samples of mature oocytes, sperm, and blastocysts that were discard material from IVF cycles. No telomerase activity was detected in the mature sperm 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 analyzed individually and 97.6% of the samples evaluated had detectable levels of telomerase. Immature oocytes and blastocysts expressed the highest levels and were greater than zygotes or any of the cleavage stage groupings.
Interestingly, there was no difference between the levels detected in the immature oocyte and the blastocyst with approximately 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). Two studies in other species, bovine (Betts and King, 1999) and rat (Eisenhauer et al., 1997), found measurable levels of telomerase activity in the mature oocyte, albeit low. The high level of expression of telomerase in the immature oocyte was also seen in the bovine (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, which differs 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 study used the relative telomerase activity (RTA) method using a positive control cell line as the method 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.

When the data for whole oocytes and embryos were evaluated on a per cell basis, telomerase declined from the immature oocyte through maturation and fertilization and leveled off to a similar value per cell through subsequent cleavages. The immature oocyte has a much larger cytoplasmic volume than cells from subsequent divisions and this is certainly part of the reason for the significantly higher levels of telomerase activity. However, the mature oocyte has a similar volume as the immature oocyte with
an apparent decline in telomerase activity and this trend continues through the one cell zygote. Perhaps this decline in telomerase activity is due to depletion of the maternal stores of proteins known to exist in the oocyte. As the shift from oocyte to embryonic genome takes places at approximately the 4-cell stage (Braude et al., 1988) there is an upregulation of critical proteins. Analysis of this data on a per cell basis does not show an upregulation of telomerase activity.

Results 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 on the high incidence of mosaicism of normal and abnormal embryos (Munne 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 of care for certain couples trying to avoid genetic disease in their offspring. Research has shown that there is no detrimental affect to the embryo by removing 1 or 2 cells from the day 3 embryo (Hardy et al., 1990). There are no publications that refer to the use of thawed pronuclear embryos cultured to day 3, biopsied, and followed to the blastocyst stage 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 rate is lower than the average reported by Hardy et al. (1990) of 71% blastocyst rate from biopsied embryos. Some differences in the study by Hardy et al. (1990) is their use of fresh embryos and they only reported on the biopsy of embryos that reached the 8-cell stage. Our laboratory
biopsied embryos when they reached the 5 to 9-cell stage.

Telomerase levels were not statistically 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. These results suggest that telomerase activity in the day 3, biopsy stage embryo, is not a predictor of blastocyst development potential.

This is the first study to evaluate telomerase activity of ICM isolated from the trophoblast cells of the whole blastocyst. Nineteen percent of blastocysts evaluated did not contain an ICM. Yet, all ICM analyzed had detectable levels of telomerase activity that were not significantly different from the whole blastocyst. This data suggests that a significant level of telomerase activity of the whole blastocyst is originating from the ICM, when one recognizes that the ICM only constitutes approximately 30% of the cell number associated with the blastocyst (Devreker et al., 1998).

The results of this study leads researchers into new territory for study of the human oocyte and preimplantation embryo. Evaluation of telomerase levels within the immature oocyte in the future could provide some beneficial information on the role of telomerase and aneuploidy that is present at a higher level in older oocytes. Measurement of the telomeric length of the immature oocytes of older verses younger patients might show evidence of shortening telomeric length, predisposing the older oocyte to inappropriate divisions leading to aneuploidy.

Culture systems for the human preimplantation embryo are constantly evolving. Research in the mouse (O’Neill, 1997; O’Neill, 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. Future studies in the human need to address the role of autocrine factors on embryo development. 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 evaluated the level of telomerase activity in discard material of whole oocytes through blastocysts, finding that all stages expressed telomerase activity. Immature oocytes and blastocysts had the highest levels of telomerase activity and were significantly greater than the levels from zygote through pre-morula stages. Variability within cells of an embryo was found to be very high. Telomerase activity measured in 1 to 2 cells biopsied, from day 3 cleaving embryos, did not predict the potential for development to blastocyst stage.
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Ms. Wright’s Bachelor of Science in Biomedical Science was completed in 1986 at Texas A&M University. She followed this degree with the pursuit of a Master of Science in Physiology of Reproduction from Texas A&M University, completed in 1990.

Following these two degrees Ms. Wright worked with Granada Bioscience, Inc. of College Station, Texas conducting research in the field of cloning cattle. After one year, a career adjustment resulted in the attainment of an embryologist position at Presbyterian Hospital of Dallas. Ms. Wright worked in this position until the Fall of 1996 when she moved to Norfolk, Virginia to begin a Doctor of Philosophy degree in Biomedical Sciences, from Eastern Virginia Medical School and Old Dominion University (Department of Obstetrics and Gynecology, Eastern Virginia Medical School, 601 Colley Ave., Norfolk, VA). While pursuing this Ph.D., Ms. Wright was elected Vice President of the Biomedical Sciences Student Organization for the school year 1998 to 1999. Ms. Wright also received the honor of election into the Phi Kappa Phi Honor Society in 1999.

Ms. Wright will move to the position of Embryology Laboratory Director of the Vincent IVF Unit at Massachusetts General Hospital in Boston and serve as a Harvard Medical School Faculty member, following completion of this degree.