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Feasibility of Human Telomerase Reverse Transcriptase mRNA Expression in Individual Blastomeres as an Indicator of Early Embryo Development

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***Purpose*:** The study was undertaken to test whether human telomerase reverse transcriptase (hTERT) transcripts in an individual blastomere could be used as an indicator for embryo development.

***Methods*:** Group A consisted of day 3 normal cleaving embryos at 4- to 8-cell stage, which were surplus and not allocated for uterine transfer. Group B consisted of arrested or fragmented embryos at the same stage, which were considered to be compromised. After blastomere dissociation, RNA purification, reverse transcription, and hTERT PCR amplification, the amplified product was separated by 3% gel electrophoresis.

***Results*:** Eighty-six (90.5%) of the 95 intact blastomeres in group A and 78 (70.9%) of the 110 blastomeres in group B demonstrated hTERT mRNA expression. The difference was statistically significant ($P < 0.05$, chi-square). Eight (38.1%) of the 21 embryos in group A and 22 (62.9%) of the 35 embryos in group B had at least one blastomere that did not express hTERT mRNA under this procedure. The difference was not significant ($P > 0.05$, chi-square).

***Conclusions*:** General hTERT mRNA transcripts can be detected in most of the individual blastomeres but cannot be used as an indicator for early embryo development. Further investigations are necessary to elucidate its clinical application.

KEY WORDS: Embryo development; hTCS; hTERT; telomerase activity.

INTRODUCTION

The ends of eukaryotic chromosomes are capped with copies of a hexamer repeat sequence and associated proteins (1). These structures are known as telomeres, and they stabilize the ends of chromosomes during replication (2). Many scientists propose the telomere as a "mitotic clock" (3). In human cells, the DNA repeats are highly conserved TTAGGG nucleotide segments (1). Its shortening correlates with the number of cell divisions. When the telomere length reaches a certain limit, it will signal the cell to undergo replicate senescence, under which the cells stop cell division, become resistant to apoptosis, and alter their normal functions (4).

Telomerase is a ribonucleoprotein (5). Its RNA subunit acts as a template for the synthesis of telomeric DNA, whereas its protein component catalyzes this process to make up for the inability of polymerase to replicate linear DNA through conventional replication (6). It has been hypothesized that telomerase activity is necessary for cellular immortalization (7). Most of the reports regarding telomerase activity

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were focused on the research of cancer biology or cell aging (8,9). In humans, telomerase activity is only found in germ cells and embryos, immortalized cell lines, and most tumor cells; it is not found in cells of most somatic tissues (7). Lack of telomerase activity in somatic cells, and the finite length of telomeric DNA would limit the number of cell divisions. Most tumor and immortal cells have telomerase turned on as a mechanism to counteract telomere loss, thus enabling them to divide infinitely (7,10). Consistent with the hypothesis, telomerase activity has been detected in extracts of human ovaries and testes (11,12). Although other reports showed that telomerase activity was relatively high in blastocyst-stage embryos (13,14), very little is known about telomerase activity and its components in human single blastomeres possibly because of difficulties in obtaining sufficient samples for analysis. But analysis of a single blastomere *is* more helpful in our clinical practice of human in vitro fertilization (IVF) system. Through embryo biopsy and analysis of a blastomere, it is *now* possible for us to evaluate an embryo prior to uterine transfer (15).

The constituents and action of the telomerase complex have recently been further identified. Human telomerase catalytic subunit (hTCS), or human telomerase reverse transcriptase (hTERT), a recently cloned enzyme, has been found (16). Its expression at the mRNA level has been reported to be strongly associated with enzyme activity and concomitant immortalization (17,18). Its introduction into normal human epithelial cells and fibroblasts was sufficient to reconstitute telomerase activity, arrest telomere shortening, and extend the life span in vitro (19).

On the basis of the importance of telomerase for cellular meiosis and mitosis, a similar process as early human embryo development, we designed this study for the assessment of hTERT mRNA in individual blastomeres. With the recent cloning of the hTERT gene, it is now possible to detect telomerase-positive cells by a more sensitive reverse transcription-polymerase chain reaction (RT-PCR), rather than the telomerase enzymatic assay called telomeric repeat amplification protocol (TRAP) (7,11), which requires more well-preserved fresh tissue that is rarely available in a routine clinical setting. The objective of this study was to demonstrate its mRNA expression in single blastomeres first, so that we can better understand embryo physiology, use it as a predictor for embryonic health and development, and select a best embryo for uterine transfer.

MATERIALS AND METHODS

Embryos Collection

Oocytes were obtained from patients undergoing assisted reproductive techniques (ART). Ovarian stimulation for ART was administered with recombinant human follicular stimulating hormone (r-FSH; Gonal-F; Serono, Zug, Switzerland) and/or human menopausal gonadotropin (HMG; Pergonal; Serono, Zug, Switzerland) after standard pituitary down-regulation with gonadotropin-releasing hormone agonist (GnRHa; Lupron; Tap Pharmaceuticals, Deerfield, IL) until at least two follicles had attained a mean diameter of 17 mm. Oocytes retrieval was performed 34–36 h after 10000 IU of human chorionic gonadotropin (hCG; Profasi; Serono, Zug, Switzerland) was administered. Fertilization was performed 4–6 h after oocyte retrieval with either conventional insemination or intracytoplasmic sperm injection (ICSI) as appropriate. The oocytes were checked for fertilization by the presence and number of pronuclei (PN). These embryos were cultured for 72 h after retrieval. Under patients' informed consent, the experiment was divided into two groups. Group A consisted of normal cleaving embryos at 4- to 8-cell stage, which were surplus and not allocated for uterine transfer. Group B consisted of arrested or fragmented embryos at the same stage, which were considered to be compromised.

Blastomeres Collection

The embryos were placed for 2 min in acid Tyrode solution for zona removal. Afterward, the zona-free embryos were placed for 3 min in calcium- and magnesium-free medium for dissociation of blastomeres. After repeated pipetting, individual blastomeres with a distinctive nucleus were separated. All blastomeres were washed thoroughly in sterile phosphate-buffered saline (PBS) in plastic dishes and then transferred to 0.2-mL tubes in as small a volume as possible. A 50 μL sample of denaturing solution (Fast-Run RT-PCR Kit; HT Biotechnology, Cambridge, UK) was added immediately after transfer and the tube was briefly vortexed. Some samples were frozen and stored at -70°C prior to RNA purification.

RNA Purification

Previously frozen samples were thawed and RNA purification was performed using the Fast-Run

RT-PCR Kit (HT Biotechnology, Cambridge, UK) according to the manufacturer's protocol. Copies (10^6) of the human epidermoid-carcinoma cells SiHa mRNA (ATCC, Rockville, MD, USA) were added as a positive control. The SiHa cell line was established from fragments of a primary tissue sample of an undifferentiated squamous carcinoma of the cervix.

Reverse Transcription

The reverse transcription reaction was performed by incubating the mRNA with 11.5 μL master mix solution consisting of 4 μL of 25 mM $MgCl_2$, 2.0 μL 10X PCR buffer (Perkin Elmer Inc, Foster City, CA, USA), 4.0 μL 100 mM dNTP, 1.0 μL RNase inhibitor (Perkin Elmer Inc), 0.5 μL 50 IU/μL Maloney murine leukaemia virus (MMLV) reverse transcriptase (Perkin Elmer Inc), and 0.5 μL random hexamers. The mRNA was reverse transcribed at 37°C for 1 h, followed by inactivation of the MMLV enzyme by incubation at 95°C for 5 min (12).

hTERT PCR Amplification

An aliquot of cDNA was used as a template for PCR amplification using a 9600 Perkin Elmer system in a total reaction volume of 50 μL containing 1.5 mmol/l $MgCl_2$, 1 mmol/l dNTP mix, 1X Q Solution, 0.5 μmol/l of each gene-specific primer, and 1.25 IU Taq DNA polymerase (Quiagen Inc, Chatsworth, CA, USA). The following amplification profile was applied: 1 cycle, 94°C for 2 min; 35 cycle of 94°C for 2 min, 58°C for 1 min, 72°C for 1 min; 1 cycle, 94°C for 7 min; hold at 4°C. Primer pair LT5 (5′-CGGAAGAGTGTCTGGAGCAA-3′) and LT6 (5′-GGATGAAGCGGAGTCTGGA-3′) were used as gene-specific PCR primers to amplify hTERT mRNA (16). The amplification would result in a PCR product size of 145 bp in all hTERT mRNA transcripts (general transcripts) (20). To obtain better resolution, PCR products were separated by 3% gel electrophoresis using NuSieve GTG agarose (FMG Inc., Rockland, ME, USA), stained with ethidium bromide and visualized under UV illumination.

RESULTS

Analysis of Each Blastomere of Entire Normal-Cleaving Embryos

Blastomeres of normal-cleaving embryos were separated and assayed individually to provide information on the variability of hTERT mRNA from one blastomere to the next within an entire embryo. Twenty-one embryos were donated from 17 patients. During isolation, 17/112 (15.2%) of the individual blastomeres lysed. Intact blastomeres were analysed for the mRNA expression of hTERT. Of the 95 intact blastomeres evaluated, 86 (90.5%) demonstrated presence of hTERT mRNA by the expression of 145 bp amplified product (Fig. 1). Eight (38.1%) of the 21 embryos had at least one blastomere that did not express presence of hTERT mRNA under this procedure.

Analysis of Each Blastomere of Entire Compromised Embryos

Blastomeres of compromised embryos were separated and assayed individually as those from

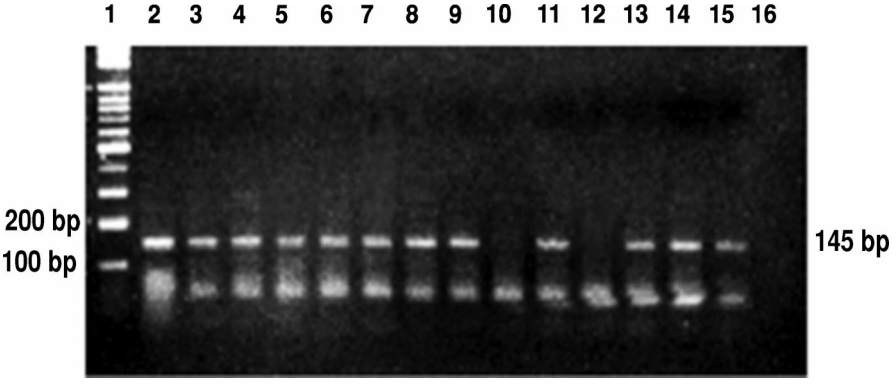

Fig. 1. Representative results for the expression of hTERT in individual blastomeres. RT-PCR was performed using primer pair LT5 and LT6 to amplify a 145-bp segment present in all transcripts. Lane 1, 100-bp DNA molecular marker; lane 2, SiHa cell cDNA (positive control); lanes 3–15, blastomeres isolated from normal-cleaving embryos; lane 16, pure water (negative control).

normal-cleaving embryos. Thirty-five embryos were donated from 22 patients. During isolation 29/139 (20.9%) of the individual blastomeres lysed. Of the 110 intact blastomeres evaluated, 78 (70.9%) demonstrated presence of hTERT mRNA. Twenty-two (62.9%) of the 35 embryos had at least one cell that did not express presence of hTERT mRNA.

Telomerase Activity in Human Blastomeres as a Predictor for Embryo Viability and Development

The difference (90.5% vs. 70.9%) in the expression of the amplified product from hTERT mRNA in the individual blastmeres from normal-cleaving and compromised embryos was significant in statistics ($P < 0.05$, chi-square). While the difference (38.1% vs. 62.9%) in the embryos that had at least one cell that did not express presence of hTERT mRNA was not significant in statistics ($P > 0.05$, chi-square).

DISCUSSION

Despite the fact that telomerase activity has been studied thoroughly in cancer behavior and cell aging (8,9), very few data in human oocytes and embryos have been published (12–14, 21). Wright *et al.* (13) analyzed single mature oocytes retrieved from ovarian tissue of patients undergoing hysterectomy and adult sperm samples. They reported 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 in the same study. With the increased sensitivity of the assay employed, many other studies have found measurable, albeit low, levels of the telomerase activity in the mature oocyte (14,22,23).

To enhance the implantation and pregnancy rate in an IVF treatment, the embryos should be carefully evaluated in order to select those with the highest implantation potential. However, the limitations of evaluating embryos based on morphological criteria alone were well recognized (24,25). Except when the embryos are clearly fragmenting, correlations between gross morphology and implantation capability are weak and inaccurate. Many centers have worked on more objective criteria for judging embryo viability and implantation potential (26–29). At this moment, nevertheless, it is difficult to incorporate these selection procedures into an IVF practice. Morphological assessment, although unsatisfactory, is quick, noninvasive, and easy to carry out in general practice.

A common belief is that telomerase activity depends on the presence of hTERT transcripts (20,30). Human oocytes and embryos have been evaluated for alternative splice variants of the telomerase catalytic subunit (hTCS, or hTERT) (12). All of them express hTERT mRNA, but expression varies dramatically. Nevertheless, a relationship between poor quality embryo and an increase in alternative splicing variants of hTERT mRNA was found. They suggested that the presence of alternately spliced mRNA variants in human preimplantation embryos may suggest a lack of telomerase activity and thus chromosomes associated with shortened telomeres. However, telomerase activity was not compared between oocytes and embryos in their study. Wright *et al.* studied samples that were discarded from IVF cycles (21). Telomerase activity was detected in all developmental stages from immature oocytes through blastocyst stage embryos. Moreover, thawed zygotes were cultured to day 3, biopsied by removing 1–2 cells, and the biopsied embryos were cultured to blastocyst stage. They found that there was no difference in telomerase activity between cells biopsied from embryos that reached the blastocyst stage and cells from those that arrested in growth. However, their samples were derived from thawed zygotes, which might be different from fresh ones in cell physiology.

With the aim to determine the possibility of using hTERT mRNA expression of single biopsied blastomeres, we conducted this study in individual human blastomeres from fresh embryos not allocated for uterine transfer. Through embryo biopsy and RT-PCR system, the expression of hTERT mRNA in individual blastomeres was determined first. Accordingly we could be able to evaluate embryo viability and predict future embryo development. In our study, two different regions of hTERT mRNA were amplified in parallel: primers LT 5 (hTERT 1784), and LT 6 (hTERT 1910) amplify a 145-bp segment present in all hTERT mRNA transcripts (general transcripts) (16,20). The result demonstrated that the difference in the expression of hTERT mRNA in the individual blastmeres from normal-cleaving and compromised embryos was significant in statistics. Our results are different from Brenner's study (12) in the aspects that we performed the study in single blastomeres instead of a whole embryo, and that all transcripts (general hTERT transcripts) were detected instead of alternately spliced variants. A full-length hTERT transcript accomplished by using primers that span

the putative splice site or a quantitative assessment of hTERT expression levels by real-time PCR, which is nonetheless delicate and sometimes impossible in a clinical setting, might be required for further verification. We did not try to employ a quantitative measure in this study partly because we would like to make the procedures more timesaving and less complicated in order to be applied clinically, and partly because previous report from blastomeres of disaggregated embryos showed a significant variability in telomerase levels within blastomeres in an embryo (21). Additionally, considering the embryos from normal-cleaving and compromised condition that had at least one blastomeres which did not express presence of hTERT mRNA, the difference was not significant in the present study. This may further explain the fact delineated by Brenner *et al.* (12) that all human preimplantation embryos express hTERT mRNA.

Although our results showed that the difference in the detection of general hTERT mRNA transcripts in the individual blastmeres from normal-cleaving and compromised embryos was significant in statistics, we could not predict embryo developmental potential simply from our results, because blastomeres from compromised embryos also showed a relatively high incidence of hTERT mRNA expression (70.6%). Besides the possibility of false negative results in our study, such a high incidence of mRNA expression in individual blastomeres would make it impossible to differentiate between good and bad embryos for uterine transfer. Furthermore, the result also implicates that telomerase and its components may play only a partial role in embryo development. In fact, not only telomerase, but also many other diverse factors, including nutrition, embryo metabolism, culture/coculture system, gamete formation, embryonic genome activation, and imprinting, are known to affect embryo viability and development (31). More comprehensive and informative assessments of an embryo are needed to explore and clarify the behavior of early human embryos in vitro.

In summary, our results demonstrate that general hTERT mRNA transcripts can be detected in most of the individual blastomeres but cannot be used as an indicator for early embryo development. The conclusion is consistent with the report by Wright *et al.* (21) that there was no difference in telomerase activity between cells biopsied from embryos that reached the blastocyst stage and cells from those that arrested in growth. Further investigations are necessary to elucidate its biological significance and clinical application.

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REFERENCES

1. Zakian VA: Telomeres: Beginning to understand the end. Science 1995;270:1601–1607
2. Preston RJ: Telomeres, telomerase and chromosome stability. Radiat Res 1997;147:529–534
3. Harley CB, Futcher AB, Greider CW: Telomeres shorten during aging of human fibroblasts. Nature 1990;345:458–460
4. Faragher RG, Kipling D: How might replicative senescence contribute to human aging? Bioessays 1998;20:985–991
5. Morin GB: The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. Cell 1989;59:521–529
6. Dahse R, Fiedler W, Ernst G: Telomeres and telomerase: Biological and clinical importance. Clin Chem 1997;43:708–714
7. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW: Specific association of human telomerase activity with immortal cells and cancer. Science 1994;266:2011–2015
8. Shay JW, Gazdar AF: Telomerase in the early detection of cancer. J Clin Pathol 1997;50:106–109
9. Fossel M: Telomerase and the aging cell: Implications for human health. JAMA 1998;279:1732–1735
10. Weinberg RA: Telomeres. Bumps on the road to immortality. Nature 1998;396:23–24
11. Fujisawa M, Tanaka H, Tatsumi N, Okada H, Arakawa S, Kamidono S: Telomerase activity in the testis of infertile patients with selected causes. Hum Reprod 1998;13:1476–1479
12. Brenner CA, Wolny YM, Adler RR, Cohen J: Alternative splicing of the telomerase catalytic subunit in human oocytes and embryos. Mol Hum Reprod 1999;5:845–850
13. Wright WE, Piatyszek MA, Rainey WE, Byrd W, Shay JW: Telomerase activity in human germline and embryonic tissues and cells. Dev Genet 1996;18:173–179
14. Xu J, Yang X: Telomerase activity in bovine embryos during early development. Biol Reprod 2000;63:1124–1128
15. Hardy K, Martin KL, Leese HJ, Winston RM, Handyside AH: Human preimplantation development in vitro is not adversely affected by biopsy at the 8-cell stage. Hum Reprod 1990;5:708–714
16. Nakamura TM, Morin GB, Chapman KB, Weinrich SL, Andrews WH, Lingner J, Harley CB, Cech TR: Telomerase catalytic subunit homologs from fission yeast and human. Science 1997;277,955–959
17. Meyerson N. Counter CM, Eaton EN, Ellisen LW, Steiner P, Caddle SD, Ziaugra L, Beijersbergen RL, Davidoff MJ, Liu Q, Bacchetti S, Haber DA, Weinberg RA: hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. Cell 1997;90: 785–795

18. Kilian A, Bowtell DD, Abud HE, Hime GR, Venter DJ, Keese PK, Duncan EL, Reddel RR, Jefferson RA: Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns in different cell types. Hum Mol Genet 1997;6:2011–2019
19. Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu C, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE: Extension of life-span by introduction of telomerase into normal human cells. Science 1998;279:349–352
20. Krams M, Hero B, Berthold F, Parwaresch R, Harms D, Rudolph P: Full-length telomerase reverse transcriptase messenger RNA is an independent prognostic factor in neuroblastoma. Am J Pathol 2003;162:1019–1026
21. Wright DL, Jones EL, Mayer JF, Oehninger S, Gibbons WE, Lanzendorf SE: Characterization of telomerase activity in the human oocyte and preimplantation embryo. Mol Hum Reprod 2001;7:947–955
22. Eisenhauer KM, Gerstein RM, Chiu CP, Conti M, Hsueh AJ: Telomere activity in female and male rat germ cells undergoing meiosis and in early embryos. Biol Reprod 1997;56: 1120–1125
23. Betts DH, King WA: Telomerase activity and telomere detection during early bovine development. Dev Genet 1999;25:397–403
24. Giorgetti C, Terriou P, Auquier P, Hans E, Spach JL, Salzmann J, Roulier R: Embryo score to predict implantation after in vitro fertilization: Based on 957 single embryo transfers. Hum Reprod 1995;10:2427–2431
25. Visser DS. Fourie FR: The applicability of the cumulative embryo score system for embryo selection and quality control in an in vitro fertilization/embryo transfer programme. Hum Reprod 1993;8:1719–1722
26. Bolton VN, Hawes SM, Taylor CT, Parsons JH: Development of spare human preimplantation embryos in vitro: An analysis of the correlations among gross morphology, cleavage rate, and development to the blastocyst. J In Vitro Fert Embryo Transf 1989;6:30–35
27. Leese HJ: Analysis of embryos by non-invasive methods. Hum Reprod 1987;2:37–40
28. Conaghan J, Hardy K, Handyside AH, Winston RM, Leese HJ: Selection criteria for human embryo transfer: A comparison of pyruvate uptake and morphology. J Assist Reprod Genet 1993;10:21–30
29. Grifo JA, Boyle A, Fischer E, Lavy G, DeCherney AH, Ward DC, Sanyal MK: Preembryo biopsy and analysis of blastomeres by in situ hybridization. Am J Obstet Gynecol 1990;163:2013–2019
30. Hisatomi H, Nagao K, Kanamaru T, Hiyama E: Levels of telomerase catalytic subunit mRNA as a predictor of potential malignancy. Int J Oncol 1999;14:727–732
31. Hartshorne GM: Factors controlling embryo viability. Hum Fertil 2001;4:225–234