Quantitative RT–PCR reveals tuberous sclerosis gene, TSC2, mRNA degradation following cryopreservation in the human preimplantation embryo

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The use of cryopreserved human embryos in gene expression studies provides an additional source to the scarce embryos available for research. To validate their use we have implemented a quantitative RT–PCR to characterize the levels of the tuberous sclerosis, TSC2 gene in fresh and frozen–thawed human embryos. Frozen embryos were thawed using two different clinical protocols. In fresh embryos 9.95 fg of TSC2 cDNA was present in the unfertilized oocyte, which was comparable to the level on day 2 of preimplantation development. On day 3 there was a significant drop (P < 0.001) to 6.8 fg, followed by an increase in cDNA levels to 10.8 fg (P < 0.01) on day 6 at the expanded blastocyst stage. Day 2 frozen embryos possessed 50% less (P < 0.001) TSC2 mRNA in comparison to the fresh embryos using thawing protocol one (from frozen to 37°C) and 25% less TSC2 mRNA (P < 0.01) with thawing protocol 2 (from frozen to room temperature). After culturing day 2 frozen embryos for an additional day they showed mRNA levels comparable with fresh day 3 embryos. There was no significant difference in the levels of TSC2 mRNA between fresh and frozen day 3 human embryos with either thawing protocol. This study demonstrates that cryopreservation does affect the normal pattern of gene expression during human preimplantation development, and that intact frozen–thawed embryos are not equivalent to their non-frozen counterparts. Furthermore human embryos frozen on day 2 appear to be more susceptible to temperature change than embryos frozen on day 3.

Key words: cryopreservation/expression/tuberous sclerosis

Introduction

The initial cleavages of the mammalian embryo are supported by the maternal proteins and mRNA laid down in the oocyte during oogenesis. During mammalian preimplantation development, activation of the embryonic genome coincides with the rapid degradation of the maternal transcripts. The timing of global embryonic gene expression is species dependent, and occurs at the 4–8-cell stage in the human (Braude et al., 1988; Tesarik et al., 1988). Examination of both global and specific gene expression enables us to investigate the process of normal human preimplantation development. This is of clinical as well as scientific importance, because of the possibility of identifying more viable embryos for IVF–embryo transfer, which is currently performed on the limited criteria of embryo morphology. In addition, gene expression studies are essential for evaluating the possibility of using RT–PCR as a diagnostic strategy for preimplantation genetic diagnosis (PGD). The diagnosis of single gene defects for PGD is typically based on PCR from the two copies of the gene present in a single cell (Wells and Sherlock, 1998). Due to the limited starting material, problems specifically associated with single cell PCR, such as failure of amplification, contamination and allele dropout arise (Findlay et al., 1995; Ray and Handyside 1996). The possibility that these complications may result in a serious misdiagnosis is more apparent when considering the diagnosis of autosomal dominant disorders. Once transcribed, multiple copies of the gene are present in each cell in the form of mRNA. RT–PCR is therefore a potentially more reliable diagnostic approach, as the increase in target molecules should reduce the incidence of these single cell PCR complications. The multiple mRNA templates, in combination with the lack of introns from the sequence, would also allow larger fragments of the gene to be amplified and therefore a larger number of different mutations to be analysed from a single cell. In addition, with the use of RT–PCR, confirmation of the diagnosis could be performed without the need for the removal of a second cell. Before RT–PCR can be considered for PGD of a specific gene, however, its pattern of gene expression throughout human preimplantation development must be established. Most of our current understanding on the changes in gene expression which occur during normal mammalian preimplantation development are based on mouse studies (Piko and Clegg, 1982; Clegg and Piko, 1983; Bachvarova et al., 1989; Taylor and Piko, 1990). Typically these studies have required hundreds of oocytes and embryos for analysis and hence cannot be performed using the limited number of human embryos donated for research. This situation is exacerbated as more IVF patients opt to freeze their good quality supernumerary embryos following the IVF–embryo transfer. Embryo cryopreservation offers a number of potential advantages in routine IVF, such as reducing the risk of multiple pregnancies (Staessen et al., 1993; 1995; Templeton and Morris, 1998), reducing the risk of ovarian hyperstimulation
syndrome by delaying embryo transfer (Wada et al., 1993), reducing patient time and expense from additional stimulation cycles, avoiding the destruction of potentially viable supernumerary embryos and increasing the number of embryo transfers and hence pregnancies per oocyte retrieval (Selick et al., 1995; Salat-Baroux et al., 1996). Once IVF patients have achieved a pregnancy, however, they are more likely to donate their frozen embryos for research. It has been reported that intact thawed embryos have the same implantation potential as stage-matched fresh embryos (Burns et al., 1999; Edgar et al., 2000).

As the thawed embryos appear comparable to fresh embryos, the use of these cryopreserved human embryos in gene expression studies and other projects provides an additional source to the scarce embryos available for research. However, there is an increasing number of studies which demonstrate that in-vitro culture and assisted reproductive techniques can result in mammalian embryonic reprogramming (Doyle et al., 1992; Dulioust et al., 1995; Niemann and Wrenzycki, 2000). Therefore we cannot assume cryopreserved embryos exhibit the same pattern of gene expression as seen in fresh embryos.

The aim of this study was to determine whether frozen embryos can be used alongside fresh embryos for expression studies, specifically for the evaluation of the use of RT–PCR as a diagnostic approach for PGD of the autosomal dominant disorder tuberous sclerosis (TSC). Tuberous sclerosis is a multisystem, autosomal dominant, hereditary disease. The disease is characterized by the widespread development of benign tumours, usually described as hamartoma. These lesions present an unpredictable distribution within a wide range of tissues, which results in a wide variety of symptoms and complications. The most serious manifestations of the disease can be mental retardation, seizures, intracerebral tumours and death (Gomez, 1988). The disease results from mutations in one of two genes, TSC1 and TSC2 which are located on chromosomes 9 and 16 respectively, leading to apparently indistinguishable phenotypes (Janssen et al., 1994; Povey et al., 1994).

To assess the extent to which temperature influences mRNA levels, we implemented a sensitive quantitative RT–PCR to compare the pattern of expression of the TSC2 gene in fresh and frozen embryos, which were thawed using two different clinical procedures.

Materials and methods

Human oocytes and embryos

Women underwent ovulation induction and oocytes were collected by transvaginal ultrasound-guided aspiration and inseminated with prepared sperm as previously described (Rutherford et al., 1988). Oocytes were examined the following morning, 19–20 h post insemination and classified as normally fertilized or polyspermic depending upon the presence of two or more pronuclei respectively. Fertilized embryos were cultured in Medicult medium (Medicult). Oocytes that failed to fertilize and surplus embryos following transfer on day 2 post insemination were used with the couple’s informed and written consent. Normally fertilized embryos were cultured individually in 1 ml of Medicult medium for up to day 6 post insemination. Embryo development was recorded every 12 h and embryos were matched for both stage of development and embryo grade prior to their use (Dawson, 1987). Embryos of grade III or less were not included in the study. All work carried out in this project had local ethical approval from the Research Ethics Committee of Imperial College School of Medicine and under license from the Human Fertilisation and Embryology Authority.

Embryo cryopreservation

Cleavage stage embryos were frozen on day 2 or day 3 using 1,2-propanediol (PROH) (Sigma) and sucrose (BDH) as cryoprotectants, made up in PBS supplemented with 20% human serum albumin (HSA) (Zenall) (Lassalle et al., 1985). Embryos were initially equilibrated in 1.5 mol/l PROH at room temperature for 10 min. Embryos were then transferred to a solution containing 1.5 mol/l PROH and 0.1 mol/l sucrose before being loaded individually or in pairs into freezing straws. The cooling was performed on a Kryo 10 series programmable freezer (Planar products, UK). The straws were cooled at a rate of −2°C/min to −7°C. Each straw was then seeded manually and the temperature maintained for 10 min. Cooling was then resumed at a rate of −0.3°C/min to −30°C and at −10°C/min to −150°C. Straws were then plunged into liquid nitrogen prior to storage.

Quantitative RT–PCR approach

The quantification assay was based on the co-amplification of a reference standard, referred to as the competitor, in the same reaction as the sequence of interest (Becker-Andre, 1989; Cross et al., 1993; Taylor et al., 2001). The competitor is a synthetic template identical to the target sequence, in both size and sequence, except for a single base pair mismatch, which introduces a novel HpaII site. A fixed amount of competitor cDNA is added to each embryo sample and the mixture is subjected to single step PCR. Both the competitor and the target sequence are amplified using the same primers, producing PCR products of identical size, therefore allowing the competitor to control for any tube-to-tube variation in amplification efficiency. The competitor template is subsequently distinguished from the embryonic product following restriction digestion with HpaII, which results in the competitor band being 23 bp smaller than the 201 bp embryonic product. The initial amount of cDNA reverse transcribed from the embryo sample can be determined by comparing amplification from the embryonic cDNA with that of the known amount of competitor template added to the PCR.

Embryo thawing

Two protocols were followed for embryo thawing. In protocol 1, embryos were thawed by removing straws from liquid nitrogen storage, exposing to air for 30 s and immersing in a 37°C water bath for 45 s. Protocol 2 was a more gentle approach as embryos were thawed by removing straws from liquid nitrogen storage and exposing to air (room temperature) for 45 s avoiding immersion in a 37°C water bath. The thawing procedure for both protocols continued as follows. Propanediol was removed in three steps (1.0 mol/l PROH for 5 min, 0.5 mol/l PROH for 5 min, 0.2 mol/l sucrose for 10 min) in the presence of 0.2 mol/l sucrose at room temperature. Rehydration was completed by incubation in sucrose-free phosphate-buffered saline (PBS) (Gibco)/bovine serum albumin (BSA; Sigma) for 10 min. Thawed embryos were either reverse transcribed immediately or cultured (extended embryo culture).

The thawed embryos used were all stage- and grade-matched to fresh embryos. Only embryos having all their blastomeres intact were used for this study. The thawing survival rates were >90% and there was no significant difference between the survival rates observed for protocols 1 and 2.

Extended embryo culture

Eight day 2 frozen embryos thawed by protocol 1 were cultured for an additional 24 h up to day 3. After completion of the thawing method as described above, the embryos were initially transferred to 10 μl drops of PBS/BSA at room temperature in dishes which were transferred to an incubator at 37°C, 5% CO2 for 10 min. Subsequently, embryos were transferred into gas- and temperature-equilibrated drops of culture medium (Medicult) at 37°C, 5% CO2 in which they were left overnight.

The development and cleavage rates observed over the 24 h period in culture for the thawed embryos were >85% and were not significantly different from those observed in fresh embryos over the same period.

Reverse transcription

After removing the zona pellucida with acidified Tyrode’s solution (pH 2.4), individual oocytes and embryos were washed in PBS supplemented with BSA (4 mg/ml) and subsequently lysed in 5 μl of lysis buffer (0.5% NP40, 10 mol/l Tris (pH 8.0), 10 mmol/l NaCl and 3 mmol/l MgCl2) on ice (Gilliland et al., 1990). The whole lysate was then made up to 20 μl with reverse transcriptase buffer (50 mmol/l Tris–HCl (pH 8.3), 75 mmol/l KCl, 3 mmol/l MgCl2), 10 mmol/l dithiothreitol (Pharmacia), 300 pmol oligo (dT)12–18 primer (Pharmacia), 0.5 mmol/l dNTP and 100 IU of Moloney murine leukaemia virus (M-MLV) reverse transcriptase enzyme. The mixture was incubated at 37°C for 1 h and the reaction was terminated by heating at 95°C for 5 min. The reverse transcriptase enzyme was omitted from the negative controls, which
were included in each experiment. The tubes were centrifuged at 300 g for 1–2 min and the resulting supernatant used for PCR. In cases where reverse transcription was not carried out immediately, the lysates were snap-frozen in liquid nitrogen and stored at −70°C. RT–PCR products were verified by sequencing analysis (data not shown).

**Nested PCR**

Following reverse transcription, 10 μl of the cDNA product from each embryo was amplified by PCR with 5 μl 10× PCR buffer (Perkin Elmer), 1.5 mmol/l MgCl2 solution (Perkin Elmer), 0.5 μl dNTP mix (10 mmol/l each), 40 pmol of each primer and 2.5 IU of Taq Polymerase (Ampli-Taq; Perkin Elmer) in a 50 μl reaction. Samples were denatured at 94°C for 3 min and subsequently underwent a 32-cycle PCR of denaturation for 45 s at 94°C, primer annealing for 60 s at appropriate temperature (60°C outer primers, 67°C inner primers) and extension for 90 s at 72°C. The reaction was completed with a final extension step at 72°C for 5 min. A nested PCR protocol was carried out only for the generation of the competitor template. For the nested reaction, 2 μl of the first amplification product was added to freshly prepared PCR mix as above. Primer positions and product sizes are given in Figure 1A.

**Generation of competitor template**

The competitor template was generated using the newly designed, degenerate primer, DP (5’-ACAGCCGGAGATGTTCTACTGCGCCG-3’). Figure 1A demonstrates how TSC2 outer primer pair OP1 (5’-CCATAACTCTGGAGCAAAAACCA-3’) and OP2 (5’-GCAGAACACAGGGCATCTGG-3’) and inner primer pair DP and IP (5’-GCCAGATGTGACACGGACTC-3’) were designed in such a way as to generate the competitor template from cDNA reverse-transcribed from a pool of unfertilized oocytes. DP is identical to the inner TSC2 primer IP1 (5’-ACAGCCGGAGATGTTCTACT-3’) apart from an extended 5 bp region possessing a single base pair mismatch, which when used to amplify cDNA introduces a novel HpaII restriction site (Figure 1B). In order to derive absolute quantitative information by competitive PCR, the principal requirement is that both the target and competitor sequences be amplified with equal efficiencies using identical primer pairs. Therefore, only the TSC2 inner primer pair IP1 and IP2 was used to amplify both the target and the competitor cDNA without affecting the HpaII restriction site previously introduced by the degenerate primer. An aliquot of the resulting cDNA was restriction-digested with HpaII to ensure that the site had been efficiently incorporated into the amplified product (Figure 2). The remaining amplified, uncalt cDNA was visualized on an agarose gel and the cDNA band was cut out and extracted from the gel according to the manufacturer’s instructions using a Stratagene® DNA gel extraction kit. The cleaned product was sequenced, to verify its identity (data not shown), and subsequently quantified using a spectrophotometer. This product was used as the competitor control template in all subsequent quantification experiments.

**Quantitative PCR**

For quantification to be accurate and absolute, data must be obtained during the exponential phase of the PCR. To characterize the kinetics of the reaction, a cycle profile was carried out using cDNA from a pool of reverse-transcribed unfertilized human oocytes. The reaction plateaus at ~38 cycles (data not shown). As a consequence, all subsequent quantitative PCR experiments were run at 32 cycles. To increase the sensitivity of the assay while remaining in the exponential phase of the reaction, a radioactive PCR protocol was followed (Shim et al., 1997). The reactions were set up as described previously with the extra addition of 0.3 μCi [32P]dCTP (10 mCi/ml, Amersham) in each tube. To obtain accurate quantification, the embryonic target and competitor sequence must be present in similar amounts. To achieve this, a concentration profile of competitor template was run alongside single unfertilized oocytes using 32 cycles. Net values for the competitor and the unfertilized eggs were obtained, the data were plotted linearly creating a standard curve and the point at which the oocytes and the competitor reached equivalent concentrations was determined. Figure 3 shows how the TSC2 cDNA from the single oocytes showed comparable amplification to 10.4 fg of competitor cDNA. This quantity of competitor was used in all subsequent competitive PCR analyses.

**Restriction digestion of PCR products**

Distinction between competitor and target (embryonic) TSC2 PCR products was achieved by digesting 50 μl of amplification product with 25 IU of HpaII restriction enzyme and its reaction buffer according to the manufacturer’s specifications (Promega). The reaction was incubated overnight at 37°C.

**Detection of competitor and endogenous TSC2 fragments**

Following restriction digestion, the competitor and target (embryonic) TSC2 PCR products were separated by 10% polyacrylamide gel electrophoresis. Gels were dried for 90 min at 80°C and directly exposed to general-purpose storage phosphor screen (Molecular Dynamics–Amersham Pharmacia Biotech). Products were visualized using the Typhoon 8600 variable mode phosphoimager.

**Quantitative data analysis**

Following application of radioactive PCR and subsequent phosphoimager analysis, quantitative data were obtained using the Image Quant software. Multiple readings from each phosphor screen were generated during the short exposure times in order to ensure that quantification was always calculated.
prior to saturation of the screens. Levels of TSC2 cDNA can be calculated by relating the quantification volume of the embryonic/oocyte band with the competitor band. Results are expressed as a percentage of the competitor cDNA. Since the competitor template is co-amplified with the embryonic cDNA and its initial amount added to the PCR is known (10.4 fg), as stated in Figure 3, we can use this to calculate the amount of TSC2 cDNA for each different sample studied. The amount of 10.4 fg is equivalent to 47,218 molecules of competitor cDNA.

Statistical analysis and interpretation of the data was performed using the InStat version 3.0 software. The statistical test used was the unpaired t-test.

**Results**

**Levels of TSC2 gene expression in fresh human embryos throughout preimplantation development**

Figure 4 is a representative selection of stage- and grade-matched fresh human preimplantation embryos, at different stages of development, used for the detection of TSC2 transcripts. Figure 5A shows results from the application of radioactive PCR and subsequent phosphoimager analysis and demonstrates the relative changes in TSC2 mRNA levels in fresh human embryos throughout preimplantation development. A total number of 46 oocytes and embryos were used for the completion of this experiment on six separate occasions. Figure 5B is a graphic representation of the fluctuation of TSC2 transcripts throughout preimplantation development. At the unfertilized oocyte stage, there is a high level of TSC2 transcripts, equivalent to 9.95 fg of TSC2 competitor cDNA. This high level is maintained at 9.83 fg after a further 24 h of in-vitro culture, on day 2, at the 3–5-cell stages. TSC2 cDNA levels present a 69% significant drop ($P < 0.001$) down to 6.8 fg on day 3 (day 3), when the embryos have reached the 6–8-cell stage. TSC2 cDNA shows an increase up to 10.8 fg at the blastocyst stage on day 6. This increase is statistically significant ($P < 0.01$).

**Levels of TSC2 gene expression in day 2 and day 3 frozen human embryos using protocol 1**

Figure 6A is a phosphoimager visualization describing the relative levels of TSC2 transcripts in day 2 and day 3 frozen human embryos following thawing using protocol 1. A total of 15 frozen embryos was used in three separate experiments. Figure 6D shows the quantitative data obtained from the three repeat experiments. Embryos frozen on day 2 and thawed using protocol 1 show a level of TSC2 cDNA equivalent to 5.03 fg of competitor cDNA ($n = 8$). This represents a 51% drop ($P < 0.001$) in mRNA levels in comparison with fresh embryos at the same stage of development. In embryos frozen on day 3 and thawed using protocol 1, TSC2 transcripts are equivalent to 7.7 fg of competitor cDNA ($n = 7$). There is no significant difference between day 3 thawed mRNA levels and the mRNA levels observed in fresh embryos. Levels of TSC2 mRNA in day 3 embryos thawed using protocol 1 are significantly higher than mRNA levels on day 2 ($P < 0.05$). This rise in transcript levels in thawed embryos is the converse of what is seen in day 2 and day 3 fresh embryos, which show a significant drop ($P < 0.001$) over the same period of in-vitro culture (Figure 5B).
Levels of TSC2 gene expression in day 2 and day 3 frozen human embryos using protocol 2

Figure 6B is a phosphoimager representation of the relative levels of TSC2 transcripts in day 2 and day 3 frozen human embryos following thawing using protocol 2. A total of 12 frozen embryos was used for the completion of this experiment on three separate occasions.

Figure 6D shows the quantitative data obtained from those three repeat experiments. For embryos frozen on day 2 and thawed using protocol 2, the levels of TSC2 transcripts were equivalent to 7.33 fg of competitor cDNA \( (n = 6) \). These day 2 levels represent a 25.4% drop \( (P < 0.01) \) in TSC2 mRNA in comparison with stage- and grade-matched fresh embryos. Embryos frozen on day 3 and thawed using
of the competitor band. Data were obtained by phosphoimager analysis and expressed as a percentage represented by D2, D3 and D6 respectively.

Restriction digestion with HpaII distinguishes between the top oocyte (O) and bottom competitor (C) templates. Days 2, 3 and 6 of in-vitro culture are represented by D2, D3 and D6 respectively. (A) Phosphoimager visualization and quantification of radioactive PCR co-amplification of competitor and TSC2 oocyte templates in oocytes subjected to increasing days of in-vitro culture. Restriction digestion with HpaII distinguishes between the top oocyte (O) and bottom competitor (C) templates. Days 2, 3 and 6 of in-vitro culture are represented by D2, D3 and D6 respectively. (B) Histogram showing relative levels of TSC2 gene expression in cultured unfertilized oocytes. Quantitative data were obtained by phosphoimager analysis and expressed as a percentage of the competitor band.

Protocol 2 possess TSC2 transcripts equivalent to 8 fg of competitor cDNA, showing no significant difference from the levels observed in fresh embryos (n = 6). There was no significant difference between the TSC2 mRNA levels of day 2 and day 3 frozen embryos when protocol 2 was applied.

Levels of TSC2 transcripts in day 2 frozen human embryos thawed using protocol 1 after an extended 24 h culture

To assess the potential long-term effects of temperature changes on mRNA levels in frozen human embryos, day 2 frozen embryos that had been thawed using protocol 1 were cultured for an additional 24 h up to day 3. Figure 6C is a phosphoimager visualization of the results obtained. The experiment was completed on two separate occasions and a total of eight embryos was used. Figure 6D includes the graphic representation of the quantitative data obtained. After 24 h of extended in-vitro culture, day 2 embryos thawed using protocol 1 showed a 30% increase (P < 0.05) in TSC2 transcript levels when compared with day 2 embryos analysed immediately after protocol 1 thawing. These day 3 levels were equivalent to levels of fresh day 3 embryos and embryos frozen on day 3.

Comparison of levels of TSC2 transcripts in day 2 and day 3 frozen embryos thawed using protocols 1 and 2

To assess the impact of different temperature changes during thawing, protocols 1 and 2 were compared. The amount of TSC2 transcripts observed on day 2 frozen embryos thawed by protocol 1 was ~25% less than the equivalent amount observed for day 2 frozen embryos thawed by protocol 2 (P < 0.001). However, there was no significant difference between TSC2 transcript levels in embryos frozen on day 3 and thawed using either protocol 1 or 2.

Stability of TSC2 gene transcripts in aged unfertilized oocytes

To assess the stability of TSC2 transcripts during the period of human preimplantation development, unfertilized oocytes were left in culture and subsequently collected and quantified on day 2, day 3 and day 6. The pattern of TSC2 mRNA observed was compared with the expression of TSC2 seen in cleavage stage embryos over the same period of in-vitro development. A total of 27 oocytes was used from three different treatment cycles. Figure 7A is a phosphoimager representation of the relative levels of TSC2 transcripts in day 2, day 3 and day 6 unfertilized oocytes while Figure 7B is the graphic representation of the results. Unlike the stage-specific profile observed in fresh and frozen cleavage stage embryos, the amount of TSC2 mRNA in unfertilized oocytes remains remarkably constant regardless of the 6 day in-vitro culture. Assuming the oocytes are transcriptionally inactive, the stability of TSC2 transcripts up to day 6 demonstrates the persistence of maternally derived mRNA in the absence of cleavage and/or fertilization.

Discussion

We have used a sensitive competitive RT–PCR to characterize the expression of the tuberous sclerosis TSC2 gene throughout human preimplantation development. There is a high level of TSC2 mRNA in the unfertilized oocyte, equivalent to 9.95 fg of competitor cDNA, which is maintained on day 2 and subsequently falls significantly to 6.8 fg (P < 0.001) on day 3. The timing of this loss coincides with the timing of global activation of the embryonic genome in the human, which occurs at the 4–8-cell stage (Braude et al., 1988; Tesarik et al., 1988). Although the nature of the lost TSC2 transcripts has not been established, it is possible they are of maternal origin. It is still unclear what is the specific signal that causes the degradation of the oocyte-derived maternal transcripts. However, no loss in mRNA was observed in the aged unfertilized oocytes, leading therefore to the conclusion that the degradation of maternally derived transcripts is likely to be a direct consequence of the activation of the embryonic genome.

The pattern of gene expression observed in this study is not uncommon as it resembles the pattern reported for the mouse β-actin gene (Taylor and Piko, 1990), the housekeeping rat insulinoma gene (rig gene), as well as the zona pellucida gene ZP3 (Roller et al., 1989; Taylor and Piko, 1991). A drop in mRNA levels following activation of the embryonic genome occurs in a number of mammalian species (Telford et al., 1990) and appears to be characteristic of normal preimplantation development, probably reflecting the successful switch from maternal to embryonic gene expression. Although this pattern of specific gene expression has been observed in other mammalian species, it contrasts with the pattern of human hypoxanthine phosphoribosyl transferase (HPRT) gene expression (Figure 5B), one of the few genes to have been quantitatively characterized throughout human preimplantation embryo development (Taylor et al., 2001). Unlike TSC2, the levels of HPRT cDNA dropped markedly between the unfertilized oocyte stage (7.7 fg) and the 4-cell stage (1.2 fg) and remained at this low level throughout the remainder of preimplantation development, even at the expanded blastocyst stage. The sharp contrast in the pattern of gene expression demonstrated between these genes may reflect their specific roles in...
preimplantation development. Although TSC2 is a cell cycle regulator (Inoki et al., 2002; Potter et al., 2002) and homozygous mutations are embryonic lethals in mice and rats (Kobayashi et al., 1999; Rennebeck et al., 1998), its role in mammalian preimplantation development is still unknown. This makes it difficult to explain why, unlike HPRT, the high levels of TSC2 are quickly restored following activation of the embryonic genome.

Following transcription, mRNA is more abundant than the two copies of the gene present in a single cell. RT–PCR therefore presents an attractive alternative to PCR-based diagnosis of single gene defects for PGD. This approach has already been used for the diagnosis of the autosomal disorder Marfan syndrome (Eldadah et al., 1995). However, the use of RT–PCR for PGD requires careful evaluation, as the oocyte-derived transcripts must be sufficiently degraded prior to the embryo biopsy. This study shows a drop in TSC2 mRNA between day 2 and day 3, probably reflecting the degradation of the oocyte-derived transcripts generally associated with activation of the embryonic genome (Telford et al., 1990). The increase in the level of TSC2 transcripts occurring between day 3 and day 6 also suggests that the embryonic genome is active at this stage of development and hence RT–PCR may be considered for PGD for tuberous sclerosis. However, before this approach can be clinically applied it is necessary to confirm when the oocyte-derived transcripts are fully degraded using a method that allows the oocyte-derived and embryonic transcripts to be clearly distinguished.

To assess the effects of cryopreservation on specific gene expression, we applied quantitative RT–PCR to examine the levels of the TSC2 gene transcripts in thawed human embryos, which had been frozen on either day 2 or day 3. For this purpose, two different thawing protocols were used. Embryos thawed using protocol 1 were removed from liquid nitrogen (−196°C) and subsequently warmed to 37°C. When applying protocol 2, the embryos were subjected to a less rapid temperature change as they were transferred from liquid nitrogen and warmed to room temperature (~20°C). Protocol 1 produced a 50% drop (P < 0.001) in TSC2 transcript levels in embryos frozen on day 2 when compared with fresh day 2 embryos. However, protocol 2 produced a smaller 25% drop (P < 0.01) in TSC2 transcript levels when compared with the fresh embryos. For both thawing protocols, embryos frozen on day 3 showed no significant effects after thawing, as TSC2 mRNA levels were equivalent to the transcript levels found in the fresh day 3 embryos. These findings suggest that TSC2 mRNA are more vulnerable to temperature change on day 2 than on day 3. It is possible that the effects of cryopreservation observed for TSC2 are a more general occurrence and hence it may be beneficial to perform cleavage stage embryo freezing procedures on day 3 rather than on day 2.

The TSC2 mRNA levels observed in the fresh embryos fall significantly between day 2 and day 3. Thawing embryos using protocol 1 produced the converse pattern of gene expression, as the TSC2 mRNA levels rose between day 2 and day 3. To determine the length of time during which this change in expression pattern was apparent, embryos thawed on day 2 were left in culture for an extended 24 h. Levels of TSC2 transcripts in the embryos cultured to day 3 were significantly higher (P < 0.05) than the levels observed in the non-cultured counterparts and were comparable to the levels seen in fresh day 3 embryos. This demonstrates the embryo’s ability to actively transcribe TSC2 mRNA in order to compensate for the loss seen on day 2, confirming that the embryonic TSC2 gene is being expressed on day 3.

In this study, we were unable to distinguish between maternal and embryonic transcripts and therefore cannot determine whether there was preferential degradation of maternally or embryonically derived TSC2 transcripts. It is clear that the embryo’s ability to restore the high levels of TSC2 transcripts can only be achieved through active transcription, and therefore on day 3, a significant proportion of the TSC2 transcripts must be derived from expression of the embryonic genome. If the embryonic TSC2 gene only becomes active late on day 2 or on day 3 then we can assume that the drop in TSC2 levels on day 2 is most likely attributable to the loss of maternal rather than embryonic transcripts. If indeed maternal mRNA is more vulnerable to temperature change then freezing at the pronucleate (PN) stage of preimplantation development, when the embryonic genome is inactive and development is supported solely by maternal transcripts, would be less successful than freezing at the cleavage stages. However, it is unclear if this is the case, as studies on cryopreserved embryo survival, morphology, pregnancy and delivery rates give conflicting evidence (Kattera et al., 1999; Senn et al., 2000; Tao et al., 2001). Preferential degradation of maternal TSC2 transcripts seems unlikely as our analysis of aged unfertilized oocytes demonstrates that these transcripts are in fact remarkably stable, assuming that the oocytes are transcriptionally inactive. However, in a number of mammalian species the activation of the embryonic genome does coincide with the degradation of maternal transcripts (Telford et al., 1990). It can therefore be argued that following activation of the embryonic genome, maternal transcripts become destabilized, increasing the probability that external factors such as cryopreservation cause their premature degradation.

It is interesting to see that a relatively small temperature difference of ~17°C between protocols 1 and 2 could account for such a significant drop (25%) in the levels of TSC2 transcripts observed on day 2 frozen embryos (P < 0.001). However, we have demonstrated that over a period of 24 h the embryos possess the ability to restore the TSC2 levels seen in the fresh embryos. This study used only intact thawed embryos and it has been reported that these embryos have the same implantation potential as stage-matched fresh embryos (Burns et al., 1999; Edgar et al., 2000). Despite human embryo cryopreservation being widely available in IVF programmes for nearly two decades, there are increasing concerns regarding the effects of assisted reproductive techniques on embryonic reprogramming which may affect health in adult life (Duloujst et al., 1995; Tamashiro et al., 2002). We have demonstrated that the expression of the TSC2 gene in day 2 intact thawed embryos is different from day 2 fresh embryos. Hence regardless of morphology, cryopreservation and subsequent thawing does appear to affect the embryo’s normal pattern of gene expression. The effects of cryopreservation on specific mRNA levels in human preimplantation embryos can become more or less profound depending on the magnitude of the temperature changes applied. Although embryos do demonstrate the ability to cope with freeze–thaw procedures, the possibility of long-term effects from the observed drop in mRNA levels cannot be ignored. As the TSC2 mRNA levels in embryos frozen on day 3 were relatively unaffected, this supports our previous argument that it may prove wise to freeze embryos on day 3 rather than day 2.

This is the first study to examine the pattern of gene expression in frozen human preimplantation embryos and it clearly demonstrates that morphology alone is a limited indicator of the effect of cryopreservation on an embryo. It is therefore clear, for research and clinical considerations, that even intact frozen–thawed embryos cannot be assumed to be equivalent to fresh embryos. From this current research we can see that day 2 embryos are more vulnerable to temperature change than day 3, which may be a consequence of the activation of the embryonic genome. To our knowledge, there has never been a study comparing the cryopreservation outcome between day 2 and day 3 frozen embryos and therefore we are unable to comment on the clinical outcome of our observations.
This study demonstrates that the pattern of TSC2 gene expression observed in normally developing embryos is altered following cryopreservation and subsequent thawing. Although it is possible that cryopreservation has a different effect on other gene transcripts, this work emphasizes the need for more studies to be conducted on the impact of established and future assisted reproductive techniques such as cryopreservation, stem cell and nuclear transfer technology prior to making assumptions of their harmless.

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References


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