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ORIGINAL RESEARCH

Embryo collection induces transient activation of XBP1 arm of the ER stress response while embryo vitrification does not

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ABSTRACT: Embryo cryopreservation has become a standard procedure in the practice of assisted reproduction. While routinely performed in IVF labs, the effects of embryo vitrification on the molecular mechanisms governing preimplantation development remain largely unknown. The endoplasmic reticulum stress (ER stress) response is an evolutionary conserved mechanism that cells employ to manage ER stress. ER stress can be defined as an imbalance between protein synthesis and secretion within the ER. The primary focus of this study was to investigate whether standard embryo manipulations, including embryo collection, culture and vitrification, result in activation of the ER stress pathway *in vitro* and to determine whether the embryo utilizes the unfolded protein response as an adaptive response. Our results indicate that the major ER stress pathway constituents are present at all stages of preimplantation development and that the activation of ER stress pathways can be induced at the 8-cell, morula and blastocyst stages. Additionally, we have demonstrated that the IRE1α arm of the ER Stress pathway is activated in freshly collected embryos but contrastingly, this ER Stress arm is not activated following embryo vitrification. It is important to understand the possible stresses that Assisted Reproductive Technologies place on the embryo and the mechanisms the embryo employs to adapt to these stresses. This study indicates that among the adaptive pathways available, cultured mammalian embryos can employ the ER stress pathway. Assisted reproduction techniques should be aware that their activities may induce the ER stress pathway in their patients' early embryos.

Key words: preimplantation development / assisted reproductive technologies / embryo culture / ER stress / blastocyst

Introduction

It is clear that mammalian gametes and early embryos are vulnerable to a variety of cellular stresses when placed into an *in vitro* environment (for review see Lane and Gardner, 2005). These stresses include shear stress during handling, temperature, culture media, altered pH, aberrant gas phases and even visible light (Leese, 1995; Gardner *et al.*, 2000; Lane and Gardner, 2000; Thompson *et al.*, 2002; Gardner and Lane, 2003; Fleming *et al.*, 2004; Gardner and Lane, 2005; Fong *et al.*, 2007; Bell *et al.*, 2009). The consequences of these stresses on gamete and embryo health manifest themselves in a number of ways, including altered gene expression, altered epigenetic mechanisms, altered metabolism, apoptosis induction and overall reduced development and embryo viability compared with their *in vivo* counterparts (Leese, 1995; Gardner *et al.*, 2000; Lane and Gardner, 2000; Thompson *et al.*, 2002; Gardner and Lane, 2003; Fleming *et al.*, 2004; Gardner and Lane, 2005; Fong *et al.*, 2007; Bell *et al.*, 2009). The early embryo is however not completely defenseless in the presence of these stresses and is able to activate adaptive mechanisms in an attempt to counter the effects of these stresses on its development. If this were not so, the success that *in vitro* culture and assisted reproductive technologies exhibit would surely have not occurred. It is important to understand how cellular stress affects early mammalian development and what mechanisms the embryo can employ to defend itself against these stresses. Our study explores the presence, activation and function of an important stress response mechanism during preimplantation development. Our findings indicate that the major components of the ER stress pathways are present

during preimplantation development, and they are activated when induced by artificial stress and embryo collection but not by vitrification.

The UPR (unfolded protein response) is an evolutionary conserved mechanism that cells employ to manage endoplasmic reticulum (ER) stress (Malhotra and Kaufman, 2007; Ni and Lee, 2007; Yoshida, 2007; Schroder, 2008; Vembar and Brodsky, 2008). ER stress can be defined as an imbalance between protein synthesis and secretion within the ER (Malhotra and Kaufman, 2007; Ni and Lee, 2007; Yoshida, 2007; Schroder, 2008; Vembar and Brodsky, 2008). The ER is the cellular organelle responsible for the production and maturation of secretory and membrane proteins. Nascent, unfolded polypeptide chains enter the ER lumen where they are folded and undergo post-translational modification by ER chaperone proteins (Malhotra and Kaufman, 2007; Ni and Lee, 2007; Yoshida, 2007; Schroder, 2008; Vembar and Brodsky, 2008). Properly folded proteins are exported to the Golgi apparatus, while malfolded or unfolded proteins are exported to the cytoplasm for degradation (Malhotra and Kaufman, 2007; Ni and Lee, 2007; Yoshida, 2007; Schroder, 2008; Vembar and Brodsky, 2008). The flux of proteins moving into the ER varies in response to a wide range of environmental and physiological conditions (Malhotra and Kaufman, 2007; Ni and Lee, 2007; Yoshida, 2007; Schroder, 2008; Vembar and Brodsky, 2008). If the amount of secretory proteins entering the ER exceeds the capacity of the ER machinery, unfolded proteins begin to accumulate in the ER, forming protein aggregates (Malhotra and Kaufman, 2007; Ni and Lee, 2007; Yoshida, 2007; Schroder, 2008; Vembar and Brodsky, 2008). These aggregates are toxic to all cell types, and this imbalance is referred to as ER stress (Malhotra and Kaufman, 2007; Ni and Lee, 2007; Yoshida, 2007; Schroder, 2008; Vembar and Brodsky, 2008).

ER stress can be induced by a variety of factors, including oxidative stress, hypoxia, altered protein glycosylation and abnormal Ca^{2+} regulation (Malhotra and Kaufman, 2007; Ni and Lee, 2007; Yoshida, 2007; Schroder, 2008; Vembar and Brodsky, 2008). In conditions of ER stress, the UPR is activated and several mechanisms are triggered. These include: (i) attenuation of protein synthesis, to decrease the protein load that enters the ER, (ii) increased expression of ER chaperone proteins, which increase the protein folding capacity of the ER and (iii) induction of ER-associated degradation, to degrade excess or defective protein (Malhotra and Kaufman, 2007; Ni and Lee, 2007; Yoshida, 2007; Schroder, 2008; Vembar and Brodsky, 2008). If these mechanisms are not able to sufficiently alleviate cellular stress, apoptotic cell death is triggered to protect surrounding cells within the tissue. The three major transducers of the UPR are PERK, IRE1 and ATF6 (Malhotra and Kaufman, 2007; Ni and Lee, 2007; Yoshida, 2007; Schroder, 2008; Vembar and Brodsky, 2008).

While the functional role of the ER stress pathways has not been directly examined during preimplantation development, its influence on embryonic development is implicated by studies that have knocked out UPR-specific genes (Harding *et al.*, 2000a, b; Luo *et al.*, 2006). Both *Grp78*$^{-/-}$ and *Ppp1r15a/Ppp1r15b*$^{-/-}$ mice are unable to survive beyond preimplantation development, suggesting a critical role for these ER chaperone proteins during this developmental period (Luo *et al.*, 2006; Harding *et al.*, 2009). Additionally, UPR pathways have been examined in extra-embryonic tissues. Studies have demonstrated that compared with normal controls, placentas from cases of intrauterine growth restriction and pre-eclampsia display elevated levels of UPR constituents, phospho-eIF2α, GRP94 and CHOP, indicating that ER stress response is inducible in diseased placentas (Burton *et al.*, 2009). Additionally, studies knocking out IRE1α demonstrate that IRE1α null mice are unable to produce functional placentas, resulting in an embryonic lethality. This indicates an important role of IRE1α in extra-embryonic tissues (Iwawaki *et al.*, 2009).

It is well established that the embryo synthesizes and secretes a wide range of growth factors to enhance development and promote survival (Watson *et al.*, 1992; Watson *et al.*, 1999; Singh *et al.*, 2011). Resultantly, many secretory proteins are trafficked through the ER during preimplantation development. Therefore, it is reasonable to propose that ER stress may occur in the embryo *in vivo*. It is known that ER stress occurs under a variety of environmental and physiological stressors (Malhotra and Kaufman, 2007; Ni and Lee, 2007; Yoshida, 2007; Schroder, 2008; Vembar and Brodsky, 2008). Therefore, it is very likely that ER stress is induced during preimplantation development *in vitro* as the application of assisted reproduction techniques (ARTs) requires the exposure of gametes and early embryos to non-optimized *in vitro* conditions (Leese *et al.*, 2008).

While routinely performed in IVF labs, the effects of embryo vitrification on the molecular mechanisms governing preimplantation development remain largely unknown. Cells have evolved the necessary mechanisms required to adapt to temperature changes in their surrounding environment. Such cellular responses include the inhibition of protein transcription and translation, changes in membrane permeability to ions and an overall slower metabolic rate (Sonna *et al.*, 2002). As the process of vitrification exposes the embryo to extremely low temperatures very quickly, it is plausible that the embryo would trigger stress response pathways to manage this shock. One potential pathway is the UPR.

The present study was conducted to directly investigate whether the various arms of the ER stress pathway are present and inducible during mouse preimplantation embryo development. Due to the widespread use of ART procedures, an increasing number of studies have been focused on understanding any effects ART techniques may have on the embryo. As a result, the primary focus of this study was to examine whether standard embryo manipulations including embryo collection, culture and vitrification, result in activation of the ER stress pathway *in vitro* and to determine whether the embryo utilizes the UPR as an adaptive response. Our results indicate that the major UPR constituents are present at all stages of mouse preimplantation development and that the activation of UPR pathways can be induced at the 8-cell, morula and blastocyst stages. Additionally, we have demonstrated that the IRE1α arm of the UPR is activated in response to embryo collection techniques but contrastingly, this arm of the UPR is not activated in response to embryo vitrification.

Materials and Methods

Mouse embryo collection, culture and manipulations

MF-1 females (4–6 weeks old, Harlan Sprague Dawley, Indianapolis, IN, USA) were superovulated by injection of 7–10 IU pregnant mares serum gonadotrophin (PMSG, Intervet, Whitby, ON, Canada). Forty-six to 48 h later, an injection of 7–10 IU human chorionic gonadotrophin

(hCG, Intervet) was administered prior to mating with a CD-1 male (Charles River Laboratories, St. Constant, QC, Canada). Successful matings were determined by the presence of a vaginal sperm plug the following morning. Preimplantation mouse embryos were collected at 18 (1-cell), 48 (2-cell), 55 (4-cell), 68 (8-cell), 80 (morulae) and 92 h (blastocysts) post-hCG. Female reproductive tracts were dissected and embryos were flushed from oviducts and uteri using M2 flushing media (Sigma-Aldrich, Oakville, ON, Canada). One-cell embryos were incubated in hyaluronidase (Sigma, St Louis, MO, USA) to remove cumulus cells, prior to further manipulations. Flushed embryos were washed three times in potassium simplex optimized medium media, containing amino acids (KSOMaa, Millipore, Burlington, MA, USA). Pools of embryos were either: (i) frozen and stored at −80°C to be used for RNA extraction, (ii) fixed in 2% paraformaldehyde (PFA) for immunostaining or (iii) cultured in either KSOMaa alone, or KSOMaa containing tunicamycin (TM). All embryos were cultured under mineral oil with an embryo-KSOMaa ratio of 1 embryo/μl in a 5% CO_2 in air atmosphere at 37°C (Bell and Watson, 2009; Bell *et al.*, 2009).

Embryo culture in TM

TM is a mixture of antibiotics that blocks glycoprotein synthesis in many cells and is commonly used to induce the UPR ER stress response for experimental purposes. TM (Sigma) was dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 0.2 mg/ml. Three different culture media were prepared: (i) KSOMaa containing DMSO at a 0.25% concentration (ii) KSOMaa containing TM at a concentration of 0.1 μg/ml and (iii) KSOMaa containing TM at a concentration of 0.5 μg/ml. Media were prepared freshly each time, at a volume of 1 ml. Pools of 20 embryos at either the 8-cell, morula or blastocyst stage were cultured in 20 μl drops of each of these three media.

RNA extraction, reverse transcription and polymerase chain reaction

Total RNA was extracted from pools of 5–20 embryos (Bell and Watson, 2009; Bell *et al.*, 2009). Prior to RNA extraction, 0.025 pg/embryo of exogenous *Luciferase* Control RNA (Promega Corporation, Madison, WI, USA) was added to samples that were intended for quantitative real-time PCR analysis. RNA extraction was carried out using the PicoPure™ RNA Isolation Kit (Arcturus Bioscience Inc., Mountain View, CA, USA), following the manufacturer's instructions. Total RNA was reverse-transcribed (RT) using Oligo(dT) Primers (Invitrogen Life Technologies, Burlington, ON, Canada), RNaseOUT™ (Invitrogen) and Sensiscript RT (Qiagen, Mississauga, ON, Canada), following the manufacturer's suggested protocol. Samples were incubated at 37°C for 90 min. Following the RT reaction, samples were subjected to PCR amplification of H2A histone family member Z (*H2afz*) to assess the efficiency of the RNA extraction and RT reaction (Bell and Watson, 2009; Bell *et al.*, 2009). The total amount of cDNA used in each PCR reaction was equal to one embryo equivalent, while the total PCR reaction volume was 50 μl. PCR reactions were carried out using 1.0 U Platinum® *Taq* DNA Polymerase (Invitrogen, Burlington, ON, Canada), 1X PCR Reaction Buffer (Invitrogen), 1.5 mM $MgCl_2$ (Invitrogen), 0.2 mM dNTP mixture (Sigma-Aldrich) and 1.0 μM of each mouse gene-specific PCR primer pair. Primer pairs were designed and synthesized (Invitrogen) for *Bip*, *Atf4*, *Chop*, *Ire1α*, *Xbp1*, *Ask1*, *Atf6*, *Gadd34* and *Perk* based on available mouse nucleotide sequences obtained from the NCBI Entrez nucleotide database (Bell and Watson, 2009; Bell *et al.*, 2009). All gene-specific primer pairs are listed in Table I.

Amplification reactions were performed using either a Techne Touchgene Gradient DNA thermal cycler (Techne, Burlington, NJ, USA) or an Applied Biosystems Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA). The reaction was run at 94°C for 2 min, followed by 42–45 cycles consisting of denaturation at 94°C for 30 s, reannealing primers to target sequence at 59–62°C (depending on primer set) for 30 s, and primer extensions at 72°C for 1 min (Bell and Watson, 2009; Bell *et al.*, 2009). The reaction concluded with a final extension at 72°C for 10 min. PCR products were run out on a 2% agarose gel containing 1.0 μg/ml ethidium bromide (Invitrogen) in 1X TAE buffer (40 mM Tris, 40 mM acetate, 1 mM EDTA). All PCR products were sequenced to confirm their identity (DNA Sequencing Facility, Robarts Research Institute, London, ON, Canada). In all reactions, a positive (pancreatic

Table I PCR primer sequences for amplification of UPR constituents.

Gene product (official symbol)	UPR gene alias	Primer	Primer sequence	Entrez gene ID	Size (bp)
Xbp1	*Xbp1*	5′ 3′	GAGCAGCAAGTGGTGGATTT CTCTGGGGAAGGACATTTGA	22 433	380
Hspa5	*Bip*	5′ 3′	TCTGGTGATCAGGATACAGGTG TTCAGCTGTCACTCGGAGAATA	14 828	327
Ern1	*Ire1α*	5′ 3′	TGCAAAGGAAACAGCATCAG TCCACAGCATTGCTAACGAG	78 943	328
Map3k5	*Ask1*	5′ 3′	GCTGAGAGAAAATGGAGCTGAT TTGCGACACGAGTAAAGAAAGA	26 408	306
Atf6	*Atf6*	5′ 3′	GAGCCGCACAGCTACCTAAC GACAGCTCTTCGCTTTGGAC	226 641	356
Atf4	*Atf4*	5′ 3′	TCGATGCTCTGTTTCGAATG AAGCAGCAGAGTCAGGCTTC	11 911	312
Ddit3	*Chop*	5′ 3′	ACAGAGGTCACACGCACATC CTTCCGGAGAGACAGACAGG	13 198	336
Eif2ak3	*Perk*	5′ 3′	GGGAAAACGGTTCTGAGACA GCTGACCAGCTAGTCTTGGG	13 666	332
Ppp1r15a	*Gadd34*	5′ 3′	CCATAGCTCCGGGATACAAA ACCGGTCTGAGCTGTGTCTT	17 872	377

cDNA) and negative control (dH_2O) sample was included. When examining *Xbp1* splicing, an additional positive control was included (cDNA from 8-cell embryos cultured in TM for 30 h).

Quantitative real-time PCR analysis

Real-time PCR reactions were performed using the ABI PRISM® 7900HT sequence detection system (Applied Biosystems) and TaqMan® Gene Expressions Assays (Applied Biosystems) (Bell and Watson, 2009; Bell *et al.*, 2009). Pre-designed and pre-optimized commercially available TaqMan® Gene Expression Assays for *Grp94* (Assay ID: Mm01253170_m1) and *Dnajc3* (Assay ID: Mm01226332_m1) were used. The custom TaqMan® primer and probe set for *Luciferase* were designed using the Assays-By-Design file builder program from Applied Biosystems (Fong *et al.*, 2007). The PCR reaction mixture (25 μl) contained 12.5 μl of TaqMan® Universal PCR Master Mix (2X concentration, containing AmpliTaq Gold® DNA Polymerase, AmpErase® UNG, dNTPs with dUTP, Passive Reference 1, and optimized buffer components; Applied Biosystems), 1.25 μl of the appropriate 20X TaqMan® Gene Expression Assay (Applied Biosystems), 1.25 μl RNase free water (UltraPure™ Distilled Water, Invitrogen) and 10 μl of embryo cDNA (at a diluted concentration of 0.1 embryo/μl) corresponding to one embryo equivalent per one reaction. Thermal cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and a combined annealing extension stage, 60°C for 1 min. Each reaction was carried out in triplicate (Bell and Watson, 2009; Bell *et al.*, 2009). Relative quantification of gene expression levels was carried out using the comparative C_T (threshold cycle) method. Transcript levels were normalized to the external control, *Luciferase*. Triplicates were averaged to determine a C_T value for each treatment (Fong *et al.*, 2007; Bell and Watson, 2009; Bell *et al.*, 2009). To determine the ΔC_T level, the *Luciferase* C_T value was subtracted from the corresponding target gene C_T value for each sample. The $\Delta\Delta C_T$ value was calculated by subtracting each sample from the control sample (either 1-cell or KSOM control sample). Finally, relative mRNA levels of each target gene were determined by using the formula $2^{-\Delta\Delta CT}$.

TUNEL assay

DNA strand breaks were detected by TUNEL staining, using the 'In Situ Cell Death Detection Kit, Fluorescein' (Roche, Laval, QC, Canada) (Bell *et al.*, 2009). Pools of treated embryos were fixed using 2% paraformaldehyde in a PBS solution for 20 min at room temperature. Fixed embryos were incubated in 100 μl of freshly prepared permeabilization solution (0.1% Triton X-100 in a 0.1% sodium citrate solution) for 1 h at room temperature. Prior to this step, a group of embryos were set aside to act as the first negative control group. This group (negative control #1) was not incubated in permeabilization solution, and was incubated in PBS instead. Following permeabilization, embryos were placed in a humidified chamber and were incubated at 4°C for 2 min, followed by two washes in PBS. At this point, a second group of embryos were removed to act as a positive control. This positive control group was treated with 100 μl DNase treatment (10 μl DNase in 990 μl 50 mM Tris–HCl at pH 7.5, containing 1 mg/ml BSA) for 10 min at room temperature, followed by one wash in PBS. A DNase treatment was carried out to induce DNA strand breaks to allow visualization of maximal DNA fragmentation. A second negative control group (negative control #2) was set aside at this point. Both negative control group #1 and negative control group #2 were incubated in 50 μl of TUNEL label solution alone (nucleotide mixture in reaction buffer). TUNEL reaction mixture was prepared following the manufacturer's instructions. The positive control and all treatment groups were incubated in 50 μl of TUNEL reaction mixture (enzyme solution + label solution; terminal deoxynucleotidyl transferase from calf thymus, recombinant in *Escherichia coli*, in storage buffer + nucleotide mixture in reaction buffer). All groups were incubated in a humidified chamber for 1 h at 37°C. Embryos were subsequently incubated in 200 μl PBS containing 0.2 μl DAPI (4′,6-diamidino-2-phenylindole dihydrochloride; Sigma-Aldrich; diluted 1:1000 from a 1 mg/ml stock solution) for 30 min at 37°C, to stain nuclei. Finally, embryos were washed two times in 200 μl PBS for 15 min each. Embryos were mounted onto glass slides, under glass cover slips, in a drop of FluoroGuard™ anti-fade reagent (Bio-Rad Laboratories, Mississauga, ON, Canada). Cover slips were sealed with nail polish (to prevent leaking), and slides were stored at 4°C for future observation (Bell and Watson, 2009; Bell *et al.*, 2009).

Embryo vitrification

Blastocysts were vitrified using the McGill Cryoleaf™ (MediCult, Innovation with Care; Meditech IST Canada Inc., Montreal, QC, Canada) (Chian *et al.*, 2009). Equilibration medium (EM), vitrification medium (VM), thawing medium (TM), diluent medium-1 (DM-1) and diluent medium-2 (DM-2) were all prepared and filtered following the manufacturer's suggestions (Chian *et al.*, 2009). At least one hour prior to embryo vitrification, 20 μl drops of EM and VM were incubated at room temperature under mineral oil. One drop of each medium was prepared for each group of embryos. Blastocysts were flushed from the uterine tract and a pool of 10 blastocysts was collected and stored at −80°C for later analysis. Pools of eight fresh flushed blastocysts were cultured in KSOMaa for 2 h. After 2 h in culture, embryos were subjected to the vitrification protocol. Each embryo pool was incubated in a drop of EM for 3–5 min at room temperature. After this incubation period, embryos were transferred to a VM drop for 30–45 s. Embryos were loaded onto the Cryoleaf™ using a glass pipette (Chian *et al.*, 2009). The loaded Cryoleaf™ was immediately submerged in liquid nitrogen. The time from when embryos were transferred into VM until Cryoleaf™ submersion in liquid nitrogen did not exceed 1 min. Once submerged in liquid nitrogen, the Cryoleaf™ was capped and stored in a liquid nitrogen tank for up to one month (Chian *et al.*, 2009).

At least 30 min prior to embryo thawing, all thawing media drops were prepared. For each Cryoleaf™, a 250 μl drop of TM was prepared and pre-warmed at 37°C (Chian *et al.*, 2009). Three 50 μl KSOMaa drops were also pre-warmed at 37°C. Twenty microliter drops of DM-1 and DM-2 were prepared for each pool of embryos, and were incubated at room temperature. All drops were kept under mineral oil with the exception of TM. Each Cryoleaf™ was removed from the liquid nitrogen tank and immediately submerged in a TM drop for 1 min. Embryos were then transferred to DM-1 for 3 min, then to DM-2 for 3 min. Embryos were finally washed 2x in KSOMaa for 3 min. Washed embryos were transferred to KSOMaa culture drops and incubated at 37°C, under mineral oil in a 5% CO_2 in air atmosphere (Chian *et al.*, 2009).

Statistical analysis

All data were statistically analyzed using the SigmaStat® 3.5 software package. To analyze the real-time PCR data obtained from embryos cultured in TM, the data were subjected to a one-way analysis of variance (ANOVA), and was followed by Dunn's method test (used to compare multiple treatment groups to a control group). A one-way ANOVA was also carried out for the TUNEL data. For all methods of analysis, a $P < 0.05$ was considered statistically significant.

Results

Detection of ER stress pathway constituents throughout preimplantation development

The detection of ER stress response-associated genes was determined throughout preimplantation development. Reverse transcription and polymerase chain reaction (RT–PCR) methods resulted in the detection of mRNA transcripts encoding *Perk*, *Ask1*, *Atf 4*, *Atf6*, *Bip* (*Grp78*), *Chop*, *Gadd34*, and *Ire1α*, at all stages of mouse preimplantation development (1-cell to blastocyst stage, $n = 3$; Fig. 1A–H). While *Xbp1* was detected at all stages of preimplantation development, its splicing pattern (indicative of IRE1α pathway activation) varied between replicates (Fig. 1I–K). To confirm the identity of each gene, purified PCR products were sequenced and analyzed using BLAST®. Products amplified from both *Atf4* and *Xbp1* primers showed 100% identity with their GenBank® mouse nucleotide sequence, while products amplified from primers *Atf6*, *Perk*, *Chop* and *Ask1* each showed 99% identity. Product amplified from *Bip* primers showed 97% identity with its GenBank® mouse nucleotide sequence, while products amplified from *Ire1α* and *Gadd34* primers showed

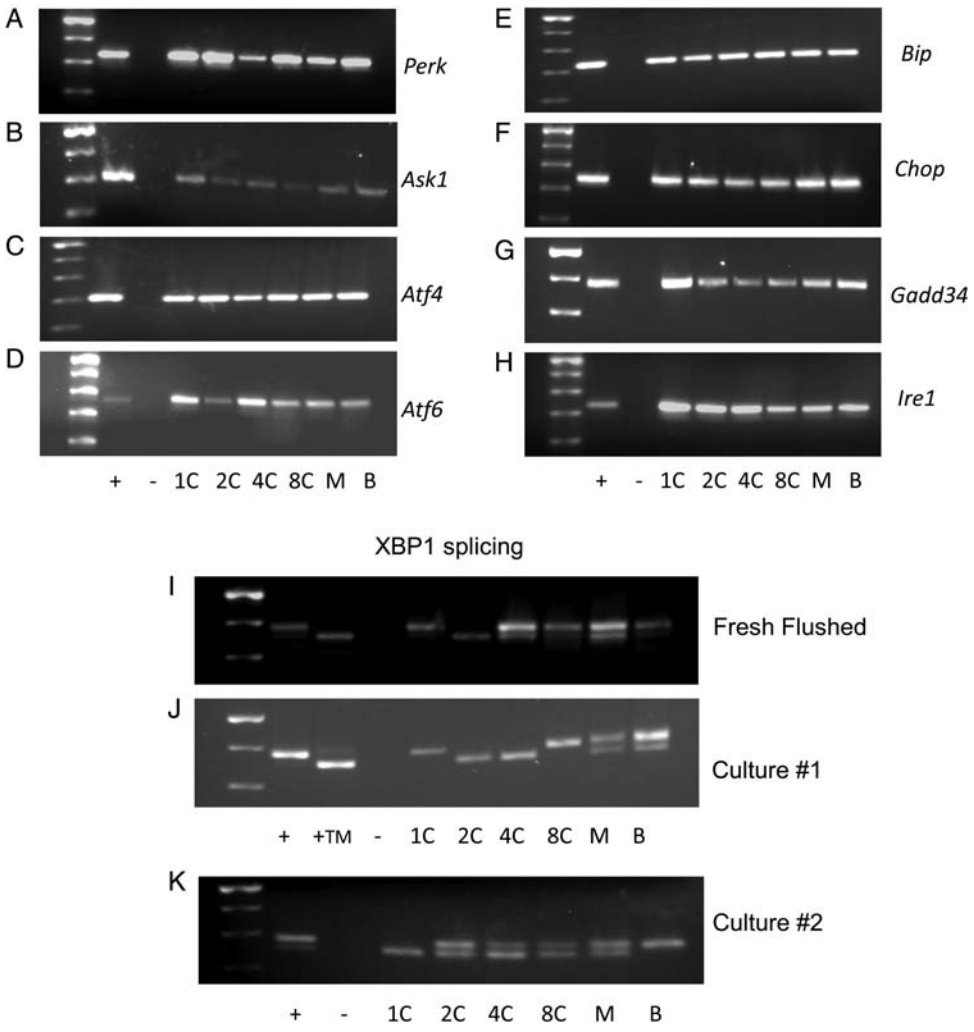


Figure 1 (**A**–**H**) Detection of mRNA transcripts encoding constituents of the ER stress pathway during mouse preimplantation development. Expression of *Perk*, *Ask1*, *Atf4*, *Atf6*, *Bip*, *Chop*, *Gadd34* and *Ire1* were detected by RT–PCR from one embryo equivalent at each stage of preimplantation development (1C, 1-cell; 2C, 2-cell; 4C, 4-cell; 8C, 8-cell; M, morula; B, blastocyst). A 100 bp ladder was used. Pancreatic tissue was used as a positive control (+) and a no cDNA negative control (−) was included. Representative of three independent replicates. (**I**–**K**) Detection of *Xbp1* mRNA throughout mouse preimplantation development. RT–PCR products encoding *Xbp1* were detected in cDNA from one embryo equivalent at each stage of preimplantation development (1C, 1-cell; 2C, 2-cell; 4C, 4-cell; 8C, 8-cell; M, morula; B, blastocyst). *Xbp1* splicing was examined in both fresh flushed generated embryo series' (I) and cultured embryo series' (J and K). A 100 bp ladder was used. Pancreatic tissue was used as a positive control (+) for unspliced *Xbp1* while TM-treated 8-cell embryos were used as a positive control (+TM) for spliced *Xbp1*. A no cDNA negative control (−) was included.

98 and 95% identity with their GenBank® mouse nucleotide sequences, respectively.

Effect of TM treatment on *Xbp1* splicing in embryos

To examine whether the IRE1 arm of the UPR can be induced in the preimplantation embryo, *Xbp1* splicing was examined in embryos treated with TM. *Xbp1* splicing was observed in 8-cell embryos cultured for 10 and for 30 h in KSOMaa, 0.1 μg/ml TM and 0.5 μg/ml TM (Fig. 2A and B). Both spliced and unspliced *Xbp1* was detected in the KSOMaa control. A greater proportion of spliced *Xbp1* was observed in embryos cultured in either 0.1 μg/ml TM or 0.5 μg/ml TM. *Xbp1* splicing was also examined in morulae cultured for 12 h in the presence of TM (Fig. 2C). Morulae cultured in KSOMaa displayed both unspliced and spliced forms of *Xbp1* (Fig. 2C). However, the unspliced band was more apparent. Conversely, while morulae cultured in the presence of either 0.1 μg/ml TM or 0.5 μg/ml TM also displayed both spliced and unspliced levels of *Xbp1*, the spliced band was more apparent in these treatment groups (Fig. 2C). The *Xbp1* splicing pattern differed for blastocysts cultured for 4 h in TM (Fig. 2D). While *Xbp1* splicing was observed in blastocysts cultured in 0.1 μg/ml TM and 0.5 μg/ml TM, no splicing was observed in the KSOMaa control (Fig. 2D and E). Furthermore, 0.1 μg/ml TM treatment for 4 h did not result the in detectable *Xbp1* splicing, whereas 0.5 μg/ml TM treatment clearly did (Fig. 2D). Both TM concentrations resulted in detection of *Xbp1* splicing in blastocyst samples treated for 10 h (Fig. 2E).

Up-regulation of ER chaperones in response to TM treatment

As ER chaperone transcriptional up-regulation is downstream of UPR activation, expression levels of two ER chaperones (*Grp94* and *Dnajc3*) were examined in morulae and blastocysts cultured in the presence of KSOMaa, 0.1 μg/ml TM and 0.5 μg/ml TM (Fig. 3A–F). Morulae cultured in each TM treatment for 12 h displayed a clear trend for rising *Grp94* and *Dnajc3* mRNA levels following treatment (Fig. 3A and B). Compared with embryos cultured in KSOMaa, morulae cultured in 0.5 μg/ml TM displayed significantly higher levels of *Grp94* ($P < 0.05$) (Fig. 3A). mRNA levels of *Grp94* and *Dnajc3* were also examined in blastocysts cultured in each treatment for 4 and 10 h. A rising trend in transcript levels for both *Grp94* and *Dnajc3* was observed in blastocysts exposed to TM, compared with the KSOMaa control (Fig. 3C–F).

Effect of TM-induced ER-stress on embryo apoptosis

To examine whether prolonged exposure to ER stress resulted in higher levels of programmed cell death, percent apoptosis was examined using the TUNEL assay, in morulae and blastocysts cultured in TM (Figs 4 and 5). Apoptosis was measured in morula cultured in either KSOM, 0.1 μg/ml TM or 0.5 μg/ml TM for 12 h (Fig. 4A–C). Totally, 92.3 ± 5.0% of the cells in the positive control group displayed TUNEL staining ($n = 17$). TUNEL analysis revealed that 2.4 ± 3.1% of cells in the KSOM control displayed apoptosis ($n = 19$). No significant difference in the percentage of apoptotic cells was observed between embryos cultured in KSOM when compared with embryos cultured in 0.1 μg/ml TM (2.2 ± 1.6%, $n = 19$). However, TUNEL analysis revealed that compared with the KSOM control, the percentage of apoptotic cells was significantly increased in morulae cultured in 0.5 μg/ml TM for 12 h (6.6 ± 4.1%, $n = 28$; $P \leq 0.05$; Fig. 4B). Overall, 36.8% of morula cultured in KSOM displayed at least one

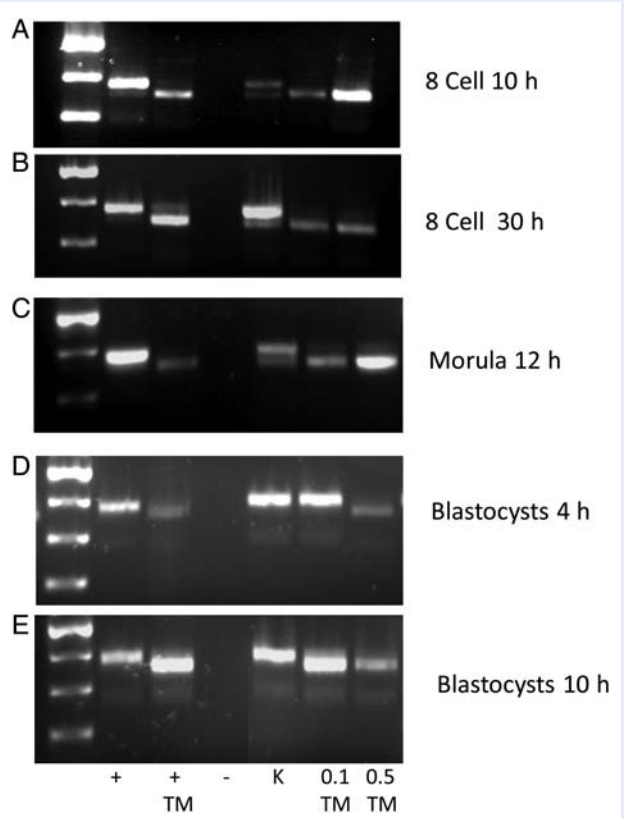


Figure 2 (**A**–**E**) *Xbp1* mRNA splicing in 8-cell embryos, morulae and blactocysts cultured in 0.1 and 0.5 μg/ml TM. Treatment groups consisted of control KSOMaa medium (K), KSOMaa + 0.1 μg/ml TM (0.1 TM) and KSOMaa + 0.5 μg/ml TM (0.5 TM). In 8-cell embryos cultured in each treatment for 10 (A) and 30 (B) hours, both unspliced and spliced *Xbp1* were detected in embryos cultured in KSOM alone, while embryos cultured in either 0.1 TM of 0.5 TM displayed a greater proportion of spliced *Xbp1* transcript. Splicing of *Xbp1* mRNA was examined by RT–PCR methods in morulae cultured in TM for 12 h (C). In morulae both unspliced and spliced *Xbp1* was detected in embryos cultured in KSOM alone, while embryos cultured in either 0.1 TM of 0.5 TM displayed a greater proportion of spliced *Xbp1* transcript. Splicing of *Xbp1* mRNA was examined by RT–PCR methods in blastocysts cultured in TM. Blastocysts cultured for 4 h (D) in KSOMaa and 0.1 TM displayed a higher proportion of unspliced *Xbp1*, while those cultured in 0.5 TM displayed a greater proportion of spliced *Xbp1* transcript. In contrast, blastocyst treated for 10 h (E) displayed a higher signal for spliced *XBP1* at the 0.1 and 0.5 TM concentrations. A 100 bp ladder was used. Pancreatic tissue was used as a positive control (+) for unspliced *Xbp1*, while TM-treated 8-cell embryos were used as a positive control (+TM) for spliced *Xbp1*. A no cDNA negative control (−) was included. Representative of three independent replicates.

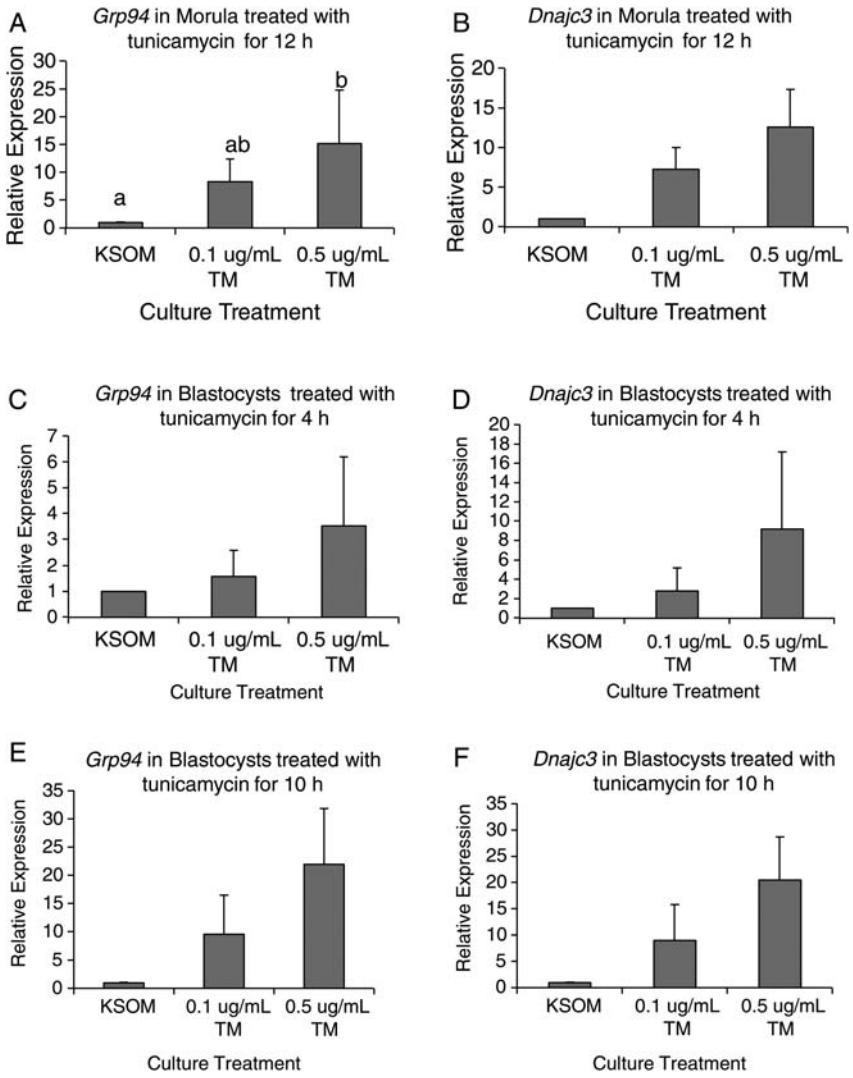


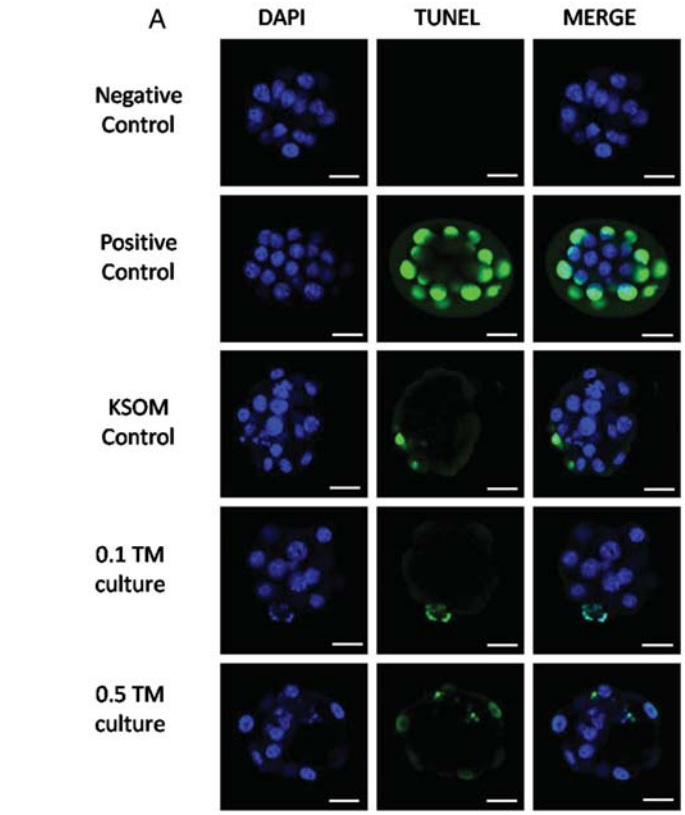
Figure 3 Relative mRNA levels of *Grp94* and *Dnajc3* transcripts in mouse morulae following 12 h of culture and blastocysts following 4 and 10 h of culture in 0.1 and 0.5 μg/ml TM. Quantitative mRNA levels were detected for *Grp94* (**A**) and *Dnajc3* (**B**) by real-time PCR in morulae treated with TM for 12 h. Real-time PCR in blastocysts treated with TM for 4 (**C**, **D**) and 10 h (**E**, **F**). Treatment groups consisted of control KSOMaa medium (KSOM), KSOMaa + 0.1 μg/ml TM (0.1 μg/ml TM) and KSOMaa + 0.5 μg/ml TM (0.5 μg/ml TM). Data were normalized to a *Luciferase* control (0.025 pg/embryo). mRNA levels presented are relative to mRNA levels detected at the 1-cell stage. Relative mRNA levels are presented as the mean ± SEM, representative of three independent replicates. Bars with different letters represent significant differences in relative mRNA levels between embryo stages ($P \leq 0.05$).

apoptotic cell, while 57.9% of embryos cultured in 0.1 μg/ml TM and 78.6% of embryos cultured in 0.5 μg/ml TM for 12 h displayed at least one apoptotic cell (Fig. 4C).

Apoptosis was also measured in blastocysts cultured in KSOM, 0.1 μg/ml TM or 0.5 μg/ml TM for 4 h (Fig. 5A–C). For blastocysts cultured in each treatment group, 95.0 ± 3.5% of cells in DNase-treated embryos displayed apoptosis ($n = 13$). TUNEL analysis revealed that 1.7 ± 1.5% of cells in blastocysts cultured in KSOM were apoptotic, while 2.1 ± 2.0% of cells in blastocysts cultured in 0.1 μg/ml TM were apoptotic ($n = 10$; Fig. 5B). Compared with the KSOM control, a significant increase in apoptosis was observed in blastocysts cultured in 0.5 μg/ml TM (4.5 ± 2.8%, $n = 16$; Fig. 5B). Overall, 25% of embryos cultured in KSOM had at least one apoptotic cell while 45.5% of embryos cultured in 0.1 μg/ml TM and 68.8% of embryos cultured in 0.5 μg/ml TM contained at least one apoptotic cell (Fig. 5C).

Effects of embryo collection on activation of the UPR

As it was demonstrated that the UPR can be activated in preimplantation mouse embryos upon exposure to TM treatment, the effects of *in vitro* manipulations on activation of the ER stress pathway were examined. To examine whether embryo flushing itself results in activation of the ER stress pathway, pools of 20 fresh flushed 8-cell stage embryos and blastocysts were collected and total RNA was extracted. Both



C

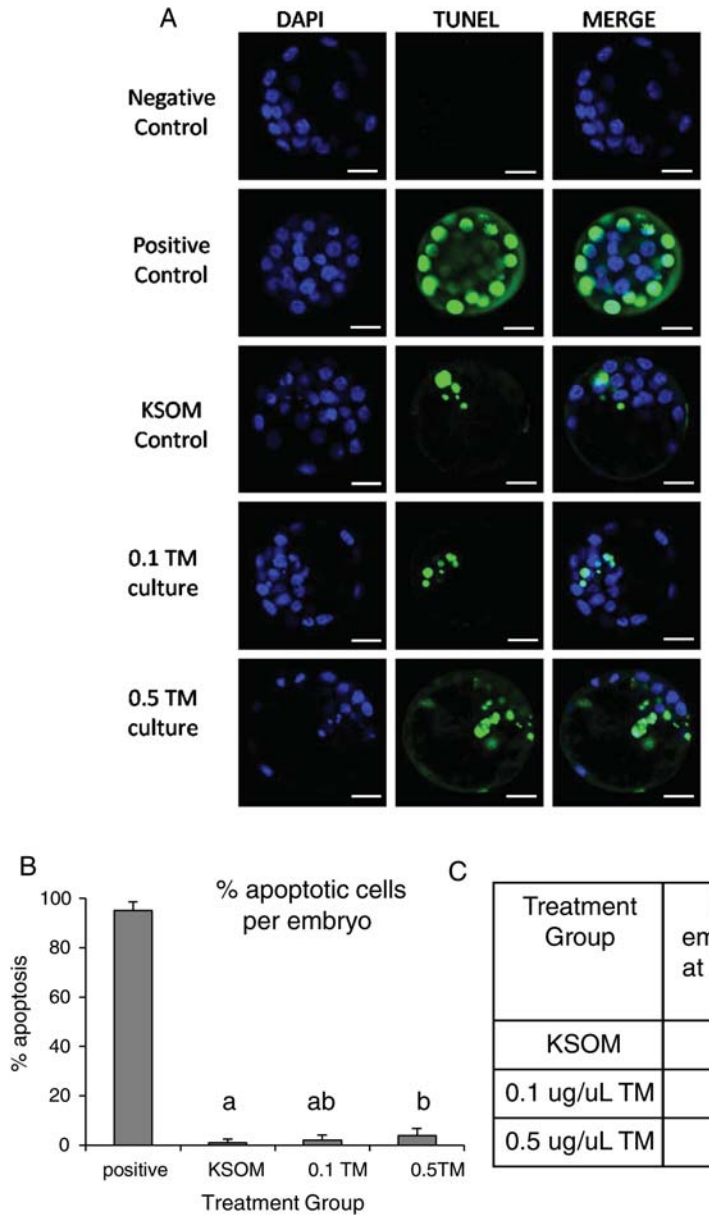
Treatment Group	Percentage of embryos displaying at least 1 apoptotic cell
KSOM	36.8 %
0.1 ug/uL TM	57.9 %
0.5 ug/uL TM	78.6 %

Figure 4 (**A**–**C**) Effect of TM on embryo apoptosis in mouse morulae cultured in treatment for 12 h. The effects of TM on apoptosis were examined by TUNEL labeling in morulae treated for 12 h (**A**). Treatment groups consisted of control KSOMaa medium ($n = 19$), KSOMaa + 0.1 μg/ml TM (0.1 TM; $n = 19$) and KSOMaa + 0.5 μg/ml TM (0.5 TM; $n = 28$). Blue represents cellular DNA (using DAPI staining), while green represents DNA breaks, indicating apoptosis (TUNEL labeling). Scale bars represent 25 μm. (**B**) Percent apoptosis in treated embryos. The percentage of apoptotic cells within treated morulae is presented. A significant increase in percent apoptosis was observed between embryos cultured in KSOMaa and embryos cultured in 0.5 TM ($P \leq 0.05$). (**C**) Percentage of morulae displaying at least one apoptotic cell. The proportion of embryos displaying at least one apoptotic cell was increased when comparing TM-treated embryos to those cultured in KSOMaa alone. DNase-treated embryos were used as a positive control and a no enzyme control (label only) was used as a negative control.

unspliced and spliced *Xbp1* transcripts were clearly detected immediately following 8 cell stage embryo (Fig. 6A) and blastocyst collection ($n = 4$; Fig. 6B). To examine whether the Xbp1 splicing detected in fresh flushed embryos was further enhanced or perturbed after exposure to standard embryo culture, fresh flushed 8-cell stage embryos and blastocysts were cultured in KSOMaa for 2 or 4 h. RT–PCR results demonstrate that although spliced *Xbp1* is detectable in fresh flushed 8-cell stage embryos and blastocysts, levels of spliced *Xbp1* are diminished after 2 h in culture and are not observed after 4 h in culture (Fig. 6A and B).

Effects of blastocyst vitrification on activation of the IRE1α arm of the UPR

To determine whether blastocyst vitrification results in activation of the ER stress pathway, *Xbp1* splicing was examined in pools of



Treatment Group	Percentage of embryos displaying at least 1 apoptotic cell
KSOM	25.0 %
0.1 ug/uL TM	45.5 %
0.5 ug/uL TM	68.8 %

Figure 5 (A–C) Effect of TM on embryo apoptosis in mouse blastocysts cultured in treatment for 4 h. The effect of TM on apoptosis was examined by TUNEL labeling in blastocysts treated for 4 h (**A**). Treatment groups consisted of control KSOMaa medium ($n = 28$), KSOMaa + 0.1 μg/ml TM (0.1 TM; $n = 10$) and KSOMaa + 0.5 μg/ml TM (0.5 TM; $n = 16$). Blue represents cellular DNA (using DAPI staining), while green represents DNA breaks, indicating apoptosis (TUNEL labeling). Scale bars represent 20 μm. (**B**) Percent apoptosis in treated embryos. The percentage of apoptotic cells within treated blastocysts is presented. A significant increase in percent apoptosis was observed between embryos cultured in KSOM and embryos cultured in 0.5 TM ($P \leq 0.05$). (**C**) Percentage of blastocysts displaying at least one apoptotic cell. The proportion of blastocyst displaying at least one apoptotic cell was increased when comparing TM treated embryos to those cultured in KSOMaa alone. DNase-treated embryos were used as a positive control and a no enzyme control (label only) was used as a negative control.

vitrified blastocysts (8 embryos/pool). As blastocyst flushing itself results in the activation of the ER stress pathway, fresh flushed embryos were cultured in KSOMaa under standard culture conditions for a minimum of 2 h prior to vitrification (Fig. 7A). Thawed embryos were placed back into culture for 0, 1 or 3 h prior to being subjected to RNA extraction (Fig. 7B–D). As reported above, RT–PCR results demonstrate that fresh flushed blastocysts show some *Xbp1* splicing but that this splicing is diminished after 2 h in culture (Fig. 7E). Interestingly, vitrified embryos displayed no *Xbp1* splicing 0, 1 or 3 h post-thaw ($n = 3$) demonstrating that this UPR pathway is not activated in response to embryo vitrification (Fig. 7E).

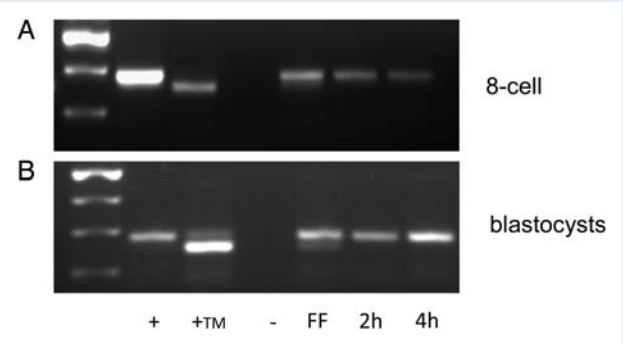


Figure 6 (**A** and **B**) *Xbp1* mRNA splicing in mouse 8-cell stage embryos and blastocysts immediately after flushing, followed by 4 h in culture. Splicing of *Xbp1* was examined in fresh flushed 8-cell embryos and blastocysts. Unspliced *Xbp1* was detected in fresh flushed (FF) 8-cell embryos (A), while both unspliced and spliced *Xbp1* was detected in FF blastocysts (**B**). After 2 and 4 h in standard KSOM culture media, only unspliced *Xbp1* was detectable in both 8-cell embryos and blastocysts (A, B). A 100 bp ladder was used. Pancreatic tissue was used as a positive control (+) for unspliced *Xbp1*, while TM-treated 8-cell embryos were used as a positive control (+TM) for spliced *Xbp1*. A no cDNA negative control (−) was included. Representative of three independent replicates.

Discussion

Although ER stress pathways are well characterized in a number of tissues, their contributions to preimplantation development have not been investigated to date. Several studies have investigated individual components of the UPR and have indicated that UPR pathway constituents are expressed during early development (Luo *et al*., 2006; Hao *et al*., 2009; Iwawaki *et al*., 2009). However, the presence or absence of the majority of UPR constituents has not been determined at all stages of preimplantation development, making the role of the UPR during this developmental period uncertain until now. In order to have a cohesive understanding of which pathway constituents are present during mouse preimplantation development, mRNA transcript analysis for several members of the ER stress response pathway was conducted in developmental series' by RT–PCR methods. mRNA transcripts for all genes of interest (*Bip*, *Atf4*, *Chop*, *Ire1α*, *Xbp1*, *Ask1*, *Atf6*, *Gadd34* and *Perk*) were detected at all stages of preimplantation development (1-cell stage to blastocyst stage).

The presence of *Bip (Grp78)*, *Ire1α*, *Perk* and *Atf6* throughout preimplantation development is not surprising, given that they are all constitutively expressed in most tissues. Additionally, knock-out studies for these genes have supported the hypothesis that these constituents are essential during early development (Luo *et al*., 2006; Iwawaki *et al*., 2009). However, the detection of *Atf4*, *Chop*, *Ask1* and *Gadd34* transcripts at all stages of preimplantation development was more interesting. Each of these genes are induced downstream of the UPR, and are not typically expressed to a high degree in unstressed cells (Wang *et al*., 1996; Harding *et al*., 2000a, b; Ma and Hendershot, 2003). This result implies that the ER stress pathway is induced at each stage of mouse preimplantation development. Interestingly, variations in the pattern of *Xbp1* splicing were observed at each stage of preimplantation development. In fact, *Xbp1* splicing differed at each stage of development between replicates. However, if all three replicates are examined as a whole, *Xbp1* splicing was observed at least once at each developmental stage. As *Xbp1* splicing is indicative of activation of the IRE1α arm of the UPR, this result suggests that this arm of the ER stress response is inducible at all stages of mouse preimplantation development. Overall, it can be concluded that the pathway constituents are present during early development, supporting the hypothesis that they are actively playing a role in mediating the embryonic response to ER stress. Furthermore, several of these observations indicate that the embryo may be experiencing some inherent level of ER stress, resulting in the activation of downstream effectors of the ER stress pathway. To address this possibility, the embryo's ability to activate the UPR in response to ER stress was directly examined.

Xbp1 is spliced exclusively under conditions of ER stress (Yoshida *et al*., 2001; Lee *et al*., 2002). Therefore, it is an appropriate marker for the induction of the IRE1 arm of the UPR. The results of this study clearly indicate that the UPR is activated in the preimplantation embryo in response to treatment with TM. Splicing of *Xbp1* was observed in 8-cell embryos, morulae and blastocysts treated with 0.5 μg/ml TM. It should be noted that the extent of splicing appeared variable between different developmental stages, as unspliced *Xbp1* was detected in some groups, and not in others. This variability could be attributed to differences in cell number, or to differences in the 'magnitude' of UPR induction experienced by each embryo.

Xbp1 splicing in embryos treated with 0.1 μg/ml TM produced different outcomes. Spliced *Xbp1* was detected in all embryos cultured in this treatment, with the exception of blastocysts cultured for 4 h. It is possible that the lower dose of TM was not potent enough to induce a high level of ER stress at the blastocyst stage. Such a mild stressor may not have affected these embryos because at this developmental time point, the embryo has sufficiently equipped itself with the proteins required for its continued growth.

As ER chaperones are targeted downstream of the ER stress pathway, the transcriptional up-regulation of ER chaperones is often used as a marker for ER stress, indicating activation of the ER stress response (Laybutt *et al*., 2007; Peyrou *et al*., 2007). All embryos cultured in TM displayed elevated levels of *Grp94* and *Dnajc3* mRNA transcripts when compared with the KSOMaa control, confirming that these embryos were in fact experiencing ER stress and that they were able to activate the UPR in response. The degree of up-regulation was proportional to the dosage of TM used. Embryos cultured in 0.5 μg/ml TM displayed higher levels of mRNA transcripts than those cultured in 0.1 μg/ml TM. This was an expected result, as it is expected that a higher degree of stress requires a greater response. Both *Grp94* and *Dnajc3* are up-regulated downstream of the ATF6 and IRE1 arms of the UPR (Wu *et al*., 2007; Yamamoto *et al*., 2007). Splicing of *Xbp1* indicates activation of IRE1. It remains unclear whether ATF6 is also being activated in response to ER stress in the preimplantation embryo. Interestingly, blastocysts cultured for 4 h in 0.1 μg/ml TM did not display *Xbp1* splicing, indicating that IRE1 is not activated in these embryos. Nevertheless, a rising trend in *Grp94* and *Dnajc3* mRNAs is still observed in these embryos. This result may indicate that ATF6 is also being activated, as transcriptional up-regulation of ER chaperones is observed, even in the absence of IRE1 activation.

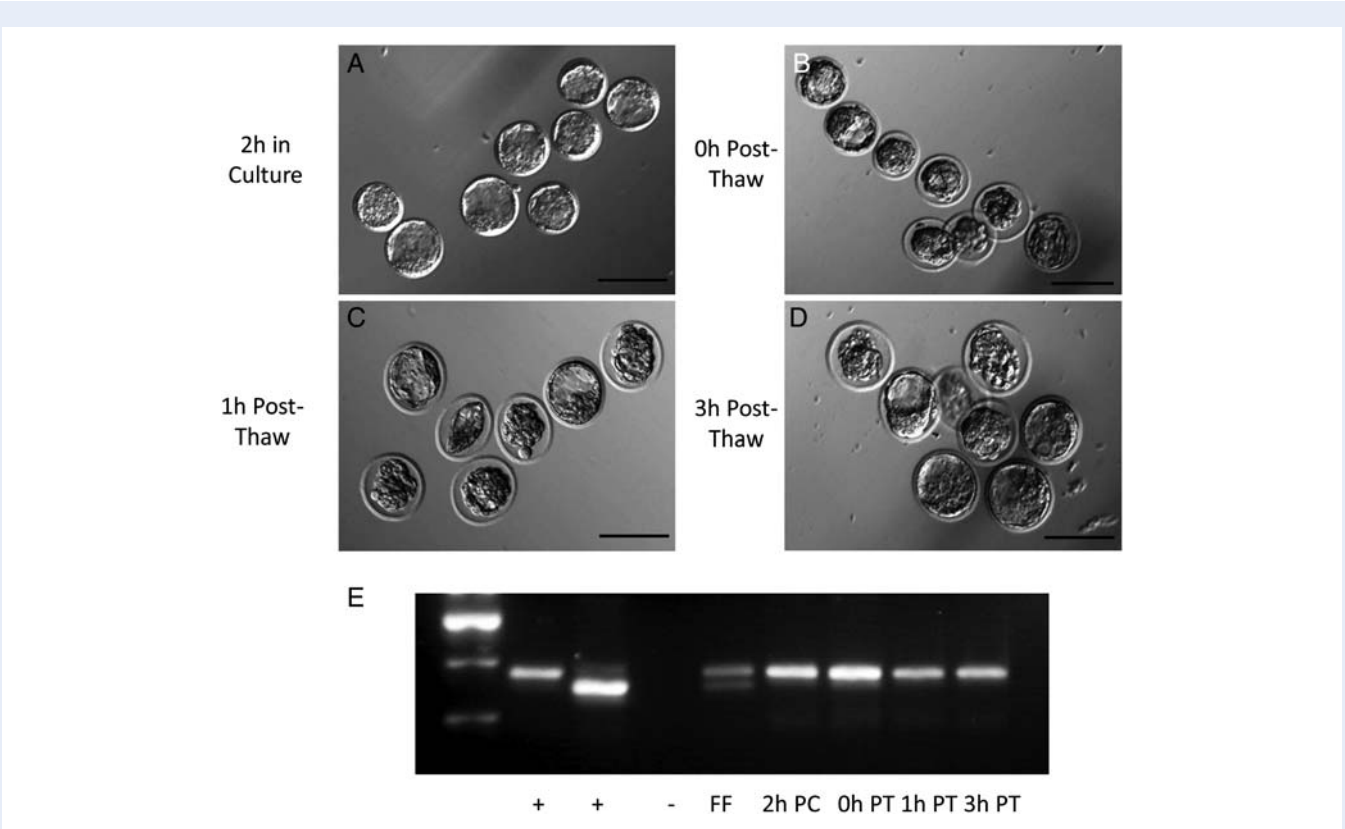


Figure 7 (**A**–**E**) Morphology of vitrified mouse blastocysts and *Xbp1* mRNA splicing in vitrified mouse blastocysts. Phase contrast imaging to compare morphology of representative groups of mouse blastocysts before vitrification (A), immediately after thawing (0 h post-thaw; **B**), 1 h post-thaw (C) and 3 h post-thaw (D). Scale bars represent 100 μm. (E) Splicing of *Xbp1* in vitrified blastocysts. Spliced *Xbp1* was detected in FF blastocysts, and this splicing was diminished after 2 h in culture (2 h PC). In thawed vitrified blastocysts, spliced *Xbp1* was not detected at any time post-thaw (PT). A 100 bp ladder was used. Pancreatic tissue was used as a positive control (+) for unspliced *Xbp1*, while TM-treated 8-cell embryos were used as a positive control (+TM) for spliced *Xbp1*. A no cDNA negative control (−) was included. Representative of three independent replicates.

Apoptosis is initiated downstream of ER stress only if all other measures taken to cope with this stress are unsuccessful, and if the cell has the capacity to activate apoptotic mechanisms (Szegezdi *et al.*, 2006). Although there are several apoptotic pathways initiated via the UPR, previous studies have demonstrated that under conditions of ER stress, mRNA transcripts for the apoptosis promoting factor CHOP are up-regulated in the mouse preimplantation embryo (Fontanier-Razzaq *et al.*, 1999). Fontanier-Razzaq *et al.* (1999) demonstrated that blastocysts cultured in 0.5 μg/ml TM displayed elevated levels of *Chop* transcript, compared with culture controls. However, any effects that elevated levels of *Chop* had on apoptosis were not examined in the study, leaving the effect of ER stress on embryo apoptosis unknown. Therefore, the effect of ER stress on embryo apoptosis was examined in the present study.

The occurrence of apoptosis during preimplantation development has been characterized in the mouse (Fabian *et al.*, 2005; Fabian *et al.*, 2007). Studies have demonstrated that apoptosis cannot be detected in the mouse embryo prior to the MZT and that *in vivo* derived embryos very rarely display apoptosis prior to the blastocyst stage. On the other hand, *in vitro* derived embryos display a higher degree of apoptosis in both cleavage stage and blastocyst stage embryos (Fabian *et al.*, 2005). A significant increase in apoptosis was observed in morulae cultured for 12 h in 0.5 μg/ml TM when compared with KSOMaa controls. A significant increase in apoptosis was also observed in blastocysts cultured for 4 h in 0.5 μg/ml TM when compared with KSOMaa controls. These results indicate that apoptosis is in fact initiated in response to conditions of ER stress in late stage embryos, when the ER stress pathway is likely able to be fully activated during preimplantation development. The level of apoptosis observed in these embryos is within the range typically observed in mouse blastocysts cultured *in vitro* (~3–6%; Fabian *et al.*, 2005). The results observed in blastocysts after 4 h in 0.5 μg/ml support the hypothesis that the significant increase of *Chop* transcript levels reported by Fontanier-Razzaq *et al.* (1999) is a precursor to an increased level of apoptosis in these embryos. Furthermore, if taken together, these results would suggest that the PERK arm of the UPR is active in the preimplantation embryo, as *Chop* is a downstream target of this effector.

Overall, these results demonstrate that the ER stress pathway is inducible and active during mouse preimplantation development. Under conditions of ER stress (treatment with TM), preimplantation embryos at the 8-cell, morula and blastocyst stage displayed activation of the ER stress pathway as indicative of *Xbp1* splicing. Furthermore, downstream targets *Grp94* and *Dnajc3* were up-regulated in TM-treated morula and blastocysts, indicating that the pathways downstream of IRE1 and ATF6 can be activated during this

developmental period. Finally, TUNEL staining revealed that apoptosis is initiated in response to ER stress in morula and blastocysts. If considered with reports that levels of *Chop* are up-regulated under similar conditions, it can be hypothesized that the PERK arm of the UPR is also being activated in these embryos under conditions of ER stress.

While it is likely that the embryo is utilizing the ER stress response to facilitate its movement through the female reproductive tract *in vivo*, this study sought to determine whether the mouse preimplantation embryo is activating the UPR in response to *in vitro* stressors. *Xbp1* splicing was observed in fresh flushed blastocysts. It is possible that even quickly flushed embryos are no longer really *in vivo* embryos. We have interpreted our outcomes as suggesting that we are inducing the spliced form during the collection period and it is lost following stabilization under normal culture conditions. However, it is equally likely that spliced variant is normally present in *in vivo* embryos in the reproductive tract and it is aberrantly disappearing once embryos have been placed in culture for a couple of hours. Our outcomes cannot distinguish these two possibilities. ER stress can occur under conditions of cellular mechanical stress (Mak *et al.*, 2008). Specifically, mechanical stress can result in an efflux of Ca^{2+} ions from the ER, which perturbs the function of ER chaperones. This inhibits protein folding in the ER, resulting in an accumulation of unfolded proteins in the ER lumen, causing ER stress (Mak *et al.*, 2008; Schroder, 2008). Flushing embryos from the reproductive tract applies a considerable amount of shear stress on the embryo. Furthermore, recent studies have demonstrated that stress-activated pathways can be induced in the preimplantation embryo due to shear stress caused by pipetting (Xie *et al.*, 2007). ART clinics are handling human preimplantation embryos in culture on a daily basis. It is important to understand the possible stresses that this requirement places on the embryo and the mechanisms the embryo employs to adapt to these stresses. This study indicates that among the adaptive pathways available, the cultured mammalian embryo can employ the ER stress pathway. ART technicians should be aware that their activities may induce the ER stress pathway in their patients' early embryos. This result indicates that the IRE1 arm of the UPR is activated to mediate stress induced by these conditions.

It is interesting to note that previous studies examining the effects of mechanical stress on UPR activation have referred to the PERK arm as the primary mediator for this type of stress (Mak *et al.*, 2008; Cheng *et al.*, 2009). This was initially hypothesized due to the high levels of cell death observed in cells subjected to mechanical stress. As PERK is upstream of ER-stress-induced apoptosis, its role in conditions of mechanical stress was characterized. In fact, *Xbp1* splicing was not observed in mouse fibroblasts subjected to mechanical stress, although the PERK arm of the UPR was deemed active (Mak *et al.*, 2008). This was a surprising result, demonstrating that specific arms of the UPR may be induced under specific conditions. Additionally, this indicates that full induction of the ER stress response is not always the case under all conditions of ER stress. The fact that IRE1 was activated in response to mechanical stress in the present study is a novel and important finding. Additional experiments examining whether the PERK and ATF6 arms of the UPR are also activated should be carried out to understand the full extent of UPR induction under conditions of mechanical stress in the mouse preimplantation embryo.

Although *Xbp1* splicing was observed in fresh flushed 8 cell stage embryos and blastocysts, this splicing was diminished after 2 h in culture, and remained undetectable after 4 h in culture. This may be a point for clinicians to take into account in their practice, as ER stress in the embryo could be mitigated by placing freshly collected embryos in culture media for a period of 'recovery' prior to further manipulation. Furthermore, this result demonstrates that short-term exposure to standard embryo culture (in KSOMaa at 37°C, under a 5% CO_2 in air atmosphere) does not result in conditions of ER stress in the preimplantation embryo or in activation of the UPR.

The advent of embryo cryopreservation has made it increasingly common for embryos to be exposed to conditions of cold stress. In particular, the process of vitrification exposes the embryo to extremely low temperatures very quickly, which could trigger stress pathways to manage this shock. Therefore, the effect of embryo vitrification on activation of the ER stress pathway was investigated. Surprisingly, *Xbp1* splicing was not observed in vitrified embryos. At 0, 1 and 3 h post-thaw, no activation of the IRE1 arm of the UPR was observed. This result suggests that severe cold stress may not result in ER stress within the mouse preimplantation embryo. On the other hand, this result may simply reflect that this particular arm of the UPR is not activated in response to this stress, or that the time course chosen was not long enough to observe the effects of this technique. Up-regulation of molecular chaperones HSP70 and HSP90 in human fibroblasts and HeLa cells upon re-warming (after exposure to cold shock) has been reported (Liu *et al.*, 1994). However, the mechanism behind this up-regulation was attributed to the activation of the heat shock response (HSR) through the activity of heat shock factor 1 (HSF1) (Liu *et al.*, 1994). The heat shock response is very similar to the UPR, as downstream effects include transcriptional up-regulation of molecular chaperones and increased protein degradation. It is primarily induced under conditions of thermal stress, to respond to protein aggregates formed in the cytoplasm. Importantly, a functional overlap has been demonstrated between the two pathways. Recently, it was shown that overexpression of HSF1 can compensate for deficiencies in the UPR (*Ire1* deletion) under conditions of ER stress (Liu and Chang, 2008). Therefore, it is possible that vitrified embryos are employing other stress response pathways (i.e. HSR) to respond to the cold stress, and that these pathways are sufficient to alleviate any resultant ER stress.

Authors' roles

T.A. conducted all of the described experiments, produced the first draft of the manuscript and contributed to the study design and directions. C.L.P. contributed directly to the study design and experimental content, and drafted the manuscript. A.J.W. supervised Ms Abraham's studies, assisted with experimental design and directions, and prepared the submitted and final revised drafts of the manuscript.

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Conflict of interest

None declared.

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