BIOCELL
2007, 31(3): 405-410

ISSN 0327 - 9545
PRINTED IN ARGENTINA

Technical Note

Prolonged exposure of human embryonic stem cells to heat shock induces necrotic cell death

Boon Chin Heng*, Kumar Jayaseelan Vinoth*, Kai Lu*, Xuliang Deng*, Zigang Ge*, Boon Huat Bay**, and Tong Cao*

* Stem Cell Laboratory, Faculty of Dentistry, National University of Singapore, 5 Lower Kent Ridge Road, 119074 Singapore.
** Department of Anatomy, Faculty of Medicine, National University of Singapore, 5 Lower Kent Ridge Road, 119074 Singapore.

Key words: embryonic, heat shock, human, necrosis, stem cells

ABSTRACT: We investigated the effects of prolonged heat shock treatment on human embryonic stem cell (hESC) viability. The hESC viability steadily declined with longer exposure to heat shock treatment (43ºC). After 4 h of exposure to heat shock at 43ºC, only 56.2 ± 1.5% of cells were viable. Viability subsequently declined to 37.0 ± 3.3% and 3.5 ± 0.7% after 8 h and 16 h, respectively of heat shock treatment at 43ºC. Transmission electron micrographs showed that the morphology of the dead/dying cells after heat shock treatment was characteristic of cellular necrosis with an uncondensed chromatin and a non-intact plasma membrane. This was further confirmed by flow cytometry analysis which showed that the DNA of the dead/dying cells was still mostly intact, unlike the characteristic DNA fragmentation observed with apoptotic cells. In conclusion, prolonged exposure to heat shock treatment was detrimental to hESC viability. Hence, any future protocols developed for either the heat shock pre-conditioning of hESC prior to transplantation or for the temporary expression of specific genes with heat shock-responsive promoters should take these results into account; to achieve an optimal balance between the duration of heat shock exposure and the attainment of the desired effects.

Introduction

Several studies have reported the beneficial effects of heat shock pre-treatment on the survival and engraftment of transplanted cells (Baumeister *et al*., 2004; Bounchentout *et al*., 2004; Kim *et al*., 2006; Nakamura *et al*., 2006; Radaelli *et al*., 2001, 2002; Terajima *et al*., 2000; Watanabe *et al*., 2005; Yamagami *et al*., 2003), particularly within an ischemic or inflammatory microenvironment at the transplantation site. Additionally, the constitutive expression of heat-shock proteins through recombinant DNA transfection has also been reported to enhance cell survival and engraftment upon transplantation (Jayakumar *et al*., 2006; Kim *et al*., 2006). However, because of current safety and regulatory concerns in human clinical trials (Nathwani *et al*., 2004; Scharschmidt and Lo, 2006), the use of heat shock pre-conditioning is favored over recombinant DNA technology and viral vectors.

Human embryonic stem cells (hESC) derived from the inner cell mass of blastocyst-stage embryos hold much promise in cell transplantation therapy because of their unlimited proliferative capacity and extensive multi-lineage differentiation capacity (Thomson *et al*., 1998; Reubinoff *et al*., 2000; Cowan *et al*., 2004). The

Address correspondence to: Dr. Tong Cao. Stem Cell Laboratory, Faculty of Dentistry, National University of Singapore. Lower Kent Ridge Road. 119074, SINGAPORE.
E-mail: *omscaot@nus.edu.sg*
Received on December 12, 2006. Accepted on June 5, 2007.

beneficial effects of heat-shock preconditioning on the survival and engraftment of hESC upon transplantation in live animal models have been reported (Laflamme *et al*., 2005); but to date, there has not been a systematic study of the prolonged effects of heat shock treatment on hESC viability. Therefore, the focus of this study was to use the MTT (Tetrazolium salt 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Mosmann *et al*., 1983) to evaluate how prolonged exposure of hESC to heat shock at 43ºC affects cellular viability at various durations up to 16 h. Subsequently, flow cytometry analysis together with transmission electron microscopy were used to characterize if any heat shock-induced loss of cell viability resulted from apoptotic or necrotic mechanisms.

Materials and Methods

hESC, Media, Reagents, and Chemicals

The hESC were obtained from the Wicell Research Institute Inc. (Madison, WI, USA), and were of the H1 line listed on the National Institute of Health (NIH) registry, which had received Federal approval for US government-supported research funding. Unless otherwise stated, all liquid media, serum, and serum replacement were purchased from Gibco BRL Inc. (Gaithersburg, MD, USA), while all other reagents and chemicals were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA).

Culture and propagation of hESC in the undifferentiated state

Undifferentiated hESC were maintained on a feeder layer of mitomycin C-inactivated murine embryonic fibroblast feeder (MEF) cells (Thomson *et al*., 1998; Reubinoff *et al*., 2000; Cowan *et al*., 2004). These cells were harvested from the CF1 inbred mouse strain purchased from Charles River Laboratories (Wilmington, MA, USA). The culture medium was DMEM/F12 supplemented with 20% (v/v) Knockout (KO) serum replacement, 1 mM L-glutamine, 1% non-essential amino acid, 100 mM β-mercaptoethanol, and 4 ng/ml bFGF. All cell cultures were carried out in 6-well culture dishes (Nunc Inc., Roskilde, Denmark) within a humidified 5% CO_2 incubator set at 37ºC. We changed the culture media daily, and passaged the hESC on a fresh MEF layer once a week. Dissociation of hESC colonies into cell clumps for serial passage was achieved through treatment with 1 mg/ml collagenase type IV for 3 to 5 min. Special care was taken to ensure that the hESC clumps were seeded at equal densities within the 12-well dishes. This was achieved by pooling all the dissociated hESC clumps into a single tube and then pipeting up and down each time before seeding each well individually with a fixed volume of hESC clump suspension.

Prolonged exposure of hESC to heat shock

After 7 days of culture following the last serial passage, the hESC colonies reached 70% to 80% confluence (1500 to 2000 cells/mm^2) in the culture dish (12-well dish). The cultures were then exposed to prolonged durations of heat shock within a humidified CO_2 incubator set at 43ºC. There were four treatment groups: (1) physiological control maintained at 37ºC, (2) exposure to 43ºC for 4 h, (3) exposure to 43ºC for 8 h, and (4) exposure to 43ºC for 16 h. Fresh culture media was changed prior to prolonged exposure to heat shock at 43ºC.

MTT assay to quantify hESC viability after heat-shock exposure

The MTT (Tetrazolium salt 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Mosmann *et al*., 1983) was performed to quantify the survival rate of hESC after prolonged exposure to heat shock (43ºC) for different durations (0, 4, 8 and 16 h). In brief, this involved placing 0.5 ml of 1 mg/ml MTT (Sigma-Aldrich Inc, St. Louis, MO, USA) constituted in hESC culture media into each well (4.8 cm^2) of the 12-well dish and then incubating the MTT solution for 4 h at 37ºC in the dark. After incubation, the MTT solution was removed, and the cells were fixed with a few drops of formol-calcium (0.4% (v/v) formaldehyde with 1.0% (v/v) anhydrous $CaCl_2$ in de-ionized water), rinsed with PBS, and air-dried. The MTT-formazan products were extracted in the dark at room temperature with 1 ml of DMSO. We then transferred 100 microliters of the supernatant into a 96-well flat-bottomed cell culture plate, which we measured spectrophotometrically at 570 nm using a Sunrise modular microplate reader (Tecan, Maennedorf, Switzerland). The survival rate after exposure to heat shock was then computed by dividing the MTT absorbance values obtained from the heat shock-treated samples with the corresponding absorbance reading obtained for the physiological control maintained at 37ºC after correction for blank absorbance readings (DMSO) by the spectrophotometer.

Preparation of an apoptotic hESC positive control for flow cytometry and transmission electron microscopy analysis

Previously, it was described that the cryopreservation of intact and adherent hESC colonies through conventional slow-cooling protocols utilizing 10% (v/v) dimethyl sulfoxide (DMSO) led to apoptosis of the majority of the cell population (Heng *et al*., 2006). This finding was used as a basis for preparing an apoptotic hESC positive control for flow cytometry and transmission electron microscopy analysis. The cryopreservation solution consisted of DMEM/F12 medium supplemented with 10% (v/v) DMSO and 20% (v/v) defined fetal bovine serum. To achieve a gentler drop in temperature within the -80ºC refrigerator, we placed the 12-well dish containing intact and adherent hESC colonies within an insulated styrofoam box instead of an isoproponal container. To facilitate rapid thawing, we added just enough cryopreservation solution (0.3 ml) to cover the adherent hESC colonies in each well of the dish. The adherent hESC colonies cryopreserved in 12-well dishes were stored in the -80ºC refrigerator for at least one week prior to thawing.

Subsequently, the hESC colonies were thawed for 1 to 2 min within a 37ºC water bath. Special care was taken to ensure that no water from the bath seeped into the dish during thawing. To minimize osmotic shock to the cells, we gradually diluted the thawed cryopreservation solution (0.3 ml) within each well by drop-wise addition of 2 ml of hESC culture medium. Subsequently, to allow apoptotic cell death to take place, each well was consecutively washed twice with 2 ml of hESC culture media and incubated at 37ºC for 4 h.

Transmission electron microscopy

The hESC culture in all heat-shock treatment groups, including the physiological (37ºC) and apoptotic controls, were fixed with 3% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer for 30 min at room temperature. After osmication in 2% osmium tetroxide, specimens were dehydrated in an ascending series of ethanol and embedded in araldite. Ultrathin sections were cut, mounted on formvar-coated copper grids, and doubly stained with uranyl acetate and lead citrate before viewing in a Phillips BioTwin CM120 transmission electron microscope (Phillips Electron Optics, Eindhoven, The Netherlands).

Cell cycle analysis by flow cytometry

The hESC colonies exposed to heat-shock treatment for 16 h, together with the physiological (37ºC) and apoptotic controls, were trypsinized into a single cell suspension and then fixed in 70% (v/v) ice cold ethanol. After two consecutive washes in phosphate buffered saline (PBS) by centrifugation, the cells were incubated for 30 min in a solution comprised of 0.1% (v/v) Triton X-100, 0.2 mg/ml RNase, and 1 mg/ml propidium iodide (PI) in PBS. Cell cycle analysis of the sample preparations was then carried out with a FACSVantage flow cytometer (Becton Dickinson Inc., Franklin Lakes, NJ, USA).

Results

Effects of heat shock on hESC viability over a time course of sixteen hours, as assessed by the MTT assay

The MTT assay showed a steady decline in hESC viability with increasing duration of exposure to heat shock (Fig. 1). After 4 h of exposure to heat shock at 43ºC, only 56.2 ± 1.5% of cells were viable. Cell viability declined to 37.0 ± 3.3% and 3.5 ± 0.7% after 8 h and 16 h of heat shock treatment respectively.

Transmission electron microscopy

The transmission electron micrograph (TEM) of viable cells within the physiological control sample maintained at 37ºC is shown in Figure 2A, while that of apoptotic cells is shown in Figure 2B. For both viable and apoptotic cells, the outer plasma membrane is vis-

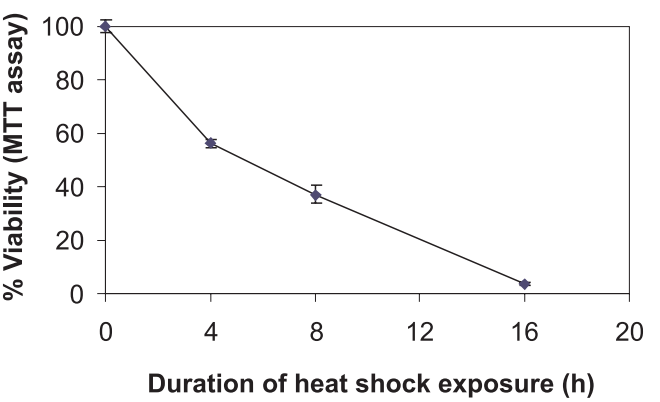

FIGURE 1. MTT assay showed a steady decrease in cell viability with longer exposure to heat shock treatment (43ºC).

ibly intact, and the only major difference is the morphological appearance of the cell nuclei. For viable cells, the chromatin is uncondensed giving a mottled appearance, and the nuclear membrane is distinctively intact and continuous (Fig. 2A). Apoptotic cells, in contrast, have condensed chromatin that marginate towards the nuclear membrane (Fig. 2B). Additionally, the nuclear membrane of apoptotic cells is distended and shows signs of degradation, which gives a faint non-continuous appearance (Fig. 2B).

The TEM of heat-shock exposed hESC are shown in Figures 2C-E. Necrotic cells can be easily distinguished from both viable and apoptotic cells by their non-intact plasma membrane, which often have a jagged discontinuous appearance under TEM (Figs. 2C-E). Moreover, the chromatin is uncondensed and not marginated towards the nuclear membrane (Figs. 2C-E), as it is in apoptotic cells (Fig. 2B). Hence, it is evident from TEM that the heat-shock exposed cells (Figs. 2C to E) are necrotic rather than apoptotic.

Cell cycle analysis by flow cytometry

The DNA of viable hESC from the physiological control sample maintained at 37ºC is mostly intact, as seen by the two distinctive prominent peaks representing cells at the G0/G1 phase (2n complement of chromosomal DNA) and G2/M phase (4n complement of chromosomal DNA) of the cell cycle (Fig. 3A). Nevertheless, a small amount of fragmented DNA is still in the physiological control (G0/G1 sub-peak), which may be the result of apoptotic cells arising from trypsinization of the hESC colonies.

By contrast, the apoptotic sample (Fig. 3B) showed a very prominent G0/G1 sub-peak, and the other two peaks representing the G0/G1 and G2/M phases of the cell cycle are relatively small and inconspicuous. This may indicate that most of the DNA is fragmented, which is what would be expected of an apoptotic sample.

For the hESC sample exposed to 16 h of heat shock (Fig. 3C), most of the DNA is still intact, as seen by the prominent G0/G1 and G2/M peaks and the relatively inconspicuous G0/G1 sub-peak. This is consistent with cellular necrosis, in which little if no DNA fragmentation occurs. Comparing the heat-shocked hESC (Fig. 3C) with the physiological control (Fig. 3B), we observed that the former had a higher G0/G1 peak and lower G2/M peak. This would indicate a lower proportion of actively mitotic cells in the heat shock-exposed sample.

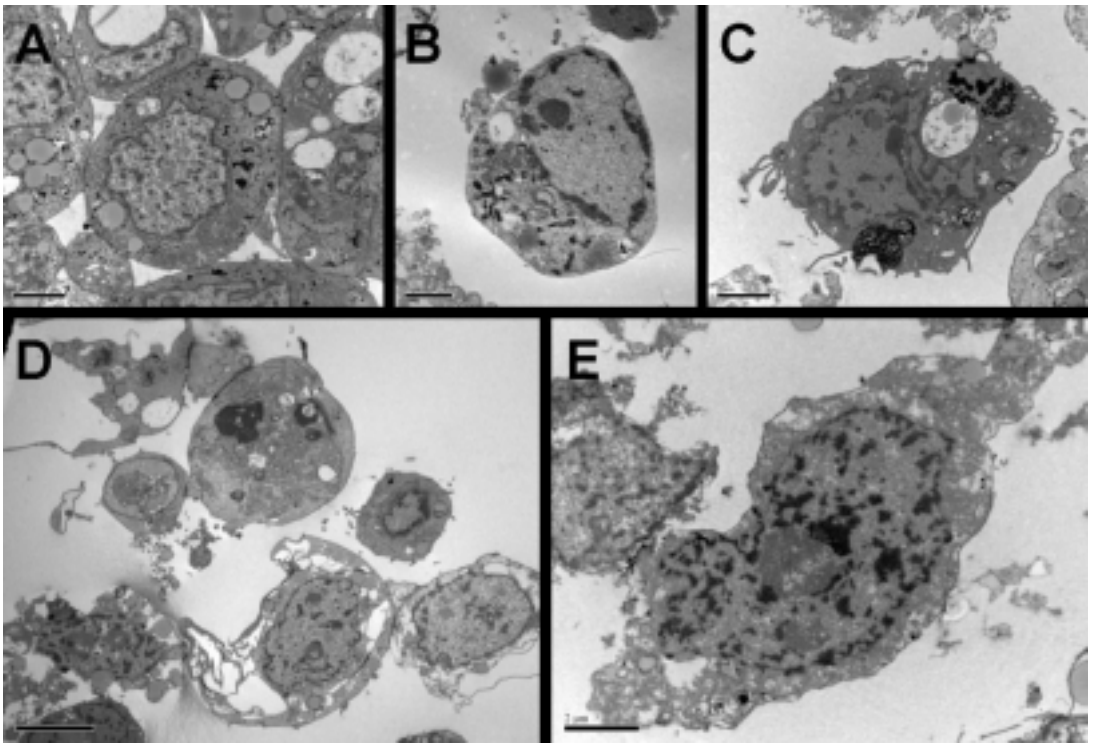

FIGURE 2. Transmission electron micrographs of (A) Physiological control maintained at 37ºC, (B) Apoptotic hESC induced by cryopreservation, (C) Heat-shock treatment at 43ºC for 4 h, (D)Heat-shock treatment at 43ºC for 8 h, (E) Heat-shock treatment at 43ºC for 16 h.

Discussion

A major challenge in cell transplantation therapy is the survival and subsequent engraftment of the transplanted cells within the hostile pathological environment of damaged/diseased tissues and organs. Several studies on animal models and phase I human clinical trials have demonstrated that the ischemic condition (Chen *et al*., 2006), as well as the strong presence of free radicals and inflammatory cytokines encountered by transplanted cells within damaged/diseased tissues, is a major factor in reducing the efficacy of transplantation therapy (Barshes *et al*., 2005; Li *et al*., 2004). Moreover, dead or dying endogenous cells within damaged/diseased tissues may possibly release metabolic toxins and apoptotic signals, which could in turn have adverse effects on the transplanted cells.

Hence, pre-conditioning of the cells prior to transplantation could be a strategy to enhance their survival and subsequent engraftment onto the host tissue/organ. One of the most common modes of pre-conditioning cells prior to transplantation is heat shock treatment for the activation of various stress-related proteins, which may aid in cell-survival within the hostile pathological environment at the transplantation site (Baumeister *et al*., 2004; Bounchentout *et al*., 2004; Kim *et al*., 2006; Nakamura *et al*., 2006; Radaelli *et al*., 2001; 2002; Terajima *et al*., 2000; Watanabe *et al*., 2005; Yamagami *et al*., 2003). Although the constitutive expression of heat shock-related proteins through recombinant DNA transfection have been reported to enhance cell survival and engraftment upon transplantation (Jayakumar *et al*., 2006; Kim *et al*., 2006); current safety and regulatory concerns favor the use of heat shock pre-conditioning, instead of recombinant DNA technology and viral vectors.

Another possible application of heat shock in cell-based therapy is the temporary expression of specific genes by using switchable heat shock-responsive promoters on recombinant DNA (Vilaboa and Voellmy, 2006). Because the constitutive expression of specific genes by transplanted cells can have detrimental effects, it may be advantageous to link gene expression to swit-

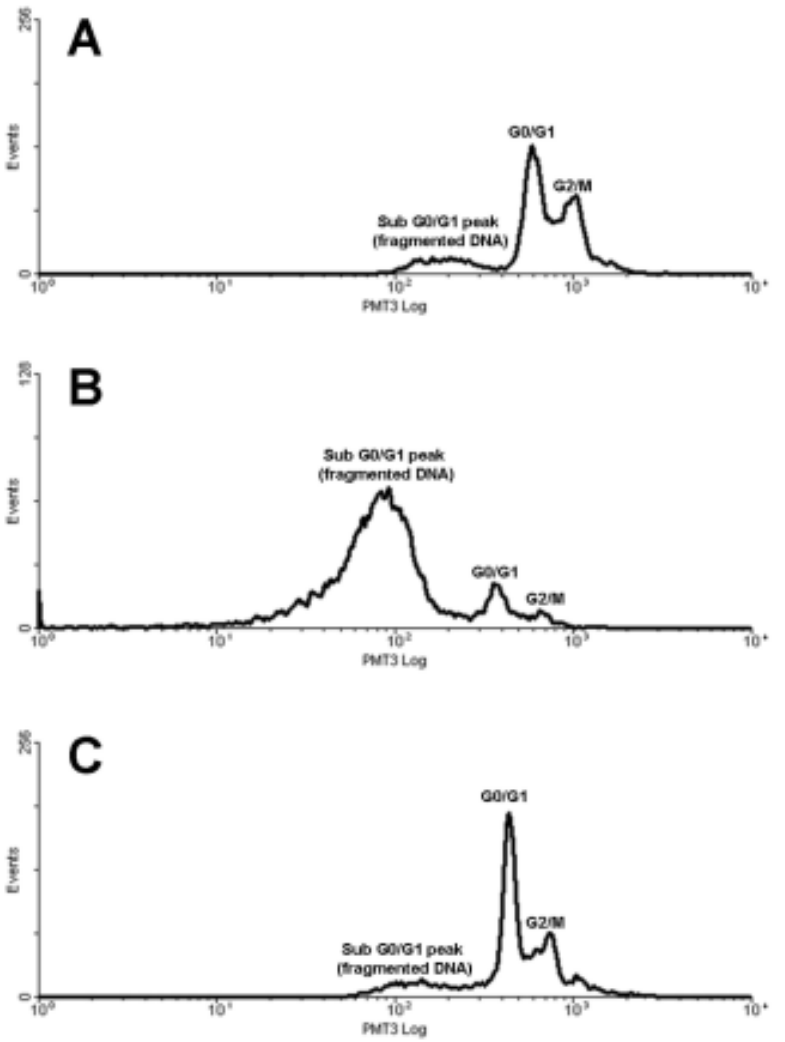

FIGURE 3. Flow cytometry analysis (propidium iodide staining) of (A) Physiological control maintained at 37ºC, (B) Apoptotic hESC induced by cryopreservation, (C) Heat shock treatment at 43ºC for 16 h.

chable promoters. For example, the temporary expression of specific transcription factors may be required to initiate stem-cell commitment or differentiation to a particular lineage, and once lineage commitment has been achieved, it may be necessary to switch-off gene expression to allow further differentiation and maturation of that particular cell lineage.

Hence, this study systematically characterized the effects of heat shock on hESC, which is currently at the forefront of new advances in cell transplantation therapy. The results (Fig. 1) of the MTT assay showed a steady, almost linear, decrease in hESC viability with increasing duration of heat-shock exposure (43ºC for up to 16 h). This finding is likely a result of the denaturation of various intracellular protein and enzymes during prolonged exposure to an elevated temperature (43ºC), which in turn disrupts normal cellular metabolism and homeostasis. Transmission electron micrographs showed that the morphology of the dead/dying cells were characteristic of cellular necrosis, rather than activation of the apoptotic cascade. This was further confirmed by flow cytometry analysis, which showed that the DNA of the dead/dying cells exposed to heat shock was still mostly intact, unlike the characteristic DNA fragmentation observed in apoptotic cells.

In conclusion, prolonged exposure to heat shock treatment is detrimental to hESC viability. Therefore, any future protocol developed for either heat shock preconditioning of hESC or for the temporary expression of specific genes with heat shock-responsive promoters should take this findings into account to achieve an optimal balance between the duration of heat shock exposure and the attainment of the desired effect.

References

Barshes NR, Wyllie S, Goss JA (2005). Inflammation-mediated dysfunction and apoptosis in pancreatic islet transplantation: implications for intrahepatic grafts. J Leukoc Biol. 77(5): 587-597.

Baumeister S, Ofer N, Kleist C, Terne P, Opelz G, Gebhard MM, Germann G, Heitmann C (2004). Reduction of skeletal muscle injury in composite tissue allotransplantation by heat stress preconditioning. Plast Reconstr Surg. 114(7): 1832-1841.

Bouchentouf M, Benabdallah BF, Tremblay JP (2004). Myoblast survival enhancement and transplantation success improvement by heat-shock treatment in mdx mice. Transplantation. 77(9): 1349-1356.

Chen CP, Lee YJ, Chiu ST, Shyu WC, Lee MY, Huang SP, Li H (2006). The application of stem cells in the treatment of ischemic diseases. Histol Histopathol. 21(11): 1209-1216.

Cowan CA, Klimanskaya I, McMahon J, Atienza J, Witmyer J, Zucker JP, Wang S, Morton CC, McMahon AP, Powers D, Melton DA (2004). Derivation of embryonic stem-cell lines from human blastocysts. N Engl J Med. 350(13): 1353-1356.

Heng BC, Ye CP, Liu H, Toh WS, Rufaihah AJ, Yang Z, Bay BH, Ge Z, Ouyang HW, Lee EH, Cao T (2006). Loss of viability during freeze-thaw of intact and adherent human embryonic stem cells with conventional slow-cooling protocols is predominantly due to apoptosis rather than cellular necrosis. J Biomed Sci. 13(3): 433-445.

Jayakumar J, Suzuki K, Khan M, Smolenski RT, Farrell A, Latif N, Raisky O, Abunasra H, Sammut IA, Murtuza B, Amrani M, Yacoub MH (2000). Gene therapy for myocardial protection: transfection of donor hearts with heat shock protein 70 gene protects cardiac function against ischemia-reperfusion injury. Circulation. 102(19 Suppl 3): III302-6.

Kim HP, Morse D, Choi AM (2006). Heat-shock proteins: new keys to the development of cytoprotective therapies. Expert Opin Ther Targets. 10(5): 759-769.

Laflamme MA, Gold J, Xu C, Hassanipour M, Rosler E, Police S, Muskheli V, Murry CE (2005). Formation of human myocardium in the rat heart from human embryonic stem cells. Am J Pathol. 167(3): 663-671.

Li X, Chen H, Epstein PN (2004). Metallothionein protects islets from hypoxia and extends islet graft survival by scavenging most kinds of reactive oxygen species. J Biol Chem. 279(1): 765-771.

Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 65(1-2): 55-63.

Nakamura Y, Yasuda T, Weisel RD, Li RK (2006). Enhanced cell transplantation: preventing apoptosis increases cell survival and ventricular function. Am J Physiol Heart Circ Physiol. 291(2): H939-H947.

Nathwani AC, Benjamin R, Nienhuis AW, Davidoff AM (2004). Current status and prospects for gene therapy. Vox Sang. 87(2): 73-81.

Redaelli CA, Tien YH, Kubulus D, Mazzucchelli L, Schilling MK, Wagner AC (2002). Hyperthermia preconditioning induces renal heat shock protein expression, improves cold ischemia tolerance, kidney graft function and survival in rats. Nephron. 90(4): 489-497.

Redaelli CA, Wagner M, Kulli C, Tian YH, Kubulus D, Mazzucchelli L, Wagner AC, Schilling MK (2001). Hyperthermia-induced HSP expression correlates with improved rat renal isograft viability and survival in kidneys harvested from non-heart-beating donors. Transpl Int. 14(6): 351-360.

Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation *in vitro*. Nat Biotechnol. 18(4): 399-404.

Scharschmidt T, Lo B (2006). Clinical trial design issues raised during recombinant DNA advisory committee review of gene transfer protocols. Hum Gene Ther. 17(4): 448-454.

Terajima H, Thiaener A, Hammer C, Messmer K, Yamamoto Y, Yamaoka Y (2000). Attenuation of hepatic microcirculatory failure during in situ xenogeneic rat liver perfusion by heat shock preconditioning. Transplant Proc. 32(5): 1111.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998). Embryonic stem cell lines derived from human blastocysts. Science. 282(5391): 1145-1147.

Vilaboa N, Voellmy R (2006). Regulatable gene expression systems for gene therapy. Curr Gene Ther. 6(4): 421-438.

Watanabe J, Kushihata F, Matsumoto K, Honda K, Matsuda S, Kobayashi N (2005). Downregulation of cytokine release by heat preconditioning of livers used for transplantation in rats. Dig Dis Sci. 50(10): 1823-1828.

Yamagami K, Enders G, Schauer RJ, Leiderer R, Hutter J, Yamamoto Y, Yamaoka Y, Hammer C, Messmer K (2003). Heat-shock preconditioning protects fatty livers in genetically obese Zucker rats from microvascular perfusion failure after ischemia reperfusion.c Transpl Int. 16(8): 456-463.