Stress response genes are suppressed in mouse preimplantation embryos by granulocyte-macrophage colony-stimulating factor (GM-CSF)

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**BACKGROUND:** Granulocyte-macrophage colony-stimulating factor (GM-CSF) is known to promote the development and survival of human and mouse preimplantation embryos; however, the mechanism of action of GM-CSF in embryos is not defined.

**METHODS:** Mouse blastocysts were cultured from zygote stage in vitro with and without recombinant mouse GM-CSF (rmGM-CSF), and in vivo developed blastocysts were flushed from Csf2 null mutant and wild-type mice. The effect of GM-CSF on blastocyst expression of stress response and apoptosis genes was evaluated by microarray, qPCR and immunochemistry.

**RESULTS:** Microarray analysis of the gene transcription profile showed suppression of stress response and apoptosis gene pathways in blastocysts exposed to rmGM-CSF in vitro. qPCR analysis confirmed that rmGM-CSF inhibited expression of heat shock protein (HSP) and apoptosis pathway genes Cbl, Hspa5, Hsp90aa1, Hsp90ab1 and Gas5 in in vitro blastocysts. Immunocytochemical analysis of HSP 1 (HSPA1A/1B; HSP70), BAX, BCL2 and TRP53 (p53) in in vitro blastocysts showed that HSPA1A/1B and BCL2 proteins were less abundant when embryos were cultured with rmGM-CSF. BAX and TRP53 were unchanged at the protein level, but Bax mRNA expression was reduced after GM-CSF treatment. In in vivo developed blastocysts, Csf2 null mutation caused elevated expression of Hsp68 but not other stress response genes.

**CONCLUSIONS:** We conclude that GM-CSF inhibits the cellular stress response and apoptosis pathways to facilitate embryo growth and survival, and the protective effects of GM-CSF are particularly evident in in vitro culture media, whereas in vivo other cytokines can partly compensate for absence of GM-CSF.

**Key words:** blastocyst / GM-CSF / heat shock proteins / stress response / apoptosis

**Introduction**

As the preimplantation embryo traverses the female reproductive tract and develops from the zygote to blastocyst stage, it experiences fluctuations in the physiochemical composition of its extracellular environment, including the availability of nutrients, growth factors and cytokines. Growth factors and cytokines mediate signaling between the maternal tissues and the embryo, and act in the embryo to modulate implantation competence and post-implantation development (Hardy and Spanos, 2002; O’Neill, 2008).

One cytokine that promotes preimplantation embryo development is granulocyte-macrophage colony-stimulating factor (GM-CSF), a cytokine originally identified as a regulator of the proliferation, differentiation and activation of myeloid hematopoietic cells (Ruef and Coleman, 1990; Robertson, 2007). GM-CSF is secreted by epithelial cells lining the oviduct and the uterus, and in mice its synthesis is up-regulated by estrogen and seminal fluid during early pregnancy (Robertson et al., 1992). Similar patterns of expression are observed in women where GM-CSF expression fluctuates over the course of the menstrual cycle (Zhao and Chegini, 1994; Giacomini et al., 1995). In the uterus, it is one of several cytokines and chemokines that influence the immune response to pregnancy through controlling the infiltration and activation of leukocyte populations (Robertson et al., 2000).

In vitro studies indicate that GM-CSF is a potent embryotrophic factor with survival- and development-promoting effects on both mouse and human embryos (Sjoblom et al., 1999; Robertson et al.,...
2001). Blastocyst stage embryos express Csf2ra mRNA encoding the GM-CSF receptor α (GM-Rα) subunit, but Csf2 mRNA encoding GM-CSF is not expressed (Robertson et al., 2001; Sjoblom et al., 2002), suggesting that in vivo, preimplantation embryos receive GM-CSF secreted from maternal uterine epithelial cells in a paracrine manner. The most obvious effect of GM-CSF on human and mouse blastocysts in vitro is an increased number of blastomeres (Sjoblom et al., 1999; Robertson et al., 2001), and blastocysts recovered from mice with a null mutation in the Csf2 gene have fewer cells (Robertson et al., 2001). Embryo transfer experiments show that GM-CSF exerts long-term programming effects in preimplantation embryos, with addition of this cytokine to culture media protecting embryos from later adverse effects of in vitro culture including fetal growth restriction and incidence of metabolic disorders in adult progeny (Sjoblom et al., 2005).

The means by which GM-CSF exerts its embryotrophic and programming influence in embryos is not defined, but several factors point to a mechanism involving protection of embryos from cell stress. Increased cell accumulation in the presence of GM-CSF suggests that this cytokine influences the rates of cell division, differentiation and/or cell death that accompany progression from zygote to blastocyst, events that are highly sensitive to disruption by environmental stressors (Xie et al., 2008). Embryo culture, particularly in suboptimal culture media, exerts cell stress in developing embryos (Wang et al., 2005), which results in fewer blastomeres due equally to retarded progression through the cell cycle and elevated incidence of apoptosis (Xie et al., 2006, 2007). Stress factors in vitro are proposed to include growth factor and cytokine deprivation (O’Neill, 2008), metabolic and substrate deficiency (Leese et al., 1998; Leese, 2002) and oxidative stress (Nasr-Esfahani and Johnson, 1991). Evidence that GM-CSF influences embryo metabolism comes from experiments showing increased glucose uptake in mouse blastocysts cultured with GM-CSF (Robertson et al., 2001). TUNEL staining in in vitro cultured human embryos shows that GM-CSF protects blastomeres from programmed cell death (apoptosis), particularly in the inner cell mass (ICM) (Sjoblom et al., 2002), and mouse embryos cultured with GM-CSF also show evidence of reduced apoptosis (Behr et al., 2005; Desai et al., 2007).

Activation of the apoptosis pathway is part of the adaptive response to culture stress in embryos where it presumably removes damaged cells (Brison and Schultz, 1997; Hardy, 1999). Apoptosis regulators are divided into suppressor, inducer, and executor molecules. Well-described members of these families are encoded by the Bcl2, Bax and Trp53 genes, respectively. Anti-apoptotic Bcl2 and pro-apoptotic BAX proteins are homologs from the Bcl-2 apoptosis regulator family and are critical factors in determining cell survival. Transformation-related protein (TRP53; p53) is a key inducer of cell cycle arrest and/or apoptosis in cells with fragmented DNA (Agarwal et al., 1998). Culture stress is known to activate expression of Bcl-2 family genes and the TRP53 stress response pathways in cultured mouse (Jurisicova et al., 1998a, b; Li et al., 2007) and human embryos (Wells et al., 2005).

Environmental insults to cells also induce activation of the heat shock protein (HSP) or stress response pathway, which acts to limit cell damage and facilitate recovery to help cells survive stress. HSPs are a group of highly conserved proteins which have homeostatic functions in normal cells as molecular chaperones that control protein folding, transport and degradation (Kregel, 2002). Several HSP genes are induced in response to cellular stressors to protect cells from cytotoxic damage by refolding and stabilizing damaged proteins (Kregel, 2002; Beere, 2004). HSPs interact with and modulate the apoptosis pathway, e.g. by inhibiting activity of pro-apoptotic Bcl-2 family proteins (Kregel, 2002; Beere, 2004), and influence commitment to either cell death or survival, depending on the severity of cell damage. HSPs comprise an essential part of the embryo response to cell stress (Leese et al., 1998), with constitutive and inducible forms of HSPs expressed from the onset of genome activation in embryos (Dvorak et al., 1995; Neuer et al., 2000). Mild thermal, oxidative and osmotic stress or culture ex vivo is sufficient to induce HSPs from as early as the blastocyst stage in mouse embryos (Wittig et al., 1983; Edwards et al., 1995; Luft and Dix, 1999) as well as in cow and pig embryos (Edwards et al., 1997; Bernardini et al., 2004).

The aims of this study were to investigate the effect of GM-CSF on expression of genes involved in the cellular stress response and apoptosis in mouse blastocysts. Both microarray and quantitative real-time PCR (qPCR) were utilized in in vitro cultured embryos and in vivo developed embryos from Cf2T mouse null mutant mice to quantify the effect of this cytokine on transcription of HSP and apoptosis genes. Proteins mediating the stress response including HSP 1 (isoforms A and B; HSPA1A/1B, also known as HSP70), BAX, BCL2 and TRP53 (p53) were assessed using immunohistochemistry in in vitro cultured blastocysts. The results indicate that GM-CSF acts in blastocysts by protecting blastomeres from the adverse effects of cell stress and that these effects of GM-CSF are particularly evident in the in vitro culture environment.

**Materials and Methods**

**Mice and mating protocols**

Pre-pubertal (3 week) CBA × C57BL/6 F1 (CBA F1) female mice mated with stud CBA F1 males were used for in vitro experiments, whereas adult (8–12 week) female wild-type (WT) C57BL/6 mice or C57BL/6 mice homozygous for a null mutation in the Cf2T gene encoding GM-CSF (Cf2T−/− mice) (Robertson et al., 1999) mated with stud C57BL/6 mice were used for the in vivo experiments. CBA F1 mice were obtained from the Laboratory Animal Services, University of Adelaide and the Animal Resource Centre, Western Australia (ARC), whereas Cf2T−/− and WT mice were bred in the Animal House, University of Adelaide. The animals were provided with food and water ad libitum and were housed in a specific pathogen-free facility under 12 h light:12 h dark cycle and were utilized according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes with approval from the University of Adelaide Animal Ethics Committee.

For natural matings, naturally cycling adult WT or Cf2T−/− mice were placed 2 per cage with individual males of the same genotype. For superovulation, pre-pubertal CBA F1 females were primed at 1230 h with 5 IU PMSG (Folligon, Intervet, Victoria, Australia) followed 46–48 h later with 5 IU hCG (Chorulon, Intervet) and then placed 1 per cage with males. The day of sighting a vaginal plug was designated day 1 post-coitum (pc). Mated females were separated from males on day 1 pc.

**Embryo culture media**

Media for embryo culture included G1.2 and G2.2, as well as MOPS handling media, were prepared in-house by a central laboratory facility according to published formulations (Gardner and Lane, 2004). Human serum was added to a final concentration of 10% in all media. Culture media included L-15 (Gibco, Invitrogen, Victoria, Australia) supplemented with 10% fetal calf serum (FCS), antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin), 0.1% gentamycin and 0.5% fungizone (Gibco, Invitrogen, Victoria, Australia) as in-house defined formulations (Gardner and Lane, 2004). Human serum served as the primary culture medium for all in vitro experiments. Human serum was added to a final concentration of 10% in all media. Culture media included L-15 (Gibco, Invitrogen, Victoria, Australia) supplemented with 10% fetal calf serum (FCS), antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin), 0.1% gentamycin and 0.5% fungizone (Gibco, Invitrogen, Victoria, Australia) as in-house defined formulations (Gardner and Lane, 2004). Human serum served as the primary culture medium for all in vitro experiments.
were euthanized at 1000 h on day 4 pc. The uterus was dissected and generated blastocysts, naturally mated WT and were used for cell number analysis or

Mouse embryo collection and in vitro culture
To generate in vitro cultured blastocysts, superfused mice were euthanized at 12:00 h on day 1 pc and the oviducts were excised. Putative zygotes were flushed from the oviducts and the cumulus cells were removed using 200 U/ml hyaluronidase in MOPS for several minutes (Sigma Chemical Company, St Louis, MO, USA). The embryos were then allocated randomly to culture in either G1.2 or G1.2 containing GM-CSF, from culture days 1–3. Embryos were then transferred to G2.2 media with or without GM-CSF from culture days 3–5. In both phases, embryos were cultured in groups of 10 embryos per 20 μl drop of culture media, under paraffin oil. In some experiments, simple media with high and low HSA were used in place of G1.2 and G2.2. Embryo development was assessed by standard morphological criteria daily (Hogan et al., 1986) and on the morning of culture day 5, blastocysts were used for cell number analysis or in situ protein imaging by immuno-fluorescence. Cultured blastocysts were also collected in groups of 30 blastocysts per tube for qPCR analysis of gene expression. For in vivo generated blastocysts, naturally mated WT and C57Bl/6 female mice were euthanized at 1000 h on day 4 pc. The uterus was dissected and blastocysts were flushed from both uterine horns, then frozen in groups of 30 blastocysts per tube for qPCR analysis.

Total cell count and differential staining
Cultured blastocysts collected on the morning of culture day 5 were stained using propidium iodide (PI) to determine total cell number. Blastocysts were permeabilized in 0.05% Triton X-100 (Sigma) then washed in MOPS handling media before incubation in 50 μg/ml PI (Sigma) in MOPS for 10 min at 37°C.

Differential staining was then performed using goat anti-OCT4 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) (1 : 100 in PBS–BSA) and rabbit anti-CDX2 (gift of J. Collins and T. Fleming, University of Southampton, UK; 1 : 100). OCT4 and CDX2 are localized in ICM and TE cells, respectively. Reactivity was detected with donkey anti-goat-TRITC (Jackson ImmunoResearch Labs, West Grove, PA, USA) (1 : 200 in PBS–BSA) and swine anti-rabbit-FITC (DAKO Corporation, Glostrup, Denmark) (1 : 200 in PBS–BSA). Stained blastocysts were examined using confocal microscopy (Nikon C1 confocal scanning head, Nikon TE2000E, Nikon Corporation, Tokyo, Japan) at ×40 magnification and a single 10 μm equatorial section was captured for each blastocyst. The total number of PI positive cells was counted (total cell number), and each PI positive cell was classified as either ICM or TE on the basis of predominant expression of either OCT4 or CDX2, respectively.

Microarray
RNA was extracted from two pools of 140 blastocysts, cultured as above in G1.2 and G2.2 with or without 2 ng/ml rmGM-CSF, using a RNeasy Micro Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer’s instructions. RNA was processed at the Australian Genome Research Facility (AGRF) in Melbourne, Australia for two-cycle target labeling and hybridization to Affymetrix GeneChip™ Mouse genome 430_2.0 chips (Affymetrix, Santa Clara, USA). This 3’ expression microarray chip is comprised >45 000 probe sets covering over 39 000 transcripts and variants (for complete list, see http://www.affymetrix.com). RNA integrity analysis, hybridization and washing were performed according to the manufacturer’s instructions. CEL files for each chip were scaled globally to a target intensity of 150 for generation of CHP files to assess quality control parameters. Data were analyzed using the algorithm MASS in GeneChip Operating Software (GCSOS v1.4, Affymetrix), and the GCRMA algorithm (Wu et al., 2004) implemented by the Bioconductor (http://www.bioconductor.org) package affy (Smyth, 2004) to quantify fold change in gene expression. Effects of treatment on gene pathways or families were identified using Pathway Express (Draghici et al., 2007) and OntoExpress Analysis (Draghici et al., 2003).

RNA isolation, reverse transcription and qPCR
RNA was extracted from groups of 30 blastocysts collected from in vitro cultured or flushed from tracts after in vivo development, using RNeasy Micro Kits (Qiagen Inc.) according to the manufacturer’s instructions. The isolated RNA in a final volume of 12 μl was stored at −80°C. Then 3 ml of RNA was reverse-transcribed with Superscript III (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions, with 200 ng random sequence oligohexamers (Geneworks, Adelaide, Australia) and 500 ng oligo-dT18 (Promega, Madison, WI, USA) containing 10 μl of SYBR Green master mix (Applied Biosystems), 5 μl of H2O. 1 μl each of 10 μM forward and reverse primers (see Table I for primer sequences, GenBank accession numbers, amplicon sizes and melting temperatures) and 3 μl of cDNA template or water (non-template negative control). qPCR reactions were then performed in a final volume of 20 μl, containing 10 μl of SYBR Green master mix (Applied Biosystems), 5 μl of H2O. 1 μl each of 10 μM forward and reverse primers (see Table I for primer sequences, GenBank accession numbers, amplicon sizes and melting temperatures) and 3 μl of cDNA template or water (non-template negative control). qPCR conditions were 10 min at 95°C followed by 40 cycles of 95°C for 15 s and 60°C for 45 s, using a Rotorgene 6000 (Corbett Life Sciences, Sydney, Australia). After DNA amplification, PCR products were subjected to melt analysis to confirm amplicon purity. Reaction products were further analyzed by electrophoresis in 2% agarose (Promega, Madison, WI, USA) gels containing 0.2 μg/ml ethidium bromide followed by visualization over an ultraviolet light box and image capture using a Kodak digital camera to confirm expected amplicon size. For each gene of interest, the relative mRNA expression was calculated by comparison with standard curves generated from serial dilutions of pooled day 13 mouse placental cDNA, and normalized to the average abundance of mRNA for two reference genes Ywhaz and Sdf1. Preliminary experiments showed that the treatments did not alter expression of these genes.

Immunofluorescence staining
For immunolocalization of BAX, BCL2 and HSPA1A/1B staining, blastocyst stage embryos were fixed in freshly prepared 4% paraformaldehyde (Sigma) in PBS for 1 h at RT, washed in PBS±2% BSA (Sigma) (PBS–BSA, by pipetting through three sequential 50 μl drops) and transferred to 0.1 M glycine (Sigma) in PBS–BSA for 5 min at RT to neutralize aldehydes. After washing in PBS–BSA (as above) embryos were permeabilized in 0.25% Triton X-100 in PBS (0.25% TX-PBS) for 30 min at RT, then washed again in PBS–BSA and incubated in FcBlock (BD Biosciences Pharmingen, San Jose, CA, USA; 50 μg/ml in PBS–BSA) for 30 min at RT. They were then incubated overnight at 4°C with rabbit anti-BAX (Santa Cruz Biotechnology, Santa Cruz, CA, USA; diluted 1 : 50 in PBS–BSA), rabbit anti-BCL2 (Santa Cruz; 1 : 50) or mouse anti-HSPA1A/HSPA1B (anti-HSPA70; Stressgen, Ann Arbor, MI, USA; 1 : 50). Negative controls included the irrelevant species-matched antibodies rabbit anti-human CSF2RB (Santa Cruz; 1 : 25) or mouse anti-human CD1A (BD Biosciences; 1 : 200) or PBS–BSA alone. Embryos were then washed and incubated.
# Table I  Primer sequences and PCR conditions for quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene name (synonym)*</th>
<th>Protein name</th>
<th>Primer sequence (5’–3’)</th>
<th>Amplicon size (bp)</th>
<th>GenBank accession</th>
<th>Amplicon Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aifm1 (Aif, Pdcd8)</td>
<td>AIFM1</td>
<td>F: CGCTAAGGCTACGCTGCA; R: CAACTTGCGGCCAACATTCTACA</td>
<td>101</td>
<td>NM_012019</td>
<td>78.5</td>
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<tr>
<td>Bax</td>
<td>BAX</td>
<td>F: GAGATGAACTGGAGAGGAG; R: GCAAAGTTGAAGGCGAAACCA</td>
<td>151</td>
<td>NM_007527</td>
<td>84.5</td>
</tr>
<tr>
<td>Bcl2 (Bcl-2)</td>
<td>BCL2</td>
<td>F: GGAGAGCGTCAACAGGGAG; R: CAGGCCAGAGAATCAAACAGAG</td>
<td>169</td>
<td>NM_009741</td>
<td>83.5</td>
</tr>
<tr>
<td>Chi</td>
<td>CBL</td>
<td>F: AAGCCTAAGGAGCTGCTG; R: AGCTTTGAGCTGCTGCTGAAC</td>
<td>82</td>
<td>NM_007619</td>
<td>80.5</td>
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<tr>
<td>Ccar1 (Carp-1)</td>
<td>CCAR1</td>
<td>F: CTTATCTACAGTATGATGATATCCACACT; R: CTTGGATTTCATCTGCTGTTTAC</td>
<td>82</td>
<td>NM_026201</td>
<td>72.5</td>
</tr>
<tr>
<td>Dusp14 (Mkp6)</td>
<td>DUSP14</td>
<td>F: GCAAACGCGCCGCTGCCCA; R: CAGGGGCAAGCAGCAGAG</td>
<td>139</td>
<td>NM_019819</td>
<td>83.5</td>
</tr>
<tr>
<td>Gas5</td>
<td>GAS5</td>
<td>F: ATGCAGTGACTGCCCTTGT; R: GTCTTTGATATTGAAGGCAAAC</td>
<td>80</td>
<td>NR_002840</td>
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<td>Gas2l3</td>
<td>GAS2L3</td>
<td>F: TGCTCAGGCCAAAGCAATG; R: AACACGTGGTGCTCCTGCTGTTG</td>
<td>101</td>
<td>NM_001033331</td>
<td>82.5</td>
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<tr>
<td>Hif1α</td>
<td>HIF1A</td>
<td>F: CCTCCGTATTAGCAGTGAAGCT; R: GCCTTTGTATGGAGCAAGGACACTTTC</td>
<td>101</td>
<td>NM_010431</td>
<td>79.5</td>
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<tr>
<td>Hsp90aa1 (Hspca, Hsp86)</td>
<td>HSP90AA1</td>
<td>F: AATGCTTAGAATATTTCTG; R: GTCTTATGGGAGGAGCTAATCCTTC</td>
<td>114</td>
<td>NM_010480</td>
<td>72.5</td>
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<tr>
<td>Hsp90ab1 (Hspcb, Hsp84)</td>
<td>HSP90AB1</td>
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<td>113</td>
<td>NM_008302</td>
<td>81.5</td>
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<tr>
<td>Hsp60 (Hsp70–3) and Hsp110 (Hsp70–1)</td>
<td>HSPA1A and HSPA1B</td>
<td>F: ATGGCAAGGCGCAACTGCC; R: CTCGACTTTGCTCCTGCTGCT</td>
<td>183</td>
<td>NM_010479/NM_010478</td>
<td>88.5</td>
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<tr>
<td>Hsp70 (Hsp70RY)</td>
<td>HSPA4</td>
<td>F: TGGAGTGAATAGAATAGCAGCT; R: TGCAATTTTCTGAGCTCTCTTAATT</td>
<td>114</td>
<td>NM_008300</td>
<td>78.0</td>
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<tr>
<td>Hsp9 (mot-2)</td>
<td>HSPA9</td>
<td>F: CAGGCGACATCTTTCCCTACAG; R: CCAAGGACAGAATTTACCTCAAA</td>
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<td>NM_010481</td>
<td>79.5</td>
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<td>Hsp10 (Hsp105/110)</td>
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<td>NM_013559</td>
<td>76.8</td>
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<td>Sdha</td>
<td>SDHA</td>
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<td>185</td>
<td>NM_023281</td>
<td>83.5</td>
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<td>Trp53 (p53)</td>
<td>TRPS3</td>
<td>F: TTATGAGCGCCACCCAGGCG; R: GTACCGCGGTGCTCTGCTGCTG</td>
<td>195</td>
<td>NM_011640</td>
<td>83.5</td>
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<td>Ywhaz</td>
<td>YWHAZ</td>
<td>F: AACACGGTCTTGATGAGCCA; R: CATCTCTGTTGGATCTCGGATG</td>
<td>129</td>
<td>NM_011740</td>
<td>77.5</td>
</tr>
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</table>

Tm, melting temperature.
*Gene symbols and synonyms are according to nomenclature specified by Mouse Genome Informatics at http://www.informatics.jax.org/.
#Reference genes.
with the relevant detection reagent, swine anti-rabbit-FITC (DAKO Corporation, Glostrup, Denmark; diluted 1:200 in PBS–BSA) or donkey anti-rabbit-FITC (Chemicon, Temecula, CA, USA; diluted 1:200 in PBS–BSA). Embryos were washed and finally counterstained with PI (50 μg/ml in PBS–BSA, 15 min) to stain all cell nuclei.

For TRP53 staining, embryos were fixed in freshly prepared 4% paraformaldehyde in PBS for 30 min at RT, then washed in 0.25% TX-PBS and transferred to 0.1 M glycine (Sigma) in PBS–BSA for 5 min at RT. Embryos were then washed in 0.25% TX-PBS, permeabilized in 0.25% TX-PBS for 15 min, washed again and blocked in 5% normal donkey serum in PBS (Jackson ImmunoResearch Labs) at RT for 30 min. After a further wash, embryos were incubated overnight at 4°C with sheep anti-TRP53 antibody (Calbiochem, San Diego, CA, USA; 1:100 in 0.25% TX-PBS), or dilution buffer alone (negative control). Embryos were then washed and incubated with anti-sheep IgG-FITC (R&D Systems; 1:150 in 0.25% TX-PBS), and counterstained with PI as above.

All stained blastocysts were examined by confocal microscopy and images were captured as above. Fluorescence intensity was quantified using Adobe Photoshop CS3 (Adobe Systems Inc., San Jose, CA, USA). A fluorescence intensity index for the ICM and TE of individual blastocysts was calculated as the mean fluorescence intensity of measurements made of each of eight TE cells and eight ICM cells, or the mean value for all 16 cells, for each blastocyst.

Statistical analysis

All data on cell counts, embryo development, fluorescence intensity and gene expression were analyzed by independent sample t-tests after confirmation of normal distribution of data by Shapiro–Wilk test of normality. When more than two groups were compared, data were analyzed by one-way ANOVA and post hoc Sidak t-test. All statistical analyses were performed using SPSS (SPSS Inc., Chicago, IL, USA), with differences considered significant when \( P < 0.05 \). Outlier values defined as \( >2 \) SD greater or lesser than the mean were excluded from the analyses, as indicated.

Results

GM-CSF promotes cell number in both ICM and TE of blastocysts

To generate pools of embryos for analysis of gene expression and protein synthesis, a total of 3572 1-cell zygotes were cultured from 1-cell to blastocyst in the presence or absence of 2 ng/ml rmGM-CSF. In all experiments, embryo development was assessed daily over the 4 day culture period. A consistent increase in the proportion of 1-cell development to morula and blastocyst was observed daily over the 4 day culture period. A consistent increase in the proportion of 1-cell development to morula and blastocyst was observed daily over the 4 day culture period. GM-CSF promotes cell number in both the ICM and TE.

Differential staining revealed OCT4+ ICM cells and CDX2+ TE cells, with the expected pattern of nuclear staining (Fig. 1A–F). Co-localization of both OCT4 and CDX2 proteins was evident in a subset of cells that were apparently incompletely differentiated (Fig. 1C and F), but no effect of this GM-CSF on the incidence of dual-labeled cells was noted. Blastocysts cultured with rmGM-CSF were found to have increased numbers of both ICM and TE cells (both \( P < 0.02 \)), giving rise to greater total cell numbers (\( P < 0.01 \)) (Fig. 1K). These results confirm previous observations (Robertson et al., 2001) and indicate that increased blastomere number after culture with GM-CSF is attributable to increases in both the ICM and TE.

GM-CSF alters blastocyst transcription profile

To explore the molecular basis of GM-CSF regulation of blastocyst cell number, we began by performing a microarray experiment on mRNA extracted from two pools of 140 blastocyst stage embryos cultured in the presence or absence of rmGM-CSF. Analysis of CHP files indicated that quality-control parameters were within expected ranges for both data sets, with scale factor = 0.93 and 1.52; background = 81.4 and 75.7; % probes present = 27.4 and 19.2 and Actin 3′/5′ = 2.1 and 3.6, for control and GM-CSF groups, respectively. When data were analyzed using the MAS5 and GCRMA algorithms, 446 probe sets with log fold change \( \geq 1.0 \) were common to both analyses including 15 probe sets that showed a significant increase with GM-CSF, and 431 probe sets that showed a significant decrease. Analysis of gene pathways or families using Pathway Express (KEGG) identified the apoptosis pathway as differentially regulated by GM-CSF (\( P = 0.019 \)). Several genes involved in cellular stress and apoptosis were found to be down-regulated in the presence of GM-CSF (Table II).

Among the most down-regulated genes were the tyrosine kinase signaling regulator Cbl, the phosphatase enzyme Dusp14, the HSPs Hsp90aa1 and Hsp5 and the cell division cycle and apoptosis regulators Ccar1 and Gas5 (for gene names in full and fold changes, see Table II).

GM-CSF suppresses stress response gene expression

To more precisely quantify the effect of GM-CSF on expression of specific stress response and apoptosis genes in in vitro cultured blastocysts, qPCR was performed on mRNA extracted from blastocysts cultured with and without GM-CSF. Of 13 genes of interest that showed >2-fold changes in expression in the microarray experiment, 5 were confirmed as down-regulated in blastocysts cultured with GM-CSF, including Cbl (\( P = 0.005 \)) (Fig. 2A), Hspa5 (\( P = 0.033 \)) (Fig. 2C), Hsp90aa1 (\( P = 0.033 \)) (Fig. 2D), Gas5 (\( P = 0.011 \)) (Fig. 2F) and Hsp90ab1 (\( P = 0.020 \)) (Fig. 2G). An additional gene Aifm1 showed a trend to decreased expression in response to GM-CSF (\( P = 0.091 \)) (Fig. 2H). Seven genes that showed smaller changes in the microarray experiment were not different when evaluated by qPCR, including Dusp14 (Fig. 2B), Ccar1 (Fig. 2E) and Gas2l3, Hsphi1, Hsap9, Hif1a and Hspa4 (data not shown).

In addition, expression of mRNA encoding the well-described HSP HSPA1A/1B was evaluated. Using primer sequences targeted to a consensus sequence present in both Hsap9a and Hsap9b genes, no effect of GM-CSF on Hsap9a / Hsap9b mRNA expression was detected (Fig. 2I). Expression of mRNAs encoding key apoptosis regulators Bax, Bcl2 and Trp53 mRNAs was also quantified. Embryos cultured with GM-CSF showed decreased expression of Bax mRNA expression compared with embryos cultured without GM-CSF (Fig. 2J). No effect of GM-CSF on Bcl2 or Trp53 mRNA expression was detected (Fig. 2K and L, respectively).
GM-CSF reduces HSPA1A/1B protein accumulation in blastocysts

Immunohistochemistry was utilized to investigate whether GM-CSF alters abundance of the HSP HSPA1A/1B in blastocysts. Diffuse staining throughout the cytoplasm and the nucleus of both ICM and TE cells was evident in all blastocysts cultured with and without GM-CSF (Fig. 3). Quantifying the intensity of staining revealed that blastocysts cultured in control media (Fig. 3A–C) contained more HSPA1A/1B than blastocysts cultured in GM-CSF (Fig. 3D–F). HSPA1A/1B intensity was reduced in both the ICM cells (P = 0.025) and TE cells (both P = 0.030) of blastocysts cultured in GM-CSF (Fig. 3J).

GM-CSF reduces BCL2, but not BAX or TRP53 protein accumulation in blastocysts

Immunohistochemistry was also utilized to investigate the effect of GM-CSF on abundance of key regulators of apoptosis BAX, BCL2 and TRP53. BAX was present in both ICM and TE cells of all blastocysts cultured with and without GM-CSF (Fig. 4). There was no effect of GM-CSF treatment on the intensity of BAX staining in blastocysts (Fig. 4J). BCL2 protein was present in both ICM and TE cells of all blastocysts with a cytoplasmic and mostly perinuclear distribution (Fig. 5). The intensity of BCL2 was significantly reduced in TE cells (P = 0.041) and total cells (P = 0.039), but not in the ICM cells of blastocysts cultured with rmGM-CSF (Fig. 5J). TRP53 immunolocalization was performed on blastocysts cultured in simple medium, or in G1.2/G2.2 medium, with and without GM-CSF. Nutrient deficient simple medium was used as a positive control since preliminary experiments showed this medium elevated the abundance of TRP53 in blastocysts. TRP53 protein was present in both TE and ICM cells of all blastocysts and the staining was cytoplasmic and mostly perinuclear (Fig. 6). Blastocysts cultured in simple medium showed more intense expression of TRP53 than other groups, but there was no significant difference between control and GM-CSF-cultured blastocysts (Fig. 6M).

Csf2−/− null mutation alters blastocyst gene expression

To investigate the effect of GM-CSF in the natural 'in vivo' environment, qPCR was performed on mRNA extracted from blastocysts flushed from WT (GM-CSF replete) and Csf2−/− (GM-CSF deficient) mice. Of the 12 genes quantified in in vitro cultured embryos, only one gene was elevated in Csf2−/− blastocysts. Hspf1 expression was increased 2.2-fold from 0.31 ± 0.10 to 0.67 ± 0.20 arbitrary mRNA units in WT
GM-CSF inhibits stress response in embryos

Table II Genes from apoptosis and cell stress response pathways identified as differentially expressed in embryos cultured with or without rmGM-CSF by Affymetrix mouse 430-2 microarray analysis

<table>
<thead>
<tr>
<th>Gene symbol*</th>
<th>Name</th>
<th>Fold changea</th>
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<tbody>
<tr>
<td>Cbl</td>
<td>Casitas B-lineage lymphoma</td>
<td>-20.8</td>
</tr>
<tr>
<td>Dusp14</td>
<td>Dual specificity phosphatase 14</td>
<td>-8.24</td>
</tr>
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<td>Hspa5</td>
<td>Heat shock protein 5</td>
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<td>Heat shock protein 90, alpha (cytosolic), class A member I</td>
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</tr>
<tr>
<td>Ccar1</td>
<td>Cell division cycle and apoptosis regulator 1</td>
<td>-4.13</td>
</tr>
<tr>
<td>Gas5</td>
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<td>-3.92</td>
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<td>Heat shock protein 90, alpha (cytosolic), class B member I</td>
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<td>Apoptosis-inducing factor, mitochondrion-associated 1</td>
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<td>Capn7</td>
<td>Calpain 7</td>
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<tr>
<td>Dffb</td>
<td>DNA fragmentation factor, beta subunit</td>
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*Gene symbols and synonyms are according to nomenclature specified by Mouse Genome Informatics at http://www.informatics.jax.org/.

aFold change in presence of GM-CSF compared with absence of GM-CSF.

and Csf2−/− blastocysts, respectively (P = 0.010; n = 8 blastocyst pools per genotype group). All of the remaining genes, Cbl, Dusp14, Hspa5, Hsp90aa1, Ccar1, Gas5, Hsp90ab1, Gas213, Hspa9, Hif1a, Aifm1 and Hsp9, were unchanged or expressed at lower levels in Csf2−/− embryos (data not shown). Similarly, blastocyst expression of Hspa1a/Hspa1b, Bax, Bcl2 and Trp53 genes was not changed in response to Csf2 null mutation.

Discussion

Compared with the in vivo environment, in vitro culture is generally detrimental for preimplantation embryo development and can adversely affect embryo programming as reflected in later fetal and post-natal growth (Thompson et al., 2002; Sjoblom et al., 2005). The disruption in embryo homeostasis induced by culture results in adaptive responses, particularly elevated expression of stress response genes and cell death, to varying extents depending on the quality of the culture media (Jurisicova et al., 1998a, b; Hardy, 1999; Li et al., 2007). GM-CSF has been shown to improve the developmental potential of blastocysts cultured in vitro (Sjoblom et al., 2005) and this is associated with increased numbers of viable blastomeres (Robertson et al., 2001). The current investigation reveals that GM-CSF promotion of blastocyst cell accumulation in cultured mouse embryos is associated with down-regulation of the cellular stress response.

Initially, we extended our previous report of increased cell number after addition of GM-CSF to culture media, by showing that GM-CSF increases cell number in both the TE and ICM compartments. The ability of GM-CSF to expand the ICM is important as the ICM forms the fetus and the number of cells within the ICM at implantation has been linked to implantation success and pregnancy outcome (Tam, 1988; Thompson et al., 2002). This is consistent with our earlier observation of larger fetal size after transfer of embryos cultured with GM-CSF (Sjoblom et al., 2005).

Using Affymetrix genome microarrays and qPCR, several genes involved in apoptosis and cell survival were found to be down-regulated in embryos cultured in the presence of GM-CSF. We therefore investigated the effect of GM-CSF on the abundance of proteins involved in the cell stress and apoptosis response. HSPA1A/1B, BAX, BCL2 and TRP53 were evaluated as they are informative markers for apoptosis and cellular stress response pathways and are known to be induced in embryos in response to stressors including heat, oxidative and cytotoxic stress (Beere, 2004).

Most notably, HSPA1A/1B protein was less abundant in blastocysts cultured with GM-CSF. The levels of HSPA1A/1B in other cell lineages reflect the extent of environmental stress, with induction of HSPA1A/1B being a pivotal mechanism for protecting cells from damage which otherwise results in apoptosis (Huang et al., 2001; Hunt et al., 2004). HSPA1A and HSPA1B are encoded by the Hspa1a and Hspa1b mRNAs, respectively, which in mouse embryos are constitutively expressed until the 8-cell stage and then become inducible in blastocysts (Snoek et al., 1993; Perry et al., 1994). Although stress-induced HSPA1A and HSPA1B are regulated transcriptionally, we did not find evidence of GM-CSF regulation of Hspa1a and Hspa1b gene expression. This is surprising since both genes have previously been shown to be induced in mouse and cow blastocysts in response to heat shock (Wittig et al., 1983; Edwards et al., 1995, 1997; Luft and Dix, 1999). Increased capacity for inducible HSPA1A expression occurs as blastocysts reach hatching stage (Christians et al., 1997), suggesting that it may be particularly important in protecting embryos from apoptosis at this vulnerable time.
The apoptotic inhibitor BCL2 protein in embryos was reduced by GM-CSF, particularly in the TE cells. Although no change in Bcl2 mRNA was found, its expression was barely detectable in a low proportion of blastocysts, and the qPCR assay was a less sensitive indicator than BCL protein detection. In contrast, although we could not detect any effect of GM-CSF on pro-apoptotic BAX protein abundance, Bax mRNA expression was inhibited in the presence of GM-CSF. Although trends to reduced TRP53 protein and Trp53 mRNA were evident in the presence of GM-CSF, neither parameter showed a statistically significant change. These inconsistencies presumably reflect the fact that these apoptotic regulators are controlled at the transcriptional as well as post-transcriptional level, as well as the technical limitations of both experimental strategies, highlighting the value of examining both gene expression and protein levels.

From the microarray data, we selected 13 genes with evidence of GM-CSF-regulated differential expression for qPCR analysis. This investigation confirmed five genes, Hsp90aa1, Hsp90ab1, Hspa5, Gas5, and Cbl, to be down-regulated by GM-CSF. Hsp90aa1 encodes HSP90AA1, an HSP that mediates protein refolding and

**Figure 2** Effect of GM-CSF on apoptosis and stress response gene expression in blastocysts. mRNA was quantified by qRT–PCR from blastocysts cultured in control medium (n = 15 pools of 30 blastocysts) or medium with GM-CSF (n = 15 pools of 30 blastocysts), and normalized to expression of reference genes Ywhaz and Sdha. Transcripts analyzed were (A) Cbl, (B) Dusp14, (C) Hspa5, (D) Hsp90aa1, (E) Ccar1, (F) Gas5, (G) Hsp90ab1, (H) Aifm1, (I) Hspa1a/Hspa1b, (J) Bax, (K) Bcl2 and (L) Trp53. Data are mean ± SEM, and the effect of treatment was evaluated by independent sample t-test. *P < 0.05, **P < 0.005, #P = 0.091.
has been detected in primordial germ cells under normal growth conditions during mouse embryo development (Vanmuylder et al., 2002). Hsp90ab1 encodes HSP90AB1, a protein with similar function to HSP90AA1 that is involved in placental development (Vanmuylder et al., 2002). Hspa5 encodes HSPA5 or GRP78, a protein that is induced by endoplasmic reticulum (ER) stress as a key part of the ‘unfolded protein response’ (Luo et al., 2006) and required for cell proliferation and protecting the ICM during early mouse embryo development (Luo et al., 2006). Abnormal accumulation of HSPA5 and inability to resolve ER stress is associated with arrest at the...

**Figure 3** Effect of GM-CSF on HSP 1 (HSPA1A/1B) synthesis in blastocysts. (A–I) Images are shown in red (PI; A, D and G), green (anti-HSPA1A/1B; B, E and H) and merged (C, F and I) channels. Images are representation of >20 blastocysts cultured in control medium (A–C) or medium with rmGM-CSF (D–F). No staining was observed in the negative control (irrelevant primary antibody) (G–I). (J) Mean fluorescence intensity of HSPA1A/1B protein in ICM and TE cells of blastocysts cultured in control medium (n = 28), or medium with rmGM-CSF (n = 27). Data are mean ± SEM, and the effect of treatment was evaluated by independent sample t-test. *P < 0.03.

**Figure 4** Effect of GM-CSF on BAX synthesis in blastocysts. (A–I) Images are shown in red (PI; A, D and G), green (anti-BAX; B, E and H) and merged (C, F and I) channels. Images are representation of >20 blastocysts cultured in control medium (A–C) or medium with rmGM-CSF (D–F). No staining was observed in the negative control (irrelevant primary antibody) (G–I). (J) Mean fluorescence intensity of BAX protein in ICM and TE cells of blastocysts cultured in control medium (n = 32), or medium with rmGM-CSF (n = 38). Data are mean ± SEM, and the effect of treatment was evaluated by independent sample t-test. There was no significant difference between the control and GM-CSF groups.
Figure 5 Effect of GM-CSF on BCL2 synthesis in blastocysts. (A–I) Images are shown in red (PI; A, D and G), green (anti-BCL2; B, E and H) and merged (C, F and I) channels. Images are representation of >20 blastocysts cultured in control medium (A–C) or medium with rmGM-CSF (D–F). No staining was observed in the negative control (irrelevant primary antibody) (G–I). (J) Mean fluorescence intensity of BCL2 protein in ICM and TE cells of blastocysts cultured in control medium (n = 32), or medium with rmGM-CSF (n = 38). Data are mean ± SEM, and the effect of treatment was evaluated by independent sample t-test. *P < 0.05.

Figure 6 Effect of GM-CSF on TRP53 synthesis in blastocysts. (A–L) Images are shown in red (PI; A, D, G and J), green (anti-TRP53; B, E, H and K) and merged (C, F, I and L) channels. Images are representation of >20 blastocysts cultured in simple medium (A–C), control medium (D–F) or medium with rmGM-CSF (G–I). No staining was observed in the negative control (irrelevant primary antibody) (J–L). (M) Mean fluorescence intensity of TRP53 protein in ICM and TE cells of blastocysts cultured in simple medium (n = 11), control medium (n = 24) or medium with rmGM-CSF (n = 24). Data are mean ± SEM, and the effect of treatment was evaluated by independent sample t-test. There was no significant difference between the control and GM-CSF groups.
morula stage in mouse embryos of the DDK strain (Hao et al., 2009). Gs5 expression has been described in mouse preimplantation embryos from the 8-cell stage but altered expression in vitro is not evident (Fleming et al., 1997). Together these observations at the mRNA and protein level are consistent with addition of GM-CSF to culture media causing down-regulation of both the stress response and apoptosis pathways in blastocysts.

The gene most highly regulated by GM-CSF in embryos was Cbl, which encodes a 120 kDa protein Cbl that in leukocytes becomes phosphorylated and mediates downstream effects of signaling by cytokines including GM-CSF (Thien and Langdon, 2001). Cbl has at least two distinct functions, serving as an E3 ubiquitin-protein ligase involved in marking protein tyrosine kinases for proteosome degradation, and an adaptor protein involved in remodeling of the actin cytoskeleton (Thien and Langdon, 2001). Cbl has not previously been detected in mouse preimplantation embryos. In GM-CSF-dependent monocytes, v-Cbl transfection confers resistance to apoptosis induced by growth factor withdrawal, as well as inhibition of differentiation (Sinha et al., 2001). This suggests that in embryos cultured without GM-CSF, elevated Cbl expression might contribute to an adaptive response to growth factor deficiency.

Blastocyst development is impaired in Cs2+/− mice in vivo and this deficiency appears causally linked with altered growth of the surviving pups, particularly in males (Robertson et al., 1999). Only one HSP gene Hsp70 showed elevated expression in embryos recovered from Cs2−/− mice. Hsp70 encodes HSPH1, an HSP that functions as an HSP70 inducer in mammalian cells (Saito et al., 2009). High Hsp70 expression has been described in post-implantation mouse embryo development, but this gene has not previously been detected in preimplantation embryos (Hatayama et al., 1997). Our results show that although GM-CSF absence in vivo might activate the stress response pathway, the effects of GM-CSF deficiency are less apparent in vivo. The lack of effect on other stress response genes suggests that in vivo, GM-CSF deprivation is less detrimental than in vitro. This might reflect a lower degree of environmental stress in vivo than in vitro, or GM-CSF deficiency may be compensated by other maternal tract cytokines present in both WT and Cs2−/− mice. This redundancy is characteristic of hemopoietic cytokines, since factors with similar functions compensate in the absence of GM-CSF in other cell lineages (Martinez-Moczygemba and Huston, 2003). However, as Cs2−/− embryo development is clearly compromised (Robertson et al., 1999), alternative cytokine pathways must be only partially redundant and this may explain the impaired reproductive outcomes in Cs2−/− mice.

There are several growth factors present in the female reproductive tract that exert paracrine and autocrine embryotrophic effects, including leukemia inhibitory factor and epidermal growth factor (Hardy and Spanos, 2002) which like GM-CSF act through the JAK/STAT signal transduction pathway. Embryotrophic factors that utilize the phosphotyrosinolinositol-3-kinase (PI3 kinase) signaling pathway include growth hormone, insulin, insulin-like growth factor-1 (IGF1), IGFII and transforming growth factor-alpha (TGFα) (Brison and Schultz, 1997; Markham and Kaye, 2003; O’Neill, 2008). Platelet activating factor also acts through the PI3 kinase signaling pathway to potent suppress apoptosis acting via the TRP53 pathway (Jin et al., 2009). In the pig, IGF protects blastocysts from thermal stress-induced developmental arrest and apoptosis (Hansen, 2007).

Evidence that GM-CSF can largely but not completely substitute for the absence of other growth factors in vitro comes from embryo transfer experiments (Sjoblom et al., 2005). Exposure of embryos to GM-CSF prior to embryo transfer results in a fetal growth trajectory which is similar to that of in vivo-developed embryos, but GM-CSF exposure does not fully prevent the increased adiposity that is observed in adulthood after embryo development in vitro (Sjoblom et al., 2005). In other cell lineages, GM-CSF has the specific capacity to inhibit apoptosis induced via the extrinsic pathway by the cytokine tumor necrosis factor-α (Quentmeier et al., 2003), a known inducer of apoptosis in embryos (Wuu et al., 1999). This provides a potential pathway through which GM-CSF might exert specific effects on embryo stress responses.

In summary, this study furthers our understanding of how GM-CSF assists in promoting development of the mouse preimplantation embryo, showing that this cytokine acts to suppress genes involved in the cellular stress response that influence the cell cycle and apoptosis. We suggest that growth factor and cytokine deprivation is likely to contribute to the elevated level of stress response gene expression seen in cultured compared with in vivo developed embryos and that GM-CSF addition to culture media can in part alleviate this culture-induced stress. In human IVF, this has important implications since it is clear that culture-induced stress can critically influence the growth parameters of the developing embryo and impact post-implantation fetal development and long-term health (Thompson et al., 2002). Our continued verification that GM-CSF affords protection against culture-induced stress raises confidence in utilizing this cytokine in human IVF culture media, particularly in protocols where elevated stress occurs, e.g. due to embryo freezing and thawing (Sjoblom et al., 1999; Papayannis et al., 2007). However, an unresolved question is whether in human embryos, fragmentation associated with apoptosis might play a normal physiological role in selective removal of abnormal blastomerers (Hardy, 1999). Although this is certainly possible, recent studies in human embryos have shown that culture with GM-CSF does not increase the incidence of aneuploidy (Agerholm et al., 2009), indicating that any protection from apoptosis afforded by GM-CSF is unlikely to result in ‘rescue’ of abnormal embryos.

### Funding

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