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Using hyperosmolar stress to measure biologic and stress-activated protein kinase responses in preimplantation embryos

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We used hyperosmolar stress to test blastocysts for their biologic and enzymatic responses to culture stress. Embryos mount dose- and time-dependent responses to hyperosmolar stress. Biological responses included slowed cavitation and cell accumulation and increased apoptosis at increasing doses. These responses were preceded by stress-activated protein kinase (SAPK) phosphorylation and nuclear translocation consistent with its causal role. For cavitation and new cell cycle initiation, 200 mM sorbitol caused stasis. Above 200 mM, sorbitol was ultimately lethal and below 200 mM, its embryos had milder effects. Phosphorylated SAPK was induced rapidly in embryos at 0.5 h in a dose-dependent manner from 0 to 600 mM sorbitol. Higher hyperosmolarity caused a biphasic peak of phosphorylated SAPK, but there was no return to baseline through 3 h. At 24 h, a dose-dependent response persisted that was linear from 0 to 200 mM sorbitol. Hyperosmolar stress rapidly induced, within 0.5 h, phosphorylated, nuclear c-Jun and decreased phosphorylated, nuclear c-Myc in a SAPK-dependent manner. The data suggest that SAPK is induced and functions on down-stream effector molecules in a temporal and quantitative manner consistent with its function in the embryonic homeostatic response to stress. The remarkable resistance of embryos to high concentrations of sorbitol suggests that part of its homeostatic response is different from that of somatic cells.

Keywords: preimplantation embryo; SAPK (stress-activated protein kinase/c-Jun N-terminal protein kinase); stress; hyperosmolar sorbitol; apoptosis

Introduction

Most human embryos (and many farm mammal species) are lost before birth and most of the loss occurs around the time of implantation (Cross *et al.*, 1994; Roberts *et al.*, 1996). Sublethal post-natal effects arise during the preimplantation period *in vivo* from diet (Kwong *et al.*, 2000) and from embryo culture (Ecker *et al.*, 2004; Fernandez-Gonzalez *et al.*, 2004). Although these studies do not emulate exact human *in vitro* fertilization (IVF) protocols, they are clinically important since transient embryonic stress may decrease implantation rates or lead to post-natal effects in the nearly two million offspring produced by IVF (Dawson *et al.*, 2005), and because most of the embryogenic period occurs before the female is aware of pregnancy. It is important to understand how the early embryo and placenta respond to stress in order to correlate duration and magnitude of embryonic stress and stress enzyme activation with mechanisms that lead to morbidity and mortality.

Stress enzymes such as stress-activated protein kinase/c-Jun kinase (SAPK/JNK1/2, MAPK8/9, but SAPK throughout text) and p38 mitogen-activated protein kinase (MAPK14) are expressed in the oocyte, early embryo and in placental stem cells of humans and mice (Natale *et al.*, 2004; Zhong *et al.*, 2004). Elevated levels of

phosphorylation of SAPK and p38MAPK correlate negatively with embryonic development rate in seven culture media (Wang *et al.*, 2005). In the most stressful of these media, both increases in apoptosis (terminal deoxynucleotidyl transferase-mediated dUDP nick-end labeling [TUNEL]) and decreases in DNA synthesis (Xie *et al.*, 2006a) contribute to slower cell accumulation and embryo development. SAPK inhibitors severely decrease embryo development in the most stressful media, but increase embryo development in the least stressful media. Therefore, SAPK may not be required for normal development, but play a role during responses to elevated stress.

However, stress-inducing components of different media are complex and may cause stress by many different mechanisms so a consistent means to apply stress over wide dose ranges at different stages of development (and in embryo-derived stem cell line models) is needed. A standardized hyperosmolar stress is advantageous as it can be used in oocytes and embryos at many stages of development and in the embryonic and placental stem cells derived from preimplantation embryos (Rappolee, 2007). In addition, stress enzymes were cloned in yeast and mammals using hyperosmolar stress and this continues to be the standard for stimulating and

assaying stress effects in somatic cells. Sorbitol and raffinose have been used to assess stress in oocytes and preimplantation embryos (Steeves *et al.*, 2003; LaRosa and Downs, 2006).

In somatic cells, p38MAPK, SAPK/JNK and MAPK/extracellular receptor kinase (ERK)—three subfamilies of the MAPK superfamily—respond to stress; MAPK/ERK also respond to mitogenic signals (Ip and Davis, 1998; Roovers and Assoian, 2000; Rappolee, 2003). SAPK/JNK and p38MAPK family members are activated by a wide range of physiological, pathological and developmental stimuli (Ip and Davis, 1998; Kyriakis and Avruch, 2001). SAPK/JNK is activated by a cascade of kinases that result in the phosphorylation of SAPK/JNK at Thr183/Tyr185 (Kyriakis and Avruch, 1996; Whitmarsh and Davis, 1999). This dual phosphorylation opens ATP and substrate binding sites on SAPK/JNK, initiating its activity.

In embryos, like somatic cells, MAPK/ERK is phosphorylated but does not accumulate in the nucleus (Corson *et al.*, 2003; Wang *et al.*, 2004), and MAPK remains phosphorylated in FGF-receptor-dependent response fields for days rather than undergoing a rapid biphasic response (Rappolee, 2007). The SAPK subfamily may also have unique properties in embryos.

We report here that hyperosmolar stress causes dose-dependent increases in apoptosis and decreases in cell accumulation and cavitation in preimplantation embryos. The dose- and time-dependent responses to stress, and nuclear translocation of phosphorylated SAPK, are similar to those observed for somatic cells but embryos tend to resist for more than a day levels of hyperosmolar stress that would be lethal to somatic cells within a few hours.

Materials and Methods

Reagents

Ham's F10 (+bovine serum albumin) and sorbitol were from Sigma Chemical Co. (St Louis, MO, USA). KSOM and KSOM+ amino acids (KSOMaa) were from Specialty Media (Phillipsburg, NJ, USA). The primary antibodies for total SAPK/JNK and phosphorylated SAPK/JNK Thr183/Tyr185 (CS9251, CS9252 and CS4671), for total c-Jun and phosphorylated c-Jun Ser63 (CS9261 and CS9262) and total c-Myc and phosphorylated c-Myc Thr58/Ser62 (CS9401, SC8000R and SC788) have been described previously (Liu *et al.*, 2004; Wang *et al.*, 2005; Xie *et al.*, 2005b) and were from Cell Signaling (CS product number prefixes) Technology (Beverly, MA, USA) and Santa Cruz (SC product number prefixes) Biotechnology (Santa Cruz, CA, USA), respectively. The SAPK/JNK inhibitor D-JNKI1 and the penetration control *twin arginine translocation* (TAT)-fluorescein isothiocyanate (FITC) (Bonny *et al.*, 2001) were from Alexis (San Diego, CA, USA). D-JNKI1 is based on the sequence of IB1/JIP1 that binds and inhibits SAPK/JNK (Bonny *et al.*, 2001; Thompson *et al.*, 2001). Some studies were done with the chemical SAPK/JNK inhibitor SP600125 (Bennett *et al.*, 2001) from Calbiochem (San Diego, CA, USA).

Collection and culture conditions for mouse embryos

Standard techniques were used for obtaining mouse embryos (Hogan, 2002). Female MF-1 mice (4–5 weeks old, Harlan Sprague Dawley, Indianapolis, IN, USA) were super-ovulated, and their embryos were obtained as described previously (Wang *et al.*, 2005; Xie *et al.*, 2005b). Animal use protocols were approved by the Wayne State University Animal Investigation Committee (AIC). In all studies, embryos were equilibrated for at least 1 h in KSOMaa or Ham's F-10 and stressed with the reagent dose for the time period indicated. Per manufacturer's specifications, the embryo culture media had ranges of 271–299 mOsmol and 250–270 mOsmol for Ham's F-10 and KSOMaa, respectively. For inhibitor studies (except where indicated), the inhibitors were preincubated with embryos 3 h before the stress and continued during the stress.

Cell lines and culture conditions

The changes in osmolality caused by sorbitol are in Table 1. Osmolality was measured by crystallizing media samples and assaying them on a model

Table 1: Osmolality of media used in experiments with embryos, HTR and TS cells

Media [sorbitol]	KSOMaa ^a (SI) mOsmol	Ham's F-10 ^b mOsmol
None	257 (1)	254
10 mM	269 (1.05)	ND ^c
25 mM	274 (1.07)	ND
50 mM	305 (1.19)	ND
100 mM	348 (1.35)	ND
200 mM	437 (1.70)	ND
400 mM	626 (2.44)	ND
600 mM	826 (3.21)	ND
1000 mM	1259 (4.90)	ND

Stimulation index with sorbitol or without sorbitol media.

^aSOM + AA, no serum.

^bHam's F10, no serum.

^cND, not done.

3W2 osmometer per manufacturer's instructions (Advanced Instruments, Inc. Needham Heights, MA, USA). In the text, the level of sorbitol (w/v) added is used to produce the given molarity of sorbitol. For inhibitor studies, the inhibitors were preincubated with embryos for 3 h before stress was added and during stress.

Indirect immunocytochemistry and western blot analysis

Indirect immunocytochemistry was performed as described previously (Liu *et al.*, 2004; Wang *et al.*, 2004; Xie *et al.*, 2005b,c). Photomicrography was done with a Leica DM IRE2 automated epifluorescence microscope (Wetzlar, Germany) controlled electronically by SimplePCI AI software (Compix Inc., Imaging Systems, Cranberry Township, PA, USA). Photomicrographs were formatted using Adobe Photoshop 6.0 (San Jose, CA, USA). FITC intensity measurement and comparison were done with SimplePCI DNN software. sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blots were done as described previously (Wang *et al.*, 2005; Xie *et al.*, 2005b).

Analysis of cell number, viability and embryo size

In studies of the dose- and time-dependent effects of sorbitol on cell numbers, embryos were incubated with stress and the dose and time period were indicated. The cell number was analysed by Hoechst-stained nuclei. In embryos, cell numbers were counted in the *z*-axis after Hoechst staining. Blastocyst expansion was measured as an increase in the radius of the blastocyst using Image J (<http://rsb.info.nih.gov/ij/>) and applying it to phase micrographs of embryos. We used several morphological measures for viability. In descending order of severity, opacity/brownness/loss of translucence > withdrawal from zona pellucida > chronic collapse (with no blastocoel), which we have over time, associated with eventual death. The key criterion is whether the embryos recover after replacing them in KSOMaa without sorbitol.

Analysis of apoptosis

Apoptosis was assayed using TUNEL (Promega Co., Madison, WI, USA) as done previously (Xie *et al.*, 2006a,b).

Statistical analysis

All experiments were repeated three times with similar results, except some western blot analyses that were performed twice. Similar results mean that the similar (proportional) stimulus response ratios were observed, although absolute values for baseline and stimulated embryos varied between experiments. One representative experiment is shown for each result. Data are presented as mean \pm SD. Embryo cell number and immunofluorescence intensity were analysed by one-way analysis of variance (ANOVA) when there was one variable and more than two groups. If ANOVA showed significant difference between groups, least significant difference *post hoc* tests were used to analyse differences between paired groups that were normally

distributed. The χ^2 -test was used to determine the amount of difference between groups measured as a percentage. Linear regression was used to define the linear relationship between SAPK/JNK phospho fluorescent intensities and doses of sorbitol. The independent-samples *t*-test was used to analyse the difference in cell numbers due to the SAPK inhibitor. Three levels of significance were determined: $P \geq 0.05$ was insignificant, $P < 0.05$ was significant and $p < 0.01$ was highly significant. All statistical analysis was performed using SPSS software v10.0 (SPSS Inc., Chicago, IL, USA).

Results

To understand the impact of stress and SAPK on early embryo development, we did three types of experiments. We first tested for the biological effects of hyperosmolar stress on embryo cavitation and lethality (Fig. 1), apoptosis (Fig. 2) and the equivalence of the least and most stressful media (as measured by cell number accumulation) (Fig. 3). In the second series of experiments, we tested for the dose- and time-dependent induction of phosphorylated SAPK by sorbitol (Figs. 4 and 5), and in the third series of experiments, we tested for the sorbitol stress-induced (insert sorbitol stress induced)

up- and down-regulation of phosphorylated nuclear transcription factors c-Jun and c-Myc (respectively, Figs. 6 and 7).

Embryos undergo slower growth, cell cycle arrest, apoptosis and slower development in response to hyperosmolar stress

We first sought to determine the homeostatic response to different quantities and qualities of stress by embryos. For mouse preimplantation embryos, we used blastocyst expansion rates to test for dose-dependent effects of stress. Sorbitol at 1000 mM (and 400–600 mM) caused an immediate collapse of embryos by 1 min, but blastocyst cavities were reforming by 5 min and by 30 min embryo size was not significantly different than that in unstressed embryos (Fig. 1A). After 48 h of 200 mM sorbitol, a state of zero embryo expansion of E3.5 blastocysts was created (Fig. 1B) that was significantly less than unstressed embryos ($P < 0.01$). At 50 mM, a significant decrease in cavitation occurred ($P < 0.05$), but blastocyst cavity size increased. At 600 and 1000 mM sorbitol, a significant decrease in blastocyst size occurred ($P < 0.05$).

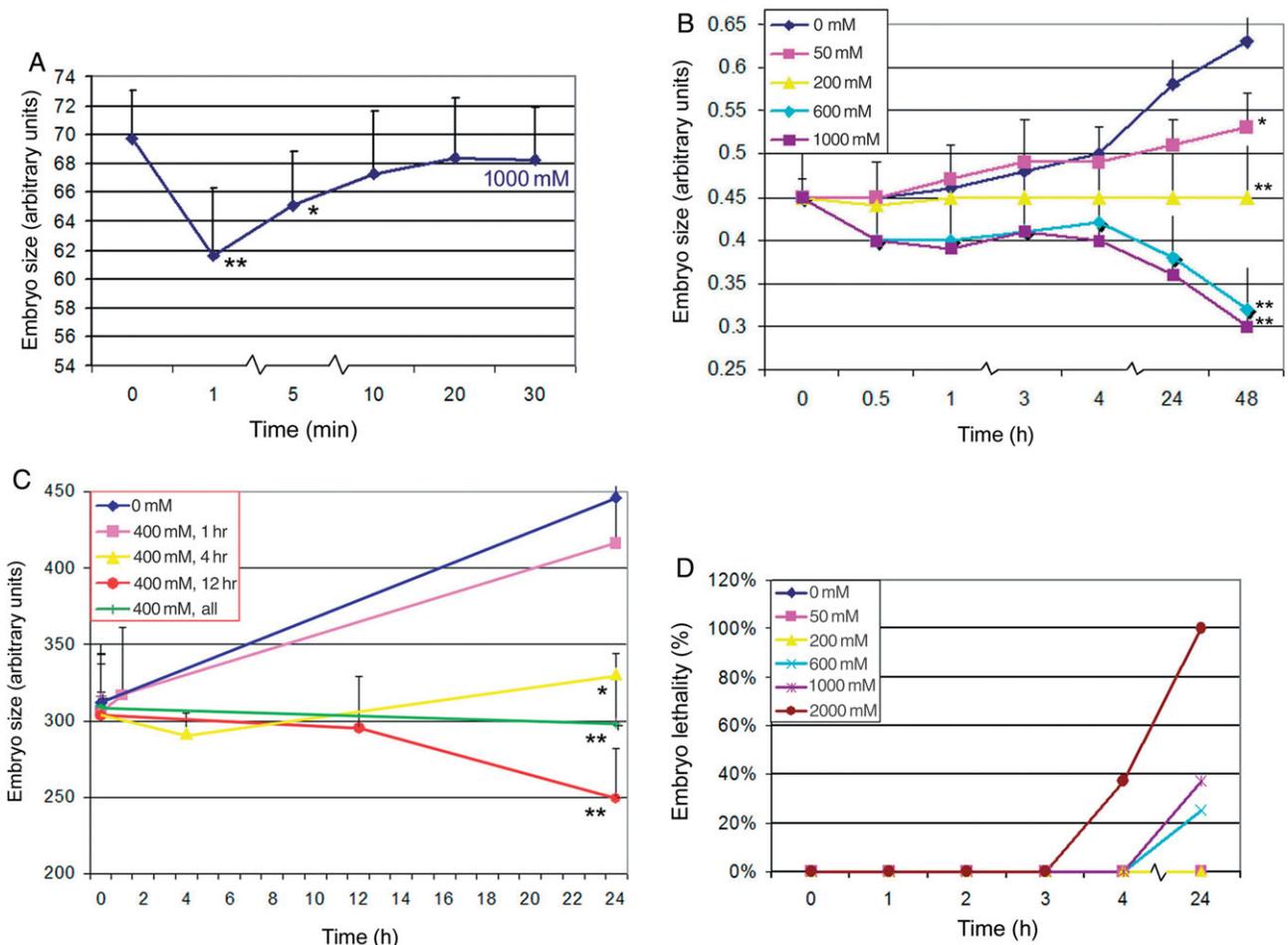


Figure 1: Dose- and time-dependent effects of sorbitol-induced hyperosmolar stress on embryo size and viability can be reversible (A) E3.5 embryos were cultured individually in 5 μ l microdrops of KSOMaa*** and 1000 mM sorbitol doses for 0–30 min and micrographed as indicated. Error flags show standard deviations. Note that the *x*-axis is not linear for A, B and D. For (A, B, C) *significant ($P < 0.05$) or **highly significant ($P < 0.01$) shows comparisons between unstressed and stressed. (B) E3.5 embryos were cultured as in (A) in 0–1000 mM sorbitol for 0–48 h, micrographed at the intervals indicated and measured for radius. (C) E3.5 preimplantation were cultured as in (A, B) with or without 400 mM sorbitol, and sorbitol-containing media was replaced with KSOMaa alone after 1, 4 or 12 h. All embryos were cultured through 24 h, and micrographs were taken at time zero and final, at the time sorbitol-containing media were removed, and embryo radii were determined. (D) E3.5 embryos were cultured as in (A–C) in 0–2000 mM sorbitol, micrographed at the times indicated and assayed for embryo death (combined collapse and opacity)

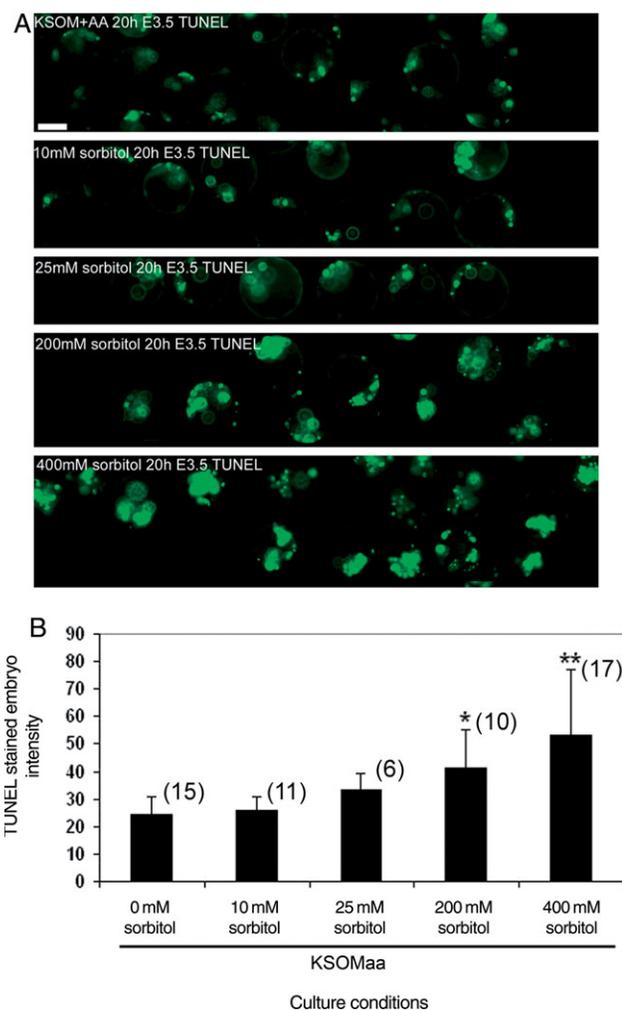


Figure 2: Sorbitol induces dose-dependent apoptosis in E3.5 embryos cultured for 24 h (A) E3.5 preimplantation embryos were incubated in KSOMaa with increasing sorbitol (0–400 mM), for 24 h and assayed for TUNEL. Micron bar = 50 μ m. (B) Histogram shows *significant ($P < 0.05$) and **highly significant ($P < 0.01$) increases of stressed embryos compared with unstressed embryos cultured in KSOMaa alone (each histogram bar is mean \pm standard deviation error flag). Numbers in parentheses indicate number of embryos in each group

The time-dependent reversibility of hyperosmolar stress was also tested by blastocyst cavitation rate. E3.5 embryos were cultured in 400 mM SAPK, which highly induces phosphorylated SAPK, but is not as toxic as 600–1000 mM sorbitol (Xie and Zhong, data not shown). Like 200 mM, 400 mM sorbitol produced nearly static size over 24 h culture (Fig. 1C). After return to media alone after 1 h in 400 mM sorbitol, embryos recovered and embryos progressed similarly to unstressed embryos (Fig. 1C). However, return to media alone after 4 h resulted in significant decrease in embryo expansion ($P < 0.05$), suggesting that between 1 and 4 h, embryos lost the ability to recover. Interestingly, return to media alone after 12 h, caused embryo collapse and significant size decrease ($P < 0.01$) that was more profound than sorbitol-containing media throughout the entire 24 h culture. These embryos returned to media alone after 12 h rapidly collapsed and died (lost translucence and became brown and opaque, when tested these embryos never recover when placed in KSOMaa alone). Finally, we found that embryos cultured in 2000 mM sorbitol began dying as early as 3 h and all embryos

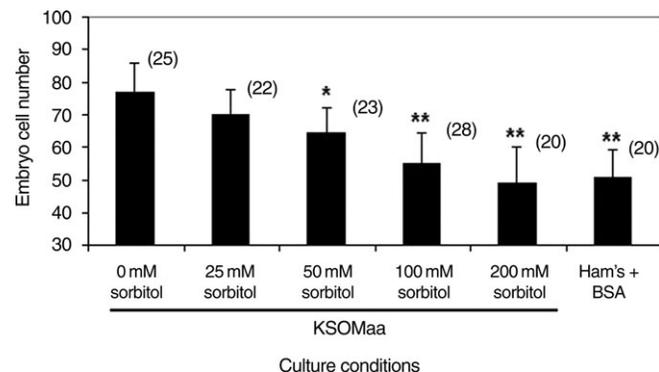


Figure 3: Sorbitol at 200 mM is sufficient to make the least stressful media into the most stressful media with regard to cell accumulation in cultured embryos. E3.5 preimplantation embryos were incubated in KSOMaa with increasing sorbitol (0–200 mM) or incubated in Ham's F10 for 24 h and assayed for cell number. *Significant ($P < 0.05$) and **highly significant ($P < 0.01$) increases from control embryos cultured in KSOMaa alone (each histogram bar is mean \pm standard deviation error flag). Note that Ham's F10 group was not significantly different than KSOMaa + 200 mM sorbitol group. Numbers in parentheses indicate number of embryos in each group

were dead by 24 h (Fig. 1D). In contrast, 1000 and 600 mM had $< 50\%$ dead at 24 h (37.5 and 25%, respectively). However, both these groups had collapsed (compare with Fig. 1B), but they had not become opaque by 24 h. No embryos at 50 or 200 mM sorbitol had collapsed or become opaque. Comparing rapid, reversible collapse at 1 min (Fig. 1A) with collapse after chronic exposure to doses above 200 mM sorbitol shows that it is chronic collapse that is associated with the inability of embryos to recover. Loss of translucence, opacity and browning are associated with inability to recover at any time during culture at any stress level.

We sought to understand dose-dependent increases in apoptosis caused by sorbitol in embryos. E3.5 embryos cultured overnight in 200 mM sorbitol produced significant ($P < 0.05$), or in 400 mM sorbitol highly significant ($P < 0.01$), increases in TUNEL intensity (Fig. 2A). The lowest hyperosmolar stresses, 10–25 mM sorbitol, produced insignificant increases ($P \geq 0.05$) in TUNEL. Therefore, optimal media are not significantly more stressful, with regards to apoptosis, until a substantial amount of sorbitol is added. It should be noted that although the relative increases in TUNEL were constant from experiment to experiment (each experiment was repeated three times), the absolute values were different. It is possible that this is due to the confounding variable of stress induced on females and their embryos in the vivarium before embryo isolation. Variation in absolute TUNEL rates may also be a function of the variation in basal KSOMaa of ~ 20 m osmolality reported by the manufacturer. In a previous report, we observed higher morbidity and mortality of embryos in one experiment when compared with its replicates. In that experiment, there was an increased number of granules of the embryos noted during isolation, before culture (Xie *et al.*, 2006a). The increased number of granules would be associated with problems with embryo health before culture and might contribute to higher morbidity during culture. But, in this experiment the relative effects of culture media and SAPK inhibitors on the embryos had similar relative proportions when compared with replicate experiments that started with embryos with less granules.

There is a negative correlation between increased SAPK phosphorylation and embryo development for seven culture media. KSOMaa and Ham's F-10 induced phosphorylated SAPK the least and most (Wang *et al.*, 2005). Compared with KSOMaa alone, the cell number in E3.5 mouse embryos cultured for 24 h was reduced

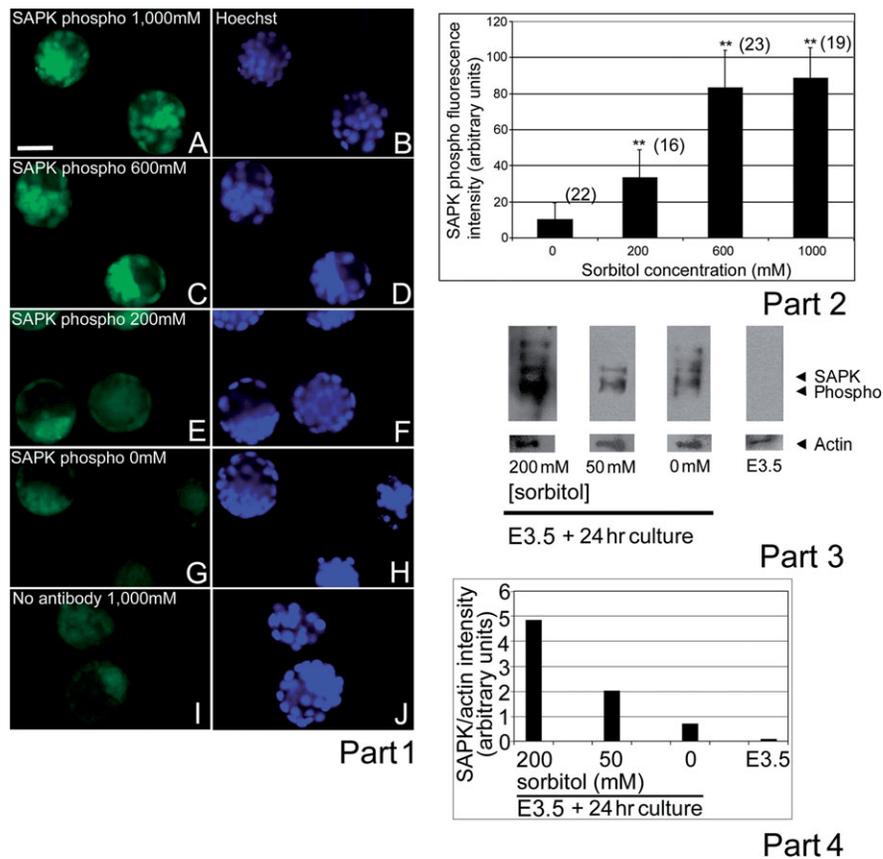


Figure 4: Dose-dependent effects of sorbitol on SAPK phosphorylation in mouse preimplantation embryos (Part 1) E3.5 preimplantation embryos were cultured in KSOMaa with the following doses of sorbitol for 0.5 h: 1000 mM (A, I), 600 mM (C), 200 mM (E) and 0 mM (G). After treatments, embryos were examined by immunocytochemical techniques using SAPK phospho antibody (A, C, E and G) or no primary antibody (I). Hoechst staining nuclei (B, D, F, H, J) are coupled with the detection of SAPK phosphorylation (A, C, E, G, I), respectively. In (A) micron bar = 50 μ m. (Part 2) Histogram shows SAPK phospho fluorescent intensity (each histogram bar is mean \pm standard deviation error flag). $^{***}P < 0.01$ SAPK phospho fluorescent intensity incubated in KSOMaa alone versus 200, 600 and 1000 mM sorbitol. Numbers in parentheses indicate number of embryos in each group. (Part 3) E3.5 embryos were cultured in 0, 25 or 200 mM sorbitol for 24 h or isolated *ex vivo*. After treatments, equal numbers of embryos were examined by western blot analysis using SAPK phospho antibody and actin antibody as indicated. (Part 4) Histogram shows SAPK phospho expression normalized to actin from the western blot in Part 3

from 76.6 ± 9.3 (unstressed) to 69.8 ± 8.0 (25 mM sorbitol), 64.5 ± 7.8 (50 mM sorbitol), 55 ± 9.3 (100 mM sorbitol) and 49.1 ± 11.1 (200 mM sorbitol), respectively (Fig. 3). The cell number in KSOMaa + 200 mM sorbitol (49.1 ± 11.1) was not significantly different ($P > 0.05$) to that from the stressful Ham's F-10 media (50.8 ± 8.4). The difference in cell number in embryos cultured with KSOMaa was significant when compared with 50 mM ($P < 0.05$) and highly significant at 100 or 200 mM sorbitol or Ham's F-10 (all $P < 0.01$). Sorbitol at 100 or 200 mM made KSOMaa media as stressful as Ham's F-10 media such that it was insignificantly different than Ham's F-10 ($P \geq 0.05$). The number of cells of E3.5 mouse embryos at 0 h was 32.1 ± 3.5 (data not shown), increasing to 76.6 ± 9.3 after 24 h of culture. Therefore embryos in all media accumulated cells during culture, but embryos in Ham's F10 or KSOMaa + 200 mM sorbitol had the smallest increases in cell number.

Dose-dependent induction of SAPK phosphorylation by sorbitol is consistent with roles in increased apoptosis and decreased cell accumulation in E3.5 embryos

SAPK phospho induction was tested to determine its consistency with roles in biologic outcomes in embryos. The same SAPK phospho-specific antibodies were used as in western blot analysis and embryos previously described (Wang *et al.*, 2005; Xie *et al.*,

2006b). Phosphorylated SAPK phospho in E3.5 mouse embryos is induced rapidly at 0.5 h (Fig. 4). E3.5 mouse embryos cultured in 1000 and 600 mM sorbitol showed higher fluorescent intensity than those cultured in media alone (Fig. 4 Parts 1 and 2; 1000, 600, 200 and 0 mM had arbitrary units of 88.4 ± 17.1 , 83.2 ± 21.1 , 33.4 ± 15.6 and 10 ± 9.1 , respectively). Sorbitol doses of 1000 and 600 mM were insignificantly different from each other ($P \geq 0.05$), but both were significantly higher compared with 200 or 0 mM (all $P < 0.01$); 200 mM was also significantly higher than 0 mM ($P < 0.01$). The response was linear between 0 and 1000 mM (Pearson's linear regression, $R^2 = 0.904$, $P < 0.05$) and similar to that observed in western blots of placental HTR and TS cells (Zhong, data not shown). Phosphorylated SAPK was also induced by 25 and 200 mM sorbitol during 24 h of culture (Fig. 4, Parts 3 and 4), compared to culture without sorbitol or *ex vivo* E3.5 embryos. The data suggest that induction of phosphorylated SAPK by stress is similar in E3.5 embryos and placental cell lines and that a linear range of increase exists for 0–50–200–600 mM sorbitol, as measured by immunofluorescence or western blot.

In somatic cells, phosphorylated SAPK is induced in a biphasic manner (Kyriakis and Avruch, 1996), so we next tested for time-dependence in embryos. Phosphorylated SAPK was induced rapidly by 15 min and significantly by 400 mM sorbitol ($P < 0.01$) (Fig. 5). A peak occurred by 30 min before a decline and leveling off by 120–180 min. There was a biphasic component an early peak

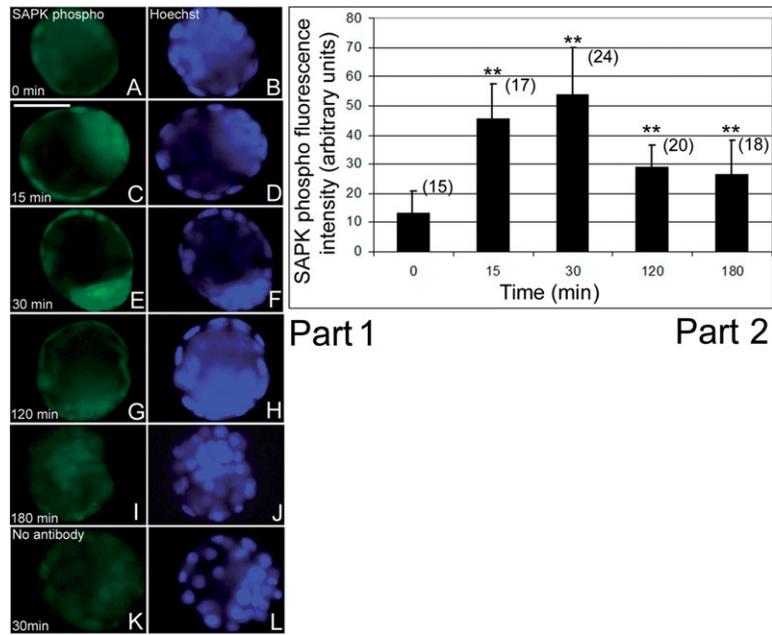


Figure 5: Time-dependent effects of sorbitol on SAPK phosphorylation in mouse preimplantation embryos (Part 1) E3.5 preimplantation embryos were cultured in KSOMaa with 400 mM sorbitol 0–180 min: 0 min (A), 15 min (C), 30 min (E, K), 120 min (G) and 180 min (I). After treatments, embryos were examined by immunocytochemical techniques using SAPK phospho antibody (A, C, E, G) or no primary antibody (K). Hoechst staining nuclei (B, D, F, H, J) are coupled with the detection of SAPK phosphorylation (A, C, E, G, I), respectively. The no antibody control for 30 min sorbitol is (K, L). Note that SAPK phospho in the figure refers to phosphorylated SAPK. In (A) micron bar = 50 μ m. (Part 2) Histogram shows SAPK phospho fluorescent intensity (each histogram bar is mean \pm standard deviation error flag). ** $P < 0.01$ SAPK phospho fluorescent intensity incubated in KSOMaa at time zero versus 15–180 min. Numbers in parentheses indicate number of embryos in each group

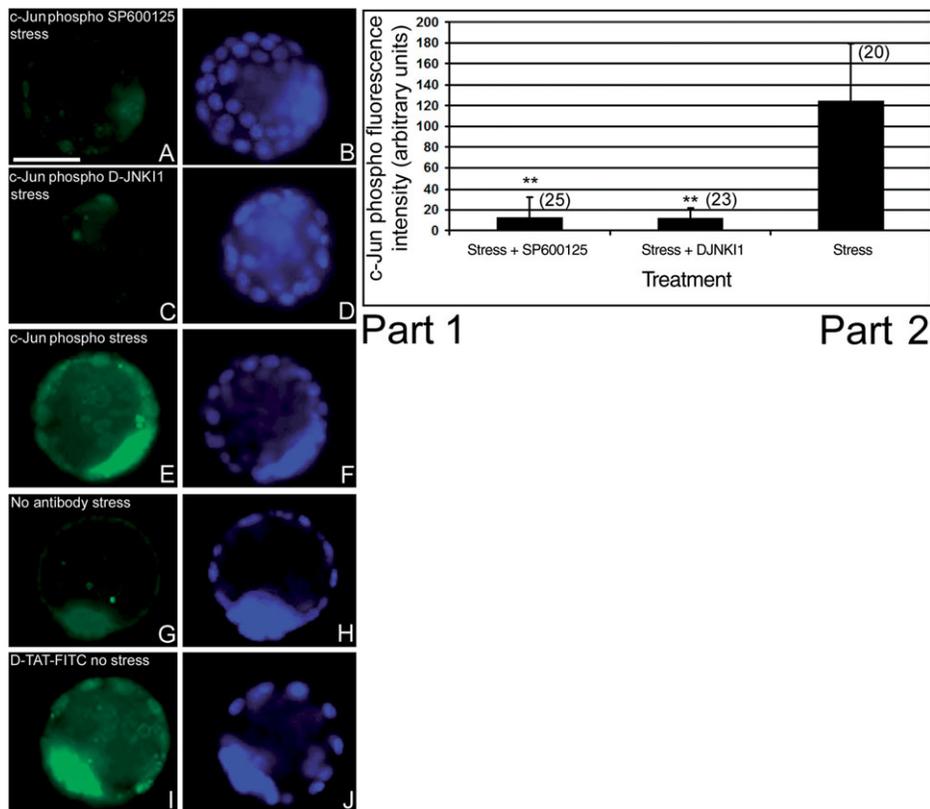


Figure 6: c-Jun phospho in E3.5 embryos is activated by 0.5 h stress in embryos in a SAPK-dependent manner. (Part 1) A, C, E and G are embryos after 0.5 h of 1000 mM sorbitol stress. A, C and E were stained for c-Jun phospho (Jun phospho in figure). A and C were inhibited by SAPK inhibitors SP600125 and D-JNK11, respectively, E was not inhibited and G had no antibody. A control TAT, conjugated with FITC instead of JNK inhibitor (I, H), was used to show uptake by the embryo. B, D, F, H and J are the Hoechst-stained replicates of the green-stained embryos to the left. In (A) micron bar = 50 μ m. (Part 2) Histogram shows c-Jun phospho fluorescent intensity (each histogram bar is mean \pm standard deviation error flag). ** $P < 0.01$ c-Jun phospho fluorescent intensity of embryos incubated with 400 mM sorbitol with SP600125, DJNK11 or in KSOMaa alone for 30 min. Numbers in parentheses indicate number of embryos in each group

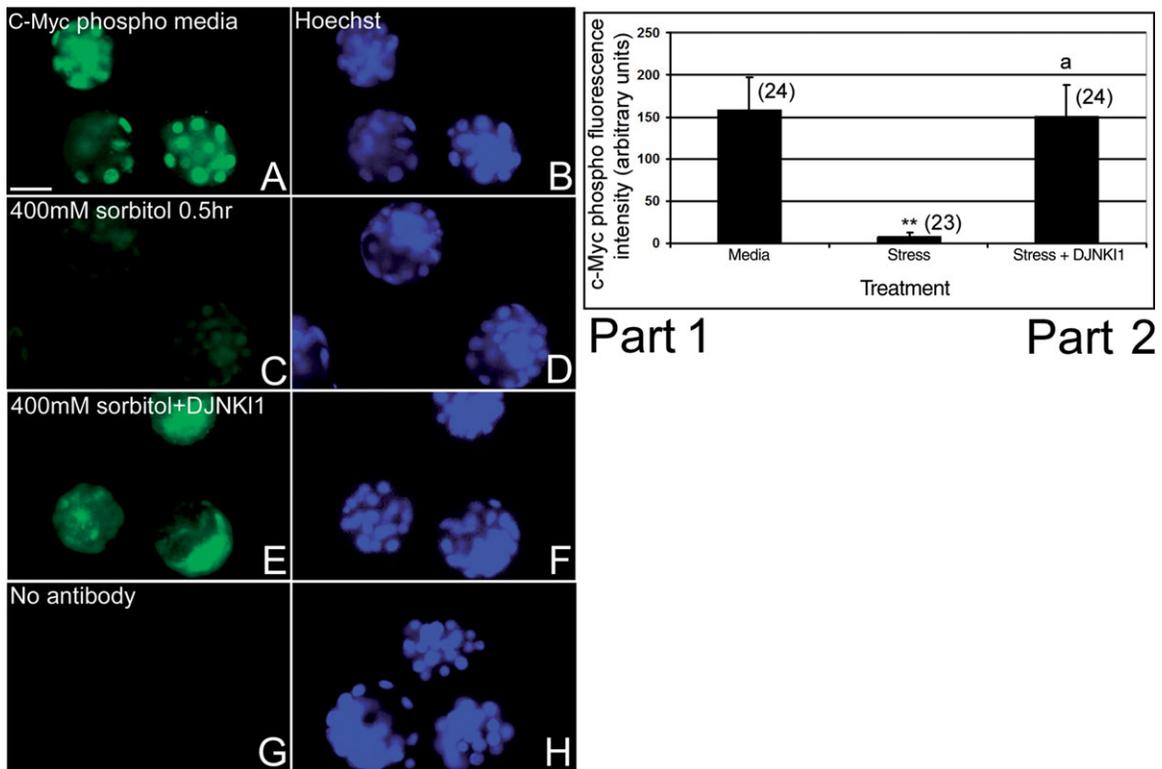


Figure 7: Sorbitol added for 0.5 h suppresses the c-Myc phospho in E3.5 embryos (Part 1) E3.5 preimplantation embryos were cultured in KSOMaa without (A, B) or with (C, D) 1,000 mM sorbitol and with sorbitol and DJNK11 (E, F) for 0.5 h, then fixed and stained for c-Myc phospho. In (G, H), embryos were probed without first antibody. In (A) micron bar = 50 μ m. (Part 2) Histogram shows c-Myc phospho fluorescent intensity (each histogram bar is mean \pm standard deviation error flag). ** $P < 0.01$, (a) media and sorbitol with DJNK11, $P = 0.6$. c-Myc phospho fluorescent intensity of embryos incubated with KSOMaa alone, with 1,000 mM sorbitol or with 1,000 mM sorbitol DJNK11 for 30 min. Numbers in parentheses indicate number of embryos in each group

occurred, but phosphorylated SAPK did not return to baseline and its expression was significantly ($P = 0.01$) above the level in unstressed embryos at 120–180 min. At 200 mM sorbitol, phosphorylated SAPK remained high through out 24 h (Fig. 4, Part 3).

Rapid responses to stress include a SAPK-dependent increase in c-Jun phospho and a decrease in c-Myc phospho

SAPK induction leads to increased phosphorylation of c-Jun at Ser63 (Smeal *et al.*, 1992; Maekawa *et al.*, 2005), and SAPK inhibitors block this increase. SP600125 (Bennett *et al.*, 2001) and D-JNK11 (Bonny *et al.*, 2001) are effective inhibitors of SAPK function, but not SAPK phosphorylation. The blockage of stress-induced c-Jun phospho is a test of the efficacy of SAPK inhibitors and demonstrates that phosphorylated nuclear c-Jun is SAPK-dependent.

As expected, 0.5 h of 1000 mM sorbitol increased c-Jun phospho in embryos (Fig. 6), but SAPK inhibitors blocked this increase. Phosphorylated c-Jun fluorescence intensity values in embryos treated with sorbitol + SP600125, sorbitol + D-JNK11 or sorbitol alone (no antibody background) were 26.0 ± 9.2 , 11.7 ± 7.3 and 130.9 ± 38.6 arbitrary units, respectively. The inhibitory effects of SP600125 or D-JNK11 were highly significant ($P < 0.01$). D-JNK11 was delivered to the embryo by the covalently bound fusogenic delivery peptide from the human immunodeficiency virus *twin arginine translocation* gene (TAT). Embryos treated with TAT-FITC had a peak fluorescence-penetration of 2 h (Fig. 4, data not shown); therefore, all embryos were pre-loaded with inhibitor for 2 h before the addition of sorbitol.

Since cell accumulation is diminished by 200 mM sorbitol (and >200 mM) (Fig. 3), we hypothesized a phosphorylated form of c-Myc corresponding to G1-S commitment (Pulverer *et al.*, 1994) and cell accumulation would be suppressed. To test this hypothesis,

we used two antibodies to c-Myc phospho at Ser58/Thr62, a site of activation of c-Myc that is highly correlated with S-phase commitment and DNA synthesis and is expressed throughout preimplantation development (Xie *et al.*, 2005a). Embryos cultured in KSOMaa are maximally growth-induced compared with other media (Wang *et al.*, 2005) and express high c-Myc phospho in the nucleus (Fig. 7). After 0.5 h of stress, phosphorylated c-Myc fluorescence intensity was decreased. However, this loss of phosphorylated c-Myc was largely reversed by SAPK inhibitor D-JNK11, suggesting that the loss is SAPK-dependent.

Discussion

We found that hyperosmolar sorbitol caused dose- and time-dependent decreases in three biological outcomes: embryo growth, cell number accumulation and apoptosis. We previously found that the stress caused by seven embryo media phosphorylated SAPK Thr183/Tyr185 to levels that were inversely correlated with rates of embryo development (Wang *et al.*, 2005). The different complex formulations of these media could have induced stress by different mechanisms. Here we used hyperosmolar sorbitol as the single stressor and studied only the two media that induce one of the most (Ham's F10) and one of the least (KSOMaa) powerful inductions of phosphorylated SAPK (Wang *et al.*, 2005). Since phosphorylated SAPK levels were induced proportionally to the amount of sorbitol added, it suggests that these levels are proportional to decreased cell accumulation and embryo cavitation and increased apoptosis. Since both phosphorylation and nuclear translocation of SAPK preceded and was simultaneous with the three biological outcomes measured, this suggests that SAPK has a causal role in these outcomes.

Consistent with a role for SAPK in induction of homeostatic responses, stress-induction of phosphorylated, nuclear c-Jun Ser63 is dependent on SAPK activity and is inhibited by two inhibitors of SAPK. SAPK-dependent activation of homeostatic responses in somatic cells is dependent on c-Jun phosphorylated at Ser63 (Smeal *et al.*, 1991; Shaulian and Karin, 2002; Morton *et al.*, 2003). Consistent with a role for SAPK in attenuating the pre-stress growth phenotype, stress-induced decrease of phosphorylated, nuclear c-Myc Thr58/Ser62 is dependent on SAPK activity. c-Myc exemplified a larger group of phosphoproteins, including Rb and b-Myc (also three other proliferation-associated phosphoproteins, data not shown), that are up-regulated in a MAPK-dependent manner during stress-free culture and that undergo down-regulation in a stress-induced, SAPK-dependent manner. Phosphorylated, nuclear c-Myc is correlated with proliferation in somatic cells (Henriksson *et al.*, 1993; Lutterbach and Hann, 1994). Mitogenic phosphorylated c-Myc is detected in the nucleus of cells in preimplantation embryos from the 2-cell stage through to the blastocyst (Xie *et al.*, 2005a). Loss of all Myc isoforms' function by knockout of its obligate heterodimer partner, Max, results in embryo death at implantation due to lack of proliferation (Shen-Li *et al.*, 2000). Decreasing c-Myc expression with anti-sense oligonucleotides blocks embryonic development at the 8-cell stage (Paria *et al.*, 1992). The data taken together for stress-induced regulation of c-Jun and c-Myc at 0.5 h by SAPK is consistent with its regulation of the biological responses to stress at 24 h.

We found that the difference measured in cell accumulation, between the most and least SAPK phosphorylation-inducing media (Wang *et al.*, 2005) is 200 mM sorbitol. This dose also causes embryonic stasis with regard to expansion in size. In addition, this level of stress creates stasis in regard to cell accumulation. Increasing stress diminished the cell accumulation. With 200 mM sorbitol in KSOMaa, embryos started culture with 32.1 cells and ended culture 24 h later with ~49 cells. However, this is likely not to be due to new proliferation. Typically, ~45% of cells in the blastocyst are brdU positive (Xie *et al.*, 2006a); so, of the 32.1 cells in embryos at the start of culture, ~14.5 cells in S phase would complete division, resulting in ~47 cells, similar to the ~49–51 cells observed in embryos cultured in KSOMaa + 200 mM sorbitol. Therefore, the increase in cell number of embryos in KSOMaa + 200 mM sorbitol may be due only to completion of cell cycle of those cells already in S/G2 + M phase at the time of sorbitol addition. The relatively small number of cells in G2 or M phase that would complete cell division would be equivalent to the cells induced to undergo apoptosis. Interestingly, 200 mM sorbitol does cause cell number stasis for 72 h in human first trimester placental cell line HTR (Zhong, data not shown). Therefore, for the E3.5 embryo, 200 mM sorbitol is a threshold level of stress that maintains size and cell number, but likely leaves insufficient energy for further growth.

We show here that time- and dose-dependent apoptotic and cell cycle arrest responses in embryos are similar to somatic cells. The induction and role of SAPK in mediating these responses is also similar. MAPK has two molecular responses that are unique in embryos and placental stem cells compared with somatic cells (Corson *et al.*, 2003; Wang *et al.*, 2004; Wang *et al.*, to be submitted). SAPK is unlike related MAPK in one response and similar in another response. Unlike MAPK, once cytoplasmic SAPK phosphorylation is induced by stress, phosphorylated SAPK does accumulate in the nucleus in embryos. But, like MAPK, phosphorylated SAPK has a mild peak at 200 mM sorbitol, but does not return to baseline. The functional significance of these molecular response differences remains to be determined. The balance between MAPK and SAPK signaling during normal and stress responses determines biological

choice of growth, cell cycle arrest or apoptosis (Bogoyevitch *et al.*, 1995; Xia *et al.*, 1995; Kang *et al.*, 2000). In the unstressed state, this balance must favor nearly constant net cell increase in the rapidly growing early embryo and placenta.

Stress-induced, SAPK-dependent, phosphorylation of c-Jun is similar to somatic cell responses. The results show that both phosphorylated SAPK and phosphorylated c-Jun are in the nucleus, suggesting that much of the regulation mediated by SAPK will be of transcription and have longer term effects. Recent reports using chromatin immunoprecipitation suggest that SAPK, p38MAPK and MAPK enzymes occupy many promoters after stress-induction in yeast and mammals [(Pokholok *et al.*, 2006), and citations therein]. Changes in nuclear activity due to stress and mediated by stress enzymes are a key area of future studies.

Surprisingly, blastocysts exposed to high stress survived and also maintained blastocoels for >24 h. The blastocysts initially collapsed but re-established blastocoels; and after 0.5 h their size was not significantly different than unstressed blastocysts. Re-establishment and maintenance of the blastocoel requires a high expenditure of energy. For example ~40–60% of ATP in the trophectoderm is used to pump water and solutes into the blastocoel during blastocyst formation (Houghton *et al.*, 2003). Forming and maintaining a blastocoel is one of the highest energy-requiring cellular events in the trophectoderm or in somatic cells in the intestinal epithelium (Leese *et al.*, 1993; Brison and Leese, 1994). Embryos stressed by 400–1000 mM sorbitol are irreversibly committed to death after 12 h (and do not recover if placed in KSOMaa alone); however, they continue to maintain the blastocoel after 12 h and many embryos have blastocoels after 24 h of stress. Maintaining a blastocoel does not appear to be a function that the preimplantation embryo is programmed to jettison in times of high stress. It will be important to measure energy consumption and test for the SAPK-dependence of the embryos ability to reform the blastocoel during stress.

As shown previously (Wang *et al.*, 2005), one of the least stressful media (KSOMaa) induces more phosphorylated SAPK in embryos after overnight culture, than in embryos isolated from the uterus. This is significant in that the SAPK-dependent modulation of homeostatic and growth programs, exemplified by c-Jun and c-Myc transcription factors, is also likely to occur during culture. In addition to growth and homeostasis, it is likely that pluripotency of stem cells is influenced by stress and stress enzymes. SAPK controls polycombs which in turn controls determination of imaginal wing discs in *Drosophila* (Rappolee, 2007). Extraembryonic ectoderm deficient/polycombs is also important in suppressing the differentiated state in mouse and human embryonic stem cells. The modulation of growth, homeostasis and pluripotency by stress enzymes during embryo culture may provide epigenetic mechanisms leading to the long-term, post-natal effects observed previously (Ecker *et al.*, 2004).

Conclusions

The work reported here suggests that biological responses of embryos can be induced by hyperosmolar stress and associated to the proportional induction of phosphorylated SAPK. The causal roles of SAPK in stress homeostasis, and its redundancy with other stress enzymes, remain to be determined. An attractive advantage of the embryo model is that it allows easy analysis of the kinetics and magnitude of the stress response, and after controlled stress in IVF culture the embryo can be reimplanted. Then the ramifications of stress and stress enzyme function in preimplantation embryos can be understood in terms of their effects on later placental, fetal and post-natal development.

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