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Effects of SAPK/JNK inhibitors on preimplantation mouse embryo development are influenced greatly by the amount of stress induced by the media

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Stress-activated protein kinase/c-Jun kinase (SAPK/JNK) is thought to be necessary for preimplantation embryonic development (Maekawa *et al*., 2005). However, media increases SAPK/JNK phosphorylation and these levels negatively correlate with embryonic development (Wang *et al*., 2005). Culture-induced stress could confuse analysis of the role of SAPK in development. In this study, we tested how SAPK/JNK inhibitors influence embryonic development in optimal and non-optimal media and define the contribution of cell survival and proliferation to the embryonic response to these media. SAPK/JNK inhibitors retard embryonic development in suboptimal Ham's F10, but improve development in optimal potassium (K+) simplex optimized media (KSOM) +AA. In KSOM + amino acids (KSOM+AA), two SAPK/JNK inhibitors increase the rate of cavitation and hatching. These data suggest that (i) SAPK/JNK mediates the response to culture stress, not normal preimplantation embryonic development and (ii) SAPK/JNK inhibitors may be useful in ameliorating embryo stress caused by culture. To define the effects of media, we assayed the contribution of cell survival and proliferation and the differences in total cell number of cultured embryos. Embryos cultured from E3.5+24 h in the suboptimal medium (Ham's F10) induced significant but small increases in TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUNEL) positive cells. Bromodeoxyuridine (BrdU) incorporation in suboptimal Ham's F10 was significantly lower than in optimal KSOM+AA, suggesting that cell cycle arrest also contributes to slower increase in cell number in stressful media. This is the first report where TUNEL and BrdU were both assayed to define the relative contribution of cell cycle/S phase commitment and apoptosis to lessened cell number increase during embryo culture.

Key words: apoptosis, brdU/cell proliferation/N-terminal Jun kinase (SAPK/JNK), SAPK inhibitor D-JNK1, TUNEL/preimplantation embryos, embryo media, stress activated protein kinase

Introduction

Stress-sensing enzymes such as p38 mitogen-activated protein kinase (MAPK) are essential during normal development in mediating embryonic progression and cavitation at the 8-cell stage (Natale *et al*., 2004; Maekawa *et al*., 2005). Stress-activated protein kinase/c-Jun kinase (SAPK/JNK) may also be important in these events (Maekawa *et al*., 2005) or in sensing cues at implantation for initiating the maternal recognition of pregnancy signal (Zhong *et al*., submitted for publication) or during endoderm differentiation (Kanungo *et al*., 2000; Wang *et al*., 2002; Lee *et al*., 2004). Placental cells and preimplantation embryos respond to culture-induced stress with increased SAPK/JNK phospho and p38MAPK phospho (Wang *et al*., 2005; Zhong *et al*., submitted for publication) and this response may also have effects on development, including slower proliferation, cell cycle arrest and apoptosis. Although none of the p38MAPK and SAPK/JNK family members are essential to preimplantation mouse embryos, as measured by lethality of null mutants, these enzymes may be important in normal or stressed development. The lack of null lethal effects may be due to residual maternal gene products or redundant or compensatory mechanisms in early embryos and placental cells. Interestingly, p38MAPKgamma is essential for placental formation after implantation (Mudgett *et al*., 2000), and JNK interacting protein (JIP)-1alpha, a SAPK/JNK scaffolding protein that prevents SAPK/JNK activation and nuclear translocation (Thompson *et al*., 2001), is essential for implantation. However, the SAPK/JNK1, 2, SAPK/JNK1, 3 and SAPK/JNK2, 3 double knockouts have no peri-implantation lethal effects [(Kuan *et al*., 1999) and citations therein], but SAPK/JNK1, 2 double knockout is lethal to the fetus. These data suggest an essential role for stress-sensing enzymes in post-implantation placental development, but not preimplantation development, and suggest that attenuating excess SAPK/JNK activation is important.

Culture of embryos itself is stressful, with a rapid metabolic response suggesting an energy-consuming homeostatic response to culture (Brison and Leese, 1991; Leese, 2002). Our previous data showed that SAPK and p38MAPK mRNA are present throughout preimplantation development and in placental cells and cell lines in mice and humans (Zhong *et al*., 2004). In addition, phosphorylated SAPK/JNK and p38MAPK levels increased in mouse embryos cultured in suboptimal medium Ham's F10 and M16 when compared to those cultured in optimal medium potassium (K+) simplex optimized media

(KSOM)+AA and Quinn's (Wang *et al*., 2005). Phosphorylation levels of SAPK/JNK and p38MAPK correlate negatively with mouse embryo development, including lower cell number, lower blastocyst formation and hatching rate (Wang *et al*, 2005). One purpose of this investigation is to answer whether SAPK/JNK activity is essential to mouse preimplantation embryo development and whether SAPK/JNK inhibitor can reverse the media stress-induced negative developmental outcomes. Another purpose of this investigation is to examine whether these negative developmental biological outcomes are mediated by apoptosis and/or cell cycle arrest.

In the current studies, we report that the SAPK inhibitors, SP600125 and DJNKl1 improve development in optimal media, but decrease developmental rates in suboptimal media, suggesting that SAPK is not required for normal preimplantation development as reported (Maekawa *et al*., 2005). These data also suggest that SAPK inhibitors may alleviate negative developmental effects of low amounts of stress during preimplantation embryo culture. The decrease in cell number accumulation in embryos cultured in the most suboptimal media was due both to decreased entrance into S phase as shown by decreased Bromodeoxyuridine (BrdU) corporation and to significant increases in TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUNEL)/apoptosis.

Materials and methods

Media

Ham's F10 was purchased from Sigma Chemical Co. (St. Louis, MO) and supplemented with 1% bovine serum albumin (BSA). M16 and KSOM+AA (supplemented with amino acids) were from Specialty Media (Phillipsburg, NJ). Quinn's Cleavage Media was purchased from SAGE Biopharma media (Bedminster, NJ). Whitten's media was a gift from Dr BC Paria (Whitten and Biggers, 1968). All media and mineral oil (Sigma) were equilibrated overnight at 37°C in 5% CO2 before embryo culture.

Collection of mouse embryos

Mouse embryos were obtained by techniques described previously (Hogan, 2002). Female MF-1 mice (4–5 weeks old, Harlan Sprague Dawley, Indianapolis, IN) were injected intraperitoneally with 7.5 IU pregnant mares' serum gonadotrophin (Sigma Chemical Co., St. Louis, Mo), followed by an injection of 7.5 IU of HCG (Sigma) 44–48 h later. After the second injection, females were housed overnight with C57BL/6 J × SJL/J F1 hybrid males (Jackson Laboratories, Bar Harbor, ME). Noon of the day following coitus was considered Day E0.5. Embryos were obtained at the following stages: Late 2-cell stage (E1.5) and morula-early blastocyst (E3.5). The protocols for animal use were approved by the Wayne State University Animal Investigation Committee (AIC).

Embryo culture and evaluation

For each analysis, groups of 10–30 embryos were cultured and collected in 40 µl of media overlayed with mineral oil at 37°C in an atmosphere of 5% CO_2 in air for either 0–24 h (E3.5 embryos) or 72 h (E1.5 embryos) after. For E1.5 embryos, embryos were cultured for 72 h, and the number of embryos developing to hatching, full blastocyst, $^1/_2$ blastocyst and collapsed or dead embryos were monitored under the microscope (Leica DM IRE2, Germany) at the end of culture. In experiments to measure apoptosis and BrdU incorporation, E3.5 embryos were cultured in groups in one of four different media, KSOM+AA, Quinn's, Whitten's and Ham's F10 for 24 h. The criteria used for evaluating compaction, morula, blastocyst formation and hatching were described previously (Hogan, 2002; Wang *et al*., 2005; Xie *et al*., 2005a; Xie *et al*., 2005b).

Use of SAPK/JNK inhibitors

Since inhibitors have varied specificities in general, we used two SAPK/JNK inhibitors. The SAPK/JNK inhibitor D-JNKI1 and the penetration control-fluorescein isothiocyanate (TAT-FITC) (Bonny *et al*., 2001) were from Alexis (San Diego, CA). D-JNKI1 is based on the sequence of IB1/JIP1 that binds and inhibits SAPK/JNK at 1 µM (Bonny *et al*., 1998; Bonny *et al*., 2000; 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). This inhibitor is used generally at 1–100 µm. It was used specifically in preimplantation embryos at 25 µM (Maekawa *et al*., 2005) and used at 10 µM here. SP600125 has been reported to have non-specific effects at higher concentrations (Bain *et al*., 2003; Joiakim *et al*., 2003), but none have been reported for DJNKl1. A 2 h pre-load was found to be required to obtain maximal inhibition of endogenous SAPK/JNK in embryos (data not shown).

TUNEL assays for apoptosis in embryos

The E3.5 embryos were cultured in KSOM+AA, Quinn's, Whitten's and Ham's F10 for 24 h and then were washed and fixed in 2% (v/v) paraformaldehyde/PBS solution for 30 min at room temperature. For membrane permeabilization, the embryos were incubated in 0.1 M glycine/PBS solution for 10 min and then incubated in 0.1% Triton X-100/PBS solution for 10 min. A TUNEL assay was used to assess the presence of apoptotic cells (DeadEnd™. Fluorometric TUNEL System, Promega, Madison, USA). Fixed embryos were incubated in TUNEL reaction medium containing Equilibration buffer, Nucleotide Mix and rTdT (terminal deoxynucleotidyltransferase dUTP nick end labelling) enzyme at 37°C for 1 h, washed and stained with 2 mg/ml of DAPI (49,6-diamidine-29-penylindole dihydrochloride, Roche). The fraction of TUNEL positive cells was quantitated in embryos by visually inspecting them using the z-axis control of an epifluorescent microscope (Leica DM IRE2, Germany). Criteria for assigning positive status were co-localization of TUNEL product around a single DAPI-stained nucleus above the background level of TUNEL staining in normal, unperturbed static culture embryos in areas that lacked any elevated TUNEL staining (Xie *et al*., 2006).

BrdU assays for cell proliferation in embryos

Cell proliferation in embryos was detected using BrdU Flow Kits (BD Biosciences, San Diego). E3.5 embryos were cultured in KSOM+AA and Ham's F10 respectively for 24 h at 37°C in a humidified atmosphere containing 5% CO_2, and then embryos were labelled with 20 µM BrdU for 40 min. Embryos were fixed with BD cytofix/cytoperm buffer for 30 min at room temperature. For membrane permeabilization, the embryos were incubated in BD Cytoperm buffer for 10 min on ice. Embryos were refixed with BD cytofix/cytoperm buffer for 10 min The DNA was denatured by incubating the embryos with 300 µg/ml DNase for 30 min at 37°C. The incorporated DNA was detected by staining embryos with fluorescent anti-BrdU antibody for 3 h at room temperature. Nuclei were stained with Hoechst for 20 min at room temperature. Fraction of BrdU positive cells was quantitated in embryos by visually inspecting them using an epifluorescent microscope (Leica DM IRE2, Germany).

Statistical analysis

The data in this study are representative of 2–3 independent studies and indicated as mean ± SD. Statistical significance of differences between different treated samples were calculated by chi-square for categorical data, and Student's *t*-test and one-way analysis of variance (ANOVA) [Statistics Package for Social Sciences (SPSS) 11.0] followed by LSD post hoc tests when pairs of continuous data were significant. Chi-square was performed on data in Tables I, and *t*-tests were performed with data in Figure 2 (part 2), Figure 3 (part 2) and Figure 4 (part 2). $P < 0.05$ indicates significant difference among groups. $P < 0.01$ indicates highly significant difference among groups.

Results

SAPK inhibitors improve development in optimal KSOM+AA and decrease development in suboptimal Ham's F10.

Our previous data showed that increases in phosphorylation of SAPK/JNK correlated negatively with preimplantation mouse developmental rates including lower blastocyst formation and hatching rate and fewer cell number in embryos (Wang *et al*., 2005; Zhong *et al*., submitted for publication). Our two goals in

Table I. SAPK inhibitors improve development in optimal KSOM+AA and decrease development in suboptimal Ham's F10

Group	Hatching	Full cavity	$^1/_2$ cavity	Collapsed/dead	Total Embryos
Cumulative outcome with SAPK inhibition* (*6 exp)					
Measures improving or worsening					
Good media	**5/6 up**	**6/6 up**	**6/6 up**	**5/6 up**	544
Bad media	6/6 down	6/6 down	4/6 down	6/6 down	577
Experiment 1					
KSOM+AA	10 (28.6%)	30 (85.7%)	3 (8.6%)	2 (5.7%)	35
KSOM+AA/DJNKI	35 **(50%)***	61 **(87.1%)**	4 **(5.7%)**	5 (7.1%)	70
Ham's	16 (26.7%)	39 (65.0%)	10 (16.7%)	11 (18.3%)	60
Ham's/DJNKI	11 (21.6%)	22 (43.1%)*	6 **(11.8%)**	23 (45.1%)*	51
Experiment 2					
KSOM+AA	4 (10.3%)	21 (53.8%)	4 (10.3%)	14 (35.9%)	39
KSOM+AA+DJNKI	13 **(30.9%)***	30 **(71.4%)**	2 **(4.8%)**	10 **(23.8%)**	42
Ham's	5 (13.2%)	6 (15.8%)	6 (15.8%)	26 (68.4)	38
Ham's +DJNKI	0 (0%)*	3 (6.7%)	3 **(6.7%)**	39 (86.7%)*	45
Experiment 3					
KSOM+AA	19 (44.2%)	35 (81.4%)	4 (9.3%)	4 (9.3%)	43
KSOM+AA/DJNKI	21 **(53.8%)**	37 **(94.8%)***	1 **(2.6%)**	1 **(2.6%)**	39
KSOM+AA/SP600125	17 (42.5%)	35 **(90%)**	2 **(5%)**	2 **(5%)**	39
Ham's	14 (36.8%)	21 (55.3%)	1 (2.6%)	16 (42%)	38
Ham's/DJNKI	5 (13.2%)*	12 (31.6%)*	3 (7.9%)	23 (60.5%)*	38
Ham's/SP600125	7 (17.5%)*	14 (35%)	2 (5%)	24 (60%)	40
M16	7 (18.4%)	19 (50%)	5 (13.2%)	14 (36.8%)	38
Experiment 4					
KSOM+AA	18 (31.6%)	47 (82.4%)	7 (12.3%)	3 (5.2%)	57
KSOM+AA/SP600125	30 **(45.5%)**	59 **(89.4%)**	6 **(9.1%)**	1 **(1.5%)**	66
Ham's	14 (26.9%)	33 (63.5)	8 (15.4%)	11 (21.2%)	52
Ham's/SP600125	7 (12.5%)	24 (42.8%)*	10 (17.8%)	22 (39.3)*	55
M16	13 (22.8%)	36 (63.2%)	9 (15.8%)	12 (21.1%)	57
Experiment 5					
KSOM+AA	18 (29.0%)	51 (82.3%)	6 (9.7%)	5 (8.1%)	62
KSOM+AA/SP600125	27 **(51.9%)***	47 **(90.4%)**	3 **(5.8%)**	2 **(3.8%)**	52
Ham's	11 (22.4%)	30 (61.2%)	7 (14.3%)	12 (24.5%)	49
Ham's/SP600125	6 (11.8%)	21 (41.2%)*	8 (15.7%)	22 (43.1%)*	51
M16	11 (21.5%)	31 (60.7%)	7 (13.7%)	13 (25.5%)	51

2-cell stage embryos were cultured for 72 h, and graded for four developmental criteria as indicated.
Boldface indicates conditions where inhibitors improved fraction of embryos hatching or at full cavity and decreased fraction of embryos at only $^1/_2$ cavity or collapsed/dead. Underlined indicates when the SAPK inhibitors had the opposite negative effect on these parameters. Note that SAPK inhibitors improved 18/20 parameters in KSOM+AA and worsened 18/20 parameters in Ham's F10.
*$P < 0.05$ significant difference compared with medium alone.

these experiments were to test whether SAPK/JNK inhibitors might improve embryonic development in media, producing different levels of stress and to ascertain whether changes in cell survival or proliferation were contributing to the slow growth reported previously.

We first tested in this investigation whether SAPK/JNK inhibitors DJNKI1 or SP600125 can improve mouse preimplantation embryo development and at the same time asked whether SAPK is required for development in the most optimal media. To do this, we cultured 2-cell stage embryos in optimal KSOM+AA or suboptimal Ham's F10 in the presence or absence of SAPK/JNK inhibitor DJNKI1 or SP600125 for 72 h. As shown in Table I and Figure 1, SAPK inhibitors improve development in optimal KSOM+AA medium and decrease development in suboptimal Ham's F10 medium. DJNKI1 significantly increases ($P < 0.05$) the hatching rate (from 28.6 to 50% in experiment 1, 10.3–30.9% in experiment 2 and 29.0–51.9% in experiment 5) and full cavity rate (53.8–71.4% in experiment 2, 81.4–94.8% in experiment 3) in KSOM+AA ($P < 0.05$), but both DJNKI1 and SP600125 significantly increased collapsed/dead rates in Ham's F10 ($P < 0.05$) (18.3–45.1% in experiment 1, 68.4–86.7% in experiment 2, 42–60.5% in experiment 3, 21.2–39.3% in experiment 4 and 24.5–43.1% in experiment 5).

In addition, four parameters [two representing poor development (1/2 cavity and collapsed/dead) and two representing good development (hatching and full cavity)] were assayed in five experiments using the two SAPK inhibitors (DJNKI1 and SP600125, SP600125 was used only in experiment 3).

The four parameters measured six times (three experiments using DJNKI1 and three using SP600125) produced 24 measurements and 22/24 measured parameters improved when SAPK inhibitors were added to optimal KSOM+AA (Table I, boldface). Conversely, when SAPK inhibitors were added to suboptimal Ham's F10, 22/24 measured parameters in the six experiments worsened (Table I, underlined). The increase in less than $^1/_2$ cavity and morbid embryos was similar to those noted in previous experiments using suboptimal M16 (Maekawa *et al*., 2005). M16 media was previously found to be suboptimal and stimulatory of SAPK/JNK phosphorylation (Wang *et al*., 2005) and was similar to Ham's F10 in its poor quality as noted here (Table I, experiment 3, 4 and 5).

Interestingly, in experiment 2, a highly significant higher percentage of embryos was observed to fail to progress and be morbid in both optimal and suboptimal media ($P < 0.01$) compared with experiments 1 and 3. It was noted that embryos were more granular (data not shown) than normal embryos at the start of the culture experiment, suggesting problems with the female mice and/or embryos preceding the culture. Despite this confounding variable, SAPK inhibitor DJNKl1 increased development in optimal media and decreased development in suboptimal media as in the other two experiments.

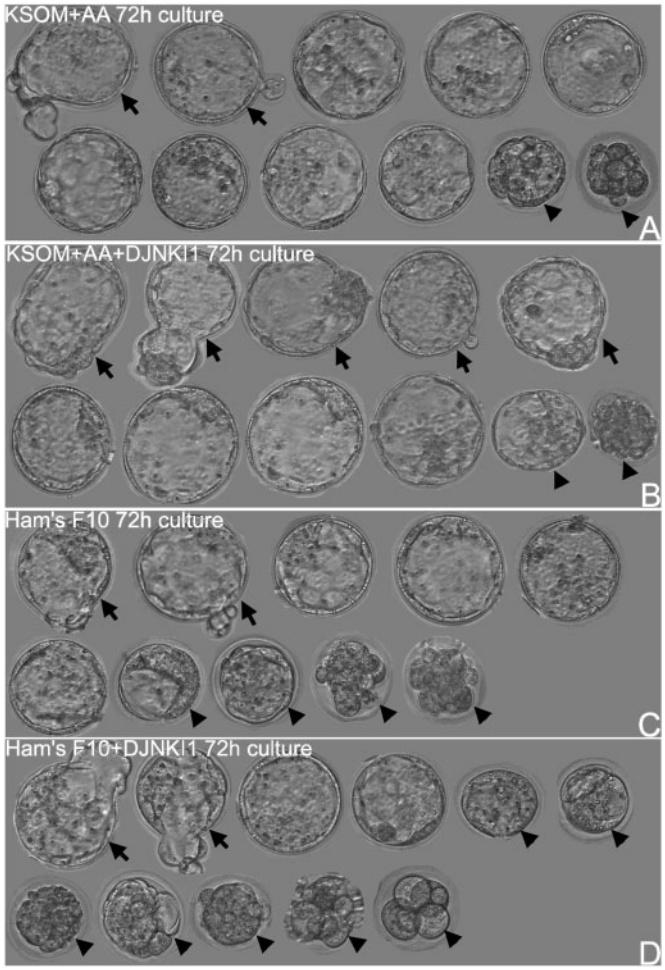

Figure 1. SAPK inhibitors improve development in optimal KSOM+AA and decrease development in suboptimal Ham's F10. Late 2-cell stage embryos were cultured in KSOM+AA or Ham's F10 with or without the presence of SAPK inhibitors DJNKI1 for 72 h. The development of embryos was observed under the microscope. (A) Embryos were cultured in optimal KSOM+AA (B) Embryos were cultured in optimal KSOM+AA with DJNKI1. (C) Embryos were cultured in suboptimal Ham's F10. (D) Embryos were cultured in suboptimal Ham's F10 with DJNKI1. Arrows show the hatching embryos while arrowheads show $^{1}/_{2}$ cavity and dead embryos.

Apoptosis increases in embryos cultured in suboptimal medium Ham's F10 for 24 h

We next tested whether apoptosis contributed to poor development of embryos in suboptimal media. In three experiments, E3.5 embryos in Ham's F10 were consistently significantly higher in TUNEL/apoptosis than KSOM+AA ($P < 0.01$) (14.1% compared to 5.1% in experiment 1, 13.3% compared to 6.4% in experiment 2 and 13.6% compared to 6.2% in experiment 3), at the end of 24 h culture [Figure 2 (part 1, 2)] compared with embryos in KSOM+AA. In one experiment, embryos in Whitten's had significantly higher TUNEL/apoptosis ($P > 0.05$) (9.7% compared to 6.4%) than embryos in KSOM+AA, but in all other experiments embryos in Whitten's or Quinn's were not significantly increased ($P = 0.05$) for TUNEL/apoptosis compared with KSOM+AA.

Time course experiments show apoptosis in embryos increased significantly after 6 h culture in suboptimal Ham's F10 medium and continued increasing through 24 h culture.

We next performed tests for the time-dependence of TUNEL development during the 24 h culture period. This was done for two reasons. First,

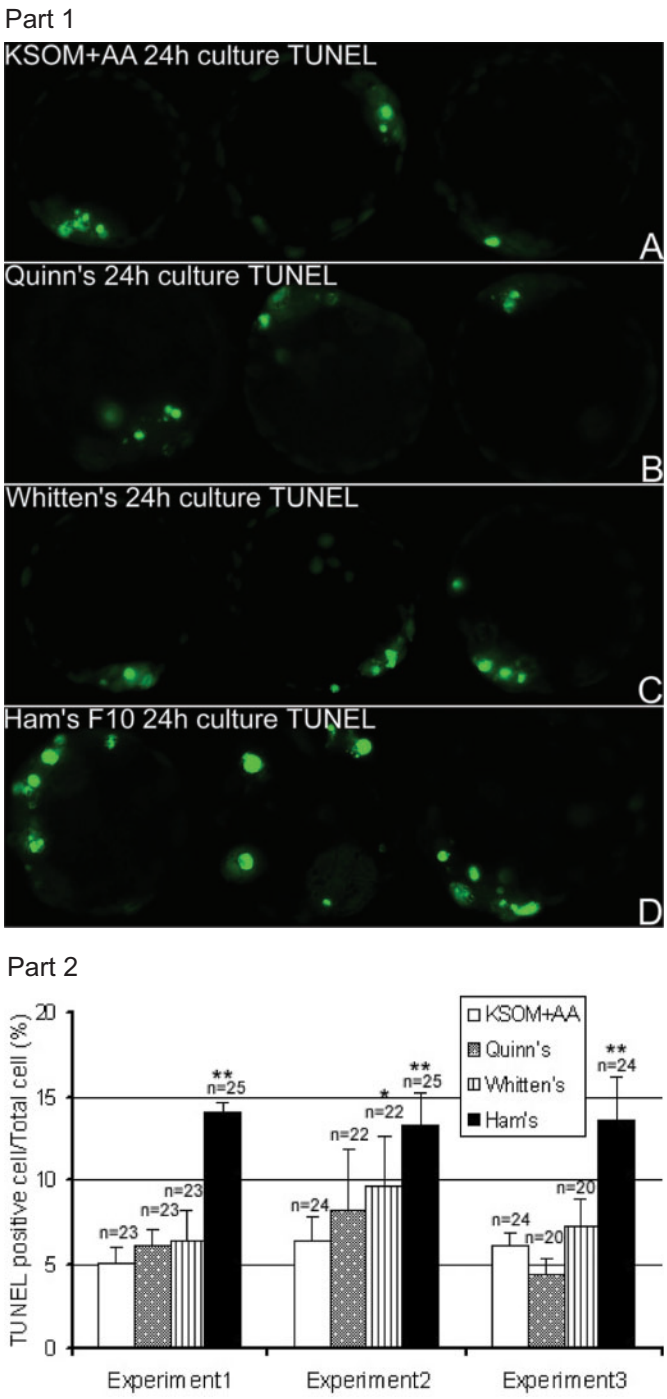

Figure 2. Percentage of apoptotic cells is increased in embryos cultured in Ham's F10 medium for 24 h. E3.5 embryos were cultured in four different medium, KSOM+AA, Quinn's, Whitten's or Ham's for 24 h, and then were tested for apoptosis by TUNEL. Part 1. Embryos were cultured in (A) KSOM+AA, (B) Quinn's, (C) Whitten's, and (D) Ham's F10. Part 2. Histogram at the bottom shows the quantitative measurements of the fraction of TUNEL positive cell in embryos treated in Part 1. The experiment was repeated three times with consistent results (experiment 1, experiment 2 and experiment 3). *P < 0.05, significant difference when compared to embryos cultured in KSOM+AA. **P < 0.01, highly significant difference when compared to embryos cultured in KSOM+AA.

previous observations had suggested that E3.5 embryos progressed from about 32.1 ± 3.5 cells at the start of culture to about 50.8 ± 8.4 cells after 24 h in Ham's F10 and to about 76.6 ± 9.3 cells after 24 h in KSOM+AA (Zhong *et al*., submitted for publication). Time-dependence of TUNEL

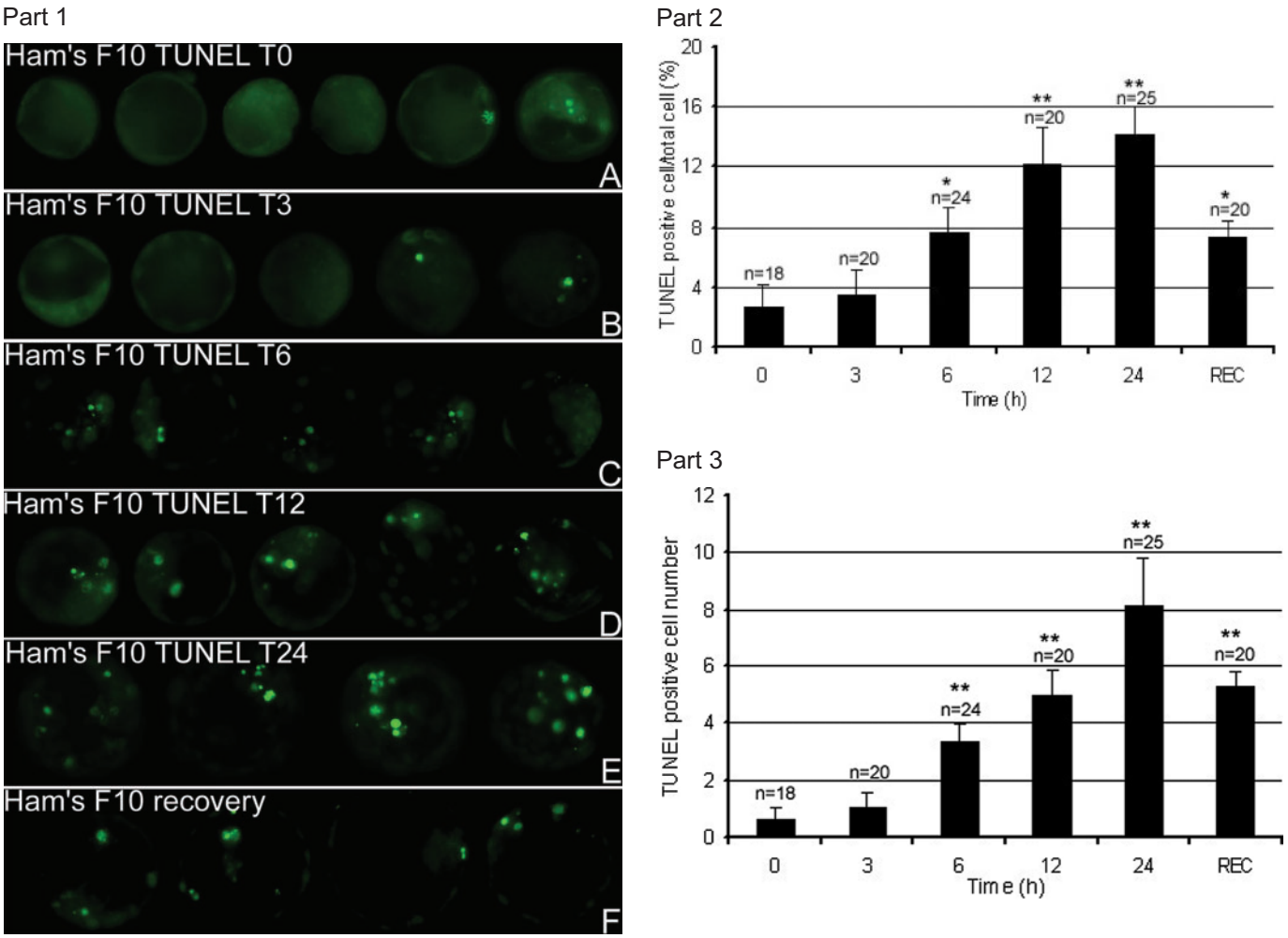

Figure 3. Time course experiment to detect the onset and progression of apoptosis in embryos cultured in Ham's medium. E3.5 embryos were cultured in Ham's F10 medium for different time periods and then were tested for apoptosis by TUNEL. Part 1. (A) Embryos ex vivo, (B) Embryos cultured in Ham's F10 for 3 h, (C) 6 h, (D) 12 h or (E) 24 h, (F) Rec, embryos were cultured for 12 h in Ham's F10 and allowed to recover in KSOM+AA for 12 h. Part 2. Histogram shows the quantitative measurements of the fraction of TUNEL positive cell in embryos treated in Part 1. *P < 0.05, significant difference when compared to 0 h **P < 0.01, highly significant difference when compared to 0 h. Part 3. Histogram shows the quantitative measurements of the absolute number of TUNEL positive cell in embryo.

would suggest whether waves of apoptotic cells might arise and cells disappear, accounting for the approximately 25 fewer cells gained for E3.5+24 h culture in suboptimal Ham's F10. Second, we wanted to find whether there was an early TUNEL peak at 3–6 h due to a general metabolic stress of explanting embryos *ex vivo* to culture (Leese, 2002). We did not detect an early peak [Figure 3 (part 1, 2)] and therefore we did not test whether there was an early TUNEL peak in the optimal media. A time-dependent increase in TUNEL occurred that was significantly higher by 6 h ($P < 0.05$) (7.6% compared to 2.7%), and highly significantly higher at 12 and 24 h ($P < 0.01$) (12.2% and 14.2% compared to 2.7%). Interestingly, embryos incubated to a highly significant increase in TUNEL at 12 h in suboptimal Ham's F10, then allowed to recover in KSOM+AA for an additional 12 h, had significantly fewer TUNEL positive cells than time-matched embryos remaining in Ham's F10 for the entire 24 h ($P < 0.05$) (5.3 compared to 8.1 per embryo). This suggests that, TUNEL positive cells at 12 h die and are phagocytized, resulting in fewer TUNEL positive cells. The data suggest that waves of TUNEL positive cells could arise, die and disappear during the 24 h culture.

Cell proliferation decreased in embryos cultured in suboptimal Ham's F10 for 24 h.

Our second goal in this study was to assay media recently studied by us and others (Ecker *et al*., 2004; Wang *et al*., 2005) for effects on cell survival. It seemed unlikely, after the analysis of TUNEL time-dependence, that the entire difference in cell number increase between optimal and suboptimal media was due to apoptosis. Therefore, we then tested differences in rates of entrance into cell cycle. Significantly ($P < 0.01$) more cells in embryos cultured in KSOM+AA (45.6%) from E3.5 for 24 h incorporated BrdU and were in S phase than embryos cultured in Ham's F10 (31.0%) (Figure 4). These data suggest that failure to enter S phase, as well as increased apoptosis, contributed to the lesser number of cells in embryos cultured in suboptimal media.

Discussion

SAPK/JNK is not required for the nearly normal embryonic development provided by optimal media such as KSOM+AA. Conversely, when suboptimal media such as Ham's F10 or M16 create abnormally high amounts of stress and SAPK/JNK phosphorylation (Wang *et al*., 2005), SAPK/JNK becomes necessary to maintain homeostasis. Clearly, p38MAPK is required for normal development as observed in

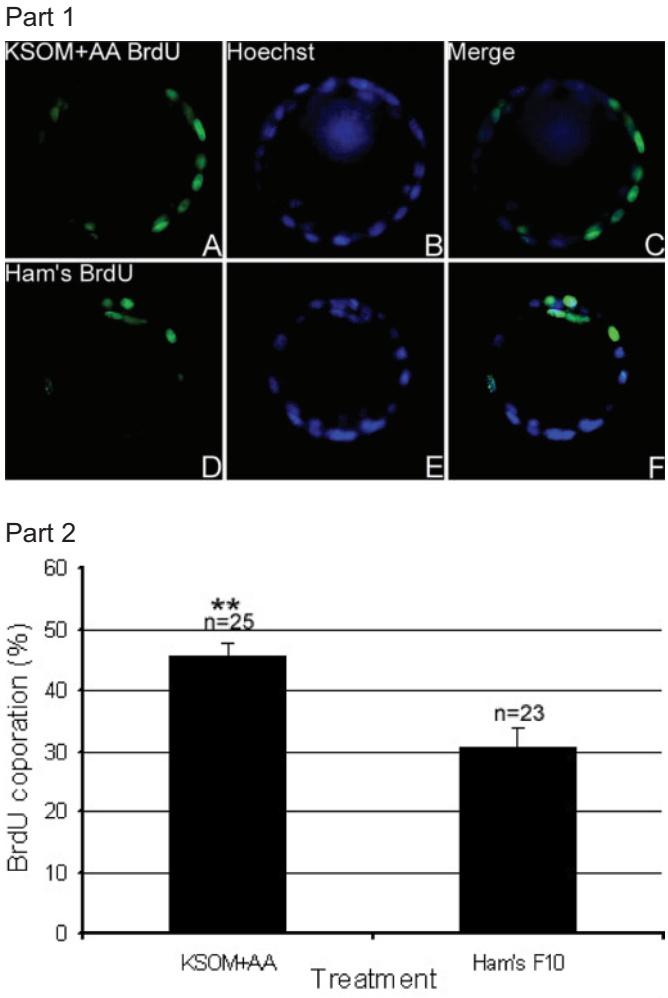

Figure 4. Percentage of BrdU cells is decreased in embryos cultured in Ham's F10 medium for 24 h. E3.5 embryos were cultured in KSOM+AA or Ham's F10 for 24 h and then were tested for entrance into the cell cycle by BrdU incorporation. Part 1. BrdU incorporation into embryos cultured in (A) KSOM+AA or (D) Ham's F10. (B, E) Hoechst stain for nuclei in (A, D), respectively. (C, F). Merge of BrdU stain and Hoechst stain in (A, D) and (B, E). Part 2. Histogram at the bottom shows the quantitative measurements of the fraction of BrdU positive cell in embryos treated in Part 1. **P < 0.01 represents highly significant differences between KSOM+AA and Ham's F10.

both optimal media (Natale *et al*., 2004) and suboptimal media (Maekawa *et al*., 2005). In contrast, SAPK/JNK is not required for embryo development in optimal media, but is important in suboptimal media (Maekawa *et al*., 2005). This contrast between differential progression rates for embryos cultured in suboptimal and optimal media for p38MAPK and SAPK/JNK suggests that p38MAPK is primarily important in normal embryo progression, and SAPK/JNK is primarily important in homeostasis for preimplantation embryos.

A total of three SAPK/JNK inhibitors SP600125, DJNKl1 and siRNA to SAPK/JNK were used in this report and the report from Maekawa and colleagues. SP600125 was unique of the three inhibitors, in being shared by the two studies. In both reports, the outcome for all three inhibitors is a phenocopy of a single outcome, slow growth and morbidity after compaction when embryos were cultured in the most suboptimal media (Ham's F10 and M16). This suggests that the results are due to shared specific inhibition of SAPK/JNK of all three inhibitors used, not the possible unique non-specific effects of each of the three inhibitors alone.

During the tests for SAPK/JNK function, it was apparent that there are doses of stress in culture where SAPK/JNK function is detrimental to embryo development and SAPK/JNK inhibitors can improve function. Interestingly, we have also observed the improvement in embryo progression and decrease in TUNEL/apoptosis when embryos are subject to shear stress SAPK/JNK function, blocked by SAPK/JNK inhibitors (Xie *et al*., 2006). For embryos, shear stress is functionally more stressful when compared with high hyperosmolar stress caused by 1 M sorbitol. In comparison, KSOM+AA can be made as poor as Ham's F10 for sustaining increase in cell number in embryos cultured for 24 h from E3.5, by adding 0.2 M sorbitol (Zhong *et al*., submitted for publication). This suggests that quality, quantity and duration of stress all make significant contributions in defining the need for SAPK/JNK-mediated homeostasis and also in defining when SAPK/JNK inhibitors may improve development of cultured embryos.

Several reviews have pointed out the importance of epigenetic effects and stress on embryonic development during culture (Summers and Biggers, 2003; Johnson, 2005; Lane and Gardner, 2005). To date, key molecular mechanisms mediating epigenetic effects of culture stress that have been studied include imprinting, heat shock pathway and mitochondria number and function. To this list, and possibility interacting with the other mechanisms, is the stress response mediated by stress enzymes such as SAPK/JNK, p38MAPK, extracellular response kinase (ERK)5 and MAPK family members that transduce negative signals (Ip and Davis, 1998; Rappolee, 2003). The importance of stress during preimplantation development *in vivo* (Kwong *et al*., 2000) and *in vitro* (Ecker *et al*., 2004; Fernandez-Gonzalez *et al*., 2004) is made apparent by recent reports on the long-term effects of perturbation on post-natal hypertension and learning. It is clear that 'memory' of stress during preimplantation development leads to long-term effects and several epigenetic mechanisms, including SAPK/JNK function, may play a role in the development of stress 'memory'. Taken together the data suggest a possible role of SAPK/JNK in long-term effects placed under the general consideration of 'Fetal Origins of Adult Disease' (FOAD), suggested by the Barker hypothesis, but also extended to the embryonic period of development (Barker *et al*., 1993; Kwong *et al*., 2004).

We have sought to account for the decrement in cell number increase in embryos during culture in suboptimal media. It seems that cell survival, suppression of TUNEL/apoptosis, cell cycle progression and fraction of cells entering S phase, all play major roles in response to suboptimal media. It is difficult to make a firm conclusion about the numerical contributions of increased apoptosis and decreased S phase commitment to the decrement of cell number increase in suboptimal media. From previous studies, we have observed approximately 25 less cells gained in 24 h culture of E3.5 embryos in suboptimal Ham's F10 compared with optimal KSOM+AA (Zhong *et al*., submitted for publication). Since the number of cells/embryo at the start of culture is about 30, then a 50% decrease in entrance into S phase would be about 15 fewer cells, assuming all cells normally enter S phase during 24 h of culture.

In our analysis of TUNEL cells per embryo, Ham's F10 developed a peak of 8 cells/embryo in 24 h, but the recovery experiments indicated that waves of TUNEL positive cells may have developed, died and been phagocytized during the 24 h. Phagocytosis of beads, and likely apoptotic cells, has been observed in E3.5 embryos (Rassoulzadegan *et al*., 2000). However, the difference between maximal TUNEL positive cells/embryo in Ham's F10 at 8 cells/embryo and KSOM+AA at about 2 cells/embryos, produces a difference of about 6 TUNEL positive cells per embryo. If these cells developed and disappeared 2.5 times during the 24 h culture period, this would produce a decrement of 15 fewer cells, similar to the decrement caused by decreased S

phase entrance measured by decreased BrdU. It, therefore, seems likely that stress-induced loss of cell survival and stress-induced decrease in S phase commitment contribute fairly similarly to the total decrement in cell number in suboptimal media.

Interestingly, in the second of three experiments testing the effects of SAPK inhibitors on development of embryos in suboptimal and optimal media, a confounding variable arose. The embryos were clearly more granular and vacuolated at the start of culture (data not shown). All embryos, in both optimal and suboptimal media, had increased fractions in the two negative parameters, fraction of $^{1}/_{2}$ cavity and morbidity in experiment 2 compared with the other experiments. This suggested that embryo quality and health was not optimal at the start of the culture. Despite this, the effects of inhibitors in optimal and suboptimal media showed the same pattern as in the other two experiments. This suggests that levels of stress during culture, and the ability of the embryo to use SAPK to mount a homeostatic response to culture stress, can be dominant to aspects of quality of the embryos prior to the culture.

In summary, the data presented here suggest caution in the design and interpretation of experiments intended to test the roles of negatively or positively acting signal transduction enzymes in normal preimplantation mouse development. The relative merits of different media provide a 'frame of reference' that must be considered carefully in these experiments. The data also suggest that SAPK/JNK inhibitors may provide a medicinal improvement in optimal culture media measured by developmental rates during and at the end of culture. Ongoing experiments are intended to test whether SAPK/JNK inhibitors may also improve long-term post-natal effects that result from preimplantation culture stress. Finally, we have sought to account for decrements in cell number increase during culture and have concluded that increase in apoptosis and decrease in S phase commitment play relatively equal roles in effects of suboptimal media.

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