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Abstract

Mitogen-activated protein kinase (MAPK) pathways mediate some important cellular processes and are likely to also regulate preimplantation development. The role of p38 MAP kinase signaling during murine preimplantation development was investigated in the present study. p38 MAPK, p38-regulated or -activated kinase (PRAK; MK5), map kinase-activated protein kinase 2 (MK2), and heat shock protein 25 (hsp25) mRNAs and proteins were detected throughout preimplantation development. Two-cell stage embryos cultured in the presence of SB220025 and SB203580 (specific inhibitors of p38 MAPK α/β), progressed to the eight-cell stage with the same frequency as controls; however, treated embryos halted their development at the 8- to 16-cell stage. In addition, embryos treated with p38 MAPK inhibitors displayed a complete loss of MK2 and hsp25 phosphorylation and also a complete loss of filamentous actin as indicated by the absence of rhodamine–phalloidin staining. In these inhibitor-treated groups, the embryos were composed of a mixture of compacting and noncompacting cells, and the embryos were one to two cell divisions behind controls. Treated embryos remained viable as the developmental blockade was rescued by removing embryos from the drug treatment and placing them in drug-free medium until they progressed to the blastocyst stage. This study demonstrates that p38 MAPK activity is required to support development through the murine preimplantation interval.

Keywords: p38 MAPK; Blastocyst; Preimplantation; Embryo

Introduction

Preimplantation development is critical to the establishment of a viable mammalian pregnancy. It is estimated that only 40% of human embryos successfully implant to establish a pregnancy (Edwards, 1997). Despite a reasonably sound understanding of the specific events associated with preimplantation development, including zygotic genome activation (Memili et al., 1998; Schultz, 1993; Zimmermann and Schultz, 1994), the onset of compaction (Collins and Fleming, 1995; Fleming et al., 2000; Sutherland and Calarco-Gillam, 1983), and blastocyst formation (Betts et al., 1998; Watson and Barcroft, 2001; Watson et al., 1990, 1992b), comparatively little is known about the intracellular signaling pathways that must regulate these events.

To identify specific signaling molecules, which may play a role in the progression of the preimplantation embryo, we used differential display-reverse transcription polymerase chain reaction (DD-RT-PCR). From this screen, we identified, among others (Natale and Watson, 2002; Natale et al., 2000, 2001), the cDNA for p38-regulated or -activated kinase (PRAK), a downstream effector of the p38 mitogen-activated protein kinase (MAPK) subfamily.

MAPK pathways mediate some cellular processes, including cell proliferation, growth, differentiation, and death (reviewed by Ono and Han, 2000). The p38 MAPK family consists of four isoforms, p38 MAPK α, β, γ, and δ, which share more than 60% amino acid identity. The p38 MAPK cascade is activated in response to many types of stress and proinflammatory cytokines. In addition, p38 MAPK isoforms act through multiple effectors (reviewed by Kyriakis and Avruch, 2001; Ono and Han, 2000), including several transcription factors as well as MAP kinase-activated

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protein kinase-2 (MK2; Huot et al., 1997) and/or PRAK (New et al., 1998), which is also designated as MK5 (Ni et al., 1998). PRAK and MK2 are specifically activated by active p38 MAPK α and/or β. These proteins in turn phosphorylate a member of the small heat shock protein family (hsp), hsp25/27 (Davidson and Morange, 2000; New et al., 1998).

Targeted inactivation of the p38 MAPK α gene resulted in embryonic lethality at or around E10.5 (Adams et al., 2000; Allen et al., 2000; Mudgett et al., 2000; Yang et al., 2000). p38 MAPK α, like an upstream regulator of the p38 cascade, Mekk3, is essential for placental morphogenesis (Adams et al., 2000; Allen et al., 2000; Mudgett et al., 2000; Yang et al., 2000). Although the $p38\alpha^{-/-}$ mutant mouse proceeds through the preimplantation period, the expression of PRAK during this interval led us to hypothesize that p38α and other members of the p38 MAPK family are required to direct the developmental program that oversees preimplantation development. We have predicted that multiple p38 MAPK family members might be expressed during the preimplantation period, permitting the $p38\alpha^{-/-}$ mutant mouse embryos to survive this early developmental interval. Thus, the primary objective of the present study was to investigate the expression profiles and function of components of the p38 MAPK signaling pathway in murine preimplantation development in vitro.

Our results demonstrate that all members of this MAPK pathway are expressed during murine preimplantation development. More importantly, inhibition of p38 MAPK activity (using cytokine suppressive anti-inflammatory drugs, CSAIDs, pharmacological inhibitors of p38 MAPK α/β; Cirillo et al., 2002; Davidson and Morange, 2000; Huot et al., 1997; Zayzafoon et al., 2002) results in a reversible arrest of preimplantation development, suggesting that p38 MAPK α/β activity is required for successful preimplantation development to occur.

Materials and methods

Superovulation and mouse embryo collection

Female CD-1 mice (Charles River, Wilmington, MA, USA), 3–4 weeks of age, were injected with 5 IU pregnant mare serum gonadotrophin (PMSG; Sigma, St. Louis, MO, USA) followed by 5 IU human chorionic gonadotrophin (hCG; Sigma) 48 h later and mated with CB6F1/J males (Charles River). Successful mating was determined the next morning (which was considered to be day 1 of development) by the presence of a vaginal plug. Embryos were collected at specified times following hCG injection, which correspond to appropriate cleavage stages: mature, unfertilized eggs, 18-h post-hCG; two-cell, 48 h; four-cell, 60 h; eight-cell, 65–68 h; morulae, 80–85 h; and blastocyst, 90 h. Zygotes through to the eight-cell stage were flushed from oviducts of female mice using flushing medium I (calcium lactate, 1.71 mM; sodium pyruvate, 0.25 mM; bovine serum albumin, 3 mg/ml; and 10× Leibovitz-modified Hank's balanced salt solution all diluted with water to 1×) (Spindle, 1980). Morulae and blastocysts were flushed from uteri on day 4 using flushing medium II ($CaCl_2$, 1.8 mM; amino acids: 0.1 mM L-arginine, 0.5 mM L-cysteine, 1.03 mM L-histidine, 0.2 mM L-isoleucine, 1 mM L-leucine, 2 mM L-lysine, 0.25 mM L-methionine, 0.5 mM L-phenylalanine, 2 mM L-threonine, 0.1 mM L-tryptophan, 0.1 mM L-tyrosine, 1 mM L-valine, 2 mM L-glutamine, and 1× BME vitamins added to 10× Leibovitz-modified HBSS diluted with water to 1×) (Spindle, 1980).

RNA extraction and reverse transcription

Total RNA was extracted from frozen pools of preimplantation embryos using the RNeasy total RNA extraction kit (QIAGEN, Mississauga, ON, Canada) and the manufacturer's suggested protocol, followed by vacuum drying to reduce the volume for reverse transcription (RT). As previously described (Barcroft et al., 1998; Offenberg et al., 2000), RT reactions were performed using oligo-dT (Gibco BRL). Samples were incubated for 90 min at 42°C in a total volume of 20 μl consisting of 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTPs, and 200 units of Superscript II (Gibco BRL). Reactions were terminated by heating the samples to 95°C for 5 min.

PCR amplification

Polymerase chain reaction (PCR) was conducted as previously described (Barcroft et al., 1998; Offenberg et al., 2000), using a total amount of cDNA equal to two embryos of each stage per reaction. Briefly, PCR reactions were carried out in a volume of 50 μl consisting of 0.2 U of AmpliTaq Gold (Perkin Elmer, PE Applied Biosystems, Mississauga, ON, Canada), 1× PCR Gold Buffer (PE Applied Biosystems), 1.5–2.0 mM $MgCl_2$, 0.2 mM of each dNTP, and 1.0 μM of each PCR primer using a Perkin Elmer GeneAmp 2400 thermal cycler. AmpliTaq Gold was activated by an initial incubation at 95°C for 10 min followed by 40 cycles of amplification: 95°C for 30 s, 52–56°C for 60 s, 72°C for 30 s, with a final extension of 10 min at 72°C. PCR products were then resolved on 2.0% agarose gels containing 0.5 μg/ml ethidium bromide (Sigma). PCR reactions were repeated a minimum of three times using cDNA prepared from embryos at each of the indicated stages and isolated from three developmental series.

PCR primers

Gene-specific primers for PCR were designed and synthesized (Gibco BRL) for p38 MAPK α, β, γ, and δ, PRAK, MK2, and hsp25 based on available human and

Table 1
Nucleotide sequences for polymerase chain reaction amplification of p38 MAPK α, β, γ, δ, MK2, PRAK (MK5), and hsp25 (shown in the 5′–3′direction)

Gene product	Primer	Primer sequence	Size (bp)
P38 MAPK α	5′	AGGCCATGGTGCATGTGTGT	320
	3′	AGTAGCTGGAGGAGGAGGAG	
P38 MAPK β	5′	GGCTGCATCATGGCTGAACT	544
	3′	TGAGAGGCGCTTCTTGAGGA	
P38 MAPK γ	5′	TCTCAGCTTCAAGCCTCCTA	402
	3′	TCCTAAGCTCTGCTCACTCT	
P38 MAPK δ	5′	GTCTGTTGGTTGCATCATGG	630
	3′	GGATCTCTTGAGTTGCAGGT	
MK 2	5′	AACGCCATCACCGACGACTAC	537
	3′	CAGGACTTCCGGAGCCACATA	
PRAK	5′	TGGCCAACATGAGAATCCAGG	330
	3′	TGAATCCACGACCATTCCAGC	
hsp25	5′	CTCTTCGATCAAGCTTTCGG	410
	3′	CTCAGGGGATAGGGAAGAGG	

Shown also is the size in base pairs (bp) of the expected amplicon.

mouse full-length and EST nucleotide sequences in GenBank nucleotide databases. PCR primer sets were designed to regions of nucleotides conserved across species. The primer pairs used to amplify p38 α, β, γ, and δ, MK2, and hsp25 are reported in Table 1. In addition, primer sets designed to bracket an intron of the β-actin gene (Watson et al., 1992a) were used to assess the efficiency of the RNA extraction or reverse transcription and to ensure the absence of DNA contamination in each sample before investigation of expression of the target genes. Positive (tissue cDNA; heart, liver, and kidney) and negative control (no cDNA template) samples were included for each primer set in each experiment.

Cloning and sequencing of RT-PCR products

RT-PCR products amplified from embryonic cDNA were selected for each target gene and purified using a QIAquick Gel Extraction Kit (QIAGEN). Purified cDNA was then cloned by TA cloning protocol into pGEM-T (Promega, Madison, WI, USA). Several clones representing each amplicon were chosen and submitted for nucleotide sequencing (DNA Sequencing Facility, Robarts Research Institute, London, ON, Canada). To confirm identity of each PCR product, an NCBI BLAST search was then carried out on each nucleotide sequence to compare it to sequences available in the GenBank nucleotide sequence databases.

Whole-mount indirect immunofluorescence and actin labeling

Characterization and localization of expression of members of the p38 MAPK signaling pathway at the protein level in preimplantation stage mouse embryos were assessed by whole-mount indirect immunofluorescence and detected by laser scanning confocal microscopy as previously described (Barcroft et al., 1998). Briefly, preimplantation embryo series, including two-cell, four-cell, eight-cell (compacting stages), morula, and blastocyst stages, were examined. Immunofluorescence experiments were repeated on a minimum of three distinct developmental series, containing at least five embryos representative of each developmental stage for each protein. To assess background and nonspecific binding of secondary antibody, embryos were exposed to the same procedure in the absence of primary antibody.

The following commercial antibodies were used: p38 MAPK and MAPKAP K5 (both are mouse monoclonal antisera raised against human protein; BD Transduction Laboratories); MK2, phospho-MK2, and phospho-hsp25 (all are rabbit anti-human polyclonal antisera; Cell Signaling Technologies); PRAK (goat anti-human polyclonal antiserum; Santa Cruz Biotechnology, sheep anti-human polyclonal; Upstate Biotechnology); and hsp25 (goat anti-human polyclonal antiserum; Santa Cruz Biotechnology). We tested all primary antibodies over a range of dilutions and found that all were most effective at a dilution of 1:100 from the commercial stock concentration. In addition, the specificity of each antiserum was verified by conducting a series of Western immunoblots applied to a selection of murine

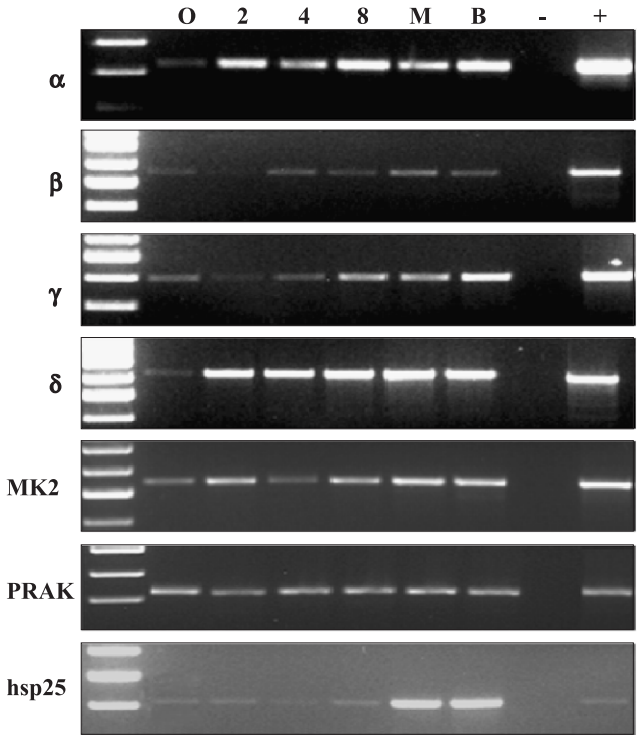

Fig. 1. Detection of transcripts encoding p38 MAPK isoforms and pathway constituents during murine preimplantation development. RT-PCR products encoding p38 MAPKα (A), 320 bp; β (B), 544 bp; γ (C), 402 bp; δ (D), 630 bp; and MK2 (E), 537 bp. PRAK (F), 330 bp, and hsp25 (G), 410 bp, were detected throughout preimplantation development. O, ovulated oocytes; 2, 4, 8, M, and B represent two-, four-, eight-, morula, and blastocyst stage embryos, respectively. (−) indicates no-RT negative control, while (+) indicates tissue RT positive control.

tissues and by immunofluorescence using mouse embryonic fibroblast cells (data not shown).

For whole-mount indirect immunofluorescence, embryos were washed two times in phosphate-buffered saline (PBS) and then fixed in 2% paraformaldehyde in PBS for 20 min at room temperature. Following fixation, embryos were washed in PBS and either processed immediately for immunofluorescence or stored at 4°C in PBS + 0.09% sodium azide for up to 3 weeks. For immunofluorescence staining, fixed embryos were permeabilized and blocked concurrently by incubation at room temperature in PBS + 5% donkey serum + 0.01% Triton X-100 for 1 h. Embryos were then washed in PBS and incubated at room temperature for 1 h in PBS + 1% Donkey Serum + 0.005% Triton X-100 and the primary antibody (1:100). This was followed by rigorously washing the embryos at 37°C with PBS, which was

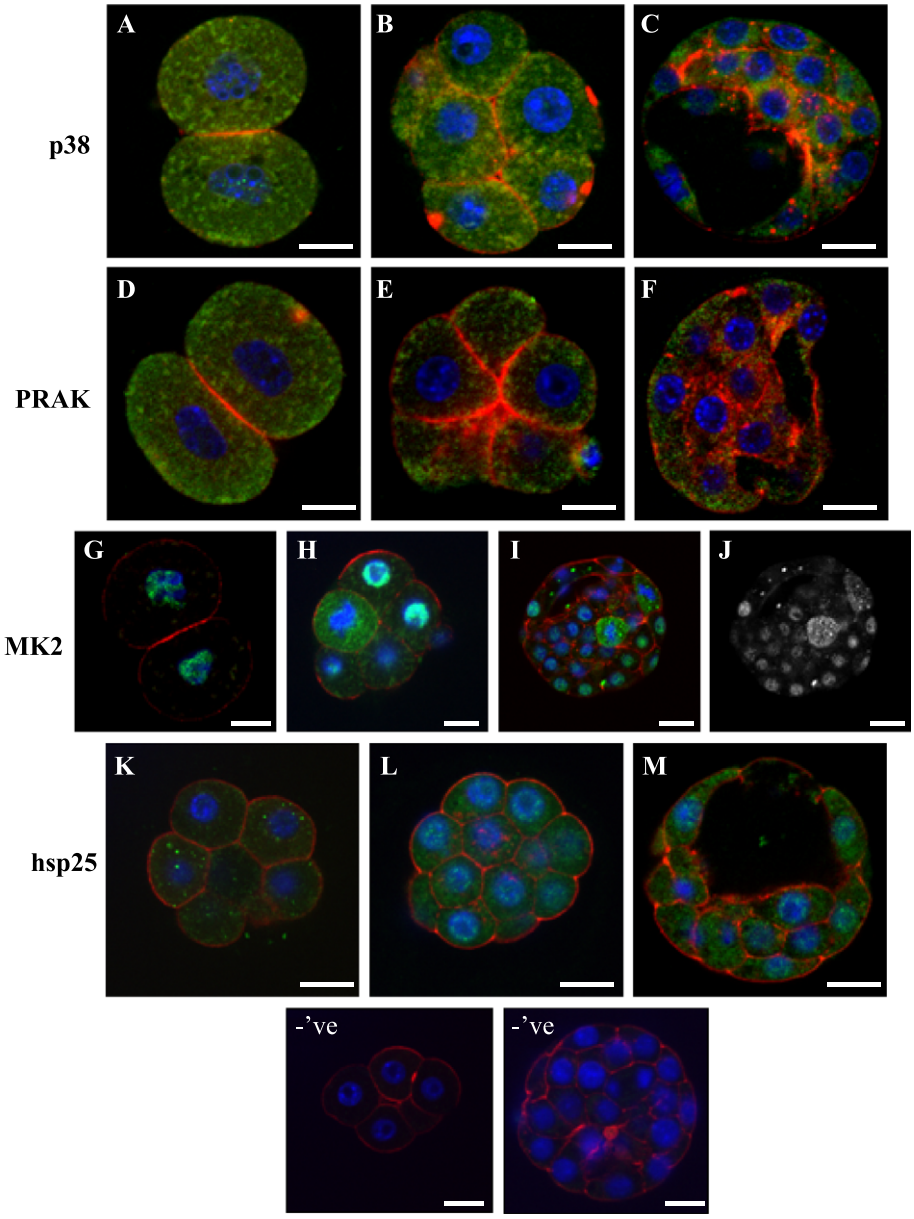

Fig. 2. Distribution of p38 MAPK pathway constituent proteins during murine preimplantation development. In all micrographs, green indicates positive staining for the respective primary antibody, red indicates F-actin (rhodamine–phalloidin), and blue indicates nuclei (DAPI). Phosphorylated p38 MAPK (A, two-cell; B, eight-cell; and C, blastocyst) and PRAK (MK5) (D, two-cell; E, eight-cell; and F, blastocyst) were detected in the cytoplasm of blastomeres throughout development. Phosphorylated MK2 (G, two-cell; H, eight-cell; I, blastocyst; and J, blastocyst-P-MK2 only) was detected primarily in the nucleus of blastomeres throughout development. In cells undergoing mitosis, MK2 was detected throughout the cytoplasm. Phosphorylated hsp25 (K, eight-cell; L, morula; and M, blastocyst) was detected in the cytoplasm and in association with some nuclei of blastomeres at all stages of development. Bottom panels (-'ve) show negative controls and are representative of embryos that have been exposed to FITC-conjugated secondary only (no primary antibody) in addition to rhodamine–phalloidin and DAPI. Scale bar = 25 μm.

changed frequently over 1 h. The washed embryos were incubated at room temperature in PBS + 1% donkey serum + 0.005% Triton X-100 and FITC-conjugated secondary antibodies (Jackson Immuno Labs, MA, USA; 1:200 dilution). Embryos were washed rigorously once again as described above and mounted in Antifade Mounting Reagent (BioRad, Mississauga, ON, Canada). To visualize F-actin localization and stain DNA within nuclei, the first wash following secondary antibody included TRITC-conjugated Phallodin (F-actin-Phalloidin-Tetramethylrhodamine B isothiocyanate, Sigma-Aldrich; diluted 1:200 from 5 μg/ml stock solution) and DAPI (nuclei-4′, 6-diamidino-2-phenylindole dihydrochloride; Sigma-Aldrich; diluted 1:2000 from 1 mg/ml stock solution). Immunofluorescence patterns were visualized by microscopy using a Zeiss compound microscope (Zeiss Axiovert; Carl Zeiss Inc., Thornwood, NY, USA) equipped with epifluorescent optics and confocal laser-scanning microscopy (Zeiss LSM 410 and computer system equipped with Zeiss LSM software; Carl Zeiss Inc.).

p38 MAPK inhibition experiments

Murine embryos were flushed from oviducts of timed-pregnant mice at the two-cell stage (48 h post-hCG; as described above), pooled, washed, and divided into one of five KSOMaa (Ho et al., 1995) microdrop culture treatments: (1) KSOMaa alone, (2) KSOMaa + 20 μM SB202474 (inactive analogue), (3) KSOMaa + 0.2 μM SB220025, (4) KSOMaa + 2.0 μM SB220025, and (5) KSOMaa + 20 μM SB220025. This experiment was also duplicated using a second inhibitor, SB203580, in place of SB220025. This class of compounds has been determined to specifically inhibit both p38 MAPK α and β at the concentrations used in this study (Cirillo et al., 2002; Davidson and Morange, 2000; Huot et al., 1997; Zayzafoon et al., 2002). Embryos were assessed for morphology and progression through cleavage divisions at 24 h postdrug treatment (72 h post-hCG), at which point half of the embryos in groups 3, 4, and 5 were removed from culture, washed, and placed in fresh KSOMaa culture drops for the remainder of the experiment. All embryos were then assessed at 48 h postdrug treatment (96 h post-hCG) to measure development to the blastocyst stage. Cell viability was determined at this time by assaying for uptake of a vital dye (trypan blue; Humason, 1979). Embryos from each treatment group were fixed as described above for whole-mount indirect immunofluorescence and stained with DAPI to count embryo cell numbers.

Statistical analysis

Statistical analysis of data was carried out using SigmaStat (Jandel Scientific, San Rafael, CA) software package. Data showed normal distribution and one-way ANOVA was used to determine treatment effects followed by Tukey's multiple comparison test to determine statistical significance. Differences of $P \leq 0.05$ were considered significant.

Results

Detection of mRNAs encoding p38 MAPK signaling pathway constituents in the preimplantation embryo

The application of RT-PCR methods to preimplantation embryo cDNA samples resulted in the detection of mRNAs

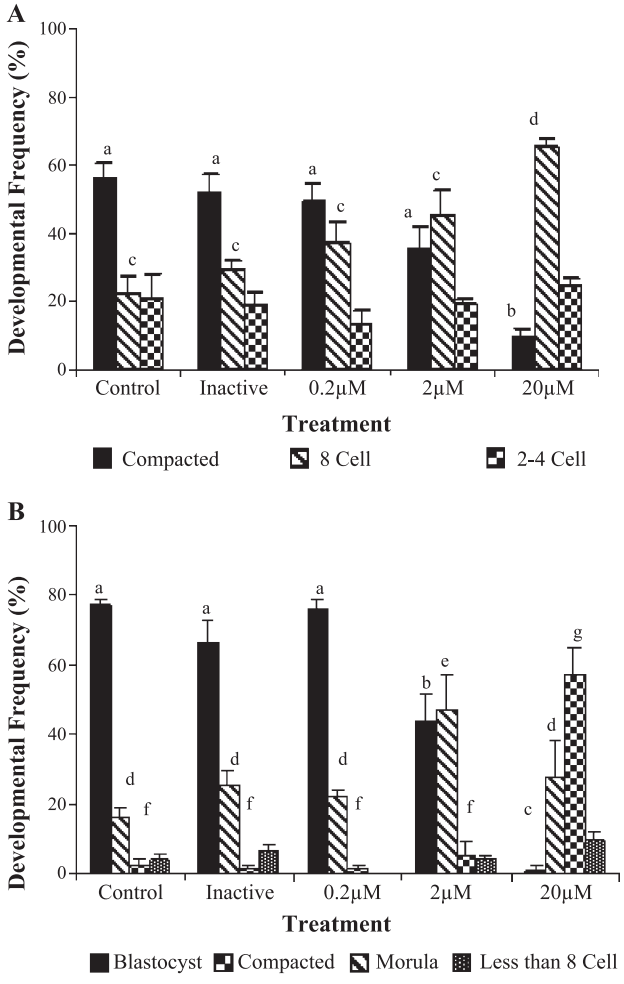

Fig. 3. The effect of treatment with SB220025 on preimplantation development. Two-cell stage murine embryos were treated in culture with 0 μM (control); 20 μM inactive analogue SB202474 (inactive); and 0.2, 2.0, and 20 μM SB220025. (A) Following 24 h of treatment with 20 μM SB220025, there was a significant reduction in the proportion of embryos undergoing compaction. a and b indicate significant differences between compacted embryo frequencies; c and d indicate significant differences between eight-cell embryo frequencies ($P \leq 0.05$). (B) Following 48 h of treatment with 2.0 and 20 μM SB220025, there was a significant reduction in the proportion of embryos that developed to the blastocyst stage. a, b, and c indicate significant differences between blastocyst frequencies; d and e indicate significant differences between morula frequencies; f and g indicate significant differences between compacted embryo frequencies ($P \leq 0.05$).

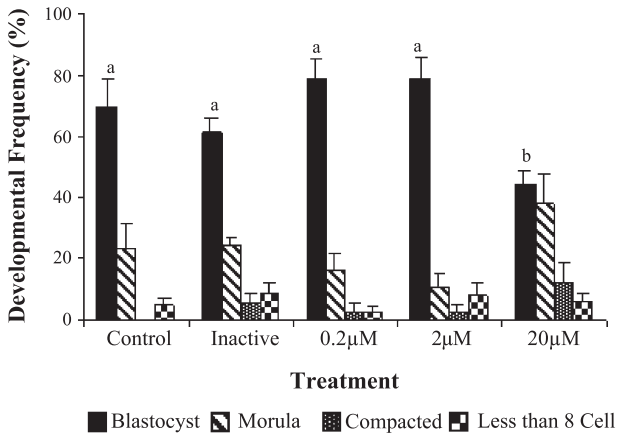

Fig. 4. The effect of 48 h of treatment with SB203580 on preimplantation development. Two-cell stage murine embryos were treated in culture with 0 μM (control); 20 μM inactive analogue SB202474 (inactive); and 0.2, 2.0, and 20 μM SB203580. Following 48 h of treatment with 20 μM SB203580, there was a significant reduction in the proportion of embryos that developed to the blastocyst stage. a and b indicate significant differences between blastocyst frequencies ($P \leq 0.05$).

encoding all four p38 MAPK isoforms (α, β, γ, and δ), PRAK (MK5), MK2, and hsp25 throughout murine preimplantation development. The expected size amplicons for each of p38 MAPK α, β, γ, and δ, PRAK, MK2, and hsp25 were detectable at each stage of the developmental series examined (oocyte, two-cell, four-cell, eight-cell, morula, and blastocyst; Table 1, Fig. 1). In all cases, the sequence of each product was identical to known murine cDNA sequences.

Distribution of p38 MAPK signaling pathway proteins in the preimplantation embryo

We next used antisera raised against p38 MAPK pathway constituents to determine the presence and localization of these proteins throughout preimplantation development by immunofluorescence. First, antisera specific for both phosphorylated p38 MAPK (Figs. 2A–C) and total p38 MAPK (data not shown) were used. p38 MAPK protein was detectable in the cytoplasm of blastomeres at all stages of preimplantation development, and this pattern consisted of both diffuse cytoplasmic staining combined with a more intense vesicular localization pattern. The distribution of phospho-p38 MAPK α/β immunofluorescence completely mirrored the distribution of total p38 MAPK α/β protein in all embryo stages. Similarly, PRAK protein was detectable at all stages of preimplantation development (Figs. 2D–F) with fluorescent foci observed in each blastomere in a diffuse cytoplasmic staining pattern in all embryos.

In contrast, at all stages of preimplantation development investigated, MK2 (data not shown) and phosphorylated-MK2 immunofluorescence were concentrated primarily in the nucleus of each blastomere (Figs. 2G–J). Interestingly, in mitotic blastomeres (observed by DNA staining with DAPI), MK2 and phosphorylated-MK2 immunofluorescence were dispersed throughout the dividing cell and consisted of a punctate distribution throughout the cytoplasm (for example, see Figs. 2H and I). This pattern of immunofluorescence staining was unique to MK2.

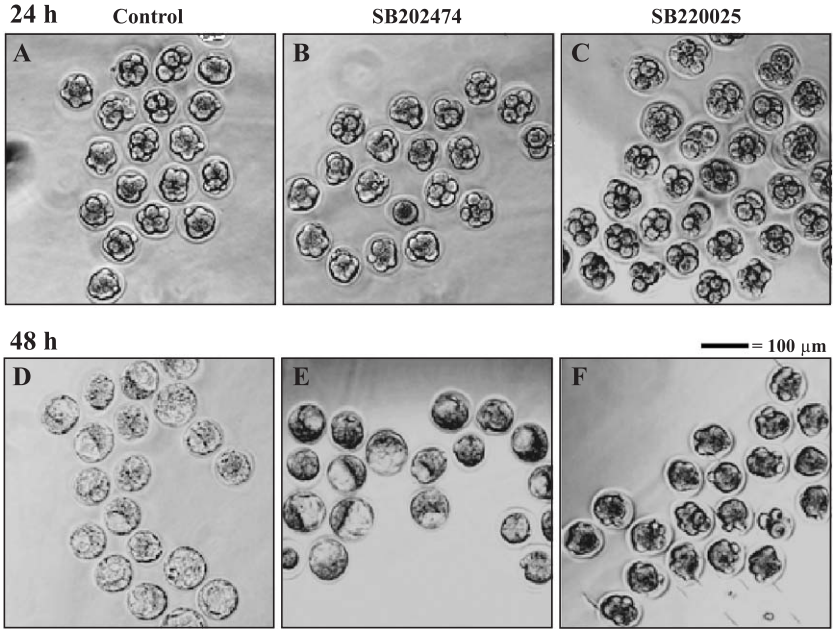

Fig. 5. Morphology of murine embryos treated with p38 MAPK α/β inhibitors. Two-cell murine embryos cultured in the absence of inhibitor (control; A) and 20 μM SB202474 (inactive analogue; B) progressed normally to the compacted eight-cell stage following 24 h in culture. Embryos treated with 20 μM SB220025 (C) were predominantly uncompacted following 24 h of treatment. Two-cell murine embryos cultured for 48 h in the absence of inhibitor (control; D) and 20 μM SB202474 (inactive analogue; E) progressed normally to the blastocyst stage. Embryos cultured in 20 μM SB220025 (F) did not develop to the blastocyst stage.

The hsp25 and phosphorylated-hsp25 were also detected throughout murine preimplantation development (Figs. 2K–M). At all developmental stages, embryos exhibited both a cytoplasmic and nuclear localization pattern for hsp25 immunofluorescence. The phosphorylated form of hsp25 had a similar cellular distribution; however, the cytoplasmic localization changed from a largely punctate pattern observed mostly in early cleavage stages (i.e., four- and eight-cell stages) to that of a more generalized even cytoplasmic distribution by the morula and blastocyst stages. This transition in hsp25-staining pattern may be indicative of a transition from polymeric to dimeric hsp, which occurs as hsp25 phosphorylation increases in other cell types (Butt et al., 2001).

Inhibition of the p38 MAPK signaling pathway

Having ascertained that all members of the p38 MAPK family were present in the preimplantation embryo, we next sought to determine whether blocking p38 MAPK activity would disrupt preimplantation development. To investigate this possibility, embryos were incubated for specific intervals in one of five treatment groups: (1) KSOMaa alone, (2) KSOMaa + 20 μM SB202474 (inactive analogue), (3) KSOMaa + 0.2 μM SB220025, (4) KSOMaa + 2.0 μM SB220025, and (5) KSOMaa + 20 μM SB220025. This experiment was then repeated using SB203580.

Treatment of two-cell stage embryos with a 20-μM concentration of the p38 MAPK α/β inhibitor SB220025 for 24 h resulted in a significant ($P \leq 0.05$) reduction in the proportion of eight-cell compacted embryos, as compared to that observed for other treatment concentration groups and untreated controls (Fig. 3A). Treated embryos continued to cleave from the two-cell stage to the eight-cell stage in the presence of the inhibitors (0.2, 2.0, and 20 μM); however, there was a delay in their progression to the 16-cell stage in the 20-μM SB220025 treatment group (Figs. 5A–C). Embryos treated with SB203580 displayed a similar trend following 24 h of treatment; however, the proportion of compacted eight-cell embryos was not reduced significantly in the SB203580 treatment groups (data not shown).

Following 48 h of culture, the frequency of development of embryos to the blastocyst stage did not vary significantly between those groups treated with 0.2 μM SB220025 or

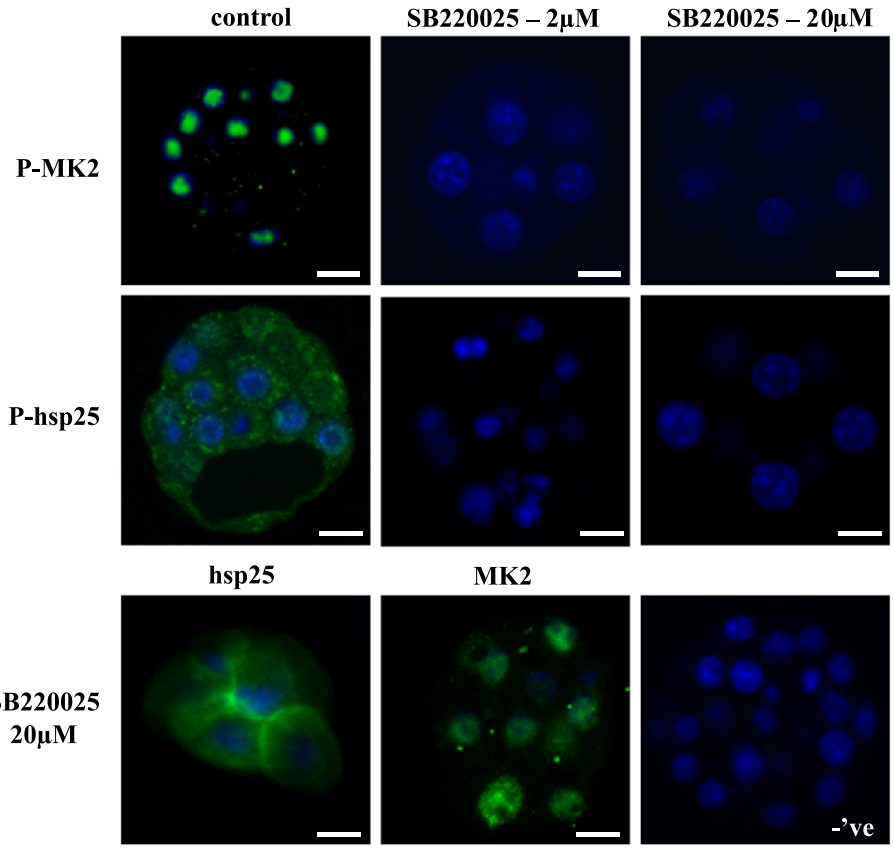

Fig. 6. Effect of p38 MAPK inhibition on the phosphorylation of MK2 and hsp25. In all micrographs, green indicates positive staining for the respective primary antibody and blue indicates nuclei (DAPI). Murine embryos cultured in the presence of 2 or 20 μM SB220025 for 24 h did not display any fluorescence following application of whole-mount immunofluorescence assays to detect phosphorylated forms of MK2 (top row) and hsp25 (middle row). Embryos cultured in the absence of the inhibitor (control) or with 20 μM SB202474 (inactive analogue; data not shown) displayed the expected fluorescence patterns for both MK2 and hsp25. In the lower panel, fluorescence was detected using antisera recognizing both phosphorylated and nonphosphorylated forms of hsp25 (hsp25) and MK2 (MK2) in embryos cultured in the presence of 20 μM SB220025 for 24 h. Scale bar = 25 μm.

SB203580, 2.0 μM SB203580 or 20 μM SB202474 (inactive analogue), and untreated controls. In contrast, a significant proportion ($P \leq 0.05$) of two-cell stage embryos treated with 2.0 μM SB220025, 20 μM SB203580, and 20 μM SB220025 failed to develop to the blastocyst stage (Figs. 3B and 4). Embryos treated with 20 μM SB220025 reached an early stage of compaction as indicated by the appearance of increased interblastomeric contact; however, only one such treated embryo was observed to develop a fluid-filled cavity (Figs. 5D–F).

Cell counts on treated embryos stained with DAPI indicated that 50% (13 of 26 examined) of the embryos in the 20-μM SB220025 treatment groups were blocked at the 16-cell stage, whereas approximately 42% (11 of 26 examined) were blocked at the eight-cell stage. The remaining embryos (8% of the total) contained fewer than eight cells. In contrast, at the same time points, the majority of embryos in control groups (KSOMaa + vehicle and 20 μM SB202474) progressed to the 32-cell stage or beyond (24/30, 80%, and 23/30, 77%, respectively).

Embryo viability was assessed for each control and treatment group immediately following the 48-h interval by assaying for the uptake of the vital dye trypan blue. Baseline dye uptake was observed in blastomeres of one to two embryos in each group including controls. We did not observe any increase in dye uptake (indicative of loss of cell integrity and thus viability) from controls in embryos exposed to either 20 μM of the inhibitors, SB203580, SB220025, or 20 μM of the inactive analogue, SB202474 (example, Figs. 8B and i).

Effect of p38 MAPK inhibitors on phosphorylation of MK2 and hsp25

To assess the ability of SB220025 and SB203580 to effectively and specifically inhibit p38 MAPK activity, we investigated the phosphorylation state of the downstream substrates, MK2 and hsp25. Following treatment with SB220025 and SB203580 for 24 h, embryos in all of the treatment groups including controls were assessed by whole-mount indirect immunofluorescence techniques using antisera specific to the total and phosphorylated forms of MK2 and hsp25. Embryos from the control groups displayed fluorescence patterns corresponding to those already described for phospho-MK2 and phospho-hsp25 (Fig. 6). In embryos treated at the 20-μM concentrations with either SB220025 (Fig. 6) or SB203580 (data not shown), fluorescence was undetectable using either anti-phospho-MK2 or

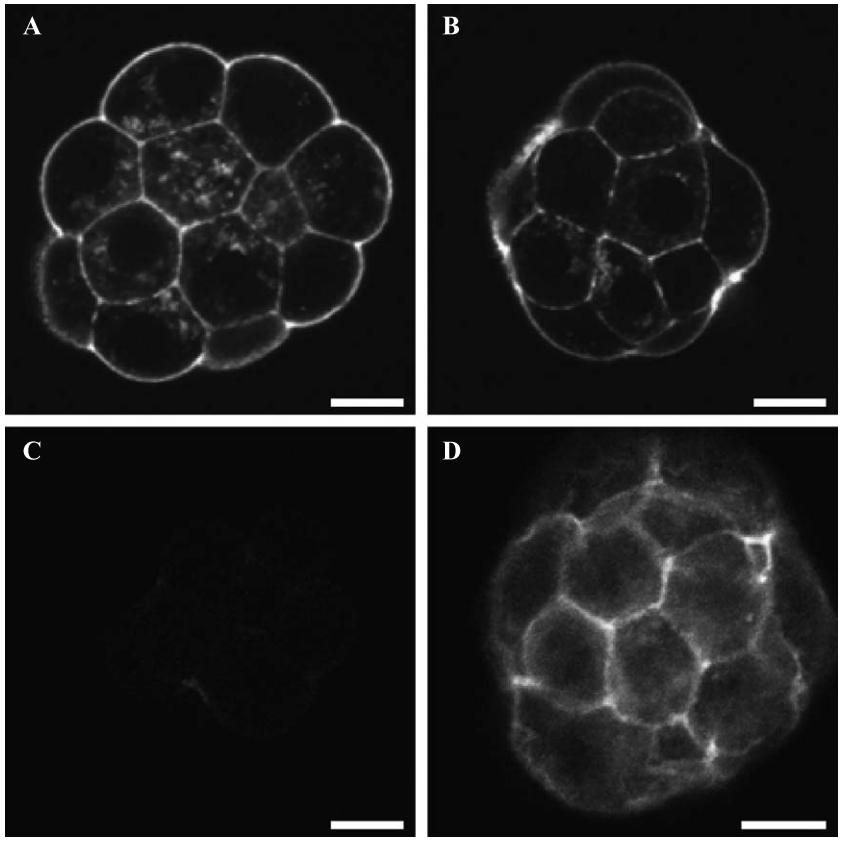

Fig. 7. Effect of p38 MAPK inhibitors on embryonic F-actin. Murine embryos assessed for filamentous actin by rhodamine–phalloidin fluorescence displayed an actin network typical of the 8- to 16-cell stage in the control group (A) and the group treated with 20 μM SB202474 (B). Embryos cultured for 24 h in the presence of 20 μM SB220025 displayed a loss of filamentous actin as indicated by the absence of rhodamine–phalloidin fluorescence (C). Following removal from SB220025 treatment, however, embryos resumed development and displayed a restored filamentous actin network (D). Scale bar = 15 μm.

anti-phospho-hsp25 antisera. Treatments with 2 μM SB220025 (Fig. 6) also resulted in a complete loss of anti-phospho-MK2 or anti-phospho-hsp25 fluorescence, while treatment with 2 μM SB203580 resulted in a marked reduction in anti-phospho-MK2 or anti-phospho-hsp25 fluorescence (data not shown). In embryos treated with either SB220025 or SB203580, expression of nonphosphorylated MK2 and hsp25 was still detectable (Fig. 6, lower panel). These data collectively demonstrate that p38 MAPK activity was suppressed in treated embryos.

Effect of p38 MAPK inhibitors on embryonic actin

Since p38 MAPK is an important regulator of filamentous actin in many cell types, we investigated the affect of treatment with p38 MAPK inhibitors on the structure of embryonic filamentous actin (Davidson and Morange, 2000; Huot et al., 1997). Treatment with a 20-μM concentration of the p38 MAPK α/β inhibitor SB220025 for 24 h resulted in a complete loss of filamentous actin in blocked 8- to 16-cell embryos as indicated by the loss of rhodamine–phalloidin fluorescence (Fig. 7C). Control embryos and embryos treated with SB202474 displayed the expected filamentous actin network that is typical of 8- to 16-cell stage embryos (Figs. 7A and B, respectively). Embryos treated with 2.0 μM SB220025 displayed an obvious reduction in rhodamine–phalloidin fluorescence but never displayed a complete loss of fluorescence (data not shown). Embryos released from p38 MAPK inhibitors resumed development as described below and also displayed a return of rhodamine–phalloidin fluorescence and a restoration of the filamentous actin network (Fig. 7D). Our results indicate that p38 MAPK is a potent regulator of filamentous actin in the preimplantation embryo.

Rescue of embryos exposed to the p38 MAPK α/β-specific inhibitors SB220025 and SB203580

We next sought to determine whether the effects of the p38 MAPK α/β-specific inhibitors, SB220025 and SB203580, were reversible. To this end, embryos were incubated in one of the five media treatments for 24 h and then washed with normal KSOMaa and maintained in this medium for an additional 24–48 h. All embryos removed from SB203580 following treatment for 24 h resumed development to the blastocyst stage with the same frequency as controls. A significant number of embryos removed from SB220025-containing media (2.0 and 20 μM SB220025; $P \leq 0.05$) after 24 h of treatment failed to progress to the blastocyst stage at the same time as embryos in untreated controls and 20 μM inactive analogue, SB202474, treatment groups (Fig. 8A). Despite removal from the inhibitor and placement in fresh

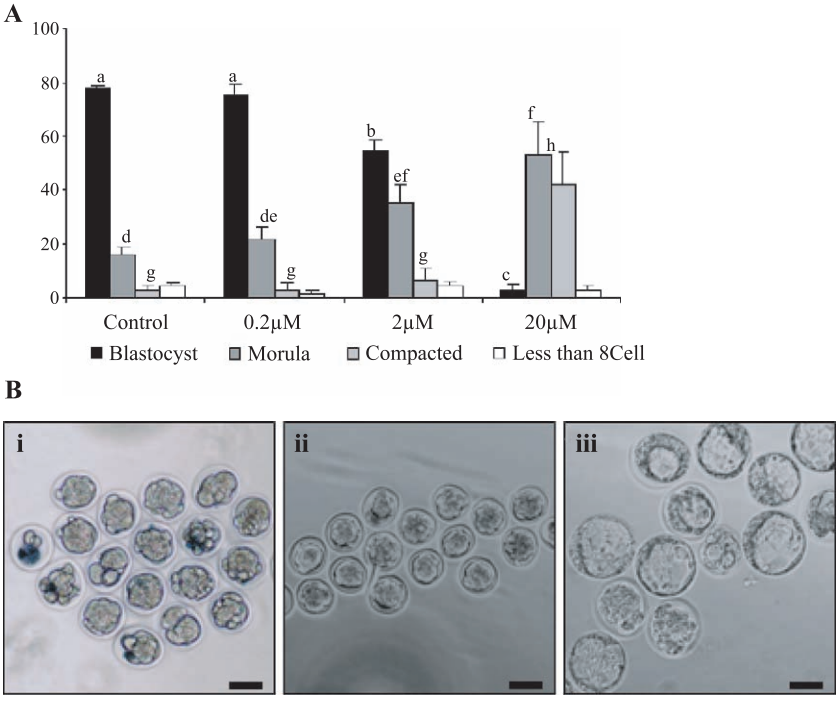

Fig. 8. Recovery of preimplantation embryos from 24 h of treatment with SB220025. Murine embryos undergoing treatment with SB220025 from the two-cell stage were removed from treatment following 24 h, washed, and placed in fresh culture drops in the absence of SB220025. Results indicate that embryos cultured initially in the presence of 2.0 and 20 μM SB220025 fail to recover completely from p38 inhibition in only 24 h. (A) There is a significant reduction in blastocyst formation in these groups (a, b, and c indicate significant differences between blastocyst formation frequencies; d, e, and f indicate significant differences between morula frequencies; g and h indicate significant differences between compacted embryo frequencies; $P \leq 0.05$). (B) Morphology of embryos treated with SB220025 for 48 h; (i) morphology of embryos removed from SB220025 treatment at 24 h and cultured for an additional 24 in drug-free medium; (ii) and morphology of embryos removed from SB220025 treatment at 24 h and cultured for an additional 48 h in drug-free medium (iii).

KSOMaa medium for 24 h, these embryos did not appear to be any further advanced than embryos continuously cultured in SB220025 for the entire 48 h (Fig. 8B and ii). However, in contrast to embryos exposed for 48 h to the inhibitor, those treated for only 24 h and then removed to regular media displayed a higher proportion of 16 cell or greater embryos in their group (9/26 20–24 cells, 35%; 12/26 16 cells, 46%; and 5/26 8 cells, 19%), suggesting that release from the inhibitor and progression towards the blastocyst stage had been initiated. To determine whether SB220025-treated embryos recovered completely, we extended the culture period in drug-free KSOMaa medium for an additional 24 h (72 h total culture time from the two-cell stage). Embryos so treated progressed to the blastocyst stage, including blastocoele formation accompanied by cell proliferation (>30 cells, 28/34, 82%; 16–24 cells, 3/34, 9%; and <16 cells, 9%), in a similar frequency to controls after 48 h of culture (28/34, 82%; Fig. 8B and iii). Therefore, none of treatments resulted in a permanent blockade of development. Embryos treated with 20 μM SB220025 simply required an additional 24 h to recover and resume their development to the blastocyst stage following treatment.

Discussion

This is the first study to investigate p38 MAPK signaling during murine preimplantation development. From our results, we can conclude that p38 MAPK signaling is required to promote development through to the blastocyst stage. We have previously applied differential display-reverse transcription polymerase chain reaction (DD-RT-PCR) to provide a global assessment of gene expression patterns throughout preimplantation development (Natale et al., 2000, 2001). From this analysis, we identified PRAK (MK5) mRNA expression during preimplantation development. Application of gene-specific RT-PCR methods in the present study indicated that mRNA encoding PRAK (MK5) and other members of the p38 MAPK signaling cascade, including p38 α, β, γ, δ, and the specific downstream effectors, MK2 and hsp25, are expressed throughout murine preimplantation development.

The p38 MAPK signaling cascade regulates a variety of cellular activities in different cell and tissue types including the cell cycle, apoptosis, and cellular differentiation, all of which must be tightly controlled in mammalian preimplantation development (Bavister, 1987; Niemann and Wrenzycki, 2000; Watson et al., 1992b). This signaling pathway is influenced by a variety of stimuli including growth factors, cytokines, and pathogens. Most notably, and of relevance to our study, p38 MAPK signals through PRAK (MK5) in response to environmental stress (New et al., 1998; Ono and Han, 2000). Changes in osmolarity, temperature, oxygen tension, and oxygen radical metabolites activate p38 MAPK α/β (reviewed by Ono and Han, 2000). Osmolarity and oxygen tension are crucial variables in embryo culture and must be tightly regulated to promote optimal frequencies of development to the blastocyst stage (Baltz, 2001; Bernardi et al., 1996; Byatt-Smith et al., 1991; Hashimoto et al., 2000; Quinn and Harlow, 1978; Wrenzycki et al., 2001). The p38 MAPK signaling system may serve an important role in mediating and transmitting signals from the environment (in vivo and in vitro) that assist in coordinating embryonic development.

Our protein localization studies indicate that p38 MAPK, PRAK (MK5), MK2, and hsp25 are present in mouse embryos throughout preimplantation development. In addition, our results indicate that each of these proteins is phosphorylated and likely active throughout murine preimplantation development.

PRAK (MK5) shares sequence homology with MK2, is activated by similar upstream activators, and shares downstream targets (Ni et al., 1998). However, our findings suggest that each protein exhibits distinct subcellular distributions in early mouse embryos. PRAK (MK5) was found in the cytoplasm of blastomeres at all developmental stages, whereas MK2 was primarily associated with the nucleus except in dividing blastomeres wherein it is located throughout the cell. Both PRAK and MK2 phosphorylate and activate hsp25 in response to p38 MAPK activity (New et al., 1998; Stokoe et al., 1992); however, it is likely that based on their subcellular distribution in preimplantation embryos, they have additional and independent functions.

Our experiments examining the consequences of p38 MAPK inhibition indicate that p38 MAPK activity is required for murine blastocyst development in vitro. A significant proportion of two-cell embryos incubated with either 2.0 or 20 μM SB220025 or 20 μM SB203580 failed to progress to the blastocyst stage and halted their development at the 8- to 16-cell stage. In addition, following 48 h of SB220025 or SB203580 treatment, these embryos were one to two cell cycles behind the control groups. Importantly, embryo viability was not compromised by these treatments. These data suggest that p38 MAPK activity is required beginning at the eight-cell stage to promote development to the blastocyst stage. This conclusion is further supported by our inhibition-recovery data. In these experiments, embryos recovered from inhibition and formed blastocysts at the same frequency as observed in controls; however, this recovery required an additional 24 h of culture (72 h of total culture time versus 48 h).

Treatment with SB220025 had a more dramatic effect than SB203580 on blastocyst development (2.0 and 20 μM SB220025 versus 20 μM SB203580 following 48 h). However, both compounds are specific inhibitors of p38 MAPK activity when used at the concentrations employed in our study and in other studies SB220025 has been demonstrated to be more potent (Wang et al., 1998). Active p38 MAPK is required to phosphorylate MK2, which in turn phosphorylates hsp25. In embryos treated with both drugs, we observed an absence of phosphorylated forms of

MK2 and hsp25. In embryos from the same treatment groups, we observed no apparent affect to total MK2 and hsp25 protein levels, demonstrating that p38 MAPK activity is blocked by these treatments.

One of the most dramatic effects of treatment with p38 MAPK inhibitors that we observed was the complete loss of filamentous actin in 8- to 16-cell-treated embryos. It is possible that many of these observations on the effects of p38 MAPK inhibition on preimplantation development could be mediated via downstream effects to the actin cytoskeleton. The adherens junction is dependent upon an interaction of E-cadherin and catenins with the actin cytoskeleton (Pratt et al., 1982; Sutherland and Calarco-Gillam, 1983). The effects to development that we observed in the presence of the inhibitor may reflect an impairment of processes that regulate the establishment of stable cell-to-cell adherens junctions in the 8- to 16-cell embryo during compaction. We expect that inhibition of p38 MAPK by CSAIDs prevents MK2 and/or PRAK phosphorylation of hsp25 resulting in an abrupt impairment of actin polymerization. Support for this hypothesis comes from studies that have implicated hsp25/27 in mediating actin stabilization in response to mitogens and stress (Benndorf et al., 1994; Butt et al., 2001; Lavoie et al., 1993). Actin polymerization, as measured by the presence of cortical F-actin, is decreased when a mutant nonphosphorylated hsp27 is expressed (Lavoie et al., 1993). When hsp27 is overexpressed, cortical F-actin expression increases. In addition, mutation of serine residues in hsp27 to mimic phosphorylation and activation by MK2 results in increased actin polymerization (Butt et al., 2001). Future studies will continue to investigate the mechanism of p38 MAPK-controlled actin polymerization in the preimplantation embryo.

Recently, p38 MAPK has been implicated as a regulator of placental morphogenesis. Targeted inactivation of p38 MAPKα is accompanied by an early embryonic lethality in mice and is associated with defects in placental development (Adams et al., 2000; Allen et al., 2000; Mudgett et al., 2000). Rescue experiments employing tetraploid wild-type cells to form embryo aggregates (Guillemot et al., 1994) with p38 MAPK α null embryos demonstrated that normal placental and embryonic development occurs, implying a trophoblast-specific phenotype for the p38 MAPKα knockout (Adams et al., 2000). Trophoblast cells mediate attachment, invasion, and establish the embryonic component of the placenta. These cells are derivatives of the first differentiated cell type of the embryo, the trophectoderm. The trophectoderm develops from the totipotent blastomeres of the eight-cell embryo. These studies define a clear role for the p38 MAPK pathway during early placentation, but they do not support our findings that p38 MAPK activity is required to support preimplantation development. This apparent variation in outcomes between the two studies may be explained by our discovery that the preimplantation murine embryo expresses multiple isoforms of p38 MAPK. The inhibitors we employed in the present study target both p38 MAPK α and β activity (Fox et al., 1998; Goedert et al., 1997; Lisnock et al., 1998; Tong et al., 1997; Wilson et al., 1997). Therefore, we contend that the exposure of all two-cell embryos to 20 μM SB203580 or 20 μM SB220025, which will inhibit both p38 MAPK α/β activity, precludes progression to the blastocyst stage in vitro, whereas targeted inactivation of p38 MAPK α alone does not. We propose that the dual inactivation of p38 MAPK α and β will result in a preimplantation lethal phenotype and a failure to support development to the blastocyst stage in vivo.

The objectives of this work were to investigate the expression and function of the p38 MAPK signaling pathway through the preimplantation development interval. We have shown mRNA expression and protein localization of PRAK, p38 MAPK, MK2, and hsp25 throughout murine preimplantation development. We have also demonstrated that active p38 α/β MAPK signaling is required for development from the 8- to 16-cell stage to the blastocyst stage and that p38 MAPK is a regulator of filamentous actin during preimplantation development.

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