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ABSTRACT Preimplantation embryos utilize mitogen-activated protein kinase signaling (MAPK) pathways to relay signals from the external environment to prepare appropriate responses and adaptations to a changing milieu. It is therefore important to investigate how MAPK pathways are regulated during preimplantation development. This study was conducted to investigate whether PP2Cδ (*Ppm1d*, WIP1) is expressed during mouse preimplantation development and to determine the influences of p38 MAPK inhibition on expression of *Trp53* (p53), *Ppm1d*, (WIP1), and *Cdkn2a* (p16) during mouse preimplantation development. Our results indicate that *Trp53*, *Ppm1d*, and *Cdkn2a* mRNAs and TRP53 and PP2Cδ proteins are expressed throughout mouse preimplantation development. Treatment of 2-cell embryos with SB220025 (potent inhibitor of p38 MAPK α/β/MAPK 14/11) significantly increased *Trp53*, *Ppm1d* and *Cdkn2a* and *Mapk14* mRNA levels at 12 and 24 hr. Treatment of 8-cell embryos with SB220025 for 12 hr increased *Trp53*, *Ppm1d*, and *Cdkn2a* mRNA levels, but not *Mapk14* mRNA levels. Treatment of 8-cell embryos for 24 hr increased *Trp53*, and *Ppm1d* mRNA levels, but decreased *Cdkn2a* and *Mapk14* mRNA levels. Therefore, blockade of p38 MAPK activity is associated with embryo stage specific influences on *Trp53*, *Ppm1d*, *Cdkn2a*, and *Mapk14* expression during mouse preimplantation development. These results define downstream targets of p38 MAPK during preimplantation development and indicate that the p38 MAPK pathway regulates *Trp53*, *Ppm1d*, and *Cdkn2a* expression. This study increases our understanding of the mechanisms controlling preimplantation development and of the interactions between preimplantation embryos and their culture environments. *Mol. Reprod. Dev. 74: 821–834, 2007.* © 2007 Wiley-Liss, Inc.

Key Words: oocyte; blastocyst; gene expression; cell signaling; in vitro fertilization

INTRODUCTION

Preimplantation development is susceptible to modification by exposure to variations in the external environment (Rinaudo and Schultz, 2004; Johnson, 2005). The consequences of environmental insults range from modification of embryonic gene expression patterns, to impaired embryo health, fetal development and greater susceptibility to disease later in life (Rinaudo and Schultz, 2004; Johnson, 2005). Research is now focused on understanding the mechanisms upon which the external environment may affect the embryonic developmental program. This research has revealed that mitogen-activated protein kinase (MAPK) signaling pathways are important mediators of environmental stresses on embryonic gene expression and development (Saba-El-Leil et al., 2003; Natale et al., 2004; Maekawa et al., 2005; Paliga et al., 2005; Wang et al., 2005).

The MAPK signal transduction pathways are well-characterized networks that regulate cellular processes including gene transcription, protein synthesis, cell cycle, apoptosis, cell differentiation, inflammation, and cytoskeletal rearrangements (Herskowitz, 1995; Marshall, 1995; Kyriakis and Avruch, 1996; Ono and Han, 2000; Kyriakis and Avruch, 2001). In general, all MAPK

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signaling pathways include a central three-tiered signaling module consisting of MAPKs, MAPK kinases (MAP2Ks) and MAPK kinase kinases (MAP3Ks). MAPK core signaling modules are regulated by a wide variety of upstream activators and inhibitors (Herskowitz, 1995; Marshall, 1995; Kyriakis and Avruch, 1996, 2001; Ono and Han, 2000).

Our studies have focused upon defining the role and regulation of the p38 MAPK pathway during preimplantation development (Natale et al., 2004; Madan et al., 2005; Paliga et al., 2005). p38 MAPK was initially identified by its ability to specifically bind pyridinyl imidazole derivatives (i.e., cytokine suppressive anti-inflammatory drugs; CSAIDS), which inhibit the production of pro-inflammatory cytokines (Lee et al., 1994). To date, four p38 MAPK members, encoded by distinct genes, have been cloned and characterized: p38α (MAPK14) (Han et al., 1994; Lee et al., 1994), p38β (MAPK11) (Jiang et al., 1997), p38γ (MAPK12) (Lechner et al., 1996; Cuenda et al., 1997), and p38δ (MAPK13) (Jiang et al., 1997). p38 MAPKs are activated by MAP2Ks including MAP2K3 (MKK3), MAP2K6 (MKK6), and MAP2K4 (MKK4) (Keesler et al., 1998; Ono and Han, 2000). The MAP3Ks upstream of the MAP2K/p38 MAPK pathway are even more expansive and include the mitogen-activated protein/extracellular signal-regulated kinase kinase kinases (MEKKKs/MAP3K1-5), the mixed lineage kinases (MLKs/MAP3K9-11) and the thousand and one kinases (TAOKs, MAP3K17) (Kyriakis and Avruch, 2001). A number of p38 MAPK substrates have been identified and include MAPK-activated protein kinase-2 (MAPKAPK2), MAPKAPK3, and p38 regulated and activated kinase (PRAK, MAPKAPK5). Once activated, MAPKAPK2, MAPKAPK3, and PRAK phosphorylate heat shock protein 27 (HSP27) (McLaughlin et al., 1996; Sithanandam et al., 1996; Huot et al., 1997; New et al., 1998; Lambert et al., 1999) which regulates the actin cytoskeleton (Huot et al., 1997; Lambert et al., 1999). p38 MAPK also enters the nucleus to phosphorylate transcription factors including activator protein 1 (AP1, JUN), CREB homologous protein (CHOP, DDIT3), and activating transcription factor 2 (ATF2) (Habener, 1990; Ono and Han, 2000; Kyriakis and Avruch, 2001).

In addition, p38 MAPK phosphorylates the tumor suppressor protein, TRP53 (p53) (Bulavin et al., 1999; Sanchez-Prieto et al., 2000; She et al., 2000; Kishi et al., 2001). *Trp53* transcripts are present throughout murine preimplantation development, and are increased in 1-cell stage embryos exhibiting programmed cell death (PCD) (Jurisicova et al., 1998). Recently it was determined that TRP53 is a key component of a p38 MAPK regulatory feedback loop (Takekawa et al., 2000). In response to UV radiation, osmotic stress, and chemotherapeutic agents, p38 MAPKs directly phosphorylate TRP53 (Bulavin et al., 1999; Sanchez-Prieto et al., 2000; She et al., 2000; Kishi et al., 2001). Active TRP53 induces the transcription of the wild-type p53-induced phosphatase 1, Wip1 (PP2Cδ or *Ppm1d*). PP2Cδ is a member of the type 2C protein phosphatase (PP2C) family (Fiscella et al., 1997). PP2Cs are implicated in stress protection, sexual differentiation, and cell-cycle control (Schweighofer et al., 2004). *Ppm1d*-null mouse embryonic fibroblasts (MEFs) display elevated TRP53 activity and its downstream targets, but also express higher levels of CDKN2A (p16-INK4A), an inhibitor of the cyclin D protein kinases, which are in turn upstream regulators of the retinoblastoma (Rb) tumor-suppressor protein (Bulavin et al., 1999, 2004). Thus, deletion of *Ppm1d* activated two distinct tumor suppressors, the TRP53 and CDKN2A/CDKN2D pathways. PP2Cδ contributes to the TRP53-p38 MAPK-PP2Cδ regulatory circuit by dephosphorylating p38 MAPK at threonine 180 and thus inhibiting p38 MAPK activity (Takekawa et al., 2000).

All principle members of the p38 MAPK family are expressed throughout preimplantation development (Natale et al., 2004; Madan et al., 2005). Inhibition of MAPK 14/11 activity using CSAIDs, results in a reversible arrest of preimplantation development at the 8–16 cell stage that is accompanied by a loss of filamentous actin, decreased HSP25/27 phosphorylated protein, and redistribution of α-catenin (Natale et al., 2004; Paliga et al., 2005). We have very recently discovered that p38 MAPK activity during preimplantation development is regulated by culture medium osmolarity and that p38 MAPK activation is associated with increased levels of Osmosensing Scaffold for MEKK3 (OSM) or CCM2 which is an osmotic responsive scaffolding protein that mediates the assembly of RhoGTPases, MAP3K, MAP2K, and p38 MAPK into phospho-relay modules to regulate actin cytoskeletal events (Uhlik et al., 2003). The present study is focused on investigating the expression and regulation of *Trp53*, *Ppm1d*, and *Cdkn2a* during preimplantation development (Bulavin et al., 1999; Sanchez-Prieto et al., 2000; Takekawa et al., 2000; Bulavin et al., 2004). Determination of the effects of p38 MAPK inhibition on their expression during early development will contribute to an understanding of p38 MAPK regulation during this developmental period and also provides a possible mechanism for the modulation of environmental influences on embryo development. We have discovered that *Trp53*, *Ppm1d*, and *Cdkn2a* mRNAs and TRP53 and PP2Cδ proteins are expressed during preimplantation development and that blockade of p38 MAPK activity is associated with embryo stage specific influences on *Trp53*, *Ppm1d*, *Cdkn2a*, and *Mapk14* expression during mouse preimplantation development.

MATERIALS AND METHODS

Materials

SB220025 and SB202474 were purchased from Calbiochem (La Jolla, CA). Both compounds were dissolved in dimethylsulphoxide (DMSO) to make stock concentrations of 10 mM and were stored at −20°C. The embryo culture medium, potassium simplex optimized media with amino acids (KSOMaa), was purchased from Chemicon (Temecula, CA). All real-time reverse tran-

scription polymerase chain reaction (RT-PCR) reagents were purchased from Applied Biosystems (Foster City, CA).

Superovulation and Mouse Embryo Collection

Female MF-1 or CD-1 mice (Harlan, Indianapolis, IN; Charles River, PQ) were injected with 5 IU pregnant mare's serum gonadotropin (PMSG; Intervet, Whitby, ON, Canada) followed by 5 IU human chorionic gonadotropin (hCG; Sigma, St. Louis, MO) 48 hr later. Female mice were then mated with CD-1 males. Successful mating was determined by the presence of a vaginal plug the next morning. Embryos were collected at specified times following hCG injection, which correspond to appropriate cleavage stages: 1-cell zygotes 24 hr post-hCG; 2-cell, 48 hr; 4-cell, 60 hr; 8-cell, 65–68 hr; morulae, 80–85 hr and blastocyst, 90 hr. Embryos at the 1-cell through morula stage were flushed from the oviducts and uteri of female mice using flushing media I (Spindle, 1980). Blastocysts were flushed from the uteri using flushing media II (Spindle, 1980). Embryos were washed several times in flushing medium and collected in pools to be either: (1) frozen and stored at −80°C for RNA extraction; (2) fixed for immunofluorescence analysis of protein distribution; or (3) cultured in 20 μl drops of EmbryoMax® KSOMaa (potassium simplex optimized medium with amino acids) Liquid Mouse Embryo Media (Chemicon International—Specialty Media, Temecula, CA) (Ho et al., 1995) under mineral oil and maintained in culture under 5% CO_2 in air atmosphere at 37°C. Animal care and treatment followed protocols established by the UWO Animal Care Committee.

RNA Extraction and Reverse Transcription

Total RNA was extracted from 40 mg pieces of adult mouse liver tissue using TRI Reagent (Molecular Research Center, Cincinnati, OH). RT reactions were performed using oligo$(dT)_{12-18}$ primers and SuperScript II reverse transcriptase (Invitrogen, Burlington, ON, Canada) as previously described (Offenberg et al., 2000; Natale et al., 2004). For embryos, 0.025 pg of luciferase RNA (Promega Corporation, Madison, WI) per embryo was added to pools of 20 (for experimental treatment trials) or 40 (for developmental series, 1-cell to blastocyst stage) embryos and total RNA was extracted using TRI Reagent and Poly Acryl Carrier (Molecular Research Center). The Sensiscript RT Kit and protocol from Qiagen was employed to reverse transcribe RNA extracted from these embryos.

PCR Primer Sets

Primer sets were designed and synthesized for mouse *Trp53*, *Ppm1d*, and *Cdkn2a* (Table 1). The web-based program Primer 3 release 1.0 (Whitehead Institute for Biomedical Research, Cambridge, MA, www.http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) was used to generate primer pairs based on available mouse nucleotide sequences in GenBank databases and were generated by Sigma Genosys, Oakville, ON, Canada. The optimal PCR conditions for all primers were characterized in adult mouse liver tissue cDNA samples by running samples at various Mg^{2+} concentrations and annealing temperatures. The RT-PCR products amplified from adult liver tissue cDNA and blastocyst cDNA were purified using a QIAquick Gel Extraction Kit (Qiagen, Maryland). Purified cDNA was then submitted for nucleotide sequencing (DNA Sequencing Facility, Robarts Research Institute, London, ON, Canada). To confirm the 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.

Qualitative RT-PCR Amplification

Standard PCR was applied as previously described (Offenberg et al., 2000; Natale et al., 2004), using a total amount of cDNA equal to one to two embryos per reaction, or approximately 200 pg adult mouse liver RNA. PCR reactions were carried out in a volume of 50 μl consisting of 0.2 U of Platinum Taq (Invitrogen), 1× PCR Platinum Buffer (Invitrogen), 2 mM $MgCl_2$, 0.2 mM of each dNTP, and 1.0 μM of each PCR primer (Table 1) using a gradient thermocycler (Techne, Burlington, NJ). Platinum Taq was activated by an initial incubation at 95°C for 5 min followed by a series of up to 45 cycles consisting of denaturation at 95°C for 50 sec, reannealing primers to target sequence at 56–64°C for 50 sec and primer extensions at 72°C for 50 sec. The amplification cycles concluded with a 10 min final extension at 72°C.

TABLE 1. Nucleotide Sequences of Primer Sets Used for Qualitative Polymerase Chain Reaction Amplification (PCR) of *Trp53*, *Ppm1d*, *Cdkn2a*, and *Luciferase* cDNAs

Gene product	Primer	Primer sequence	Product size (bp)	GenBank accession number
Trp53	5′	AGAAAATTTCCGCAAAAAGGA	450	NM_011640
	3′	GCTGAGCCCTAGCTACAAGGT		
Ppm1d	5′	AAGAAGCAGAAAGGCTTCACC	445	NM_016910
	3′	TTGCTACAGCCAGAAAAGGAA		
Cdkn2a	5′	TGATGATGATGGGCAACG	248	NM_009877
	3′	ACGGGAACGCAAATATCG		
Luciferase	5′	TTGACAAGGATGGATGGCTAC	395	Sequence provided by Promega
	3′	TTCGGTACTTCGTCCACAAAC		

PCR products were resolved on a 2.0% agarose gel with 1.0 μg/ml ethidium bromide (Invitrogen). PCR reactions were repeated a minimum of three times using cDNA prepared from embryos at each of the indicated stages and isolated from a minimum of three separate developmental series. Positive (tissue cDNA: liver) and negative control (no cDNA template) samples were included for each primer set in every experiment.

Quantitative Real-Time PCR Amplification

Real-time RT-PCR was performed using the ABI PRISM 7900HT sequence detection system (Applied Biosystems). Predesigned and qualified TaqMan® Gene Expression Assays for the genes of interest *Trp53*, *Ppm1d*, *Cdkn2a*, and *Mapk14*, were employed (Applied Biosystems) (Jeong et al., 2005). The probe sequence for *luciferase* was directed against a target site 550 bp into the full-length sequence used to generate the Luciferase Control RNA (Promega Corporation). The target site specifies an approximate location for generation of a TaqMan® probe, and each target site was verified to be unique by performing BLAST® analysis. Dual-labeled probes were synthesized (Applied Biosystems) to contain the reporter dye 6-carboxyfluorescein (6-FAM) at the 5′ end and a nonfluorescent quencher dye at the 3′ end. A list of assay ID's and accession numbers of the target sequences used to design the primer-probes sets are shown in Table 2. PCRs were carried out in a 96-well plate in a 50 μl reaction containing 25 μl TaqMan® Universal PCR Master Mix (2× concentration solution optimized for TaqMan® reactions and contains AmpliTaq Gold DNA Polymerase, AmpErase UNG, dNTPs with UTP, Passive Reference, and optimized buffer components), 2.5 μl of 20× TaqMan® Gene Expression Assay Mix (unlabeled PCR primers and FAM dye-labeled TaqMan MGB probe), 10 μl of appropriate dilution of cDNA (0.1 embryo/μl), and 10 μl of water. Amplification conditions were 2 min at 50°C (UNG activation), 5 min at 95°C, 60 cycles of 15 sec at 95°C and 60 sec at 60°C. Each reaction was run in triplicate on the plate, and three developmental series and three experimental replications were completed. All three genes of interest were validated against the exogenous control to ensure equal amplification efficiency.

Final quantification was conducted using the comparative C_T method (Leutenegger et al., 2000). Within the log-linear phase of the amplification curve, the difference between each cycle was equivalent to a doubling of the amplified product of the PCR. The ΔC_T value was determined by subtracting the luciferase C_T value for each sample from the target gene C_T value of the sample. Calculation of $\Delta\Delta C_T$ used either the 1-cell or DMSO cultured sample ΔC_T value as a constant to subtract from all other ΔC_T sample values. Fold-changes in the relative mRNA expression of the target gene were determined using the formula $2^{-\Delta\Delta CT}$.

Antisera

We employed TRP53-phospho which is a mouse antiserum raised against a synthetic phospho-peptide corresponding to residues surrounding Ser15 of human TRP53 (Cell Signaling, Danvers, MA). PP2Cδ is a rabbit antiserum raised against amino acids 306–605 mapping at the C-terminus of human PP2Cδ (Santa Cruz Biotechnology, Santa Cruz, CA). Both primary antibodies were tested over a range of concentrations and were most effective at a dilution of 1:100 from the commercial stock concentration. Primary antibodies were labeled using fluorescein isothiocyanate-conjugated (FITC)-donkey anti-mouse secondary antiserum, for TRP53-phospho, or FITC-donkey anti-rabbit secondary antiserum for PP2Cδ 1 (Jackson Immuno Labs, La Jolla, MA).

Whole-Mount Indirect Immunofluorescence

Localization of TRP53-phospho and PP2Cδ in preimplantation stage mouse embryos (1-, 2-, 4-, 8-cell, morula, and blastocyst) was assessed by whole-mount indirect immunofluorescence methods combined with observation using a laser scanning confocal microscope. The method was applied to a minimum of three replicate embryo developmental series representing a minimum of 10 embryos for each stage per replicate. Negative controls were also conducted in which embryos were exposed to the same procedure in the absence of primary antibody to assess the levels of background and nonspecific binding of secondary antibody. After collection, embryos were fixed in 2% paraformaldehyde (PFA) in PBS for 20 min at room temperature. Following fixation, embryos were processed for immediate application of immunofluorescence methods or were stored at 4°C in 1× PBS +0.09% sodium azide (Embryo Storage Buffer) for a maximum of 2 weeks. For immunofluorescence, fixed embryos were permeabilized and blocked in 1× PBS +5% donkey serum +0.01% Triton X-100 for 1 hr at room temperature. Embryos were washed in 1× PBS and incubated with primary antibody diluted 1:100 in 1× PBS +0.5% donkey serum +0.005% Triton X-100

TABLE 2. Assay ID's and Accession Numbers for Real-Time Polymerase Chain Reaction (PCR) Primer Sets Used to Amplify *Trp53*, *Ppm1d*, *Cdkn2a*, *Mapk14*, and *Luciferase* cDNAs

Gene product	Applied biosystems assay ID	GenBank accession number
Trp53	Mm00441964_g1	NM_011640
Ppm1d	Mm00450393_m1	NM_016910
Cdkn2a	Mm00494449_m1	NM_009877
Mapk14	Mm00442497_m1	NM_011951.1
Luciferase	Custom-made primer/probe set	Sequence provided by Promega

for 1 hr at 37°C, followed by three 30 min washes at 37°C. Primary antibodies were detected following incubation for 1 hr in FITC-conjugated secondary antibodies diluted to 1:200. To visualize F-actin localization and to stain nuclear DNA, the first 30 min wash following secondary antibody incubation included rhodamine-conjugated phalloidin (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada), diluted to 1:200 from 5 μg/ml stock solution, and DAPI (Sigma-Aldrich Canada Ltd.), diluted to 1:2,000 from 1 mg/ml stock solution. Embryos were then subjected to three additional 30 min washes at 37°C. Embryos were finally mounted in Fluro-Guard Antifade Mounting Reagent (BioRad, Mississauga, ON, Canada). Fluorescence patterns were examined by confocal microscopy using a Zeiss LSM 510 (laser scanning microscope) based on an inverted Axiovert 100 microscope using a Zeiss 40× water Plan Apochromat objective lens with a numerical aperture of 1.2. The confocal microscope used cooled photomultiplier tubes produced by Hamamatsu (PMT). The images were then captured and stored as TIFF files by the Zeiss LSM software package. To capture confocal images, saturation levels were optimized using control embryos and all subsequent embryos were scored using absorbance levels established form control exposures.

Assessment OF *Trp53*, *Ppm1d*, *Cdkn2a*, and *Mapk14* mRNA Levels Following p38 MAPK Inhibition

Embryos were flushed from oviducts of timed-pregnant mice at the 2-cell (48 hr post-hCG) and 8-cell stages (72 hr hCG) as described (Natale et al., 2004; Paliga et al., 2005). Groups of 20 embryos each were pooled and washed. Groups were cultured for 12 or 24 hr in either a 20 μl drop of KSOMaa plus 0.1% DMSO, KSOMaa plus 10 μM SB220025 (CSAID, active MAPK14/11 inhibitor), or KSOMaa plus 10 μM SB272474 (CSAID, inactive analog) (Natale et al., 2004; Paliga et al., 2005). Following each treatment period, embryos were placed in 500 μl TRI-Reagent, frozen in liquid nitrogen and stored at −80°C until processed for RNA extraction and application of real-time RT-PCR analysis of transcript abundance. Three experimental replicates were conducted for each set of embryo stages.

Statistical Analysis

Statistical analysis of data were carried out using SPSS 14.0 (SPSS, Inc., Chicago, IL) 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 *Trp53*, *Ppm1d*, and *Cdkn2a* Transcripts in Mouse Preimplantation Embryos

The application of qualitative RT-PCR methods to embryo cDNA samples resulted in the detection of *Trp53*, *Ppm1d*, and *Cdkn2a* transcripts throughout mouse preimplantation development (Fig. 1A). The RT-PCR products amplified from adult liver tissue cDNA and blastocyst cDNA were purified and sequenced to confirm the identities of each RT-PCR product. The *Trp53* and *Cdkn2a* mouse embryo RT-PCR products displayed 99% sequence identity to their respective GenBank mouse nucleotide sequences. The *Ppm1d* embryo RT-PCR product displayed a 100% sequence identity to its GenBank mouse nucleotide sequence.

After determining that *Trp53*, *Ppm1d*, and *Cdkn2a* mRNAs were present during preimplantation development, we next assessed the variations in their relative abundance throughout preimplantation development by applying real-time RT-PCR methods. Fold-changes in the relative mRNA levels of the target genes were determined using the formula $2^{-\Delta\Delta CT}$ as described. *Trp53* mRNA levels displayed an accumulation pattern where they were elevated at the 1-cell stage, then dropped significantly ($P \leq 0.05$) by the 2-cell stage to reach 16% of the 1-cell levels (Fig. 1B). *Trp53* transcript levels remained low until the morula stage when they increased to reach a level that was 23% higher than 1-cell levels (Fig. 1B). The *Trp53*, mRNA levels at the blastocyst stage increased to become 176% of the levels observed at the 1-cell stage (Fig. 1B). In contrast to the accumulation pattern displayed by *Trp53* mRNAs, *Ppm1d*, and *Cdkn2a* mRNA levels were highest at the 1-cell stage, dropped precipitously at the 2-cell stage (16 and 4% of 1-cell levels, respectively) and remained low but detectable through to the blastocyst stage (Fig. 1C,D, respectively). Small but significant variations ($P \leq 0.05$) were observed for both gene transcripts between the 2-cell and blastocyst stages. *Ppm1d* transcript levels dropped significantly to reach 3% of the 1-cell levels at the 8-cell stage and then rose again at the morula stage to become 13% of the 1-cell levels (Fig. 1C). *Cdkn2a* mRNAs rose at the 8-cell stage to reach 20% of the 1-cell levels, and then decreased to become 9 and 11% of the 1-cell levels at the morula and blastocyst stages, respectively (Fig. 1D).

Distribution of TRP53 AND PP2Cδ Polypeptides Throughout Preimplantation Development

The protein distribution patterns of TRP53-phospho and PP2Cδ were examined using whole-mount indirect immunofluorescence applied to three independent developmental series of embryos (1-, 2-, 4-, 8-cell, morula and blastocyst). TRP53-phospho protein was detected throughout mouse preimplantation development (Fig. 2A–G). TRP53-phospho immunofluorescence was predominantly cytoplasmic, however nuclear fluorescence was also observed (but not in all blastomeres) beginning at the 4-cell stage and continuing onto the blastocyst stage (Fig. 2C–F). TRP53-phospho immunofluorescence was detected in both trophectoderm and inner cell mass cell lineages. PP2Cδ protein was also detected throughout mouse preimplantation development (Fig. 2H–N). PP2Cδ immunofluoresence was predominantly cytoplasmic with some punctate fluorescent

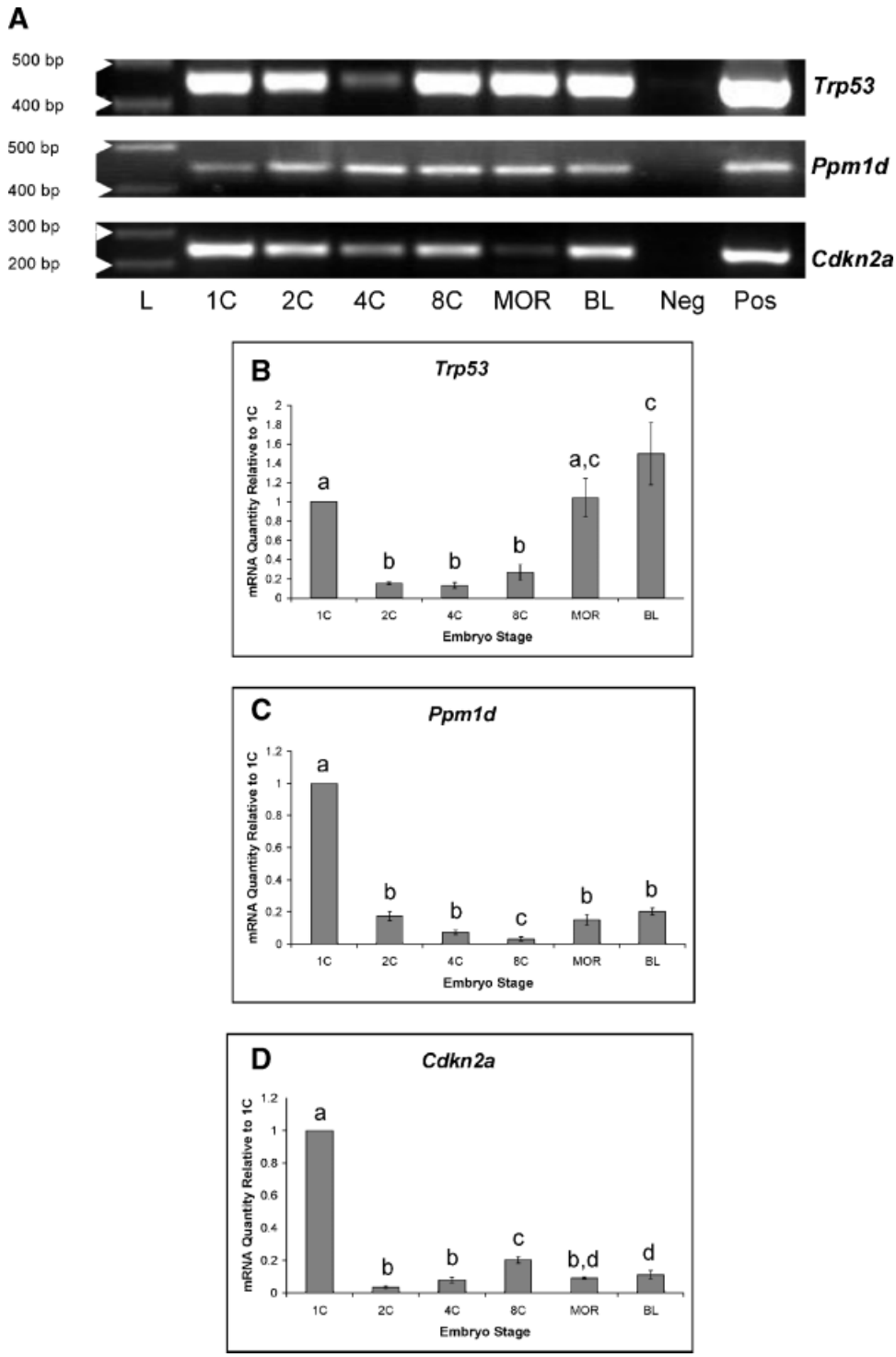

Fig. 1. mRNA transcripts encoding TRP53, PP2Cδ, and CDKN2A in 1-cell (1C), 2-cell (2C), 4-cell (4C), 8-cell (8C), morula (MOR), and blastocyst (BL) stage embryos. Gel representative of three independent developmental series depicting qualitative detection of transcripts throughout mouse preimplantation development (**A**). Positive control (Pos) is adult liver tissue, and negative control (Neg) is no transcript added to the PCR reaction. To confirm identities of the PCR products, samples of blastocyst PCR products were sequenced. **B**–**D**: Illustrate quantitative real-time PCR analysis of the *Trp53*, *Ppm1d*, and *Cdkn2a*. Values shown are normalized to exogenous *luciferase* added before RNA extraction and set relative to the amount of the transcript of interest in the 1-cell sample. Lowercase letters denote significant differences ($P \leq 0.05$).

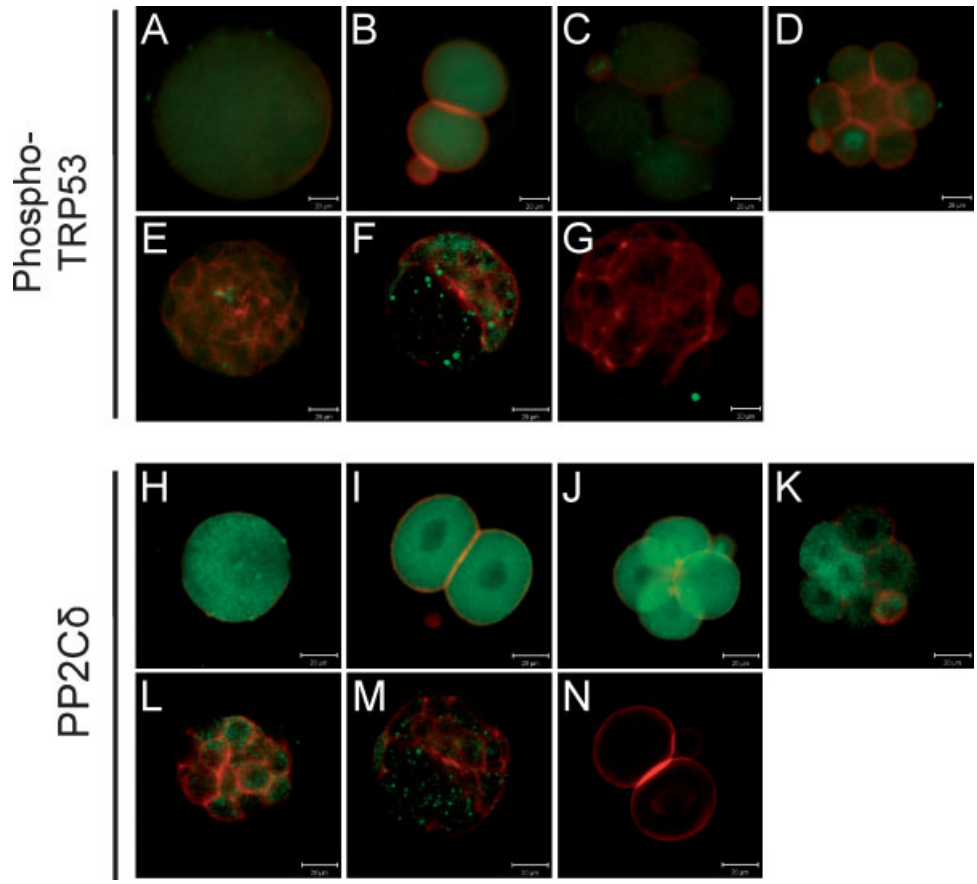

Fig. 2. Localization of TRP-Phospho and PP2Cδ polypeptides during mouse preimplantation development. 1-cell (**A**,**H**), 2-cell (**B**,**I**), 4-cell (**C**,**J**), 8-cell (**D**,**K**), morula (**E**,**L**), and blastocyst (**F**, **M**) stage embryos were flushed at timed stages of development and processed for application of indirect immunofluorescence methods for the detection of TRP53-phospho (A–G) and PP2Cδ (H–N) polypeptides. Three independent replications were completed and 8–10 embryos of each stage were examined per replication. Panels G and N depict the negative controls. Protein of interest in represented in green and red indicates rhodamine phalloidin.

foci appearing near the nuclei in each blastomere (Fig. 2H–N). Although not a quantitative assay PP2Cδ immunofluorescence was consistently more intense at the 1-, 2-, and 4-cell stages and lower at the 8-cell and morula stages (Fig. 2H–L). Furthermore, the cytoplasmic immunofluorescence declined over these stages and the punctate fluorescent pattern became more dominant by the morula stage (Fig. 2L). At the blastocyst stage, PP2Cδ immunofluorescence was barely detectable in both trophectoderm and inner cell mass cell lineages (Fig. 2M).

Effects of MAPK14/11 Inhibition on Development, and *Trp53*, *Ppm1d*, *Cdkn2a*, and *Mapk14* mRNA Levels

Our primary hypothesis states that p38 MAPK regulates *Trp53*, *Ppm1d*, and *Cdkn2a* and its own transcript levels during preimplantation development. To test this hypothesis, 2-cell and 8-cell embryos were flushed and cultured in KSOMaa medium plus 0.1% DMSO, KSOMaa medium plus 10 μM SB202474 (inactive CSAID analog), or KSOMaa medium plus 10 μM SB220025 (active CSAID) for 12 and 24 hr prior to assessment of outcomes that included observing effects on embryo development and measuring effects on *Trp53*, *Ppm1d*, *Cdkn2a*, and *Mapk14* mRNA levels by the application of real-time RT-PCR methods. Three independent trials were completed. We applied treatments to both 2-cell and 8-cell stage embryos to replicate the experimental design used in our earlier studies (Natale et al., 2004; Paliga et al., 2005) investigating effects of CSAID treatment on mouse preimplantation development. By using the same stages and experimental design we have maintained a consistent design across studies to increase our overall understanding of p38 MAPK function during preimplantation development.

Treatment with SB220025 did not significantly affect ($P \leq 0.05$) development of 2-cell stage embryos at either the 12 or 24 hr time points (data not shown). However, *Trp53* mRNA levels were significantly ($P \leq 0.05$) elevated (10 and 150% increases, respectively) over control levels (DMSO and SB202474 treatment) following 12 and 24 hr treatment with SB220025 (Fig. 3A,B). *Ppm1d* mRNA levels displayed a more rapid increase than *Trp53* mRNAs as *Ppm1d* mRNAs were significantly

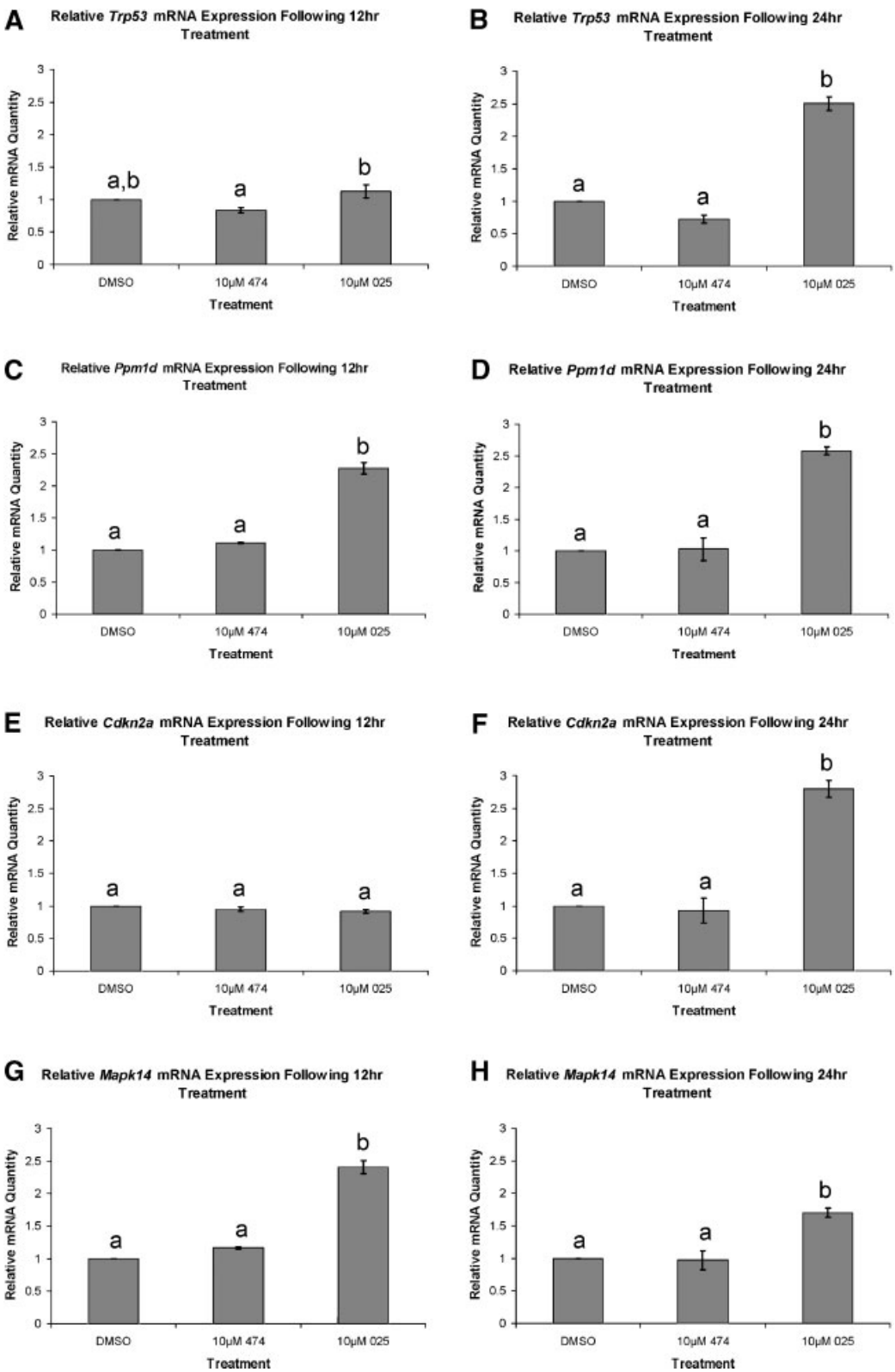

Fig. 3. Variations in *Trp53* (**A**,**B**), *Ppm1d* (**C**,**D**), *Cdkn2a* (**E**,**F**), and *Mapk14* (**G**,**H**) mRNA levels following 12 and 24 hr CSAID treatment of 2-cell embryos. 2-cell embryos were collected and treated for 12 hr (A,C,E,G) and 24 hr (B,D,F,H) in 0.1% dimethylsulphoxide (DMSO), 10 μM SB202474 (inactive CSAID, 474), and 10 μM SB220025 (active CSAID, 025) prior to application of Real-Time RT-PCR methods. mRNA values were normalized to exogenous luciferase mRNA and set relative to control DMSO cultured embryos. Error bars are ±SE. Lowercase letters denote statistically significant differences ($P \leq 0.05$).

($P \leq 0.05$) elevated (130 and 160%, respectively) over control levels following both 12 and 24 hr treatment with SB220025 (Fig. 3C,D). In contrast to these results *Cdkn2a* mRNA levels did not display a significant increase over that of control levels following 12 hr treatment with SB220025 (Fig. 3E,F), however by 24 hr of treatment *Cdkn2a* mRNA levels were significantly ($P \leq 0.05$) higher (180% increase) in the SB220025 treatment group over that observed for controls (Fig. 3E,F). *Mapk14* transcripts were also significantly elevated following treatment with 10 μM SB220025 for 12 and 24 hr (Fig. 3G,H). However, in contrast to the other genes of interest, *Mapk14* mRNAs displayed a maximal elevation compared to control levels following 12 hr of treatment with SB220025 (140% increase over controls) (Fig. 3G). By 24 hr of treatment with SB220025 *Mapk14* levels were still significantly ($P \leq 0.05$) higher than that of controls but the fold difference between groups was much lower than that observed following 12 hr of SB220025 treatment (70% increase over controls Fig. 3H).

In contrast to results following treatment of 2-cell stage embryos with SB220025, the majority of 8-cell stage embryos treated with 10 μM SB220025 for 12 hr remained at the 8-cell stage (68%), while most of the embryos in control groups compacted (65%) (data not shown). After 24 hr of CSAID treatment, the variation in development was even more striking, with only 5% of 8-cell embryos placed in the 10 μM SB220025 treatment group progressing to the blastocyst stage, compared to 43–48% of the embryos placed into the control groups (data not shown). These results have replicated outcomes reported in Natale et al. (2004) and demonstrate the consistency of our experimental design and the treatment effects of SB220025 on preimplantation development. As observed following the treatment of 2-cell stage embryos, *Trp53* and *Ppm1d* mRNAs were significantly ($P \leq 0.05$) elevated above control levels following 12 and 24 hr treatment of 8-cell embryos in 10 μM SB220025 (60 and 50% above control levels for *Trp53*, and 60 and 40% above control levels for *Ppm1d*; Fig. 4A–D). However, in contrast to the 2-cell experimental results, *Cdkn2a* mRNA levels increased significantly ($P \leq 0.05$) by 140% over the control levels after a 12 hr treatment of 8-cell embryos in 10 μM SB220025, but decreased significantly ($P \leq 0.05$) to become 60% of control levels by 24 hr of CSAID treatment (Fig. 4E,F). *Mapk14* mRNA levels did not vary significantly among treatment groups following 12 hr of CSAID treatment, but they were observed to decline significantly ($P \leq 0.05$) to become 50% of control levels following 24 hr of treatment in 10 μM SB220025 (Fig. 4G,H). In total our results indicate that *Trp53*, *Ppm1d*, *Cdkn2a*, and *Mapk14* transcripts are affected by p38 MAPK inhibition during preimplantation development and that these effects vary across embryo stages.

DISCUSSION

This study has characterized the expression of *Trp53*, *Ppm1d*, and *Cdkn2a* gene products during mouse preimplantation development. Using a pharmacological inhibitor of p38 MAPKα/β-MAPK14/11, we have demonstrated that *Trp53*, *Ppm1d*, *Cdkn2a*, and *Mapk14* mRNA levels are affected by p38 MAPK inhibition and that outcomes following p38 MAPK inhibition on *Cdkn2a* and *Mapk14* mRNAs vary between the 2-cell and the 8-cell embryo stages. This work serves to increase our understanding of the molecular signaling pathways that regulate and assist in responding to stressful conditions that preimplantation embryos face in culture.

Recent research is focused on defining the specific cellular pathways that regulate stress responses in the early embryo, to understand how the early embryo copes with and responds to the culture environment. This research has revealed that MAPK signaling pathways are important mediators of environmental stress influences on embryonic gene expression and development (Saba-El-Leil et al., 2003; Natale et al., 2004; Maekawa et al., 2005; Paliga et al., 2005; Wang et al., 2005). Interestingly, Natale et al. (2004), discovered that inhibition of MAPK14/11 activity by employing CSAID treatment, results in a reversible arrest of preimplantation development at the 8–16 cell stage in the mouse. Importantly, treated embryos recovered completely from p38 MAPK blockade and proceeded to the blastocyst stage at the same frequency as controls, however, this recovery was delayed by the same time period in which the embryos were exposed to CSAID treatment (Natale et al., 2004).

The present study was directed at expanding our understanding of p38 MAPK function and regulation during preimplantation development by characterizing the expression and responsiveness of p38 MAPK targets, *Trp53*, *Ppm1d*, and *Cdkn2a* during preimplantation development (Bulavin et al., 1999; Sanchez-Prieto et al., 2000; Takekawa et al., 2000). Studies have indicated that p38 MAPK directly phosphorylates and activates TRP53 (Kishi et al., 2001). TRP53 in turn upregulates the expression of the protein phosphatase PP2Cδ. PP2Cδ has been recently characterized as an endogenous protein phosphatase inhibitor of p38 MAPK as it selectively dephosphorylates p38 MAPK on threonine 180 (Takekawa et al., 2000). In the absence of PP2Cδ, p38 MAPK activity increases, as well as the expression of *Trp53 Cdkn2a*, an upstream regulator of retinoblastoma (RB) protein (Bulavin et al., 2004).

The first objective was to determine the developmental expression profiles of *Trp53* and *Ppm1d* mRNAs and proteins and *Cdkn2a* mRNAs throughout preimplantation development. Qualitative reverse-transcription PCR, quantitative real-time RT-PCR and whole-mount immunofluorescence methods with confocal microscopy were employed to determine that mRNAs encoding all three of these genes of interest and phospho-TRP53 and PP2Cδ polypeptides were present throughout mouse preimplantation development. *Trp53* mRNA levels were high at the 1-cell stage, dropped significantly at the 2-cell stage, then rose steadily from the 8-cell stage to the blastocyst stage. These results are in close agreement

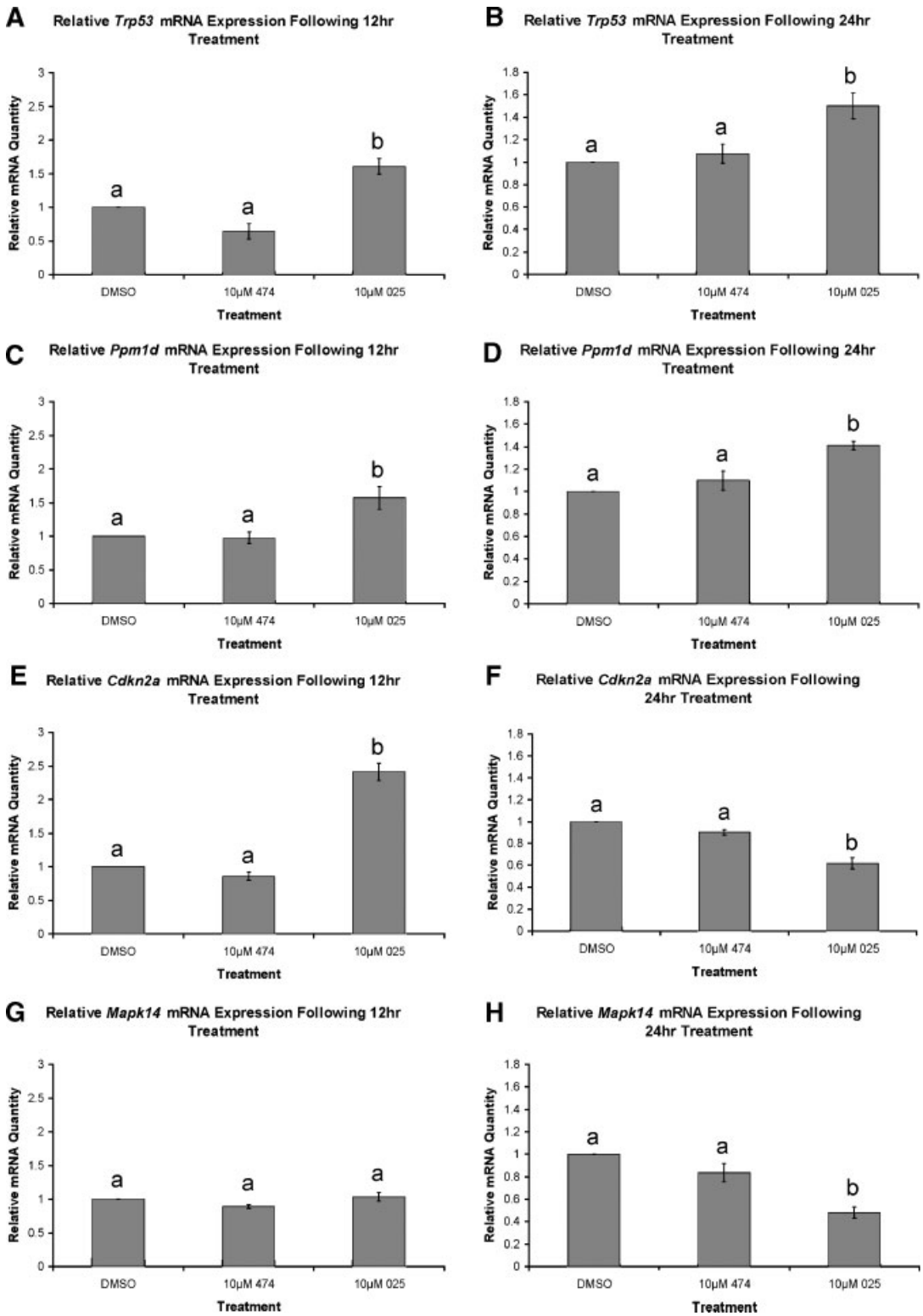

Fig. 4. Variations in *Trp53* (**A**,**B**), *Ppm1d* (**C**,**D**), *Cdkn2a* (**E**,**F**), and *Mapk14* (**G**,**H**) mRNA levels following 12 and 24 hr CSAID treatment of 8-cell embryos. 8-cell embryos were collected and treated for 12 hr (A,C,E,G) and 24 hr (B,D,F,H) in 0.1% dimethylsulphoxide (DMSO), 10 μM SB202474 (inactive CSAID, 474), and 10 μM SB220025 (active CSAID, 025) prior to application of Real-Time RT-PCR methods. mRNA values were normalized to exogenous luciferase mRNA and set relative to control DMSO cultured embryos. Error bars are ±SE. Lowercase letters denote statistically significant differences ($P \leq 0.05$).

with (Jurisicova et al., 1998) who reported *Trp53* levels in CD-1 and C57BL/6 mouse embryos at 18, 49, 57, 67, 88, and 97 hr post-hCG using quantitative dot blot hybridization. This pattern of mRNA accumulation is commonly reported for transcripts during mouse preimplantation development and likely demonstrates the turnover of large pools of maternal transcripts in the 1-cell stage that become degraded at the 2-cell stage prior to replacement via activation of zygote genome transcriptional activity and accumulation of gene transcripts through development to the blastocyst stage (Nothias et al., 1995; Hamatani et al., 2004; Alizadeh et al., 2005). The main variation that TRP53 mRNAs displayed from this pattern is that their reaccumulation following the 2-cell low did not begin immediately and was delayed until the 8-cell stage. Phosphorylated TRP53 protein was detected in all preimplantation embryo stages primarily confined to the cytoplasm, however, phosphor-TRP53 immunolocalization was detected in the nuclei in a few blastomeres beginning at the 4-cell stage and continuing to the blastocyst stage. Studies have determined that TRP53's cellular localization is tightly linked to the cell cycle (Liang and Clarke, 2001; O'Brate and Giannakakou, 2003; Lavin and Gueven, 2006). TRP53 accumulates in the cytoplasm during the G_1 phase and enters the nucleus during the G_1/S phase transition (Liang and Clarke, 2001). A short period after the beginning of S phase, TRP53 cycles back to the cytoplasm (Liang and Clarke, 2001). Since cell division in cleavage stage embryos is asynchronous, one would not expect all of the blastomeres to be at the same phase of the cell cycle. The presence of nuclear phospho-TRP53 beginning at the 4-cell stage then may simply reflect this asynchronous nature of the cleavage divisions during preimplantation development and also indicates the proportion of the blastomeres at each embryonic stage that are engaged in the G_1/S phase of the cell cycle.

Ppm1d and *Cdkn2a* both displayed a very different pattern of mRNA accumulation during preimplantation development from that observed for *Trp53* transcripts, which consisted of high levels at the 1-cell stage that dropped at the 2-cell stage and remained low but detectable through to the blastocyst stage. This pattern of mRNA accumulation could represent a sustained oogenetic transcript pattern in which the maternal transcripts are stabilized through development to the blastocyst stage. Alternatively it may reflect very low but sustained transcription of these gene products by the embryonic genome following the degradation of maternal transcripts during the 1- to 2-cell transition. The unusual accumulation profile displayed by *Ppm1d* and *Cdkn2a* mRNAs has been observed by others for additional genes including *stat3*, *N-cadherin*, and *rac1* (Hamatani et al., 2004).

PP2Cδ protein was detected in all preimplantation stages by the application of immunofluorescence methods. PP2Cδ fluorescence was primarily cytoplasmic with punctate fluorescent foci accumulating near the nucleus in each blastomere. Overall a qualitative assessment of PP2Cδ fluorescence indicated that there was a reduction in PP2Cδ fluorescence from the 8-cell stage onward. By the blastocyst stage, PP2Cδ fluorescence was only faintly detectable. These data correspond well with the mRNA expression pattern of PP2Cδ. It is intriguing to report that PP2Cδ protein expression appears to decrease as a developmental dependence on p38 MAPKα/β emerges at the 8- to 16-cell stage (Natale et al., 2004; Paliga et al., 2005). It is possible that PP2Cδ is primarily a regulator of p38 MAPK during the early cleavage stages prior to when p38 MAPKα/β activity is absolutely required for normal developmental progression.

In a pioneering paper on the structure and expression of PP2Cδ, (Choi et al., 2000) reported PP2Cδ expression in embryo stages from E8.5 to E19 and also demonstrated that its expression was ubiquitous in all adult tissues suggesting a role in basic cellular functions shared by all cells. Although the physiological role of PP2Cδ in cell cycle control is not fully understood, it has been suggested that PP2Cδ regulates the TRP53-induced growth arrest response. By dephosphorylating critical target proteins such as p38 MAPK and uracil DNA glycosylase 2 (UNG2), an important base excision repair protein, PP2Cδ may enhance the cell-cycle inhibitory activities of its targets or it may contribute to a regulatory feedback mechanism that acts to reduce cell-cycle inhibitory activity following an initial activation period. In conjuction with the TRP53 results, the presence of both phosphorylated TRP53 and PP2Cδ suggests that the regulatory negative feedback mechanism for p38 MAPK could be active in mouse preimplantation embryos.

Interestingly, it has been previously reported that *Cdkn2a* is not expressed during mouse development and that transcripts for this gene only appear following birth (Zindy et al., 1997). However, Zindy et al. (1997) employed Northern Blot analysis and only examined expression from days 7 to 17 of embryogenesis, far after the preimplantation stages that we investigated in the present study. It is possible that *Cdkn2a* transcript levels continue to drop off further into development after the blastocyst stage to a point where they are undetectable. In fact, *Cdkn2a*-null animals develop normally (Serrano et al., 1996). Zindy et al. (1997) showed that *Cdkn2a* expression is low throughout early postnatal life, and rises as mice age suggesting a role for *Cdkn2a* in cell senescence. The most likely reason for the discrepancy between studies is that we employed a much more sensitive method of transcript analysis and it is conceivable that the low but steady levels of *Cdkn2a* mRNA that we detected using real-time RT-PCR are not contributing an indispensable role during preimplantation development. Regardless, we did not investigate CDKN2A protein distribution due to its low mRNA expression after the 1-cell stage.

The main hypothesis of our study is that p38 MAPK regulates *Trp53*, *Ppm1d*, *Cdkn2a*, and possibly its own transcript levels during preimplantation development. To test this hypothesis, 2-cell and 8-cell embryos were

cultured in 0.1% DMSO (control), 10 μM SB202474 (inactive CSAID), or 10 μM SB220025 (active CSAID) for 12 and 24 hr then assessed for morphology prior to the application of real-time RT-PCR methods to contrast treatment effects on *Trp53*, *Ppm1d*, *Cdkn2a*, and *p38 mapkα* mRNA levels. No significant effects to development of 2-cell stage embryos were observed at 12 or 24 hr. Treatment of 8-cell embryos did result in arrested development at the 8-cell to 16-cell stages at 12 and 24 hr. These results are in complete agreement with the findings reported in Natale et al. (2004) and Paliga et al. (2005). These studies reported that a CSAID concentration as low as 2 μM SB220025 was sufficient to eliminate phospho-MAPKAPK-2 (a direct downstream target of activated p38 MAPK) immunostaining (Natale et al., 2004; Paliga et al., 2005). However, this low dose only resulted in a reduced developmental delay. The 10 μM SB220025 concentration employed in the present study was an intermediate level between the 2 μM dose and the 20 μM dose utilized in these earlier studies (Natale et al., 2004; Paliga et al., 2005).

One of the most intriguing outcomes from the present study is the variation in stage-specific effects of CSAID treatment on *Trp53*, *Ppm1d*, *Cdkn2a*, and *Mapk14* mRNA levels. Since p38 MAPKα/β activity is required for development past the 8- to 16-cell stage it is possible that the mechanisms used to regulate the activity of this MAPK pathway vary between the 2-cell and 8-cell stages of development. *Trp53* and *Ppm1d* mRNAs may be regulated similarly at the 2-cell and 8-cell stages as they were both elevated following CSAID treatment at both time points in 2-cell and 8-cell embryos. These results do not agree with the negative feedback model proposed by Bernards (2004) where p38 MAPK is predicted to activate TRP53, which in turn activates PP2Cδ, to downregulate p38 MAPK activity. Our results raise the possibility that this pathway and the proposed interactions of p38 MAPK, TRP53, PP2Cδ, and CDKN2A do not occur in the early embryo as has been reported for established cell lines (Bernards, 2004). Instead the results suggest that p38 MAPKα/β inhibition for 12 and 24 hr is associated with upregulation of *Trp53* mRNA levels which are also associated with increased *Ppm1d* mRNA levels. The regulation of *Ppm1d* mRNA levels in conjunction with elevated TRP53 expression is consistent with the proposed model (Bernards, 2004). Recently Fabian et al. (2006) reported that ERK is transiently activated following p38 MAPK inhibition in PC12 cell lines (rat adrenal tumor) and this is associated with increased TRP53 protein and mRNA. However, caution must be exercised when attempting to correlate outcomes between post-translational activation of proteins and mRNA abundance.

Cdkn2a mRNA was elevated over controls following treatment of 2-cell embryos for 24 hr. It was also elevated following treatment of 8-cell embryos for 12 hr, but below control levels after 24 hr. These results suggest that *Cdkn2a* mRNA is differentially regulated at the 8-cell stage in response to decreased p38 MAPKα/β activity. In the absence of active p38 MAPKα/β at the 8-cell stage, *Cdkn2a* levels may at first rise in an response to CSAID treatment, however this is not maintained, and its levels decline. The 2-cell stage embryo, however, maintains these elevated *Cdkn2a* levels. Another reasonable possibility is that since *Cdkn2a* is expressed at such a low levels in preimplantation embryos, and *Cdkn2a*-null mice exhibit normal prenatal development, the observed differences in *Cdkn2a* mRNA expression simply have little consequence to early development (Serrano et al., 1996).

The final important outcome from this study is that *Mapk14* mRNA expression is regulated by blocking MAPK 14/11 activity, and *Mapk14* mRNA was differentially regulated at the 2-cell and 8-cell stages following CSAID treatment. At the 2-cell stage, inhibition of MAPK14/11 activity was linked with elevated levels of *Mapk14* mRNAs after 12 and 24 hr of CSAID treatment. In contrast, at the 8-cell stage, after 12 hr in CSAID treatment, *Mapk14* mRNA levels did not differ significantly from controls, but was significantly decreased from control levels after 24 hr of CSAID treatment. These results indicate that at the 2-cell stage *Mapk14* gene expression is responsive to changes in MAPK 14/11 activity. Thus, at the 2-cell and 4-cell stages it may be possible to compensate for reduced MAPK14/11 activity by increasing *Mapk14* expression. At the 8-cell stage this mechanism does not assert itself to the same degree as *Mapk14* levels do not increase and in fact decline in the presence of MAPK 14/11 inhibitors. This result is especially intriguing as this stage also represents the time of first developmental sensitivity to CSAID treatment. Perhaps this arises due to an inability of the embryo at this stage to maintain required *Mapk14* transcript levels in the presence of the inhibitor. These studies certainly support the vulnerability of 8- to 16-cell stage embryos to CSAID treatment which has now been associated with reduced *Mapk14* transcript levels in addition to loss of filamentous actin (Paliga et al., 2005). Why *Mapk14* gene expression displays a greater dependence on p38 MAPK activity at the 8-cell stage is unknown at this time but this discovery certainly represents an important avenue for further experimentation in the future.

In conclusion, p38 MAPKs regulate many important cellular functions such as inflammation, cell proliferation, and apoptosis in several different cellular systems (Enslen et al., 2000; Ono and Han, 2000; Kyriakis and Avruch, 2001). This study has characterized the expression of *Trp53*, *Ppm1d*, and *Cdkn2a* gene products during preimplantation development. This is the first study to show the presence of PP2Cδ gene products in preimplantation embryos. As a predicted endogenous inhibitor of p38 MAPK activity, it is interesting to discover that PP2Cδ protein expression may decrease around the 8-cell stage, when p38 MAPK activity is absolutely required for developmental progression. As well, this study demonstrated a stage-specific regulation of *Cdkn2a* and *Mapk14* mRNA expression following treatment of 2-cell and 8-cell embryos for 12 and 24 hr with the MAPK 14/11 inhibitor, SB220025.

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