

# Requirement of the MAP kinase signaling pathways for mouse preimplantation development

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## Summary

Mammalian preimplantation development involves several crucial events, such as compaction and blastocyst formation, but little is known about essential genes that regulate this developmental process. Here, we have focused on MAP kinase signaling pathways as potential regulatory pathways for the process. Our results show that inhibition of the JNK pathway or of the p38 MAP kinase pathway, but not of the ERK pathway, results in inhibition of cavity formation, and that JNK and p38 are active during mouse preimplantation development. Our subsequent microarray analyses show that, of about 39,000 transcripts analyzed, the number of those genes whose expression level is sensitive to the inhibition of the JNK or the p38 pathway, but insensitive to the inhibition of the ERK pathway, is only

156. Moreover, of the 156 genes, expression of 10 genes (two genes upregulated and eight genes downregulated) is sensitive to either inhibition of the JNK or p38 pathways. These 10 genes include several genes known for their function in axis and pattern formation. Downregulation of some of the 10 genes simultaneously using siRNA leads to abnormality in cavity formation. Thus, this study has successfully narrowed down candidate genes of interest, detailed analysis of which will probably lead to elucidation of the molecular mechanism of preimplantation development.

Key words: JNK, Microarray analysis, p38, Preimplantation development, Signal transduction

## Introduction

Preimplantation development is a mammalian-specific event, and is vital for successful implantation and pregnancy. This process involves many crucial events with cavity formation occurring during morula to blastocyst stages being one of the most important. Recently, several reports focusing on preimplantation development, especially cavity formation, have appeared (Fleming et al., 2001; Barcroft et al., 2003; Salas-Vidal and Lomeli, 2004). However, neither the detailed molecular mechanisms nor the crucial genes that regulate preimplantation developmental processes have been clarified. To address these issues, we have focused on intracellular signal transduction pathways that might regulate gene expression during cavity formation.

The mitogen-activated protein kinase (MAPK) cascades have central roles in diverse cellular functions (Sturgill and Wu, 1991; Ahn et al., 1992; Nishida and Gotoh, 1993; Davis, 2000; Ono and Han, 2000; Chang and Karin, 2001; Kyriakis and Avruch, 2001; Pearson et al., 2001). The MAPK pathways include the extracellular signal-regulated protein kinase (ERK) pathway, the Jun N-terminal kinase (JNK) pathway and the p38 pathway. Each member of MAPK is activated in response to various extracellular stimuli, and controls, mainly through regulating gene expression, various biological processes such

as cell proliferation, cell differentiation, cell cycle arrest, apoptosis, etc. As previous genetic and biochemical studies have revealed the crucial involvement of the MAP kinase family molecules in early embryonic development, it has been expected that they might act also in preimplantation development. However, difficulties in manipulating preimplantation embryos have delayed progression in this field.

Recent development of specific inhibitors of each MAPK pathway, however, enabled us to test our hypothesis that several of the MAPK family molecules may be involved in processes during preimplantation development. In this study, the use of the specific inhibitors has firstly shown that inhibition of the p38 pathway or the JNK pathway, but not the ERK pathway, leads to abnormality in mouse preimplantation development, especially in cavity formation. Immunostaining with anti-phospho-specific antibodies has then shown the existence of activated forms of p38 and JNK from four-cell to blastocyst stages. These results strongly suggest the involvement of JNK and p38 in mouse preimplantation development. As for p38, Natale et al. (Natale et al., 2004) reported, just after completion of this work, that p38 MAPK activity is required to support successful murine preimplantation development, in agreement with our results. Our experiments with actinomycin D then

show that gene expression is essential for compaction and cavitation, in accordance with previous reports with another inhibitor (Khidir et al., 1995; Kidder and McLachlin, 1985). Moreover, recent microarray analyses during mouse preimplantation development revealed global gene expression changes, which could be associated with various signaling pathways (Hamatani et al., 2004; Wang et al., 2004). These observations prompted us to examine expression profiles of genes that are regulated by the JNK or the p38 pathway, but not by the ERK pathway. Our microarray analyses with Affymetrix GeneChips then show that of over 39,000 transcripts (45,000 probe sets) analyzed, there are only 156 transcripts (161 probe sets) whose expression is enhanced or decreased significantly (by at least twofold) by the inhibition of the JNK or the p38 pathway but is insensitive to the inhibition of the ERK pathway. Of the 156 genes, 10 genes are regulated by both the JNK and p38 pathways. These genes include several genes known for their function in axis and pattern formation. Targeting some of these 10 simultaneously by RNAi results in abnormality in cavity formation. Thus, our study reveals involvement of the MAPK pathways in mouse preimplantation development and identifies a limited number of genes that could be crucial for this developmental process.

## Materials and methods

### Embryo collection and culture

One-cell stage embryos were released from swollen ampullae of ICR mice (Japan SLC), and follicle cells were removed with hyaluronidase (Sigma). Eggs were incubated in M2 medium (Sigma) plus hyaluronidase (about 300  $\mu\text{g}/\text{ml}$ ) at room temperature for several minutes until the cumulus cells fell off, and transferred to fresh M2 to rinse off the hyaluronidase. Two- to eight-cell stage embryos were flushed from oviducts using M2 medium. Embryos were cultured in M16 medium (Sigma) at 37°C in 5% CO<sub>2</sub>. PD98059, SB203580 and SP600125 were purchased from Calbiochem. U0126 was from Promega and actinomycin D was from Sigma. In some experiments, the zona pellucida was removed by the method using acid tyrode. Embryos were transferred in acid tyrode (Sigma) at room temperature, and observed continuously under the stereomicroscope. As soon as the zona was dissolved, embryos were collected and transferred back to M16 medium.

### Immunofluorescence confocal microscopy

Prior to fixation, the zona pellucida was removed by treatment with acid tyrode, and then embryos were washed twice in M16 medium. Then, the embryos were fixed overnight in 4% paraformaldehyde in PBS at 4°C and washed in 2% BSA in PBS. The fixed embryos were permeabilized and blocked by incubation for 1 hour in 2% BSA in PBS plus 0.01% Triton X-100 at room temperature. The embryos were then washed in 2% BSA in PBS and incubated with anti-phospho-p38 antibody (Promega) (1:250), anti-phospho-Jun (Ser 73) antibody (Cell Signaling) (1:500), anti-phospho-MAPKAPK-2 (Thr 334) antibody (Cell Signaling) (1:50), anti-phospho-HSP27 (Ser 82) antibody (Cell Signaling) (1:50), anti-Dkk-1 (H-120) antibody (Santa Cruz) (1:250), or anti-CDX1 antibody (Bai et al., 2002) (1:250) in 2% BSA in PBS for 16 hours at 4°C. Embryos were washed three times with 2% BSA in PBS, and incubated with anti-rabbit IgG secondary antibody in 2% BSA in PBS for 2 hours at room temperature. After three washes with 2% BSA in PBS, fluorescence was viewed with a BioRad confocal microscope (Radiance 2000).

### Generation of microarray data

We used Affymetrix GeneChip for microarray analysis. Three

independent experiments were carried out: one using GeneChip Mouse Expression Set 430 (MOE 430) and two using GeneChip Mouse Genome 430 2.0 Array (Mouse 430 2.0) (Affymetrix). For each microarray experiment, we collected two sets of 40 embryos from four kinds of pools: wild-type embryos, SB203580-treated embryos, SP600125-treated embryos and U0126-treated embryos. Eight-cell stage embryos were treated with these drugs, and blastocyst stage embryos were collected and stored at -80°C for RNA extraction. Total RNA was isolated by following the manufacturer's instructions (Isogen, Nippon Gene). Eighty embryos were used for one array. All amplifications started with 100 ng whole-embryo total RNA. Two rounds of amplifications were performed for each replicate following the protocol 'Two-Cycle Target Labeling Assays' by Affymetrix. For each replicate, 10  $\mu\text{g}$  cRNA was fragmented and hybridized following Affymetrix instructions. The microarrays were then washed and stained using the GeneChip fluidics station, according to the manufacturer's instructions.

### Microarray data analysis

Hybridized arrays were scanned using an Affymetrix GeneChip Scanner. We used the GeneChip Operating Software 1.0 (Affymetrix) to analyze the data. For clustering, the first variation was the detection call with the statistical algorithms (Affymetrix software). The detection call indicates whether a transcript is reliably detected (present) or not detected (absent). Genes deemed absent in all four conditions in any one experiment were excluded from further analysis. Second, to generate change significance and change quantity metrics for every probe set, we used a comparison analysis (Affymetrix software). Those genes that are increased or decreased by at least twofold over baseline (control, wild-type embryos) with a statistical significance are judged as increased or decreased, respectively. The data generated from the above process were imported into GeneSpring 6.1 (Silicon Genetics, Redwood City, CA) for making a gene list. For hierarchical clustering, the cosine similarity method was used and the distance metric (1-correlation) was calculated (Fig. 3B). The array data have been deposited into the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>) (series Accession Number GSE2229; sample Accession Numbers GSM40799, GSM40801, GSM40802, GSM40803, GSM40865, GSM40866, GSM40867, GSM40868, GSM40869, GSM40870, GSM40871, GSM40872, GSM40873, GSM40874, GSM40875 and GSM40876).

### RT-PCR

Embryos were obtained at the appropriate stages and stored in ISOGEN at -80°C. Total RNA was isolated by following the manual of ISOGEN (Nippon Gene). Forty embryos were used in isolation of RNA and reverse-transcription reaction with SuperScript II reverse transcriptase (Invitrogen) in a 40  $\mu\text{l}$  reaction. Prepared cDNA was purified and subjected to quantitative PCR analysis by using Light Cycler (Roche Diagnostics) with SYBR Green PCR Kit (Qiagen). The sequences of the PCR primer pairs (5' to 3') that were used are as follows: G3PDH, CATCCACTGGTGTGCTGCCAAGGCTGT and ACAACCTGGTCCTCAGTGTAGCCCA; CD24a antigen, CTCC-TACCCACGCAGATTTA and TGGTGGTAGCGTTACTTGGA; Map2k6, GCAAACCATACATGGCTCCT and GCGTTCGCCAA-GAATCATAA; Tctst1, GATGCAGTCCATTCCCAGTT and TGCC-TAAACAAAGTGCCTCA; Cdx1, CACTACAGCCGGTACATCA and AGAAGGCCAGCATTAGTAGG; Glipr1, GGTTGTTTGGGCA-GACAGTT and TGGGTAAATTCCTGCTGGTC; and Aqp8, TTGC-TACCTTGGGGAACATC and CCAAATAGCTGGGAGATCCA. These primers, except for Tctst1, were designed to contain intron. For Tctst1, our result (no-RT negative control) verified that there was no contamination of genome (data not shown).

### siRNA

Chemically synthesized 21 nucleotide siRNAs were commercially obtained (Japan Bio-service). These RNAs were designed to form 19

bp dsRNA with 2 nucleotide deoxynucleotide overhangs at both 3' ends. The targeting sequences of the siRNA are as follows: siJNK1-1, GAAGCUCAGCCGGCCAUUUTT; siJNK1-2, GCGAGCCUACCGAGAACUATT; siJNK2-1, GUCGUCCUUUCAGAACATT; siJNK2-2, CGCACGAAAGAGAGCCUATT; siCdx1-1, CUACUAAUGCUGGCCUUCUTT; siCdx1-2, CAACGCCUAGAGCUGGAAATT; siDkk1-1, CCAACGCGAUCAAGAACCUTT; siDkk1-2, GAACCACACUGACUCAAATT; siFoxq1-1, GCAAGGACAACUACUGGAUTT; siFoxq1-2, AGAUCAACGAGUACCUCAUTT; siSox7-1, AAGCCGAGCUGUCGGAUGGTT; siSox7-2, ACCUUCUUCUGCCUCAUTT. siRNA duplexes were generated by mixing 20  $\mu$ M sense and antisense ssRNA oligomers in the annealing buffer that has been previously described (Elbashir et al., 2001).

### Microinjection

Microinjection was performed under inverted microscope using a mechanical micromanipulator (Narishige). We injected about 10 pl of 20  $\mu$ M siRNA duplexes into the cytoplasm of one-cell stage embryo. The injected embryos were cultured in M16 medium.

## Results and discussion

To see possible involvement of the MAPK signaling pathways in mouse preimplantation development, we examined the effect of the specific inhibitors of the four major MAPK pathways, the ERK1/2 pathway, the JNK pathway, the p38 pathway and the ERK5 pathway, on the development. The inhibitors were added at the eight-cell stage, and developmental processes were examined. MEK specific inhibitors U0126 and PD98059 (Davies et al., 2000; Dudley et al., 1995; Alessi et al., 1995; Favata et al., 1998; Kamakura et al., 1999; Mody et al., 2001), which inhibit the activation of ERK1/2 and ERK5, had no apparent effect on the preimplantation development at even higher concentrations, up to 50  $\mu$ M. Apparent morphology of the inhibitor-treated embryos under a conventional stereomicroscope was indistinguishable from that of control embryos at the compaction stage, the morula stage and the blastocyst stage (data not shown). The indirect immunostaining pattern for junctional proteins, E-cadherin (adherens junction), ZO-1 (tight junction), Connexin-43 (gap junction) or occludin (tight junction) was essentially normal in the inhibitor-treated embryos (data not shown). Even when the fresh inhibitors were repeatedly added at every 7 hours, the embryos were indistinguishable from control embryos (data not shown). These results indicate that neither the ERK1/2 pathway nor the ERK5 pathway plays an essential role in mouse preimplantation development.

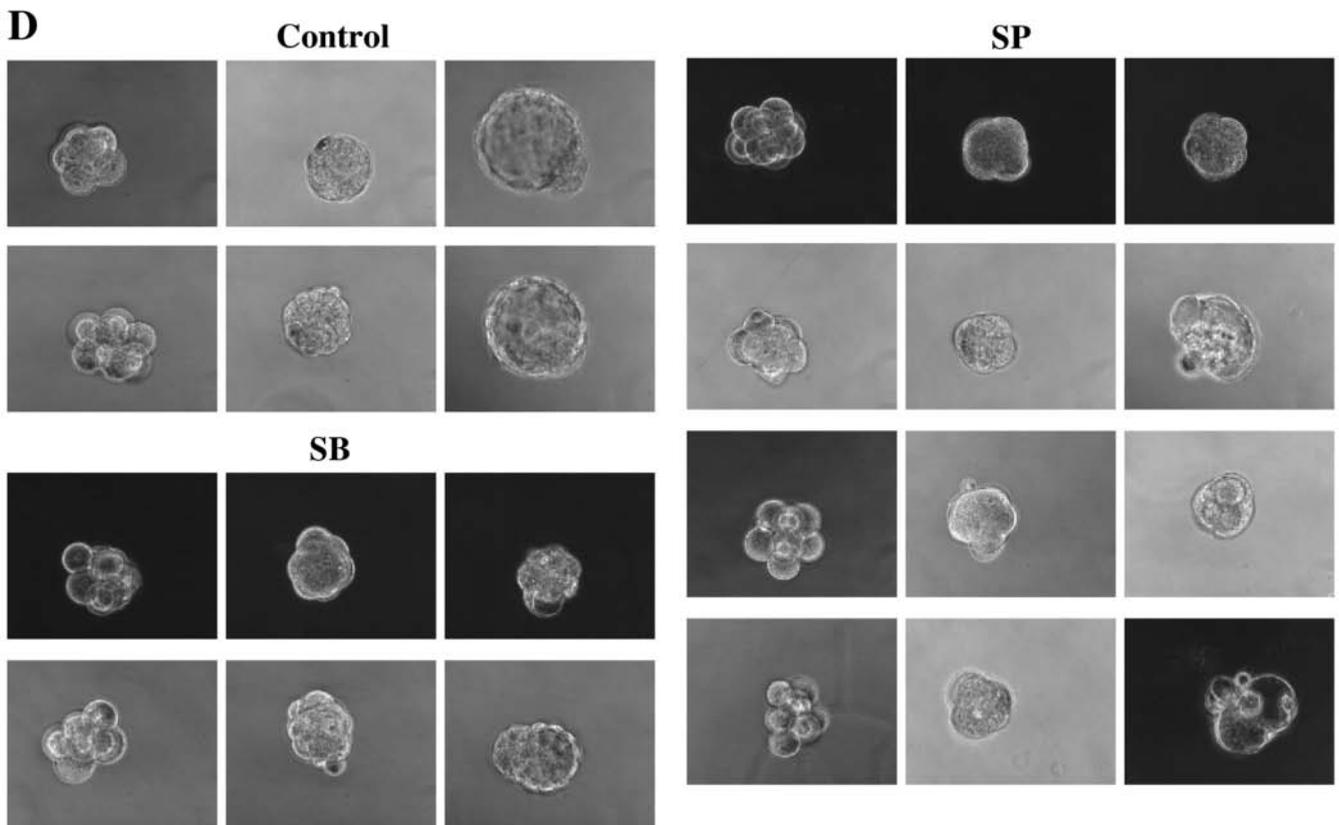
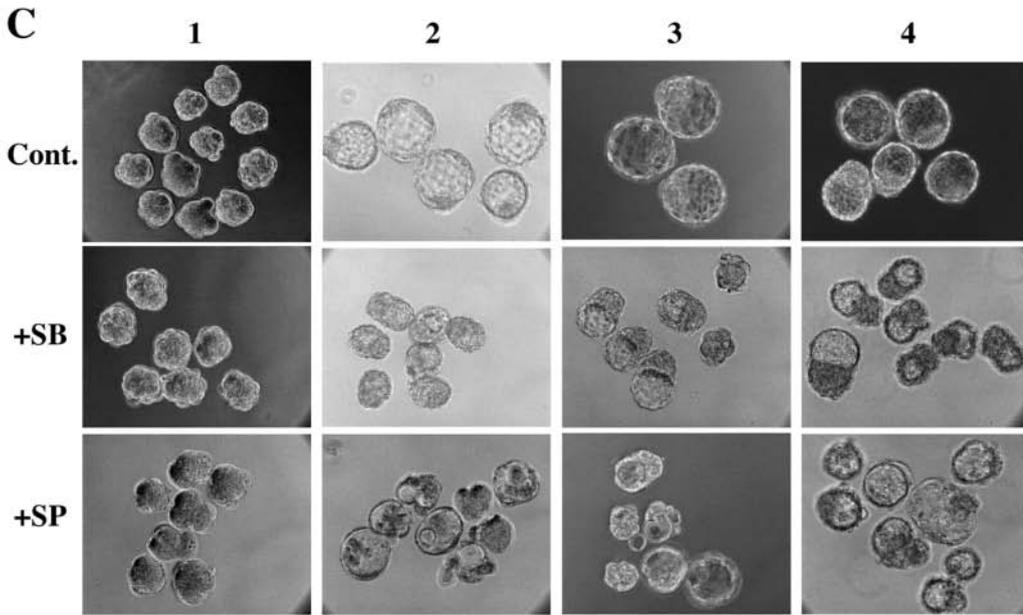
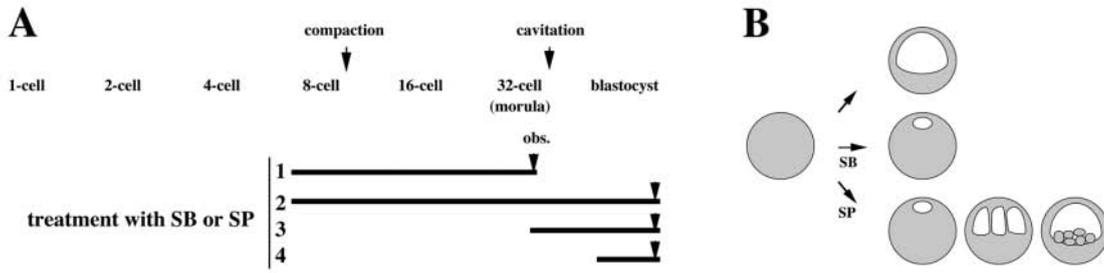
In contrast to the treatment with the ERK pathway-specific inhibitors, treatment with SB203580, a specific inhibitor of p38 $\alpha$  and  $\beta$  MAPKs (Cuenda et al., 1995), caused a severe defect in blastocyst formation. The embryos treated with SB203580 at 20  $\mu$ M were not distinguishable morphologically from control embryos until the cavitation started (Fig. 1C). They had undergone compaction normally. The indirect immunostaining pattern for E-cadherin, connexin-43, ZO-1 or occludin was also apparently normal (data not shown). However, the embryos treated with SB203580 did not form a cavity, or formed a much smaller cavity than did control embryos (Fig. 1C). The cavity formation was inhibited markedly even when SB203580 was added at the pre-cavitation stage (late morula) or after initiation of cavitation (Fig. 1C),

and the cavity was not formed or remained small. There are four isoforms in the p38 MAPK family, p38 $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  (Ono and Han, 2000; Pearson et al., 2001). p38 $\alpha$  and  $\beta$  are thought to play redundant roles.  $\gamma$  or  $\delta$  is different from  $\alpha$  and  $\beta$  in their upstream activating kinases, downstream targets and inactivating MAPK-specific dual specificity phosphatases (Ono and Han, 2000; Pearson et al., 2001). Thus, functions of p38 $\gamma$  and  $\delta$  appear to be different from those of p38 $\alpha$  and  $\beta$ . SB203580 specifically inhibits p38 $\alpha$  and  $\beta$ , but not p38 $\delta$  or  $\gamma$ . Therefore, our results here suggest that p38 $\alpha$  and  $\beta$  are crucially involved in cavitation during preimplantation development. Possible roles of p38 $\gamma$  and  $\delta$  in the preimplantation development, however, remain to be elucidated in the future studies.

Treatment with SP600125, an inhibitor of JNK1, 2 and 3 (Han et al., 2001; Bennett et al., 2001), also caused severe defects in the cavity formation. The inhibitor was added at the eight-cell stage. The overall morphology of embryos treated with the inhibitor was normal until cavitation started (Fig. 1C). However, the cavitation was severely impaired. Like the treatment with SB203580, defects in cavity formation were caused even when SP600125 was added at the pre-cavitation stage (32 cells) or after initiation of cavitation (Fig. 1C).

To eliminate the possibility that the sensitivity of the preimplantation stages to these drugs is merely a reflection that the cultured embryos are stressed, we performed uterine transfer experiments. Eight-cell stage embryos were treated with or without the MEK-specific inhibitor U0126, that has no effect on preimplantation development in culture (see Fig. 1), and then the blastocyst stage embryos were transferred back to 2.5-day p.c. pseudopregnant recipients. Both control embryos and U0126-treated embryos implanted and developed to term (data not shown), suggesting that our drug treatment in culture per se is not severely toxic.

Apparent phenotypes of the embryos treated with SP600125 are similar to, but not completely the same as, those treated with SB203580. Differences were seen in observations of time-dependent morphological changes of a single embryo under a stereomicroscope. Each embryo was cultured in separate dish, and was examined for its apparent phenotypic changes from the eight-cell stage when the inhibitor was added (Fig. 1D). The phenotypes of embryos treated with SB203580 can be categorized into two types: no cavity formation or a small cavity. When SB was added at eight-cell, morula or blastocyst, no cavity formation was found in about 90%, 60% or 20% of the embryos, respectively. The phenotypes of embryos treated with SP600125 can be roughly categorized into three types (see Fig. 1B): a small cavity (the first type), apparent multiple small cavities in an embryo instead of a single cavity (the second type) or a normal size cavity with abnormality in the inner-cell mass (the third type). In the third type, cells were partially detached from the inner-cell mass. As these three phenotypes are overlapping one another, exact classification was difficult. In the case of the SB203580 treatment, a much severer defect in cavitation was observed when the drug was added at the eight-cell stage. By contrast, in the case of the SP600125 treatment, marked defects in blastocyst were found even when the drug was added after initiation of cavitation. These results suggest that p38 and JNK are simultaneously, but slightly differently, involved in blastocyst formation during preimplantation development.



We then examined whether p38 and JNK are activated during mouse preimplantation development. Activation of p38 $\alpha$  can be assessed by using anti-phospho-specific p38 $\alpha$  antibody. Indirect immunofluorescence showed that p38 $\alpha$  began to be activated from the four-cell stage and remained active during morula and blastocyst stages (Fig. 2A). The phosphorylated form of p38 $\alpha$  was detected in dots or patches near the plasma membrane (Fig. 2A). The activation of p38 $\alpha$  showed no apparent asymmetry within the cells. As confirmation of the activation of p38, we investigated whether SB203580 inhibition of the kinase activity of p38 abolished the staining of phospho-MAPKAPK-2 and phospho-HSP27. MAPKAPK-2, MAP kinase-activated protein kinase 2, is a direct target of p38 MAPK (Rouse et al., 1994), and HSP27 is a substrate of MAPKAP kinase 2 (Landry et al., 1992; Rouse et al., 1994). Treatment with SB203580 markedly decreased the staining intensity of phospho-MAPKAPK-2 and phospho-HSP27 (Fig. 2B), confirming that p38 MAPK is activated during mouse preimplantation development. The activation of JNK can be assessed by using anti-phospho-specific antibody for Jun transcription factor. Jun is a well-known substrate of JNK. Indirect immunofluorescent staining showed that nuclear existence of phosphorylated Jun was detected from the four-cell stage to the blastocyst stage (Fig. 2C). Furthermore, to verify that the staining of phospho-Jun was caused by the activation of JNK, we examined whether treatment with SP600125, a JNK inhibitor, abolished this staining. The result, shown in Fig. 2D, demonstrated that the SP600125 treatment inhibited almost completely the phosphorylation of Jun, indicating that JNK is activated during the four-cell to the blastocyst stages. These results taken together show that both p38 $\alpha$  and JNK are activated in mouse preimplantation development.

As SP600125 was recently reported not to be totally specific (Bain et al., 2003), we used the RNAi approach to inhibit the JNK pathway. We chemically synthesized four sets of complementary 21 nucleotide RNA oligonucleotides, each producing a 19 nucleotide RNA duplex. siJNK1-1 and siJNK1-2 are two RNA duplexes for mouse JNK1. siJNK2-1 and siJNK2-2 are for mouse JNK2. These RNA duplexes were injected into one-cell zygotes and the embryos were cultured

in vitro for three days. Embryos that were not injected or injected with distilled deionized water (DDW) developed normally (Fig. 2E). Likewise, siJNK1-1 and siJNK2-1 had no effect on preimplantation development. By contrast, embryos injected with siJNK1-2 and siJNK2-2 showed abnormality in cavity formation: apparent multiple cavities in an embryo instead of one cavity, a normal size cavity with abnormality in the inner-cell mass or a small cavity (Fig. 2E). These phenotypes are similar to those of SP600125-treated embryos. Importantly, the siJNK1-2 and siJNK2-2-injected embryos were not stained by anti-phospho-Jun (Ser 73) antibody, while uninjected or DDW-injected embryos were stained (data not shown). This suggests that the two siRNAs, siJNK1-2 and siJNK2-2, specifically inhibit the expression of JNK1 and JNK2. Taken together, these results clearly indicate that JNK plays an essential role in preimplantation development.

Phenotypes of mice carrying a single or doubly targeted deletion of the molecules comprising the MAPK cascades have previously been reported (Pearson et al., 2001). A defect in cavitation during preimplantation development, however, has not been observed in any of these mice. This may be due to redundancy of the function of multiple isoforms of the family members. Moreover, when deletion of one gene leads to embryonic lethality, preimplantation developmental processes may not be examined. Thus, possible roles of members of the MAPK family in mammalian preimplantation development have not been addressed. Our present results have revealed crucial involvement of two subfamily members of the MAPK family, p38 and JNK, in mouse preimplantation development. After completion of this work, a paper by Natale et al. (Natale et al., 2004) appeared, reporting requirement of p38 activity for mouse preimplantation development. Although there are subtle differences between our experiments and theirs, such as the period of the inhibitor treatment and the focused events in preimplantation development, the obtained conclusions are essentially the same.

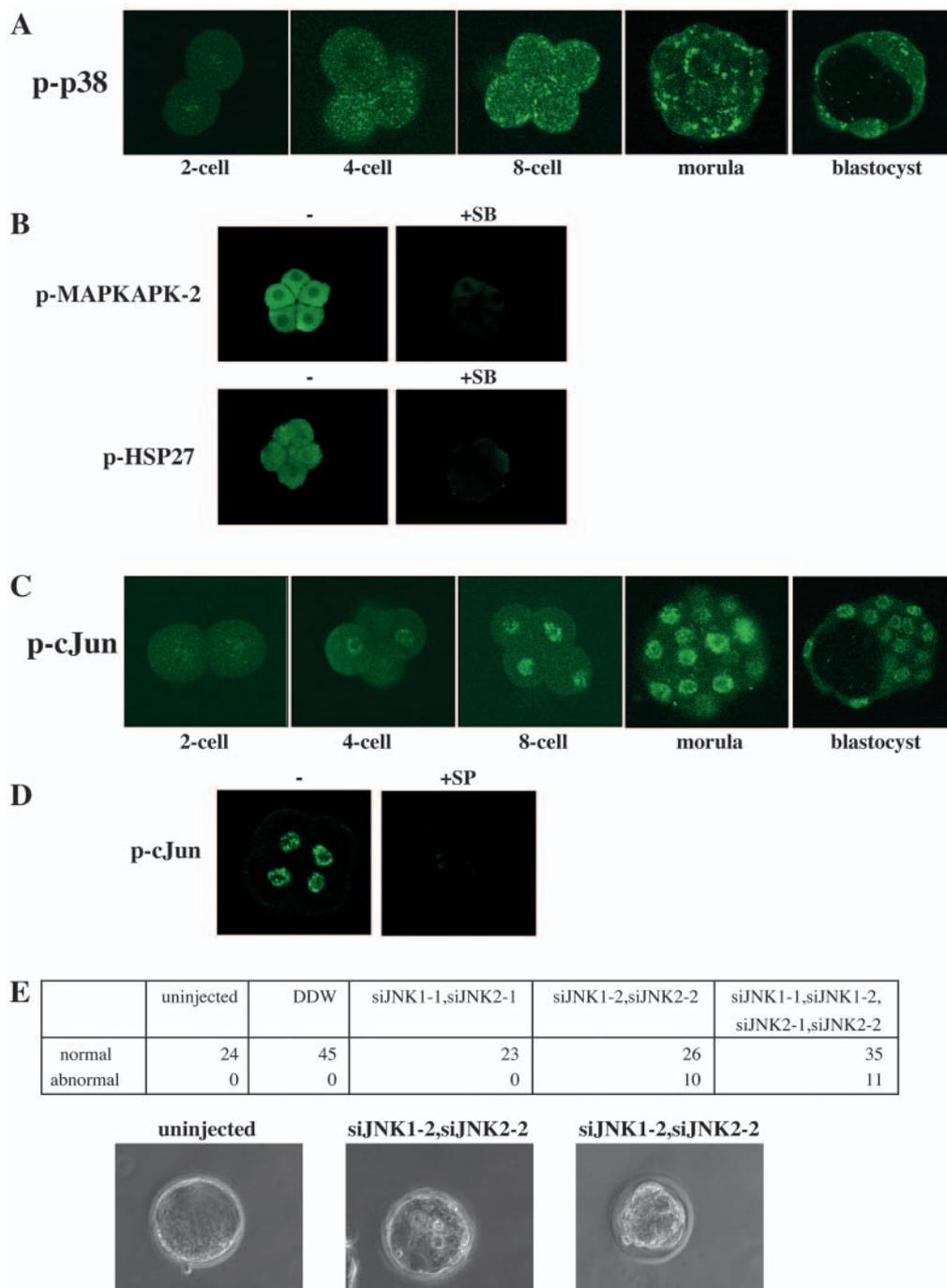
As the MAPK signaling pathways are known to regulate gene expression in the nucleus, we next asked whether gene transcription is required for preimplantation development. Previous experiments with  $\alpha$ -amanitin showed that gene expression is necessary for preimplantation development (Khidir et al., 1995; Kidder and McLachlin, 1985). To confirm this, we examined the influence of transcription inhibitor, actinomycin D, on preimplantation development. Treatment of eight-cell stage embryos with actinomycin D (0.05  $\mu$ g/ml or 0.5  $\mu$ g/ml) completely inhibited compaction, and embryos were unable to develop into morula (Fig. 3A, left). When we added actinomycin D to morula stage embryos, dose-dependent effects were observed (Fig. 3A, right). At 0.05  $\mu$ g/ml actinomycin D, the cavity formation was completely blocked in about 60% of embryos. In about 40% of embryos, the smaller cavity was formed. At 0.5  $\mu$ g/ml actinomycin D, the complete inhibition of cavitation was observed in about 85% of embryos, and the remaining embryos showed a much smaller cavity. These results, together with the previous studies (Khidir et al., 1995; Kidder and McLachlin, 1985), suggest that both compaction and cavity formation require de novo synthesis of mRNAs. It is likely that the p38 and JNK signaling pathways are involved in these processes during preimplantation development through regulation of gene expression.

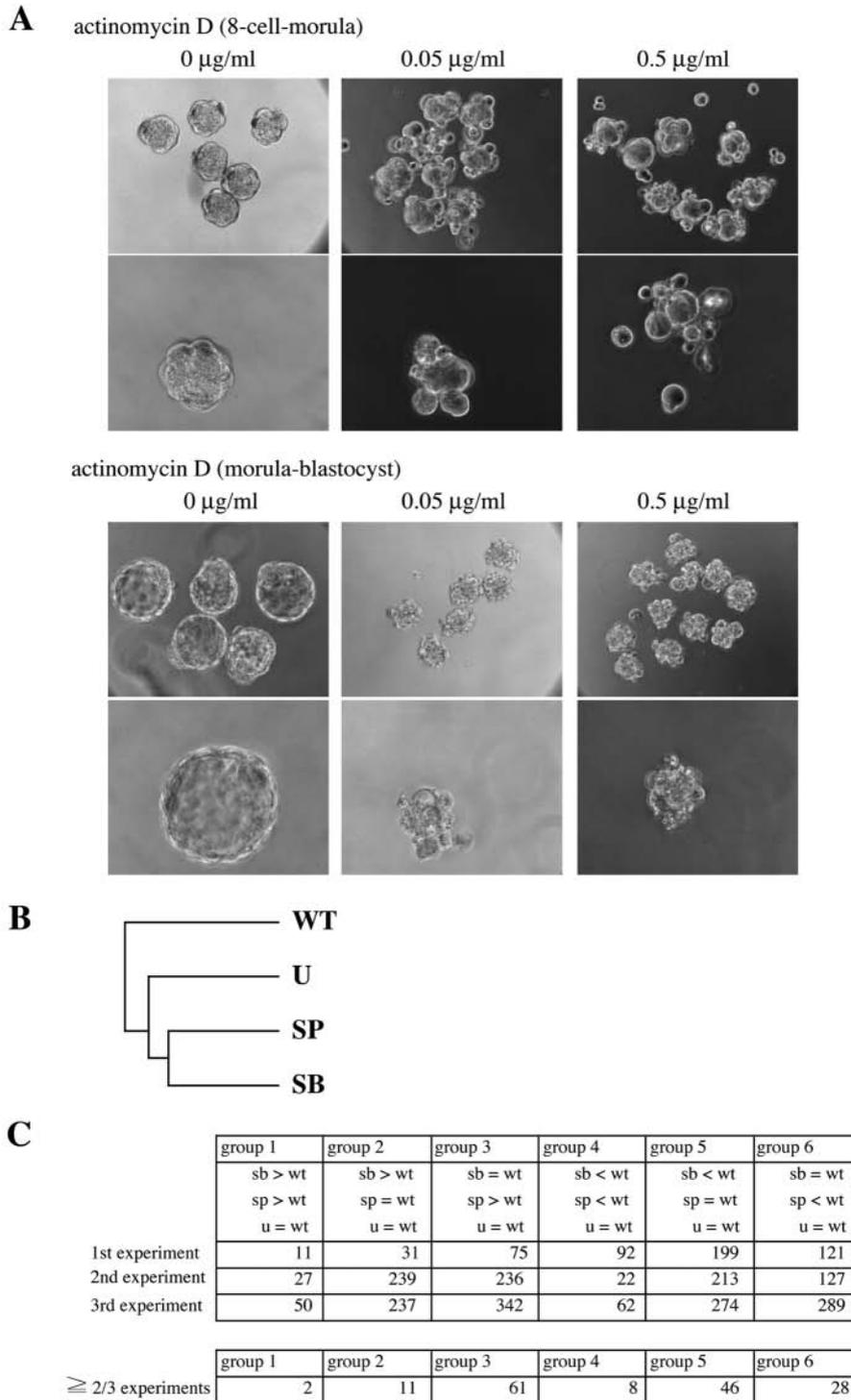
**Fig. 1.** SB203580 and SP600125 inhibit normal blastocoel formation. (A) Schedule of inhibitor treatment and analysis of preimplantation embryos. The program of developmental processes is shown at the top. Arrowheads indicate timing of compaction and that of cavitation. Bars in scheme 1, 2, 3 and 4 indicate the duration of the inhibitor treatment. At the end of the bars (indicated by arrowheads), embryos were observed and the typical images are shown in B. The zona pellucida was removed before the inhibitor was added to M16 medium. The inhibitor was added at the early eight-cell stage, and embryos were examined before the cavitation in scheme 1. In scheme 2, the inhibitor was added at the early eight-cell stage, and embryos were examined at the blastocyst stage. In scheme 3, the inhibitor was added at the pre-cavitation stage. In scheme 4, the inhibitor was added after initiation of cavitation. (B) Schematic representation of the phenotypes of embryos treated with each inhibitor. (C) Embryos treated with 20  $\mu$ M SB203580 (+SB) or 25  $\mu$ M SP600125 (+SP) were compared with control embryos (Cont.). 1, 2, 3 and 4 correspond to those in A. (D) Time-dependent observations of control, SB203580-treated or SP600125-treated embryos were performed. The inhibitor (SB or SP) was added at the eight-cell stage.

Based on the above observations, we examined expression profiles of genes that could be regulated by the MAPK pathways during preimplantation development. We used Affymetrix GeneChips for microarray analysis. Three independent experiments were carried out. For each experiment, we collected eighty embryos from four kinds of pools: wild-type embryos, SB203580-treated embryos, SP600125-treated embryos and U0126-treated embryos. Eight-cell stage embryos were treated with these drugs, and blastocyst stage embryos were collected for RNA extraction, labeling and hybridization. The used array comprised over 39,000 transcripts (45,000 probe sets) and about 60% (about

24,000 transcripts) were judged to be expressed in the wild-type sample by the GeneChip Operating Software 1.0 determination. In any of the SB203580-treated, SP600125-treated and U0126-treated samples, the percentages of transcripts that were judged to be expressed were about 60%. Rather surprisingly, the expression level of most of the transcripts was not changed by the drug treatment. Only ~1-4% of transcripts showed the sensitivity to the inhibition of MAPK pathways; the expression level of these genes was increased or decreased by either of SB203580, SP600125 or U0126 at least by twofold compared with baseline (wild type). Hierarchical clustering was performed with those transcripts

**Fig. 2.** p38 and JNK are active during mouse preimplantation development. (A) The activated form of p38 was detected by using anti-phospho-specific p38 $\alpha$  MAP kinase antibody. Embryos at the four-cell, eight-cell, morula or blastocyst stage were stained with anti-phospho-specific p38 $\alpha$  MAP kinase antibody. Fluorescence was viewed with a confocal microscope. (B) Phosphorylation of MAPKAPK-2 or HSP27 with or without SB203580. The eight-cell stage embryos were treated with SB203580 for 1.5 hours, and then the embryos were fixed and stained with anti-phospho-specific MAPKAPK-2 (Thr 334) antibody or anti-phospho-specific HSP27 (Ser 82) antibody. (C) Activation of JNK was assessed by using anti-phospho-specific Jun (Ser 73) antibody. Embryos at the four-cell, eight-cell, morula and blastocyst stage were stained with anti-phospho-specific Jun (Ser 73) antibody. Fluorescence was viewed with a confocal microscope. (D) Phosphorylation of Jun was examined with or without SP600125. SP600125 was added at the eight-cell stage. After 2 hours of incubation, the embryo was fixed and stained with anti-phospho-specific Jun (Ser 73) antibody. (E) Effect of JNK1/2-targeting siRNAs on mouse preimplantation development. One-cell stage embryos were not injected or injected with DDW or dsRNA oligonucleotides as indicated, and cultured for 3 days. The numbers of morphologically normal or abnormal embryos were counted. Bright-field microscopic photographs are shown.

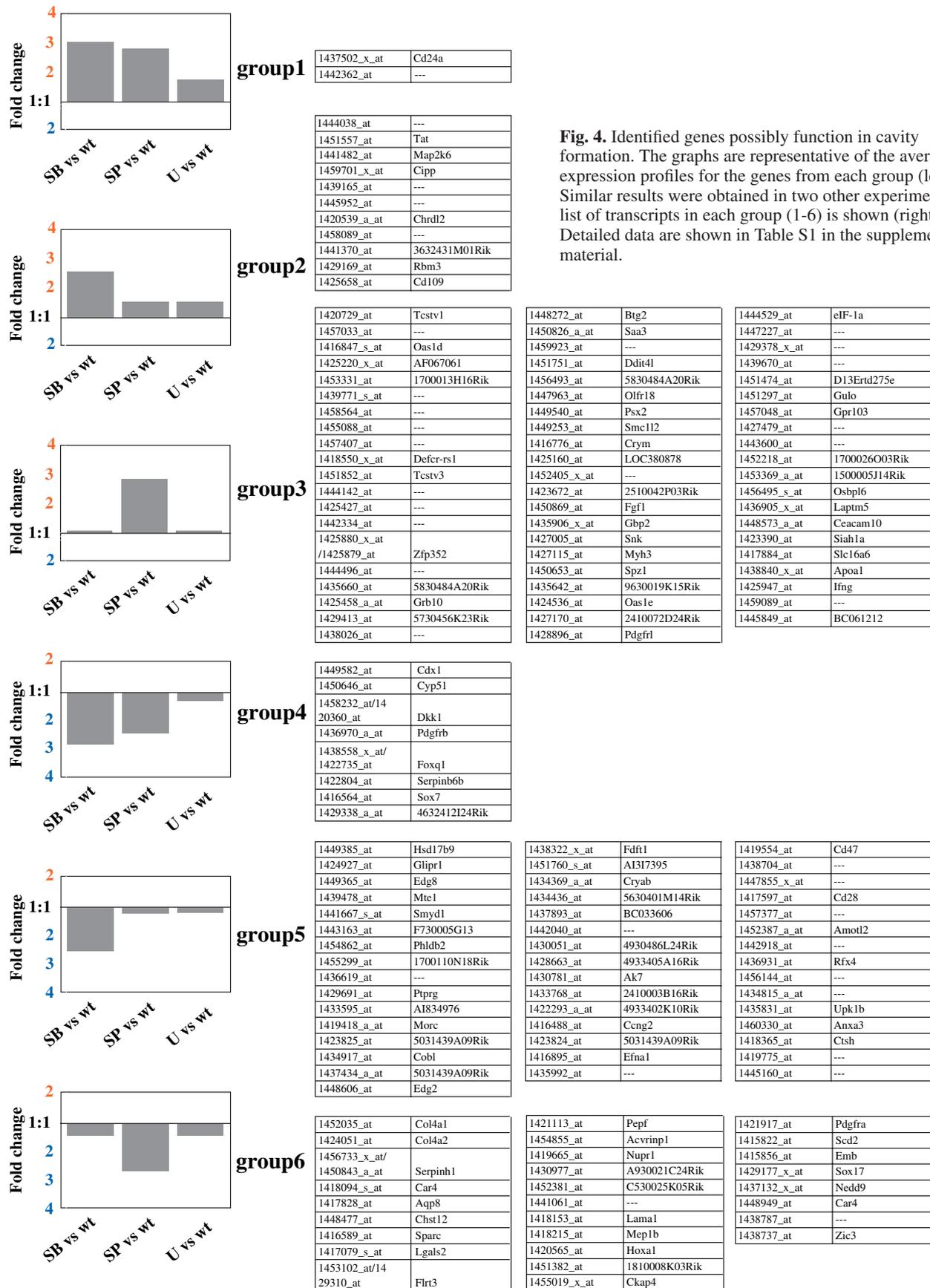




that were sensitive to these drugs by GeneSpring 6.1. The result shows that the effect of SB203580 is most similar to that of SP600125, and the effect of U0126 is not so similar to that of SB203580 or SP600125 (Fig. 3B), as was the case for the effect of these drugs on preimplantation development (see Fig. 1). Thus, we extracted 156 transcripts (161 probe sets) whose changes in mRNA levels were more than twofold in at least two out of the three independent experiments; 74 transcripts (groups 1, 2 and 3) showed an increased level of expression while the remaining 82 transcripts (groups 4, 5 and 6) showed a decreased level of expression by SB203580 or SP600125 treatment but not by U0126 treatment (Fig. 3C). We classified these 156 transcripts into six groups based on the sensitivity to the drugs (Fig. 3C, Fig. 4). In Fig. 4 (left), the average expression levels of genes in each group were shown. Expression levels of genes in group 1, 2, or 3 were increased by the inhibition of the p38 or the JNK pathway, but rather insensitive to the inhibition of the ERK pathway. By contrast, expression levels of genes in group 4, 5, or 6 were decreased by SB203580 or SP600125, but insensitive to U0126.

Group 1 includes two transcripts, whose expression levels are increased at least twofold when compared with baseline by either of the SB203580 and SP600125 treatments. Group 2 (11 transcripts) shows at least a twofold increase only by SB203580. The effect of SP600125 was minimal in this group. Group 3 comprises 61 transcripts (62 probe sets). Transcripts in this population are increased at least twofold by SP600125 treatment, but insensitive to SB203580 treatment. Group 4 is interesting, as expression of genes in this group is decreased by either SB203580 or SP600125 treatment but is insensitive to U0126. Thus, it is likely that the function of the genes in this group is required for successful preimplantation development. This group contains eight transcripts (10 probe sets). *Cdx1*, caudal type homeobox 1, has been described as an intestine-specific transcription factor. Because many homeobox family genes play key roles in determining cell fate, *Cdx1* may also have a role in preimplantation development. *Foxq1*, forkhead box Q1, is a member of the evolutionarily conserved winged helix (WH)/forkhead transcription factor gene

**Fig. 3.** Gene transcription is essential for preimplantation development. (A) Eight-cell stage embryos were treated with actinomycin D (0.05  $\mu\text{g/ml}$  or 0.5  $\mu\text{g/ml}$ ), and compared with control embryos (0  $\mu\text{g/ml}$ ) at morula stage (upper panel). In addition, morula stage embryos were treated with actinomycin D, and observed at blastocyst stage (lower panel). Low and high magnifications are shown. (B) Hierarchical clustering analysis. The transcripts used in this analysis were increased or decreased by SB203580, SP600125 or U0126 at least by twofold over baseline (wild type). (C) The transcripts were classified into six groups based on the sensitivity to the drugs as shown. The numbers of transcripts in each group in each experiment are shown (upper). The numbers of the transcripts that were classified into each group in at least two out of the three independent experiments are shown (lower).

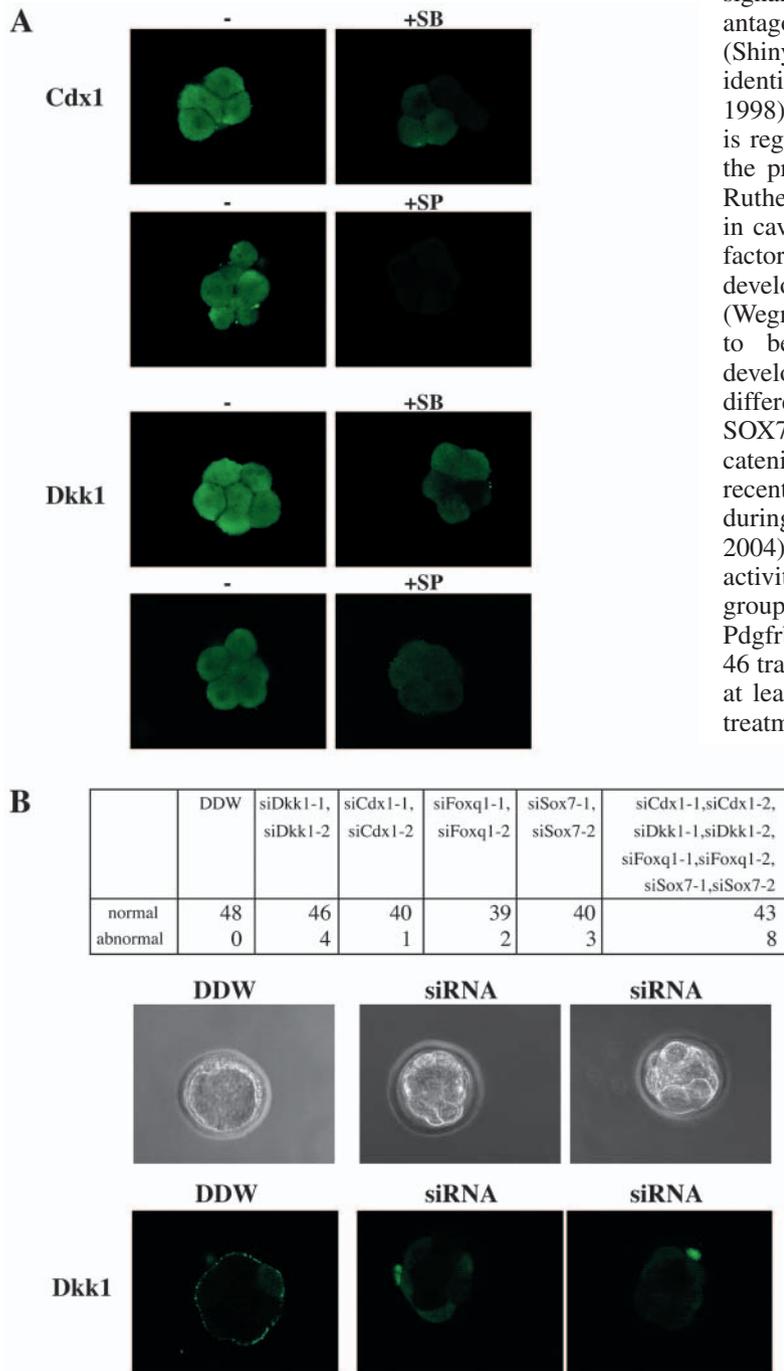


**Fig. 4.** Identified genes possibly function in cavity formation. The graphs are representative of the average expression profiles for the genes from each group (left). Similar results were obtained in two other experiments. A list of transcripts in each group (1-6) is shown (right). Detailed data are shown in Table S1 in the supplementary material.

family, and has been shown to have a unique and distinct function in differentiation and development of the hair shaft (Hong et al., 2001). Foxq1 is expressed throughout embryonic

development (Hong et al., 2001), but its role during embryogenesis remains unclear. Dkk1, Dickkopf-1, is a secreted protein that specifically inhibits Wnt/ $\beta$ -catenin signaling (Mao et al., 2001; Zorn, 2001), and Wnt-antagonizing function of Dkk1 is conserved in vertebrates (Shinya et al., 2000; Marvin et al., 2001). It was originally identified as a strong head inducer in *Xenopus* (Glinka et al., 1998). A recent report has shown that the expression of Dkk1 is regulated by Jun and that Dkk1 has a potential function in the process of vertebrate limb development (Grotewold and Ruther, 2002). It is possible that Dkk1 plays an important role in cavitation. Sox proteins, SRY box-containing transcription factors, are key players in the regulation of embryonic development and in the determination of different cell fates (Wegner, 1999; Soullier et al., 1999). Sox7 has been shown to be expressed rather ubiquitously during embryonic development, and has been suggested to function in several differentiation processes (Takash et al., 2001). In addition, SOX7 protein has the ability to significantly reduce Wnt/ $\beta$ -catenin-stimulated transcription (Takash et al., 2001). As the recent report has suggested that Wnt signaling may operate during mouse preimplantation development (Wang et al., 2004), it is notable that two genes known for Wnt-antagonizing activity, Dkk1 and Sox7, are identified as a member of the group 4 in our present study. Other genes in group 4 are Cyp51, Pdgfrb, Serpinb6b and one unknown gene. Group 5 contains 46 transcripts. Expression of transcripts in this group is halved at least by SB203580 treatment but insensitive to SP600125 treatment. Expression of transcripts in the last group (group 6), including 28 transcripts (30 probe sets), is halved at least specifically by SP600125 treatment. The most notable transcript in group 6 should be Hoxa1, homeobox A1, which is a transcription factor and known to function in patterning of the segments in the developing hindbrain. Because Hox genes, in general, play a role in the processes that control the AP identity of the embryo, Hoxa1 may have a role in preimplantation developmental processes.

Representative transcripts in each group (CD24a in group 1, Map2k6 in group 2, Testv1 in group 3, Cdx1 in group 4, Glipr1 in group 5 and Aqp8 in group 6) were examined for their sensitivity to each drug by RT-PCR to confirm the microarray data (data not shown). The obtained results completely conform to the classification (see Fig. 3C, Fig. 4). Recently, two reports performing microarray analysis revealed that there are two major phases of stage-specific gene activity, which precede blastocoel formation (Hamatani et al., 2004; Wang et al., 2004). In addition Hamatani et al. (Hamatani et al., 2004) have classified genes in terms of their expression pattern (clusters 1 to 9). Our rough analysis has suggested that there exists little or no specific correlation between their clustering and our grouping (1 to 6); for example, any of the genes in our group 4 could not be classified into any of nine clusters. Thus, at present we are unable to characterize stage-specific expression patterns of our 156 transcripts identified. Further studies should identify transcriptional regulation mechanisms of these genes. In this context, it has been shown that



**Fig. 5.** Functional analysis of several genes in group 4. (A) Eight-cell stage embryos were treated with or without SB203580 or SP600125 for 1 hour, and stained with anti-CDX1 antibody or anti-Dkk1 antibody. Fluorescence was viewed with a confocal microscope. (B) Effect of siRNA-based inhibition of genes in group 4. One-cell stage embryos were injected with dsRNA oligonucleotides as indicated, and cultured for 3 days. The numbers of morphologically normal or abnormal embryos were counted, and embryos were stained with anti-Dkk1 antibody. Bright-field (upper) and fluorescence (lower) micrographs of DDW-injected (left) and siCdx1/siDkk1/siFoxq1/siSox7-injected (middle and right) embryos are shown.

the expression of *Dkk1* is dependent on the Ap-1 family member Jun (Grotewold and Ruther, 2002). We were able to find ten possible Ap-1 sites in 1 kb upstream from the ORF of *Dkk1* (data not shown). Other members of group 4 also have 5-19 possible Ap-1 sites. Moreover, several members of group 4 may have other transcription factor binding sites, e.g. MEF and CRE. Because Ap-1, MEF and CRE are known to be regulated by the p38 and JNK pathways, it is possible that the expression of genes in group 4 could be regulated directly by the p38 and JNK pathways.

Last, we performed two types of experiments to assess the possible function of several genes in group 4. First, we carried out immunofluorescent examination for *Cdx1* and *Dkk1* in eight-cell embryos. Both proteins were clearly detected at this stage, and their expression level was decreased by SB203580 or SP600125 treatment (Fig. 5A). This result is consistent with the p38- and JNK-dependent increase of the mRNA level of both genes. Next, siRNA experiments were performed to examine the function of *Cdx1*, *Dkk1*, *Foxq1* and *Sox7*. siCdx1-1 and siCdx1-2 are two RNA duplexes for mouse *Cdx1*. siDkk1-1 and siDkk1-2 are for mouse *Dkk1*. siFoxq1-1 and siFoxq1-2 are for mouse *Foxq1*. siSox7-1 and siSox7-2 are for mouse *Sox7*. These RNA duplexes were injected into one-cell zygotes and 3 days later, the embryos were examined. Embryos injected with DDW developed normally and were stained by anti-*Dkk1* antibody (Fig. 5B). At this stage, *Dkk1* protein appeared to localize at or near plasma membrane. siRNA of individual genes had little effect on preimplantation development. However, when all these siRNAs for the four genes were injected together, about one fifth of the injected embryos showed abnormality in cavity formation (Fig. 5B). These results suggest that at least several of the group 4 genes in combination may play a role in preimplantation development.

In summary, our microarray analyses, based on the finding of differential involvement of various MAPK signaling pathways in preimplantation development, have identified a small number of genes, the function of which could be important for developmental processes. This is a crucial step for our understanding of the molecular mechanisms of preimplantation development in mammals. Detailed functional analysis of these genes will be the next challenge.

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### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/132/8/1773/DC1>

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