A JNK1/AP-1–Dependent, COX-2 Induction Is Implicated in 12-O-Tetradecanoylphorbol-13-Acetate–Induced Cell Transformation through Regulating Cell Cycle Progression

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Abstract
Cyclooxygenase-2 (COX-2) is reported to be one of the early-response gene products induced by 12-O-tetradecanoylphorbol-13-acetate (TPA). However, the relevance of COX-2 in TPA-induced cell transformation and the underlying mechanisms remains to be explored. Initially, we verified COX-2 induction after TPA treatment in mouse embryonic fibroblasts (MEF) and mouse epidermal cells Cl 41. More importantly, introduction of COX-2 small interfering RNA in MEFs or Cl 41 cells suppressed the cell transformation caused by TPA treatment. This inhibition could be reversed by overexpression of human full-length COX-2, indicating that COX-2 is at least one of the critical molecules involved in TPA-induced cell transformation. We further showed that TPA-promoted cell cycle progression was partially suppressed by COX-2 small interfering RNA, indicating that COX-2 also participated in TPA-associated cell cycle progression. Investigation of the upstream signaling pathways revealed that c-Jun-NH2-kinase 1 (JNK1), but not JNK2, played important roles in COX-2 induction, because knockout of JNK1 gene rather than JNK2 gene markedly impaired COX-2 induction. Furthermore, inhibition of c-Jun/activator protein 1 pathway or JNKs/c-Jun pathway by overexpression of dominant negative mutants of c-Jun, or MKK4 and MKK7 together, resulted in impairment of COX-2 induction, suggesting that JNK1/c-Jun/activator protein 1 pathway is involved in TPA-associated COX-2 induction. In contrast, IKK/p65 nuclear factor-κB pathway was not implicated because knockout of IKKα, IKKβ, or p65 gene did not affect COX-2 induction although nuclear factor-κB was activated by TPA. In addition, the TPA-promoted cell cycle progression was found impaired in JNK1-deficient, but not in JNK2-deficient, MEFs. Our results show that JNK1-associated COX-2 induction is implicated in TPA-associated cell transformation and cell cycle progression. (Mol Cancer Res 2008;6(1):165–74)

Introduction
A growing body of compelling evidences shows the close association of inflammation with carcinogenesis (1, 2). Cyclooxygenase-2 (COX-2), the inducible isoform of cyclooxygenases, is one of the major inflammatory mediators, and has also been found overexpressed in both premalignant and malignant tissues (3–7). COX-2 knockout mice have been reported to develop fewer skin papillomas than controls (8), and dysregulation of COX-2 expression is a crucial cause-and-effect in carcinogenesis (9). 12-O-tetradecanoylphorbol-13-acetate (TPA) is a well-known tumor promoter, widely used in two-stage skin carcinogenesis animal models in vivo and in cultured cell transformation assay (10-12). TPA has also been shown to induce COX-2 gene expression (13-15). Although accumulating evidence has suggested that COX-2 plays an important role in tumor development, its function in TPA-induced carcinogenesis seems conflicting. Bol et al. (16) found that the transgenic mice that overexpressed COX-2 under the control of the keratin 14 promoter were unexpectedly very resistant to TPA tumor promotion. This resistance phenotype seems to be restricted to TPA promotion because the same mice developed six times more tumors than wild-type mice when anthralin was used as the tumor promoter, and ~ 3.5 times more tumors when they were treated only with 7,12-dimethylbenz(α)anthracene (11), suggesting that the role of COX-2 in skin tumorigenesis is context dependent. To further clarify the complicated role of COX-2 in TPA response, we investigate the molecular mechanisms involved in COX-2 expression and its involvement in TPA-induced cell transformation in both mouse epidermal cells Cl41 and mouse embryonic fibroblasts (MEF).

Results
COX-2 Is Involved in TPA-Induced Cell Transformation
Previous studies have shown that COX-2 is involved in cell proliferation and growth in both normal and tumor cells (17). COX-2 knockout mice developed ~ 75% fewer chemically
induced skin papillomas than control mice (18, 19). However, in the tumor promotion potential of TPA, the role of COX-2 still remains unknown. To test this, the COX-2 induction by TPA was initially verified in MEFs and mouse epidermal cells CI 41. As expected, TPA treatment induced COX-2 protein expression in both MEFs (Fig. 1A) and CI 41 cells (Fig. 1B). The result of reverse transcription-PCR assay showed that TPA treatment also elevated the mRNA level of COX-2 (Fig. 1C), which resulted from the increased COX-2 transcription because TPA treatment enhanced COX-2 promoter-driven luciferase activity in both MEFs (Fig. 1D) and CI 41 cells (Fig. 1E). These data suggest that TPA induces COX-2 transcription and protein expression in both MEFs and CI 41 cells.

To determine the relevance of COX-2 in TPA-induced cell transformation, COX-2 small interfering RNA (siRNA) construct was transfected into MEFs and CI 41 cells. The stable transfectants of COX-2 siRNA was identified in MEFs (Fig. 2A) and CI 41 cells (20). The soft agar assay was then carried out to measure the ability of cells to grow in an anchorage-independent manner promoted by TPA treatment. As expected, both MEFs (Fig. 2B) and CI 41 cells (Fig. 2C), which were treated with TPA for 30 days, developed the transformed phenotype of colony formation in soft agars, which were suppressed by the introduction of COX-2 siRNA in MEFs (Fig. 2B) and CI 41 cells (Fig. 2C). To eliminate the concern over an off-target effect or colonial variation, human full-length COX-2 was reintroduced into the cells already transfected with COX-2 siRNA. Because COX-2 siRNA was designed specifically for mouse COX-2, the exogenous human COX-2 was not affected by this siRNA (Fig. 2C, left). Therefore, expression of human COX-2 in COX-2 siRNA-transfected cells restored the ability of the cell to grow in soft agar (Fig. 2C). Moreover, human full-length COX-2 was also transfected into CI 41 parental cells, and the stable transfectants were established and identified as in Fig. 2D. Overexpression of COX-2 alone resulted in the anchorage-independent cell growth in the absence of TPA; however, its colony number was relatively fewer than that induced by TPA in parental cells (Fig. 2D). Collectively, our results suggest that COX-2 is at least one of the key molecules implicated in TPA-induced cell transformation.

**COX-2 Regulated TPA-Induced Cell Cycle Progression**

Dysregulation of cell proliferation and cell cycle progression is associated with extracellular signaling leading to oncogenesis (21). TPA has been reported to promote cell proliferation and cell cycle progression in A549 cells (22). COX-2 is associated with cell proliferation and growth in some cancerous conditions (23), and the inhibitors of COX-2 are able to interfere cell cycle progression by regulating the cell cycle–related proteins, such as cyclins (24), which are critical for oncogenic transformation (21). Thus, our next investigation is to test the possibility that the augmented expression of COX-2 induced by TPA might participate in the regulation of cell cycle progression. Treatment of parental MEFs with TPA for 24 h resulted in an increase in the proportion of cells in S phase and G2-M phase compared with those of medium control cells; meanwhile, it led to a decrease in the proportion of cells in the quiescent phase (G0-G1 phase; Fig. 3A), suggesting that TPA treatment enabled the promotion of cell cycle progression. Importantly, knockdown of COX-2 by its siRNA suppressed the increased entry of the TPA-treated cells into the S phase and the G2-M phase (Fig. 3A). The similar results were observed in CI 41 cells (data not shown). Furthermore, we also used chemical inhibitor nocodazole to synchronize cells in M phase to determine the function of COX-2 in the regulation of cell cycle progression. As shown in Fig. 3B (left two panels), nocodazole treatment for 16 h resulted in >52% cells arresting in M phase. Twenty-four hours after nocodazole withdrawal, TPA treatment led to an increase in proportion of cells in S phase compared with that of medium control, whereas this effect was dramatically inhibited by expression of COX-2 siRNA (Fig. 3B, right two panels). Those results suggest that COX-2 induction plays a critical role in TPA-induced cell cycle progression.

**c-Jun-NH2-Kinase 1, but not c-Jun-NH2-Kinase 2, Is Required for the COX2 Induction in Response to TPA Treatment**

Previous studies have indicated that c-Jun-NH2-kinase (JNK) as well as extracellular signal-regulated kinases and p38 are respectively or cooperatively responsible for COX-2 induction under diverse stimuli and different cellular contexts (25-30). However, the differential roles of JNK1 and JNK2 in the regulation of COX-2 expression have not yet been investigated. To address this point, genetically disrupted MEFs for JNK1 gene (JNK1–/–) and for JNK2 gene (JNK2–/–) were used.
FIGURE 2. COX-2 induction was involved in TPA-induced cell transformation in MEFs and Cl 41 cells. COX-2 siRNA was transfected into WT MEFs (A and B) or Cl 41 cells (C). The stable transfectants were established and named as COX-2 siRNA. The COX-2 siRNA efficiency was identified by Western blotting for the basal COX-2 expression (cells in 10% FBS) or upon TPA treatment (A and C). Human COX-2 expression vector was transfected into Cl 41 parental cells (D) or Cl 41 COX-2 siRNA cells (C), and the stable transfectants were named as COX-2 and COX-2 siRNA(COX-2), respectively. Western blotting was carried out for identification of these transfectants (C and D). The cells as indicated were subjected to soft agar assay as described in Materials and Methods to compare their anchorage-independent growth capability. The pictures were taken and the numbers of colonies were counted 30 d later (B and D). *, P < 0.05, significant increase compared with medium control. †, P < 0.05, significant decrease compared with the siRNA control transfectants.
were used (Fig. 4A). TPA treatment for 30 min caused obvious activation of JNK1/2 as indicated by dual phosphorylations at Thr183/Tyr185 in wild-type (WT) MEFs (Fig. 4B). JNK1 and JNK2 deficiency impaired JNK1 and JNK2 phosphorylation and expression, respectively, whereas activation of the extracellular signal-regulated kinases and p38 pathways was not affected in JNK1/2–deficient MEFs (Fig. 4B). Treatment of WT MEFs and JNK2–/– MEFs with TPA for 12 and 24 h led to a marked increase in COX-2 protein level, whereas TPA-induced COX-2 expression was impaired in JNK1–/– MEFs (Fig. 4C). To verify that in JNK1–/– MEFs, the lack of COX-2 induction in response to TPA stimulus is specifically due to the deficiency of JNK1 protein, the HA-JNK1/pcDNA3 plasmid was transfected into JNK1–/– MEFs and the JNK1/C0 (HA-JNK1) stable transfectants were established (Fig. 4D). Reconstitutive expression of JNK1 entirely rescued the COX-2 induction (Fig. 4E), attesting that in WT and JNK2–/– cells the lack of COX-2 induction by TPA was strictly due to the absence of JNK1 activity. All of these data suggest that JNK1, but not JNK2, contributes to COX-2 induction in response to TPA exposure. We also noticed that not only the inducible but also the basal COX-2 protein expression level was lower in JNK1–/– cells than that in WT and JNK2–/– cells (Fig. 4C). To unravel whether the low basal COX-2 expression in JNK1–/– cells is due to more protein degradation or less gene transcription, we used proteasome inhibitor, MG132, to see whether blocking protein degradation can rescue the COX-2 protein expression in JNK1–/– cells. As shown in Fig. 4F, MG132 treatment obviously increased COX-2 protein expression in WT and JNK2–/– cells, indicating the quick COX-2 protein degradation in these quiescent cells. However, MG132 only modestly affected COX-2 protein expression in JNK1–/– cells. Therefore, it is suggested that the low protein expression level of COX-2 in JNK1–/– cells is mainly due to the inefficiency of gene transcription or mRNA instability rather than affecting preexisting protein stability.

The c-Jun/Activator Protein 1 Pathway, but not the IKK/p65 Nuclear Factor-κB Pathway, Was Essential for TPA-Induced COX-2 Expression

COX-2 induction was reported to be regulated by transcription factors such as nuclear factor-κB (NF-κB), activator protein 1 (AP-1), NF-IL6, NFAT, and PEA3, either independently or in combination (31-34). The NF-κB binding sequences have now been identified in over 400 different genes, including inflammatory enzymes, such as COX-2 (34). Thus, we examined whether NF-κB is a JNK1 downstream transcription factor responsible for COX-2 induction by TPA. As shown in Fig. 5A, TPA treatment resulted in an increase in NF-κB–dependent transcriptional activity in both WT and JNK2–/– cells, but not in JNK1–/– cells, which was similar to the COX-2 protein induction pattern in these cell lines (Fig. 4C). We, therefore,

FIGURE 3. COX-2 regulated TPA-promoted cell cycle progression. A. MEFs and the stable transfectants with COX-2 siRNA were seeded into each well of six-well plates and cultured in 10% FBS-DMEM at 37°C. After the cell density reached 80% to 90%, the cells were treated with TPA for 24 h and collected for flow cytometry assay. B. CI 41 cells and the COX-2 siRNA transfectants were pretreated with or without 0.5 μg/mL nocodazole (Noc) for 16 h. After nocodazole withdrawal, the cells were exposed to TPA or left untreated for another 24 h. The cell cycle was analyzed by flow cytometry.
as well as c-Jun activation could also affect COX-2 induction (DN-MKK4) and dominant negative MKK7 (DN-MKK7) confirm this notion, we transfected dominant negative MKK4 necessary for TPA-induced COX-2 expression. To further show that c-Jun/AP-1 is the downstream regulator of JNK1 expression upon TPA treatment (Figs. 6D and E). These results dramatically reduced COX-2 promoter activity and protein transcriptional activity (Fig. 6C). Accordingly, expression of TAM67 was cotransfected with AP-1 luciferase reporter or COX-2 in MEFs, dominant negative mutant of c-Jun (TAM67; ref. 36) was observed in both WT and JNK2−/− MEFs, whereas it was blocked in JNK1−/− cells (Fig. 6A). To further evaluate whether AP-1 was required for TPA-induced COX-2 expression in MEFs, dominant negative mutant of c-Jun (TAM67; ref. 36) was cotransfected with AP-1 luciferase reporter or COX-2 luciferase reporter into WT MEFs. Overexpression of TAM67 significantly reduced the basal and inducible levels of c-Jun expression (Fig. 6B), as well as AP-1-dependent transcriptional activity (Fig. 6C). Accordingly, expression of TAM67 dramatically reduced COX-2 promoter activity and protein expression upon TPA treatment (Figs. 6D and E). These results show that c-Jun/AP-1 is the downstream regulator of JNK1 necessary for TPA-induced COX-2 expression. To further confirm this notion, we transfected dominant negative MKK4 (DN-MKK4) and dominant negative MKK7 (DN-MKK7) together into WT MEFs to see whether blocking JNK activity as well as c-Jun activation could also affect COX-2 induction (Fig. 6F). As expected, blockade of MKK4 and MKK7 activities significantly decreased JNK/c-Jun activation, which subsequently resulted in the reduction of COX-2 induction by TPA (Fig. 6G). Our data therefore show that the JNK1/c-Jun/AP-1 pathway is critical for TPA-induced COX-2 expression.

**JNK1, but not JNK2, Was Essential for TPA-Induced Cell Cycle Progression**

JNKs participate in the regulation of various aspects of mammalian physiology, including cell proliferation (37). Thus, it is of interest to investigate whether JNKs are also involved in the regulation of the cell cycle progression caused by TPA stimulation. As shown in Fig. 7A and B, TPA-promoted cell cycle progression was observed in WT and JNK2−/− cells in the same pattern as described above (Fig. 3A). However, in JNK1−/− cells, TPA treatment did not lead to obvious enhancement of DNA synthesis, and it actually reduced the proportion of the cells in S phase from 6.04% to 2.59%. In addition, there were no obvious alternations in the G0-G1 phase (82.12% versus 80.49%) and G2-M phase (13.69% versus 11.8%) in TPA-treated JNK1−/− MEFs, suggesting that JNK1 deficiency also blocked TPA-promoted cell cycle progression.

**Discussion**

Our current studies for the first time show that in MEF and Cl 41 cells the induction of COX-2 is implicated in the cellular transformation potential of TPA via regulating cell cycle progression. Moreover, the activation of the JNK1/c-Jun/AP-1 signaling pathway, but not the JNK2 or IKK/p65 NF-κB pathway, is necessary for TPA-induced COX-2 expression. Together, our studies indicate that the cascade activation of the
JNK/c-Jun/COX-2 module is at least one of the major events involved in TPA-induced cell cycle progression as well as cell transformation.

COX-2 is one of the key enzymes required for prostaglandin synthesis, converting the arachidonic acid released by phospholipase A2 into prostaglandin, which participate in cell growth and proliferation (28). Although the implications of COX-2 in cell proliferation, cell transformation, and carcinogenesis have been evident in most of the previous studies (4, 20), there is still some evidence showing that COX is not involved in biological effects in some systems (38, 39). For example, Lu et al. (38) has reported that overexpressing human cDNA of COX-2 in human breast epithelial cells inhibited cell proliferation by delaying cell cycle progression through the G1 phase. Our recent findings show that reducing epidermal growth factor–induced cell cycle progression of COX-2 siRNA reduces the transformation potential of TPA. Mechanism studies further reveal that the expression of COX-2 siRNA reduces the increased proportion of cells entering the DNA synthesis phase, keeping more cells in the quiescent phase, demonstrating that COX-2 is implicated in TPA-induced cell cycle progression. We anticipate that implication of COX-2 in TPA-induced cell transformation may be associated with its regulation of cell cycle progression.

Three genes (JNK1, JNK2, and JNK3) with distinct expression patterns encode the JNK protein kinases (40). In contrast to JNK1 and JNK2, which are ubiquitously expressed, JNK3 is largely restricted to the brain and is involved in the neuron diseases, such as Parkinson’s disease (41). In some cases, JNK2 seems to participate in the pathology of the brain too. Stéphane Hunot and colleagues show that both JNK2 and JNK3, but not JNK1, are required for 1-methyl-4-phenyl-1,2,4,6-tetrahydropyridine–induced c-Jun activation, which leads to the occurrence of Parkinson’s disease in mouse model. Furthermore, they have identified COX-2 as a molecular target of JNK activation and showed that COX-2 is dispensable for 1-methyl-4-phenyl-1,2,4,6-tetrahydropyridine–induced Parkinson’s disease (41). In the present study, we provide definite evidence for a central role of JNK1 in induction of COX-2 expression in response to TPA exposure, whereas JNK2 plays no relevant role in the COX-2 induction. Those data further widen our understanding of the roles of different JNK isoforms in COX-2 expression (42). We also identify that the c-Jun/AP-1 pathway, but not the NF-κB pathway, is critical for COX-2 induction by TPA because impairment of IKKα, IKKβ, or p65 gene expression only marginally affects COX-2 induction, whereas overexpression of TAM67 or DN-MKK4 and DN-MKK7 obviously reduces COX-2 induction. In addition, our results show that the JNK1 pathway plays a critical role in cell cycle progression induced by TPA, because deficiency of JNK1 expression results in an obvious blockage of cell cycle progression, especially S-phase cell accumulation, which is consistent with the effect of introduction of COX-2 siRNA.

It has been reported that COX-2 transcription could be activated in cell response to tumor promoters, growth factors, oncogenes, and cytokines via PKC and Ras (43). In the present study, we further exploit the JNK1 downstream pathway leading to the transactivation of COX-2. NF-κB pathway of JNK1 downstream pathway, but not the NF-κB pathway, is critical for COX-2 induction by TPA because impairment of IKKα, IKKβ, or p65 gene expression only marginally affects COX-2 induction, whereas overexpression of TAM67 or DN-MKK4 and DN-MKK7 obviously reduces COX-2 induction. In addition, our results show that the JNK1 pathway plays a critical role in cell cycle progression induced by TPA, because deficiency of JNK1 expression results in an obvious blockage of cell cycle progression, especially S-phase cell accumulation, which is consistent with the effect of introduction of COX-2 siRNA.

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methods has no obvious inhibitory effect on TPA-associated COX-2 expression. Therefore, we conclude that NF-κB is not involved in the COX-2 induction by TPA although it is activated by TPA. In rat smooth muscle cells, the activation of NF-κB also seems not related to the expression of COX-2 induced by tumor necrosis factor-α because calpain inhibitor I blocks the activation of NF-κB, but not the expression of COX-2 mRNA or protein (46). Wadleigh et al. (47) has reported the similar phenomena that inhibiting endotoxin-induced NF-κB activation by expression of an inhibitor-κB α mutant does not block endotoxin-dependent COX-2 reporter activity. Our results supports the notion that the role of NF-κB in COX-2 induction may depend on cell type and stimulus (47, 48).

c-Jun is a major component of AP-1 complex in many cells. In most cells, c-Jun is a moderately labile protein (with a half-life of ~2 h), which is subject to polyubiquitination on multiple lysine residues and degraded via the 26S proteasome. JNKs mediate phosphorylation of Ser63/73 in c-Jun and are shown to inhibit both ubiquitination and degradation of c-Jun, leading to the accumulation of protein and increased transcriptional activity. The promoter region of c-Jun contains the TRE binding domain, so c-Jun regulates self-transcription (49). Therefore, ablation of c-Jun transactivation domain in TAM67 interfered with c-Jun protein function and decreased c-Jun transcriptional activity, which subsequently led to the reduction of both basal and inducible c-Jun protein expression as well as AP-1 transcriptional activity in TAM67-overexpressed cells. Meanwhile, TAM67 also dramatically reduced COX-2 protein induction, suggesting the essential role of c-Jun/AP-1 in COX-2 induction by TPA. Because the AP-1 transactivation by TPA was blocked in JNK1−/−/JNK2−/− cells compared with the inductions in WT and JNK2−/−/JNK2−/− cells, our results suggested that the c-Jun/AP-1 pathway was the downstream mediator of JNK1 for COX-2 induction. To further confirm this hypothesis, we overexpressed dominant negative MKK4 and MKK7 in WT cells, and find that it blocks the cascade of JNK/c-Jun/COX-2, demonstrating that the JNK1/c-Jun/AP-1 pathway is required for TPA-induced COX-2 expression.

In summary, our present study shows that TPA induces COX-2 expression through a JNK1/c-Jun/AP-1–dependent,
and JNK2- and NF-κB–independent, pathway. JNK1-implicated COX-2 induction is at least one of the essential events in TPA-associated cell cycle progression and cell transformation. These findings provide a new insight into the molecular mechanisms of tumor promotion effects of TPA.

**Materials and Methods**

**Cell Culture**

MEFs, including WT, JNK1−/− (50), JNK2−/− (51), IKKα−/− (52), IKKβ−/− (53), and p65−/− (54), as well as their stable transfectants, were maintained at 37°C in 5% CO2 incubator with DMEM supplemented with 10% fetal bovine serum (FBS), 2 mmol/L l-glutamine, and 25 μg/mL of gentamicin. Mouse epidermal cells Cl41 and their stable transfectants were cultured in 5% FBS MEM. The cultures were dissociated with trypsin and transferred to new 75-cm² culture flasks (Fisher) twice weekly. FBS was purchased from Nova-Tech, and the other cell culture reagents were obtained from Sigma.

**Plasmids and Construction of siRNA Vector**

The AP-1– and NF-κB-luciferase reporter plasmids were purchased from Stratagene. The COX-2–luciferase reporter plasmid containing the upstream 5’-flanking region of the COX-2 gene promoter linked to the luciferase reporter was described in our previous studies (20). A pcDNA3.1 plasmid containing c-Jun dominant negative mutant (pcDNA3.1/TAM67) was kindly provided by Drs. Tim G. Bowden (College of Pharmacy, University of Arizona, Tucson, AZ) and Matthew Young (Center for Cancer Research, National Cancer Institute, Frederick, MD). HA-tagged murine full-length JNK1 cDNAs was subcloned into pcDNA3 expression vector, confirmed by DNA sequencing and then named as HA-JNK1.pcDNA3. Dominant negative mutant of MKK4 (DN-MKK4) and of MKK7 (DN-MKK7) were generous gifts from Dr. Han-Ming Shen (Department of Community, Occupational and Family Medicine, Faculty of Medicine, National University of Singapore, Singapore, Singapore). Human COX-2 expression vector was a gift from Dr. Kotha Subbaramaiah (Weill Medical College of Cornell University, New York, NY). The COX-2 siRNA expression plasmid was made by using GeneSuppressor System (Imgenex Co.). The siRNA target sequence for COX-2 was 5’-AGACAGATCATAAGCGAGGA-3’ (siRNA: bases 686-705 of BC052900, mouse COX-2 mRNA; ref. 20). Construct containing the reversed target sequences was used as negative siRNA control.

**Generation of Stable Transfectants**

Transfection experiments were done with Lipofectamine 2000 (Invitrogen, Inc.) according to instructions from the manufacturer. For the transfection of AP-1–, NF-κB–, COX-2–luciferase reporter plasmids in Cl41 cells or WT MEFs, 5 μg of these plasmids were cotransfected with 0.4 μg of pcDNA3, and the stable transfectants were generated by G418 selection. For

**FIGURE 7.** JNK1, but not JNK2, was critical for TPA-induced cell cycle progression. A. WT, JNK1−/−, and JNK2−/− MEFs were seeded into each well of six-well plates and cultured in 10% FBS-DMEM at 37°C. After the cell density reached 80% to 90%, the cells were treated with TPA for 24 h and collected for flow cytometry assay. B. The graphic presentation of cell cycle progression.
the transfection of COX-2 siRNA alone or combined with human COX-2 expression construct in CI 41 cells and WT MEFs, or transfection of pcDNA3.1/His-TAM67 in WT MEFs, 5 µg of plasmid DNA were used and the stable transfectants were generated by G418 selection. For transfection of AP-1–, NF-κB–, COX-2–luciferase reporter plasmids in JNK1–/– or JNK2–/– cells, and for transfection of HA-JNK1/pcDNA3 in JNK1–/– cells, 5 µg of these plasmids were cotransfected with 0.4 µg of the hygromycin-resistant plasmid, and the stable transfectants were generated by hygromycin selection.

Gene Reporter Assay

Confluent monolayer of AP-1–, NF-κB–, or COX-2 luciferase reporter stable transfectants were trypsinized, and 8 × 10⁴ viable cells suspended in 100 µL of medium were added to each well of 96-well plates. After the cell density reached 80% to 90% confluence, the cells were treated with 20 ng/mL of TPA for times as indicated in the figure legends. The cells were then lysed with 50 µL lysis buffer, and the luciferase activity was measured using Promega Luciferase assay reagent with a luminometer (Wallac 1420 Victor2). The Student’s t test was used to determine the significance of differences of COX-2 induction and AP-1 or NF-κB activation relative to the medium control (relative AP-1 or NF-κB activation) or COX-2 induction relative to the medium control (relative COX-2 induction; refs. 55, 56).

Anchorage-Independent Colony Assays

Soft agar colony formation assays were done as described previously (12). Briefly, 2.5 mL of 0.5% agar in BMEM supplemented with 10% FBS with or without 20 ng/mL of TPA were layered onto each well of six-well plates. One milliliter of MEFs or CI 41 cells or its stable transfectants with COX-2 siRNA construct alone or combined with human COX-2 expression vector (1 × 10⁴) was mixed with 2 mL of 0.5% agar in BMEM supplemented with 10% FBS with or without 20 ng/mL TPA and layered on top of the 0.5% agar layer. Plates were incubated at 37°C in 5% CO₂ for 30 days, and the number of colonies was scored as described previously (12).

Flow Cytometry

To analyze cell cycle distribution with propidium iodide staining, WT MEFs, COX-2 siRNA transfectants, and JNK1–/– or JNK2–/– MEFs were plated in six-well plates with the density of 2 × 10⁵ per cell and cultured in normal 10% serum medium until 70% to 80% confluence. After being treated as described in figure legends, the cells were collected by centrifugation, fixed in ice-cold 75% ethanol at −20°C overnight. The fixed cells were stained in the buffer containing 100 mM/L sodium citrate, 0.1% Triton X-100, 0.2 mg/mL RNase A, and 50 µg/mL propidium iodide at 4°C for 1 h, and then analyzed by the Epics XL fluorescence-activated cell sorting (Beckman Coulter) as described in our previous publication (57).

Reverse Transcription PCR

Three or 6 h postexposure to TPA, total RNA was extracted from the cells using Trizol reagent (Invitrogen). Total cDNAs were synthesized by ThermoScript reverse transcription-PCR system (Invitrogen). COX-2 mRNA amount presented in the cells was measured by semiquantitative reverse transcription-PCR using a pair of oligonucleotides (5'-TCTCCTGGGA-CATGGACTC-3' and 5'-GCTCGGCTTCCAGATTGGAG-3'). The control mouse β-actin mRNA was also detected by reverse transcription-PCR using the primers 5'-GACGATGATATTGCGCGACT-3' and 5'-GATAACAGCCTTTGCTTGAG-3'. The PCR products were separated on 2% agarose gels and stained with ethidium bromide; the images were then scanned from a UV light.

Western Blotting

Cells were plated in six-well plates and cultured in 10% FBS-DMEM until 70% to 80% confluence. After exposure to TPA for various times as indicated in the figure legends, the cells were washed once with ice-cold PBS and collected with SDS-sample buffer (58). The cell extracts were sonicated, denatured by heating at 100°C for 5 min, and quantified with a detergent-compatible protein assay kit (Bio-Rad). Equal aliquots of cell extracts were separated on SDS-polyacrylamide gels. The proteins were then transferred to polyvinylidene difluoride membranes (Bio-Rad), blocked, and probed with one of the antibodies against COX-2 antiserum (Cayman Chemical Co.), phosphospecific c-Jun (Ser⁷³), JNKs (Thr¹⁸³/Tyr¹⁸⁵), p38 (Thr¹⁸⁰/Tyr¹⁸²), and extracellular signal-regulated kinases (Thr²⁰²/Tyr²⁰⁴), or nonphosphorylated c-Jun, JNKs, p38, extracellular signal-regulated kinases, HA, IkKβ, Cell Signaling Technology), p65 (Santa Cruz Biotechnology), or β-actin (Sigma). Primary antibody-bound proteins were detected by using an IgG alkaline phosphatase–linked secondary antibody and an ECF Western blotting system (Amersham; ref. 59).

Statistical Analysis

The Student’s t test was used to determine the significance of differences of COX-2 induction and AP-1 or NF-κB activation between TPA-treated cells and medium control or among various cell lines. The differences were considered significant at P < 0.05.

References

10. Huang C, Ma W-Y, Dawson MI, Riscon M, Flavell RA, Dong Z. Blocking activator protein-1 activity, but not activating retinoic acid response element, is


Correction: Article on the Role of COX-2 in TPA-Induced Cell Transformation

In the article by Zhang and colleagues on the role of COX-2 in TPA-induced cell transformation in the January 2008 issue of Molecular Cancer Research, Chuanshu Huang’s affiliation was incorrect. It is Nelson Institute of Environmental Medicine, New York University School of Medicine, New York, NY.

Errors were also found in the β-actin bands of Figures 4E and 6F and the cropped bands of COX-2 and β-actin in Figure 2D. The corrected figures appear here:

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**FIGURE 2D.** C141 cells were transfected with human COX-2 expression vector or exposed to TPA (20 ng/ml) for 12 hrs as indicated. COX-2 protein expression was evaluated by Western blotting using specific antibodies against COX-2. β-Actin was used as protein loading control.

**FIGURE 4E.** WT, JNK1−/− and JNK1−/−(HA-JNK1) MEFs were treated with TPA for 6 hrs, and the cell extracts were subjected to Western blotting using specific antibody against COX-2. β-Actin was used as protein loading control. Lanes irrelevant to the figure have been cropped as indicated.

**FIGURE 6F.** WT and DN-MKK4+7 cells were treated with TPA for 60 min. The extracts were subjected to Western blotting with specific antibodies as indicated. β-Actin was used as protein loading control. Lanes irrelevant to the figure have been cropped as indicated.

Molecular Cancer Research

A JNK1/AP-1–Dependent, COX-2 Induction Is Implicated in 12- O-Tetradecanoylphorbol-13-Acetate–Induced Cell Transformation through Regulating Cell Cycle Progression

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