ASK1 and ASK2 differentially regulate the counteracting roles of apoptosis and inflammation in tumorigenesis

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Apoptosis and inflammation generally exert opposite effects on tumorigenesis: apoptosis serves as a barrier to tumour initiation, whereas inflammation promotes tumorigenesis. Although both events are induced by various common stressors, relatively little is known about the stress-induced signalling pathways regulating these events in tumorigenesis. Here, we show that stress-activated MAP3Ks, ASK1 and ASK2, which are involved in cellular responses to various stressors such as reactive oxygen species, differentially regulate the initiation and promotion of tumorigenesis. ASK2 in cooperation with ASK1 functioned as a tumour suppressor by exerting proapoptotic activity in epithelial cells, which was consistent with the reduction in ASK2 expression in human cancer cells and tissues. In contrast, ASK1-dependent cytokine production in inflammatory cells promoted tumorigenesis. Our findings suggest that ASK1 and ASK2 are critically involved in tumorigenesis by differentially regulating apoptosis and inflammation.

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Introduction

It has been widely recognised that a variety of physicochemical and biological stressors initiates and promotes tumorigenesis (Evan and Vousden, 2001; Murakami *et al*, 2007). Multiple stress-activated signalling systems induce apoptosis in damaged cells, and thereby function as critical defense systems against tumour initiation. On the other hand, such systems are often commonly used as signalling intermediates for inflammation that plays beneficial roles in wound healing and infection. Ironically, however, accumulating evidence has suggested that inflammation, particularly its chronic form, can also be a critical component for tumour promotion (Coussens and Werb, 2002). Therefore, pathophysiological roles of stress-activated signalling systems that regulate apoptosis and inflammation in tumorigenesis have received much attention as therapeutic targets for cancer.

Among the various stress-activated signalling systems, stress-activated mitogen-activated protein (MAP) kinase cascades that converge on c-Jun N-terminal kinases (JNK) and p38 MAP kinases have been characterised as regulators of cellular functions, including apoptosis and inflammation, in response to a wide variety of environmental and biological stressors (Kyriakis and Avruch, 2001). Critical roles of JNK and p38 in tumorigenesis have been revealed recently in mouse models such as those of liver and lung tumorigenesis in p38α-deficient mice (Hui *et al*, 2007; Ventura *et al*, 2007) and skin tumorigenesis in mice deficient in JNK2 or p38-regulated/activated protein kinase (Chen *et al*, 2001; Sun *et al*, 2007). Nevertheless, it has not been fully understood whether and how JNK and p38 regulate apoptosis and inflammation in tumorigenesis.

It has been proposed for decades that oxidative stress triggered by accumulation of reactive oxygen species (ROS) is implicated in tumour initiation and promotion (Benhar *et al*, 2002). ROS production is increased in many types of cancer cells, and elevated ROS levels appear to induce cellular proliferation through constitutive activation of transcription factors such as NF-κB and AP-1 and even induce DNA damage that causes genomic instability, accelerating tumour

progression (Hsu *et al*, 2000; Marnett, 2000). In these ROS-medicated oncogenic responses, JNK and p38 have been shown to play multiple roles (Benhar *et al*, 2002; Han and Sun, 2007). Recently, it has been shown that p38α specifically prevents ROS-dependent transforming activity of oncogenic Ras, which not only shows the tumour suppressive role of p38 but also provides indirect evidence of the relevance of ROS in tumorigenesis (Dolado *et al*, 2007). Therefore, ROS-mediated JNK and p38 activation appears to be a key mechanism in stress-associated tumorigenesis.

Activities of JNK and p38 are tightly regulated by their upstream MAP kinase kinases and MAP kinase kinase kinases (MAP3Ks). Among a growing number of MAP3Ks, apoptosis signal-regulating kinase 1 (ASK1) has been widely accepted as a major player in the regulation of ROS-induced JNK and p38 activation (Ichijo *et al*, 1997; Sumbayev and Yasinska, 2005; Takeda *et al*, 2008). ASK1 is preferentially activated by ROS and is required for ROS-induced JNK and p38 activation as well as apoptosis (Tobiume *et al*, 2001). ROS-induced activation of ASK1 is tightly regulated by a redox protein thioredoxin (Trx) and TNF-receptor-associated factors TRAF2 and TRAF6 (Saitoh *et al*, 1998; Tobiume *et al*, 2002; Noguchi *et al*, 2005; Fujino *et al*, 2007). Trx, in its reduced form, binds to ASK1 and inhibits ASK1 activity. In response to ROS, Trx is converted from the reduced form to the oxidised form and dissociates from ASK1. TRAF2 and TRAF6 are then recruited to ASK1 and the activating autophosphorylation of the critical threonine residue in the activation segment of ASK1 is induced. Interestingly, it has been shown recently that ROS-dependent activation of ASK1 also plays a critical role in innate immune responses through production of proinflammatory cytokines in splenocytes and macrophages (Matsuzawa *et al*, 2005). These findings raise the possibility that ASK1 may be involved in apoptosis- and inflammation-related tumorigenesis through ROS-dependent regulation of the JNK and p38 pathways.

As the involvement of ASK1, as well as of other MAP3Ks in the JNK and p38 pathways, in tumorigenesis is largely unknown, we investigated the roles of ASK family kinases, ASK1 and a recently characterised MAP3K, ASK2, in chemically induced skin tumorigenesis in mice, which is an established animal model linking inflammation to tumorigenesis (Mueller, 2006). ASK2 is closely related to ASK1 but can activate the JNK and p38 pathways only by forming a heteromeric complex with ASK1 (Takeda *et al*, 2007). Here, we show that ASK1 and ASK2 are critically involved in tumorigenesis by differentially regulating apoptosis and inflammation in the initiation and promotion stages of tumorigenesis.

Results

ASK2 deficiency promotes chemically induced skin tumorigenesis

To establish an animal model in which we can evaluate the involvement of ASK1 and ASK2 in tumorigenesis, we initially examined the tissue distribution of ASK1 and ASK2 proteins in mice. In contrast to the ubiquitous expression of ASK1, ASK2 protein was highly expressed in the skin as well as the gastrointestinal tract and lung (Figure 1A). ASK2 mRNA was expressed homogeneously throughout the epidermal layer in mouse skin (Figure 1B), and, consistent with this, ASK2

protein was highly expressed in primary keratinocytes derived from the epidermis, compared with macrophagic RAW264.7 cells and primary mouse skin fibroblasts (Figure 1C). We thus explored the possibility of involvement of ASK2 in the development of epidermal tumour using ASK2-deficient ($ASK2^{-/-}$) mice (Supplementary Figure S1).

$ASK2^{-/-}$ mice were viable and fertile and exhibited no apparent abnormalities in the tissues and organs examined including the skin, and no spontaneous tumorigenesis was induced. However, in two-stage skin tumorigenesis experiments, in which the dorsal skin of mice was treated once with DMBA (7,12-dimethylbenz(a)anthracene) and then continually treated with TPA (12-O-tetradecanoylphorbol-13 acetate), papillomas were induced significantly more in $ASK2^{-/-}$ mice than in wild-type (WT) mice (Figure 1D). No difference in the size or histology of papillomas was observed between these mice. The incidence and frequency of papillomas in $ASK2^{+/-}$ mice were intermediate between those in $ASK2^{-/-}$ and WT mice, suggesting that ASK2 gene dosage plays a critical role in tumorigenesis. These results suggested that ASK2 functions as a tumour suppressor in the skin.

ASK2 functions as a tumour suppressor by facilitating DMBA-induced apoptosis in epidermal keratinocytes

To investigate the possible involvement of ASK2 in DMBA-induced tumour initiation, we examined whether DMBA activates ASK2 by immunoblotting using phospho-ASK antibody that specifically recognises the activating phosphorylation of the critical threonine residue conserved between ASK1 and ASK2 (Tobiume *et al*, 2002). Consistent with the previous finding that ASK2 exhibited responsiveness to oxidative stress such as H_2O_2 only by forming a complex with ASK1 (Takeda *et al*, 2007), DMBA-induced activating phosphorylation of ASK2 was induced in HEK293 cells co-expressing ASK2 and kinase-negative mutant of ASK1 (ASK1-KN), but not in those expressing only ASK2 (Figure 2A). Moreover, reactiveness to phospho-ASK antibody (p-ASK1/2) increased in response to DMBA in primary keratinocytes derived from WT mice, which was strongly reduced in those derived from $ASK2^{-/-}$ mice (Figure 2B), indicating that ASK2, probably as well as ASK1, was activated by treatment with DMBA. Importantly, DMBA-induced activation of JNK and p38 was also reduced in $ASK2^{-/-}$ primary keratinocytes, compared with WT keratinocytes (Figure 2B). These results suggested that the ASK2-JNK and -p38 pathways are activated by DMBA in keratinocytes.

In the initiation stage in two-stage skin tumorigenesis, elimination of cells severely damaged by DMBA, so called 'initiated' cells, is a prerequisite for tumour suppression. Given that ASK2 in a complex with ASK1 had potential to induce apoptosis (Takeda *et al*, 2007), we expected that ASK2 contributed to DMBA-induced apoptosis in keratinocytes. Whereas no significant differences were observed between the viability of WT and $ASK2^{-/-}$ keratinocytes in the absence of DMBA treatment, $ASK2^{-/-}$ cells were more resistant to the toxic effects of DMBA than WT cells (Figure 2C). We also found that DMBA-induced DNA fragmentation was significantly reduced in $ASK2^{-/-}$ keratinocytes, compared with WT cells (Figure 2D). These results suggested that ASK2 is required for DMBA-induced apoptosis at least in primary keratinocytes.
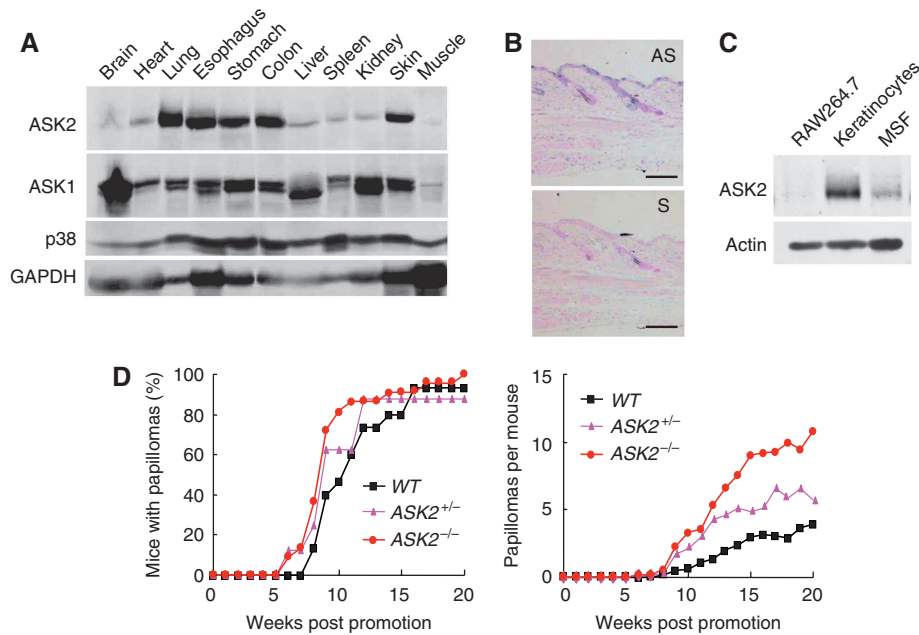
Figure 1 ASK2 deficiency promotes chemically induced skin tumorigenesis. (**A**) Normal tissue distribution of ASK2 in mice. Cell lysates were obtained from the indicated mouse organs and levels of protein expression of ASK1 and ASK2 were detected by immunoblotting. (**B**) Expression of ASK2 mRNA in mouse skin. ASK2 mRNA was detected by *in situ* hybridisation of adult mouse skin with anti-sense (AS) and sense (S) riboprobes. Scale bar = 100 μm. (**C**) ASK2 protein is highly expressed in primary keratinocytes. ASK2 protein was detected by immunoblotting in various cultured mouse cells. RAW264.7, a macrophagic cell line; Keratinocytes, primary keratinocytes; MSF, mouse skin fibroblasts. (**D**) Chemically induced skin tumorigenesis is promoted in $ASK2^{-/-}$ mice. Wild-type (WT; $n = 15$), $ASK2^{+/-}$ ($n = 7$) and $ASK2^{-/-}$ mice ($n = 22$) were treated once with DMBA (100 μg in 200 μl of acetone) and then continually treated with TPA (10 μg in 200 μl of acetone) twice a week for 20 weeks. The percentage of mice with papillomas (left) and the average number of papillomas per mouse (right) are shown.
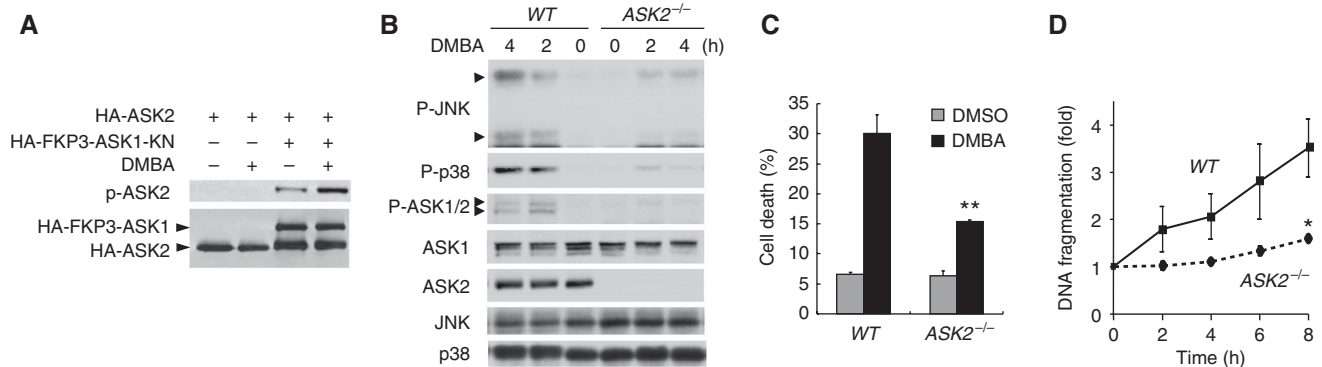

Figure 2 ASK2 facilitates DMBA-induced apoptosis in primary keratinocytes. (**A**) ASK2 is activated in response to DMBA. HEK293 cells transfected with HA-ASK2 in combination with (+) or without (−) HA-FKP3-ASK1-kinase-negative (KN) were treated (+) or not (−) with 100 μM DMBA for 1 h. Cell lysates were subjected to immunoblotting. (**B**) Immunoblotting of primary keratinocytes treated with 100 μM DMBA for the indicated periods. Phospho-ASK antibody recognised activation states of both ASK1 and ASK2 (p-ASK1/2). (**C**) $ASK2^{-/-}$ primary keratinocytes were more resistant to the toxic effects of DMBA than WT cells. WT and $ASK2^{-/-}$ keratinocytes were treated with DMSO or 100 μM DMBA for 8 h. Live and dead cells were simultaneously stained with 0.5 mM calcein AM and 0.5 mM EthD-1, respectively. Percentage of dead cells (at least 200 cells were counted for each group) is shown. Data are mean ± s.e.m. ($n = 3$). **$P < 0.01$, compared with DMBA-treated WT cells. (**D**) DMBA-induced apoptosis is suppressed in $ASK2^{-/-}$ primary keratinocytes. DNA fragmentation in WT and $ASK2^{-/-}$ primary keratinocytes treated with 100 μM DMBA for the indicated periods was quantified. Data are presented as the fold increases relative to the value of unstimulated cells and are mean ± s.e.m. ($n = 3$). *$P < 0.05$, compared with WT cells treated with DMBA for 8 h.

To examine whether ASK2 is indeed required for DMBA-induced apoptosis in epidermal cells *in vivo*, apoptotic cells in the DMBA-treated dorsal skin were detected by staining for active caspase-3 and TUNEL. Both active caspase-3-positive and TUNEL-positive cells were fewer in number in $ASK2^{-/-}$ mice than in WT mice (Figure 3). On the other hand, there was no obvious difference in proliferation of epidermal keratinocytes, as assessed by Ki67 staining, between WT and $ASK2^{-/-}$ mice treated with DMBA and/or TPA (Supplementary Figures S2). Taken together with the finding that the TPA-induced inflammation in $ASK2^{-/-}$ skin was comparable to that in WT skin (see below), these results suggested that ASK2 primarily functions, through its proapoptotic activity, as a tumour suppressor by eliminating damaged keratinocytes in the initiation stage.
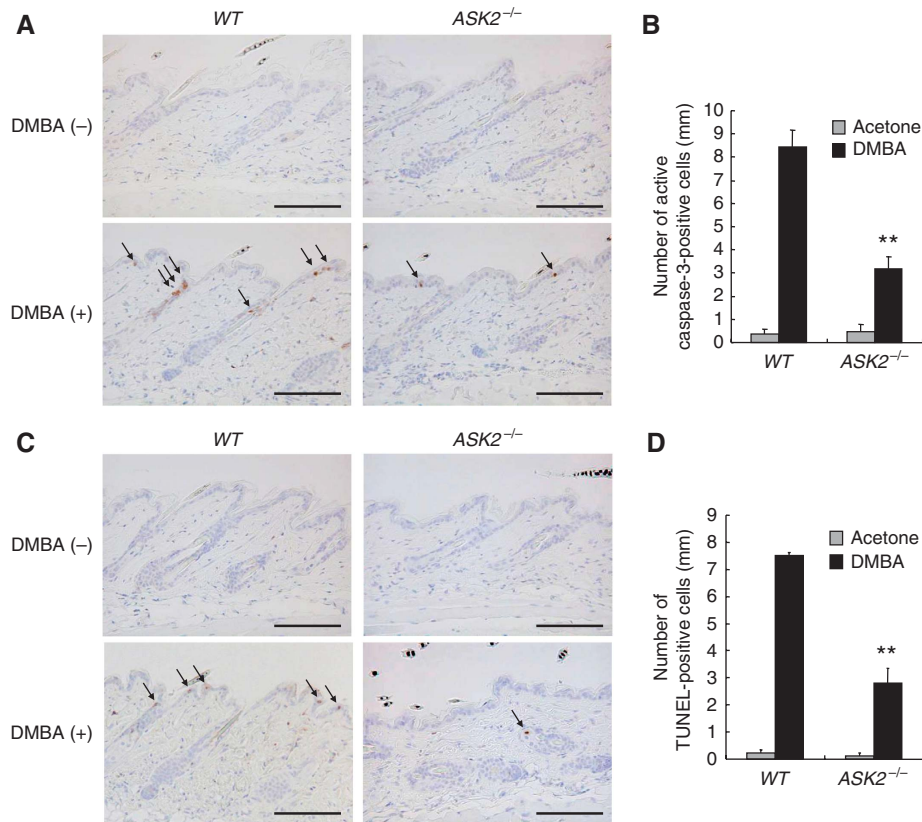
Figure 3 ASK2 facilitates DMBA-induced apoptosis in the epidermis. (**A**) Immunohistochemical staining of DMBA-treated skin sections with an antibody against active caspase-3. Skin samples were taken 24 h after the application of acetone (DMBA (−)) or 100 μg DMBA. Images are representatives of three independent experiments. Arrows indicate active caspase-3-positive cells. Scale bar = 100 μm. (**B**) Quantification of active caspase-3-positive cells. The number of active caspase-3-positive cells in the hair follicles and interfollicular epidermis was counted over a linear distance of approximately 15 mm, and averaged for each 1-mm interval. Values are the mean ± s.e.m. ($n = 3$). **$P < 0.01$, compared with DMBA-treated WT mice. (**C**) TUNEL staining of sections as in (**A**). Arrows indicate TUNEL-positive cells. Scale bar = 100 μm. (**D**) Quantification of TUNEL-positive cells. The number of TUNEL-positive cells was counted and averaged as in (**B**). Values are the mean ± s.e.m. ($n = 3$). **$P < 0.01$, compared with DMBA-treated WT mice.

ASK2 is critically involved in the signalling evoked by ROS-producing carcinogenic stimuli

DMBA is metabolised to toxic metabolites and ROS, the latter of which causes oxidative DNA damage in the skin (Frenkel *et al*, 1995; Muqbil *et al*, 2006). We found that ROS were indeed produced by DMBA treatment in human HaCaT keratinocytes, which was significantly inhibited by antioxidants *N*-acetyl-L-cysteine and propyl gallate (PG) (Figure 4A). Importantly, DMBA-induced ASK2 activation was inhibited by PG in HEK293 cells stably co-expressing ASK2 and ASK1-KN (293-ASK2/1KN cells), indicating that ROS were involved in DMBA-induced ASK2 activation (Figure 4B). Consistent with this finding, DMBA-induced activation of endogenous JNK, p38 and ASK2, as well as ASK1, was also inhibited by antioxidants in keratinocytes (Figure 4C). As DMBA-induced ROS production itself was not attenuated in the absence of ASK2 (Supplementary Figure S3), and ASK2 was required for ROS-induced JNK and p38 activation in keratinocytes (Supplementary Figure S4), ROS produced by DMBA appeared to activate the ASK2-JNK/p38 cascades. We also found that antioxidants significantly reduced DMBA-induced apoptosis in WT keratinocytes (Figure 4D). These findings indicated that ROS played a key role in ASK2-dependent apoptosis in DMBA-treated keratinocytes.

We next examined the involvement of ASK2 in ultraviolet (UV) response as a more physiologically relevant stimulant of human carcinogenesis. UV is classified into three types based on wavelengths, UVA (320–400 nm), UVB (290–320 nm) and UVC (200–290 nm). Among these, UVA reaches the earth's surface and is, therefore, a major skin carcinogen (Agar *et al*, 2004). As has been reported previously (Valencia and Kochevar, 2006), UVA, but not UVC, induced ROS production in HaCaT cells (Supplementary Figure S5A). Physiologically relevant doses of UVA was used in this and latter experiments (Bode and Dong, 2003). In 293-ASK2/1KN cells, ASK2 was strongly activated by UVA, but to a lesser extent by UVC, in a time- and dose-dependent fashion (Supplementary Figure S5B). Although UVB is also known as a strong skin carcinogen, UVA was a stronger activator of ASK2 than UVB, at least in our assay system. Pretreatment with the antioxidants suppressed the UVA-induced activation of ASK2 in 293-ASK2/1KN cells (Figure 4E), indicating that ASK2 was activated in response to ROS induced by UVA. Moreover, UVA-induced activation of JNK and p38 was reduced in *ASK2*$^{−/−}$ keratinocytes (Figure 4F). We also found that *ASK2*$^{−/−}$ keratinocytes were more resistant to UVA-induced cell death than WT cells (Figure 4G). These results suggested that ASK2 widely plays a proapoptotic role in response to ROS-producing carcinogenic stimuli.
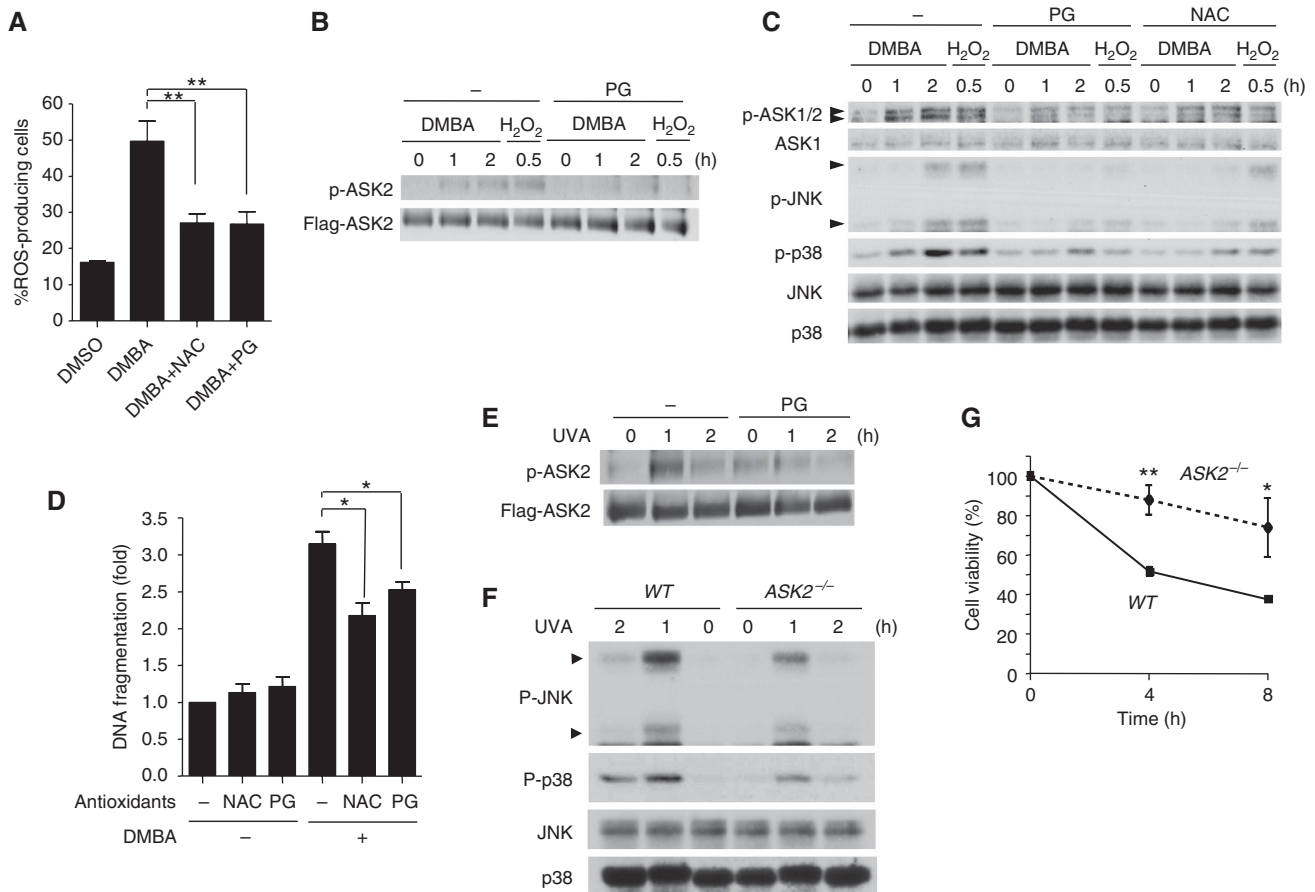
Figure 4 ASK2 is critically involved in the signalling evoked by ROS-producing carcinogenic stimuli. (**A**) DMBA induces intracellular ROS production. HaCaT cells were pretreated with 1 mM *N*-acetylcysteine (NAC) or 20 μM propyl gallate (PG) for 15 min and then stimulated with DMSO or 100 μM DMBA for 30 min. ROS production was measured by FACS analysis and the percentage of ROS-producing cells was calculated. Values are the mean ± s.e.m. ($n = 5$). **$P < 0.01$. (**B**) DMBA-induced activation of ASK2 is inhibited by antioxidants. 293-ASK2/1KN cells were pretreated with ($+$) or without ($-$) 20 μM PG for 15 min and then stimulated with 100 μM DMBA or 0.3 mM H_2O_2. Cell lysates were subjected to immunoblotting. (**C**) Immunoblotting of WT primary keratinocytes pretreated with antioxidants (20 μM PG or 1 mM NAC) for 15 min and stimulated with 100 μM DMBA or 0.3 mM H_2O_2 for the indicated periods. (**D**) DMBA-induced apoptosis in primary keratinocytes is reduced by antioxidants. DNA fragmentation in WT primary keratinocytes pretreated with 20 μM PG or 1 mM NAC for 15 min and stimulated with ($+$) or without ($-$) 100 μM DMBA for 8 h. Data are presented as the fold increases relative to the value of untreated cells and are mean ± s.e.m. ($n = 4$). *$P < 0.05$. (**E**) UVA-induced activation of ASK2 is mediated by ROS. 293-ASK2/1KN cells were pretreated with ($+$) or without ($-$) 20 μM PG for 15 min and then stimulated with 80 kJ/m^{2} UVA for the indicated periods. Cell lysates were subjected to immunoblotting. (**F**) UVA-induced activation of JNK and p38 is reduced in *ASK2*$^{-/-}$ primary keratinocytes. WT and *ASK2*$^{-/-}$ primary keratinocytes were treated with 80 kJ/m^{2} UVA for the indicated periods. Cell lysates were subjected to immunoblotting. (**G**) UVA-induced cell death is reduced in *ASK2*$^{-/-}$ primary keratinocytes. Viability of WT and *ASK2*$^{-/-}$ primary keratinocytes treated with 80 kJ/m^{2} UVA was measured by MTT assay. Data are presented as the percentage of viable cells and are mean ± s.e.m. ($n = 3$). *$P < 0.05$, **$P < 0.01$, compared with WT cells.

ASK2 appears to function as a tumour suppressor in human epithelial cancers

To explore the roles of ASK2 in human carcinogenesis, we examined ASK2 expression in various human cancer cells and tissues. In contrast to cervical and ovarian cancer cell lines, in which mRNA expression of ASK2 was inconsistent and varied, ASK2 expression was reduced in many cancer cell lines derived from the gastrointestinal tract (Figure 5A). In representative cell lines derived from esophageal cancer (KYSE30, KYSE110, KYSE850 and KYSE1250 cells), levels of expression of ASK2 mRNA were correlated well with those of ASK2 protein (Figure 5B). Immunohistochemical analysis of esophageal squamous cell carcinoma (ESCC) specimens revealed that ASK2 level was indeed reduced in 46 of 100 cases examined (Figure 5C). Moreover, a specimen of the boundary of the normal tissue and ESCC in an ASK2-negative case clearly showed that ASK2 protein was specifically reduced in

ESCC (Figure 5D). These findings together suggested that ASK2 functions as a tumour suppressor in human epithelial cancers.

Tumour-promoting activity of ASK1 counteracts with the proapoptotic activity of ASK1 and ASK2 in keratinocytes

The stability and activity of ASK2 protein are preserved only with formation of a heteromeric complex with ASK1 (Takeda *et al*, 2007). As ASK2 expression was strongly reduced in *ASK1*$^{-/-}$ keratinocytes (Figure 6A), it is possible that *ASK1*$^{-/-}$ mice are also prone to skin tumorigenesis. This was supported by the findings that DMBA-induced JNK and p38 activation was inhibited in *ASK1*$^{-/-}$ keratinocytes (Figure 6A) and that DMBA-induced apoptosis in *ASK1*$^{-/-}$ keratinocytes was suppressed to a level similar to that in *ASK2*$^{-/-}$ cells (Figure 6B). Intriguingly, however, no major
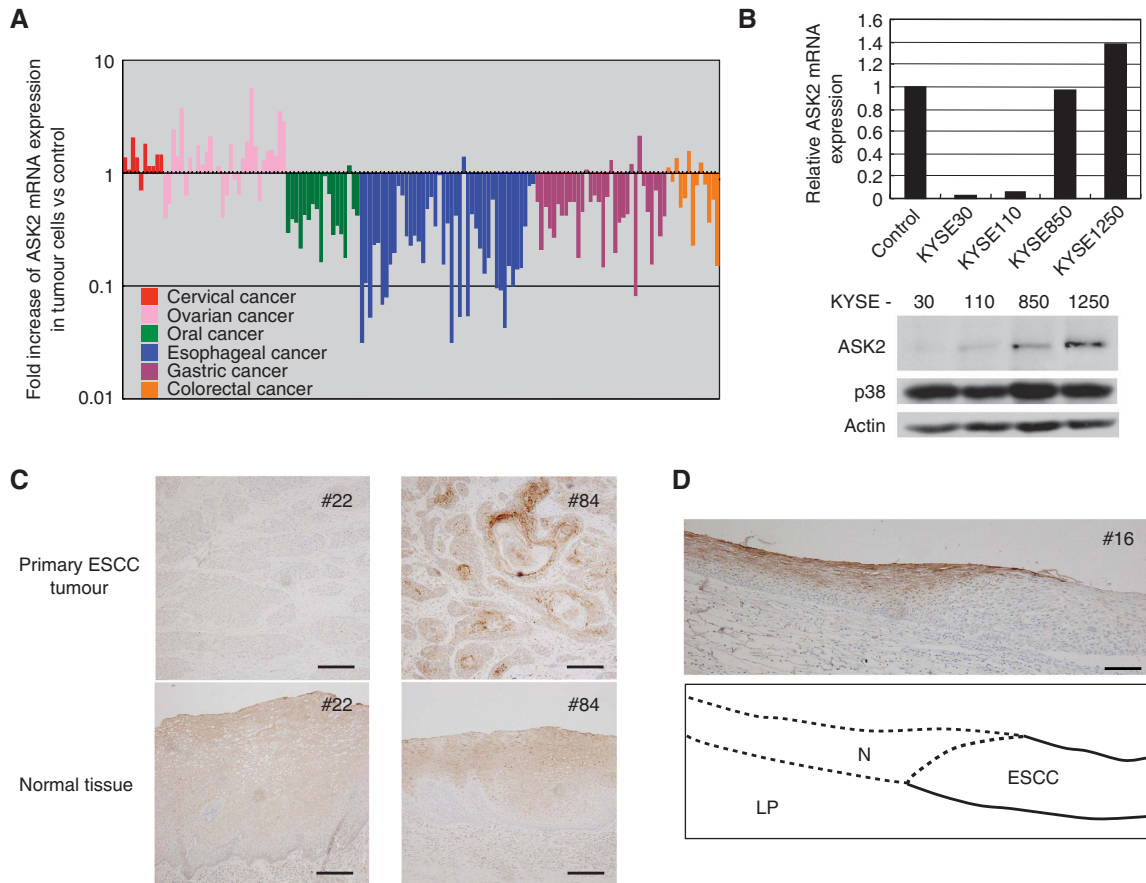
Figure 5 ASK2 expression is reduced in human cancer cell lines and tissues. (**A**) Quantification of levels of ASK2 mRNA expression in various human cancer cell lines. Real-time RT–PCR of ASK2 mRNA in cancer cell lines and respective normal tissues was carried out. After normalisation to GAPDH, levels of ASK2 mRNA expression in each human cancer cell line are indicated as fold increase relative to that in the respective normal tissue. (**B**) mRNA and protein expression of ASK2 in esophageal cancer cell lines, KYSE30, KYSE110, KYSE850 and KYSE1250. Data of mRNA expression of ASK2 was extracted from Figure 5A. Protein expression of ASK2 was detected by immunoblotting. (**C**, **D**) Immunohistochemical staining of ASK2 protein in esophageal cancer specimens. Representative ASK2-negative (#22) and ASK2-positive (#84) cases are shown in (C). The boundary of the normal tissue (N) and ESCC in an ASK2-negative case (#16) is shown in (D). LP, lamina propria. Scale bar $= 100\,\mu m$.
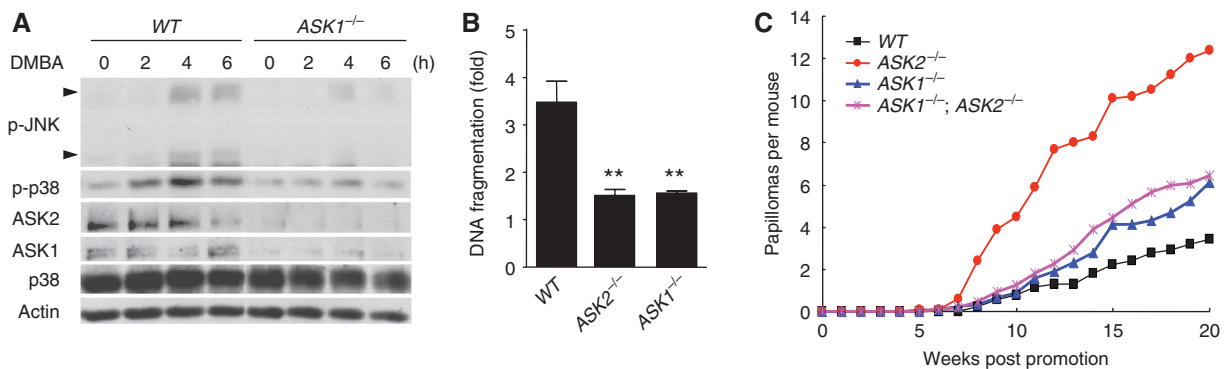

Figure 6 Tumour-promoting activity of ASK1 counteracts with the proapoptotic activity of ASK1 and ASK2 in keratinocytes. (**A**) DMBA-induced activation of JNK and p38 is reduced in $ASK1^{-/-}$ primary keratinocytes. Immunoblotting of WT and $ASK1^{-/-}$ primary keratinocytes treated with $100\,\mu M$ DMBA for the indicated periods. (**B**) DMBA-induced apoptosis is suppressed in $ASK1^{-/-}$ primary keratinocytes to a similar level to that in $ASK2^{-/-}$ cells. DNA fragmentation in WT, $ASK1^{-/-}$ and $ASK2^{-/-}$ primary keratinocytes treated with $100\,\mu M$ DMBA for 8 h was quantified. Data are presented as the fold increases relative to the value of respective unstimulated cells and are mean $\pm$ s.e.m. ($n = 4$). **$P < 0.01$, compared with WT cells. (**C**) Two-stage skin tumorigenesis induced by DMBA and TPA in WT ($n = 14$), $ASK2^{-/-}$ ($n = 10$), $ASK1^{-/-}$ ($n = 9$) and $ASK1^{-/-}$; $ASK2^{-/-}$ ($n = 11$) mice. The average number of papillomas per mouse is shown.

increase in skin tumorigenesis was observed in $ASK1^{-/-}$ mice (Figure 6C). More convincingly, mice deficient in ASK1 and ASK2 ($ASK1^{-/-}$; $ASK2^{-/-}$ mice) exhibited an extent of

tumorigenesis similar to that in $ASK1^{-/-}$ mice (Figure 6C), suggesting that ASK1 is required for the increased tumorigenesis in $ASK2^{-/-}$ mice. Thus, ASK1 appeared to play two

opposite roles in skin tumorigenesis: a tumour suppressive function in cooperation with ASK2 and a tumour promoting function independent of ASK2.

ASK1-dependent inflammatory response is required for tumour promotion

Therefore, we examined whether and how ASK1 and ASK2 contribute to TPA-dependent tumour promotion. TPA is recognised as the most potent tumour promoter, which induces pleiotrophic tissue response encompassing a strong inflammatory reaction, leading to the proliferation of initiated cells (Mueller, 2006). After topical treatment of the dorsal skin with TPA, the epidermis of WT and $ASK2^{-/-}$ mice exhibited dramatic hyperplasia with extensive infiltration of inflammatory cells, whereas the epidermis of $ASK1^{-/-}$ mice exhibited considerably milder hyperplasia (Figure 7A). This was supported by the significant difference in epidermal thickness between WT or $ASK2^{-/-}$ mice and $ASK1^{-/-}$ mice treated with TPA (see the graph in Figure 7A). Proliferation of basal keratinocytes in $ASK1^{-/-}$ and $ASK1^{-/-}; ASK2^{-/-}$ mice

was less prominent than that in WT and $ASK2^{-/-}$ mice (Supplementary Figure S6). Moreover, p38 activation was not induced in TPA-treated $ASK1^{-/-}$ skin (Figure 7B). Consistent with the well-established roles of p38 in cytokine production (O'Neill, 2006) and the requirement of proinflammatory cytokines such as TNF-α and IL-6 for chemically induced skin tumorigenesis (Moore et al, 1999; Suganuma et al, 1999; Ancrile et al, 2007), we found that TPA-induced production of TNF-α and IL-6 was diminished in the skin of $ASK1^{-/-}$ and $ASK1^{-/-}; ASK2^{-/-}$ mice (Figure 7C) and also in papillomas extirpated from $ASK1^{-/-}$ mice (Supplementary Figure S7). These results suggested that ASK1, but not ASK2, played a critical role in TPA-induced inflammation, at least in part through cytokine production.

Continual application of TPA to the skin is known to induce excess production of ROS (Nakamura et al, 1998; Ha et al, 2006). Thus, we expected that ASK1 regulated TPA-induced inflammation in a ROS-dependent fashion and that the cells, in which ASK1, but not ASK2, was required for ROS-induced cellular responses, played a critical role in TPA-
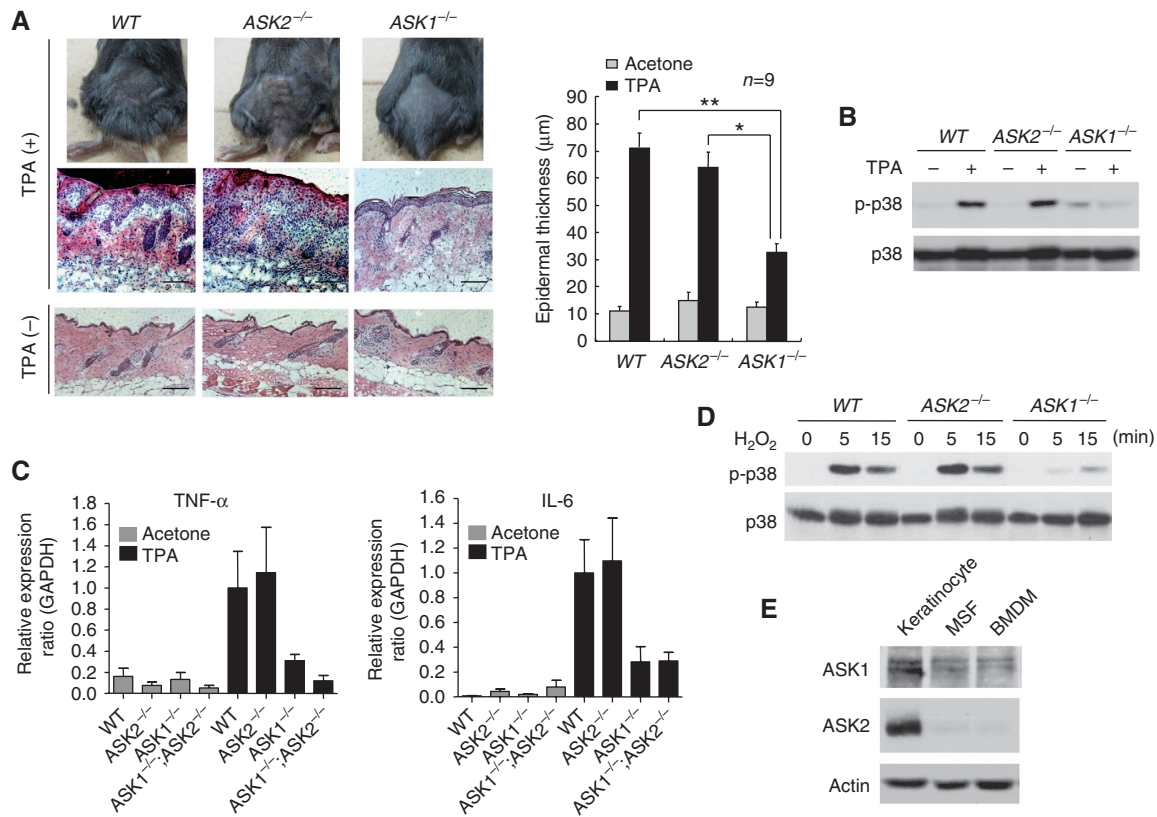

Figure 7 ASK1-dependent inflammatory response is required for tumour promotion. (**A**) TPA-induced inflammatory response is reduced in $ASK1^{-/-}$ mice. The dorsal skin of WT, $ASK2^{-/-}$ and $ASK1^{-/-}$ mice was treated twice with acetone (TPA (−)) or TPA (10 μg each) with 24 h interval. Mice were killed 48 h after the latter treatment, and skin sections were processed for hematoxylin-eosin staining. Images are representatives of nine mice for each genotype. Scale bar = 100 μm. Macroscopic observations of the dorsal skin of TPA-treated mice are also shown (left top panels). Quantification of epidermal thickness of these mice is shown in a graph on the right. Values are the mean ± s.e.m. ($n = 9$). *$P < 0.05$, **$P < 0.01$, compared with TPA-treated WT mice. (**B**) TPA-induced activation of p38 is attenuated in $ASK1^{-/-}$ skin. Acetone and 10 μg TPA were independently applied to separate areas of the dorsal skin of the same mouse. Mice were killed at 24 h after treatment, and each treated region of skin was excised and lysed and then subjected to immunoblotting. (**C**) Induction of TNF-α and IL-6 is attenuated in TPA-treated skin of $ASK1^{-/-}$ and $ASK1^{-/-};ASK2^{-/-}$ mice. The dorsal skin of WT, $ASK2^{-/-}$, $ASK1^{-/-}$ and $ASK1^{-/-};ASK2^{-/-}$ mice was treated with acetone or 10 μg TPA. Mice were killed 24 h after the treatment, and RNA was extracted from the treated skin. mRNA expression of TNF-α and IL-6 was quantified using real-time RT–PCR. Data are mean ± s.e.m. ($n = 4$ for TPA-treated mice and $n = 3$ for acetone-treated mice). (**D**) H_2O_2-induced activation of p38 is reduced in bone marrow-derived macrophages (BMDMs) from $ASK1^{-/-}$ mice, but not from $ASK2^{-/-}$ mice. WT, $ASK2^{-/-}$ and $ASK1^{-/-}$ BMDM were treated with 0.3 mM H_2O_2 for the indicated periods. Cell lysates were subjected to immunoblotting. (**E**) Comparison of ASK1 and ASK2 protein expression among primary cultured mouse cells. Cell lysates from primary keratinocytes, mouse skin fibroblasts (MSF) and BMDM were subjected to immunoblotting.

induced inflammation. Reductions in H_2O_2-induced p38 activation in both $ASK1^{-/-}$ and $ASK2^{-/-}$ keratinocytes indicated the similar roles of ASK1 and ASK2 in response to ROS in keratinocytes (Supplementary Figure S4), suggesting that ASK1 exerts its specific function in TPA response in other cell types than keratinocytes. This hypothesis, together with the ROS-dependent requirement of ASK1 for lipopolysaccharide (LPS)-induced p38 activation and cytokine production in splenocytes and macrophages (Matsuzawa *et al*, 2005), raised the possibility that ASK1 was selectively required for cytokine production in inflammatory cells. In fact, ROS-induced p38 activation was reduced in bone marrow-derived macrophages (BMDMs) from $ASK1^{-/-}$ mice, but not from $ASK2^{-/-}$ mice (Figure 7D). Consistent with this, ASK2 expression was much lower in BMDMs than in keratinocytes, whereas similar levels of ASK1 were expressed in these cells (Figure 7E). These findings suggested that, in inflammatory cells such as macrophages, ASK1 contributed to TPA-induced inflammatory response, which was found to be a prerequisite for promotion of skin tumorigenesis.

Discussion

In this study, our findings provide evidence for novel functions of stress-activated MAP3Ks in tumorigenesis (Figure 8). Proapoptotic activity of ASK2 in cooperation with ASK1 in epithelial cells and ASK1-dependent cytokine production in inflammatory cells were found to be critical determinants of chemically induced skin tumorigenesis in mice. Intriguingly, ROS appear to play critical roles in the regulation of the ASK family kinases in both these tumour-suppressing and tumour-promoting processes.

ASK2 is a recently identified MAP3K that interacts with ASK1, but functions of ASK2 have not been well understood, except that it activates the JNK and p38 pathways, induces apoptosis, and is required for ROS-induced activation of JNK (Takeda *et al*, 2007). By the two-stage skin tumorigenesis experiment using $ASK2^{-/-}$ mice, ASK2 was found to function as a tumour suppressor primarily by inducing apoptosis in epidermal keratinocytes in the initiation stage. Here, we propose that this function of ASK2 is also relevant to human cancers, as expression of ASK2 was strongly reduced in various human gastrointestinal cancer cells and tissues compared with their normal counterparts. This reduced ASK2 expression may be related, in part, to the fact that the human

ASK2 gene is located on chromosome 1p36.1, a region that is frequently deleted in a variety of human tumours (Kaghad *et al*, 1997).

As the stability and activity of ASK2 protein is preserved only with formation of a heteromeric complex with ASK1 (Takeda *et al*, 2007), proapoptotic activity of ASK2 in keratinocytes appeared to depend on ASK1. In fact, DMBA-induced apoptosis in $ASK1^{-/-}$ keratinocytes was reduced to a similar extent to that in $ASK2^{-/-}$ keratinocytes (Figure 6B), suggesting that ASK1 functions cooperatively with ASK2 in DMBA-induced apoptosis in keratinocytes. The finding that reactiveness to phospho-ASK antibody was abolished in DMBA-treated $ASK2^{-/-}$ keratinocytes, even though ASK1 protein still existed (Figure 2B), also suggests the cooperative regulation of activity of ASK1 and ASK2. This cooperation may be accounted for by the previous finding that ASK1 and ASK2 in their heteromeric complex activated each other not only by stabilisation of ASK2 by ASK1, but also by direct activating phosphorylation of ASK1 by ASK2 (Takeda *et al*, 2007).

Proapoptotic activity of ASK2 was found to be evoked by ROS production upon DMBA, although the source of ROS has not been specified. In $ASK2^{-/-}$ keratinocytes, DMBA-induced activation of JNK and p38 was reduced, suggesting that ASK2, as well as ASK1, has an important function in the regulation of ROS-induced JNK and p38 activation. Taken together with the accumulating evidence suggesting the multiple roles of JNK and p38 in ROS-medicated oncogenic responses (Benhar *et al*, 2002; Han and Sun, 2007), the ROS-activated ASK1–ASK2 complex appears to exert its proapoptotic activity through the JNK and/or p38 pathways. However, the underlying mechanism by which JNK and p38 induce apoptosis in DMBA-treated keratinocytes has not been elucidated so far. Considering the recent understandings that JNK and p38 exhibit pro- or anti-apoptotic activity depending on cell type and cellular context (Liu and Lin, 2005; Zarubin and Han, 2005), JNK and p38 may be required, but not sufficient, components for DMBA-induced apoptosis in keratinocytes. Critical roles of ASK2 in the signalling evoked by ROS-producing carcinogenic stimuli was also suggested by the findings that ASK2 was activated by UVA in a ROS-dependent fashion and that ASK2 was required for UVA-induced cell death. Given that UVA is regarded as a physiologically relevant stimulant of human carcinogenesis, we propose again that ASK2 may be a tumour suppressor in humans.

Compared with ASK2, the roles of ASK1 in tumorigenesis are more divergent. ASK1 exerts tumour-suppressive activity in cooperation with ASK2 in the initiation stage, whereas ASK1 accelerates tumorigenesis by facilitating production of inflammatory cytokines such as TNF-α and IL-6 in the promotion stage (Figure 8). Mice deficient in these cytokines have been shown to be resistant to two-stage skin tumorigenesis, supporting our hypothesis (Moore *et al*, 1999; Suganuma *et al*, 1999; Ancrile *et al*, 2007). The predominant expression of ASK1 in inflammatory cells such as BMDMs appears to be a plausible explanation for the fact that the proinflammatory role was assigned specifically to ASK1, but not to ASK2. Importantly, it has been shown recently that ASK1 mediates LPS-induced p38 activation and cytokine production in a ROS-dependent fashion in splenocytes and macrophages (Matsuzawa *et al*, 2005), showing the critical role of ASK1 in innate immunity. Taking into account
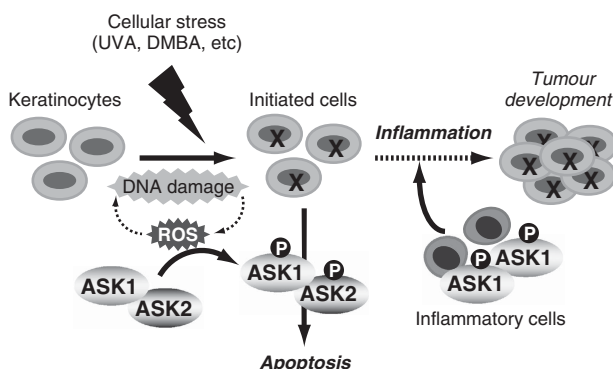

Figure 8 A schema of the roles of ASK family kinases in two-stage skin tumorigenesis. See Discussion for details.

evidence suggesting that excess ROS are produced upon continual treatment of the skin with TPA (Nakamura et al, 1998; Ha et al, 2006), the ROS-mediated ASK1-p38 pathway, which otherwise plays a beneficial role as a host defense system against pathogen, may play an adverse role in inflammation-induced tumorigenesis.

In addition to the skin, ASK2 is highly expressed in the gastrointestinal tract and lung, which are constantly exposed to a variety of stress stimuli such as toxic agents and invading pathogens from external environments. In these organs, correlation between inflammation, particularly chronic inflammation, and cancer has been established (Azad et al, 2008; Roessner et al, 2008). For instance, Helicobacter pylori gastritis and inflammatory bowel disease increase risks of developing gastric and colorectal cancers, respectively, and inflammation induced by cigarette smoke and ambient air pollutants is a major risk factor of lung cancer. Under such inflammatory conditions, inflammatory cells such as neutrophils and macrophages produce large amounts of ROS, which further enhance inflammation in part through upregulation of proinflammatory cytokines and predispose cells to malignant transformation. In this regard, proapoptotic function of ASK2 in cooperation with ASK1 in epithelial cells and proinflammatory function of ASK1 in inflammatory cells may also be involved in inflammation-related carcinogenesis in the gastrointestinal tract and lung.

In conclusion, genetic findings in this study strongly suggest that ASK1- and ASK2-dependent apoptosis of keratinocytes in the initiation stage and ASK1-dependent cytokine production in inflammatory cells in the promotion stage are critical steps in tumorigenesis. These stage- and cell type-dependent counteracting functions of stress-activated MAP kinase pathways thus appear to be critical determinants of apoptosis- and inflammation-related carcinogenesis and may have implications for cancer prevention and treatment.

Materials and methods

Mice
Generation of mice deficient for the ASK1 (MAP3K5) gene has been described (Tobiume et al, 2001). The 6.5 and 1.5 kb fragments of the ASK2 (MAP3K6) gene were used as homologous regions for recombination. pBluescript SK (Stratagene) was used as a backbone to construct the targeting vector with a DT-A cassette for negative selection. The first (3′ of the initiation codon) to fifth exons were replaced by NLS-LacZ (coding sequence for β-galactosidase containing nuclear localisation signal sequence followed by poly A signal) and a reverse-oriented PGK-neo cassette. The linearised targeting vector was electroporated into E14 ES cells. G418-resistant ES clones with the intended recombination were screened by Southern blot analysis. Heterozygous mutant ES cells were injected into C57BL/6J blastocysts. Germline transmission of mutated alleles to F1 mice obtained by intercross of resultant male chimeras and female C57BL/6J was confirmed by Southern blot analysis. Homozygous mutant mice were obtained by F1 heterozygous intercrosses. Deficiency of ASK2 polypeptide was confirmed by immunoblot analysis. $ASK1^{-/-}$ and $ASK2^{-/-}$ mice were back-crossed onto the C57BL/6J strain for 12 and 10 generations, respectively. All experiments accorded with protocols approved by the Animal Research Committee of the Graduate School of Pharmaceutical Sciences, The University of Tokyo (Tokyo, Japan).

Two-stage skin tumorigenesis model
The dorsal skin of 8–9-week-old female mice was treated once with DMBA (100 μg in 200 μl of acetone). One week later, it was continually treated with TPA (10 μg in 200 μl of acetone) twice a week for 20 weeks.

Cell culture, antibodies and reagents
See Supplementary information.

Expression plasmids and transfection
Epitope-tagged mammalian expression plasmids for ASK1 and ASK2 were described previously (Takeda et al, 2007). Transfection of these expression plasmids into HEK293 cells was performed with FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions.

In situ hybridisation
For generation of anti-sense and sense RNA probes, a 429 bp DNA fragment corresponding to nucleotide positions 697–1125 of mouse ASK2 (AB021861) was subcloned into pGEM-T Easy vector (Promega). For in situ hybridisation, skin sections from untreated WT mice were hybridised with digoxigenin-labelled RNA probes at 60°C for 16 h. Bound label was detected using NBT-BCIP, an alkaline phosphate colour substrate. The sections were counterstained with Kernechtrot (MUTO PURE CHEMICALS).

FACS analysis
For measurement of ROS production, HaCaT keratinocytes were pretreated with 2 μM DCHF-DA and then incubated with DMSO or 100 μM DMBA for 30 min. Fluorescence intensity was measured by flow cytometry with an excitation wavelength of 488 nm and emission wavelength of 580 nm. Fluorescent cells were detected by FACScalibar (BD Biosciences) and analysed using FlowJo software (Tree Star).

Immunoblotting
Cells were lysed with a lysis buffer containing 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 5 μg/ml aprotinin. Excised mouse skin was lysed in a buffer containing 1% Triton-X, 50 mM HEPES-KOH, pH7.5, 10 mM KCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM MgCl2 and 10% glycerol supplemented with a phosphatase inhibitor cocktail (PhosSTOP; Roche). Lysates were resolved on SDS–PAGE and electroblotted onto polyvinylidene difluoride membranes. After blocking with 5% skim milk in TBS-T (50 mM Tris–HCl, pH 8.0, 150 mM NaCl and 0.05% Tween 20), the membranes were probed with antibodies. The antibody/antigen complexes were detected using the ECL system (GE Healthcare).

Assessment of cell death of keratinocytes
Induction of death of primary keratinocytes by DMBA was determined using ToxCount (Active Motif) according to the manufacturer's instructions. Briefly, primary keratinocytes were seeded onto glass-bottomed dishes. Cells treated with DMBA were incubated with 0.5 mM calcein AM and 0.5 mM EthD-1 for 30 min at 37°C. Fluorescence images were acquired using an LSM510 laser-scanning unit coupled to an Axiovert 100 M inverted microscope with a C Apochromat × 63 objective lens (Carl Zeiss). DNA fragmentation in primary keratinocytes was quantified using the Cell Death Detection ELISAPLUS Kit (Roche Diagnostics) according to the manufacturer's instructions. The viability of primary keratinocytes treated with UVA was determined using the MTT assay-based Cell Counting Kit-8 (Dojindo) according to the manufacturer's instructions.

TUNEL staining and immunohistochemical staining for active caspase-3
Samples were fixed with Tissue Fixative (Genostaff), embedded in paraffin, and sectioned at 4 μm. Tissue sections were de-paraffined with xylene, and rehydrate through a ethanol series and TBS. After blocking the endogenous peroxidase with 3% H_2O_2 in methanol for 15 min, TUNEL staining was performed using the ApopTag Peroxidase in situ Apoptosis Detection Kit (Chemicon, S7100) according to manufacturer's instructions. Peroxidase activity was visualised with diaminobenzidine. The sections were counterstained with Mayer's Hematoxylin (Muto), dehydrated, and then mounted with Malinol (Muto). For active caspase-3 immunostaining, antigen retrieval was performed by microwave treatment in 1 mM EDTA buffer, pH 9.0. Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 30 min, followed by incubation with Protein Block (Dako). The sections were incubated with rabbit polyclonal antibody to Caspase 3 (R&D Systems) at 4°C overnight. After washing with TBS, the sections were treated with the Biotin

blocking system (Dako) and biotin-conjugated goat anti-rabbit Ig (Dako) diluted 1:600 for 30 min at room temperature, followed by addition of peroxidase conjugated streptavidin (Nichirei) for 5 min. Peroxidase activity was visualised with diaminobenzidine. The sections were counterstained with Mayer's Hematoxylin (Muto), dehydrated, and then mounted with Malinol (Muto). For image acquisition, a microscope (DM4000B; Leica) equipped with a digital camera (DC300FX; Leica) was used.

Analysis of expression of ASK2 mRNA in human cancer cell lines
To examine the expression of ASK2 mRNA in human cancer cells (cervical cancer, 10 lines; ovarian cancer, 30 lines; oral cancer, 18 lines; esophageal cancer, 43 lines; gastric cancer, 32 lines; colorectal cancer, 13 lines), quantitative real-time RT–PCR analysis was performed with TaqMan universal PCR master mix using the ABI Prism 7000 Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. The human GAPDH gene was used as an endogenous control. TaqMan probes and primers for ASK2 (No.Hs00177968_m1) and GAPDH (No.4326317E) were assay-on-demand gene expression products (Applied Biosystems). All samples were tested in duplicate. To normalise the relative expression of ASK2 to the GAPDH control, standard curves were prepared for ASK2 and GAPDH in each experiment.

Histology and immunohistochemistry
Surgically resected specimens were obtained from patients who received esophagectomy to ESCC in the National Defense Medical College Hospital (Saitama, Japan) between 1985 and 2003. Resected tissue specimens were formalin-fixed, paraffin-embedded, and routinely processed for pathological diagnosis. Written consent was acquired from each patient in the formal style, and the local ethics committees approved this study. These tissue blocks were cut into 4-μm thick sections and subjected to immunohistochemistry. Indirect immunohistochemistry of specimens using ASK2 antibody was performed as follows. Briefly, antigens were retrieved by autoclave in citrate buffer (pH 6.0) for 10 min at 120°C. After blocking in 2% normal swine serum, the slides were incubated with 3.5 μg/ml human ASK2 antibody overnight at 4°C, and then reacted with a dextran polymer reagent combined with secondary antibody and peroxidase (Envision Plus; DAKO). Antigen–antibody reactions were visualised with 0.2% diaminobenzidine tetrahydrochloride and hydrogen peroxide. The slides were counterstained with Mayer's hematoxylin. For morphological analysis of the TPA-treated mouse skin, the dorsal skin of WT, $ASK2^{-/-}$ and $ASK1^{-/-}$ mice was treated twice with acetone or TPA (10 μg each time) with a 24-h interval. Mice were killed at 48 h after the latter treatment. Sections (8 μm) of fresh–frozen TPA-treated skin were stained with hematoxylin and eosin. Images were acquired and processed as described in the section on TUNEL staining and immunohistochemical staining for active caspase-3.

Analysis of expression of cytokine genes in mouse skin
To quantify mRNA expression of TNF-α and IL-6, total RNA was isolated from mouse skin using ISOGEN (Nippon Gene) and reverse-transcribed with the QuantiTect Reverse Transcription Kit. Quantitative reverse-transcription PCR was performed with SYBR Green PCR Master Mix using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). To normalise the relative expression of TNF-α and IL-6 to the GAPDH control, standard curves were prepared for each gene and GAPDH in each experiment.

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