Suppression of Tumor Necrosis Factor-mediated Apoptosis by NF-κB-independent BMP/Smad Signaling

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Abstract

The activation of nuclear factor kappa B (NF-κB) plays a pivotal role in the regulation of tumor necrosis factor (TNF)-mediated apoptosis. However, little is known about the regulation of TNF-mediated apoptosis by other signaling pathways or growth factors. Here, unexpectedly, we found that bone morphogenetic protein (BMP)-2 and -4 inhibited TNF-mediated apoptosis by inhibition of caspase-8 activation in C2C12 cells, a pluripotent mesenchymal cell line which has potential to differentiate into osteoblasts depending on BMP stimulation. Utilizing both a trans-dominant IκBα inhibitor of NF-κB expressed in C2C12 cells, and IκB kinase β (IKKβ)-deficient embryonic mouse fibroblast, we show that BMP-mediated survival was independent of NF-κB activation. Rather, the anti-apoptotic activity of BMPs functioned through the Smad signaling pathway. Thus, these findings provide the first report of a BMP/Smad signaling pathway which can inhibit TNF-mediated apoptosis, independent of the pro-survival activity of NF-κB. Our results suggest that BMPs not only stimulate osteoblast differentiation but also can promote cell survival during the induction of bone formation, offering new insight into the biological functions of BMPs.
The abbreviations used are:

BMPs, bone morphological proteins; NF-κB, nuclear factor kappa B; TNF, tumor necrosis factor; tBid, truncated Bid; SR-IκBα, super-repressor of IκBα.
NF-κB is a stress-responsive transcription factor that plays important roles in development and immunity (1-3). Classical NF-κB is a heterodimer composed of p50 and p65/RelA, which is sequestered in the cytoplasm by the IκB group of inhibitory proteins. Pro-inflammatory cytokines such as tumor necrosis factor (TNF) activate IκB kinase complex (IKK) to phosphorylate the conserved N-terminal region of IκB proteins (1-3). The phosphorylated IκB is ubiquitinated and subsequently degraded by the 26S proteasome. This results in the nuclear translocation of NF-κB, and binding to NF-κB-responsive elements, followed by NF-κB-dependent transcriptional activation (1-6).

We and others demonstrated that NF-κB plays a critical role in inhibiting TNF-mediated apoptosis (7-9). In the absence of NF-κB activation, TNF can trigger the caspase cascade by interacting with FADD (Fas-associated death domain protein), which then recruits and activates caspase-8. (11-13). Active caspase-8 promotes cell death by either directly processing other downstream caspases or cleaving the cytosolic Bid protein, a pro-apoptotic family member of Bcl-2 (12, 14, 15). Truncated Bid (tBid) translocates to mitochondria, resulting in the release of cytochrome c from mitochondria into the cytosol and the subsequent activation of apoptosis (14, 15). However, in the presence of NF-κB activation, the caspase-8-mediated apoptotic pathway is suppressed (4). Several important anti-apoptotic molecules have been identified that are transcriptionally regulated by NF-κB. These molecules include the Bcl-2 family members A1 and Bcl-xL, IAP family proteins, TRAF family proteins, IEX-1L, and the recently elucidated NDED protein (4, 16, 17-19). Although several growth factors have been found to inhibit TNF-mediated apoptosis through the activation of NF-κB (20), little is
known regarding the regulation of TNF-mediated apoptosis by NF-κB-independent signaling.

Bone morphogenetic proteins (BMPs), members of the transforming growth factor (TGFβ) superfamily, were originally identified by their unique ability to induce bone formation in vivo (21-23). BMPs initiate a signaling cascade through the ligand-dependent activation of a complex of heteromeric transmembrane serine-threonine kinase receptors, type I and type II (24). The activated BMP type I receptor phosphorylates Smad1 and Smad5, resulting in their dissociation from the receptor complex. The phosphorylated Smad1 and Smad5 then form hetero-oligomeric complexes with Smad4 and translocate into the nucleus to activate the transcription of target genes (25-27). Interestingly, the BMP/Smad signaling pathway is also negatively regulated by a structurally and functionally divergent Smad protein of the subfamily of inhibitory Smads, Smad6 and Smad7 (25-27).

BMPs induce osteoblast differentiation of mesenchymal cells and are involved in postnatal bone remodeling (28, 29). Several studies demonstrate that BMPs control digit numbers in the limbs, possibly through the induction of apoptosis in the interdigital and anterior tissue (30, 31). BMPs can inhibit proliferation and induce apoptosis of multiple myeloma cells, indicating the therapeutic potential of BMPs in cancer treatment (32).

Given that the cell death machinery can be modulated by multiple factors or signaling pathways, we hypothesized that NF-κB-independent mechanisms may exist to suppress TNF-mediated apoptosis. During the search for regulators of TNF-mediated apoptosis, unexpectedly, we found that, in contrast to previous studies implicating BMPs in pro-apoptotic mechanisms (31, 32), BMPs possessed a novel anti-apoptotic activity that
promoted survival of mesenchymal cells during BMP-induced osteoblast differentiation. The results reported here reveal a unique BMP/Smad signaling pathway that suppresses TNF-mediated apoptosis in an NF-κB-independent manner. Given the fact that apoptosis plays an important role in osteoporosis and other inflammatory-related bone disorders (33), our results suggest that the utilization of BMP for bone regeneration and repair may have dual benefits: stimulation of osteoblast differentiation and inhibition of apoptosis.

**Experimental Procedures**

*Cell culture*--C2C12 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 15% heat-inactivated fetal bovine serum, penicillin (100 µg/ml), streptomycin (100 µg/ml), at 37°C under 5% CO₂ and at 95% humidity. IKKβ-/- mouse embryonic fibroblasts were grown in DMEM with 10% heat-inactivated fetal bovine serum.

*Trypan blue exclusion, Cell death ELISA, Annexin V staining and EGFP survival assay*-- One hundred thousand cells were plated onto 6-well plates the day before stimulation. Cells were pre-treated with the indicated concentrations of BMP2 or BMP4 for 2 hr and subsequently killed with tumor necrosis factor (20 ng/ml) for 24 hr. Cell viability was determined by trypan blue exclusion. The supernatant was collected and cell death ELISA was performed as described before (Roche).

For Annexin V staining, 2 x 10⁴ cells were plated on a microscope cover slip in 24-well plates the day before stimulation. Sixteen hours after stimulation, cells were gently washed once with 1 x binding buffer, and stained with Annexin V conjugated with EGFP (an enhanced variant of the green fluorescent protein) solution (1: 40, Clontech) and
propidium iodide (50 mg/ml, Sigma) for 15 minutes at room temperature in the dark. After staining, cells were washed twice with 1 x binding buffer and fixed in 2% formaldehyde in PBS (pH 7.4) for 20 minutes. The cover slips were inverted on a drop of Vectashield mounting media (Fisher Scientific) on slides, examined and photographed under a fluorescence microscope using a filter set for FITC.

For EGFP survival assay, cells were co-transfected with pCMV-GFP (Enhanced Green fluorescent protein) vector and either pCMV-Smad-7 or control vector with Superfect (Qiagen). Twenty-four hours after transfection, cells were pre-treated with BMP-2 or BMP-4 for 2 hr and then killed with TNF for an additional 24 hours. The GFP-expressing cells were directly examined under a fluorescent microscope.

**Western blot analysis**-- Two million cells were plated in a 100 mm plate the day before stimulation. Cells were pre-treated with BMP-2 or -4 at concentration of 200 ng/ml for the indicated times. The detached and attached cells were collected. Whole cell extracts were prepared with RIPA buffer containing 1% NP-40, 5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 100 mM sodium orthovanadate and 1: 100 protease inhibitors cocktail (Sigma). The proteins were resolved in a sodium dodecyl sulfate (SDS)-10% polyacrylamide gel (PAGE) and transferred to PVDF membrane by semi-dry transferer apparatus (Bio-Rad). The membrane was probed with polyclonal antibodies against caspase-8 (Santa Cruz) and visualized using an enhanced chemiluminescence (ECL) reagent (Amersham) according to the manufacturer’s recommendation. (10).

**In vitro caspase-3 and caspase-8 activity assay**--Cells were treated as described above for Western blot. The detached and attached cells were collected, washed with
PBS and lysed in 200 µl ice cold hypotonic lysis buffer provided by the manufacturers (R&D Systems, Clontech or Promega). The cell extracts were centrifuged and supernatants were collected. Two to three hundred µg of protein extracts were incubated in reaction buffer containing IETD-pNA (colorimetric caspase-8 substrate, R&D systems) or DEVD-pNA (colorimetric caspase-3 substrate, Promega) at 37°C for 2 to 3 hours. The samples were analyzed with a plate reader by measuring the optical density (OD) at a wavelength of 405 nm.

**Electrophoretic Mobility Shift Assays (EMSAs) and NF-κB luciferase reporter assays**

-- Cells were treated with TNF (10 ng/ml) for the indicated time points. Nuclear extracts were prepared for EMSAs as described previously (15, 22). Five µg aliquots of nuclear extracts were pre-incubated with 1 µg of poly(dI-dc) in binding buffer (10 mM Tris, pH 7.7, 50 mM NaCl, 20 % glycerol, 1 mM DTT, 0.5mM EDTA) for 10 min at room temperature. Approximately 20,000 cpm of 32P-labeled DNA probe containing the class I MHC NF-κB site (5’-CAGGGCTGGGGATTCCC CATCTCCACAGTTTCACTTC-3’) was then added and binding proceeded for 15 minutes. The complexes were separated on a 5 % polyacrylamide gel and exposed for autoradiography (5, 17).

To determine NF-κB transcription activity, cells were transfected with 2 x κB-dependent luciferase reporter constructs using the Superfect reagent (Qiagen) according to the manufacturer’s instructions (20). PRL-TK Renilla luciferase reporter was co-transfected to normalize for transfection efficiency. Luciferase activities were measured using a dual luciferase system (Promega).
RESULTS

**BMP-2 and -4 inhibits TNF-mediated apoptosis** -- C2C12 cells are pluripotent mesenchymal cells which are widely used for the study of cell differentiation in vitro (6, 28). In order to study the role of NF-κB in cell growth and apoptosis, we have established a C2C12I cell line stably expressing a modified form of the NF-κB inhibitor, super-repressor-IκBα (SR-IκBα, figure 1A). SR-IκBα contains serine-to-alanine mutations at residue 32 and 36, which prevent signal-induced phosphorylation and subsequent proteasome-mediated degradation of IκBα. As shown in figure 1A, TNF rapidly induced the phosphorylation and degradation of endogenous IκBα in C2C12 control cells (C2C12V) but not SR-IκBα in C2C12I cells following TNF stimulation. As predicted, SR-IκBα inhibited the nuclear translocation of NF-κB induced by TNF (figure 1B). Consistent with our previous studies (10), C2C12I cells were sensitive to TNF-mediated killing, confirming that NF-κB inhibits TNF-mediated apoptosis in cells (figure 2A).

Given the fact that the cell death machinery could be modulated by different factors and stimuli, we hypothesized that an NF-κB-independent pathway might be able to regulate TNF-mediated apoptosis. Thus, we utilized C2C12I cells to screen new growth factors or signaling molecules which could regulate TNF-mediated apoptosis in an NF-κB-independent fashion. Because C2C12 cells are a well-established model system to study BMP-induced osteoblast differentiation, we sought to determine if BMPs would modulate TNF-mediated apoptosis. Previously, Lopez-Rovira et al have found that TGFβ activates NF-κB which is independent of IκB degradation (34). Thus, to confirm that BMPs did not activate NF-κB in C2C12I cells, NF-κB-dependent luciferase reporter assays were performed. As shown in figure 1C, BMP4 did not induce NF-κB
transcriptional activities in C2C12I cells. To test whether BMPs promoted cell survival, C2C12I cells were pretreated with BMP-4 or vehicle control for 2 hr and then treated with TNF for 24 hr. As shown in figure 2A, trypan blue exclusion analysis found that TNF stimulation caused 70% to 80% cell death of C2C12I cells. In contrast, only approximately about 20% cells were killed by TNF when cells were pretreated with BMP-4. BMP-4-mediated protection against TNF killing was dose-dependent as shown in figure 2B. Furthermore, we also examined whether other BMP family member could inhibit TNF killing. As shown in figure 2C and D, like BMP-4, BMP-2 also potently inhibited TNF killing in a dose-dependent manner.

To further confirm the results described above, DNA fragmentation and histone release from cell culture supernatant were also measured with cell death ELISA (4). As shown in figure 3A and B, pretreatment with BMP-2 and –4 significantly inhibited DNA fragmentation induced by TNF. To determine whether BMP-mediated survival was due to the modification of apoptosis, EGFP-conjugate Annexin V staining in conjunction with propidium iodide (PI) was performed. Changes in the plasma membrane of the cell surface are one of the earliest features of cells undergoing apoptosis. After initiating apoptosis, phosphatidylserine (PS) is translocated from the inner face of the plasma membrane to the cell surface (11-13). Because Annexin V has a high affinity for PS and can bind to cells with exposed PS, it has been used to detect the early stage of apoptosis (4, 17). As shown in figure 3C, compared to numerous Annexin V positive-stained cells in the TNF-treated cells, only limited staining was detected in cells pretreated with BMP-2 or -4. Annexin V-positive cells failed to take up propidium iodide (data not shown), indicating cell death via apoptosis but not necrosis. Taken together, these results suggest
BMPs activate a NF-κB-independent signaling pathway to suppress TNF-induced apoptosis.

_BMP signaling blocks caspase-8 activation and the subsequent cleavage of bid_—Next, we examined the molecular mechanism by which BMP signaling inhibited TNF-mediated apoptosis. To initiate apoptosis, TNF binds its receptor to recruit TRADD and FADD to activate caspase-8 (4, 8, 11). To determine whether BMPs inhibited TNF-induced caspase-8 activation, cells were pretreated with BMP-2 and subsequently killed by TNF. As shown in figure 4A, Western blot analysis found that the processing of caspase-8 induced by TNF was strongly inhibited by BMP-2. Because we could not detect the active subunit of caspase-8 by Western blot analysis, we performed the caspase-8 enzymatic assay to determine whether BMP-2 suppressed caspase-8 activity induced by TNF. As shown in figure 4B, caspase-8 enzymatic activity induced by TNF was inhibited by BMP-2. A pro-apoptotic family member of Bcl-2, Bid, has been found to be a specific substrate of caspase-8 during death-receptor-mediated apoptosis. The truncated Bid promotes and/or amplifies apoptosis by inducing the release of cytochrome c from mitochondria to the cytosol (14, 15). To confirm that caspase-8 activity was inhibited by BMP-2, we examined the cleavage of Bid. As shown in figure 4A, a substantial amount of tBid was detected in cells following TNF stimulation, but not in cells pretreated with BMP-2. In addition to the cleavage of Bid, caspase-8 can also directly activate executing caspases such as caspase-3 to induce apoptosis (11, 13). Thus, we also determined whether caspase-3 activity was affected by the inhibition of caspase-8. As predicted, significantly lower DEVDase activity was present in the cells pretreated with BMP-2 compared with cells without BMP-2 pretreatment following TNF stimulation (figure 4C).
These results demonstrated that BMP-2 suppressed TNF-mediated apoptosis by inhibiting caspase-8 activation.

**BMP-mediated cell survival is dependent on SMAD signaling**—Since the results presented above were from C2C12 cells in which NF-κB activities were suppressed, it indicated that BMP-2 or -4-mediated survival was independent of NF-κB. Biochemical and genetic studies have demonstrated BMPs exhibit their biological functions through the Smad signaling pathway (35-37). Thus, we examined whether BMPs-mediated cell survival was dependent on the Smad signaling pathway. To block BMPs-mediated Smad signaling, an inhibitory Smad, Smad7 was utilized. Smad7 interacts with activated BMP type I receptors and inhibits BMP-mediate signaling by inhibiting Smad1 and Smad5 phosphorylation (37). Cells were co-transfected with pEGFP (enhanced green fluorescent protein) expression vector and pCMV-Smad7 expression vector or a control vector. Twenty-four hr after transfection, cells were pretreated with BMP-2 for 2 hr and TNF subsequently added to induce cell death. As shown in figure 5A and B, cells transfected with control vector had no effect on BMP-mediated protection against TNF killing. In contrast, cells transfected SMAD7 were sensitive to TNF killing regardless of BMP pretreatment, indicating that BMP-mediated survival was dependent on SMAD signaling.

To rule out non-specific effect of SR-IκBα, we also determined whether BMP inhibited TNF-mediated apoptosis in mouse embryonic fibroblasts from IκB kinase β-deficient mice (IKKβ-/- cells). IKKβ is a key component of IKK complex for phosphorylation of IκBs and has been found to play an essential role in NF-κB activation (38). TNF is unable to activate NF-κB transcription in those cells due to lack of IKK activity and subsequent absence of IκB degradation. Therefore, similar to SR-IκBα-
expressing cells, IKKβ−/− fibroblasts are also capable of undergoing TNF-mediated apoptosis (38). To test this effect, IKKβ−/− cells were pretreated with or without BMP-2 for 2 hr and then killed with TNF for 24 hr. As shown figure 6A, BMP-2 also significantly inhibited TNF-mediated apoptosis in IKKβ−/− cells. Similarly, the ectopic expression of Smad7 also rendered BMP-2-pretreated cells sensitive to TNF-mediated apoptosis (figure 6B), confirming that BMPs-mediated survival was dependent on SMAD signaling but not on NF-κB activation.

**DISCUSSION**

Our results reveal a unique property of BMPs that inhibit TNF-mediated apoptosis in an NF-κB-independent manner. Furthermore, we found that BMP-mediated survival was dependent on Smad signaling, indicating that other survival pathways can replace NF-κB anti-apoptotic function to suppress TNF-mediated apoptosis. BMPs suppressed TNF-mediated apoptosis by inhibiting caspase-8 activation. Since NF-κB-inducible genes including TRAF-1 and –2, c-IAP-1 and –2, c-Flip and NEDD play a critical role in inhibition of TNF-mediated caspase-8 activation (4), we examined whether the expression of those genes were regulated by BMPs. Western blot or Northern blot analysis found that none of these genes were induced by BMPs. Additionally, we also found that the expression of Bcl-2 family proteins, including Bcl-2, Bcl-xL, Bax, Bad, and Bik, were not modulated by BMPs (unpublished observation). Thus, these results indicate BMP/Smad signaling may regulate other anti-apoptotic molecules yet to be
identified. However, we cannot rule out the possibility that BMP/Smad signaling may posttranslationally modify anti-apoptotic proteins.

BMPs are multifunctional cytokines which are widely expressed and play important role in morphogenesis and development (23-24). Contrary to our results, several reports indicate that BMPs function in a pro-apoptotic manner, perhaps required for interdigit cell death (30, 31). BMPs have been found to induce apoptosis in multiple myeloma (32). These seemingly contradictory findings may be due to cell-type specific effects. However, of interest is that both C2C12 cells and embryonic fibroblasts which were used for our studies have the potential to differentiate into osteoblasts upon on BMPs stimulation (28, 29). The process of osteoblast differentiation may be a stress stimulus which requires a survival signal to prevent cell from undergoing apoptosis (33). Consistent with our results, Yang et al (39) have found that deletion of Smad5 led to apoptosis in mesenchymal cells and angiogenesis defects in during early mouse development by unknown mechanisms. Given the fact that Smad5 is a key component of BMPs signaling (35, 36), the result implicates that BMP/Smad signaling may play a role in cell survival during embryogenesis and development. In conclusion, our results suggest that BMPs not only stimulate osteoblast differentiation but also can prevent apoptosis and provide new insight into biological functions of BMPs.
Acknowledgments

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REFERENCES


Figures Legends

**Figure 1. SR-1kBα suppresses the activation of NF-κB in C2C12 cells.** (A) SR-1kBα inhibited the phosphorylation and degradation of 1kBα. C2C12 cells were transduced with retroviruses expressing human SR-1kBα or control vector and selected with neomycin (400 µg/ml). The resistant clones were pooled and designated as C2C12I cells or control cells (C2C12V), respectively. Of note, the molecular weight of human SR-1kBα was slightly larger than that of endogenous mouse 1kBα. C2C12I cells or control cells were treated with TNF (20 ng/ml) for the indicated time points. The whole extracts were probed with polyclonal antibody against 1kBα or phosphorylated 1kBα (p-1kBα). For the loading control, the blot was stripped and re-probed with monoclonal antibodies against α-tubulin. (B) SR-1kBα inhibited the nuclear translocation of NF-κB. The nuclear extracts were prepared as described in the materials and methods. Five µg of aliquots of protein were incubated with P32-labeled NF-κB probe. The reaction was resolved in 5% PAGE and exposed to film. (C) BMP4 did not activate NF-κB in C2C12I cell. Cells were transfected with 2 x κB-dependent luciferase reporter constructs. Twenty-four hours after transfection, cells were treated with BMP4 for 16 hr. Luciferase activities were measured as described in materials and methods.

**Figure 2. BMP-2 and -4 inhibits TNF-mediated apoptosis through an NF-κB-independent mechanism.** (A) BMP-4 inhibited TNF killing. Cells were un-pretreated or pretreated with BMP-4 for 2 hr (100 ng/ml) and then treated with TNF (20 ng/ml) for 24 hr. Cell viability was determined by trypan blue exclusion. The assays were performed in triplicate, and the results represent the mean value from three independent experiments. Statistical differences between each group were determined by Student’s t-test. *: P<0.01.
(B) BMP-4 inhibited TNF killing in a dose-dependent fashion. Cells were pretreated with the indicated concentrations of BMP-4 and then killed by TNF. (C) and (D) BMP-2 inhibited TNF killing. The experiments were performed as described in (A) and (B), respectively. Statistical differences between each group were determined by Student’s t-test. *: P<0.01.

**Figure 3. BMP-2 and -4 inhibit TNF-mediated apoptosis.** (A) and (B) BMP-2 and -4 inhibited DNA fragmentation. Cell treatment was performed as described in figure 1. Twenty µl of cell supernatant from each cell group were incubated with anti-histone and -DNA antibody at room temperature for 2 hours. The reaction was measured with a microplate reader at wavelength of 405 nm. The assays were performed in triplicate, and the results represent the mean value from three independent experiments. Statistical differences between each group were determined by Student’s t-test. *: P<0.01. (C) Annexin V staining of apoptotic cells. Cells were untreated or pre-treated with BMP-2 (200 ng/ml) or -4 (100 ng/ml) for 2 hr and then killed by TNF for 14 to 16 hr. Cells were washed and incubated with EGFP-Annexin V (1:40) and propidium iodide (PI). The cells were examined by fluorescence microscopy.

**Figure 4. BMPs inhibit TNF-induced caspase-8 activation.** (A) BMPs inhibited the processing of caspase-8. Cells were un-pretreated and pretreated with BMP-2 (200 ng/ml) for 2 hr and then killed with TNF for the indicated time points. The detached and attached cells were harvested and the whole cell extracts were prepared. Fifty µg aliquots of protein extracts were resolved on 10% SDS-PAGE and probed with monoclonal antibodies against caspase-8 or polyclonal antibody against tBid. For the loading control, the blots were stripped and reprobed with monoclonal antibody against α-tubulin. (B)
BMPs inhibited caspase-8 activation. Cell were pretreated with BMP-2 (200 ng/ml) for 2 hr and then killed by TNF for 14 hr. Two hundred µg aliquots of protein extracts were incubated with caspase-8 substrate IETD-pNA (200 µM) for 2 to 3 hr at 37°C. The reaction was measured with a plate reader at the wavelength of OD 405 nm. The results represent the average values from the two independent experiments. (C) The caspase-3 activity induced by TNF was suppressed by BMPs. Whole cell protein extracts were prepared as described in (B). Two hundred µg aliquots of protein extracts were incubated with DEVD-pNA substrate for 3 hr at 37°C, and the reaction was recorded at a wavelength of 405 nm.

**Figure 5. BMPs-mediated survival is dependent on SMAD signaling.** (A) Smad7 abolished BMPs-mediated survival. Cells were co-transfected with pCMV-EGFP vector and either pCMV-Smad7 or control vector. Twenty-four hours after transfection, cells were treated with TNF (20 ng/ml) for additional 24 hours. The GFP-expressing cells were examined by fluorescence microscopy. The assays were performed in duplicate. The results represent the average value of three independent experiments. Statistical differences between each group were determined by Student’s t-test. *: P<0.01. (B) Photograph of EGFP-positive cells. Cell transfection was performed as described above. The surviving cells (EGFP-positive cells) were examined by fluorescence microscopy.

**Figure 6. BMP/Smad signaling inhibits TNF-mediated apoptosis in IKK-/- mouse embryonic fibroblasts.** (A) IKK-/- cells were un-pretreated or pretreated with BMP-2 for 2 hr and then killed by TNF for 24 hr. The cell viability was determined as described in figure 1. The assays were performed in triplicate. The results represent the mean value from three independent experiments. Statistical differences between each
group were determined by Student’s t-test. *: P<0.01. (B) Smad7 abolished BMP-mediated survival. Cell transfection was performed as described in figure 4. Twenty-four hr after transfection, cells were un-pretreated or pretreated with BMP-2 (200 ng/ml) and then killed by TNF (20 ng/ml) for 24 hr. The EGFP-positive cells were counted by fluorescence microscopy. The assays were performed in triplicate and the results represent the mean value from three independent experiments. Statistical differences between each group were determined by Student’s t-test. *: P<0.01.
Fig. 1  Chen et al

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- IκBα
- p-IκBα
- α-tubulin

B

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NF-κB

C

Luciferase Activity (Fold)

- Control
- TNF
- BMP4
- BMP4+TNF
Fig. 2  Chen et al
Fig. 3  Chen et al

(A) DNA fragmentation (OD_{405nm})

(B) DNA fragmentation (OD_{405nm})

(C) Control BMP-2 BMP-4

-TNF  +TNF

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Fig. 4  Chen et al

A

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Caspase-8

tBid
α-tubulin

hr 0 8 16 0 8 16 0 8 16

B

Caspase-8 Activity (fold)

Control BMP TNF TNF+BMP

C

Caspase-3 Activity (fold)

Control BMP TNF TNF+BMP
Fig 5. Chen et al

Panel A: Graph showing cell survival (%) under different conditions: Control, TNF, and TNF+BMP2. The control group shows high cell survival, while the TNF group shows significantly lower survival, and the TNF+BMP2 group shows intermediate survival with a * indicating statistical significance.

Panel B: Images showing cell morphology under different conditions: Vector and SMAD-7. Controls show characteristic cell structure, while BMP + TNF conditions show altered morphology, particularly in SMAD-7.
Fig. 6  Chen et al
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