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Loss of Ikkβ Promotes Migration and Proliferation of Mouse Embryo Fibroblast Cells*

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The IκB kinase complex (IKK) is central to the activation of NF-κB, a critical transcription factor governing expression of genes involved in cell proliferation and anti-apoptotic responses. Mice with genetic disruptions of the Ikkβ or Ikkγ gene loci die during embryogenesis because of severe hepatic apoptosis. We now show that Ikkβ gene deficiency promotes migration and proliferation of mouse embryo fibroblast cells. Morphological analyses revealed an unusual protrusion of the cytoplasm in Ikk$\beta^{-/-}$ cells when cultured at a lower density. In a Boyden chamber assay, Ikk$\beta^{-/-}$ cells exhibited a high rate of invasion and migration. Enhanced formation of actin stress fibers was also observed in the Ikk$\beta^{-/-}$ cells. Mechanistic studies indicated that IKKβ affects the expression of proteins involved in the assembly of cytoskeleton and cell movement. Furthermore, re-expression of Ikkβ and antioxidant treatment in Ikk$\beta^{-/-}$ cells caused a reversal of protrusive phenotype and high motility, respectively. Furthermore, elimination of reactive oxygen species (ROS) blocked expression of snail and subsequently derepressed E-cadherin expression. Although the underlying mechanism is likely entangled and complicated, the data presented indicate that generation of ROS played a key role in the morphological and mobility changes in Ikk$\beta^{-/-}$ cells. These data thus suggest that IKKβ provides inhibitory signals for cell mobility and growth. Deficiency in the Ikkβ gene promotes cell mobilization, at least partially, through a ROS-dependent mechanism.

The IκB kinase (IKK)[3] complex is composed of two kinase subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ/NEMO (1). In addition to these key components, recent studies have indicated association of IKK with cdc37, hsp90 (2), and ELKS, a coiled-coil protein (3). In response to inflammatory signals, the IKK is activated through a Lys63 polyubiquitination of IKKγ/NEMO subunit (4), whereas DNA damaging signals induce monoubquitination of IKKγ/NEMO in an ataxia telangiectasia mutation-dependent manner (5). The activated IKK complex phosphorylates IκBα, leading to its ubiquitination and degradation and subsequent activation of NF-κB (4). In addition, the IKK complex is able to modulate the transcriptional activity of the activated NF-κB in the nucleus by directly phosphorylating NF-κB dimers or recruiting co-factors to the gene promoter region (1). This may also enhance the promoter clearance of certain NF-κB complexes (6). Mice with genetic disruptions in genes encoding key subunits of IKK or NF-κB die during embryogenesis or postnatally because of severe liver failure or other aberrations (7, 8). Cells derived from NF-κB-deficient mice are prone to be apoptotic in response to a variety of stress signals (9–11). Thus, the activation of NF-κB has been generally viewed as a major promoter of cell survival or proliferation by inducing either anti-apoptotic proteins including cIAPs, BclX, XIAP, and Bcl2, or proliferative molecules, such as cyclin D1, CoxII, and interleukin 6 (12). Sustained activation of NF-κB can result in insensitivity to growth inhibition, evasion of apoptosis, angiogenesis, and metastasis that contributes to tumor progression (13). It is not surprising, therefore, that a plethora of endogenous and exogenous molecules that inhibit the activation of NF-κB are being considered as potential chemotherapeutical or chemoprevention agents (14–16).

Thus far, the majority of evidence indicating an anti-apoptotic or carcinogenic role of IKK or its downstream effecter, NF-κB, is from gain- or loss-of-function of molecules important in the activation or activity of NF-κB in human cancer cell lines. In addition to these well characterized growth regulatory roles, it is still debatable whether the IKK-NF-κB pathway can affect other cellular functions in cells that are under a relatively native state. In the present study, we describe a previously undefined role for IKKβ in the regulation of cell morphology and mobilization. We show that loss of the Ikkβ gene in mouse embryonic fibroblasts results in a spindled morphologic change with an enhanced formation of actin stress fibers. The alteration in cell morphology and reorganization of stress fibers may further promote cell migration and invasion.

EXPERIMENTAL PROCEDURES

Cell Morphologic Analysis and Immunoblotting—The mouse embryo fibroblasts derived from wt and Ikk$\beta^{-/-}$ mice within 5–10 passages were cultured in DMEM supplemented with 10% FCS (Invitrogen) as described (9). For cell morphologic analy-

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[3] The abbreviations used are: IKK, IκB kinase; ROS, reactive oxygen species; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; wt, wild type; FITC, fluorescein isothiocyanate; siRNA, small interfering RNA; FAK, focal adhesion kinase; pFAK, phosphorylated form of FAK; EMT, epithelial-mesenchymal transition; JNK, c-Jun N-terminal kinase; NAC, *N*-acetyl-L-cysteine; MMP, matrix metalloproteinase.

sis, the cells were seeded in 6-well tissue culture plates for the indicated time and stained with 0.4% of crystal violet in 10% ethanol for 10 min. The total cell lysates were used to determine the expression of proteins involved in the regulation of cytoskeleton and mobility using the indicated antibodies purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Immunofluorescence—The cells were seeded on glass coverslips in 6-well tissue culture plates and maintained at 37 °C for 24 h. At the end of incubation, the cells were washed twice with phosphate-buffered saline, fixed in 3.7% formaldehyde solution in phosphate-buffered saline for 10 min at room temperature, permeabilized with 0.1% Triton X-100, and stained with FITC-phalloidin (Molecular Probes) in a humidified chamber. The actin structure was analyzed using a Zeiss microscope (Axiovert100) equipped with a Pixera Pro150ES digital camera.

Cell Invasion and Migration—In vitro invasion and migration were determined by a modified Boyden chamber assay using cell culture inserts with a polycarbonate filter coated with Matrigel (BD Biosciences) in a 24-well format. Briefly, 20,000 cells in 250 μl of serum-free DMEM were added to the inserts. The lower chambers were filled with 1% FCS in DMEM. The chambers were incubated at 37 °C in a 5% CO_2 atmosphere for 24 h. The cells that did not migrate through the filter were removed from the insides of the inserts by cotton swab. The cells migrated to the underside of the coated membrane were fixed with 3.7% paraformaldehyde for 20 min, washed three times with phosphate-buffered saline, and then stained with crystal violet. The membranes were cut from the inserts and examined under a microscope.

RNA Interference and Overexpression—To knock down the endogenous Ikkβ mRNA in wt cells, siRNA that targets nucleotides 1559–1577 of the mouse Ikkβ mRNA (GenBank™ accession number: AF088910) was synthesized and high pressure liquid chromatography-purified. The wt cells were seeded in six-well plates on the day before transfection at a concentration of 5×10^5/ml cells. The transfection of siRNA was performed by using RNAiFect reagent (Qiagen) according to the manufacturer's instructions at a final siRNA duplex concentration of 100 nM. Overexpression of Ikkβ in the Ikk$\beta^{-/-}$ cells was performed by transfecting the cells with an expression vector containing a full-length Ikkβ cDNA as previously described (17).

RESULTS

Ikk$\beta^{-/-}$ Cells Show Morphological Changes—Much attention has been focused on the effects of IKK or its downstream target, NF-κB, on cell growth or survival. However, by using mouse embryo fibroblast cells derived from wt and Ikkβ gene knock-out (Ikk$\beta^{-/-}$) mice, we have unexpectedly noted an effect of IKKβ in cell migration and proliferation. WT and Ikk$\beta^{-/-}$ cells show similar morphology when cultured at the regular density (Fig. 1*A*, *left panels*). Surprisingly, a profound change in cell morphology was observed when Ikk$\beta^{-/-}$ cells were cultured at a lower density. As shown in Fig. 1*A*, Ikk$\beta^{-/-}$ cells exhibited an unusual extended phenotype that was distinct from that of wt cells cultured at the same density (Fig. 1*A*, *right panels*). In addition, the majority of Ikk$\beta^{-/-}$ cells displayed exaggerated lamellipodia that are often concurrently observed at the leading edge of cells, some of which form multiple microruffles. In wt cells, the ratio between the long and short axes of the cells was approximately 2:1. This ratio was increased to 4–10:1 in Ikk$\beta^{-/-}$ cells because of the extended protrusion of the cytoplasm.

The morphological difference between wt and Ikk$\beta^{-/-}$ cells might be a result of alteration in cell spreading. To test this, we seeded the cells sparsely and stained the cells at the selected time points. The cytoplasm of wt cells spread evenly around the cell center on the surface of the culture plate at 20, 40, 80, or 240 s (Fig. 1*B*, *upper panels*). These cells form the shape of a "fried egg" at 20, 40, or 80 s of plating. In comparison with wt cells, the spreading of Ikk$\beta^{-/-}$ cells was notably delayed at 20, 40, or 80 s. After 240 s, however, the majority of Ikk$\beta^{-/-}$ cells exhibited an ameboid shape with aberrant large protrusions that were unevenly distributed (Fig. 1*B*, *bottom panels*).

Despite the extended protrusion of the cell membrane, analysis of cell adhesion capacity by a trypsin detachment revealed faster detachment of Ikk$\beta^{-/-}$ cells. Both wt and Ikk$\beta^{-/-}$ cells were seeded in culture plates and kept to confluence. The adhesive capacity of Ikk$\beta^{-/-}$ cells was compared with that of wt cells by incubation of these cells with 0.01% of trypsin. As shown in Fig. 1*C*, Ikk$\beta^{-/-}$ cells display less adhesion than wt cells. Complete detachment of Ikk$\beta^{-/-}$ cells was observed after 3 min of trypsin treatment. In contrast, wt cells appeared to be resistant to trypsin detachment. More than 80 and 50% of wt cells remained attached after 3 and 8 min of trypsin treatment, respectively. Therefore, these data suggested that Ikkβ deficiency weakens the adhesive ability of the cells, which might correlate with an enhanced mobility of these cells (see below).

Enhanced Invasion and Migration in Ikk$\beta^{-/-}$ Cells—The unusual change in the morphology of Ikk$\beta^{-/-}$ cells indicates possible alterations in actin cytoskeleton organization, which provides the protrusive force required for the outgrowth of lamellipodia or microruffles. Therefore, we examined the status of actin stress fibers by staining both wt and Ikk$\beta^{-/-}$ cells with FITC-phalloidin and assessed by fluorescence microscopy. Stress fibers are much less apparent in wt cells (Fig. 2*A*, *upper panels*). In contrast, a substantial enhancement of actin stress fiber formation could be observed in Ikk$\beta^{-/-}$ cells (Fig. 2*A*, *lower panels*). To evaluate formation of actin stress fibers at the level of individual cells, the sparsely seeded cells were stained with FITC-phalloidin and monitored microscopically. Actin stress fibers that were roughly parallel to the long axe of the cell were visible inside Ikk$\beta^{-/-}$ cells, whereas this characteristic was barely seen in wt cells (Fig. 2*A*, *right panels*).

The alterations in cell membrane protrusion, adhesion, and the formation of actin stress fibers in Ikk$\beta^{-/-}$ cells are indicative of an invasive phenotype. Invasive cells are able to penetrate a reconstituted extracellular matrix barrier. In a modified Boyden chamber assay in which the inserted filter membrane was coated with Matrigel, 1% of serum in the lower chamber efficiently promoted migration and invasion of Ikk$\beta^{-/-}$ cells (Fig. 2*B*). Quantification of cell invasion revealed that more than 30% of Ikk$\beta^{-/-}$ cells penetrated through the Matrigel and transferred to the underside of the coated filter (data not shown). Interestingly,

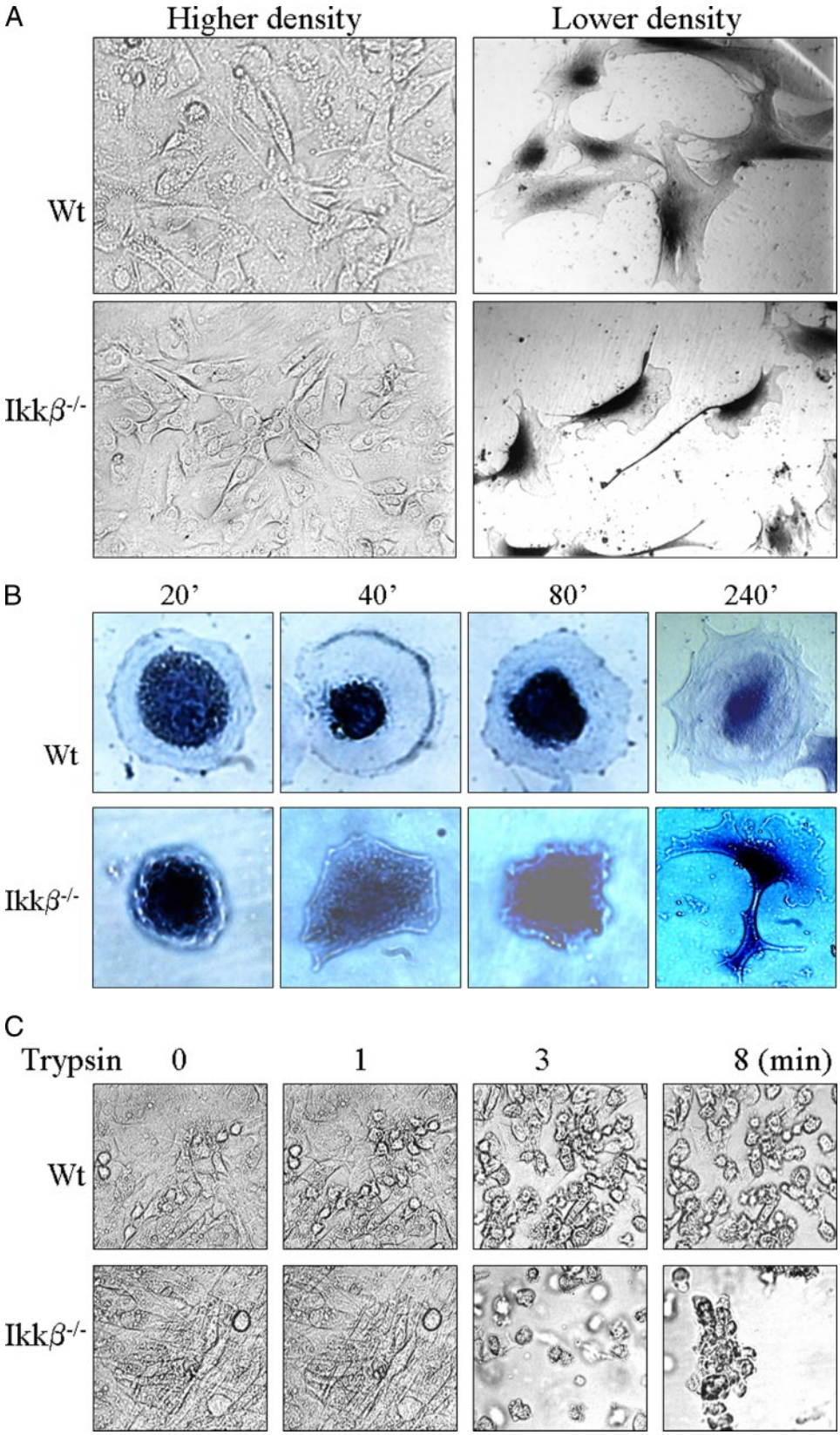

FIGURE 1. **Disruption in the Ikkβ gene changes cell morphology and adhesion.** *A*, wild type (*Wt*) and Ikk$\beta^{-/-}$ cells were seeded in 6-well tissue culture plates at concentrations of 5×10^5/ml (*left panels*) and 1×10^4/ml (*right panels*), respectively, and incubated in 10% FCS for 24 h. The cells were either unstained (*left panels*) or stained with crystal violet as described under "Experimental Procedures" at the end of the culture. *B*, wild type and Ikk$\beta^{-/-}$ cells were seeded sparsely and stained with crystal violet as in *A* at the indicated times. The spreading of the cells was evaluated by microscopy and photography. *C*, both wild type and Ikk$\beta^{-/-}$ cells were seeded in 6-well tissue culture plates at concentrations of 5×10^5/ml and cultured for 48 h to reach confluence. The adhesive capability was determined by incubating the cells with 0.01% of trypsin for the indicated times. All of these photos are representative of at least three experiments.

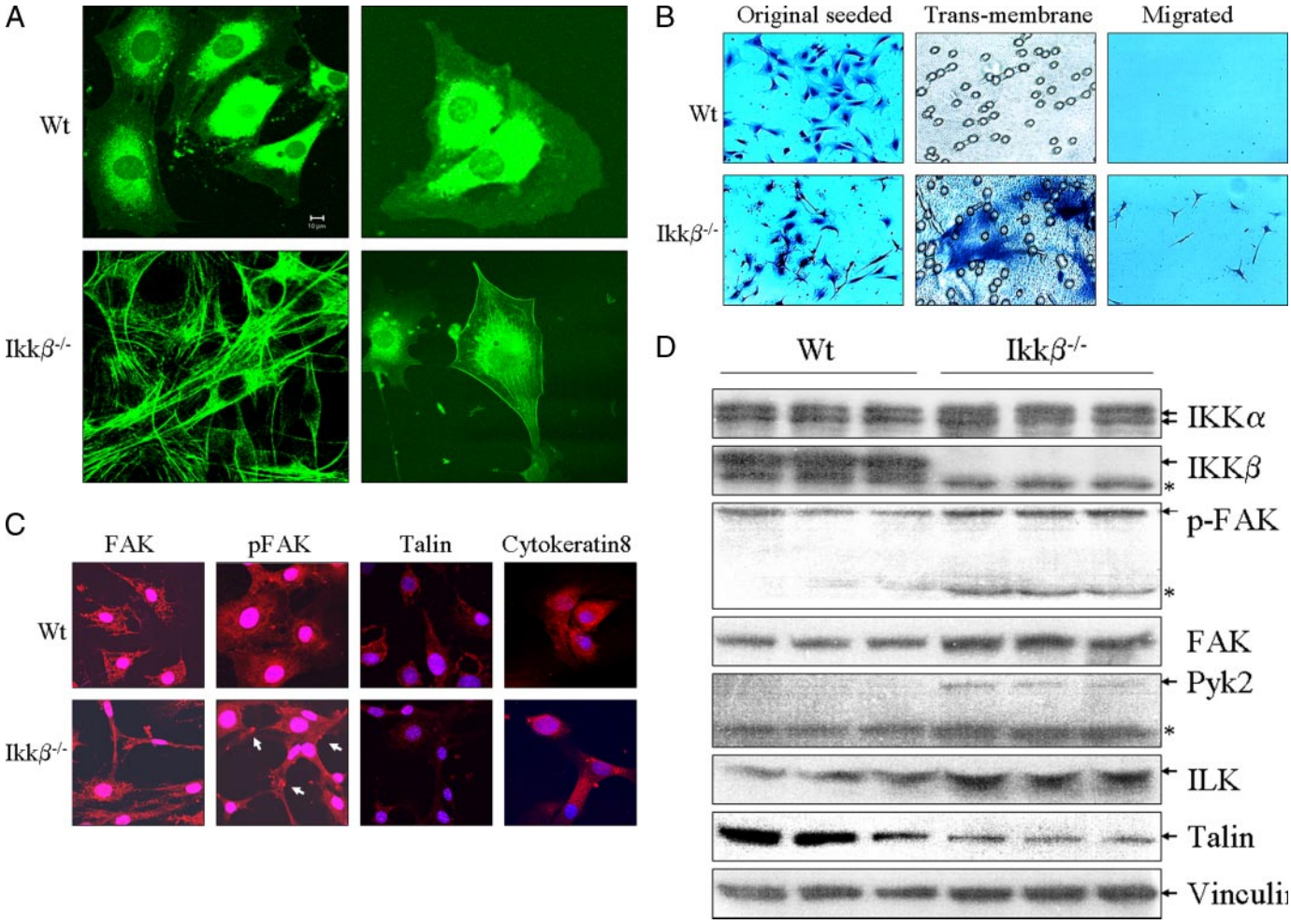

FIGURE 2. **Deficiency in Ikkβ enhances formation of actin stress fibers and cell motility.** *A*, wild type (Wt) and Ikk$\beta^{-/-}$ cells were seeded in coverslides in 6-well tissue culture plates at concentrations of 5×10^5/ml (*left panels*) and 1×10^4/ml (*right panels*), respectively, and incubated in 10% FCS for 24 h. At the end of the culture, the cells were stained with FITC-phalloidin as indicated under "Experimental Procedures." The formation of actin stress fibers was monitored by confocal microscopy. *B*, modified Boyden chamber assay for cell invasion and migration. The *upper chambers* were filled with 20,000 cells in 250 μl of serum-free DMEM. The *lower chambers* were filled with 1% FCS in 250 μl of DMEM. The invasive cells on the underside of the insert (the *blue color* in the *middle panels*) and the cells migrated to the surface of the bottom chambers were stained with crystal violet and photographed. *C*, the expression of total FAK, pFAK, talin, and cytokeratin 8 in both wild type and Ikk$\beta^{-/-}$ cells were determined by immunostaining of the proteins with rhodamine-labeled antibodies and confocal microscopy. *D*, the protein levels of IKKα, IKKβ, pFAK, total FAK, Pyk2, ILK, Talin, and vinculin in the total cell lysates were determined by Western blotting. The *arrows* indicate specific bands. *Asterisk* indicates nonspecific bands. The data are representative of at least three experiments.

~10% of these cells even mobilized through the gap between the filter and the surface of the bottom chamber (Fig. 2*B*, *Migrated*). No appreciable invasion and migration was observed with wt cells, indicating that these cells do not respond to the chemotactic force of the serum (Fig. 2*B*, *Migrated*).

To determine the molecular mechanisms of how the loss of IKKβ affects cell morphology, invasion, and migration, we next investigated the levels of several kinases or proteins that affect the motility and reorganization of cytoskeleton by either fluorescence-based immunostaining or immunoblotting. As can be seen in Fig. 2 (*C* and *D*), although wt and Ikk$\beta^{-/-}$ cells expressed a comparable level of total focal adhesion kinase (FAK) and its phosphorylated form (pFAK), a notable increase of pFAK staining was observed in peripheral focal adhesion sites of Ikk$\beta^{-/-}$ cells (Fig. 2*C*, indicated by *white arrows*). The levels of cytokeratin 8 (Fig. 2*C*) and vinculin (Fig. 2*D*) were equivalent between wt and Ikk$\beta^{-/-}$ cells. However, a decreased expression of talin (Fig. 2, *C* and *D*) and a marginal increase in expression of Pyk2 and ILK were observed in Ikk$\beta^{-/-}$ cells. It has been known that pFAK, Pyk2, and ILK are kinases linked to the cytoskeletal contractile machinery that provides the cells with mobility force. In addition, a globe gene profiling indicated an increased expression of several genes involved in assembly of actin filaments in Ikk$\beta^{-/-}$ cells (Table 1). These genes include cardiac ankyrin repeat protein, protheymosin β4, and tropomyosin 2, which can regulate the remodeling of actin cytoskeleton during cell migration (18), and versican, an anti-cell adhesion molecule (19). In contrast, a more than 100-fold decrease in the expression of IGFBP2, a previously identified NF-κB-regulated gene involved in the inhibition of mitogenesis (20, 21), was observed in Ikk$\beta^{-/-}$ cells. Furthermore, Ikk$\beta^{-/-}$ cells exhibited a decreased expression of genes associated with inhibition of matrix metalloproteinases (TIMP-2 and TIMP-3) and cell adhesion (VCAM and integrin β5) (Table 1). Thus, alterations in expression or activity of these kinases and proteins might explain the higher motility of Ikk$\beta^{-/-}$ cells.

TABLE 1

-Fold changes in genes encoding proteins involved in cell growth and motility in Ikkβ$^{-/-}$ cells compared with wt cells

Gene	Ikkβ$^{-/-}$ *vs.* wt	GenBank^{TM} accession number	Function
CARP	156.3	AF041847	Cardiac ankyrin repeat protein, binding to myopalladin
Versican	9.7	D45889	Anti-cell adhesive molecules
α-Actin	2.6	X13297	Cytoskeleton, stress fibers
Protheymosin β4	2.34	U38967	Prevents actin polymerization
S100A4	2.2	M36579	Tumor metastasis, calcium-binding protein
Tropomyosin2	2.0	M22479	Actin-binding protein
IGFBP2	−134	X81580	Inhibitor of cell mitogenesis
TIMP-2	−5.8	X62622	Inhibitor of matrix metalloproteinases
TIMP-3	−4.0	U26437	Inhibitor of matrix metalloproteinases
VCAM	−2.9	M84487	Cell adhesion
Integrin β5	−2.0	AF022110	Cell adhesion

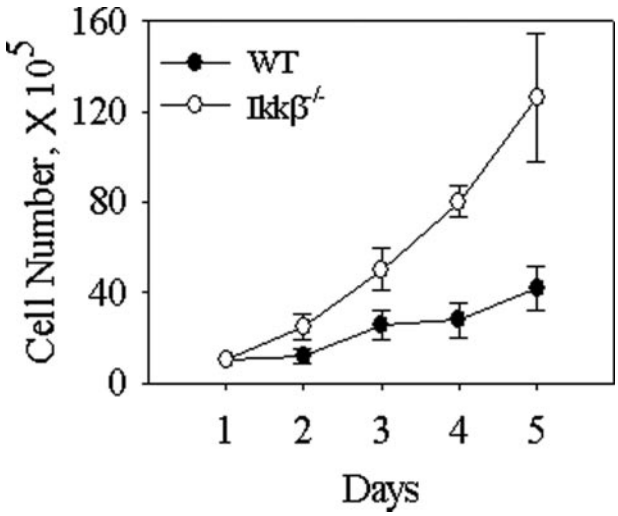

FIGURE 3. **Cell proliferation was enhanced in Ikkβ-deficient cells.** Wild type (*WT*) and Ikkβ$^{-/-}$ cells were seeded in 6-well tissue culture plates at a total cell number of 1 × 10^6/well in 10% FCS. The cells were trypsinized and counted after 2, 3, 4, and 5 days. The data are shown as the means of four wells with standard deviations.

Ikkβ$^{-/-}$ Cells Exhibited a Faster Growth Rate than That of wt Cells—It is believed that deficiency in NF-κB signaling will generally diminish the anti-apoptotic potency of the cells caused by the reduced expression of several pro-survival and anti-apoptotic genes. These genes include bcl-x, c-FLIP, cIAP1, cIAP2, XIAP, Bfl-1, and IEX-1. The product of these pro-survival or anti-apoptotic genes may either directly block caspase activation or provide unique protection of mitochondrial membrane. Indeed, we and others have previously demonstrated that Ikkβ deficiency sensitizes mouse embryo fibroblast cells to apoptotic or necrotic death in response to stress signals (9–11, 17, 22). Unexpectedly, the Ikkβ$^{-/-}$ cells exhibited an ~3–4-fold increase in cell proliferation compared with wt cells when cultured in the normal medium containing 10% FCS (Fig. 3).

IKKβ Inhibition Alters Morphology and Mobility of Human Epithelial Cells—It is unclear whether the increased cell motility and proliferation in Ikkβ$^{-/-}$ cells is a result of IKK-NF-κB deficiency or an artifact of the cellular responses during immortalization of embryonic cells. To address this issue, we stably transfected a human bronchial epithelial cell line (BEAS-2B) with control vector, wild type Ikkβ, or kinase-mutated Ikkβ (Ikkβ-KM). The BEAS-2B cell line is derived from human bronchial epithelial through transformation with an adenovirus-12-SV40 hybrid virus (23). These cells retain morphologic features of epithelial cells without tight junction under normal culture conditions (23). Previous studies indicated substantial inhibition of the endogenous IKKβ kinase and NF-κB activities in the cells stably expressing Ikkβ-KM (17, 22). Transfection of the cells with wild type human Ikkβ cDNA neither changed their morphology nor increased cell invasion/proliferation in a soft agar-based colony formation assay (Fig. 4*A*). However, the cells transfected with a kinase-mutated Ikkβ, Ikkβ-KM, exhibited a much prolonged spindled morphology, a characteristic feature of the cells during epithelial-mesenchymal transition (EMT) in tumorigenesis (24). In addition, the growth of the cells expressing Ikkβ-KM is distinct from the cells transfected with a control vector or wild type Ikkβ vector. The Ikkβ-KM-expressing cells showed an enhanced invasiveness and proliferation as indicated by the formation of giant colonies in soft agar (Fig. 4*A*, *bottom panels*). Because the BEAS-2B cell line was established by a virus-mediated transformation, these cells showed basal activity of migration in a Boyden chamber assay (Fig. 4*B*). As seen in Ikkβ$^{-/-}$ mouse embryonic fibroblasts, the BEAS-2B cells stably expressing Ikkβ-KM exhibited an enhanced motility in a migration assay, whereas expression of the wild type Ikkβ neither enhances nor reduces such activity in both Boyden chamber and wound healing assays (Fig. 4, *B* and *C*). Thus, these data provide complementary evidence indicating that Ikkβ deficiency does affect the cell morphology, enhance cell growth, and promote cell migration. In addition, the results also suggest that the observed effects may not be restricted to mouse embryo fibroblast cells.

Re-expression of Ikkβ Reversed Morphological Changes in Ikkβ$^{-/-}$ Cells—The influence of Ikkβ on the cell morphology was further determined by siRNA-based silencing of the endogenous Ikkβ mRNA in wt cells or transfection of Ikkβ$^{-/-}$ cells with an expression vector containing wild type Ikkβ cDNA. Ikkβ siRNA silenced Ikkβ in ~40–50% of wt cells as determined by a fluorescent staining for IKKβ protein. Although there was no remarkable morphological change in these cells where Ikkβ was silenced by Ikkβ siRNA, the majority of Ikkβ-silenced cells exhibited an increased ratio between the long and short axes (pointed by *red arrows* in Fig. 5*A*). In Ikkβ$^{-/-}$ cells, re-expression of Ikkβ partially reversed the protrusive phonotype in ~20% of the cells (Fig. 5*B*, *Ikkβ-transfected panel*).

Reactive Oxygen Species Mediate Morphological and Mobility Changes in Ikkβ$^{-/-}$ Cells—Recent studies demonstrated that deficiency in Ikkβ or NF-κB relA gene caused a spontane-

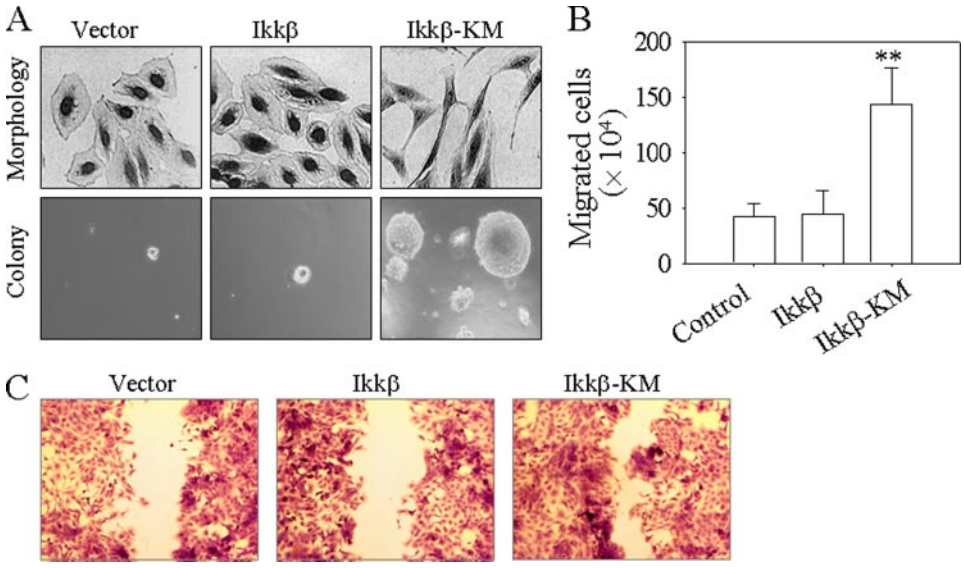

FIGURE 4. **IKKβ inhibition changes morphology and mobility of the human epithelial cells.** *A*, the human bronchial epithelial cell line, BEAS-2B, was transfected with a control vector, wild type Ikkβ, or a kinase-mutated Ikkβ (Ikkβ-KM). The cell morphology was determined by staining of the cells with crystal violet (*upper panels*). The *lower panels* show formation of colonies of the indicated cells cultured in soft agar for 10 days. *B* and *C*, the mobility of the BEAS-2B cells transfected with the indicated vectors was determined by both Boyden chamber assay (*B*) and wound healing assay (*C*).

ous elevation of ROS, especially H_2O_2, and a sustained stress-induced activation of c-Jun N-terminal kinase (JNK) (9, 25–27). This increase in ROS generation might be a result of either enhanced expression of cytochrome P-450 1b1 (9) or a decrease in the expression of an antioxidant protein, ferritin heavy chain (28). Thus, we postulated that the increased invasion and migration of the Ikk$\beta^{-/-}$ cells might be a result of oxidative stress or JNK activation. Analysis of H_2O_2 generation confirmed our previous findings, which showed that Ikk$\beta^{-/-}$ cells are ~2–3-fold more potent than wt cells in H_2O_2 generation, and this correlates with the increased expression of cyp1b1, a P-450 family member involved in the cellular oxidative stress responses (Fig. 5, *C* and *D*). Conversely, the Ikk$\beta^{-/-}$ cells are less efficient at migration when they were treated with 20 mM *N*-acetyl-L-cysteine (NAC) for 12 h (Fig. 5*E*). However, the JNK inhibitor (SP600125) failed to inhibit the migration of these Ikk$\beta^{-/-}$ cells, despite the fact that previous studies suggested a prolonged activation of JNK in Ikk$\beta^{-/-}$ cells.

The alterations in cell morphology and mobility of the Ikk$\beta^{-/-}$ cells resemble the processes of EMT during tumorigenesis or embryogenesis (24). The key molecules regulating EMT include the transcription factor snail and the tight junction protein E-cadherin, which are inversely correlated. To determine whether deficiency in Ikkβ affects these EMT markers, we measured the protein levels of snail and E-cadherin, respectively. Comparing to wt cells, the Ikk$\beta^{-/-}$ cells exhibited an increased expression of snail and a decreased expression of E-cadherin (Fig. 5*F*). Treatment of the Ikk$\beta^{-/-}$ cells with antioxidant, NAC, reduced the level of snail protein and reversed the inhibition of E-cadherin expression in a dose-dependent manner (Fig. 5*F*). Thus, these data clearly suggested to us that ROS played a central role in mediating Ikkβ deficiency-induced changes in cell migration through affecting the levels of snail and, subsequently, the expression of E-cadherin.

DISCUSSION

We observed that Ikkβ deficiency promotes morphological changes and enhances cells migration and invasion, which might result from the accumulation of ROS. The Ikk$\beta^{-/-}$ cells also exhibited an increased expression of snail, a transcriptional repressor of E-cadherin. Elimination of ROS by supplying the cells with NAC reduced Ikk$\beta^{-/-}$ cell migration significantly, possibly through blocking snail expression and subsequently, derepressing the expression of E-cadherin. Inhibition of JNK, on the other hand, appears to be unable to suppress the increased migration rate of the Ikk$\beta^{-/-}$ cells. This was not surprising because an enhanced activation of JNK in Ikk$\beta^{-/-}$ cells could be seen only in cellular response to stress but not under the basal condition (9). An alternative explanation for this is that JNK might be a minor rather than a major factor contributing to the mobilization of the Ikk$\beta^{-/-}$ cells, although involvement of JNK in cytoskeletal reorganization had been demonstrated in some other experimental systems (29).

The most important function of IKKβ is its kinase activity for the activation of NF-κB transcription factor. Indeed, a substantial decrease in the basal NF-κB activation was observed in Ikk$\beta^{-/-}$ cells (30). It was very likely, therefore, that the observed changes in cell motility and proliferation of Ikk$\beta^{-/-}$ cells were due to impaired basal activation of NF-κB. Several lines of evidence support this possibility. First, both Ikk$\beta^{-/-}$ and NF-κB relA$^{-/-}$ cells showed an enhancement of ROS generation spontaneously or in response to stress (9, 27). ROS are potent activators of receptor and nonreceptor tyrosine kinases required for the assembly of actin filaments and the cell motility. ROS are also tumorigenic by virtue of their ability to induce genetic lesions and cell proliferation (31). Second, a decreased expression in IGFBP2, TIMPs, and cell adhesion moleculars in Ikk$\beta^{-/-}$ cells (Table 1) is in agreement with earlier reports suggesting NF-κB-dependent expression of IGFBP2, TIMP-3, integrin, and VCAM (20, 21, 32–34). Because IGFBP2 is a key component of the major inhibitory factors of cell mitogenesis, decreased expression of this protein may help in explaining the faster proliferation rate of the Ikk$\beta^{-/-}$ cells. Lastly, cells derived from NF-κB relA$^{-/-}$ mice exhibited a spindled morphology and transformative phenotype (35, 36), which morphologically resembles Ikk$\beta^{-/-}$ cells as presented in this report. However, it will be too simple to attribute all of these changes in Ikk$\beta^{-/-}$ cells to the lack of basal NF-κB activity. Some alternations, such as the increased expression of cardiac ankyrin repeat protein and actin filament-regulating proteins, cannot be explained by the impairment of NF-κB. In addition, IKKβ might modulate cellular responses in a NF-κB-independ-

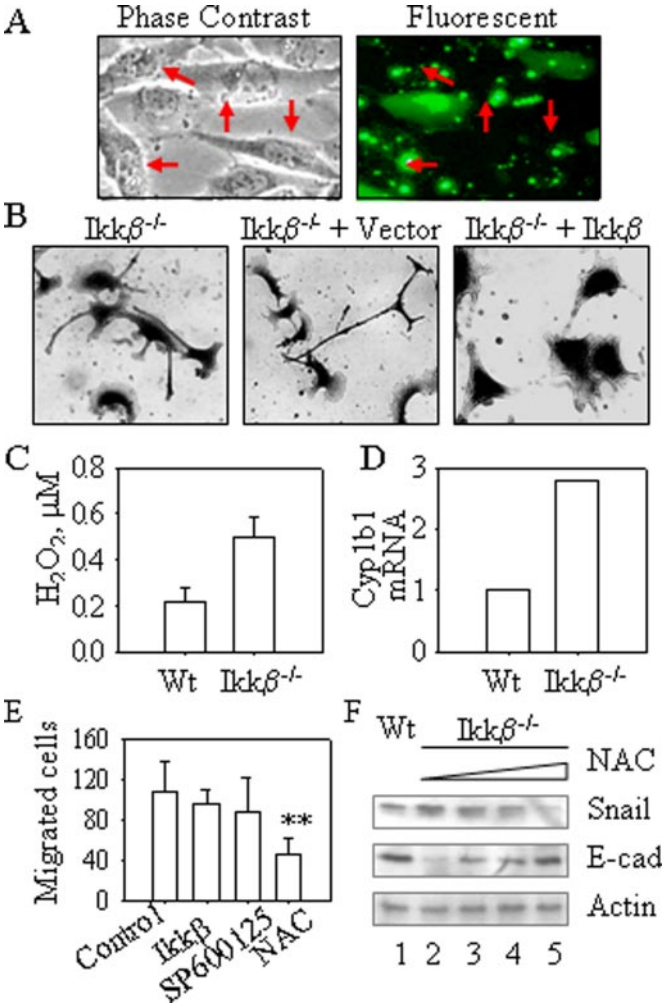

FIGURE 5. **Ikkβ affects cell morphology through reactive oxygen species.** *A*, wild type cells were transfected with siRNA targeting mouse Ikkβ and cultured for 24 h. The level of IKKβ expression was then determined using anti-IKKβ antibody and FITC-labeled second antibody. The stained cells were photographed by fluorescent microscopy. The *arrows* indicate cells where the expression of IKKβ was silenced by siRNA. *B*, reintroduction of Ikkβ by transient transfection of the cells with an Ikkβ expressing vector reverses protrusive morphology. Cell morphology was determined by staining the cells with crystal violet. The data are representative of three experiments. *C*, the level of H_2O_2 in cell culture supernatants of the wild type (*Wt*) and Ikk$\beta^{-/-}$ cells was estimated colorimetrically using H_2O_2 detection kit. *D*, relative level of cyp1b1 mRNA in wild type and Ikk$\beta^{-/-}$ cells. *E*, the migration of the Ikk$\beta^{-/-}$ cells was determined after the cells transfected with Ikkβ for 48 h, 10 μM SP600125, or 20 mM NAC for 12 h. The data show the means of three experiments with standard deviation. The *asterisks* indicate statistically significance with a p value < 0.05. *F*, the protein levels of snail and E-cadherin were determined in wild type cells and Ikk$\beta^{-/-}$ cells by immunoblotting. To determine the role of ROS on the expression of snail and E-cadherin in Ikk$\beta^{-/-}$ cells, the cells were incubated with 10, 20, or 30 mM NAC (*lanes 3–5*).

ent manner. Evidence supporting this hypothesis is from the fact that IKKβ can directly phosphorylate 14-3-3β (37), dok1 (38), Forkhead transcription factor FoxO3a (39), and the interferon regulatory factor 7 (40). These additional substrates of IKKβ might be important in the regulation of cell proliferation and/or motility.

The appearance of the Ikk$\beta^{-/-}$ cells in morphology and mobility is similar to the cells undergoing EMT during tumorigenesis (24). The mesenchymal cells generally have a more extended and elongated shape without regimented structure or tight intercellular adhesion. Because of a weakened cell-to-cell or cell-to-matrix adhesion, the mesenchymal cells are more motile and invasive than their epithelial counterparts. Thus, the EMT has been considered as a key step in tumor metastasis. Although a number of molecules, including snails, cadherins, and vimentin, have been implicated as critical regulators of EMT, a detailed signaling pathway of EMT has not been fully elucidated so far. A major breakthrough in understanding the molecular mechanisms of EMT was made recently by demonstrating the contribution of ROS to EMT in mammary epithelial cells (41). Treatment of the cells with matrix metalloproteinase-3 (MMP-3) induces expression of an alternatively spliced Rac1, Rac1b, leading to intracellular accumulation of ROS. Blockade of ROS by SOD2 or NAC inhibited MMP-3-induced cell motility, invasion, and morphological changes (41). Accordingly, the data in this report are consistent with these findings, suggesting that a rise in intracellular ROS levels mediates changes of morphology, mobility, and the expression of snail and E-cadherin in the cells where IKKβ or NF-κB is deficient. Furthermore, evidence complementarily supporting this notion is the observation of a diminished expression of TIMP-2 and TIMP-3 in Ikk$\beta^{-/-}$ cells (Table 1). Both TIMP-2 and TIMP-3 are major inhibitors for MMP-3 in a given cell or tissue. It is anticipatable, therefore, that a decrease in TIMPs will result in an elevation of MMP-3 activity, leading to an excessive generation of ROS.

From the long list of its target genes to the unprecedented role in cell migration and proliferation, the effect of IKK-NF-κB in cellular behaviors is still somehow controversial. Sustained activation of NF-κB by a ubiquitous deletion of IκBα in mice causes premalignant hematopoietic proliferation (42). Deletion of Ikkβ in intestinal epithelial cells leads to a dramatic decrease in the incidence of inflammation-dependent adenomas (43). In collaboration of the Ha-Ras oncoprotein and transforming growth factor-β, NF-κB appears to be a key factor for the induction and maintenance of EMT during mammary carcinogenesis (44). In numerous cellular experimental systems, unequivocal evidence indicates that IKK-NF-κB pathway promotes tumorigenesis or oncogenesis (44–47). Thus, the data presented in this report are somehow provocative to the established role of IKK or NF-κB played in the development of the human cancers. The protrusive and invasive phenotype of the Ikk$\beta^{-/-}$ cells implied a possible suppressive effect of Ikkβ or its downstream target, NF-κB, on tumor cell proliferation and metastasis. A similar conclusion had been made earlier from studying relA$^{-/-}$ epidermal cells, which showed that NF-κB antagonized proliferative signals derived from TNFR1 and JNK (48). Congruously, inhibition of NF-κB in the murine epidermis by either targeted overexpression of a super-repressor form of IκBα or pharmacological agent induced epidermal hyperplasia or squamous cell carcinomas (49, 50). In human keratinocytes, NF-κB appeared to be a repressor for the cell cycle transition because blockade of NF-κB produced large and invasive neoplasms resembling human squamous cell carcinomas (51). Most recently, a study using mice with hepatocyte cell-specific deletion in the Ikkβ gene demonstrated that lacking Ikkβ in hepatocytes promotes chemical carcinogen-induced hepatocarcinogenesis (25). Therefore, a careful re-evaluation is certainly required to define the role of NF-κB on the various stages of malignant transformation and tumorigenesis.

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Loss of Ikkβ Promotes Migration and Proliferation of Mouse Embryo Fibroblast Cells

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