NF-κB is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression

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The transcription factor NF-κB is activated in a range of human cancers and is thought to promote tumorigenesis, mainly due to its ability to protect transformed cells from apoptosis. To investigate the role of NF-κB in epithelial plasticity and metastasis, we utilized a well-characterized in vitro/in vivo model of mammary carcinogenesis that depends on the collaboration of the Ha-Ras oncoprotein and TGF-β. We show here that the IKK-2/1xBo/NF-κB pathway is required for the induction and maintenance of epithelial-mesenchymal transition (EMT). Inhibition of NF-κB signaling prevented EMT in Ras-transformed epithelial cells, while activation of this pathway promoted the transition to a mesenchymal phenotype even in the absence of TGF-β. Furthermore, inhibition of NF-κB activity in mesenchymal cells caused a reversal of EMT, suggesting that NF-κB is essential for both the induction and maintenance of EMT. In line with the importance of EMT for invasion, blocking of NF-κB activity abrogated the metastatic potential of mammary epithelial cells in a mouse model system. Collectively, these data provide evidence of an essential role for NF-κB during distinct steps of breast cancer progression and suggest that the cooperation of Ras- and TGF-β–dependent signaling pathways in late-stage tumorigenesis depends critically on NF-κB activity.

Introduction
Cancer development and metastasis are multistep processes that involve local tumor growth and invasion followed by dissemination to and re-establishment at distant sites. The ability of a tumor to metastasize is the major determinant of the mortality of cancer patients. Thus, elucidating the molecular pathways essential for tumor metastasis is of high priority in cancer biology and provides a basis for novel therapeutic targets for the development of anti-metastatic cancer treatments.

Initially discovered and studied as a major activator of immune and inflammatory functions via its ability to induce expression of genes encoding cytokines, cytokine receptors, and cell-adhesion molecules, the transcription factor NF-κB has recently been implicated in the control of cell proliferation and oncogenesis (reviewed in ref. 1). NF-κB transcription factors bind to DNA as hetero-or homodimers that are composed of five possible subunits in mouse and human (RelA/p65, c-Rel, RelB, p50, and p52). These proteins are characterized by their Rel homology domains, which mediate DNA binding, dimerization, and interactions with inhibitory factors known as inhibitor κB (IκB) proteins. Whereas the Rel/p65 and p50 subunits are ubiquitously expressed, the p52, c-Rel, and RelB subunits are more functionally important in specific differentiated cell types (reviewed in ref. 2). In most unstimulated cells, NF-κB dimers are inactive because of association with IκB proteins that mask the nuclear localization sequence of NF-κB, thereby retaining it in the cytoplasm and preventing DNA binding. Several IκB proteins are involved in the control of NF-κB activity: three of which (IκBα, IκBβ, and IκBε) act as negative regulators in a stimulus-dependent manner. Stimulation of cells, for example, by proinflammatory cytokines such as TNF-α and IL-1, results in the phosphorylation of IκB at two serine residues located within the N-terminal domain of the proteins (reviewed in refs. 3, 4). This phosphorylation of IκB results in ubiquitination of nearby lysine residues, which releases the signal for degradation by the 26S proteasome. Degradation of the IκB proteins results in the liberation of NF-κB, allowing nuclear translocation and binding to cognate DNA motifs in the regulatory regions of a host of target genes. As a consequence, transcription of these genes, which are involved in immune and inflammatory responses and regulation of apoptosis, as well as in cell growth control, is induced (reviewed in refs. 3, 4). The critical step in NF-κB activation is the phosphorylation of IκB by a high-molecular-weight IκB kinase (IKK) complex. This complex consists of two kinase proteins, IKK-1 and IKK-2, (also called IκKα and IκKβ, respectively), as well as a regulatory component called NF-κB essential modulator (NEMO; also called IκKγ) (reviewed in ref. 4).

Ample evidence linking NF-κB activity to oncogenesis has accumulated in the past years (reviewed in refs. 1, 2). A link between aberrant NF-κB activity and cancer was initially suggested by the identification of v-Rel, a viral homolog of c-Rel, as the transforming oncogene of an avian retrovirus that causes aggressive tumors in chickens (5). Moreover, oncogenic viruses, such as human T cell leukemia virus I or Epstein-Barr virus, activate NF-κB as part of the transformation process (6, 7). Translocation of the NF-κB gene NF-κB2/p52 and the IκB family member Rel-3 was observed in some lymphoid neoplasms (reviewed in ref. 8). High levels of NF-κB were shown to be essential for the transformed phenotype

Nonstandard abbreviations used: benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK); constitutively active (CA); electrophoretic mobility-shift assay (EMSA); epithelial-mesenchymal transition (EMT); IκB kinase (IKK); inhibitor κB (IκB); NF-κB essential modulator (NEMO); TGF-β1–activated kinase 1 (TAK1); trans-dominant (TD).

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NF-κB activity is induced during EMT. (A) Schematic illustrates the morphology and epithelial/mesenchymal marker redistribution or expression found in the cell types used in our study. Nontransformed EpH4 mammary epithelial cells were stably transfected with the Ha-Ras oncogene to yield transformed epithelial EpRas cells that undergo EMT upon treatment with TGF-β, resulting in mesenchymal EpRasXT cells further stabilized by an autocrine TGF-β loop. DPP-IV, dipeptidyl peptidase IV; ZO-1, zona occludens 1. (B) EMSAs of whole-cell extracts (6 μg) of exponentially growing EpRas and EpRasXT cells were performed with an NF-κB–specific probe (upper panel) and with an octamer-specific probe (Oct; lower panel) used as a control. Quantified relative DNA-binding levels are indicated below the EMSAs. Similar data were obtained using different protein extract preparations (see also Figure 2A).

Figures 1

Table 1

NF-κB–regulated genes induced during EMT in the EpRas/EpRasXT cell pair

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Fold change</th>
<th>Category</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vimentin*</td>
<td>141.9</td>
<td>Mesenchymal marker</td>
<td>50</td>
</tr>
<tr>
<td>Placental proliferin 2</td>
<td>108.3</td>
<td>Hormone</td>
<td>51</td>
</tr>
<tr>
<td>Cholecystokinin*</td>
<td>38.6</td>
<td>Hormone</td>
<td>52</td>
</tr>
<tr>
<td>JE/MCP-1*</td>
<td>32.6</td>
<td>Chemokine</td>
<td>53</td>
</tr>
<tr>
<td>MMP-13</td>
<td>16.6</td>
<td>Protease</td>
<td>54</td>
</tr>
<tr>
<td>Tenasin-C*</td>
<td>11.0</td>
<td>Mesenchymal marker</td>
<td>55</td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>10.8</td>
<td>Protease</td>
<td>51</td>
</tr>
<tr>
<td>Stat-1</td>
<td>6.8</td>
<td>Transcription factor</td>
<td>56</td>
</tr>
<tr>
<td>MMP-2</td>
<td>6.3</td>
<td>Protease</td>
<td>57</td>
</tr>
<tr>
<td>β2-Microglobulin*</td>
<td>5.7</td>
<td>Binds MHC class I</td>
<td>58, 59</td>
</tr>
<tr>
<td>Interleukin 11*</td>
<td>5.1</td>
<td>Cytokine</td>
<td>60</td>
</tr>
<tr>
<td>Cathepsin Z</td>
<td>4.1</td>
<td>Protease</td>
<td>51</td>
</tr>
<tr>
<td>KC/Gro1*</td>
<td>3.1</td>
<td>Chemokine</td>
<td>61</td>
</tr>
</tbody>
</table>

Data are based on the expression profiling analysis described in ref. 18. *Genes previously described to contain functional NF-κB–binding sites in their promoter/enhancer regions are indicated with an asterisk (*); genes lacking asterisks have also been described as genes regulated by NF-κB, but either they are indirectly regulated or further experiments are required to demonstrate direct regulation. **Fold change indicates fold upregulation of gene expression in EpRasXT versus EpRas cells (see Table 2 in ref. 18). References cite the first publication reporting regulation of gene in question by NF-κB.
Results

Upregulation of NF-κB signaling during EMT. EpRas cells represent oncogenic, fully polarized, Ha-Ras–transformed EpH4 mammary epithelial cells that undergo EMT in response to TGF-β both in tumors as well as in collagen gels, giving rise to mesenchyme-like cells (EpRasXT) in both cases. EpRasXT cells are characterized by a spindle-like morphology and gain of mesenchymal marker proteins, a phenotype stabilized by an autocrine TGF-β loop in vitro and in vivo (refs. 19, 20; see Figure 1A). We wanted to determine whether NF-κB might play a role in the EMT process. We therefore analyzed whole-cell extracts from exponentially growing EpRas and EpRasXT cells by electrophoretic mobility-shift assay (EMSA). We detected some NF-κB DNA-binding activity in EpRas cells even without stimulation by known inducers of NF-κB and consistently observed a 3- to 4-fold increase in NF-κB DNA-binding activity in EpRasXT cells (Figure 1B).

Based on those observations, we next asked whether NF-κB target genes were induced in mesenchymal EpRasXT cells to the same degree as their epithelial counterpart, EpRas cells. To address this question, we reanalyzed the data from a previously reported expression profile, in which we had performed microarray analyses of polysome-bound mRNAs to identify genes differentially expressed in EpRasXT compared with EpRas cells (Tables 2 and 3 in ref. 18). Interestingly, 13 of the 75 annotated genes upregulated in EpRasXT compared with EpRas cells (Tables 2 and 3 in ref. 18) were transcriptionally active. EpRas cells were transiently transfected in triplicate with a 3xκB.luc or β-globin-TATA reporter construct. At 20 hours after transfection, cells were treated with TGF-β1 (5 ng/ml) for the indicated times. Then, the luciferase activities of extracts were determined and were normalized based on Renilla luciferase expression. The ratio of 3xκB.luc and β-globin-TATA is shown. The expression level of unstimulated empty vector-infected EpRas cells was used as the reference luciferase activity and was arbitrarily set to 1. Means and standard deviations are representative of two independent experiments carried out in triplicate. Bars represent standard deviations.

NF-κB is essential for EMT. To study the contribution of the IKKβ/IκBβ/NF-κB signaling module in the regulation of EMT and metastasis, we used retroviral gene transfer to express dominant interfering mutants of this pathway in EpRas cells. Infections were performed using retroviruses expressing a trans-dominant (TD) IκBβ protein (TD-IκBβ), in which serine residues at positions 32 and 36 are mutated to alanine residues, resulting in a nondegradable repressor, a constitutively active (CA) IκK-2 protein (CA-IKK-2, in which two serine residues in the activation loop are mutated to phosphomimetic glutamic acid residues), or an empty vector control. Stably infected cells were visualized by immunofluorescence microscopy, as the retroviruses coexpress enhanced GFP. Expression of TD-IκBβ and CA-IKK-2 in cells from the φNX producer line and in stably infected EpRas cells was

Figure 2
TGF-β induces NF-κB activity in EpRas cells. (A) EpRas and EpRasXT cells were stimulated with TGF-β1 (5 ng/ml) for the indicated times. EMSA with whole-cell extracts (6 μg) was performed with an NF-κB–specific probe (upper panel) and with an octamer-specific probe (lower panel) used as a control. (B) NF-κB transcriptional activity. EpRas cells were transiently transfected in triplicate with a 3xκB.luc or β-globin-TATA reporter construct. At 20 hours after transfection, cells were treated with TGF-β1 (5 ng/ml) for the indicated times. Then, the luciferase activities of extracts were determined and were normalized based on Renilla luciferase expression. The ratio of 3xκB.luc and β-globin-TATA is shown. The expression level of unstimulated empty vector-infected EpRas cells was used as the reference luciferase activity and was arbitrarily set to 1. Means and standard deviations are representative of two independent experiments carried out in triplicate. Bars represent standard deviations.
assessed by Western blot (Figure 3, A and B). This analysis revealed strong overexpression of the mutant proteins compared with that of the endogenous counterparts. As previously observed in another cellular system (29), the presence of high levels of exogenous TD-IkBα resulted in reduced expression of endogenous IκBα, most likely due to inhibition of NF-κB activity. The effects of TD-IkBα and CA-IKK-2 expression in EpRas cells on NF-κB DNA-binding activity were analyzed by EMSA. In EpRas cells infected with TD-IkBα, no NF-κB DNA-binding activity was observed, regardless of whether cells were left unstimulated or were stimulated with TGF-β (Figure 3C), TNF-α, or PMA (data not shown). In contrast, cells infected with CA-IKK-2 exhibited 2- to 3-fold increased DNA-binding activity in the unstimulated state (compared with unstimulated EpRas control cells), and showed a roughly 2-fold higher induction of NF-κB activity after being stimulated with TGF-β, compared with TGF-β–treated EpRas control cells. Transient transfection of these cells with 3xκB-luc and subsequent luciferase assays revealed a 3- to 4-fold induction of luciferase activity in untreated EpRas cells infected with CA-IKK-2 and a more than 2-fold higher induction upon treatment with TGF-β, compared with that of unstimulated or TGF-β–treated EpRas control cells, respectively. In contrast, NF-κB transcriptional activity was virtually completely inhibited before and after treatment with TGF-β in EpRas cells expressing TD-IkBα (Figure 3D). In addition, RT-PCR analysis of a subset of NF-κB–regulated target genes that are associated with EMT (Table 1) (18) was performed with the EpRas mutants before and after TGF-β–induced EMT. This analysis showed induction of MMP-13, MCP-1, cholecystokinin, and β-actin, as described in Methods.
shaped, vimentin-positive cells only weakly expressing E-cadherin (Figure 4, A, B, and D). No phenotypic changes or changes in marker expression compared with EpRas control cells were observed in TD-IκBα-expressing cells in the absence of TGF-β (Figure 4, A–D). Upon treatment with TGF-β, however, a considerable proportion of these TD-IκBα-overexpressing EpRas cells with blocked NF-κB activity rapidly detached from porous supports as a consequence of cell death. The remaining cells almost completely failed to undergo EMT (Figure 4A). TGF-β–treated TD-IκBα–expressing EpRas cells lacked the strands of spindle-shaped mesenchymal cells that were abundant in empty virus–infected EpRas control cultures (Figure 4, A and B). The same cells failed to upregulate vimentin and retained high levels of E-cadherin, which was partially redistributed to the cytoplasm, indicating some loss of polarity (Figure 4D). Surprisingly, CA–IKK-2–overexpressing EpRas cells with increased NF-κB activity were able to undergo EMT at a considerable rate even in the absence of TGF-β (Figure 4, A and B). After cells had grown for 6 days on porous support, we observed strands of spindle-shaped, E-cadherin–negative and vimentin-positive cells that covered more than 10% of the total surface area (Figure 4, A, B, and D). When we analyzed bulk cultures of these cells by Western immunoblot, we observed a strong reduction in E-cadherin levels (Figure 4C). Consistent with these observations, we noted enhanced EMT in CA–IKK-2–expressing cells upon TGF-β treatment compared with that of control EpRas cells, as indicated by a higher percentage of spindle-shaped cells with cytoplasmic (depolarized) or no E-cadherin expression, and strong vimentin expression (Figure 4, A, B, and D). Importantly, very similar results were obtained when the CA-IKK-2 and TD-IκBα transgenes were introduced into V12S35Ras cells. These cells represent another
monogenic transformed cell line, independently generated from the original EpH4 cells and transformed by an effector mutant of a different oncogenic Ras that induces hyperactive ERK/MAPK but not PI3K signaling (17). We observed an inhibition of EMT in TD-\(\kappa\)B\(\alpha\)-expressing cells and a considerable rate of spontaneous EMT in the absence of TGF-\(\beta\) in CA–IKK-2–expressing V12S35Ras cells that occurred even more frequently than in CA–IKK-2–expressing EpRas cells (see Supplemental Figure 1; supplemental material available at http://www.jci.org/cgi/content/full/114/4/569/DC1). Thus, the role of NF-\(\kappa\)B in the regulation of EMT is not limited to a single cell line.

A more physiological culture system to analyze epithelial cell behavior and plasticity are three-dimensional serum-free collagen I gel cultures (15). In these cultures, fully polarized EpRas control...
cells form tubular and alveolar structures with large lumina (Figure 5A). These cells show basolateral membrane (polarized) expression of E-cadherin, but no vimentin expression (Figure 5C). Upon addition of TGF-β1, EpRas control cells underwent EMT, as shown by a spindle-shaped and migratory phenotype, loss of E-cadherin, and de novo expression of vimentin after 6 days of treatment (Figure 5, A and C). Untreated TD-IκBα cells resembled control EpRas cells in that they formed epithelial structures (tubular structures with lumina) and showed basolateral E-cadherin staining and no vimentin expression. Despite this resemblance, TD-IκBα epithelial structures appeared more compact and smaller in size (Figure 5, A and C). Upon treatment with TGF-β, epithelial structures formed

Figure 6
Suppression of NF-κB activity in EpRas cells leads to apoptosis and prevents EMT. (A) EpRas cells expressing the empty vector control, TD-IκBα, or CA-IKK-2 were seeded into collagen gels, were allowed to form structures for 3 days, and were treated with TGF-β for 6 days or were left untreated. Collagen cultures were subjected to in situ TUNEL staining (red) and DAPI staining (blue, indicating living cells). Light microscopy images of collagen gels from the same experiment photographed prior to TUNEL staining are also shown. Arrows indicate TUNEL-positive nuclei. Inset, right middle panel: another structure with TUNEL-positive nuclei. Original magnification, ×200. (B) Quantification of TUNEL-positive cells from collagen gel structures shown in A. TUNEL staining of nuclei was assessed in at least 300 cells from three to six randomly chosen fields. The average from two to three collagen gels was used to calculate the apoptotic index and standard deviation. Average percentage of apoptotic cells, assessed in 2–3 collagen gels (at least 300 cells per gel) for each cell type, are indicated above bars. (C) EpRas cells expressing the empty vector control or TD-IκBα were cultivated for 5 days on porous support in the presence or absence of 25 μM Z-VAD-FMK (Z-VAD; days 0–5) and/or TGF-β (5 ng/ml; days 1–5). The quantification of the area on porous support covered by mesenchymal strands is shown as the percentage relative to the total area covered by adherent cells (at day 4, after 3 days with or without TGF-β treatment). (D) Cells as indicated (with or without 25 μM Z-VAD-FMK; days 0–5) were cultivated on porous support for 5 days in the presence or absence of TGF-β (5 ng/ml; days 1–5). Cells were immunostained for E-cadherin or vimentin (red) plus DAPI counterstaining for DNA (blue) after 5 days of culture on porous support. Original magnification, ×400.
by TD-IkBα–expressing EpRas cells retained E-cadherin expression (Figure 5C), but rapidly disintegrated (Figure 5, A and C). Only a very small fraction of the structures (about 0.5%) formed unordered cell strands before disintegration (data not shown). In contrast, untreated CA–IKK-2–expressing cells with increased NF-κB activity formed two types of structures. While a large proportion of epithelial tubular structures with lumina were apparent (Figure 5A), a significant number of the structures consisted of unordered cell strands with spindle-like cellular morphology, resembling control EpRas cells treated with TGF-β (Figure 5, A and B). Interestingly, among the epithelial structures resembling control EpRas cells (E-cadherin positive and vimentin negative), a large percentage had either lost or downregulated expression of CA–IKK-2, as indicated by low levels of the coregulated GFP expression (Figure 5C, bottom). In contrast, mesenchymal structures generated by untreated CA–IKK-2–expressing cells were E-cadherin negative, expressed vimentin, and showed strong GFP staining, indicative of high transgene expression (Figure 5C). Thus, high levels of CA–IKK-2 expression appear to promote EMT even in the absence of high transgene expression (Figure 5C). As expected, TGF-β–treated EpRas cells showed cell disintegration due to apoptosis, as determined by in situ TUNEL staining (Figure 6A). As shown in Figure 6B, approximately 55% of these cells were TUNEL positive and were thus apoptotic after 6 days of TGF-β treatment. Moreover, TD-IkBα cells showed a slight elevation in induction of apoptosis compared with control EpRas cells even in the absence of TGF-β (4.5% versus 0.6%), possibly explaining the smaller size of epithelial structures formed by TD-IkBα–expressing cells in collagen gels. Finally, EpRas cells expressing CA–IKK-2 showed low levels of apoptosis, comparable to that of EpRas control cells. We then asked whether the observed failure of TD-IkBα–expressing EpRas cells to undergo EMT (Figures 4 and 5) was exclusively due to inhibition of the antiapoptotic function of NF-κB in these cells. EpRas control cells and TD-IkBα–expressing derivatives were cultured on porous support. Treatment of TD-IkBα–expressing EpRas cells with 25 μM cell-permeable caspase inhibitor benzoyloxy carbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK) strongly suppressed the TGF-β–induced apoptosis seen in these cells (on porous support as well as in collagen gels; Figure 6B), to a level that was comparable to that of EpRas control cells (data not shown). Importantly, neither mesenchymal structures (as seen in TGF-β–treated EpRas control cells) nor loss of polarized E-cadherin expression or de novo vimentin expression was observed in these Z-VAD-FMK–treated TD-IkBα–expressing EpRas cells after 4 days of stimulation with TGF-β (Figure 6, C and D). Notably, due to the significant fraction of apoptotic cells following TGF-β treatment, small areas of irregular structures were detected in the TD-IkBα–expressing EpRas cultures (data not shown). These were not observed when apoptosis was blocked by Z-VAD-FMK. In summary, NF-κB signaling is required in Ras-
transformed cells for protection from TGF-β-induced apoptosis during EMT. Moreover, NF-κB plays an additional role as a direct regulator of the EMT program, as blockade of apoptosis does not restore the ability of TD-IκBα-expressing EpRas cells to undergo EMT in response to TGF-β.

Inhibition of NF-κB activity in mesenchymal EpRasXT cells causes reversal of EMT. We next addressed whether interference with NF-κB activity would also affect the mesenchymal EpRasXT cells that have completed EMT. EpRasXT cells were again stably infected with retroviruses expressing TD-IκBα or CA–IKK-2 or with a GFP-only empty control vector. Transgene expression, as assessed by Western blot analysis, is shown in Figure 7A. EMSA showed complete inhibition of NF-κB DNA-binding activity in untreated and TNF-α- or PMA-stimulated EpRasXT cells expressing TD-IκBα. The expression of CA–IKK-2 resulted only in a moderate (less than 2-fold) enhancement of NF-κB activity in untreated cells (Figure 7B). Transient transfection with 3xRelA-luc and subsequent luciferase assays revealed a strong blockade of NF-κB transactivation activity in EpRasXT cells expressing TD-IκBα, while a moderate increase of luciferase activity was observed in EpRasXT cells infected with CA–IKK-2.

To test whether TD-IκBα was able to revert EMT, we cultured EpRasXT cells expressing TD-IκBα and CA–IKK-2, or the control vector on porous support. As expected, CA–IKK-2– and control vector–expressing EpRasXT cells showed a mesenchymal, spindle-shaped phenotype and expressed high levels of vimentin, but no E-cadherin (Figure 8, A and B). Interestingly, however, a large percentage of TD-IκBα–expressing cells reverted to an epithelial phenotype, in which the cells formed compact structures, regained marked E-cadherin expression at the plasma membrane, and almost completely lost expression of vimentin, as demonstrated by immunofluorescence (Figure 8A) and Western blot analysis (Figure 8B). Similar results were obtained under different culture conditions (data not shown). TD-IκBα–expressing EpRasXT cells showed no obvious signs of cell death when cultured on porous support (e.g., condensed nuclei, disintegrated cells, and detachment from porous support). Annexin V staining (Figure 8C) as well as cell cycle analysis (data not shown) of TD-IκBα–expressing EpRasXT cells that had reverted to an epithelial phenotype demonstrated that reverted epithelial cells were still viable and healthy. These results indicate that NF-κB activity is required for maintenance of the mesenchymal phenotype of Ras-transformed cells that have undergone EMT.

NF-κB is required for the metastatic potential of EpRas cells in vivo. EpRas cells undergo EMT in vivo in response to endogenous TGF-β (19). TGF-β–induced EMT of EpRas cells is tightly linked to their ability to form lung metastases, evident after tail vein injection of cultured EpRas cells (S. Grünert and H. Beug, unpublished data) or cells recultivated from EpRas-induced primary mammary tumors (17). Because NF-κB activity was found to be essential for both the induction and maintenance of EMT, we sought to determine whether NF-κB signaling is also required for metastatic potential induced by Ras plus TGF-β in vivo and whether inhibition of NF-κB would abrogate this metastatic ability. The metastatic potential of TD-IκBα–expressing EpRas cells and EpRas control cells was assayed by injection of cultured cells into the tail vein of nude mice. Mice injected with EpRas control cells rapidly died from numerous large metastases (on average, 3–4 weeks after tail vein injection), while mice receiving TD-IκBα–expressing EpRas cells appeared healthy at the time of death of the mice injected with EpRas control cells. Mice injected with EpRas cells with blocked NF-κB activity showed a 2-fold decrease in lung weight (compared with that of control mice of similar age; data not shown) and had only a few small (micro-) metastases (average number, 16.6 metastases per lung) by histological analysis, compared with an average number of 171.0 metastases per lung in animals injected with EpRas control cells (Figure 9, A–C). To verify that TD-IκBα–expressing EpRas cells were still able to form primary tumors in a fashion similar to that of EpRas control cells, we injected the cell types described above (in each case from the same batches as used for tail vein injections) into mammary gland fat pads of nude mice. After 3 weeks, both EpRas control cells as well as EpRas cells expressing TD-IκBα formed tumors, which differed...
NF-kB is required for distinct aspects of tumor progression. Our observation that the antiapoptotic role of NF-kB plays a critical role in late-stage tumorigenesis (EMT) is consistent with many reports in the literature. Indeed, NF-kB positively regulates a plethora of antiapoptotic genes (30), and constitutive NF-kB activity has been found in breast, prostate, colorectal, and ovarian cancers and in certain forms of leukemia and lymphoma (reviewed in ref. 2). Inhibition of NF-kB in many human tumor–derived cell lines, including malignant Reed-Sternberg cells of Hodgkin disease, induces spontaneous apoptosis and/or sensitizes cells to killing by TNF-α or anticancer drugs (30). Several studies have demonstrated that inhibition of NF-kB results in an increased sensitivity of tumor cells to cancer therapy–induced apoptosis. For example, delivery of a recombinant TD-IκBα to chemoresistant tumors in mouse xenograft models induced tumor regression by sensitizing them to chemotherapeutic treatment with the topoisomerase I inhibitor CPT-11 (31, 32). Our results clearly establish a critical role for NF-kB as an important regulator of the EMT gene program that goes beyond its well established function in apoptosis protection. This is based on several lines of evidence. First, NF-kB activation is sufficient to induce EMT in a considerable proportion of epithelial EpRas cells. Second, NF-kB inhibition results in a blockade of EMT (under conditions in which apoptosis was efficiently suppressed). Third, NF-kB is a critical factor for the activation of a subset of endogenous genes in the TGF-β–induced gene program. Finally, NF-kB blockade can partially revert EMT, resulting in viable and healthy epithelial cells.

Here we demonstrate for the first time to our knowledge that NF-kB plays a causal role in the induction and maintenance of EMT in Ras-transformed mammary epithelial cells, mediating an invasive/metastatic tumor phenotype. These findings add mechanistic insight regarding the role of NF-kB in late-stage mammary tumorigenesis and metastasis. Our observations regarding a detectable or further elevated baseline activity of NF-kB in unstimulated EpRas and EpRasXT cells, respectively, are consistent with reports demonstrating constitutive activation of NF-kB factors in breast cancer (33, 34). Inhibition of the constitutive NF-kB activity in human breast cancer cell lines induced apoptosis (34) or led to reduced tumorigenicity (35). Furthermore, mouse mammary tumor virus c-rel–transgenic mice develop late-onset mammary tumors of diverse histology (36). Interestingly, some of the tumors identified in the study by Romieu-Mourez et al. were spindled cell carcinomas, a tumor type possibly generated by EMT (12, 22).

Recent studies have suggested that NF-kB regulates the expression of multiple genes involved in tumor spread and metastasis, including those encoding MMPs, IL-8, VEGF, and CXCR4 (37, 38). Several NF-kB target genes we have reported to be induced during EMT of EpRas cells, such as those encoding various MMPs and cathepsin family members, chemokines/cytokines, tenascin-C, etc. (see Table 1 and Figure 3E), could contribute to the NF-kB–dependent metastatic capacity we observed in our study. Suppression of metastasis upon blockade of NF-kB activity has also been reported in human prostate cancer cells (37), in human melanoma cells (39), and in murine lung alveolar carcinoma cells (40). Collectively, our work and work by others clearly show the importance of NF-kB signaling for tumor progression and metastasis in multiple tumor model systems. It should be noted, however, that while NF-kB contributes to oncogenesis in many cell types, its IκBα-mediated suppression in keratinocytes was recently dem-

Discussion

Activation of NF-kB signaling is increasingly being recognized as a key mechanism for tumorigenesis and is thought to act mainly by conferring apoptosis resistance to transformed cells. In this report, we used a well characterized combined in vitro/in vivo model of mammary carcinogenesis (EpRas) to determine the function of NF-kB in the regulation of epithelial plasticity and metastasis. First, we showed that NF-kB activity is required together with oncogenic Ras for efficient protection of mammary epithelial cells from TGF-β–induced apoptosis, as a prerequisite for these cells to undergo an EMT toward an invasive, metastatic tumor phenotype. Second, NF-kB can, in part, induce EMT in Ras-transformed cells in the absence of TGF-β, suggesting that NF-kB signaling can mediate important aspects of TGF-β signaling essential for inducing EMT. Third, NF-kB activity is necessary for cells to be maintained in a mesenchymal state, as its inhibition causes reversal of EMT; and finally, in agreement with its essential role in the regulation of at least these three distinct aspects of tumor progression in vitro, NF-kB signaling is required for metastasis of Ras-transformed epithelial cells in vivo.

mainly in size (Figure 9D). In conclusion, inhibition of NF-kB activity strongly affects the metastatic potential of EpRas cells in vivo, while primary tumor formation is affected only moderately.
onstrated to be necessary for Ras-mediated induction of invasive epithelial tumors resembling squamous cell carcinomas (41). The role of TGF-β signaling in the oncogenic Ras/IKKβ tumors, however, has not been addressed.

Role of NF-κB in the cooperation of Ras- and TGF-β-dependent signaling pathways during tumor progression. Previous studies by Janda et al. (17) addressed the effect of Ras signaling pathways on epithelial plasticity in the EpH4/Erap model, using Ras mutants that specifically activate only the Raf/MEK/Erk pathway (S35Ras) or the PI3K/Akt pathway (C40Ras). Furthermore, specific inhibitors that block Ras, MEK1, or PI3K were used to interfere with EMT induction or maintenance in Eparas cells (17). Both approaches showed that Ras-dependent signaling of the Raf/MEK/Erk pathway in combination with TGF-β signaling is required for EMT (17). In contrast, C40-induced PI3K/Akt signaling protected EpH4 cells from TGF-β–induced apoptosis, but failed to induce EMT. In vivo, only a Ras mutant able to activate the Raf/MEK/Erk pathway (S35Ras), but not the C40Ras mutant, was able to generate metastases, strictly correlating with the potential of these mutants to induce EMT (17). A number of reports have shown that oncogenic Ras stimulates NF-κB–dependent transcription and that NF-κB is required for Ras-mediated transformation (reviewed in ref. 1). Several lines of evidence indicate that NF-κB acts in a common pathway with PI3K/Akt, leading to suppression of TGF-β–induced apoptosis in Ras-transformed mammary epithelial cells. First, the rate of TGF-β–dependent apoptosis induction is very similar in T-kin–expressing Eparas cells and in Eparas cells treated with a specific PI3K inhibitor (17). Second, Akt has been shown in several cellular systems to stimulate signaling pathways that upregulate the activity of NF-κB. Importantly, the antiapoptotic signals elicited by PDGF have been shown to require Akt-induced NF-κB transcriptional activity (42). Several reports have indicated that IKK activity is involved in the ability of Akt to stimulate NF-κB transcriptional activity (42–44), while others have found that PI3K or Akt can also stimulate NF-κB activity through signaling pathways targeting the p65 subunit of NF-κB (45). Our finding that modulating NF-κB activity has the same effects in Eparas cells and V12S35 cells would be consistent with the interpretation that the PI3K/Akt signaling may be upstream of NF-κB activity. Collectively, further experiments are required to elucidate how NF-κB precisely mediates Ras-dependent effects in the Eparas cellular system.

Our observation that TGF-β–dependent induction of EMT depends at least in part on NF-κB activity raises the question of how these signaling pathways may collaborate. Induction of NF-κB activity and transcription of target genes by TGF-β, as observed in our study, are in line with observations by Arsura et al. (28) in liver epithelial cells. TGF-β can signal in a Smad-independent manner through TGF-β1–activated kinase 1 (TAK1). Interestingly, TAK1 has been shown to directly phosphorylate the IKK complex in response to TGF-β treatment, promoting NF-κB activation (28). In addition, several Smad proteins, acting as transcription factors defined as the major responders to TGF-β signaling, can function as transcriptional coactivators through physical interaction with NF-κB subunits to stimulate transcription via κB sites (46). It will be important to further analyze at which levels these two pathways intersect to abrogate the classical functions of TGF-β in the induction of apoptosis and how they cooperate in enhancing the role of TGF-β in the induction of EMT. A potential novel point of intersection has been discovered very recently. TGF-β induces transcription of the cell cycle inhibitor p21cip1 by a mechanism, in which Smad proteins cooperate with FoxO transcription factors (47). Three strategies have been described to overcome this cell cycle inhibition. The first is counteraction of FoxO function by another member of the Fox transcription factor family, namely FoxG1 (47). The second is inactivation of FoxO proteins by the PI3K/Akt pathway, which results in relocalization of FoxO proteins to the cytoplasm (48). Third, in breast tumors, upregulation of IKK-2 has been observed and IKK-2 was shown to directly phosphorylate and functionally inactivate the FoxO proteins (49).

In summary, our study provides a functional dissection of the requirement for NF-κB in several aspects of breast cancer progression in this combined in vitro/in vivo model system. It identifies NF-κB as a pivotal regulator of the EMT process, which by itself is a critical prerequisite for metastasis. Further characterization of the mechanisms by which NF-κB contributes to the invasion and metastasis of mammary carcinomas and other malignant tumors will provide important targets for drug discovery, which should lead to new therapeutic approaches for antitumor metastatic cancer treatments.

Methods

Cells and cell culture. Origin and culture conditions for Eparas and EparasXT cells were described earlier (19, 20). The generation and culture conditions of φNX amphotropic retrovirus producer cells expressing TD-1κBα, CA–IKK-2, and parental vector, coupled to the expression of a GFP–zeocin-resistance fusion gene through an internal ribosome entry site were described previously (29).

Retroviral infection of Eparas and EparasXT cells with supernatant from φNX producer cells. Eparas and EparasXT cells were infected with parental vector or retroviruses expressing the dominant interfering mutants as described earlier (29). Briefly, 24 hours prior to infection, Eparas and EparasXT cells were seeded in six-well plates at a density of 2 × 10⁶ cells per well, and φNX cells were seeded at a density of 3 × 10⁶ cells per 10-cm plate. For infection, φNX cell supernatants were obtained and filtered through a 0.45-μm filter, and 5 μg/ml polybrene (Sigma-Aldrich, St. Louis, Missouri, USA) was added to the filtrate. Thereafter, medium was removed from the Eparas and EparasXT cells and was replaced with φNX cell supernatants containing the retrovirus. Culture plates were centrifuged at 750 g for 2 hours, the supernatants were removed and replaced with propagation medium. Then, 48 hours later, the efficiency of infection was monitored by fluorescence microscopy (Improvement, Heidelberg, Germany) and infected cells were selected with zeocin at concentrations of 1,500 μg/ml (Eparas) and 1,700 μg/ml (EparasXT).

NF-κB assay. Western immunoblot analysis for monitoring protein expression levels of dominant interfering NF-κB mutants was performed as described earlier (29) using antibodies specific for κB (sc-371; Santa Cruz Biotechnology, Santa Cruz, California, USA), IKK-2 (sc-7607; Santa Cruz Biotechnology), and RelA (sc-372; Santa Cruz Biotechnology). NF-κB DNA-binding activity was measured by EMSA. Whole-cell extracts were prepared by the “freeze-thaw” method and EMSAs were performed as described earlier (29). In all cases, whole-cell extracts were incubated for 20 minutes at room temperature with radiolabeled double-stranded oligonucleotides containing an Ig enhancer consensus NF-κB site, an octamer-specific site (’’-ATGCAAAT-3’’), or an Sp-1–specific site (’’-ATTCGATCGGGGCGGGCGAGC-3’’), and the DNA-protein complexes formed were then separated from free oligonucleotides by electrophoresis through a native 4% polyacrylamide gel.

Modulations of NF-κB transactivation activity were measured by stable transfection of Eparas and EparasXT parental and retrovirus-transduced

clones with the 3xSB luciferase reporter (3xSB.luc) and β-globin-TATA, and subsequent luciferase activity assays. In brief, cells were transfected with 10 μg of the 3xSB.luc reporter plasmid together with 100 ng of a thymidine kinase Renilla luciferase reporter under the control of the herpes simplex virus thymidine kinase promoter. Transfections were performed by incubation of cells for 4 hours with a mixture of DNA and lipofectamine at a ratio of 1:2.5 (Lipofectamine 2000; Invitrogen, Carlsbad, California, USA) in serum-free media. At 20–28 hours after the start of transfection, luciferase activity was determined with the Lumat LB 9507 lumimeter (Berthold Technologies, Bad Wildbad, Germany).

Semiquantitative RT-PCR. Total RNA was extracted and semiquantitative RT-PCR was carried out as described earlier (29). Mouse MMP-13 was amplified with primers 5′-CACTCCAGGAGCCCAGGACCC-3′ (sense) and 5′-GCTGAGGTTGACAGGGCCCAAA-3′ (antisense; 28 cycles); mouse MCP-1 was amplified with primers 5′-CGGCTTGGAGATCCAGTTGTTG-3′ (sense) and 5′-GTCTGGACCCATTTCTCTTGGGG-3′ (antisense; 28 cycles); mouse obfaturin was amplified with primers 5′-CCAGGCCGG TAGTCCTCTGCGAA-3′ (sense) and 5′-CCATCGACCCAGCATGTATCGCC- GG-3′ (antisense; 28 cycles); and mouse β-actin was amplified with primers 5′-GGTCAAGAAGACTCTATTGG-3′ (sense) and 5′-AGAGCAACATAGCACAGCTTC-3′ (antisense; 28 cycles).

Marker analysis of cells grown on porous support. Cells were seeded on porous supports (cell culture inserts; pore size, 0.4 μm; BD, San Jose, California, USA) at densities of 0.5 × 10⁵ cells/well for EpRas cells and 1 × 10⁶ cells/well for EpRasXT cells. In some experiments, cells were incubated with the indicated concentration (see Figure 6C) of the broad-range caspase inhibitor Z-VAD-FMK (R&D Systems, Minneapolis, Minnesota, USA). Fresh inhibitor was supplied with each medium change. The cells were treated for 6 hours with a mixture of DNA and lipofectamine (on average, 3 weeks after mammary gland injection) or if tumors ulcerated or the mice showed significant morbidity. Then, tumors were excised and collected in PBS, and lung weight was determined. Total numbers of metastases per lung were determined by collection of serial lung sections, selection of sections at approximately 0.3 mm apart, and counting of metastatic lesions, with correction for the contribution of large metastatic lesions that appeared on more than one section.

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