INTRODUCTION

The predilection of some cancers to metastasize to specific secondary sites has been recognized for decades and its molecular basis remains the subject of intense investigation.1-3 Recent evidence suggests that the ultimate site of metastases is regulated by unique gene expression signatures of clonal subpopulations that exist within primary tumors. The genes constituting these distinct signatures generally encode proteins known to mediate cell growth, motility and invasion, implying that different organs have distinct requirements for tumor expansion within their microenvironment.4

The matrix metalloproteinases (MMPs) have been identified in several gene expression signatures associated with site-specific metastasis.1,2 These zinc-dependent peptidases and their endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), play a central role in the extracellular matrix remodeling required for tumor invasion, expansion, angiogenesis and metastasis, and have also been identified as regulators of tumor survival and growth.4,5 MMP-3 is a 54-kDa stromelysin (stromelysin 1) with a broad substrate specificity that can cleave fibronectin and several collagens. MMP-3 has been implicated in the progression of primary malignancies of the lung,6-8 but its role in site-specific lung metastasis has not been defined. Huang et al.9 recently identified MMP-3 as one of several proteins that contribute to vascular destabilization in the lung during the pre-metastatic phase of melanoma dissemination.

Under physiological conditions, MMP-3 is produced by several types of cells including fibroblasts and macrophages10 and its expression can be induced by inflammatory mediators such as IL-1 and TNF-α. The MMP-3 promoter contains an AP-1 and a PEA3 binding site that act together to activate MMP-3 transcription.11,12 MMP-3 expression can also be upregulated downstream of NFkB activation.13,14 Interestingly, Borghaei et al.15,16 have also identified a polymorphic site in the MMP-3 promoter to which the NFkB subunits p50 and p65 can bind to inhibit transcription. IKKe is a phorbol 12-myristate 13-acetate (PMA)-inducible IκB kinase (IKK)-related kinase.17,18 Although a homolog of IKKα and IKKβ, its role in signaling in the canonical NFkB pathway, appears to be distinct. Unlike IKKα and IKKβ, which phosphorylate IκBα on serines 32 and 36, resulting in IκBα proteosomal degradation and NFkB activation, IKKe phosphorylates IκBα only on Ser36, although it can also phosphorylate Ser32 when PMA-activated.17,19-22 In some cells, PMA-induced NFkB activation can be blocked by a dominant negative mutant of IKKe,17 identifying it as essential for NFkB activation by phorbol esters. IKKe can also directly phosphorylate the NFkB subunits cRel23 and p65 and can translocate to the nucleus to co-activate transcription of NFkB-target genes.13 Uniquely to IKKe, it has been identified as a central mediator of the interferon response to viral infection.24

Other known substrates of IKKα are Akt, a mediator of cell survival,25-27 and the transcription factor cJun.28 Recently, IKKe was identified as an oncogene in breast cancer,29 where it was shown to phosphorylate the tumor suppressor CYLD,30 leading to cellular transformation. Although activation of any of these pathways could contribute to malignant transformation, the contribution of IKKe to the metastatic phenotype has not, to date, been elucidated.

The type 1 insulin-like growth factor (IGF) system is known to contribute to malignant transformation and promote tumor progression. Ligand binding to the IGF-I receptor (IGF-IR) activates
several signal transduction pathways known to play a role in cellular transformation and maintenance of the malignant phenotype, including the PI3K/Akt and MAPK pathways, which are known to be deregulated in multiple cancers. Altered expression of IFG system components can therefore be an initiating event in cellular transformation and/or contribute to cancer progression.

Previously, we have shown that the overexpression of IGF-IR in murine lung carcinoma M27 cells (M27R cells) altered the metastatic phenotype of the cells, resulting in the acquisition of a liver-colonizing potential. As we show here, this was associated with an unexpected loss of the lung-colonizing potential of these cells. Gene expression profiling previously revealed profound changes to expression levels of multiple genes in these cells, including genes coding for MMPs. In particular, we found that MMP-3, -9 and -13 levels were significantly reduced and subsequently identified PKC-α downregulation as the major mechanism underlying reduced MMP-9, but not MMP-3 expression. Here, we investigated the signaling pathway involved in the downregulation of MMP-3 in M27R cells and evaluated the effect that altered MMP-3 expression has on the metastatic phenotype.

RESULTS

Loss of the lung-metastasizing potential in carcinoma cells ectopically expressing IGF-IR

M27 cells are a non-clonal subline of the Lewis lung carcinoma with high lung-metastasizing potential from primary subcutaneous tumors or following tail vein injection. We previously reported that the ectopic expression of hIGF-IR in these cells (M27R) resulted in the acquisition of a liver-metastasizing potential. Intriguingly, however, we found that these cells when inoculated via the tail vein, had a markedly reduced ability to colonize the lung, relative to wild-type M27 cells, as reflected in reduced numbers of visible metastases (Figures 1a and b) and lung weights (Figure 1c) and confirmed in formalin-fixed, paraffin-embedded lung sections stained with hematoxylin and eosin (Figure 1d). These results suggested that the ectopic expression of IGF-IR, although providing a growth advantage to these tumor cells in the liver, also impaired their lung-metastasizing ability.

Downregulation of IKKε in tumor cells with ectopic overexpression of IGF-IR

The MMP profiles of tumor cells are known to dictate their ability to invade and expand in a target organ. We previously reported that MMP-3 expression levels in M27R cells were markedly reduced. Here, we used these cells to investigate signaling pathways involved in the altered expression of MMP-3 and the link between reduced MMP-3 expression levels and the observed reduction in lung metastasis. Because MMP-3 was shown to be regulated downstream of the NFκB pathway, we first compared expression levels of mediators of this pathway in M27 and M27R cells, using reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative real time PCR (qPCR). We found that IKKε expression levels but not those of other mediators of the NFκB signaling pathway were significantly downregulated in these cells relative to controls (Figures 2a and b), as was also confirmed by western blotting (Figure 2c).

Constitutive and PMA-inducible MMP-3 expression are restored by reconstitution of IKKε expression

We next asked whether reduced IKKε expression could be the underlying cause for the observed reduction in MMP-3 levels. M27R cells were stably transfeced with full-length miKKε CDNA and increased IKKε expression was confirmed by immunoblotting (M27R/iKKε cells, Figure 3a). When MMP-3 expression in the miKKε overexpressing cells was measured by qPCR, we found a sevenfold increase in mRNA expression levels relative to vector control cells (M27R/MOCK) (Figure 3b), confirming that IKKε in these cells was functional and acted as a transcriptional activator of MMP-3. This basal increase in MMP-3 expression, although it was not reflected in a measurable increase in MMP-3 production levels (Figure 4b) could be significantly augmented when M27R/iKKε cells were stimulated with PMA (30-fold increase in expression relative to PMA-stimulated M27R cells, Figure 4a) and this also translated into a measurable increase in MMP-3 production, as revealed by immunoblotting performed on tumor-cell-conditioned medium (Figure 4b). This effect of PMA was not observed in wild-type or control, vector-transfected M27R cells, suggesting that IKKε was essential for the stimulatory effect of PMA. IKKε expression also sensitized MMP-3 expression to induction by TNF-α. When treated with TNF-α, MMP-3 expression in M27R/iKKε but not in M27R/MOCK cells, was increased twofold relative to basal levels (Figure 4c). Interestingly, M27R cells overexpressing IKKε did not have a significantly increased ability to colonize the lungs (Supplementary Figure S1). This suggests that in the absence of pre-activation of IKKε signaling by an inflammatory mediator, MMP-3 production in these cells may not have reached the levels required to significantly alter the course of lung metastasis.

IKKε-dependent MMP-3 induction requires PKCα activity

To identify the signal transduction pathway(s) activated by PMA (a known inducer of PKCs) in our cells, we first analyzed the effect of PKC inhibitors on this activation. Cells were treated prior to PMA stimulation with Ro 31–8220—a broad-spectrum PKC inhibitor (Supplementary Figure S2A) and subsequently with the inhibitor Go6976 that more specifically targets the activities of PKCa, PKCB and PKCd (Figure 5a). These inhibitors blocked MMP-3 induction by PMA in M27R/iKKε cells, in a dose-dependent manner and the same effects were seen in wild-type M27 cells (Supplementary Figures S2B and C), indicating that similar regulatory mechanisms were likely at play in both cell types. Furthermore, the silencing of PKCa by short interfering RNA (siRNA) (Supplementary Figure S3) significantly reduced the ability of PMA to stimulate MMP-3 expression (Figure 5b) in M27R/iKKε cells, identifying PKCa as the major PKC involved in transcriptional activation of MMP-3 in these cells. Interestingly, we observed that the silencing of PKCa in M27 cells had no measurable effect on basal MMP-3 levels (Supplementary Figure S4), suggesting that the mediators involved in maintenance of basal MMP-3 expression levels and those regulating PMA-inducible MMP-3 transcription may be distinct.

PKA-induced MMP-3 upregulation in M27R/iKKε cells involves both Akt and MEK signaling

Multiple signal transduction pathways can be activated downstream of PKCa. We used chemical inhibitors of MEK (PD98059) and PI3K (LY294002) to evaluate their role in signaling upstream of MMP-3. A dose-dependent inhibition of MMP-3 upregulation was observed with both inhibitors (Figures 6a and d), implicating both pathways in transcriptional activation of MMP-3. This was confirmed when the cells were stimulated with PMA and ERK (Figures 6b and c) and Akt (Figures 6e and f) phosphorylation levels were analyzed by immunoblotting, revealing an increase in p-ERK2 and p-Akt levels in M27R/iKKε cells relative to M27R cells.

The transcription factor p65 is also involved in PMA-induced MMP-3 expression

IKKε can activate several transcription factors including p65. When JSH-23—an inhibitor of the nuclear translocation of p65—was used to treat PMA-stimulated M27R/iKKε cells, MMP-3 induction was...
Figure 1. Loss of the lung-metastasizing potential in M27 cells ectopically expressing IGF-IR. Mice were injected via the tail vein with $10^5$ tumor cells and the lungs removed 18 days later and fixed in Bouin’s solution. Shown are the numbers of visible metastases counted on the surface of the lungs (a and b) and lung weights (c). Representative images of hematoxylin and eosin-stained sections prepared from formalin-fixed, paraffin-embedded lung fragments are shown in (d). Mag: images on left: $\times$ 50; images on right (enlarged view of same metastases): $\times$ 400. LU: lung. T: tumor. *$P < 0.001$.

Figure 2. Tumor cells ectopically expressing IGF-IR have reduced IKKε expression levels. Expression of NFκB signal transduction mediators was measured using RT–PCR (a) and for a more selected group qPCR (b). Shown in (a) are representative results of three RT-PCR assays performed and in (b) means and s.d. of results obtained in three separate qPCR assays (each performed in triplicates), normalized to GAPDH and expressed as a ratio relative to wild-type M27 cells that were assigned a value of 1. Shown in (c) are representative results of three immunoblots performed on total cell lysates. *$P < 0.05$. 
inhibited in a dose-dependent manner (Figure 6g). A similar effect was seen in (wt) M27 cells, where a reduction was also observed in the high basal MMP-3 levels following inhibitor treatment (Supplementary Figure S5). This indicated that p65 activation was a requirement for the basal expression as well as for PMA-mediated upregulation of MMP-3.

MMP-3 expression is required for lung colonization by M27 cells

To determine whether the loss of the lung-metastasizing potential in M27R cells was due to reduced MMP-3 expression levels, the cells were transduced with a retrovirus expressing full-length murine MMP-3 cDNA (M27R/MMP-3/RV cells) or a β-galactosidase cDNA (M27R/βgal), as control. Increased MMP-3 expression in the transduced cells was confirmed by immunoblotting and zymography (Figure 7a). When these cells were injected into mice via the tail vein, their ability to generate visible lung metastases was significantly increased relative to controls (Figures 7b and c), as also confirmed by histology (Figure 7d). This increase was sitespecific, because the number of liver metastasis formed by these cells when injected intrasplenic/portal route was not significantly altered (Supplementary Figure S6), suggesting that MMP-3 facilitates metastasis, selectively in the lung. Increased lung metastasis was also observed with a clonal subpopulation of M27R cells that were transfected with a plasmid vector expressing MMP-3 cDNA (but not M27R cells transfected with an empty vector—M27R/CONT cells) and consequently produced increased MMP-3 levels, as confirmed by immunoblotting (Figures 7e–g).

Conversely, M27 cells in which MMP-3 expression was silenced using shRNA had a significantly reduced ability to form lung metastasis following intravenous injection of the cells, as compared with control cells transfected with a scrambled

**Figure 3.** Increased MMP-3 expression levels in M27R cells ectopically expressing IKKε. M27R cells were transfected with a plasmid vector expressing full-length murine IKKε (M27R/IKKε) or an empty vector (M27R/MOCK). Shown in (a) is a representative result of two immunoblots performed using 200 μg of cell lysate per lane. Shown in (b) are MMP-3 expression levels expressed as the means (± s.e.m.) of five experiments normalized to GAPDH and expressed relative to non-transfected M27R cells that were assigned a value of 1. *P < 0.05.

**Figure 4.** Ectopic IKKε expression sensitizes MMP-3 transcription to induction by PMA and TNF-α. Shown in (a) are MMP-3 expression levels measured by qPCR following a 4h stimulation of the cells with 100 nM PMA. Shown in (b) is a representative result of two immunoblots performed on 200 μg of conditioned media proteins that were obtained from cells stimulated (or not) for 24 h with 500 nM PMA and in (c) results of qPCR performed on tumor cells stimulated (or not) for 4 h with 10 nM TNF-α. Results in a and c are expressed as means (± s.e.m.) of three experiments, normalized to GAPDH and relative to non-transfected and non-stimulated M27R cells that were assigned a value of 1. *P < 0.05, **P < 0.01.
sequence (Figures 8a–c). Together, these results identify MMP-3 as essential for the growth of the tumor cells in the lung.

**DISCUSSION**

Taken together, our results show that the loss of the lung-metastasizing potential in M27ε cells was due, at least in part, to reduced MMP-3 expression levels and that this reduction, in turn, was the consequence of the downregulated expression of IKKe in these cells.

Gene expression profiles3,37 have consistently identified MMPs in gene sets associated with site-specific metastasis, indicating that distinct MMPs can contribute to tumor cell potential to colonize specific sites and implying that the unique extracellular matrix composition in different organ sites dictates the requirement for specific extracellular matrix degrading proteinases for tumor cell expansion. Our results suggest that MMP-3 plays a role in facilitating tumor growth preferentially in the lung. Huang et al.9 have shown that human breast carcinoma MBA-MB-231 cells had reduced spontaneous lung metastasis in mice with targeted reductions in MMP-3, MMP-10 and angiopoietin 2 expression levels in the lungs because of reduced vascular permeability and tumor extravasation. Several other correlative studies have also linked MMP-3 expression levels and lung cancer growth.5,7 The dependency on MMP-3 for expansion in the lung could be because of the ability of this proteinase to degrade several major components uniquely present in the lung extracellular matrix, including proteoglycans and to a lesser extent, elastin,38,39 or to an MMP-3-mediated vascular destabilization that renders the lung microenvironment more amenable to tumor infiltration, as suggested by Huang et al.9 MMP-3 may also enhance lung metastasis indirectly by activating other MMPs such as MMP-9 in a proteolytic cascade.40 While stroma-derived MMP-3 may play an important role in preparing the microenvironment during the premetastatic stages of spontaneous metastasis, as shown by Huang et al.9 for B16/F10 and MDA-MB-231 cells, MMP-3 produced by the tumor cells could further accelerate tumor expansion once dissemination to the lungs has occurred.

In a previous study, we have shown that the ectopic expression of the hIGF-IR in lung carcinoma M27 cells altered their MMP profile so that MMP-3, -9 and -13 expression levels were downregulated, whereas MMP-2 and -14 levels were upregulated, resulting in altered invasive/metastatic phenotypes of the cells.34–36 We have subsequently shown that the expression of MMP-9, but not of MMP-3, in M27ε cells could be partially restored by ectopic expression of PKCa.34 Our present results identify IKKe as the additional mediator required for transcriptional activation of MMP-3 in these cells, and show that it mediated this effect by enhancing ERK, Akt and NFκB signaling.

In agreement with our results, Sweeney et al.28 also documented a downregulation of MMP-3 expression in IKKe−/− synoviocytes. In that study, cJUN, but not IκB phosphorylation by IKKe was observed downstream of TNF-α stimulation,28 whereas in M27ε cells, we observed that transcriptional activation of MMP-3 by PMA was dependent on NFκB signaling. This suggests that the function of IKKe in MMP-3 regulation may be cell context- and also stimulus-dependent.

Peters et al17 identified IKKe as part of a novel, PMA-inducible IκB kinase complex and have shown that a dominant-negative IKKe could inhibit PMA, but not TNF-α-induced NFκB activation. Their data also demonstrated that in the absence of PMA, IKKe could only phosphorylate IκB on Ser36 and that following PMA stimulation, both Ser36 and Ser32 were phosphorylated, resulting in a fully activated kinase.17 However, in that study, the signal transduction pathway(s) leading to IKKe activation downstream of PMA were not identified.

Our results identify PKCa as a mediator of NFκB activation downstream of PMA. This is consistent with other studies where PKCs have been implicated in NFκB activation,41–43 although the precise mechanism(s) have not been elucidated. In a study by Shinohara et al.,44 PKCβ2 was shown to mediate B-cell receptor-dependent NFκB activation through the formation of a protein complex that recruits and activates the IKK complex. Data from other studies also suggest that scaffolding proteins are assembled in a stimulus-specific manner to direct non-canonical IKK activity.45 This raises the possibility that in M27ε cells, PKCα may be involved in mobilizing IKKe into a protein complex, where it can be activated and contribute to transcriptional regulation of MMP-3 (a proposed model is depicted in Figure 9).

Our results implicate both Akt and ERK in IKKe-mediated MMP-3 upregulation in our cells. In other studies, IKKe was shown to directly activate Akt in a PI3K-dependent27 or independent27 manner. Our findings that ERK and Akt activation downstream of PMA were detectable in cells with low IKKe expression but increased further in the presence of increased IKKe level suggests that IKKe-dependent and independent (possibly IGF-IR mediated) activation of these pathways may have occurred in parallel and this was required for increased MMP-3 production.

The MMP-3 promoter has AP-1 and NFκB binding sites. We found that JSH-23, an inhibitor of p65 translocation, blocked

**Figure 5.** MMP-3 induction by PMA requires PKC-α activity. M27ε cells were serum-starved overnight, treated (or not) with the indicated concentration of Go6976 for 30 min and stimulated with 100 nM PMA for 4 h in the presence of the inhibitor. Shown in (a) are results of qPCR expressed as the means (± s.e.m.) of three experiments, normalized to GAPDH and relative to non-stimulated and untreated cells that were assigned a value of 1. Shown in (b) are MMP-3 mRNA levels as measured by qPCR in cells that were transiently transfected for 48 h with 30 nM PKCα siRNA followed by a 4 h-stimulation with 100 nM PMA. The results are expressed as the mean ratios (± s.e.m.) of MMP-3 mRNA levels relative to non-stimulated M27ε cells that were transiently transfected with a control, scrambled sequence and assigned a value of 1 (n = 6). *P < 0.05, ***P < 0.005.
PMA-induced MMP-3 synthesis in M27R/IKKε cells, implicating p65 in transcriptional activation of MMP-3 downstream of IKKε signaling. The activation of p65 downstream of PI3K/Akt46,47 and the Raf/MEK/ERK pathways 48,49 has been reported by others. Interestingly, however, in our cells, ERK and Akt phosphorylation were not in themselves sufficient to trigger MMP-3 synthesis in response to PMA in the absence of IKKε. This suggests that p65 activation occurred downstream of IKKε, with ERK and Akt enhancing the p65-initiated signal.

Various physiological stimuli could mimic the effect of PMA in vivo, in the lung, during tumor growth. Included among them are inflammatory cytokines that can activate and act in concert with PKC to induce intracellular signaling and gene expression.50,51 Therefore, changes in the tumor microenvironment, including the accumulation of inflammatory cells such as neutrophils and macrophages52,53 could contribute to increased MMP-3 production in tumor cells that already express high IKKε levels, enhancing their invasion and local expansion in a selective manner.

Taken together, our data identify IKKε as a molecule involved in integrating and amplifying signals generated by different signal transduction pathways and show that this can result in important

Figure 6. ERK, Akt and NFκB signaling are involved in MMP-3 induction by PMA. M27R/IKKε cells were serum-starved overnight and treated (or not) with the indicated concentrations of PD98059 (a), LY294002 (d) or JSH-23 (g) for 3 h (a and d) or 90 min (g) prior to stimulation with 100 nM PMA for 4 h (a, d and g) or the indicated time intervals (b, c, e and f). Results of qPCR (a, d and g) are expressed as the means (± s.e.m.) of three experiments, normalized to GAPDH. Results of immunoblotting (b and e) are representative of three experiments and are expressed as mean fold increase (± s.e.m.) relative to non-stimulated cells that were assigned a value of 1. *P < 0.05, **P < 0.01.
phenotype changes, such as an altered MMP profile. They reveal therefore a hitherto unappreciated role for IKKε as a potential regulator of tumor cell invasion and site-specific metastasis.

MATERIALS AND METHODS

Cells
M27 and M27R (previously M27IGFIR) cells are variant cell lines derived from the Lewis lung carcinoma that were generated in our laboratory. Their origin, culture conditions and metastatic phenotypes have been previously described.33,35 M27R/IKKε cells were generated by stably transfecting M27R cells stably transfected with pcDNA3.1 expressing full-length murine IKKε (Invitrogen Life Technologies, Eugene, OR, USA) and cloned into the XbaI and EcoRV sites of pcDNA3.1 (Invitrogen Life Technologies, Eugene, OR, USA). To generate retroviral particles expressing MMP-3, a MMP-3 cDNA fragment was extracted from pCMV6 (Origene, Rockville, MD, USA) and cloned into the XbaI and EcoRI sites of pcDNA3.1 (Invitrogen Life Technologies, Eugene, OR, USA) and transduced with retroviral particles expressing MMP-3, a MMP-3 cDNA fragment was extracted from pCMV6 (Origene, Rockville, MD, USA) and cloned into the XbaI and EcoRV sites of pcDNA3.1 (Invitrogen Life Technologies, Eugene, OR, USA). The inhibitors Ro31–8220, Go6976 and JSH-23 were from Calbiochem (Gibbstown, NJ, USA) and PDD8059 and LY294002 were from Cell Signaling Technology.

Antibodies and reagents
The mouse monoclonal anti-MMP-3 antibody was from R&D Systems (Minneapolis, MN, USA), the rabbit polyclonal antibody to USF2 and p65 from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and all antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). All restriction enzymes were obtained from New England Biolabs (Ipswich, MA, USA). PMA was from Sigma-Aldrich Canada (Oakville, ON, Canada). The inhibitors Ro31–8220, Go6976 and JSH-23 were from Calbiochem (Gibbstown, NJ, USA) and PDD8059 and LY294002 were from Cell Signaling Technology.

Plasmids and transfection
Full-length murine MMP-3 cDNA was extracted from pCMV6 (Origene, Rockville, MD, USA) and cloned into the XbaI and EcoRI sites of pcDNA3.1 (Invitrogen Life Technologies, Eugene, OR, USA). To generate retroviral particles expressing MMP-3, a MMP-3 cDNA fragment was extracted from the pCMV6 plasmid using Phusion high fidelity polymerase (New England Biolabs) and the primer set described in Supplementary Table 1. The fragment was digested with BamHI and EcoRI, cloned into the pQxiPuro vector and virus particles were produced using the LnxE packaging cell line (as described by Geiling et al.54).

All transfections were performed using Lipofectamine 2000 (Invitrogen). M27R cells stably transfected with pcDNA3.1 expressing full-length murine MMP-3 were selected using 200 μg/ml hygromycin that was added 48 h after transfection and the transfectants cloned by the limiting dilution and screened by qPCR to select clones with high MMP-3 expression levels. MMP-3 expression and activity levels were confirmed by western blotting and zymography, respectively (see below).

To generate a population of cells stably expressing MMP-3, we used retroviral particles produced in the LnxE cells. The cells were grown to 80% confluency and transfected with the pQxiPuro vector expressing full-length mouse MMP-3 or β galactosidase cDNA. Supernatants containing virus particles were collected 48 and 62 h later, filtered and added to M27R cells.
The transduced cells were selected by the addition of 2 μg/ml puromycin, 48 h post infection.

To silence MMP-3 expression, M27 cells were transfected with MMP-3 shRNA (Origene, Rockville, MD, USA). A scrambled shRNA sequence was used as control. Transfectants were selected using 2 μg/ml puromycin and the drug-resistant cells cloned and screened by RT-PCR to identify clones with reduced MMP-3 expression. MMP-3 silencing was confirmed by immunoblotting performed on conditioned media.

Figure 8. MMP-3 is required for lung metastasis of M27 cells. Mice were injected via the tail vein with 2 × 10^5 M27 cells that were stably transfected with MMP-3 shRNA or a scrambled sequence as control. Lungs were removed 19 days later and fixed in Bouin's solution prior to enumeration of lung metastases. Shown in (a) are the results of immunoblotting performed on media conditioned by the indicated cells. Shown in (b) are the numbers of visible metastases counted per lung in each group (bars denote medians) and in (c) the lungs obtained from each injection group. **P < 0.01.

Figure 9. A proposed model for the coordinated regulation of MMP-3 transcription by IKKe. A diagrammatic representation is shown based on our and other data for potential mechanisms of action of IKKe in transcriptional regulation of MMP-3. PMA-mediated activation of PKCα results in the formation of a protein complex that recruits IKKe to the plasma membrane where it can be further activated and directly phosphorylate Akt. PI3K activity is required for the recruitment of Akt to the plasma membrane and this may be provided by the presence of IGF-IR. Phospho-Akt can activate the canonical IKK pathway, resulting in nuclear translocation of the p65 transcription factor. The requirement for ERK pathway activation in MMP-3 induction by PMA may indicate that it (1) acts together with p65 to enhance signaling and/or (2) it is required for Akt-mediated activation of p65, as was also shown with the p38 MAPK.
Polymerase chain reaction
RNA was extracted using Trizol (Invitrogen) and cDNA synthesized as we previously described.3,5 Semiquantitative RT-PCR was performed using a standard procedure, as we previously described.34 qPCR was performed using the MyqQ2 Real-time PCR Detection system (BioRad, Hercules, CA, USA). The cDNAs were diluted 1:10 (MMP-3 and PKC-α) or 1:100 (GAPDH) and 2 μl added to 23 μl of the qPCR mix containing the BioRad iQ SYBR green supermix (BioRad) and the primers (Supplementary Table S1) at a concentration of 320 nM. Samples were denatured for 3 min at 95 °C, amplified for 40 cycles (denaturation at 95 °C for 10 s followed by annealing at 57 °C (GAPDH), 59 °C (PKC-α) or 60 °C (IKKε and MMP-3) for 30 s) followed by a final extension step for 1 min at 95 °C. The normalized expression levels (ddCt) were calculated using the BioRad iQ5 software with the modified equation initially introduced by Livak et al.36 Each experiment was performed in triplicates.

Short interfering RNA
PKC-α siRNA (s71688) and a scrambled control (negative control #1 siRNA, cat # 4390843) were obtained from Ambion (Foster City, CA, USA) and used at the indicated concentrations to transfect tumor cells that were plated in 6-well plates at a density of 2 × 10⁵ cells/well, 24 h earlier. RNA was extracted 48 h later and PKC-α expression levels were analyzed by qPCR.

Immunoblotting
Cells were lysed as we described in detail elsewhere57 or tumor-conditioned media were collected and concentrated 100-fold using centrifugal filters (Millipore, County Cork, Ireland). Proteins were separated by polyacrylamide gel electrophoresis on 10% SDS gels, transferred onto a nitrocellulose membrane and the blots incubated overnight with the primary antibodies diluted 1:1000 followed by a 2-h incubation with the secondary antibodies diluted 1:10,000. The gels were imaged and densitometry performed on the bands using an Alpha Imager gel documentation system (Alpha Innotech, San Leandro, CA, USA) or an LAS4000 imagequant system (GE Healthcare, Baie d’Urfe, QC, Canada).

Zymography
Concentrated (100 ×) conditioned media proteins were separated by SDS polyacrylamide gel electrophoresis using 10% polyacrylamide gels containing also 0.1% β casein. The gels were washed twice for 30 min in a 50 mM Tris HCl buffer containing 0.2 mM NaCl, 5 mM CaCl₂, 0.02% NaN₃ and 2.5% Triton X-100, pH 7.5 and then overnight at 37 °C, with shaking, in the same buffer but without Triton X-100. Gels were stained with 0.5% Coomassie brilliant blue R-250 for 1 h and destained with a solution of 40% methanol and 10% acetic until clearings indicating β casein proteolysis were visible. The gels were imaged using the Alpha Imager gel documentation system.

Cell stimulation and inhibitor treatment
Cells were serum-starved for 18 h prior to PMA or TNF-α (Invitrogen) stimulation. Stimulation was with 100 nM PMA for 5 h prior to RNA extraction and with 500 nM PMA for 18 h prior to protein analysis. TFN-α was added at a concentration of 10 nM and the cells incubated for 4 h prior to RNA extraction. Where indicated, the cells were treated with the specified concentrations of the chemical inhibitors for 30 (Ro31–8220 and Go6976), 90 (JSH-23) or 180 (LY294002 and PD98059) min prior to PMA stimulation.

Experimental lung metastasis assay
All mouse experiments were carried out in strict accordance with the recommendations as outlined in the Canadian Council on Animal Care (CCAC) ‘Guide to the Care and Use of Experimental Animals’ and under the conditions and procedures approved by the Animal Care Committee of McGill University (AUP number: S260). To generate experimental lung metastases, female C57BL/6 mice (8–12-week old) obtained from Charles River Laboratories (St Constant, QC, Canada) were injected via the tail vein with 2 × 10⁶ cells in RPMI and euthanized 19 days later. Lungs were fixed in Bouin’s fixative and lung metastases visible on the surface of the lungs were enumerated. Paraffin sections of formalin-fixed lung fragments were hematoxylin and eosin-stained and used to compare tumor loads.

Experimental liver metastasis assay
Experimental liver metastases were generated by intrasplenic/porta injections of 2 × 10⁵ tumor cells, followed by splenectomy, as previously described.58 Animals were sacrificed 19 days later and visible metastases on the surface of the liver were enumerated without prior fixation.

Statistical analyses
All data obtained from in vitro experiments were analyzed using the Student t-test. The non-parametric Mann–Whitney test was used to analyze metastasis data.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

REFERENCES
1 Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu WP, Giri DD et al. Genes that mediate breast cancer metastasis to lung. Nature 2005; 436: 518–524.
2 Bos PD, Zhang XHF, Nadal C, Shu WP, Gomis RR, Nguyen DX et al. Genes that mediate breast cancer metastasis to the brain. Nature 2009; 459: 1005–1013.
3 Kang YB, Siegel PM, Shu WP, Drobniak MJ, Kakonen SM, Cordon-Cardo C et al. A multigenic program mediating breast cancer metastasis to bone. Cancer Cell 2003; 3: 537–549.
4 Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011; 144: 646–674.
5 Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. Cell 2010; 141: 52–67.
6 Thomas P, Khokha R, Shepherd FA, Feld R, Tsao MS. Differential expression of matrix metalloproteinases and their inhibitors in non-small cell lung cancer. J Pathol 2000; 190: 150–156.
7 Michael M, Babic B, Khokha R, Tsao M, Ho J, Pintillie M et al. Expression and prognostic significance of metalloproteinases and their tissue inhibitors in patients with small-cell lung cancer. J Clin Oncol 1999; 17: 1802–1808.
8 Beom JI, Sung SJ, Song YC, Choong SL. Expression of metastasis-associated molecules in non-small cell lung cancer and their prognostic significance. Mol Med Rep 2010; 3: 43–49.
9 Huang YJ, Song N, Ding YP, Yuan SP, Li XH, Cai HC et al. Pulmonary vascular destabilization in the premetastatic phase facilitates lung metastasis. Cancer Res 2009; 69: 7529–7537.
10 Constantan A, Lauwers-Cances V, Navaux F, Abbal M, Van Meervijk J, Mazieres B et al. Stromelysin 1 (matrix metalloproteinase 3) and HLA-DRB1 gene polymorphisms: Association with severity and progression of rheumatoid arthritis in a prospective study. Arthritis and Rheum 2002; 46: 1754–1762.
11 Sharrocks AD, Brown AL, Ling Y, Yates PR. The ETS-domain transcription factor family. Int J Biochem Cell Biol 1997; 29: 1371–1387.
12 Carrere S, Verger A, Flourens A, Stehelin D, Dutereque-Coquillaud M. Erg proteins, transcription factors of the Ets family, form homo, heterodimers and ternary complexes via two distinct domains. Oncogene 1998; 16: 3261–3268.
13 Moreno R, Sobotzik J-M, Schultz C, Schmitz ML. Specification of the NF-κappa B transcriptional response by p65 phosphorylation and TNF-induced nuclear translocation of IκκB epsilon. Nucleic Acids Res 2010; 38: 6029–6044.
14 Bond M, Chase AJ, Baker AH, Newby AC. Inhibition of transcription factor NF-κB reduces matrix metalloproteinase-1, -3 and -9 production by vascular smooth muscle cells. Cardiovasc Res 2001; 50: 556–565.
15 Borchgrevik RC, Rawlings Jr PL, Javadi M, Woloshin J. NF-κB binds to a polymorphic repressor element in the MMP-3 promoter. Biochem Biophys Res Commun 2004; 316: 182–188.
16 Borchgrevik RC, Gorski G, Javadi M, Chambers M. NF-κB AND ZBP-89 regulate MMP-3 expression via a polymorphic site in the promoter. Biochem Biophys Res Commun 2009; 382: 4.
17 Peters RT, Liao S-M, Maniatis T. IKKβ is Part of a Novel PMA-Inducible IκB Kinase Complex. Mol Cell 2000; 5: 513–522.
18 Shimada T, Kawai T, Takeda K, Matsumoto M, Inoue JI, Tatsumi Y et al. IKK-I, a novel lipopolysaccharide-inducible kinase that is related to IkB kinases. Int Immunol 1999; 11: 1357–1362.
19 Brockman JA, Scherer DC, McKinsey TA, Hall SM, Qi X, Lee WY et al. Coupling of a signal response domain in I kappa B alpha to multiple pathways for NF-kappa B activation. Mol Cell Biol 1995; 15: 2809–2818.

20 Brown K, Gersterberger S, Carlson L, Fransozo G, Siebenlist U. Control of I-kappa-B-alpha proteolysis by site specific, signal-induced phosphorylation. Science 1995; 267: 1485–1488.

21 Traenckner EB, Pahl HL, Henkel T, Schmidt KN, Wilk S, Baueerre PA. Phosphorylation of human I-kappa-B-alpha on serine 32 and serine 36 controls I-kappa-B-alpha proteolysis and NF-kappa-B activation in response to diverse stimuli. EMBO J 1995; 14: 2876–2883.

22 DiDonato J, Mercuro F, Rosette C, Wu Li J, Suyang H, Ghosh S. 40 Vandooren J, Van den Steen PE, Opdenakker G. Biochemistry and molecular biology of IL-1 receptor intermediate chain. J Immunol 2000; 164: 2757–2535.

23 Harris J, Oliere S, Sharma S, Sun Q, Lin R, Hiscott J et al. Nuclear accumulation of cRel following C-terminal phosphorylation by TBK1/IKK epsilon. J Immunol 2006; 177: 2527–2535.

24 Sharma S, tenDever BR, Grandvaux N, Zhou G-F, Lin R, Hiscott J. Triggering the interferon antiviral response through an IKK-related pathway. Science 2003; 300: 1148–1151.

25 Xie Y, Zhang D, Zhao B, Lu M-K, You M, Condorelli G et al. Ikkb kinase α and TANK-binding kinase 1 activate Akt by direct phosphorylation. Proc Natl Acad Sci 2011; 108: 6474–6479.

26 Ou Y-H, Torres M, Ram R, Formstecher E, Roland C, Cheng T et al. TBK1 directly engages Akt/IKKβ survival signaling to support oncogenic transformation. Mol Cell 2011; 41: 468–470.

27 Guo JP, Coppola D, Chen JQ, IKBKE protein activates akt independent of phosphatidylinositol 3-kinase/PDK1/mTORC2 and the pleckstrin homology domain to sustain malignant transformation. J Biol Chem 2011; 286: 37389–37398.

28 Sweeney SE, Hammaker D, Wu Li J, Suyang H, Ghosh S. Degen J, Shen RR, Abbott DW, Zhou AY, Sprott KM, Asara JM. 47 Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB. NF-kappa B activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature 1999; 401: 82–85.

29 Wang XF, Wang QD, Hu WQ, Evers BM. Regulation of phosphor-ester-mediated TRAF1 induction in human colon cancer cells through a PKC/RAF/ERK/NF-kappa B-dependent pathway. Oncogene 2004; 23: 1885–1895.

30 Hwang YP, Yun HJ, Kim HG, Han EH, Lee GW, Jeong HG. Suppression of PMA-induced tumor cell invasion by dihydroartemisinin via inhibition of PKC alpha/ Raf/MAPKs and NF-kappa B/AP-1-dependent mechanisms. Biochem Pharmacol 2010; 79: 1714–1726.

31 Kim H, Zamet R, Bai XH, Liu M. PKC activation induces inflammatory response and cell death in human bronchial epithelial cells. PLoS ONE 2013; 8: e64182.

32 Holden NS, Squires PE, Kaur M, Bland R, Jones CE, Newton R. Phorbol ester-induced NF-kappa B-dependent transcription: Roles for isofoms of novel protein kinase C. Cell Signal 2008; 20: 1338–1348.

33 Krappmann D, Patke A, Heissmeyer V, Scheideret C. B-cell receptor- and phorbol ester-induced NF-kappa B and c-jun N-terminal kinase activation in B cells requires novel protein kinase C’s. Mol Cell Biol 2001; 21: 6640–6650.

34 Zhang J, Peng PP, Vondriska TM, Tang XL, Wang GW, Cardwell EM et al. Cardio-protection involves activation of NF-kappa B via PKC-dependent tyrosine and serine phosphorylation of I kappa B-alpha. Am J Physiol Heart Circ Physiol 2003; 285: H1753–H1758.

35 Shinohara H, Yasuda T, Alba Y, Sanjo H, Hamadate M, Watarai H et al. PKC beta regulates IKK-mediated IKK activation by facilitating the interaction between TAK1 and CARMA1. J Exp Med 2005; 202: 1423–1431.

36 Zhang J, Ping PP, Vondriska TM, Tang XL, Wang GW, Cardwell EM et al. Cardio-protection involves activation of NF-kappa B via PKC-dependent tyrosine and serine phosphorylation of I kappa B-alpha. Am J Physiol Heart Circ Physiol 2003; 285: H1753–H1758.

37 Vandooren J, Van den Steen PE, Opdenakker G. Biochemistry and molecular biology of IL-1 receptor intermediate chain. J Immunol 2000; 164: 2757–2535.

38 Murphy G, Cockett MI, Ward RV, Docherty AJ. Matrix metalloproteinase activity of human lung carcinoma cells: A quantitative comparison of the activities of 95 kDa and 72 kDa gelatinases, stromelysins-1 and -2 and punctuated metalloproteinase (PUMP). Biochemical J 1991; 277: 277–279.

39 Park JB, Kong CG, Suh KI, Chang ED, Riew KD. The increased expression of matrix metalloproteinases associated with elastin degradation and fibrosis of the ligamentum flavum in patients with lumbar spinal stenosis. Clin Orthop Surg 2009; 1: 81–89.

40 Vandooren J, Van den Steen PE, Opdenakker G. Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9): the next decade. Crit Rev Biochem Mol Biol 2013; 48: 222–272.

Supplementary Information accompanies this paper on the Oncogenesis website (http://www.nature.com/oncsis)