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ORIGINAL ARTICLE

The inhibitor of kappa B kinase-epsilon regulates MMP-3 expression levels and can promote lung metastasis

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The factors that determine the ability of metastatic tumor cells to expand and grow in specific secondary site(s) are not yet fully understood. Matrix metalloproteinases (MMP) were identified as potential regulators of the site-specificity of metastasis. We found that lung carcinoma cells ectopically expressing high levels of the receptor for the type I insulin like growth factor receptor ($M27^R$ cells) had a significant reduction in MMP-3 expression levels and this coincided with reduced metastasis to the lung. We used these cells to further investigate signaling pathways regulating MMP-3 expression and the role that MMP-3 plays in lung metastasis. We show that ectopic IκB kinase ε (IKKε) expression in these cells partly restored MMP-3 expression levels and also sensitized MMP-3 transcription to induction by phorbol 12-myristate 13-acetate (PMA). This increase in MMP-3 production was due to increased activation of several signal transduction mediators, including protein kinase C alpha, ERK2, Akt and the transcription factor p65. Furthermore, reconstitution of MMP-3 expression in $M27^R$ cells restored their ability to colonize the lung whereas silencing of MMP-3 in M27 cells reduced metastases. Collectively, our results implicate IKKε as a central regulator of PMA-induced cell signaling and MMP-3 expression and identify MMP-3 as an enabler of tumor cell expansion in the lung.

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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 macrophages[10] 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 NFκB activation.[13,14] Interestingly, Borghaei *et al.*[15,16] have also identified a polymorphic site in the MMP-3 promoter to which the NFκB subunits p50 and p65 can bind to inhibit transcription.

IKKε 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 NFκB pathway, appears to be distinct. Unlike IKKα and IKKβ, which phosphorylate IκBα on serines 32 and 36, resulting in IκBα proteosomal degradation and NFκB activation, IKKε phosphorylates IκBα only on Ser36, although it can also phosphorylate Ser32 when PMA-activated.[17,19–22] In some cells, PMA-induced NFκB activation can be blocked by a dominant negative mutant of IKKε,[17] identifying it as essential for NFκB activation by phorbol esters. IKKε can also directly phosphorylate the NFκB subunits cRel[23] and p65 and can translocate to the nucleus to co-activate transcription of NFκB-target genes.[13] Uniquely to IKKε, it has been identified as a central mediator of the interferon response to viral infection.[24]

Other known substrates of IKKε are Akt, a mediator of cell survival,[25–27] and the transcription factor cJun.[28] Recently, IKKε 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 IKKε 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

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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,[31] which are known to be deregulated in multiple cancers. Altered expression of IGF system components can therefore be an initiating event in cellular transformation and/or contribute to cancer progression.[32]

Previously, we have shown that the overexpression of IGF-IR in murine lung carcinoma M27 cells ($M27^R$ cells) altered the metastatic phenotype of the cells, resulting in the acquisition of a liver-colonizing potential.[33] 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.[34] 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.[34] Here, we investigated the signaling pathway involved in the downregulation of MMP-3 in $M27^R$ 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.[35] We previously reported that the ectopic expression of hIGF-IR in these cells ($M27^R$) resulted in the acquisition of a liver-metastasizing potential.[36] 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 $M27^R$ cells were markedly reduced.[34] 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,[13] we first compared expression levels of mediators of this pathway in M27 and $M27^R$ 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. $M27^R$ cells were stably transfected with full-length mIKKε cDNA and increased IKKε expression was confirmed by immunoblotting ($M27^{R/IKKε}$ 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 ($M27^{R/MOCK}$) (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 $M27^{R/IKKε}$ cells were stimulated with PMA (30-fold increase in expression relative to PMA-stimulated $M27^R$ 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 $M27^R$ 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 $M27^{R/IKKε}$, but not in $M27^{R/MOCK}$ cells, was increased twofold relative to basal levels (Figure 4c). Interestingly, $M27^R$ 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 PKCα, PKCβ and PKCμ (Figure 5a). These inhibitors blocked MMP-3 induction by PMA in $M27^{R/IKKe}$ 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 PKCα by short interfering RNA (siRNA) (Supplementary Figure S3) significantly reduced the ability of PMA to stimulate MMP-3 expression (Figure 5b) in $M27^{R/IKKe}$ cells, identifying PKCα as the major PKC involved in transcriptional activation of MMP-3 in these cells. Interestingly, we observed that the silencing of PKCα 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.

PMA-induced MMP-3 upregulation in $M27^{R/IKKε}$ cells involves both Akt and MEK signaling

Multiple signal transduction pathways can be activated downstream of PKCα. 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 $M27^{R/IKKe}$ cells relative to $M27^R$ 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 $M27^{R/IKKε}$ cells, MMP-3 induction was

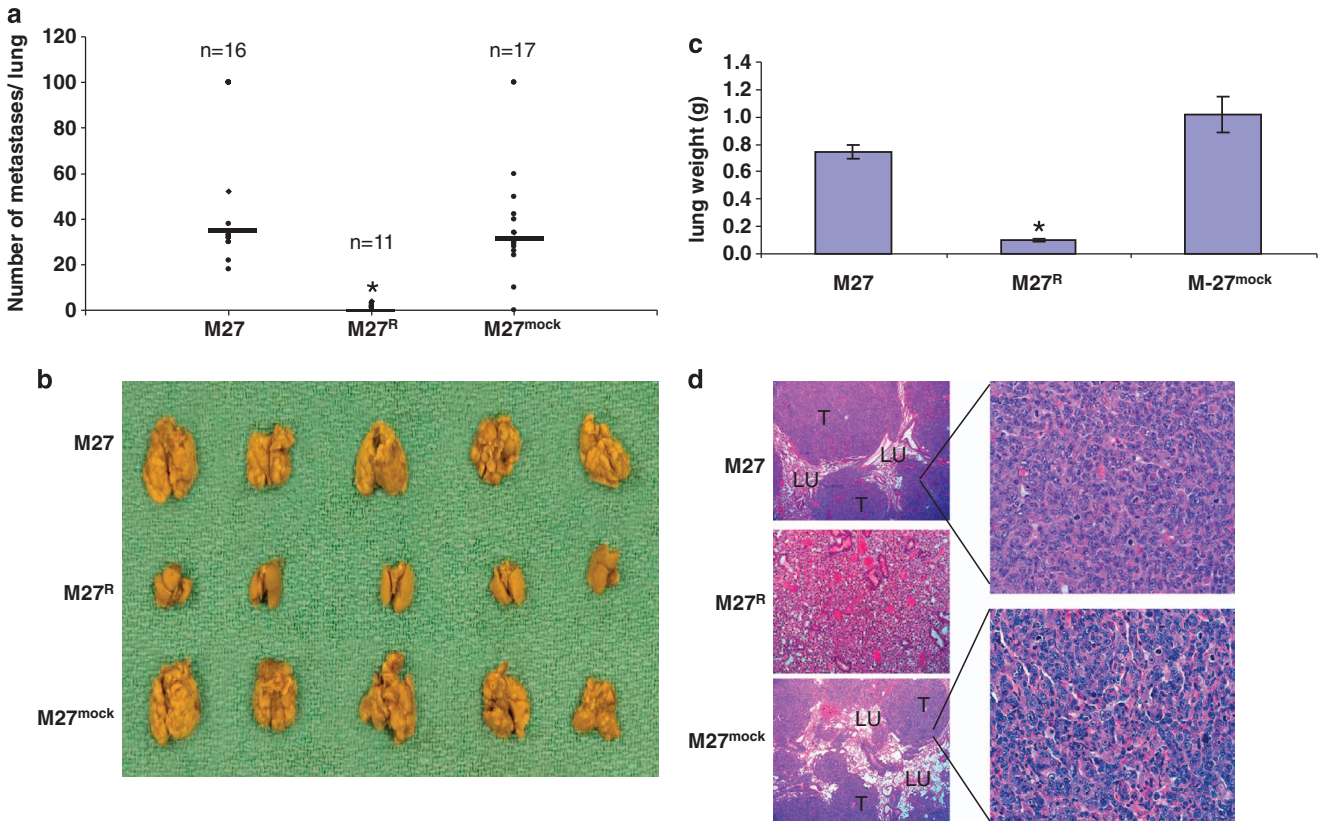

Figure 1. Loss of the lung-metastasizing potential in M27 cells ectopically expressing IGF-IR. Mice were injected *via* the tail vein with 10^5 tumor cells and the lungs removed 18 days later and fixed in Bouin's solution. Shown are the numbers of visible metastases counted on the surface of the lungs (**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- × 50; images on right (enlarged view of same metastases)- × 400. LU: lung. T: tumor. *$P < 0.001$.

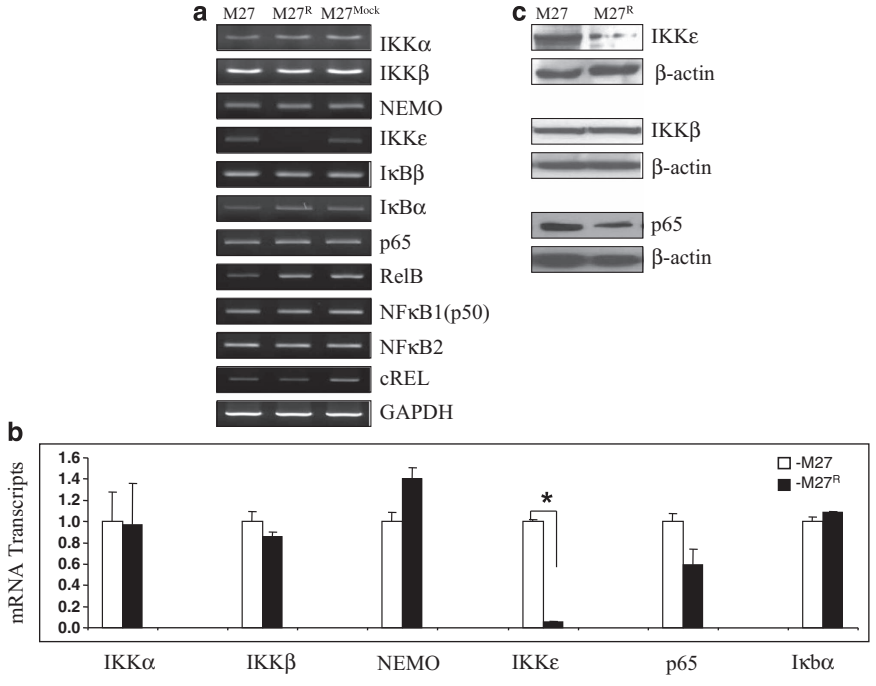

Figure 2. Tumor cells ectopically expressing IGF-IR have reduced IKKε expression levels. Expression of NFκB signal transduction mediators was measured using RT–PCR (**a**) and for a more selected group qPCR (**b**). Shown in (**a**) are representative results of three RT-PCR assays performed and in (**b**) 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. *$P < 0.05$.

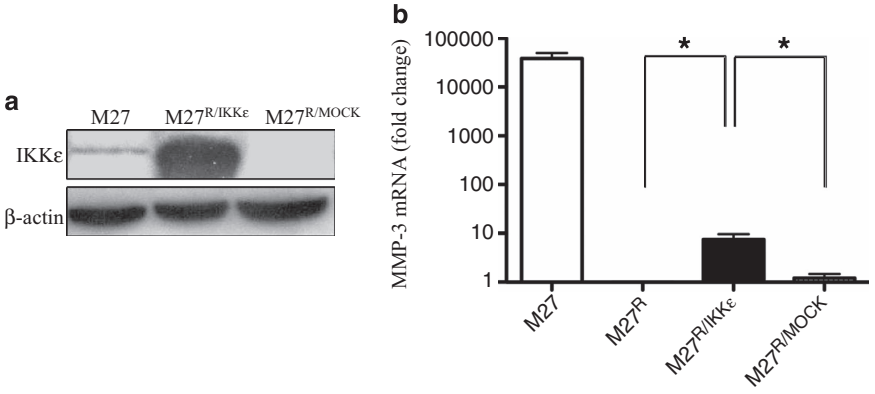

Figure 3. Increased MMP-3 expression levels in M27^{R} cells ectopically expressing IKKε. M27^{R} cells were transfected with a plasmid vector expressing full length murine IKKε (M27$^{R/IKK\varepsilon}$) or an empty vector (M27$^{R/MOCK}$). Shown in (**a**) is a representative result of two immunoblots 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 M27^{R} cells that were assigned a value of 1. *$P < 0.05$.

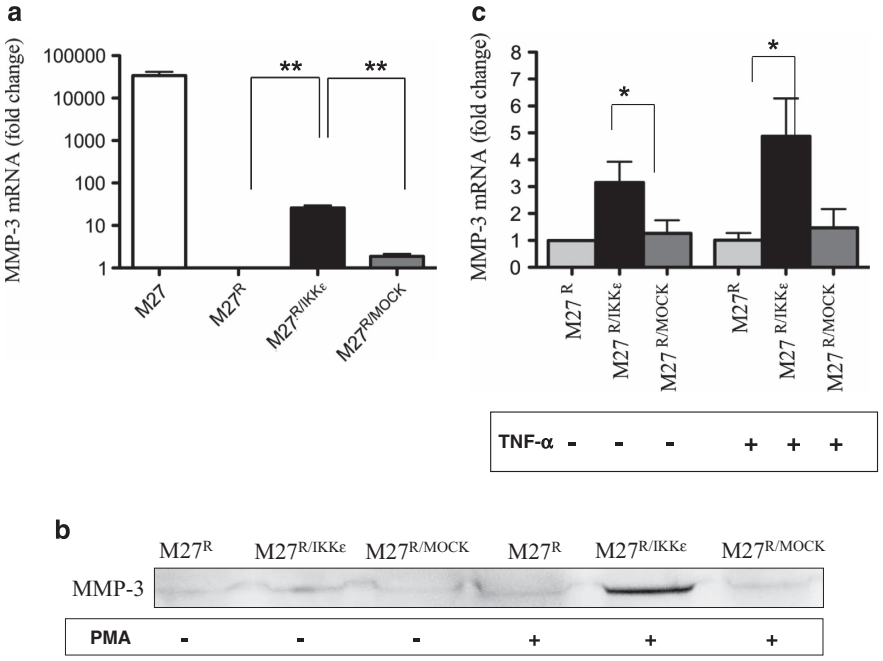

Figure 4. Ectopic IKKε expression sensitizes MMP-3 transcription to induction by PMA and TNF-α. Shown in (**a**) are MMP-3 expression levels measured by qPCR following a 4h stimulation of the cells with 100 nM PMA. Shown in (**b**) is a representative result of two immunoblots performed on 200 μg of 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 M27^{R} cells that were assigned a value of 1. *$P < 0.05$, **$P < 0.01$.

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 M27^{R} cells was due to reduced MMP-3 expression levels, the cells were transduced with a retrovirus expressing full-length murine MMP-3 cDNA (M27$^{R/MMP\text{-}3/RV}$ cells) or a β-galactosidase cDNA (M27$^{R/\beta gal}$), 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 site-specific, because the number of liver metastasis formed by these cells when injected *via* the 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 M27^{R} cells that were transfected with a plasmid vector expressing MMP-3 cDNA (but not M27^{R} cells transfected with an empty vector—M27$^{R/CONT}$ 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 metastases following intravenous injection of the cells, as compared with control cells transfected with a scrambled

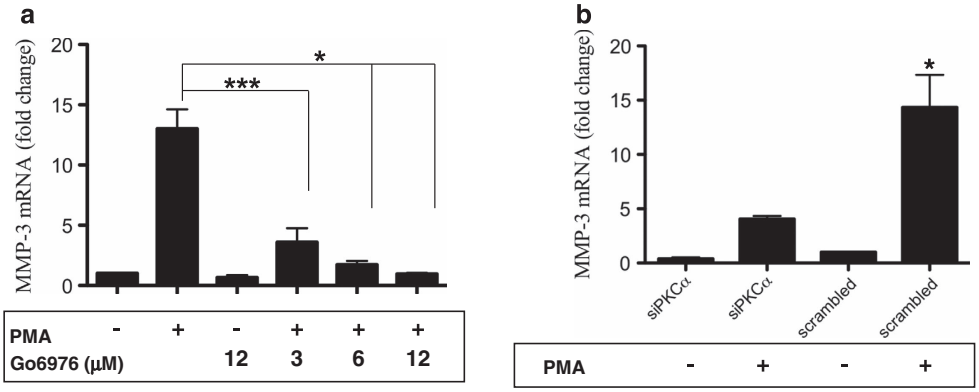

Figure 5. MMP-3 induction by PMA requires PKC-α activity. M27$^{R/IKK\varepsilon}$ cells were serum-starved overnight, treated (or not) with the indicated concentration of Go6976 for 30 min and stimulated with 100 nM PMA for 4 h in the presence of the inhibitor. Shown in (**a**) are results of qPCR expressed 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 M27$^{R/IKK\varepsilon}$ cells that were transiently transfected with a control, scrambled sequence and assigned a value of 1 ($n=6$). *$P<0.05$, ***$P<0.005$.

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 M27^{R} 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 IKKε in these cells.

Gene expression profiles[1–3,37] 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.[6,7] 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 M27^{R} cells could be partially restored by ectopic expression of PKCα.[34] Our present results identify IKKε 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 IKKε$^{-/-}$ synoviocytes. In that study, cJUN, but not IκBα phosphorylation by IKKε was observed downstream of TNF-α stimulation,[28] whereas in M27$^{R/IKK\varepsilon}$ cells, we observed that transcriptional activation of MMP-3 by PMA was dependent on NFκB signaling. This suggests that the function of IKKε in MMP-3 regulation may be cell context- and also stimulus-dependent.

Peters *et al.*[17] identified IKKε as part of a novel, PMA-inducible IκB kinase complex and have shown that a dominant-negative IKKε could inhibit PMA, but not TNF-α-induced NFκB activation. Their data also demonstrated that in the absence of PMA, IKKε could only phosphorylate IκBα 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 IKKε activation downstream of PMA were not identified.

Our results identify PKC-α 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] PKCβ 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 M27$^{R/IKK\varepsilon}$ cells, PKC-α may be involved in mobilizing IKKε 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 IKKε-mediated MMP-3 upregulation in our cells. In other studies, IKKε was shown to directly activate Akt in a PI3K-dependent[25] or independent[27] manner. Our findings that ERK and Akt activation downstream of PMA were detectable in cells with low IKKε expression but increased further in the presence of increased IKKε level suggests that IKKε-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

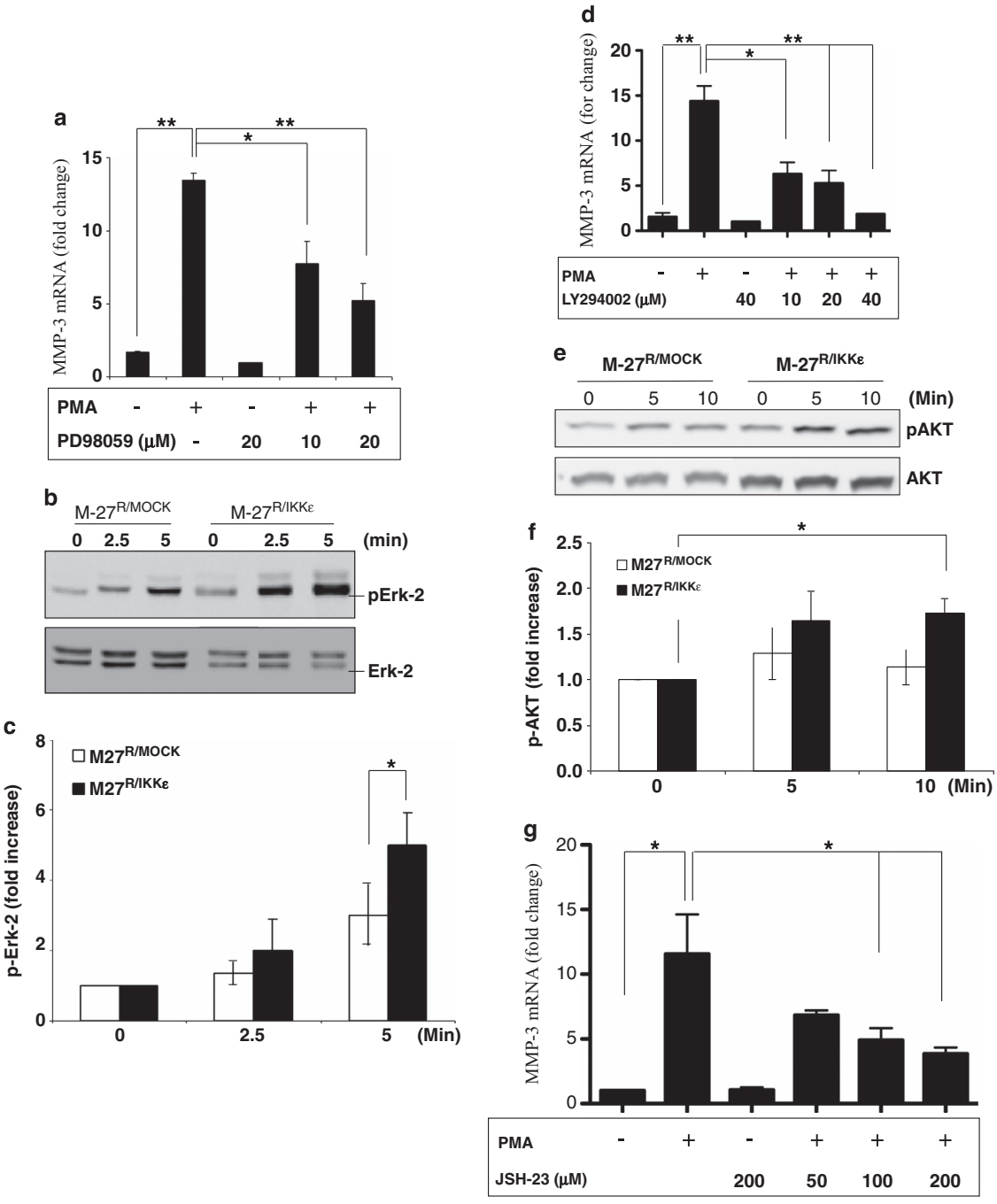

Figure 6. ERK, Akt and NFκB signaling are involved in MMP-3 induction by PMA. M27$^{R/IKK\varepsilon}$ cells were serum-starved overnight and treated (or not) with the indicated concentrations of PD98059 (**a**), LY294002 (**d**) or JSH-23 (**g**) for 3 h (**a** and **d**) or 90 min (**g**) prior to 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. *$P < 0.05$, **$P < 0.01$.

PMA-induced MMP-3 synthesis in M27$^{R/IKK\varepsilon}$ cells, implicating p65 in transcriptional activation of MMP-3 downstream of IKKε signaling. The activation of p65 downstream of PI3K/Akt[46,47] 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 macrophages[52,53] 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

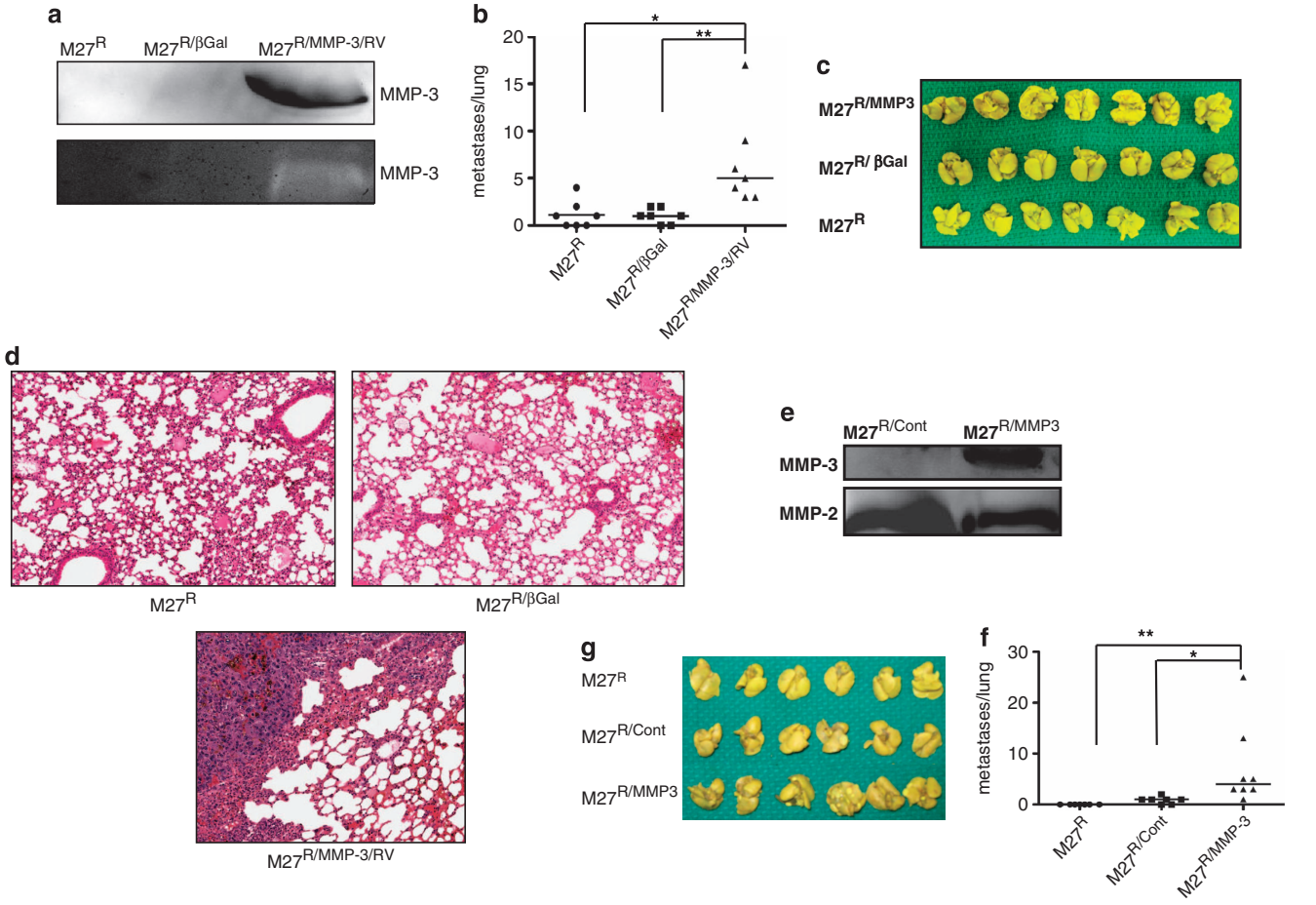

Figure 7. Increased MMP-3 expression restores the ability of $M27^R$ cells to colonize the lungs. Mice were injected intravenouly with 2×10^5 $M27^R$ cells that were transduced with retroviral particles ($M27^{R/MMP-3/RV}$, **a**–**d**) or with a clonal subpopulation selected from mMMP-3 transfectants ($M27^{R/MMP-3}$, **e**–**g**). Cells transduced with retroviral particles expressing β galactosidase ($M27^{R/βgal}$, **a**–**d**) or a vector-transfected population (**e**–**g**), were used as controls, respectively. Shown in (**a**-top and **e**) are results of immunoblotting and in (**a**-bottom) of zymography performed on tumor-conditioned media. Shown in (**c**) and (**g**) are the lungs of tumor-injected mice removed 24 (**c**) or 29 (**g**) days following tumor injection and in (**b**) and (**f**) the number of visible metastases counted per lung (bar denotes median). Representative images obtained from hematoxylin and eosin-stained formalin-fixed, paraffin-embedded sections of the lungs are shown in (**d**) Mag. ×20 $*P < 0.05$ $**P < 0.01$.

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 $M27^R$ (previously $M27^{IGFIR}$) 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] $M27^{R/IKKε}$ cells were generated by stably transfecting $M27^R$ cells with the full-length murine IKKε cDNA expressed in the pUNO plasmid (Invitrogen, Carlsbad, CA, USA). $M27^{R/MOCK}$ cells were transfected at the same time with the empty vector and used as a negative control in all the experiments. The transfected cells were selected with 100 μg/ml of blasticidin (InvivoGen, San Diego, CA, USA).

Antibodies and reagents

The mouse monoclonal anti MMP-3 antibody was from R&D Systems (Minneapolis, MN, USA), the rabbit polyclonal antibody to IKKε from Cell Signaling Technology (Danvers, MA, USA), the mouse monoclonal antibody to β-actin from Sigma Aldrich (St Louis, MO, USA), antibodies to Akt, p-Akt, ERK and p-ERK from Cell Signaling Technology and the polyclonal rabbit antibodies to USF2 and p65 from 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 PD98059 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 Xbal 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 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). $M27^R$ 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 $M27^R$ 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 sequences 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.

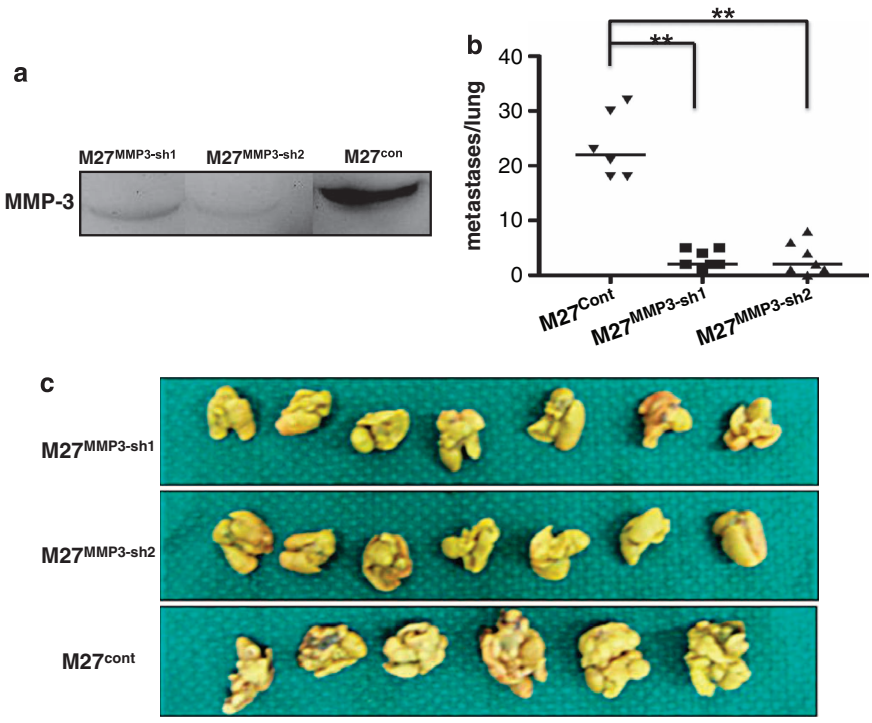

Figure 8. MMP-3 is required for lung metastasis of M27 cells. Mice were injected *via* the tail vein with 2×10^5 M27 cells that were stably transfected with MMP-3 shRNA or a scrambled sequence as control. Lungs were removed 19 days later and fixed in Bouin's solution prior to enumeration of lung metastases. 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. $^{**}P < 0.01$.

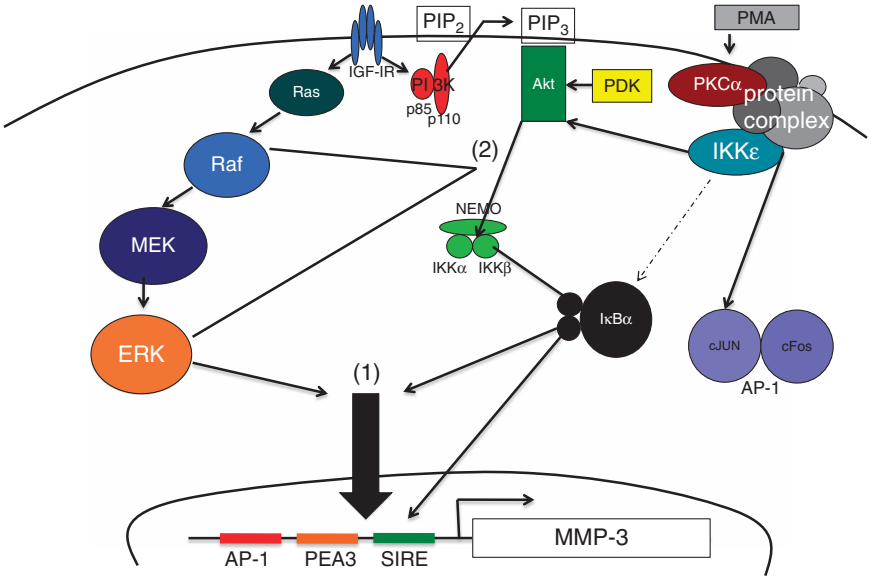

Figure 9. A proposed model for the coordinated regulation of MMP-3 transcription by IKKε. A diagrammatic representation is shown based on our and other data for potential mechanisms of action of IKKε in transcriptional regulation of MMP-3. PMA-mediated activation of PKCα results in the formation of a protein complex that recruits IKKε 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.[58]

Polymerase chain reaction

RNA was extracted using Trizol (Invitrogen) and cDNA synthesized as we previously described.[55] Semiquantitative RT–PCR was performed using a standard procedure, as we previously described.[34] qPCR was performed using the MyiQ2 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 (ddC_t) were calculated using the BioRad iQ5 software with the modified equation initially introduced by Livak *et al*.[56] 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 2×10^5 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 elsewhere[57] 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 M NaCl, 5 mM $CaCl_2$, 0.02% NaN_3 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% Coomasie 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. TNF-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: 5260). 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 2×10^5 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/portal injections of 2×10^5 tumor cells, followed by splenectomy, as previously described.[55] 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.

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