



# Blockade of NF- $\kappa$ B activity in human prostate cancer cells is associated with suppression of angiogenesis, invasion, and metastasis

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Since the NF- $\kappa$ B/relA transcription factor is constitutively activated in human prostate cancer cells, we determined whether blocking NF- $\kappa$ B/relA activity in human prostate cancer cells affected their angiogenesis, growth, and metastasis in an orthotopic nude mouse model. Highly metastatic PC-3M human prostate cancer cells were transfected with a mutated I $\kappa$ B $\alpha$  (I $\kappa$ B $\alpha$ M), which blocks NF- $\kappa$ B activity. Parental (PC-3M), control vector-transfected (PC-3M-Neo), and I $\kappa$ B $\alpha$ M-transfected (PC-3M–I $\kappa$ B $\alpha$ M) cells were injected into the prostate gland of nude mice. PC-3M and PC-3M-Neo cells produced rapidly growing tumors and regional lymph node metastasis, whereas PC-3M–I $\kappa$ B $\alpha$ M cells produced slow growing tumors with low metastatic potential. NF- $\kappa$ B signaling blockade significantly inhibited *in vitro* and *in vivo* expression of three major proangiogenic molecules, VEGF, IL-8, and MMP-9, and hence decreased neoplastic angiogenesis. Inhibition of NF- $\kappa$ B activity in PC-3M cells also resulted in the downregulation of MMP-9 mRNA and collagenase activity, resulting in decreased invasion through Matrigel. Collectively, these data suggest that blockade of NF- $\kappa$ B activity in PC-3M cells inhibits angiogenesis, invasion, and metastasis. *Oncogene* (2001) 20, 4188–4197.

**Keywords:** prostate cancer; NF- $\kappa$ B; angiogenesis; invasion; metastasis

## Introduction

Most deaths from prostate cancer are due to metastases that are resistant to therapy (Greenlee *et al.*, 2000). The pathogenesis of cancer metastasis consists of a series of sequential and selective steps that include tumor cell proliferation, angiogenesis, detachment, invasion, intravasation, survival in the circulation, adhesion to endothelial cells, extravasation, and growth in distant organs (Fidler, 1990). Numerous reports have demonstrated that in prostate cancer as well as in other tumor types, the metastatic potential of

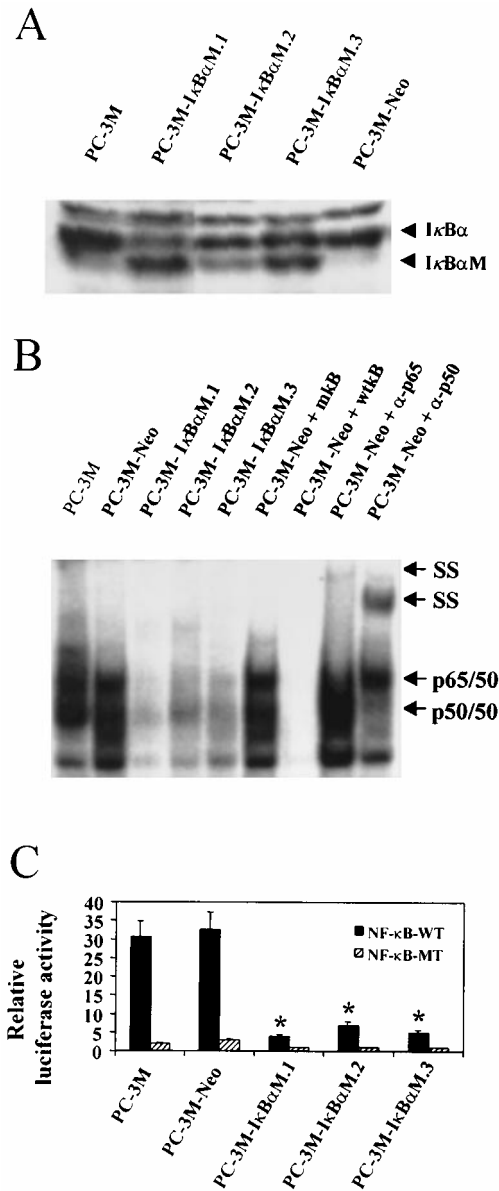
tumor cells directly correlates with the expression level of several angiogenic genes, including vascular endothelial growth factor (VEGF), also called vascular permeability factor (VPF) (Balbay *et al.*, 1999; Duque *et al.*, 1999; Melnyk *et al.*, 1999); basic fibroblast growth factor (bFGF) (Nakamoto *et al.*, 1992; Giri *et al.*, 1999; Melnyk *et al.*, 1999), interleukin-8 (IL-8) and, type IV collagenases (MMP-2 and MMP-9) (Hamdy *et al.*, 1994; Greene *et al.*, 1997; Melnyk *et al.*, 1999; Kuniyasu *et al.*, 2000).

Many of those molecules function as pro-angiogenic factors. VEGF has been shown to induce the proliferation of endothelial cells, to increase vascular permeability, to increase endothelial cell survival, and to induce the production of plasminogen activator by these cells (Senger *et al.*, 1983; Leung *et al.*, 1989). IL-8, a chemoattractant cytokine, has been shown to attract and activate neutrophils in inflammatory regions, to upregulate expression of MMPs and to directly induce angiogenesis (Koch *et al.*, 1992; Mukaida *et al.*, 1994). The proteolytic activity of MMP-9, the 92-kd type IV gelatinase/collagenase, facilitates the migration of endothelial cells through the altered extracellular matrix toward the source of the angiogenic stimulus (Hiraoka *et al.*, 1998; Puyraimond *et al.*, 1999).

How the constitutive expression of these genes is regulated in malignant prostate cancer cells is not clear. Recent studies have demonstrated that the pleiotropic transcription factor NF- $\kappa$ B regulates the expression of multiple genes including IL-8 and MMP-9 in several types of cells (Mukaida *et al.*, 1994; Vilarete *et al.*, 1996; Yokoo and Kitamura, 1996; Bond *et al.*, 1998). Importantly, NF- $\kappa$ B has been shown to be constitutively activated in prostate cancer cells (Palayoor *et al.*, 1999; Pajonk *et al.*, 1999), and elevated NF- $\kappa$ B activity is also sustained in androgen-responsive human prostate cancer cells by androgen treatment (Ripple *et al.*, 1999).

NF- $\kappa$ B is an inducible dimeric transcription factor that belongs to the Rel/NF- $\kappa$ B family of transcription factors whose prototype in most nonlymphoid cells is a heterodimer composed of the RelA (p65) and NF- $\kappa$ B1 (p50) subunits (Verma *et al.*, 1995; Barnes *et al.*, 1997). NF- $\kappa$ B activation involves its release from its inhibitor, I $\kappa$ B, and its subsequent translocation from the cytoplasm to the nucleus, where it binds to cognate

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**Figure 1** I $\kappa$ B $\zeta$ M transfection and NF- $\kappa$ B activity. (a) Exogenous I $\kappa$ B $\zeta$ M expression. Cytosolic protein was extracted from PC-3M cells, PC-3M cells transfected with pLXSN (PC-3M-Neo), and PC-3M cells transfected with pLXSN-I $\kappa$ B $\zeta$ M (PC-3M-I $\kappa$ B $\zeta$ M.1, I $\kappa$ B $\zeta$ M.2 and I $\kappa$ B $\zeta$ M.3). Western blot analysis using specific anti-I $\kappa$ B $\zeta$  antibody detected endogenous I $\kappa$ B $\zeta$  (higher mol wt) and exogenous I $\kappa$ B $\zeta$ M (lower mol wt) (arrows). (b) NF- $\kappa$ B binding activities. Nuclear protein was extracted from PC-3M (lane 1), PC-3M-Neo (lane 2), PC-3M-I $\kappa$ B $\zeta$ M.1 (lane 3), PC-3M-I $\kappa$ B $\zeta$ M.2 (lane 4), and PC-3M-I $\kappa$ B $\zeta$ M.3 (lane 5) cells. EMSA was performed as described above. For competition reactions, nuclear protein was extracted from PC-3M-Neo cells, and unlabeled cold wild-type NF- $\kappa$ B (lane 6) or mutant NF- $\kappa$ B (lane 7) oligonucleotide were added to the reaction mixture. For supershift reactions, specific anti-p65 (lane 8) and anti-p50 (lane 9) antibodies were added to the reaction mixture. (c) NF- $\kappa$ B-Luc reporter activity. Luciferase reporters driven by either wild-type (2x NF- $\kappa$ B-wt-luc) or mutant (2x NF- $\kappa$ B-mt-luc) NF- $\kappa$ B response elements were cotransfected with pBAActin-RL into the different prostate cancer cells. Firefly and Renilla luciferase activities were quantified using the dual-luciferase reporter assay system. Luciferase activity in test samples was compared with the activity in PC-3M cells transfected with 2x NF- $\kappa$ B-mt-luc, which was assigned the value of 1. Note that I $\kappa$ B $\zeta$ M transfection suppressed

sequences in the promoter region of multiple genes. Regulation of gene expression by NF- $\kappa$ B is controlled mainly by the inhibitory I $\kappa$ B proteins, which include I $\kappa$ B $\alpha$  (Beg and Baldwin, 1993; Verma *et al.*, 1995; Barnes *et al.*, 1997). Upon stimulation, I $\kappa$ B $\alpha$  is rapidly phosphorylated and degraded via the ubiquitin-proteasome pathway, permitting activation and nuclear import of NF- $\kappa$ B. Dominant-negative mutant forms of I $\kappa$ B $\alpha$  (I $\kappa$ B $\alpha$ M) have been engineered that cannot be phosphorylated and degraded, and thus the nuclear importation and DNA binding of NF- $\kappa$ B/reI $\alpha$  complexes are repressed constitutively in stably transfected as well as in transiently transfected cells (Brockman *et al.*, 1995; Brown *et al.*, 1995; Traenckner *et al.*, 1995; Van Antwerp *et al.*, 1996; Reuther *et al.*, 1998).

Numerous *in vitro* and *in vivo* studies have suggested that NF- $\kappa$ B/reI $\alpha$  plays an important role in the regulation of cell proliferation, apoptosis, and adhesion (Higgins *et al.*, 1993; Beg *et al.*, 1996; Gilmore *et al.*, 1996; Van Antwerp *et al.*, 1996; Wang *et al.*, 1996; Reuther *et al.*, 1998; Duffey *et al.*, 1999; Yoshida *et al.*, 1999; Huang *et al.*, 2000). Whether NF- $\kappa$ B/reI $\alpha$  also regulates angiogenesis, tumorigenicity, invasion, and metastasis of prostate cancer is not known. In the present study, highly malignant human prostate cancer cells PC-3M constitutively expressed high levels of NF- $\kappa$ B/reI $\alpha$  activity. Transfection of these cells with I $\kappa$ B $\zeta$ M, which inhibits NF- $\kappa$ B activity (Van Antwerp *et al.*, 1996), was associated with decreased expression of VEGF, IL-8, and MMP-9 and hence decreased angiogenesis, invasion, and metastasis of human prostate cancer cells growing in the prostate of nude mice.

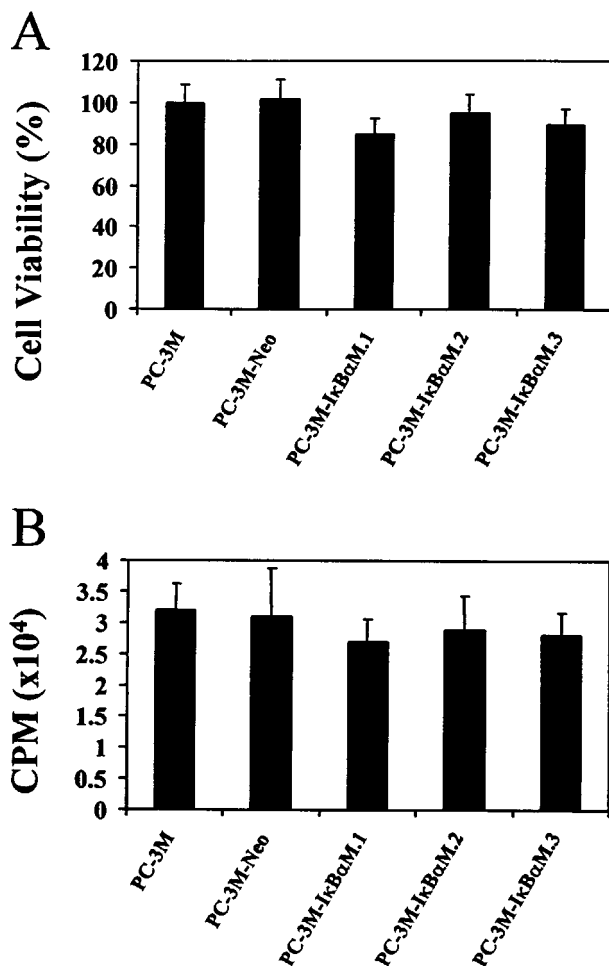
## Results

### *Downregulation of constitutive NF- $\kappa$ B activity in metastatic human prostate cancer cells by transfection with I $\kappa$ B $\zeta$ M expression vector*

In the first set of experiments, highly metastatic PC-3M human prostate adenocarcinoma cells were stably transfected with the I $\kappa$ B $\zeta$ M expression vector, which encodes a mutated I $\kappa$ B $\zeta$ . Western blot analysis detected expression of endogenous I $\kappa$ B $\zeta$  in PC-3M, control pLXSN-transfected (Neo), and I $\kappa$ B $\zeta$ M-transfected (I $\kappa$ B $\zeta$ M.1, I $\kappa$ B $\zeta$ M.2 and I $\kappa$ B $\zeta$ M.3) PC-3M cells, whereas it detected exogenous mutant I $\kappa$ B $\zeta$  only in the I $\kappa$ B $\zeta$ M-transfected PC-3M cells (Figure 1a).

To determine whether the expression of I $\kappa$ B $\zeta$ M led to a block of nuclear translocation of NF- $\kappa$ B, we probed nuclear extracts of PC-3M, PC-3M-Neo, and I $\kappa$ B $\zeta$ M-transfected PC-3M cells for NF- $\kappa$ B activity by EMSA. Antibodies detecting either the p65 or p50 components of NF- $\kappa$ B were used to confirm the

NF- $\kappa$ B activities (\* $P$  < 0.01). This is one representative experiment of two



**Figure 2** *In vitro* growth of human prostate cells transfected with I $\kappa$ B dominant-negative expression vector. (a) MTT assay. PC-3M, PC-3M-Neo, PC-3M-I $\kappa$ B $\alpha$ M.1, PC-3M-I $\kappa$ B $\alpha$ M.2, and PC-3M-I $\kappa$ B $\alpha$ M.3 cells were cultured *in vitro* for 48 h. The number of metabolically active cells ( $5 \times 10^3$ ) was determined by the MTT assay. (b) Uptake of [ $^3$ H]TdR. Cells were plated in 96-well plates for 24 h, at which point  $0.5 \mu\text{Ci/ml}$  of [ $^3$ H]TdR was added. [ $^3$ H]TdR incorporation was measured 12 h later. No discernible differences in proliferation were found among the different groups of cells. This is one representative experiment of three

specificity of the EMSA analysis. As shown in Figure 1b, constitutive NF- $\kappa$ B binding activity was present in PC-3M cells. The expression of I $\kappa$ B $\alpha$ M significantly inhibited the NF- $\kappa$ B activity in I $\kappa$ B $\alpha$ M transfected cells (I $\kappa$ B $\alpha$ M.1, I $\kappa$ B $\alpha$ M.2 and I $\kappa$ B $\alpha$ M.3) but not in the control Neo cells. The specificity of the observed bandshift was checked by supershift experiments with anti-p65 and anti-p50 antibodies, which indicated that the NF- $\kappa$ B complexes contained both p50 and p65 components.

Next, we confirmed the suppressive effect of I $\kappa$ B $\alpha$ M transfection on the constitutive levels of NF- $\kappa$ B/reIA activity by using NF- $\kappa$ B-dependent luciferase reporter activity assay. A 2x NF- $\kappa$ B-Luc (wild-type) or a 2x NF- $\kappa$ B-mut-luc (mutant) reporter (DiDonato *et al.*, 1997) was transiently transfected into the PC-3M cells.

As shown in Figure 1c, constitutive NF- $\kappa$ B reporter activity was significantly decreased in PC-3M I $\kappa$ B $\alpha$ M-transfected I $\kappa$ B $\alpha$ M.1, I $\kappa$ B $\alpha$ M.2 and I $\kappa$ B $\alpha$ M.3 cells, agreeing with the EMSA results (Figure 1b). Collectively, the data show that PC-3M cells constitutively express NF- $\kappa$ B/reIA activity, which can be inhibited by transfection with I $\kappa$ B $\alpha$ M expression vector.

#### *In vitro* growth of human prostate cancer cells transfected with I $\kappa$ B $\alpha$ M expression vector

Next, we tested whether stable transfection of PC-3M cells with I $\kappa$ B $\alpha$ M affected cell proliferation *in vitro*. PC-3M, PC-3M-Neo, and I $\kappa$ B $\alpha$ M-transfected (I $\kappa$ B $\alpha$ M.1, I $\kappa$ B $\alpha$ M.2 and I $\kappa$ B $\alpha$ M.3) cells were seeded into 96-well plates ( $2 \times 10^3$  cells/well) for 48 h in 10% CMEM. Methylthiotetrazole (MTT) assay and [ $^3$ H]TdR incorporation demonstrated that the *in vitro* growth rate of all the lines was very similar (Figure 2a,b), suggesting that the stable expression of I $\kappa$ B $\alpha$ M did not alter the *in vitro* growth of PC-3M cells.

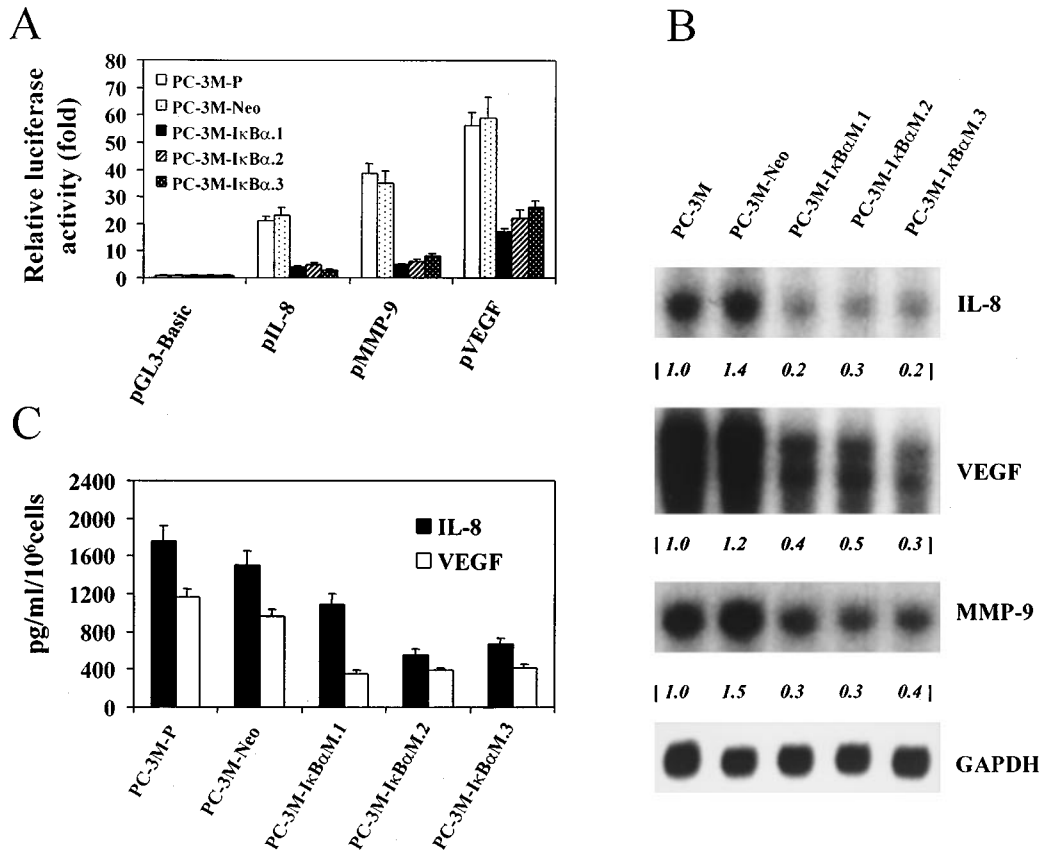
#### *Transfection of PC-3M cells with I $\kappa$ B $\alpha$ M expression vector downregulates expression of proangiogenic molecules*

The effect of NF- $\kappa$ B inhibition on the expression of proangiogenic molecules including VEGF, IL-8, and MMP-9 was studied in the I $\kappa$ B $\alpha$ M-transfected PC-3M cells. The data shown in Figure 3a demonstrate that, consistent with a decreased NF- $\kappa$ B promoter activity, VEGF, IL-8, and MMP-9 promoter activities were significantly suppressed in I $\kappa$ B $\alpha$ M-transfected PC-3M cells as compared to parental PC-3M and Neo cells. These results suggest that in addition to IL-8 and MMP-9, NF- $\kappa$ B may directly regulate VEGF expression at the transcriptional level.

The expression of VEGF, IL-8, and MMP-9 genes in I $\kappa$ B $\alpha$ M-transfected cells was further determined at both the mRNA and protein levels. Cellular mRNA was extracted from prostate cancer cells and subjected to Northern blot analysis. As shown in Figure 3b, VEGF, IL-8, and MMP-9 mRNA expression was significantly lower in I $\kappa$ B $\alpha$ M.1, I $\kappa$ B $\alpha$ M.2 and I $\kappa$ B $\alpha$ M.3 cells than in control PC-3M and Neo cells. Consistently, I $\kappa$ B $\alpha$ M-transfected PC-3M cells secreted significantly decreased levels of VEGF and IL-8 into the culture supernatant as determined by quantitative IL-8 and VEGF ELISA (Figure 3c).

#### *Suppression of human prostate cell invasion by transfection with I $\kappa$ B $\alpha$ M*

Next we analysed whether the expression of MMP-9 in I $\kappa$ B $\alpha$ M-transfected PC-3M cells correlated with their ability to invade through basement membrane. The level of MMP-9 protein levels were measured by zymographic assay of collagenase activity with the volume of culture supernatant normalized for cell number. Densitometric measurements indicated that the 92-Kb MMP-9 collagenase activity of PC-3M-



**Figure 3** Decreased expression of proangiogenic molecules in metastatic human prostate cells transfected with I $\kappa$ B $\alpha$ M expression vector. (a) IL-8, MMP-9, and VEGF promoter activity. pGL2-IL-8, pGL2-MMP-9 and PGL2-VEGF reporters were cotransfected with pB-Actin-RL (Renilla) reporter into the prostate cancer cells. The pGL2-basic luciferase reporter served as negative control. Luciferase activity was determined as described in Figure 1c. The increase in luciferase activity was calculated relative to the luciferase activity of pGL2-basic in PC-3M cells which was assigned the value of 1. (b) Northern blot. Cellular mRNA was extracted and Northern blot analysis was performed using IL-8, VEGF, and MMP-9 cDNA probes. Equal loading of mRNA was monitored by hybridizing the same membrane filter with a GAPDH cDNA probe. (c) ELISA. IL-8 and VEGF proteins in the culture supernatants determined by ELISA were expressed as pg/10<sup>6</sup> cells/48 h. Note that I $\kappa$ B $\alpha$ M transfection suppressed IL-8 and VEGF protein production (\**P*<0.01)

I $\kappa$ B $\alpha$ M.1, I $\kappa$ B $\alpha$ M.2, and I $\kappa$ B $\alpha$ M.3 was decreased 3.0 or 4.0-fold, compared with PC-3M or Neo cells, respectively (Figure 4a). In contrast, 72-Kb MMP-2 collagenase activity was unaffected by I $\kappa$ B $\alpha$ M transfection. As shown in Figure 4b, transfection of PC-3M cells with I $\kappa$ B $\alpha$ M produced a 60–70% decrease in invasion through Matrigel-coated filters as compared with PC-3M or Neo cells.

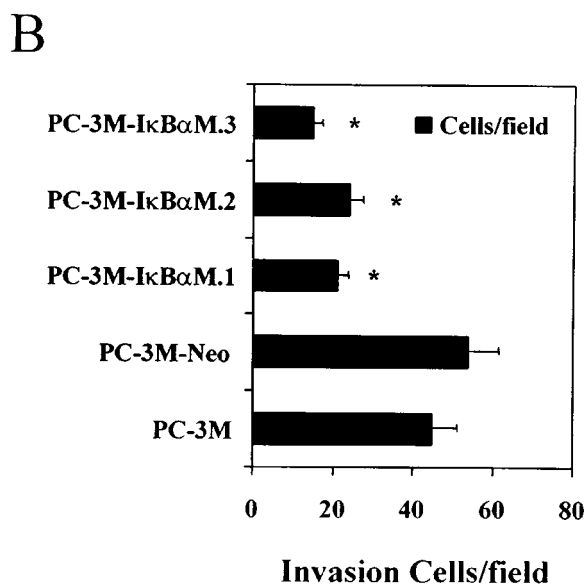
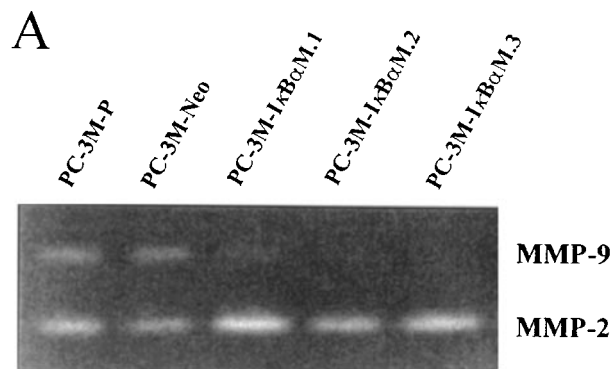
#### Suppression of tumorigenicity and metastasis of human prostate cancer cells by I $\kappa$ B $\alpha$ M transfection

PC-3M, PC-3M-Neo, and PC-3M-I $\kappa$ B $\alpha$ M cells ( $5 \times 10^5$ /inoculum) were injected into the prostate of nude mice. The mice were euthanized 6 weeks later and necropsied. Tumorigenicity (incidence, tumor weight) and lymph node metastasis were recorded. In contrast to PC-3M and PC-3M-Neo cells, PC-3M-I $\kappa$ B $\alpha$ M cells produced small tumors and a low incidence of spontaneous lymph node metastasis (Table 1). Therefore, inhibition of NF- $\kappa$ B activity by I $\kappa$ B $\alpha$ M transfection was associated with

suppression of tumorigenicity and metastasis in this androgen-independent prostate cancer system.

#### Inhibition of NF- $\kappa$ B activation and decreased expression of VEGF, IL-8, and MMP-9 in I $\kappa$ B $\alpha$ M-transfected tumors

Immunohistochemical (IHC) analyses were undertaken to determine whether I $\kappa$ B $\alpha$ M transfection suppressed NF- $\kappa$ B activity in prostate cancer cells growing in the prostate of nude mice. Analysis of tumors using antibodies that recognize the nuclear localization sequence of the activated form of NF- $\kappa$ B p65 demonstrated inhibition of activated NF- $\kappa$ B in the PC-3M-I $\kappa$ B $\alpha$ M tumors (Figure 5). The *in vivo* protein expression of VEGF, IL-8, and MMP-9 protein was also evaluated by IHC. Significant staining for IL-8, MMP-9, and VEGF was found in untransfected and, Neo-transfected tumors, but not in the I $\kappa$ B $\alpha$ M-transfected tumors. Thus, expression of I $\kappa$ B $\alpha$ M in prostate cancer cells inhibited constitutive activation of



**Figure 4** Suppression of human prostate cancer cell invasion through Matrigel-coated filters by I $\kappa$ B $\alpha$ M transfection. (a) Collagenase activity. Collagenase activity of conditioned medium of PC-3M, PC-3M-Neo, PC-3M-I $\kappa$ B $\alpha$ M.1, PC-3M-I $\kappa$ B $\alpha$ M.2, and PC-3M-I $\kappa$ B $\alpha$ M.3 cells was analysed by zymography. I $\kappa$ B $\alpha$ M-transfected (PC-3M-I $\kappa$ B $\alpha$ M.1, I $\kappa$ B $\alpha$ M.2 and I $\kappa$ B $\alpha$ M.3) cells displayed one-third to one-fourth the MMP-9 activity as PC-3M and PC-3M-Neo cells. The activity of the  $M_r$  72 000 (MMP-2) collagenase was unchanged and served as an internal control for equal loading. (b) The number of migrated cells that penetrated through the Matrigel-coated filters expressed as the average number of cells in 10 random fields identified on the bottom surface of the filter. The reduction in number of invading cells in I $\kappa$ B $\alpha$ M-transfected (PC-3M-I $\kappa$ B $\alpha$ M.1, I $\kappa$ B $\alpha$ M.2, and I $\kappa$ B $\alpha$ M.3) groups was statistically significant ( $*P < 0.01$ ) as compared with PC-3M and PC-3M-Neo cells. This is one representative experiment of three

NF- $\kappa$ B, which was in turn associated with reduced expression of VEGF, IL-8, and MMP-9.

#### Inhibition of angiogenesis in I $\kappa$ B $\alpha$ M-transfected human prostate cancers

Finally, we determined whether decreased NF- $\kappa$ B/reI $\alpha$  activity and VEGF, IL-8, and MMP-9 production correlated with suppression of tumor angiogenesis.

**Table 1** Suppression of tumorigenicity and production of lymph node metastasis by PC-3M human prostate cancer cells transfected with I $\kappa$ B $\alpha$ M

Cell line	Primary tumor		Incidence of lymph node metastasis
	Incidence	Weight (mg)	
PC-3M	8/9	495 $\pm$ 313	6/9
PC-3M-Neo	9/10	543 $\pm$ 354	7/10
PC-3M-I $\kappa$ B $\alpha$ M.1	6/10	142 $\pm$ 26*	1/10
PC-3M-I $\kappa$ B $\alpha$ M.2	8/10	220 $\pm$ 82*	2/10
PC-3M-I $\kappa$ B $\alpha$ M.3	6/10	168 $\pm$ 55*	1/10

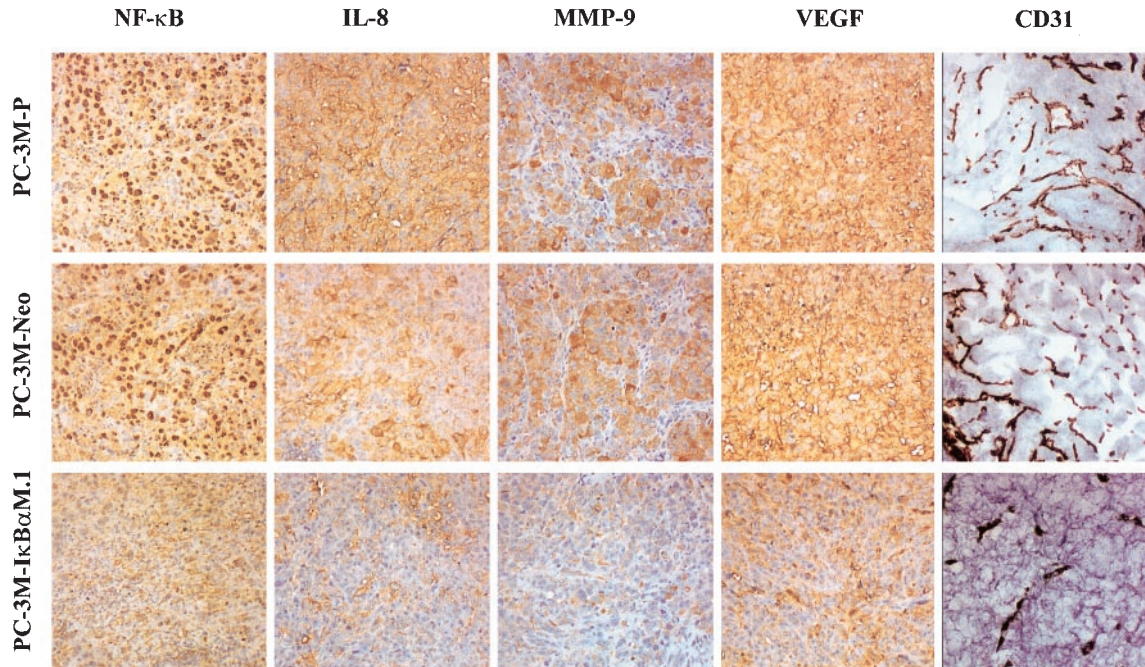
PC-3M, PC-3M-Neo, PC-3M-I $\kappa$ B $\alpha$ M.1, PC-3M-I $\kappa$ B $\alpha$ M.2, PC-3M-I $\kappa$ B $\alpha$ M.3 cells ( $1 \times 10^6$ ) were injected into the prostate of nude mice. Primary tumors in prostates were weighed, and lymph node metastasis was determined by histology.  $*P < 0.001$ , Mann-Whitney *U*-test

Mean vessel density was determined by IHC using anti-CD31 antibodies (Figure 5). Tumors formed by PC-3M and Neo-transfected cells contained numerous blood vessels, whereas the tumors formed by I $\kappa$ B $\alpha$ M-transfected cells did not.

#### Discussion

The present results demonstrate that suppression of NF- $\kappa$ B activity in human prostate cancer cells by I $\kappa$ B $\alpha$ M transfection inhibits their tumorigenic and metastatic properties in nude mice by suppressing angiogenesis and invasion. I $\kappa$ B $\alpha$ M transfection-blocked NF- $\kappa$ B activity was associated with down-regulation of VEGF, IL-8, and MMP-9 promoter activities and decreased expression of these genes in cultured cells and in cells implanted into the prostate gland of nude mice. The decreased expression of VEGF, IL-8, and MMP-9 *in vivo* directly correlated with decreased neovascularization and production of lymph node metastasis. Our results, therefore, provide direct evidence for the involvement of NF- $\kappa$ B in the regulation of angiogenesis and metastasis of prostate cancer cells.

The progressive growth and metastasis of prostate cancers are angiogenesis-dependent. Tumor angiogenesis is mediated, in part, by the secretion of angiogenic factors by tumor cells and host cells (Folkman, 1992; Fidler and Ellis, 1994). Prostate cancer cells can secrete a variety of proangiogenic molecules, including VEGF, IL-8, and collagenases. Expression of VEGF by prostate cancer cells has been shown to directly correlate with malignant potential (Ferrer *et al.*, 1997; Jackson *et al.*, 1997), and the administration of anti-VEGF antibodies to mice injected with human prostate cancer cells has been shown to inhibit neovascularization within the tumors (Borgstrom *et al.*, 1998). Previous studies have shown that serum concentrations of IL-8 associated with increasing prostate cancer stage (Moore *et al.*, 1999; Veltri *et al.*, 1999). Further IL-8 expression is associated with increased tumorigenicity, angiogenesis, and metastasis of prostate cancer cells (Inoue *et al.*, 2000). MMP activity also appeared to be



**Figure 5** Inhibition of activity of NF- $\kappa$ B and decreased expression of VEGF, IL-8, and MMP-9 and vessel formation in PC-3M I $\kappa$ B $\zeta$ M transfectant tumors, as demonstrated by immunohistochemistry of human prostate cancers growing in the prostate of nude mice. PC-3M, PC-3M-Neo, PC-3M-I $\kappa$ B $\zeta$ M.1, PC-3M-I $\kappa$ B $\zeta$ M.2, and PC-3M-I $\kappa$ B $\zeta$ M.3 cells were injected into the prostate of groups of nude mice. Forty-five days later, the mice were killed and necropsied. Prostate tumors of similar size were processed for immunohistochemical analysis. Blood vessels were counted using a light microscope after immunostaining of tissue sections with anti-CD31 antibodies. NF- $\kappa$ B (p65), VEGF, MMP-9, and IL-8 immunostaining was performed as described in Materials and methods. Note that tumors formed by I $\kappa$ B $\zeta$ M-transfected cells expressed lower levels of activated NF- $\kappa$ B, MMP-9, VEGF, and IL-8 and were less vascularized than tumors produced by PC-3M or Neo-transfected cells

increased in malignant prostatic tissue compared with BPH. MMP-9 was shown to be exclusively expressed by malignant human prostate cancers (Hamdy *et al.*, 1994) and murine prostate model systems (Sehgal *et al.*, 1998). The mechanism that is responsible for over-expression of these metastasis-related genes in prostate cancer cells is unknown. Since NF- $\kappa$ B is a pleiotropic transcription factor that controls the expression of many genes, including inducible and constitutive IL-8 (Mukaida *et al.*, 1994; Vilarete and Remick 1996) and MMP-9 (Yokoo and Kitamura, 1996; Bond *et al.*, 1998), we determined whether it regulates the expression of these angiogenic molecules in prostate cancer cells.

The significant decrease in activity of VEGF, IL-8, and MMP-9 promoters in the I $\kappa$ B $\zeta$ M-transfected cells suggested that NF- $\kappa$ B regulates constitutive expression of these genes in PC-3M cells, at least in part, at the transcription level. Our data agree with previous reports that both IL-8 and MMP-9 promoters contain NF- $\kappa$ B binding elements (Matsusaka *et al.*, 1993; Sato *et al.*, 1993). The human VEGF promoter was shown to contain multiple binding sites for Sp-1, AP-1, and AP-2 transcription factors and hypoxia-regulated elements (Garrido *et al.*, 1993; Levy *et al.*, 1995). More recently, it has been shown that induction of VEGF in UV-irradiated skin cells can be blocked by

treatment with NF- $\kappa$ B decoy oligodeoxynucleotides (Abeyama *et al.*, 2000), suggesting that NF- $\kappa$ B could play a role in the regulation of VEGF expression. Our finding that constitutive VEGF expression is down-regulated by blocking NF- $\kappa$ B activity in human prostate cells supports this concept. Studies are under way to define the NF- $\kappa$ B binding sites in the 5'- and/or 3'-regulatory region(s) of the VEGF gene.

MMP-9 and one of its indirect activators, uPA, are critical elements for the intravasation of tumor cells (Kim *et al.*, 1998). The local production of MMP-9 by prostate cancer or stroma facilitates the local degradation of the extracellular matrix and results in tumor invasion and subsequent metastasis (Ray and Stetler-Stevenson, 1994). In the present study, we found that I $\kappa$ B $\zeta$ M transfection, which blocked NF- $\kappa$ B activity, inhibited the expression of MMP-9 by PC-3M cells, resulting in decreased invasion through basement membrane.

Although our data clearly showed that blocking NF- $\kappa$ B activity suppressed tumor growth and metastasis of human prostate cancer by inhibiting of angiogenesis and invasion, we cannot exclude contributions from other mechanisms. For example, it has been shown that blocking NF- $\kappa$ B results in inhibiting cell adhesion (Higgins *et al.*, 1993) and induces production of proinflammatory cytokines (Duffey *et al.*, 1999) and

plasminogen activator uPA (Wang *et al.*, 1999). NF- $\kappa$ B activation is also involved in the regulation of cell proliferation and apoptosis (Van Antwerp *et al.*, 1996; Beg and Baltimore, 1996; Wang *et al.*, 1996). Our studies, however, are consistent with previous reports showing that inhibition of NF- $\kappa$ B in cancer cells by stable transfection of I $\kappa$ B $\alpha$ M does not inhibit cell growth (Bentires-Alj *et al.*, 1999; Pajonk, 1999), and hence the decrease in tumorigenicity was probably due to inhibition of angiogenesis.

The present results extend our previous report that in metastatic human melanoma cells blocking NF- $\kappa$ B activity decreases angiogenesis and metastasis of human melanoma cells (Huang *et al.*, 2000). Many different neoplasms have been shown to express high levels of NF- $\kappa$ B/relA (Gilmore *et al.*, 1996; Nakshatri *et al.*, 1997; Dong *et al.*, 1999; Meyskens *et al.*, 1999; Wang *et al.*, 1999), as do androgen-independent human prostate cells (Pajonk *et al.*, 1999; Palayoor *et al.*, 1999), and NF- $\kappa$ B activity can be induced by androgen in androgen-sensitive prostate cells (Ripple *et al.*, 1999). Moreover, many cytokines, present in the prostate cancer microenvironment can activate NF- $\kappa$ B (Barnes and Karin, 1997). NF- $\kappa$ B activation in prostate cancer may provide a growth advantage by increasing angiogenesis and invasiveness as well as by suppressing apoptosis.

## Materials and methods

### Cell lines and reagents

PC-3M human prostate carcinoma cells (Stephenson *et al.*, 1992) were maintained in culture (5% CO<sub>2</sub> and 95% air at 37°C) as adherent monolayers in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), sodium pyruvate, nonessential amino acids, L-glutamine, and vitamin solution (Flow Laboratories, Rockville, MD, USA). All cultures were free of mycoplasma and pathogenic murine viruses. All reagents used in tissue culture were free of endotoxins as determined by the *Limulus ameobocyte* lysate assay (sensitivity limit of 0.125 ng/ml) purchased from Associates of Cape Cod (Falmouth, MA, USA).

### Animals

Male athymic BALB/c nude mice were purchased from the Animal Production Area of the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD, USA). The mice were housed in laminar flow cabinets under specific pathogen-free conditions and used at 10 weeks of age. Animals were maintained according to institutional regulations in facilities approved by the American Association for Accreditation of Laboratory Animal Care in accordance with current regulations and standards of the United States Department of Agriculture, Department of Health and Human Services, and National Institutes of Health.

### ELISA for human IL-8 and VEGF expression

The level of IL-8 and VEGF protein in culture supernatants was determined by using a quantitative immunometric sandwich enzyme immunoassay (ELISA) kit (Quantikine

IL-8 and VEGF ELISA kits, R&D Systems, Minneapolis, MN, USA). The absorbance of the samples was compared with the standard curve (Huang *et al.*, 2000).

### Northern blot analysis

Cellular mRNA was extracted from prostate cancer cells by using the FastTrack mRNA isolation kit (Invitrogen Co., San Diego, CA, USA). The mRNA (2  $\mu$ g) was separated electrophoretically on 1% denaturing formaldehyde agarose gel, transferred to a GeneScreen nylon membrane (DuPont Co., Boston, MA, USA) in 20 $\times$  standard saline citrate, and cross-linked with a UV-Stratalinker 1800 (Stratagene, La Jolla, CA, USA). The cDNA probe used in the analysis was a 0.5-kb *Eco*RI cDNA fragment corresponding to human IL-8, a 1.0-kb cDNA fragment corresponding to human MMP-9 (Inoue *et al.*, 2000), and a 0.204-kb *Bam*HI/*Eco*RI cDNA fragment corresponding to human vascular endothelial growth factor (VEGF/VPF) (Balbay *et al.*, 1999). The cDNA probes were labeled with <sup>32</sup>P-deoxycytidine triphosphate using a random labeling kit (Roche, Indianapolis, IN, USA). The equivalence of mRNA sample loading was monitored by hybridizing the same membrane filter with a GAPDH cDNA probe.

### Promoter reporters and dual luciferase assays

Luciferase reporters driven by either two-copy wild-type (2x NF- $\kappa$ B-Luc) or mutant (2x NF- $\kappa$ B-mt-Luc) NF- $\kappa$ B responsive elements (DiDonato *et al.*, 1997) were used in this study. pGL2-IL8 is a pGL2-basic reporter containing a full-length firefly luciferase gene under the control of an IL-8 promoter flanking region from +44 to -1481 from pxp2-IL8 (Mukaida *et al.*, 1994). pGL2-MMP-9 is a pGL2-basic reporter containing a full-length firefly luciferase gene under the control of MMP-9 5'-flanking region from +53 to -670 (Gum *et al.*, 1997). The pGL2-VEGF is a pGL2-basic reporter containing a full-length firefly luciferase gene under the control of both a VEGF 5'-flanking region from +50 to -2274 and a 3'-UTR region from +1 to +1921 (Tischer *et al.*, 1991). Prostate cancer cells (1  $\times$  10<sup>5</sup>/well) growing in 6-well tissue culture plates were transfected with the indicated reporter plasmids using the Lipofectin reagent (Life Technologies, Inc., Gaithersburg, MD, USA). Transfection efficiency was normalized by cotransfection with a pB-Actin-RL reporter containing a full-length renilla luciferase gene (Promega, Madison, WI, USA) under the control of a human  $\beta$ -actin promoter (Huang *et al.*, 1998). Forty-eight hours after transfection, the cells were harvested in passive lysis buffer (Promega Corp., Madison, WI, USA). Firefly luciferase and renilla luciferase activities were quantified using the dual luciferase assay system (Promega). Changes in luciferase activity were calculated relative to the luciferase activity of pGL2-basic in PC3-M cells, which was given the reference value of 1 as described (Huang *et al.*, 1998).

### Stable transfection of prostate cancer cells with I $\kappa$ B $\alpha$ M and control vector

The cDNA plasmid pLXSN-I $\kappa$ B $\alpha$ M contains mutations at S32 and S36 of the NH<sub>2</sub>-terminus and a COOH-terminal PEST sequence mutation (Van Antwerp *et al.*, 1996). PC-3M cells (1  $\times$  10<sup>6</sup>) were transfected using 15  $\mu$ l of lipofectin reagent (Life Technologies, Inc., Gaithersburg, MD, USA) and 4  $\mu$ g of pLXSN-I $\kappa$ B $\alpha$ M expression vector or control pLXSN vector. Transfections were carried out according to the manufacturer's instructions. Cells were selected with

standard medium containing 600  $\mu$ g/ml G418. Fourteen days later, neo-resistant colonies were isolated by trypsinization and established as subcultures.

#### Western blot analysis

Cytosolic protein was isolated from control and transfected prostate cancer cells. The soluble protein was separated on 10% SDS-PAGE by electrophoresis and electrophoretically transferred onto Immobilon-P transfer membrane (Millipore, Bedford, MA, USA). The endogenous and mutant I $\kappa$ B $\alpha$  were probed with a polyclonal rabbit anti-human and anti-mouse I $\kappa$ B $\alpha$  (C-21, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The probe proteins were detected with the Amersham ECL system according to the manufacturer's instructions.

#### Electrophoretic mobility gel shift assay (EMSA)

Nuclear protein extracts were prepared as described before (Huang *et al.*, 1998). The NF- $\kappa$ B oligonucleotide probe was 5'-AGTTGAGGGACTTTCCAGGC-3'. EMSA was performed as described previously with minor modifications (Huang *et al.*, 1998). Five micrograms of nuclear extract protein and 30 000 c.p.m. of end-labeled double-stranded DNA probe were added to the mixture. The binding reaction was allowed to proceed for 25 min at 22°C. For supershift reactions, extracts were preincubated with anti-p65 or anti-p50 antibody (Calbiochem, San Diego, CA, USA) for 20 min on ice. For competition reaction using unlabeled wild-type NF- $\kappa$ B (wt $\kappa$ B) or mutant NF- $\kappa$ B (m $\kappa$ B) probes, nuclear extracts were incubated with 50-fold molar excess of unlabeled probes before the addition of the labeled probes.

#### Orthotopic implantation of tumor cells

Cultured PC-3M, PC-3M-neo, and I $\kappa$ BM-transfected cells were prepared for injection as described previously (Stephenson *et al.*, 1992). Mice were anesthetized with Nembutal and placed in the supine position. A lower-midline incision was made, and viable tumor cells ( $5 \times 10^5/40 \mu$ l) in HBSS were implanted into the dosal prostate lobes using a 30-gauge needle with a 1-ml disposable syringe and a calibrated, push button-controlled dispensing device (Hamilton Syringe Co., Reno, NV, USA). The prostate was returned to the abdominal cavity, and the abdominal wall was closed with a single layer of metal wound clips. Mice were killed 45 days after the intraprostatic implantation of tumor cells. The primary tumors were resected and weighed. Regional lymph node metastasis was assessed by microscopic examination of H&E-stained formalin-fixed paraffin-embedded tissues.

#### Immunohistochemistry and quantitation of microvessel density

Prostate tumors harvested at autopsy were processed for immunostaining using anti-NF- $\kappa$ B (p65) (Boehringer Mannheim, Indianapolis, IN, USA), anti-IL-8 (Biosource International, Camarillo, CA, USA), anti-VEGF (Santa Cruz Biotechnology), anti-MMP-9 (Oncogene Research Products, Cambridge, MA, USA), and anti-CD31/PECAM-1 (Pharmingen, San Diego, CA, USA) antibodies and appropriate peroxidase-conjugated secondary antibodies. The slides were examined under a bright-field microscope. A positive reaction was indicated by a reddish-brown precipitate in the cytoplasm. Negative controls were done using nonspecific IgG. Microvessel density of tumor was analysed by anti-CD31 immunostaining. Images were digitized using a Sony

3CD color video camera (Sony Corp., Tokyo, Japan) and a personal computer equipped with Optimas Image Analysis Software (Optimas Corp., Bothell, WA, USA).

#### Collagenase activity

Cells ( $5 \times 10^4$ ) were seeded in six-well plates and grown to 60–70% confluence. The cells were washed with HBSS twice and grown for 24 h in serum-free medium. The supernatant was collected for collagenase activity, and the remaining cells were counted to confirm the cell number. Collected samples were concentrated 50-fold using a Microcon microconcentrator (Amicon, Inc., Beverly, MA, USA). The samples were separated on 10% SDS-polyacrylamide gel containing 1 mg/ml gelatin (Sigma) by electrophoresis. After electrophoresis, the gel was washed at room temperature for 30 min in wash buffer (50 mM Tris-Cl, pH 7.5, 15 mM CaCl<sub>2</sub>, 1  $\mu$ M ZnCl<sub>2</sub>, 2.5% Triton X-100) and incubated 24 h at 37°C with shaking in the same buffer except for a 1% concentration of Triton X-100. The gel was stained with a solution of 0.1% Coomassie brilliant blue R-250. Clear zones against the blue background indicated the presence of gelatinolytic activity.

#### Matrigel-coated filter-invasion assay

Polyvinylpyrrolidone-free polycarbonate filters (8  $\mu$ m pore size; Nucleopore; Becton Dickinson Labware, Franklin Lakes, NJ, USA) were coated with a mixture of basement membrane components (Matrigel, 50  $\mu$ g/filter) and placed in modified Boyden chambers. The tumor cells ( $1 \times 10^5$ ) were seeded in the upper compartment of the Boyden chamber with MEM containing 0.1% BSA for 6 h at 37°C. NIH3T3 fibroblast-conditioned medium in the lower compartment served as a chemoattractant. The cells that migrated to the lower surface of the filter were stained with Diff-Quick (American Scientific Products, McGraw Park, IL, USA). Cells were counted at a 400 $\times$  magnification, and the mean numbers of cells per field in 10 random fields were recorded. Duplicate filters were used. The experiments were carried out three times.

#### Statistical analysis

The significance of the *in vitro* results was determined by the Student's *t*-test (two-tailed); the significance of the *in vivo* metastasis results was determined by the Mann-Whitney *U*-test.

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