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Sustained inhibition of PKCα reduces intravasation and lung seeding during mammary tumor metastasis in an *in vivo* mouse model

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Abstract

Metastasis is the major reason for breast cancer-related deaths. Although there is a host of indirect evidence for a role of PKCα in primary breast cancer growth, its role in the molecular pathways leading to metastasis have not been comprehensively studied. By treating mice with αV5-3, a novel peptide inhibitor selective for PKCα, we were able to determine how PKCα regulates metastasis of mammary cancer cells using a syngeneic and orthotopic model. The primary tumor growth was not affected by αV5-3 treatment. However, the mortality rate was reduced and metastasis in the lung decreased by more than 90% in the αV5-3-treated mice relative to the control-treated mice. αV5-3 treatment reduced intravasation by reducing MMP-9 activities. αV5-3 treatment also reduced lung seeding of tumor cells and decreased cell migration, effects that were accompanied by a reduction in NFκB-activity and cell surface levels of the CXCL12 receptor, CXCR4. αV5-3 treatment caused no apparent toxicity in non-tumor bearing naïve mice. Rather, inhibiting PKCα protected against liver damage and increased the number of immune cells in tumor-bearing mice. Importantly, αV5-3 showed superior efficacy relative to anti-CXCR4 antibody in reducing metastasis, *in vivo*. Together, these data show that pharmacological inhibition of PKCα effectively reduces mammary cancer metastasis by targeting intravasation and lung seeding steps in the metastatic process and suggest that PKCα-specific inhibitors, such as αV5-3, can be used to study the mechanistic roles of PKCα specifically and may provide a safe and effective treatment for the prevention of lung metastasis of breast cancer patients.

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Conflict of interest

DMR is the founder of KAI Pharmaceuticals, Inc. However, none of the work at her laboratory was supported by the company and the company had no access to information about unpublished research. Drs. Kim, Thorne, Huang and Mochly-Rosen and Lihan Sun declare no conflict of interest.

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Keywords

bioluminescence; mammary cancer; metastasis and protein kinase C

Introduction

Breast cancer is the leading cause of cancer-related deaths among women in the developed world and metastasis is the most common cause for the morbidity and mortality (Christofori, 2007; Podsypanina *et al*., 2008). Formation of metastasis is a multi-step process associated with breakdown of extracellular matrix surrounding the primary tumor, intravasation of tumor cells into the surrounding blood vessels, as well as migration, adhesion, survival and proliferation of these cancer cells at secondary sites (Christofori, 2007; Muller *et al*., 2001). The identification of new treatments that target critical events in the formation of metastasis without causing systemic toxicity is likely to benefit patients with advanced breast cancer.

Protein kinase C (PKC), a family of highly homologous serine/threonine protein kinases, has been implicated in a variety of processes important to tumor progression, including cancer cell proliferation, migration, invasion and survival (Griner and Kazanietz, 2007). Among the PKC isozymes, up-regulation and activation of PKCα has been suggested to be important in breast cancer (Assender *et al*., 2007; Borner *et al*., 1987; Frankel *et al*., 2007; O'Brian *et al*., 1989; Tonetti *et al*., 2003; Ways *et al*., 1995). Specifically, (i) PKCα expression is higher in human breast cancer tissues compared to normal tissues from the same patients (O'Brian *et al*., 1989); (ii) there is a negative correlation between PKCα levels and estrogen receptor α levels (a marker of positive prognosis) in human breast cancer cell lines (Assender *et al*., 2007; Borner *et al*., 1987); (iii) PKCα activity is significantly higher in tamoxifen-resistant human breast cancer cells lines as compared to tamoxifen-responsive parental cells (Frankel *et al*., 2007); (iv) accordingly, patients with PKCα-positive breast tumors were found to be resistant to tamoxifen treatment earlier than those with PKCα-negative tumors (Tonetti *et al*., 2003). Furthermore, PKCα over-expression reduced mRNA levels of estrogen receptors and increased the invasiveness of human MCF-7 breast cancer cells, as measured *in vitro* and in a xenograft model (Ways *et al*., 1995). However, a comprehensive syngeneic *in vivo* study investigating the signaling events involving PKCα in the molecular pathways leading to metastasis has not been carried out due to the lack of isozyme-specific tools to selectively inhibit the activity of this isozyme without toxicity. Therefore, we set out to define the steps where PKCα activity is critical during metastasis and to investigate the mechanisms by which PKCα regulates these steps, using *in vivo* imaging in a syngeneic orthotopic tumor model in immunocompetent mice.

We used a novel isozyme-specific inhibitor peptide of PKCα, designed from its V5 region, based on a rational approach that we described before (Mochly-Rosen and Gordon, 1998; Stebbins and Mochly-Rosen, 2001). Briefly, the PKCα inhibitor, αV5-3, is derived from a unique sequence in the highly variable region, V5, of this enzyme. We already found that PKC-derived peptides corresponding to the same position in the V5 region of PKCβI and βII serve as selective inhibitors for the corresponding isozyme (Stebbins and Mochly-Rosen, 2001).

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Until recently, the details of the metastatic processes *in vivo* remained vague due to the lack of imaging techniques with sufficient sensitivity and resolution to monitor cells engaged in the metastatic processes (Sahai, 2007). Here, we expressed firefly luciferase in mouse and human breast cancer cells and used whole body/tissue bioluminescence imaging techniques to detect the appearance of lung metastases and to follow the progression of the disease over time, in the same animal (Thorne and Contag, 2005). Bioluminescence imaging allows non-invasive *in vivo* imaging of metastatic sites with a high level of sensitivity (Sahai, 2007).

We found that PKCα inhibition with αV5-3 *in vivo* almost completely abrogates metastasis of breast cancer to the lungs and other organs in mice, which correlated with increased survival of these tumor-bearing animals. The PKCα antagonistic peptide inhibits intravasation, cell migration and lung seeding of tumor cells that lead to lung metastasis. We further demonstrated that treatment with αV5-3 not only shows no discernable toxicity in naïve, non-tumor bearing mice, but also shows potential benefits by protecting against cancer-induced liver damage and normalization of blood cell counts in tumor-bearing animals. The pharmacological efficacy of αV5-3 was compared to an anti-metastatic drug that is currently being developed for human clinical experiments. The relevance of our findings to human breast cancer is discussed.

Materials and methods

Cell lines

4T1, mouse tumor endothelial cells (2H-11) and MDA-MB-231 cells were obtained from the American Type Culture Collection (ATCC, Manassas,VA); JC cells were provided by the Cancer Research UK cell bank. The 4T1, JC and MDA-MB-231 cells were labeled to stably express firefly luciferase using retroviral infection, as described (Yee *et al*., 1987).

Antibodies

For Western blot analyses, rabbit antibodies directed against PKC isozymes and Gαi-3 (C-10) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), anti-GAPDH antibody, clone 6C5 from Advanced Immunochemical (Long Beach, CA), antibodies for phospho-IκBα and IκBα from Cell Signaling (Danvers, MA), anti-CXCR4 antibody from BioLegend (San Diego, CA) and polyclonal anti-rat CXCR4 neutralizing antibody for the efficacy study was from Torrey Pines Biolabs (East Orange, NJ).

Peptide synthesis and drug administration

The PKCα-selective translocation inhibitor (αV5-3) was derived from the PKCα V5 region ([QLVIAN] from amino acids 642–647 of PKCα, a position in the V5 domain that is homologous to the position of βI and βII peptides (Stebbins and Mochly-Rosen, 2001). Peptides were synthesized and, for intracellular delivery, were conjugated to a membrane-permeable TAT protein-derived carrier peptide (residues 47–57, [YGRKKRRQRRR]) as previously described (Begley *et al*., 2004; Chen *et al*., 2001). TAT carrier peptide or saline were used as negative controls. Peptides were delivered *in vivo* using Alzet osmotic mini-pumps (Alzet model 2001), as described (Inagaki *et al*., 2005). The peptides were dissolved in saline and administered at a constant rate (0.5μl/hr) corresponding to 24 mg/kg/day

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(30mM TAT) and 36 mg/kg/day (30mM αV5-3-TAT conjugate or αV5-3, in short). In some experiments, pyrrolidine dithiocarbamate (PDTC, Sigma, P-8765, 50 mg/kg/day) or anti-CXCR4 antibody (10mg/ml) was also delivered in these Alzet osmotic pumps. Pumps were replaced every 2 weeks, which corresponds to the stability of the peptides in the pump (Inagaki *et al*., 2005). MMP-2/9 inhibitor (SB-3CT *p*MS, Calbiochem, #444285) was used at 10μM *in vitro*.

Animal Tumor Models

All animal experimentation was conducted according to protocols approved by the Stanford University Institutional Animal Care and Use Committee (IACUC).

Six week old female BALB/c mice were purchased from Charles River laboratories (Wilmington, MA), housed at the animal care facility at Stanford University Medical Center (Stanford, CA) and kept under standard temperature, humidity, and timed lighting conditions and provided mouse chow and water *ad libitum*. 4T1-luc, JC-luc or MDA-MB-231-luc tumor cells were injected directly into the mammary fat pad of the mice in 0.1mL of sterile DMEM (100,000 or 2,500,000 cells/animal). Peptide treatment began when the tumors reached an average size of $100mm^3$, which occurred after about 1 week, unless otherwise stated. For the lung seeding study, 100,000 4T1-Luc cells in sterile PBS were injected *via* the tail vein. Animals were treated with PBS, peptide inhibitors, PDTC or anti-CXCR4 antibody delivered in osmotic pumps as described above.

Bioluminescence Imaging

Mice received luciferin (300 mg/kg, 10 minutes prior to imaging) and were anesthetized and imaged in an IVIS100 imaging system (Xenogen, part of Caliper Life Sciences). Images were analyzed with Living Image software (Xenogen, part of Caliper Life Sciences). Bioluminescent flux (Photons/sec/sr^2/cm^2) was determined for the lungs and rib cages (upper abdominal region of interest), or the primary tumors.

Immunoblot analysis

Tumors were processed as previously described (Kim *et al*., 2008). Translocation of PKCα, βII and ε was measured by determining their levels in cytosolic and particulate fractions from tumor samples, as described (Begley *et al*., 2004; Inagaki *et al*., 2005).

RNA interference

Small interfering RNA (siRNA) duplexes targeting PKCα were obtained from Santa Cruz Biotech (mouse, sc-36244, Santa Cruz, CA). This siRNA product consists of pools of three target specific 19–25 nucleotide siRNAs for reducing PKCα gene expression (see sequences in the supplemental materials). Cells at ~95% confluency were transfected with control siRNA and PKCα siRNA using transfection reagents from Gene Silencer (San Diego, CA) and Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The cells and medium were collected 48 hours after transfection.

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In vitro intravasation assay

Primary human endothelial cells (HUVEC) cells (Lonza) or mouse tumor endothelial cells (2H-11, ATCC) were grown on top of a Matrigel plug in tissue culture inserts in 24-well plates. Intravasation assays were carried out as previously described (Kim *et al*., 2000).

In vitro invasion assay

The assay was carried out according to the manufacturer's instructions (Becton Dickinson 354483). The same number of control inserts without matrigel coating (Becton Dickinson 354578) was used for assessing migration of the cells.

Immunohistochemistry

Freshly obtained lungs were fixed in 4% paraformaldehyde and transferred to 70% ethanol after 24 hours. Lungs were then embedded in paraffin, cut into 5μm sections and mounted on glass slides. Tissue sections in the slides were deparaffinized with xylene, hydrated using a diluted alcohol series, immersed in 3% H_2O_2 in distilled water for 15 minutes to quench endogenous peroxidase activity and stained with hematoxylin.

Flow Cytometry

Cells were obtained from tissue culture plates or from mechanical dissociation of tumors (through cell filters). Following antibody staining, cells were analyzed on a FACScaliber (BD Pharmigen).

Statistical analysis

Data are expressed as mean±SEM. Unpaired *t* tests for differences between 2 groups were used to assess significance using Prism 4 software (GraphPad Software, La Jolla, CA, $p<0.05$).

Results

PKCα is more active in metastatic mammary cancer cells relative to non-metastatic cancer cells

4T1 and JC are both mouse mammary cancer cell lines, syngeneic for BALB/c mice. Following orthotopic implantation of 4T1 or JC cells into BALB/c mice, the size of the respective primary tumors increased at similar rates (Figure 1A, upper panel). However, whereas 4T1 cells were highly metastatic (primarily in the lungs), as monitored by *in vivo* bioluminescence imaging, JC cells did not metastasize, even at late time points (Figure 1A, lower panel and right panel, two-tailed unpaired *t* test, $p<0.05$). Among the dozen PKC isozymes, PKCα, βII and ε were previously reported to regulate the metastatic behavior of breast cancer cells (Martiny-Baron and Fabbro, 2007; Pan *et al*., 2005; Sledge and Gokmen-Polar, 2006). We therefore compared the level of their activation in the highly metastatic 4T1 cells and the non-metastatic JC cells by measuring their subcellular distribution; the presence of PKC in the particulate fraction is a measure of activation of the kinase (Kraft *et al*., 1982). Of the PKC isozymes studied, only the levels of PKCα in the particulate fraction were significantly higher in 4T1 cells as compared with JC cells (Figure 1B, left, two-tailed

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unpaired *t* test, $p<0.05$). In contrast, the levels of PKCβII in the particulate fraction were six times lower in the metastatic 4T1 cells as compared with non-metastatic JC cells (Figure 1B, middle) and the percent activation of PKCε was not significantly different between these two cell lines (Figure 1B, right). Subcellular distribution of PKCα, βII and ε in 4T1 cells recovered from orthotopic tumors isolated from mice 4 weeks after implantation showed that PKCα is also the most active form in the 4T1 tumors, *in vivo* (Figure 1C, n=4 each, *; $p<0.05$ *vs*. PKCα). Because 4T1 cells are highly metastatic, we set out to determine if the increased level of PKCα activation in 4T1 cells plays a role in regulating mammary cancer metastasis.

Inhibition of PKCα reduces tumor metastasis, *in vivo*

To determine if PKCα plays a role in the metastasis of 4T1-luc cells *in vivo*, we treated mice with control, carrier peptide (TAT_{47-57}; 24mg/kg/day), or with equimolar concentration of αV5-3-TAT_{47-57} (αV5-3; a PKCα inhibitory peptide that is conjugated to cell permeable TAT_{47-57} for intracellular delivery; 35mg/kg/day) for four weeks, starting a week after orthotopic tumor cell implantation in the mammary fat pad (Figure 2A). This time point was chosen because we found 4T1-luc cells in the lungs 7–10 days after cancer cell implantation (Figure 1A). Treatment for 4 weeks with αV5-3 almost completely prevented metastasis to the lungs and caused a significant reduction in metastasis to the rib cages (Figure 2B, top and bottom), two of the major sites of metastasis (Smith *et al*., 2004). These data indicate that PKCα plays a major role in metastasis in this model.

We confirmed the isozyme-specific inhibition following αV5-3 treatment by determining subcellular distribution of PKC isozymes in primary tumor lysates from the treated mice. Whereas PKCβII activation was unaffected, the active level of PKCα was ~65% lower in the tumor of the αV5-3-treated mice relative to that from the control-treated mice, (Figure 2C and D). Same loading controls were used for Figures 2C and D. (Note also that αV5-3 treatment did not affect the total cellular levels of these isozymes; see the sum of cytosolic and particulate fractions in the blots of Figures 2C and D). Together, these results suggest that metastasis of 4T1-Luc tumors requires PKCα activity and that αV5-3 specifically inhibits this activity.

Inhibition of PKCα blocks metastasis at the intravasation stage

Metastasis is a multi-step process, and it is likely that different cellular processes are important for these different stages. We therefore set out to identify the step(s) regulated by PKCα. We broadly divided the metastasis process into two phases: escape of tumor cells from the primary tumor and seeding of secondary organs. We first examined the effects of PKCα inhibition on tumor intravasation, using an *in vitro* assay. This assay measures the movement of tumor cells through an endothelial cell layer and thus mimics the movement of tumor cells out of the primary tumor and into circulation, *i.e*. intravasation. We treated 4T1-luc cells with TAT or αV5-3 and measured passage through a mouse endothelial cell layer and into a matrigel plug (Figure 3A). αV5-3 treatment reduced the ability of 4T1 cells to cross the endothelial cell barrier by more than 75%, relative to control treatment (Figure 3A).

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We also determined the effects of αV5-3 on human breast cancer cells, MDA-MB-231-luc and their ability to pass through a HUVEC monolayer (Figure 3B). This was both to verify that the data obtained with the mouse cell line were relevant to the human disease, and to ensure that the effects were not influenced by the use of a transformed endothelial cell line. We found that αV5-3 treatment reduced the intravasation of the highly metastatic MDA-MB-231 cells, by ~55% as compared with TAT treatment (Figure 3B). Based on these *in vitro* assays, it appears that PKCα activation is required for the movement of tumor cells out of the primary tumor and into the vessels.

Next, we examined the molecular mechanisms behind this protection against intravasation conferred by PKCα inhibition. Matrix metalloproteinases (MMPs) are known to be major regulators of intravasation through their degradation of matrix proteins (Noel *et al*., 2008; Zucker *et al*., 2000) and proteinases MMP-2 and -9 and the serine protease, urokinase type plasminogen activator (uPA) have been reported to regulate breast cancer metastasis (Chakraborti *et al*., 2003; Ke *et al*., 2006; Mi *et al*., 2006; Tester *et al*., 2000). We therefore determined MMP-2, -9 and uPA activities in both the primary tumors and metastasized tumors in lungs of TAT or αV5-3 treated animals; uPA was not significantly affected in either the primary or the metastatic tumor (data not shown) but treatment with αV5-3 reduced the activities of MMP-9 in the primary tumors by about 40% (Figure 3C). These data indicate that αV5-3 inhibition of MMP activities may result in decreased intravasation of mammary tumor cells.

We then treated 4T1-luc cells with a MMP-9 inhibitor (SB-3CT) and compared their MMP-9 activities with cells transfected with siRNA of PKCα. We found that SB-3CT decreased MMP-9 activities by 60±6% (Figure 3D, n=4) and cells transfected with siRNA of PKCα had 50±3% lower activity of MMP-9 (Figure 3D, n=4). Taken together, these data suggest that PKCα regulates intravasation of cancer cells from the primary tumors mediated by MMP-9 activity.

Inhibition of PKCα protects against seeding of 4T1 cancer cells to the lungs

We next determined if PKCα regulates seeding of cells in the secondary organs. We used a ‘lung seeding’ assay *in vivo* (Smith *et al*., 2004), where intravenous injection of tumor cells results in seeding of the cells in the lung. This assay is designed to mimic migration, adhesion to target organ vasculature and survival, all the events that occur after intravasation of tumor cells into circulation.

TAT and αV5-3 peptides were administered continuously using an osmotic pump that was implanted in the mouse flank 2 days prior to tumor cell injection. The 4T1 tumor cells labeled with luciferase were injected through the tail vein and the animals were imaged 5 days later. Treatment with αV5-3 significantly reduced the presence of cancer cells in the lungs as determined on day 5 (Figure 4A). The 45% reduction in tumor burden in the lung on day 5 after cell injection was encouraging, as this may represent reductions in tissue adhesion and/or invasion to the metastatic site and/or survival of tumor cells in that site, all leading to reduced tumor burden in the lung.

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To determine whether the reduction in tumor burden following lung seeding was due to reduced numbers of tumor nodules or a reduced tumor nodule size, we carried out histological examination of the lung tissues obtained from animals treated in the same way and analyzed the lung tissue 2 weeks after treatments (Figure 4B). Treatment with αV5-3 greatly reduced the number of lung nodules as compared with the TAT-treated group (arrows; nodules, $p<0.05$), but the size of the individual nodules did not appear to be significantly affected (supplemental Figure 3). These data are consistent with lack of an effect of the PKCα inhibitor on the rate of tumor cell proliferation (supplemental Figure 7A–D) and again implies a selective inhibitory effect of this treatment on metastasis.

We further determined survival of the mice in the two treatment groups following lung seeding and found that mice injected intravenously with the aggressive 4T1 tumor cells and treated with αV5-3 survived significantly longer as compared with the TAT-treated mice (Figure 4C, $p=0.027$); 70% of the αV5-3-treated animals survived for 30 days whereas only 10% of the TAT-treated animals were alive at that time. These data suggest that inhibition of PKCα also increases survival of mammary tumor-bearing mice by decreasing lung seeding of the tumor cells.

αV5-3 reduces cell migration, reduces cell surface levels of CXCR4 and produces a greater reduction in metastasis than anti-CXCR4 antibody *in vivo*

Using an *in vitro* assay with Becton Dickinson chambers, we found that αV5-3 treatment significantly inhibited cell migration as well as invasion as compared with TAT controls (Figure 5A). In conjunction with its cognate ligand, CXCL12, CXCR4 regulates normal lymphocyte trafficking as well as metastatic migration of mammary tumor cells, other cancer cells and lymphocytes (Muller *et al*., 2001; Smith *et al*., 2004; Taichman *et al*., 2002). We therefore determined the levels of the chemokine receptor, CXCR4. After treatment of mice bearing 4T1 tumors with αV5-3 for 4 weeks, the cell surface CXCR4 levels in the tumors were 80% lower as compared with those in the TAT-control group (Figure 5B). Of note, CXCR4 was not detected on the surface of 4T1 cells grown in culture [unpublished data and (Smith *et al*., 2004)], highlighting the importance of using *in vivo* models to accurately determine the relative importance of signaling events during metastasis.

Relevant to this study, members of the classical PKC family (to which PKCα belongs) have been shown to phosphorylate IκBα, induce its degradataion and activate NF-κB in Jurkat cells (Steffan *et al*., 1995) and specifically, PKCα was found to regulate NF-κB activity in monocytes and normal human epithelial cells (Kawakami *et al*., 2007; Shin *et al*., 2007). Therefore, we determined if PKCα regulates NF-κB activity in 4T1 mammary cancer cells. Because the levels of phosphorylated IκBα are inversely correlated with NF-κB levels and activity (Gross and Piwnica-Worms, 2005), we measured phosphorylated IκBα levels in tumors from mice after 4 week-treatment with αV5-3 or with TAT control. αV5-3 increased the levels of phosphorylated IκBα (normalized with IκBα) by ~60% (Figure 5C). These data suggest that PKCα regulates NF-κB activity in 4T1 mammary cancer *in vivo*.

We then compared the anti-metastatic efficacy of αV5-3 with both a NF-κB inhibitor (PDTC) and an anti-CXCR4 neutralizing antibody. Currently, NF-κB inhibitors and anti-

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CXCR4 antibodies are in preclinical and clinical development as anti-metastatic therapies for melanoma, renal cell, thyroid and pancreatic cancers and for lymphomas (Alsayed *et al*., 2007; Molckovsky and Siu, 2008). αV5-3 treatment was more effective than the anti-CXCR4 neutralizing antibody and showed equivalent anti-metastatic effects to the NF-κB inhibitor, PDTC, when used in 4T1 tumor-bearing mice (Figure 5D). Taken together, these data suggest that PKCα regulates NF-κB activity and CXCR4 levels in 4T1 mammary cancer, *in vivo*.

We confirmed the regulation of CXCR4 surface expression by PKCα by measuring cell surface levels of CXCR4 on dissociated primary tumor cells after the above treatments. We found that αV5-3 treatment reduced CXCR4 levels by 60%, NF-κB inhibitor by 65% whereas anti-CXCR4 antibodies reduced CXCR4 levels by only 30% (Figure 5E). We also found that PKCα regulates NFκB activity and transcription of CXCR4 (supplemental Figure 2) in MDA-MB-231 cells *in vitro*. These data suggest that αV5-3 inhibits cell migration, at least in part, by effectively reducing CXCR4 levels and by inhibiting NF-κB activity. Figure 6 summarizes the stages of metastasis that are regulated by PKCα.

Discussion

Using a syngeneic orthotopic model of breast cancer metastasis in mice, we have shown that pharmacological inhibition of PKCα using a specific inhibitor, αV5-3, almost abrogates metastasis to the lungs. This was evident both in a spontaneous metastasis model (in mice bearing implanted primary tumors) and in a model in which seeding of the lung is measured after intravenous injection of 4T1 breast cancer cells. Figure 6 represents a summary of how PKCα regulates intravasation of cancer cells by increasing MMP-9 activity and migration of mammary tumor cells by increasing the CXCR4 cell surface levels. We also show that PKCα regulates seeding of cancer cells to the lungs. αV5-3 effectively inhibited activity of MMP-9 and NF-κB, inhibited tumor cell migration, seeding and survival of these cancer cells in the secondary organs.

The αV5-3 peptide used in our study was derived from a unique short sequence within the V5 region of PKCα (Mochly-Rosen and Gordon, 1998; Stebbins and Mochly-Rosen, 2001). In PKCβII, another member of the classical PKC isozymes, interaction of the C2 and V5 domains of PKC with the receptor for active C kinase (RACK) is needed for PKC activation (Ron *et al*., 1995; Stebbins and Mochly-Rosen, 2001) and peptides derived from either the C2- or the V5-domains have been proven to be effective inhibitors of PKCβII functions (Kim *et al*., 2008; Ron *et al*., 1995; Stebbins and Mochly-Rosen, 2001). Therefore, we rationally designed a PKCα-inhibitor peptide reasoning that a peptide derived from the position in V5 that is homologous to the PKCβII-specific inhibitor, βIIV5-3, would be a specific isozyme-selective inhibitor of translocation and function of PKCα.

CXCR4, a seven transmembrane G protein-coupled receptor, is known to be critical in directional migration and survival of breast cancer cells during the metastatic process, as demonstrated in studies using both human and mouse breast cancer cells (Harvey *et al*., 2007; Helbig *et al*., 2003; Mimeault and Batra, 2007; Smith *et al*., 2004). Here we found that PKCα is an up-stream regulator of CXCR4 levels in the regulation of cell seeding and

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survival in 4T1 mammary cancer cells. The reduction in the levels of CXCR4 following αV5-3 treatment may partly explain the decreased migration of the cancer cells and the reduced metastasis observed in our study (Figures 2 and 4).

It is possible that the PKCα inhibitor αV5-3 inhibits interaction of the regulatory domain of PKCα with CXCR4 and therefore blocks the recycling of CXCR4 between the cell surface and the intracellular compartment (Peter *et al*., 2005). In that case, CXCR4 will mainly remain in the intracellular compartment and the cell surface level will be reduced. However, here we also showed that αV5-3 reduces mRNA levels of CXCR4, suggesting that the actions of αV5-3 could not be solely due to direct interaction between CXCR4 and PKCα.

In terms of therapeutic potential, we found that treatment with αV5-3 significantly increased the survival of tumor-bearing mice, which was associated with a better inhibition of metastasis as compared to treatment with CXCR4 antibodies. Importantly, no signs of toxicity were observed with the αV5-3 treatment *in vitro* and *in vivo*. Therefore, αV5-3 represents a promising agent for the prevention and treatment of mammary tumor metastasis. Treatment with selective PKCα inhibitors, such as αV5-3, prior to or immediately after the surgical removal of the primary tumor may reduce the rates of relapse, by inhibiting tumor metastasis formation.

In conclusion, inhibition of PKCα by αV5-3 increased survival rate and inhibited intravasation, cell migration and lung seeding that lead to metastasis in a syngeneic orthotopic mouse model of breast cancer. With efficacy of chemotherapy against advanced or metastatic tumors being generally low and toxic, these findings suggest that a PKCα inhibitor, such as αV5-3, may augment current therapies of this disease in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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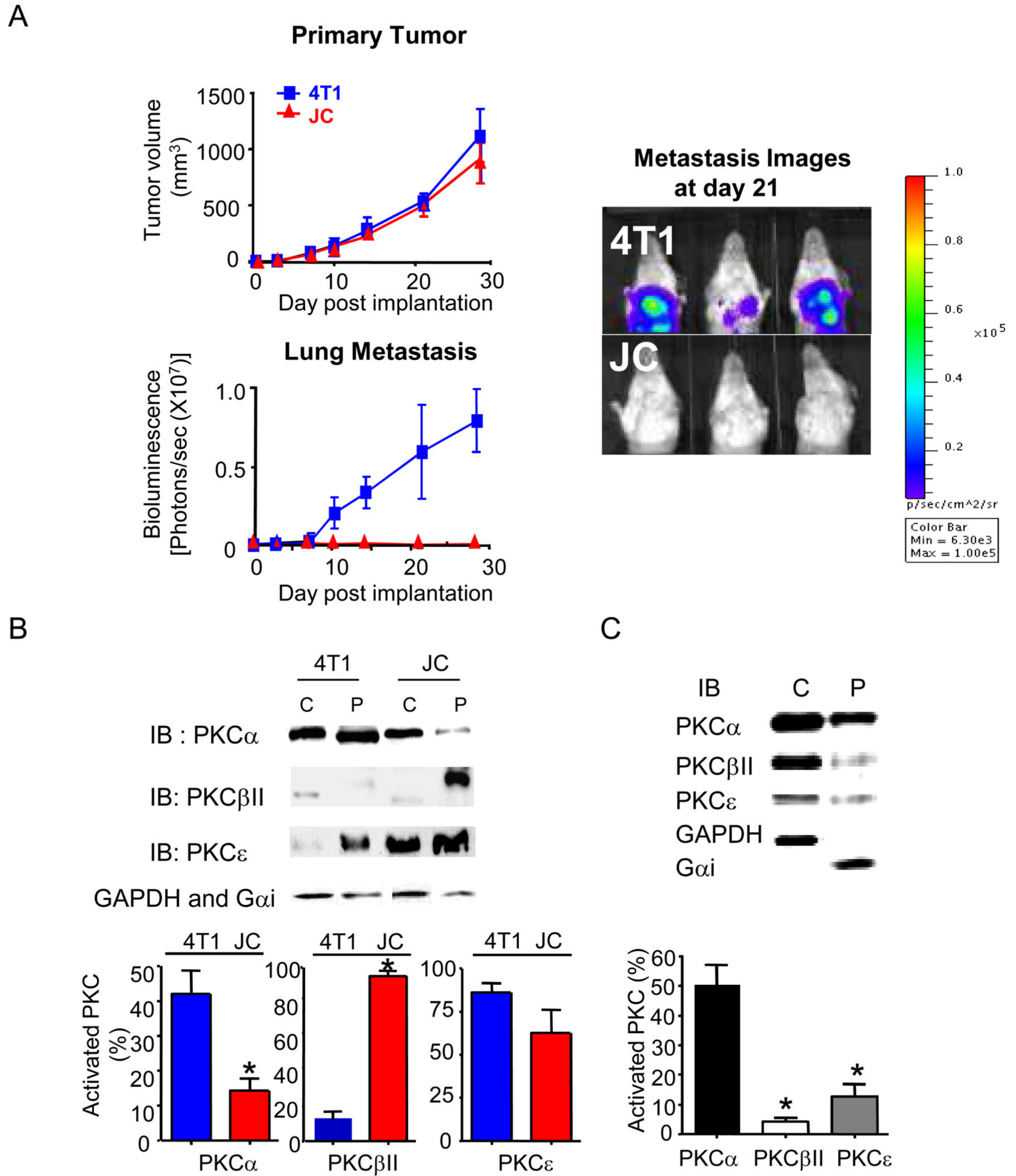

Figure 1. PKCα is more active in metastatic 4T1 mammary cancer cells relative to non-metastatic cancer cells

(A) 4T1 and JC cells were transfected with firefly luciferase and 100,000 cells were injected orthotopically into the mammary fat pad of 6 week old female BALB/c mice (n= 12 each). Primary tumor growth (Figure 1A, top) and lung metastases (bottom) of the two cell lines were compared *in vivo* by bioluminescence imaging using an IVIS100. 4T1-luc is shown in blue and JC-luc in red. Sample bioluminescence images of lung metastases are shown on right. (B) Subcellular distribution of PKCα, βII and ε between the cytosolic (C) and particulate (P) fractions (expressed as percent enzyme in the particulate fraction, a measure

of activation of PKC) in 4T1-luc *vs*. JC-luc cells, was determined by Western blot analysis (IB) of cultured cell lysates. (n=3 each, *; $p<0.05$; NS for PKCε). (C) Subcellular distribution of PKCα, βII and ε between the cytosolic and particulate fractions in 4 week-old 4T1 tumors grown in BALB/c mice (n=4 each, *; $p<0.05$ *vs*. PKCα). Loading controls for cytosolic and particulate fractions (GAPDH and Gαi) are shown.

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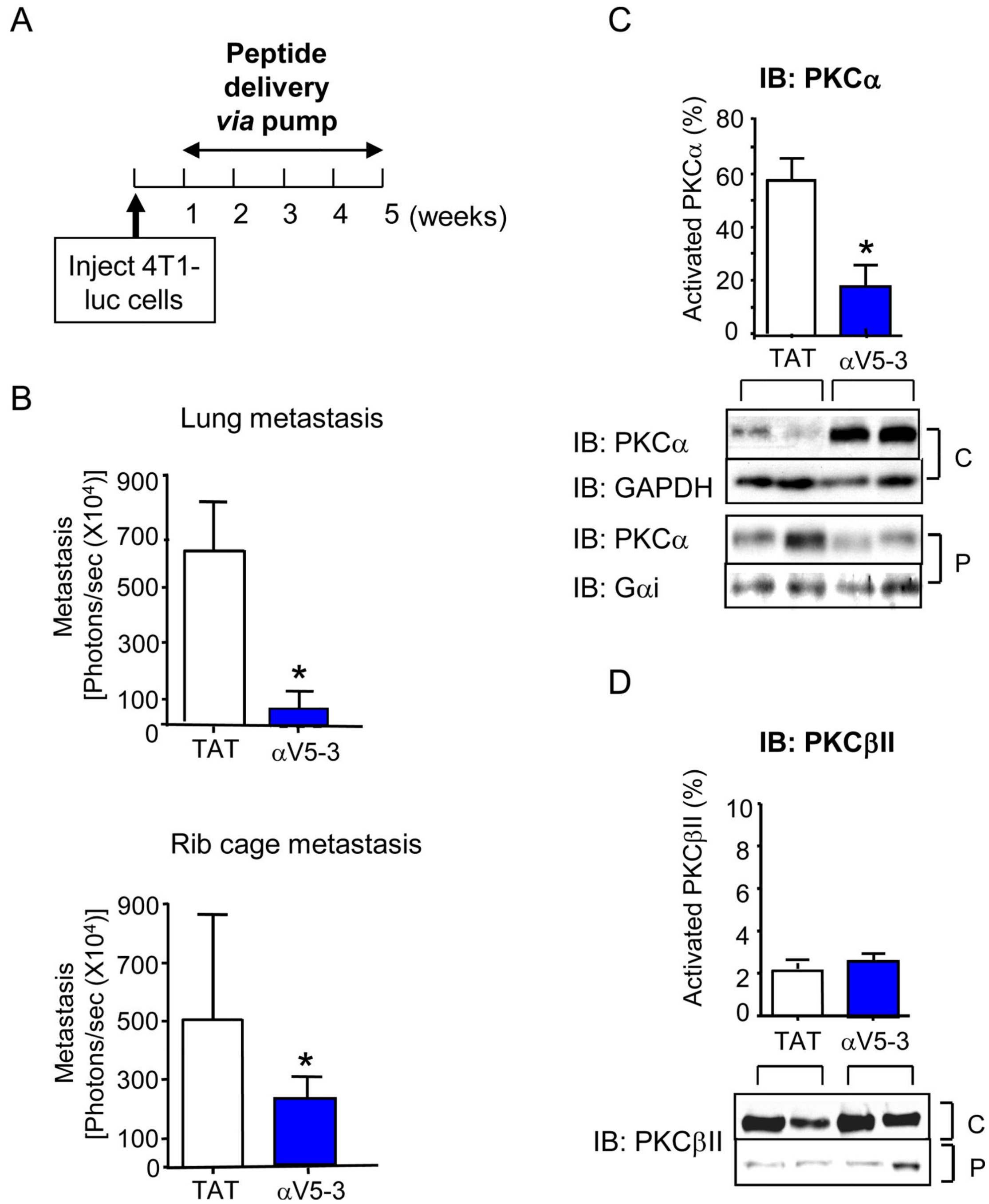

Figure 2. Inhibition of PKCα reduces tumor metastasis

(A) Experimental protocol: 4T1-luc tumor cells were injected (100, 000/0.1mL) into the mammary fat pad of BALB/c female mice (n=6–12). One week after cell injection, treatment with peptides was started using Alzet mini pumps for 4 weeks and mice were subsequently sacrificed. Treatment with αV5-3 for 4 weeks significantly reduced metastasis to lungs (B, top) and rib cages (B, bottom *; $p<0.05$, unpaired *t* test). (C) Treatment with αV5-3 decreased active levels of PKCα in the tumors as measured by translocation assay. GAPDH and Gαi are used as loading controls for cytosolic (C) and particulate (P) fractions,

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respectively. (D) αV5-3 treatment did not affect the active level of PKCβII as measured by translocation assay (Figure 2D, n=4 each, *; $p<0.05$, unpaired *t* test). Same loading controls were used for Figures 2C and D.

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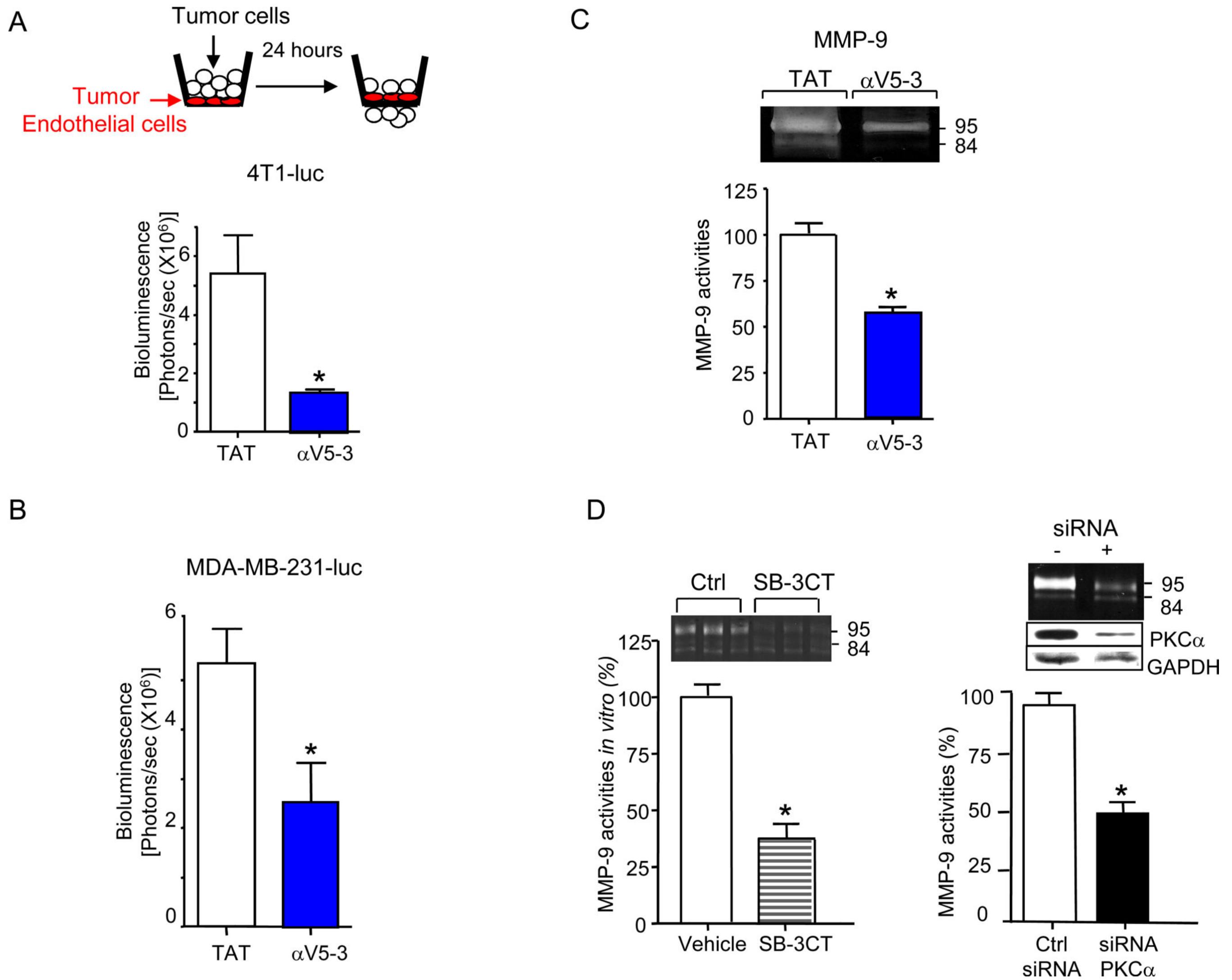

Figure 3. Inhibition of PKCα blocks metastasis at the intravasation stage

(A) Intravasation of 4T1-luc cells through a mouse endothelial cell layer and into a matrigel layer was measured using bioluminescence imaging (IVIS50, Xenogen, part of Caliper Life Science, n=4). Mouse tumor endothelial cells (2H-11, ATCC) were grown on top of a Matrigel plug in tissue culture inserts in 24-well plates until they formed a confluent monolayer. Breast cancer cells (1,000,000 cells/well) expressing luciferase were then added above the endothelial cell layer and peptides were added as indicated, to a final concentration of 10μM. Peptides were re-applied every 2h for 10 hours and the cells were then incubated for a further 14 hours (a total of 24h). At the end of this time, cell media was aspirated and a cotton swab was used to remove the endothelial cell layer. The matrigel plug was then imaged (IVIS50; Xenogen, part of Caliper Life Sciences) after addition of luciferin. Bioluminescence produced was used to quantify the number of labeled tumor cells that had crossed the endothelial cell layer and entered the matrigel plug. (B) Experiment was repeated looking at invasion of human MDA-MB-231-luc breast cancer cells across primary human endothelial HUVEC cells (TAT treatment in clear bars and αV5-3 treatment in blue

bars, n=4). (C) Activities of MMP-9 in primary tumors were measured by in-gel zymography in homogenates of tumors isolated from mice treated as described in Figure 2. Molecular weights of pro- and active-forms of MMP-9 are shown. (D) Activities of secreted MMP-9 were measured from cultures of 4T1-luc cells treated with vehicle (DMSO, control) and SB-3CT (an MMP-9 inhibitor, 10μM in DMSO) (n=3 for each). Cells were treated for 24 hours and media was collected and analyzed for MMP-9 activities. Also, MMP-9 activities were measured in the medium from cells treated with control (ctrl) siRNA and siRNA of PKCα for 48 hours and cultured for 2 more days.

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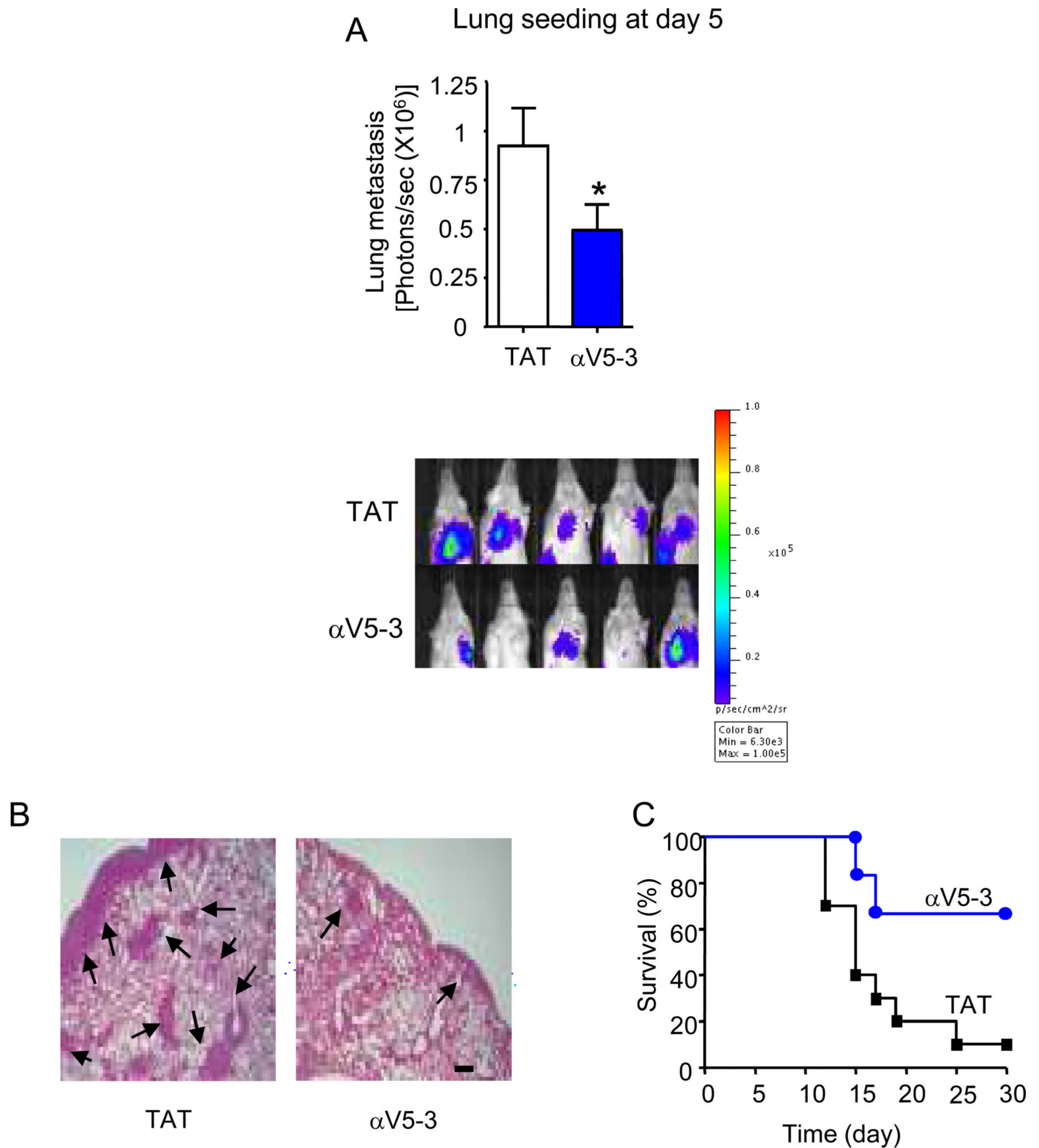

Figure 4. Inhibition of PKCα protects against lung seeding of cancer cells

(A) 4T1-luc tumor cells were injected *via* the tail vein (100,000 cells/0.1mL PBS, n=9–10 each). Administration of peptides by osmotic pumps was begun 2 days before the tumor cell injection. Animals were then imaged on day 5 post-tumor cell injection to measure the extent of lung seeding and metastasis. (B) Lungs from this study were recovered 14 days post-treatment, stained with Hematoxylin to identify number and size of 4T1-tumor nodules (n=3 each, representative images are shown at 200X; arrows indicate tumor nodules, scale bar; 10μm). (C) Comparison of survival rate between TAT-treated and αV5-3-treated

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groups. Animals were monitored up to 30 days after the tumor cell injection for survival analysis as plotted on Kaplan-Meier survival curves (n=4–6, each).

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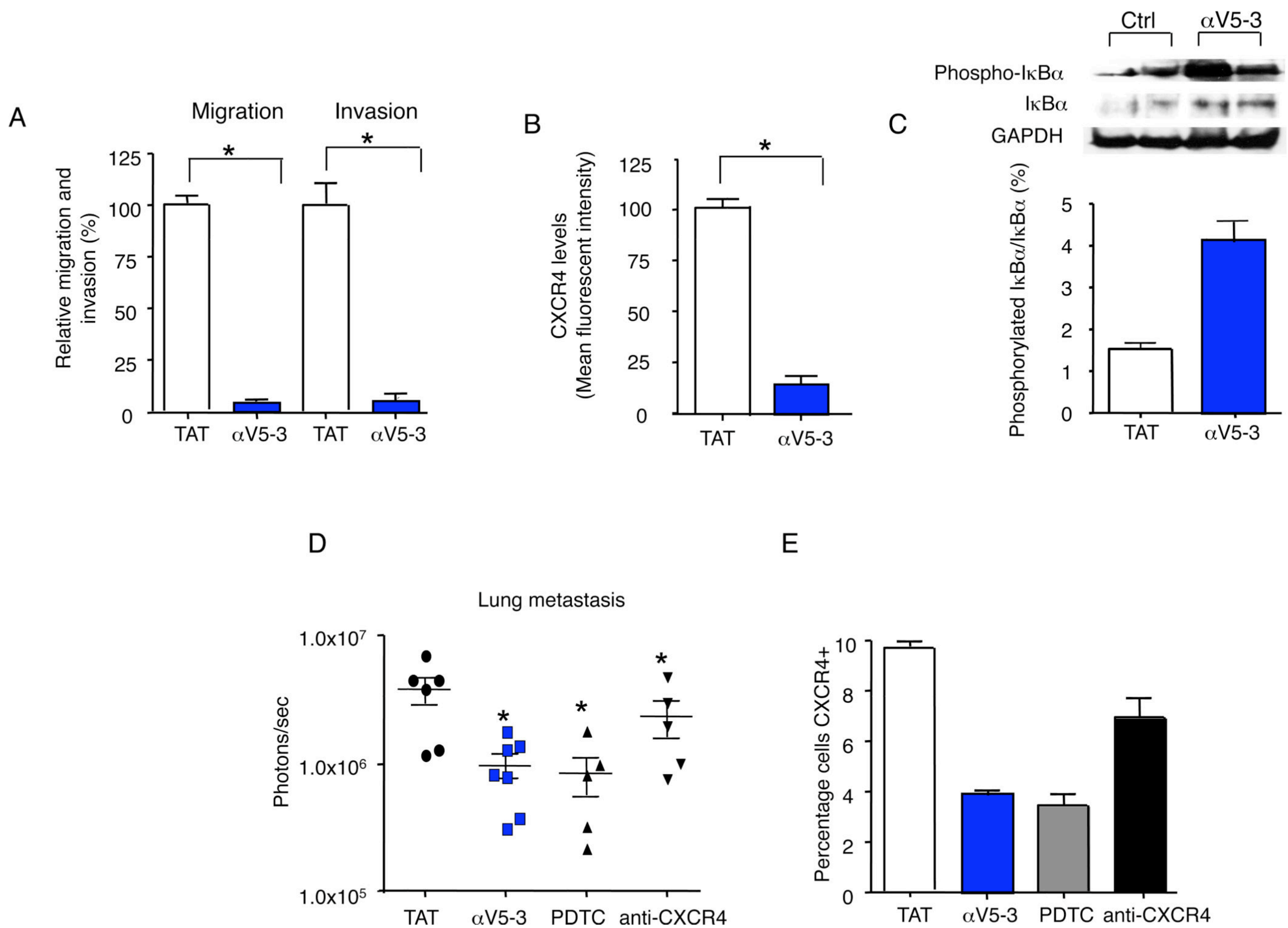

Figure 5. αV5-3 reduces cell migration, reduces cell surface levels of CXCR4 and produces a greater reduction in metastasis than anti-CXCR4 antibody *in vivo*

(A) 4T1 Cells (2.5×10^4 in 0.5ml of media) were serum starved and were pre-treated for one day every 3 hours for 9 hours with TAT or αV5-3 at 1μM. On the day of the experiment, the inserts were placed in wells with serum containing (10% FBS) media. The same number of control inserts was placed in empty wells of the BD companion plates. Cells were added on top of the wells and incubated with TAT or αV5-3 at 1μM for 24 hours. After incubation, cells migrated (to control inserts) and invaded (to matrigel inserts) were counted and percentage of cells invaded/migrated was calculated for each group. (B) Primary tumor lysates from animals treated as in Figure 2 were isolated, dissociated, stained with anti-CXCR4-FITC antibody and were analyzed by flow cytometry to measure the cell surface levels of CXCR4 (n=4 each). (C) Tumors from mice treated with TAT or αV5-3 for 4 weeks were analyzed for phospho-IκBα and non-phosphorylated IκBα levels (n=4 each). The ratio of phospho-IκBα to IκBα is shown. GAPDH was used as a loading control. (D) Comparison of the anti-metastatic effects of αV5-3 with PDTC (NF-κB inhibitor) or anti CXCR4 antibody. Peptides were dissolved in saline and administered at a constant rate (0.5μl/hr) corresponding to 24 mg/kg/day (30mM TAT) and 36 mg/kg/day (30mM αV5-3-TAT conjugate or αV5-3, in short). Pyrrolidine dithiocarbamate (PDTC, Sigma, P-8765, 50 mg/kg/day) or anti-CXCR4 antibody (10mg/ml) was also delivered in osmotic pumps.

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Bioluminescence imaging (BLI) was used to measure extent of upper abdomen metastasis after 14 days (n=5–7 each, *; $p<0.05$ *vs*. TAT). (E) Primary tumor lysates from animals treated as in (D) were isolated, dissociated, stained with anti-CXCR4-FITC antibody and were analyzed by flow cytometry to measure the cell surface levels of CXCR4 (n=5–7 each).

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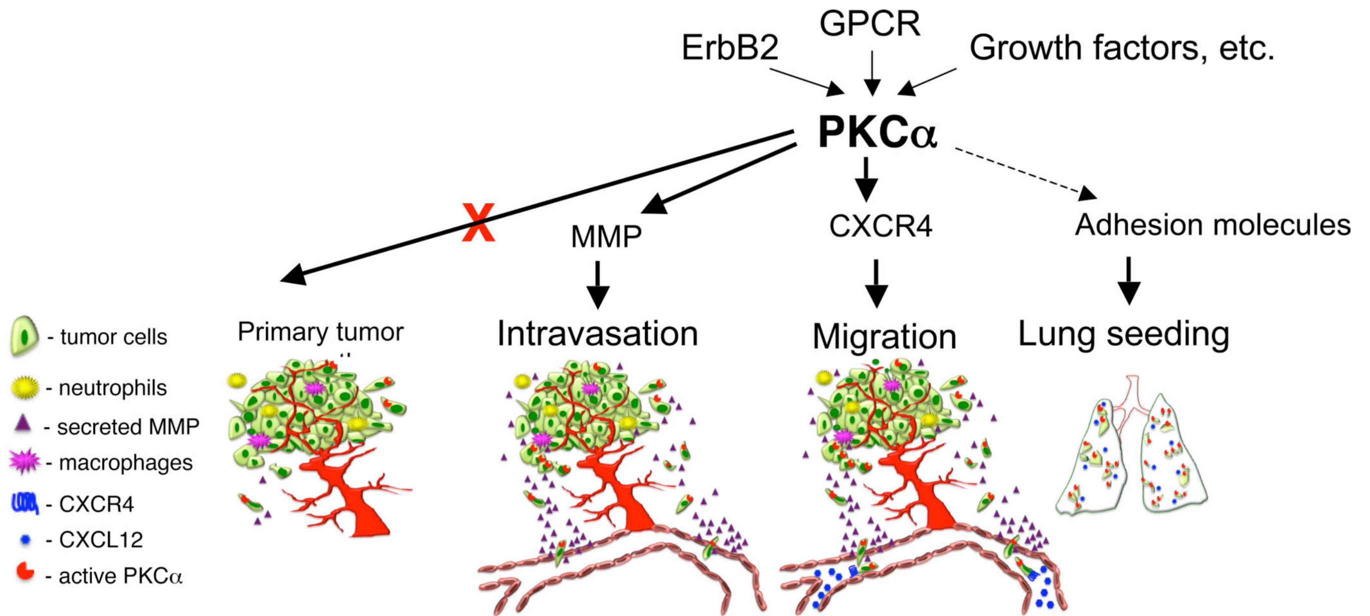

Figure 6. Summary of PKCα-mediated metastatic pathways in mammary cancer cells

A scheme summarizing the PKCα-mediated mechanisms in metastasis. A summary of potential sites of action of PKCα during metastatic processes is shown. PKCα can be activated by various factors, including activation of ErbB2 or G protein-coupled receptor (GPCR) or growth factors, etc. PKCα regulates intravasation *via* MMP-9 activity and cell surface CXCR4 levels, inducing higher migration and possibly more survival of cancer cells. Lung seeding and metastatic spread in the lungs and other secondary sites are also regulated by active PKCα. Inhibitory peptide against PKCα, αV5-3, inhibited intravasation, migration and lung seeding/metastases of mammary cancer cells *in vivo*. Green cells: tumor cells, yellow cells: neutrophils, dark pink cells: macrophages, coiled receptor: CXCR4 and blue hexagon: CXCL12. Active PKCα is shown in red.