Metastasis suppressor genes: a role for raf kinase inhibitor protein (RKIP)

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The metastatic cascade is a complicated process that involves many steps from gain of the metastatic phenotype in the primary tumor cells through establishment of macroscopic tumor at the distant target organ. A group of genes, termed metastasis suppressor genes (MSG), encode for proteins that inhibit various steps of the metastatic cascade. Accordingly, loss of MSG promotes the metastatic phenotype. Although several MSG have been identified, the mechanisms through which they enhance metastasis are not clearly defined. Gene array analysis of a low metastatic LNCaP prostate cancer cell line compared to its highly metastatic derivative C4-2B prostate cancer cell line revealed decreased expression of raf kinase inhibitor protein (RKIP) in the C4-2B cell line. RKIP blocks the activation of several signaling pathways including MEK, G-proteins and NFκB. Immunohistochemical analysis of prostate cancer primary tumors and metastases revealed that RKIP protein expression was decreased in metastases. Restoration of RKIP expression in the C4-2B cell line diminished metastasis in a murine model. These results demonstrate that *RKIP* is a MSG. Loss of RKIP enhanced both angiogenesis and vascular invasion, and protected against apoptosis. These findings suggest that targeting the RKIP pathway may diminish the metastatic cascade. However, challenges exist as to the best method to target RKIP expression. Restoration of RKIP expression in all cancer cells *in vivo* is challenging. A plausible strategy is to use small molecules that target proteins in signaling pathways that are dysregulated due to loss of RKIP. *Anti-Cancer Drugs* 15:663–669 © 2004 Lippincott Williams & Wilkins.

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Introduction

Prostate cancer is a multifactorial disease with genetic and environmental components involved in its etiology [1]. It is characterized by heterogeneous growth patterns that range from slowly growing tumors to very rapidly growing, highly metastatic tumors [1]. The heterogeneity of prostate cancer makes it difficult to define genetic markers for this disease.

Since prostate cancer is initially dependent on androgens, standard therapies include removing sources of androgen or blocking the action of androgens [2]. In most cases, androgen deprivation results in clinical regression of the cancer. However, the majority of cancers recur and metastasize [3]. Current therapies for metastatic prostate cancer are for the most part ineffective, resulting in a significant mortality rate of men with prostate cancer. Clearly, detection of prostate cancer alone provides little prognostic value; the aggressiveness and extent of the cancer must be measured as well before any treatment decisions can be made. In other words, it is critical to accurately distinguish those histologically localized cancers which will complete the metastatic process from those that will remain indolent. Although we have abundant clinical and biological information on prostate cancer, a large percentage of apparently resectable and theoretically curable lesions are found to be more advanced at the time of resection than envisaged, resulting in a substantial failure rate after attempted curative surgery [4,5]. In order to improve the ability to diagnose a potentially curable cancer or treat metastatic prostate cancer, an increasing understanding of the genes which regulate the metastatic ability of cells is required.

The metastatic cascade

Metastasis is defined as the formation of progressively growing secondary tumor foci at sites discontinuous from the primary lesion [6]. The metastatic process is a complex cascade. In brief, a metastatic cancer cell must escape from the primary tumor, enter the circulation, arrest in the microcirculation, invade a different tissue compartment and then grow at that secondary site. At the present time, metastasis is poorly understood at the molecular and mechanistic level in most cancers, including prostate cancer. Theoretically it should be possible to block metastasis by inhibiting a single gene that allows

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the completion of any one of these steps in the metastatic cascade [6]. In support of this possibility is evidence that loss-of-function of specific genes called metastasis suppressor genes (MSG) is an important event during the progression towards a malignant phenotype [7–10].

MSGs

MSGs suppress the formation of overt metastases without affecting the growth rate of the primary tumor [7]. These genes are different from tumor suppressor genes, which suppress growth of primary tumors. To date, only a handful of MSGs have been identified. Furthermore, the mechanisms through which these genes and their protein products suppress metastasis *in vivo* are not well defined and demand extensive investigation due to their importance in cancer pathophysiology.

Several MSG's have been identified, although little is known about the exact mechanism through which they suppress metastasis. Some may act to prevent the early steps of the metastatic cascade (i.e. initial migration and intravasation), whereas other may act to prevent late steps (i.e. endothelial adhesion, extravasation, angiogenesis). We will summarize some of these MSGs below.

Nm23

The first reported MSG, *nm23* RNA levels were initially described as highest in cells and tumors of relatively low metastatic potential in two experimental systems: (i) murine K-1735 melanoma cell lines, in which the gene was identified, and (ii) *N*-nitroso-*N*-methylurea-induced rat mammary carcinoma [10]. Transfection of Nm23 cDNA into the human DU 145 prostate carcinoma cell line did not alter growth rate; however, the Nm23-transfected lines displayed decreased colonization in soft agar and adhesion to extracellular matrix components when compared with the control transfected line. This result suggests that Nm23 suppresses the metastatic potential of prostate carcinoma cells by inhibiting their ability in anchorage-independent growth and extracellular matrix adhesion [11]. Immunohistochemical staining of prostate cancers revealed a negative correlation between Nm23 H1 staining and tumor stage and grade [12,13]. Overexpression of Nm23 in Rat1 fibroblasts reduced adhesion-stimulated membrane ruffles apparently through inhibition of Rac1 GTPase activation [14]. Thus, this may be one mechanism through which Nm23 acts as a MSG although this has not been proven *in vivo*.

KAI1

Identified on human chromosome 11p11.2, KAI1 was shown to suppress metastasis when introduced into rat AT6.1 prostate cancer cells [15]. Although several studies have attempted to determine how KAI1 expression is down-regulated [16–19] (with no definitive answer), there are no studies that clearly characterize its function in metastasis suppression.

KiSS1

Initially identified as a MSG in melanoma [20], KiSS1 has been shown to repress 92-kDa type IV collagenase expression by down-regulating NFκB binding to the collagenase promoter [21]. KiSS1 encodes a peptide ligand of a G-protein-coupled receptor [22,23]. In addition to its ability to regulate NFκB, it appears that a 54-amino-acid peptide of KiSS1, termed metastin, interacts with the MAP kinase pathways and modulates many different metastatic phenotypes including invasion, motility, chemotaxis and adhesions (reviewed in [24]).

CD44

CD44 is a glycosylated integral membrane adhesion molecule that binds to hyaluronic acid and other extracellular matrix proteins such as collagen and osteopontin [25,26]. CD44 expression is inversely correlated with tumor grade and progression [27–31]. Although CD44 suppresses metastasis in several model systems [32], this activity is independent of its ability to bind hyaluronic acid [33]. However, because of its importance in cell-to-cell adhesion, it is likely that loss of CD44 results in the cancer cells ability to detach from the primary tumor. Thus, it may work at the early steps of metastasis.

BRMS1

Breast-cancer metastasis suppressor 1 (BRMS1) diminishes metastasis from breast cancer cells in murine models [34]. It appears to achieve this through restoring cell–cell communication via gap junctions via modulation of connexin expression [35]. An additional mechanism of metastasis suppression may be through BRMS1's ability to participate in transcriptional regulation through its ability to interact with retinoblastoma binding protein 1 and members of the mSin3 histone deacetylase (HDAC) complex [36].

MKK4

Mitogen-activated protein kinase kinase 4 (MKK4) suppresses AT6.1. Dunning rat prostate cancer metastases *in vivo* [37] and ovarian carcinoma [38]. MKK4 expression is inversely correlated with Gleason pattern in prostate cancer [39] and was identified on chromosome 17 as a factor that invoked dormancy in metastases [40]. This finding suggests that MKK4 works on the late steps of metastasis.

Maspin

Maspin, a member of the serpin family, is a secreted protein. Maspin was inversely correlated with p53 expression and prostate tumor grade [41]. Maspin has been reported to inhibit the invasiveness and motility of prostate cancer tumor cells and angiogenesis from breast

cancer cells [41–43]. Maspin appears to suppress metastasis, in part, through inhibition of urokinase-type plasminogen activator system [44].

Summary

In summary, in spite of both the contribution towards understanding the pathophysiology of metastasis and the potential therapeutic importance of MSGs only a few have been identified. Furthermore, of the known MSGs, the mechanisms how they suppress metastasis are not well defined. Thus, identification of any new MSG and delineating the mechanism through which MSGs suppress metastasis is a fertile area of research that promise to shed light on the metastatic process and potential therapies to target metastasis.

RKIP biology

To begin to identify prostate cancer MSGs, we examined the difference in gene expression between a non-metastatic prostate cancer cell line and a metastatic prostate cancer cell line. We found that RNA expression of several genes was altered between these two lines [45]. One gene in particular, Raf kinase inhibitor protein [RKIP, also called phosphatidyl ethanolamine binding protein (PEBP)] was found to be expressed at a lower level in the metastatic compared to the non-metastatic cell line. This suggested the possibility that loss of RKIP was associated with the development of metastasis.

RKIP is synthesized locally in many tissues where it has been shown to be present in the cytoplasm and at the plasma membrane as determined by immunohistochemical staining [46]. While RKIP homologs can be found in testicular and epididymal luminal secretions, it is not found in blood, saliva, milk, uterine fluid, parotid fluid, prostate secretions or seminal vesicle secretions [47].

Rat RKIP expression has been found in oligodendrocytes and Schwann cells of the neuronal tissue; spermatids, Leydig cells and epididymal epithelium of the testis; steroidogenic cells of the adrenal gland zona fasciculata; proximal kidney tubule epithelium; enterocytes, goblet cells and plasma cells of the small intestine; plasma cells of the lymph node; plasma cells and megakaryocytes of the spleen; heart; liver; and epididymis [48]. Some expression has also been found in bronchioles of the lung, mesenteric lymph node, oviduct, ovary, lactating mammary glands, uterus and thyroid. RKIP expression has also been found in normal tissue and non-metastatic prostate cancer cells, but is expressed weakly in metastatic prostate cancer cells [49].

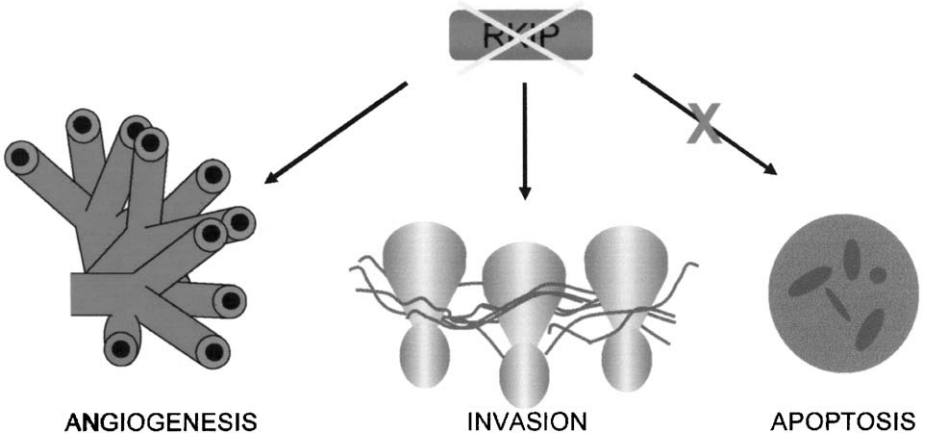
RKIP appears to have a variety of functions depending on the tissue in which it is localized. Several lines of evidence suggest that it is involved with mammalian spermatogenesis and male fertility. For example, rat epididymal secretions and sperm plasma membranes contain proteins with sequences similar to bovine brain RKIP [50]. Furthermore, RKIP released from spermatozoa may be involved with membrane biogenesis and maintenance of antigen segregation in spermatozoa [51]. Studies in the rat testis show that RKIP proteins may be involved in organization of the seminiferous epithelium or the transfer of phosphatidylethanolamine to other germ cells [52]. Due to its presence in Leydig cells, Frayne *et al.* suggested a role for RKIP as a lipid carrier or binding protein within the rat testis that contributes to membrane organization during spermatogenesis [53].

Although RKIP is expressed in multiple tissues of the rat, higher expression levels can be found in the testis, brain oligodendrocytes, Schwann cells, Purkinje cells of the cerebellum, and within cortical and hippocampal layers of the brain [48,53–57]. In rat medial septal nuclei, RKIP was found to enhance *in vitro* acetylcholine synthesis by up-regulating choline acetyltransferase and possibly stimulating cholinergic neuronal pathways [57–60].

RKIP interacts with small GTP-binding proteins, yet not GTP itself [61] and can be purified along with μ opioid receptors via morphine affinity chromatography using tissue derived from rat brain [62]. Grandy *et al.*, speculated that RKIP was a membrane-associated protein which may alter opioid binding via an enzymatic- or structural-induced reaction [62]. Using hydrophobic cluster analysis and molecular modeling, Schoentgen *et al.* showed that the bovine RKIP may [63] possess a potential nucleotide binding site, and suggested that it may belong to the kinase family and promote the transfer of hydrophobic ligands to the plasma membrane [64]. Co-expression of human RKIP with human opioid or somatastatin receptors (G-protein-coupled receptors) in *Xenopus laevis* oocytes provided *in vivo* evidence that RKIP could modulate G-protein-coupled signaling [65]. These studies, along with its widespread distribution in tissues and multiple species, provide evidence that RKIP is involved with cell regulatory and cell signaling mechanisms.

The role of RKIP in cell signaling was identified in a yeast two-hybrid assay for screening clones from a human T cell library that bound to Raf-1 kinase binding domains [66]. RKIP was shown to bind Raf-1, MEK-1 and weakly bind to ERK-2, interfering with MEK phosphorylation and activation by Raf-1. However, RKIP was not a substrate for Raf-1 or MEK. RKIP did not bind to Ras nor possess kinase activity. It appears that RKIP acts to set the threshold for Raf-1 activation and subsequent activation of the MEK/ERK pathway. Raf-1 dissociates from its complex with MEK in the presence of RKIP (summarized in Fig. 1). As a result, downstream mitogen-activated

Fig. 1



How loss of RKIP may promote metastasis. Decreased RKIP promotes angiogenesis and the ability of tumor cells to invade through extracellular matrix, and diminishes chemotherapy-induced apoptosis.

protein kinase (MAPK) signaling is interrupted and diminished. As stated earlier, RKIP can bind to Raf-1 or MEK, yet not at the same time, and binding to either one is enough to cause downstream inhibition [67]. In addition, it was postulated that RKIP may be involved in growth, transformation and differentiation [66], as these pathways are often deregulated in cancer.

Protein kinase C, which phosphorylates target proteins that control growth, differentiation and transcription, can inactivate RKIP through phosphorylation of RKIP on serine 153 and alleviate its inhibition of Raf-1 [68]. Protein kinase C is normally recruited to the plasma membrane and activated by diacylglycerol. Its location near the plasma membrane may place it in close proximity to RKIP, which also binds to phospholipids [69]. As a result, protein kinase C along with RKIP, function as unique selective regulators of the Raf-1/MEK/ERK growth factor signaling cascade. When RKIP is phosphorylated, it releases from Raf-1 and can bind onto G-protein-coupled receptor kinase-2 (GRK-2), preventing GRK-2's ability to inhibit G-protein-coupled receptor activity [70].

RKIP as a MSG

The previous studies using low versus high metastatic cell lines suggested that RKIP expression was decreased in prostate cancer metastases [45]. To explore the relation of RKIP expression to progression clinical prostate cancer, we examined RKIP protein expression in non-neoplastic prostate tissue, primary prostate cancer and prostate cancer metastases. RKIP was detectable in all non-cancerous prostate tissue and primary prostate cancers examined, but was undetectable in all prostate cancer metastases examined [49]. Specifically, RKIP expression level was highest for benign tissue, lower for cancerous tissue (declining with increasing Gleason score) and absent in metastases. These results provided strong evidence that loss of RKIP is associated with the development of prostate cancer metastases. However, these data do not demonstrate that RKIP functionally contributes to the metastatic process.

To examine the function of RKIP during prostate cancer progression, we modulated RKIP expression in prostate cancer cells to determine the effect of different RKIP levels on the prostate cancer cells metastatic ability. Specifically, we increased RKIP expression in the metastatic prostate cancer cell line C4-2B, which has low RKIP expression, by stably transfecting C4-2B cells with sense cDNA RKIP vector (or empty expression vector for control) and we reduced RKIP expression in the non-metastatic prostate cancer cell line LNCaP by stably transfecting LNCaP cells with antisense cDNA RKIP vector (or empty expression vector for control). The sense RKIP vector-transfected C4-2B cells demonstrated increased RKIP expression and the antisense RKIP vector-transfected LNCaP cells demonstrated decreased RKIP expression compared with the corresponding control vector-transfected cells.

We then examined whether modulation of RKIP expression influenced the tumorigenic properties of the prostate cancer cells. We measured the *in vitro* proliferation rates and the ability to form colonies in soft agar (an indication of the cells ability to grow independently of a basement membrane which is a property of cancer cells) of the cells with different RKIP levels. Modulating RKIP expression had no effect on the ability of the cells to grow *in vitro* or on their ability to form colonies in soft

agar. These results suggest that modulation of RKIP expression has no effect on these two primary tumorigenic properties of human prostate cancer cells.

Invasion is one of the key components of the metastatic cascade. Accordingly, to examine whether changes of RKIP expression are associated with cancer cell invasiveness, we measured the invasive ability of the cells. Increased RKIP expression in the metastatic cells was associated with an average decrease of 48.5% of the cells *in vitro* invasive ability. Conversely, decreased RKIP expression in non-metastatic cells was associated with an average increase of 102.3% of the cells *in vitro* invasive ability. These results suggest that RKIP expression is inversely associated with the invasiveness of prostate cancer cells *in vitro*.

Although the *in vitro* data provide a clue that RKIP regulates an important metastatic phenotype (i.e. invasive ability) it is still critical to determine if this affects metastasis *in vivo*. Accordingly, to determine if increasing RKIP expression decreases metastasis *in vivo*, we implanted either (i) metastatic C4-2B transfected with empty vector so they expressed basal levels of RKIP or (ii) C4-2B cells that were engineered to express increased levels of RKIP into mice prostates. We then evaluated a variety of parameters including the number of mice that developed metastases and the growth of the tumor at the primary injection site. Tumor growth at the injection site in the prostates was identical between both groups. In contrast, increased RKIP expression in the tumor cells resulted in decreasing the number of mice that developed lung metastases by 70%. Furthermore, in the mice that had received cells expressing increased RKIP and that developed metastases, the number of metastases was far fewer than in the mice that had received the cells expressing low levels of RKIP. Taken together, these results suggest that RKIP functions as a suppressor of metastasis.

The identification of RKIP as a putative MSG provides a rationale to pursue the mechanism through which it achieves this affect. Such information may lead to specific therapies to prevent metastasis. Towards that goal, we examined if RKIP's ability to inhibit kinase activity may contribute to its anti-invasive properties. The observation that diminished RKIP expression, which results in increased *in vitro* invasive ability, also promotes MEK activation is consistent with the possibility that decreased RKIP expression promotes invasiveness through activation of MEK. To test this possibility, we evaluated the effect of inhibiting MEK activity with the MEK kinase inhibitor PD098059, on the *in vitro* invasion ability of C4-2B cells. Furthermore, to determine if our results were specific to MEK or involved other signaling pathways we used kinase inhibitors that blocked several non-MEK signaling pathways including protein kinase A, protein kinase C and phosphatidylinositol-3 kinase. PD098059 decreased the invasive ability of C4-2B cells, whereas the other kinase inhibitors had no effect. These results demonstrate that MEK contributes to *in vitro* invasion which is consistent with the possibility that RKIP regulates tumor invasion through MEK activity.

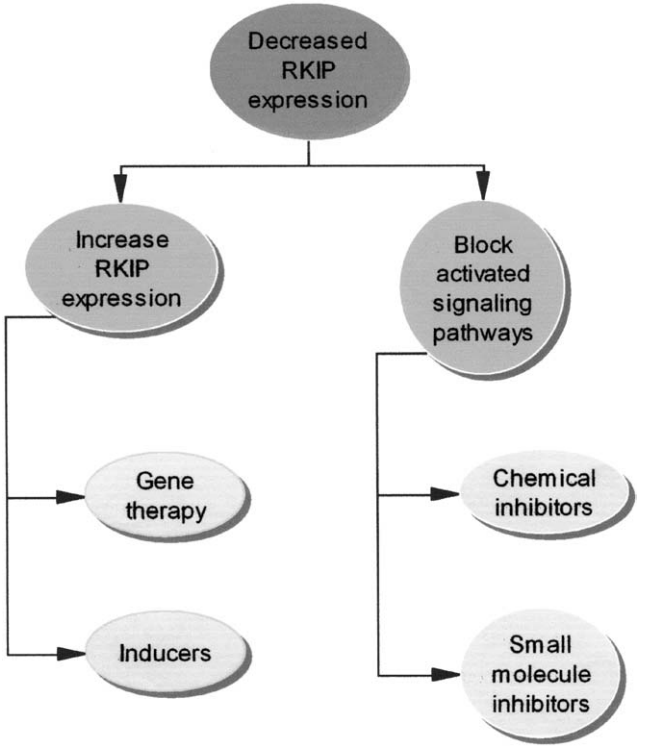
Invasion typically occurs through the blood vessels. Thus, we evaluated the degree of vascular invasion associated with the primary orthotopic tumors in mice to determine if this could contribute to the difference in metastatic rates between mice injected with C4-2B cells stably transfected with control vector or sense RKIP vector. All the mice bearing tumors derived from control vector-transfected C4-2B cells had vascular invasion, compared to only 40% mice bearing tumors derived from sense RKIP vector-transfected C4-2B cells. Thus, it appears that RKIP regulates the cancer cell's ability to invade blood vessels. Additionally, we observed that loss of RKIP was associated with increased blood vessel numbers in the tumors. Taken together, these data suggest that loss of RKIP promotes metastasis through enhancing vascular invasion.

In summary, these studies demonstrate that RKIP functions as a MSG (summarized in Fig. 1). Furthermore, our data demonstrate that decreased RKIP expression is associated with increased invasive ability, vascular invasion and angiogenesis. This was the first study to document the association between a cancer progression-associated decreased expression of a molecule that inhibits signal transduction and increased metastasis.

Targeting the RKIP pathway

RKIP presents a challenge in terms of therapeutic targeting (Fig. 2). Specifically, in the case of metastasis, RKIP expression is decreased in cancer cells. Thus, to reverse the phenotype associated with decreased RKIP, one could consider increasing RKIP in cancer cells, e.g. through gene therapy. However, this is challenging because it may be necessary to target all cancer cells as even one cell with low RKIP has the potential to become metastatic. Unfortunately, there currently are no therapies that can efficiently replace specific gene expression in all cancer cells *in vivo*. Several alternative options exist. One potential method is to identify compounds that induce RKIP expression. The ability to chemically induce RKIP expression has the potential to impact all cells. In addition to the decreased metastatic potential, inducing RKIP expression may sensitize cells to chemotherapy-induced apoptosis [71]. An alternative strategy to consider would be to target pathways that are increased due to diminished RKIP expression. For example, decreased expression of RKIP induces MEK, NFκB and G-protein activation; thus, targeting these signaling

Fig. 2



Potential mechanisms to target the metastatic phenotype due to diminished RKIP expression. Either increasing RKIP expression or blocking signaling pathways that are activated due to decreased RKIP expression. Increasing RKIP expression may be accomplished through transducing the RKIP gene into cancer cells using a gene therapy approach, but this suffers from inefficiency in current methods of gene therapy. Alternatively, identification of compounds (e.g. chemicals or cytokines) that induce RKIP expression may be useful. Inhibition of signaling pathways may be accomplished using chemical kinase inhibitors or design of small molecule inhibitors that target specific kinases.

pathways has the potential to diminish the pro-metastatic activity associated with decreased RKIP expression. Currently it is not clear how these different pathways interact to confer the metastatic phenotype and which of these pathways is the most important to target. Further exploration of the biology of RKIP and its role in the pathogenesis of cancer progression and metastasis is necessary to define potential therapeutic agents that can take advantage of RKIP's role in metastasis to impact the development of prostate cancer metastasis.

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