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Identification and Characterization of Determinants of Head and Neck Tumor Cell Invasion

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IDENTIFICATION AND CHARACTERIZATION OF DETERMINANTS OF HEAD AND NECK TUMOR CELL INVASION

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ABSTRACT

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Head and neck squamous cell carcinoma (HNSCC) is a common malignant disease with poor prognosis. The majority of patients die from local invasion or lymphatic metastasis. The mechanism(s) underlining the invasiveness of HNSCC are poorly understood. Utilizing a panel of HNSCC cell lines previously established in our laboratory, we tested the application and relevance of the three-step hypothesis of tumor invasion to HNSCC and investigated the mechanism(s) pertaining to the regulation of each step in the invasive process. Data presented in this thesis demonstrated that tumor cell invasion in HNSCC is a complex process involving three repeated sequential steps: adhesion, proteolytic degradation of the basement membrane and other extracellular matrixes (ECMs), and cell migration. These three steps are linked and interdependent. For example, cell adhesion to the ECM triggers a series of signal transduction pathways that involve calcium mobilization, focal adhesion kinase (FAK) activation, and downstream events leading to alteration of cell function manifested by migration and matrix metalloproteinase (MMP) production. Our working model extends the previous three-step hypothesis for tumor invasion by underscoring FAK as an integrator of multiple signaling pathways. Efforts to determine the role of FAK in the integration and propagation of signal transduction pathways should lead to a better understanding of the

molecular mechanisms for generating the invasive phenotype of HNSCCs which may, in turn, lead to the discovery of new targets for therapy of invasive HNSCC.

This work is dedicated to my wife Ping Yin and my daughter Miaoting Wu, for their love, support and encouragement.

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CHAPTER I

INTRODUCTION

Head and neck squamous cell carcinoma is a common malignant disease. Nearly 42,000 new cases of HNSCC and approximately 13,000 HNSCC deaths are estimated in the United States in 1998 (1). Globally, HNSCC accounts for 500,000 new cases annually and is the fifth most frequent cancer overall, comprising 1 of 12 total malignancies. Despite improvements in current therapies, including surgery, radiation, and conventional chemotherapy, 5-year survival rates are low and have remained unchanged over the past 20 years in whites but have decreased in African Americans (1-3). The major obstacle for the management of HNSCC is local-regional invasion and lymph node metastasis (3).

1.1 Overview of the metastatic process

Metastasis is the spread of tumor from a primary lesion to distant sites of the body and represents the hallmark of tumor malignancy. Successful metastasis formation involves multiple sequential steps (Fig.1-1). At the cellular level, tumor cells initiate metastasis by detachment from the primary tumor and invasion of the surrounding extracellular matrix (ECM), including the basement membrane and interstitial compartment, followed by intravasation of the nourishing blood vessels or draining lymphatic vessels. Cells are then carried in the circulation until lodging in a distal capillary bed. The survivors of the arrested cells then extravasate from the blood vessel, invade the surrounding tissues and

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The journal model for this thesis was Clinical Cancer Research.

begin to proliferate and form secondary tumors in the new organ environment (4). Angiogenesis, the recruitment of new blood vessels, is required for primary and metastatic tumors to grow to a clinically detectable size since the solid tumor never grows beyond 1 mm^3 due to lack of nutrition. Tumor-induced angiogenesis not only allows for expansion of the primary tumor, but also permits easy access to the vascular compartment as a result of defects in the basement membrane of the newly formed vessels. Thus, angiogenesis may facilitate hematogenous spread of some tumors (5,6).

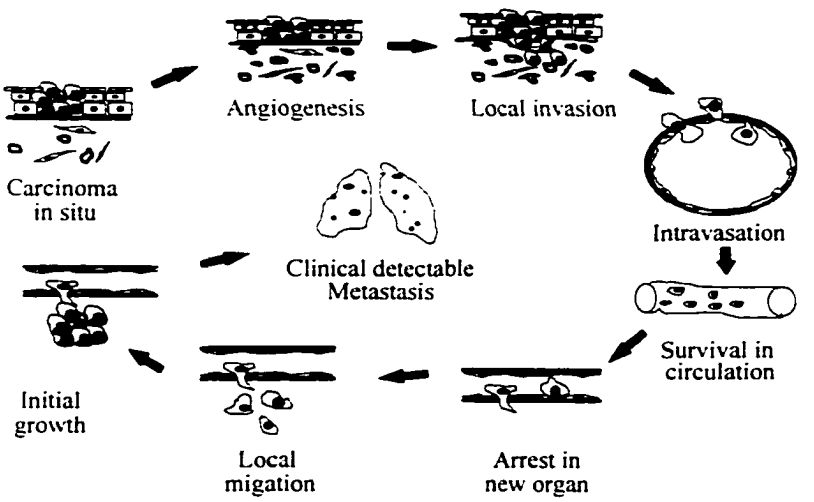

Fig1-1. Multi-step cascade of tumor cell metastasis.

Many of the steps in metastasis formation require specific interactions of tumor cells with the extracellular matrix and the nature and degree of these matrix interactions will vary from step to step during the metastasis process (7,8). Escape of tumor cells from the primary tumor may require decreased adhesion to the tumor or stromal matrix. However, cell arrest in the target organ that results in tumor-specific patterns of

metastasis formation may be mediated by specific tumor-endothelial interactions and selective binding to specific matrix components. Only the metastatically competent cells, which exhibit different responses to various ECM and stromal cells, will successfully navigate each of these interactions (7,8). Metastasis is known to be an inefficient process. Both clinical observations and experimental studies have demonstrated that large numbers of tumor cells are shed into the blood circulation, but only a small fraction of the cells succeed in forming metastases (9). One of the major contributors to this inefficiency is thought to be the traversal of the connective tissue barrier, an active process that is mediated by a specific cell-ECM interactions. Therefore, metastasis can be viewed as a process of selection of cells exhibiting invasive behavior characterized by their tendency to cross tissue boundaries and a subsequent growth advantage over normal tissue. To support this point, studies have demonstrated that individual metastases are likely to be clonal in origin (10).

1.2 The invasive phenotype: A three-step hypothesis

Tumor invasion is a complex and important process and marks the beginning of metastasis. It has been proposed that tumor invasion involves three sequential steps: cell adhesion, proteolytic degradation of the ECM, and cell migration through the ECM tunnel created by enzymatic proteolysis (Fig.1-2) (11). The invasive process of tumor cells shares many characteristics similar to normal physiological processes. Attachment, proteolysis, and migration are steps in trophoblast implantation, mammary gland involution, embryonic morphogenesis, and tissue remodeling. The difference between

normal physiological processes and the pathological process of tumor cell invasion lies in its regulation. Physiological processes are usually tightly regulated and self- limited.

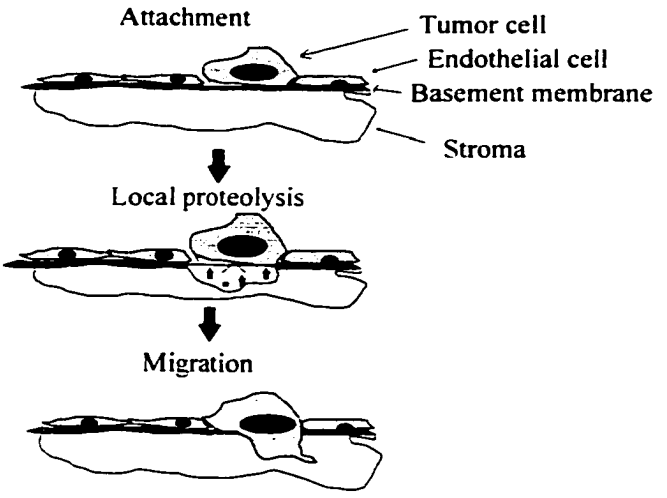

Fig. 1-2 Three step hypothesis of invasion.

whereas tumor invasion is unrestricted relative to the benefit of the host (12). Basement membrane is a special kind of ECM upon which a variety of cell types including keratinocytes in skin and endothelial cells in blood vessels rest. It serves as the tissue barrier at multiple key points in the metastatic cascade, namely, escape from the primary tumor in carcinoma, intravasation and extravasation during hematogenous dissemination, and perineural and muscular invasion. Thus, tumor invasion of the basement membrane was thought to be the rate-limiting step in metastasis. Composed of a dense meshwork of collagen type IV, laminin, and proteoglycans, the basement membrane does not normally contain pores that would allow passive tumor cell migration. Therefore, traversal of the basement membrane requires an active process defined by the invasive phenotype of tumor cells that is dominated by the ability of tumor cells to attach to, degrade, and

migrate through the ECM (reviewed in 13). In past decades, increasing interest was focused on deciphering the molecular mechanisms underlying the invasive process. The proposed three-step hypothesis has received extensive experimental support and has elucidated molecular events related to each step of tumor invasion and subsequent metastasis formation (13). However, the step-wise process of invasion has not been tested systematically in the context of HNSCCs. **We propose to test the application and relevance of this model to HNSCC. To do this, cell adhesion to ECM proteins, matrix metalloproteinase (MMP) production, chemoinvasion, and cell migration will be assessed utilizing HNSCC cell lines developed in our laboratory. (Aim 1, Fig. 1-7.1)**

1.3 Cell adhesion and biological consequences

Cell adhesion including cell-cell and cell-ECM adhesion represents the most direct form of tumor cell-host interaction. The capacity of tumor cells to adhere to other tumor cells, host cells, or components of ECM could affect multiple steps of the metastatic cascade. Cell adhesion is mediated by a series of cell adhesion receptors. Several families of cell surface adhesion molecules have been identified, each with different functions and specificities (14,15) These molecules include the cadherins, the immunoglobulin cell adhesion molecule (Ig-CAM) superfamily, selectins, and integrins. Cadherins are calcium-dependent molecules that mediate homophilic cell-cell adhesion. Three subtypes (E-, N-, and P-cadherins) have been characterized. Loss of the expression of cadherins results in the detachment of tumor cells from the primary tumor site thus

facilitating metastasis (16,17). The Ig-CAM superfamily, including N-CAM, I-CAM-1, I-CAM-2 V-CAM-1, CEA, and DDC, consists of divalent cation-independent receptors that have several repeats of immunoglobulin-like folds characterized by β-pleated sheets held together by disulfide bonds. These molecules mediate both homotypic and heterotypic cell-cell adhesion (18). Selectins, acting through a terminal calcium-dependent lectin domain, are involved in heterotypic cell-cell adhesions between blood cells and endothelial cells. CD44, a homologue of cartilage link protein, is a transmembrane GTP binding protein with intrinsic GTPase activity and functions as a cell surface receptor for hyaluronate. Under physiological conditions, most members of Ig-CAM family, selectin, and CD44 function as regulators of white blood cell trafficking and lymphocyte homing, whereas in the pathological process of tumor invasion, they determine organ- specific metastasis (19).

Like cell-cell interactions, cell adhesion to the ECM is also accomplished by cell recognition of ECM proteins via cell surface receptors and represents the initial step in the process of tumor invasion (13). The most important cell surface receptors in cell-ECM interaction are integrins, heterodimers of α and β subunits that can pair to form more than 20 unique receptors (20). Different integrin receptors interact with different components of the ECM (eg., fibronectin, laminin, collagen type IV) by binding to integrin recognition sites consisting of a short linear amino acid sequence, e.g. arginine-glycine-aspartic acid (RGD) (21). Integrin binding to the ECM and subsequent clustering leads to the formation of focal adhesions – specialized sites of contact – where integrins link to intracellular cytoskeletal complexes and bundles of actin filaments. This complex

of proteins plays an important role in modulating cell adhesion and regulating cell shape and mobility. Studies have shown that integrin-mediated cell-ECM interaction stimulates numerous complex biological processes including MMP production (22), cell migration (23-26), and changes in phosphorylation of a number of structural and signaling proteins.

a. *Integrin-mediated cell ECM interaction regulates cell migration*

Several lines of evidence support the notion that integrin mediated cell-ECM interaction can regulate tumor cell migration. First, some ECM proteins including fibronectin, laminin, and type IV collagen were found to stimulate tumor cell locomotion (23-26). Fibronectin is a large multidomain glycoprotein in the connective tissue matrix. Each domain of the fibronectin molecule is composed of a set of type I, II, and III repeats, which together confer specific binding activities. The central cell binding domain contains the RGD sequence in repeat III_{10} , which is essential for interaction with a number of integrins including $\alpha5\beta1$, $\alpha v\beta3$, $\alpha IIb\beta3$, and $\alpha v\beta6$ (27). Laminins are cross-shaped heterotrimers composed of α, β, and γ chains, which exist as ten different variants and can assemble into eleven isoform combinations, laminin-1 to laminin-11. The RGD containing sequence is located in the α chain. Apart from RGD, 8 other recognition sequences were also identified in laminin. These sequences mediate the interaction with integrins including $\alpha2\beta1$, $\alpha3\beta1$, and $\alpha6\beta1$ (28). Type IV collagen is a polypeptide composed of repeating hydroxyproline and hydroxylysine residues that, when polymerized, forms a sheet like network which is the fundamental structure of most basement membranes. Type IV collagen preferentially binds to $\alpha1\beta1$ integrin, however,

a recognition sequence other than RGD may be required for interaction (29, 30). Fibronectin, laminin, and type IV collagen either in solution (chemotaxis) or coated on a solid membrane support (haptotaxis) induced tumor cell migration by a number of tumor types (23-26). Secondly, expression of certain integrins was associated with tumor cell motility. Studies have shown that expression of integrins $\alpha 2\beta 1$, $\alpha 3\beta 1$ and $\alpha 6\beta 1$ correlated with invasive potential and cell motility of melanoma tumor cells. Moreover, anti-$\beta 1$ monoclonal antibody inhibits melanoma cell migration (31). Similarly, expression of $\alpha 3\beta 1$ was found to be associated with cell migration of malignant glioma (26), and expression of $\alpha 6\beta 1$ is essential for migration of breast carcinoma since knocking out $\alpha 6\beta 1$ by a dominant deletion mutant reduced the motility of breast cancer cells (32). Third, disruption of integrin mediated cell-ECM interaction inhibits tumor migration. The synthetic RGD containing peptide, which blocks integrin-ligand interaction, successfully inhibits melanoma cell migration, and subsequently inhibits tumor invasion. However, relative high concentrations (1mg/ml) are required to achieve these effects (33). Similarly, dysintegrin, a RGD containing peptide, obtained from snake venom, was found to have similar inhibitory activity (33).

b. Integrin mediated cell-ECM interaction regulates MMP production

Like cell migration, ECM proteins were also found to stimulate MMP production. Incubation of ovary carcinoma cells in soluble fibronectin increased MMP-9 production by two-fold (34). Fibronectin was also found to stimulate MMP production by fibroblasts. Exposure of osteogenic sarcoma cells to type I collagen induced elevated secretion of

MMP-1 (35). Similar to ligand stimulation, cross-linking integrin receptors also increases MMP production. For example, antibody cross-linking of integrin αvβ3 enhanced MMP-2 production by melanoma cells (36). Taken together, accumulating evidence supports the concept that integrin mediated cell-ECM interaction regulates MMP production.

Although substantial evidence supports the notion that cell migration and MMP secretion were regulated by integrin mediated cell-ECM interaction in other cell types, little is known about cell binding to ECM proteins and promotion of gene expression and cell migration of head and neck tumor cells. **We hypothesize that MMP production and cell migration of HNSCC are regulated by cell-ECM interactions and propose to test this possibility by evaluating the alteration of MMP production and cell migration of HNSCC following exposure to ECM proteins. (Aim 2, Fig. 1-7.2).**

1.4 Proteolytic degradation of the ECM

Breachment of the basement membrane and proteolytic degradation of the ECM is thought to be a rate-limiting step in tumor invasion (13, 37-39). Tumor cells employ proteases to accomplish this key step. Four families of proteases have been implicated in tumor invasion: (a) cysteine proteases (eg., cathepsin B,L), (b) aspartyl proteases (cathepsin D, E), (c) serine proteases (eg., urokinase-type plasminogen activator), and (d) matrix metalloproteinases (MMPs). However, much of the attention has centered on the MMP family.

a. *MMP family*

MMPs are members of a increasing family of Zn2+-dependent endopeptidases characterized by secretion as a zymogen, activation achieved by proteolytic removal of the propeptide, function extracellularly at neutral pH, and inhibition by a specific class of biological inhibitors referred to as the tissue inhibitors of metalloproteinases (TIMPs) (40). Structurally, MMPs contain the following basic domains: a signal sequence; a

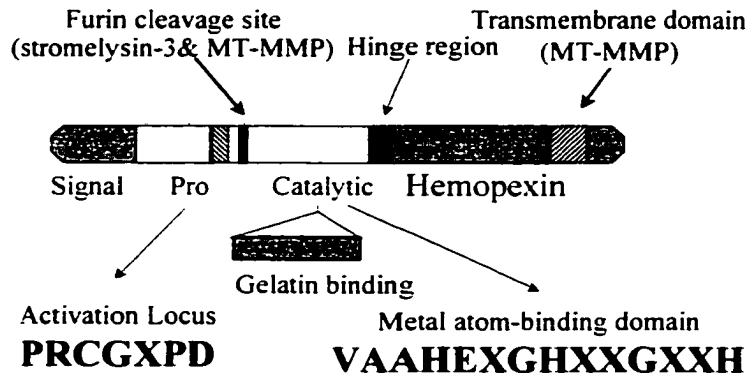

Fig. 1-3 Domains of the matrix metalloproteinases (reviewed in 41). Each member of the MMP family has at least four domains (1). The signal sequence directs the translation production to the endoplasmic reticulum; (2) The propeptide domain that is lost following enzyme activation. (3) The catalytic domain that contains the conserved sequenceVAAHEXGHXXGXXH in which the three histidine residues coordinate the active site zinc (4) the carboxyl-terminal domain that has homology to hemopexin and vitronectin, is severely truncated in matrilysin, or contains a transmembrane domain in MT-MMP. In addition, there is a fifth domain that shows homology to the type II fibronectin repeats that bind gelatin and is found only in gelatinases.

propeptide domain; a catalytic domain containing a zinc-binding site; a 54 amino acid long proline rich region homologous to the α2 chain of type V collagen; and a hemopexin like carboxyl-terminal domain (Fig. 1-3). C-terminal domains are varied to confer substrate specificity. N-terminal domains are more conserved especially at the following

regions: the amino-terminal profragment 'activation locus'(PRCGxPD) and the catalytic domain of the zinc binding site (HExGHxxHS/T) (40,41) . Three histidines in the conserved catalytic sequence complex with Zn atoms to form the active site, whereas a free cysteine in the propeptide motif binds to the same Zn atom and maintains latency of MMPs. Activation is achieved by dissociation of the propeptide from the catalytic domain. This dissociation represent a fundamental process for regulation of MMP activity. MMP activation can be achieved by auto-activation and by the action of other proteinases such as furin, urokinase, and plasmin (42). Activation of MMPs is subject to regulation by association with TIMPs, and once activated, may be inactivated by TIMPs and other plasma proteins such as α2-macroglobulin. Thus, the level of ECM degradation reflects a local balance of MMP expression and activation versus the level of TIMPs. The four different TIMPs identified so far are expressed in different tissues and exhibit different specificities to complex and inhibit MMPs (41-43). Cell surface localization of MMPs may achieve a highly regulated focus of activity while widespread degradation may result from over-expression of unchecked secreted MMP. These features of the MMP axis are significant in studying their contribution to both normal and pathological remodeling of tissues (41-44).

To date, there are 20 members of MMPs divided into six classes according to their substrate specificity and internal homologies: (a) the interstitial collagenases which degrade fibrillar collagens including type I, II, and III collagen; (b) the gelatinases which degrade denatured forms of collagen (gelatin) and type IV collagen, the main form of collagen in basement membrane; these MMPs also degrade type V, VII, IX and X

collagens and fibronectin; (c) the stromelysins which have broad substrate specificity and degrade various components of the ECM including fibronectin, laminin and the core protein of proteoglycans; (d) elastases which degrade elastin; (e) stromelysin-3 which targets alpha-1-antitrypsin, and (f) membrane type (MT) MMPs. The last two groups are distinguished by the insertion of a furin cleavage site, RXKR, between the prodomain and catalytic domain. Presence of this domain is important for the intracellular activation. Most MMPs are secreted while MT-MMPs have been shown to be membrane associated via a transmembrane domain at the carboxyl terminus of the molecule (41,42).

b. *Multilevel regulation of MMP-2 and MMP-9*

MMP-2 and MMP-9 are two well characterized forms of type IV collagenases (gelatinases) which share four common domains: the amino-terminal and zinc-binding domains shared by all members of the MMP family, a collagen-binding fibronectin-like domain, and a carboxyl-hemopexin-like domain. The main structural difference between MMP-2 and MMP-9 is the presence of a unique proline-rich domain homologous to the α2-chain of type V collagen in the hinge region of MMP-9 (13). MMP-2 is secreted as a 72 kDa proenzyme which is activated by a recently identified membrane type-MMP (MT-MMP) to generate 62 kDa, 59kDa and 41-45 kDa active species(45,46). MMP-9 is secreted as a 92 kDa proenzyme and activated by plasmin, stromelysin, tissue kallikrein, and MMP-2 to generate a 82 kDa active species (Fig. 1-4) (47). Activated forms of MMP-2 and MMP-9 are capable of digesting mainly gelatin and type IV collagen. Therefore, MMP-2 and MMP-9 are also called 72 kDa type IV collagenase (or gelatinase

A) and 92 kDa type IV collagenase (or gelatinase B), respectively. Both latent and active forms of MMP-2 and MMP-9 can interact with TIMPs (48). TIMP-1, a 28 kDa glycoprotein initially isolated from rabbit bone and human fibroblasts, forms a stable complex with proMMP-9 and blocks the activation of MMP-9 by stromelysin (49, 50).

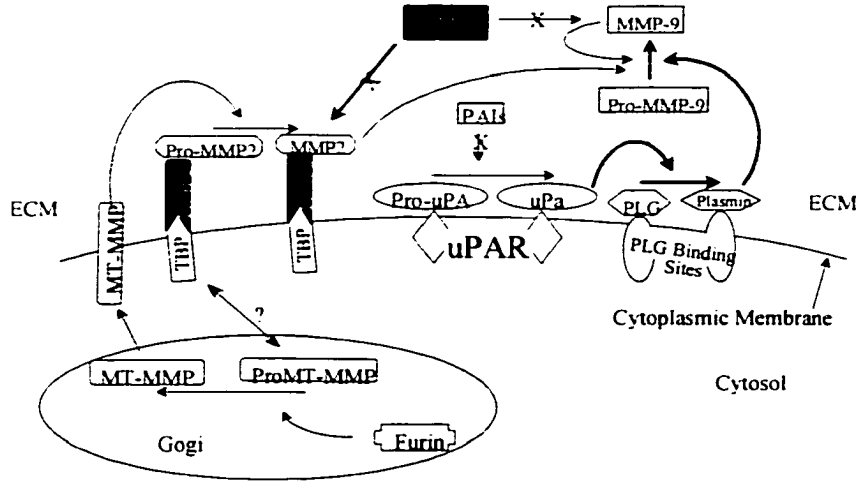

Fig.1-4 Activation of gelatinases on the cell surface. TIMP, tissue inhibitor of metalloproteinase; TBP TIMP-2 binding protein; uPAR, urokinase type plasminogen activator receptor; uPA urokinase type plasminogen activators; PAIs; plaminogen activator inhibitors; PLG, plasminogen; X indicats inhibition of the process.

TIMP-2 is a 21 kDa nonglycosylated protein that shows 66 percent overall homology to TIMP-1. TIMP-2 can form a complex with proMMP-2 and inhibits MMP-2 activation (51, 52). Both TIMP-1 and TIMP-2 can form a tight 1:1 stoichiometric noncovalent complex with MMP-9 and MMP-2, respectively (53). Formation of TIMP-MMP complexes inhibits MMP activity and prevents autoproteolytic degradation of MMPs, thus maintaining their integrity and substrate specificity (54).

Recent studies demonstrated that inhibition of MMP-2/MMP-9 is mediated by interaction between the N-terminal region of MMP and the inhibitory TIMPs. Interestingly, the C-terminus of TIMP-2, and only TIMP-2, was found to also interact with the C-terminus of proMMP-2. However, this interaction does not inhibit MMP-2 activity; instead, it is important for proMMP-2 activation. C-terminal TIMP-2 interacting with proMMP-2 and subsequent binding to a TIMP-2 binding protein or MT-MMP, anchors the proMMP-2 to the cell surface in close proximity to MT-MMP which is activated intracellularly by a serine protease, furin. The intimate relationship between active MT-MMP and proMMP-2 significantly increases the efficiency for MMP-2 activation (Fig.1-4). Therefore, the effect of TIMP-2 on MMP-2 is paradoxical. At low concentrations, it stimulates MMP-2 activity, whereas at high concentrations, it inhibits MMP-2 activity. Pericellular activation of MMP-2 is a good example of spatial regulation (41).

MMPs are also subject to transcriptional regulation. Transcriptional regulation of MMP-2 and MMP-9 is different due to unique elements within each promoter. MMP-2, whose promoter lacks an AP-1 site, was found to be constitutively expressed in a wide range of tissues, whereas expression of MMP-9 can be stimulated by a variety of growth factors, cytokines and ECM proteins in different cell types (43). Multilevel regulation of MMP-2 and MMP-9 is biologically significant. Since MMP-2 and MMP-9 play important roles in both physiological (e.g angiogenesis) and pathological matrix remodeling (e.g tumor invasion), multilevel regulation of MMP-2/MMP-9 allows precise control of MMP activity under physiological conditions. However, excess production and

activation of MMP or downregulated TIMP is thought to be a key feature of the pathological process, notably tumor invasion (42).

c. *MMP-2/MMP-9 and tumor invasion*

The role of MMP-2/MMP-9 in tumor invasion is well established. Secretion of MMP-2/MMP-9 is correlated with the invasive and metastatic phenotype of several tumor types (55-57). Inhibition of the expression or activity of gelatinases by the synthetic MMP inhibitor (batimastat), conserved MMP peptide sequences, (58-60) or by a ribozyme directed against MMP RNA (61) leads to reduced invasive and metastatic capacity. The role of MMP-2/MMP-9 in tumor invasion and metastasis is further supported by recent clinical studies. Increased gelatinase A (MMP-2) activity was found in invasive tumor regions of micro-dissected human colon cancer samples as compared to a matched number of normal epithelial cells from the same patient (62). Consistent with this result, immunohistochemical studies have shown that most invasive colonic and gastric adenocarcinomas or desmoplastic stroma are immunoreactive for MMP-2, whereas benign proliferative disorders of the breast and colon, and normal colorectal and gastric mucosa, show decreased or negative staining for this enzyme (63-66). Recent studies using a combination of immunohistochemistry and zymography demonstrated that not only expression of MMP-2/9 but, more importantly, activation of MMP-2 was associated with tumor invasion and metastasis of gastric, cervical and endometrial cancer (67,68). In contrast to MMPs, decreased TIMP expression has been asociated with invasive tumors (69,70). Exogenous TIMP-1 has been shown to inhibit in vitro tumor cell

invasion of human amniotic membranes and in vivo metastasis in animal models (71,72). Down-regulation of TIMP-1 expression by transfection of antisense RNA into mouse 3T3 cells enhanced their ability to invade human amniotic membranes and to form metastatic tumors in athymic mice (73). TIMP-2 has also been shown to successfully inhibit in vitro tumor invasion of the ECM (74). Overexpression of TIMP-2 in invasive and metastatic Ras-transformed rat embryo fibroblasts resulted in suppression of the ability of these cells to form lung colonies following intravenous injection into nude mice (75). An increased ratio of MMP to TIMP has been detected in cervical cancer and stroma by in situ RT-PCR and found to correlate with poor prognosis (76).

Accumulating data demonstrated that MMP-2 and MMP-9 play an important role in the invasive process of a variety of tumors (55-76). However, the role of MMP-2/MMP-9 in the invasive phenotype of HNSCCs has not been fully explored (67, 77). Preliminary studies from our laboratory demonstrated that HNSCC cell lines produce MMP-2 and/or MMP-9 and that secretion of MMP-2 is associated with Matrigel invasion. The results link MMP-2 expression with a more aggressive tumor cell phenotype (78). **To determine if MMP-2 is required for invasion of HNSCC, MMP-specific antibody was assessed for inhibition of Matrigel invasion and MMP activity. (Aim 3 Fig.1. 3).**

1.5 Signaling pathways in tumor cell invasion and migration

As discussed above, cell-ECM interactions are mediated by specific classes of

integrins. Recent studies have shown that integrins not only regulate cell adhesion, but are also capable of transducing signals across the plasma membrane (79,80) and regulating gene expression. To date, many components of the integrin signaling pathway have been identified (81,82), but little is known about the precise molecular mechanisms by which integrin-mediated signal transduction mediates and/or regulates cell migration and MMP production.

a. Calcium and invasion

Calcium is one of the important intracellular second messengers. It participates in cell signaling by binding to specific cellular proteins including calmodulin and protein kinase C (PKC). Calmodulin is a calcium-regulated protein that is present in all cells. Although this protein does not have intrinsic catalytic activity, it does regulate other cellular enzymes such as adenylyl cyclase and Ca2+/calmodulin protein kinase. PKC is a serine/threonine protein kinase whose activation depends on binding of calcium and diacylglycerol (DAG). Activation of PKC leads to phosphorylation of specific cellular proteins in the target cell (83).

The concentration of cytosolic calcium in a resting cell is approximately 100 nM. In the endoplasmic reticulum (ER) and extracellular fluids, the calcium concentration is in the millimolar range. The calcium gradient is maintained by an ATP-dependent calcium pump. After cell stimulation, calcium levels in the cytosol transiently rise, enabling this cation to act on several calcium dependent proteins in the cytoplasm (84)

Integrin stimulation was found to generate a rise in intracellular calcium. Cross-linking of or adhesion via integrins has been shown to induce an increase in the intracellular calcium concentration in platelets with characteristics similar to inositol-1.4.5-trisphosphate (IP3)-mediated calcium increases in other systems (85). Ligation of $\alpha v\beta 3$ integrin on osteoclasts triggers mobilization of calcium in the nucleus (86), whereas activation of integrins on endothelial cells results in elevation of intracellular calcium by an influx of calcium through the plasma membrane (87). These findings indicate that calcium participates in integrin signaling. Accumulating evidence demonstrates that calcium homeostasis and calcium-regulated cellular events are important in the generation and maintenance of the malignant phenotype (88). We speculate that integrin mediated-calcium signaling may play a role in generating the invasive phenotype of HNSCCs. Recently, work of others has shown that inhibition of calcium influx by carboxyamidotriazole (CAI) attenuates focal adhesion kinase (FAK) phosphorylation and activation, indicating that mobilization of intracellular calcium may be upstream of FAK (89).

b. FAK and invasion:

Focal adhesion kinase was originally identified as one of several proteins that were highly phosphorylated in src-transformed cells (90). Subsequent cloning of this protein showed that it was a novel tyrosine kinase which localized to cellular focal adhesions (91). Focal adhesion kinase becomes phosphorylated upon adhesion of fibroblasts and transformed epithelial cells to the ECM. Temporally, FAK

phosphorylation is coincident with cell spreading (92), and inhibitors of cytoskeletal polymerization inhibit FAK phosphorylation. Additionally, FAK becomes phosphorylated on tyrosine in response to certain neuropeptides and growth factors, indicating that FAK may be involved in cross talk between integrin and growth factor signaling (93).

1. Structure of FAK

FAK is a member of a growing family of structurally distinct cytoplasmic protein tyrosine kinases consisting of three functional domains (94). The catalytic domain, which exhibits most of the structural hallmarks of typical tyrosine kinases, is flanked by two-

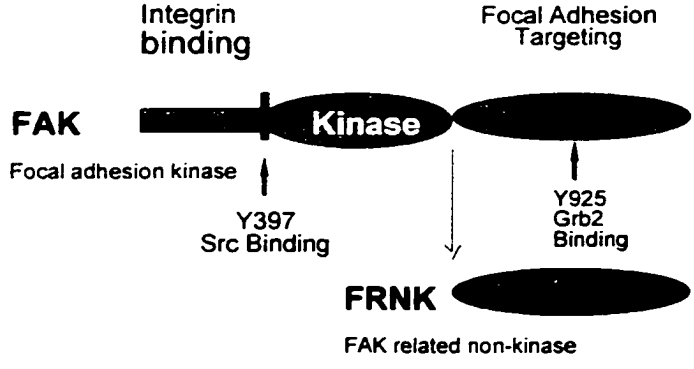

Fig. 1-5 Functional domains of FAK and FRNK (reviewed in 94).

noncatalytic domains that exhibit little sequence similarity to other proteins (Fig.1-5).The FAK amino terminal domain is responsible for integrin binding, while the carboxyl-

terminal domain mediates direct interaction with the adhesion-associated protein, paxillin, and targeting to the focal adhesion, defined as integrin-mediated sites of contact between the cell and the ECM (94). Unlike many other nonreceptor tyrosine kinases, FAK lacks Src homology 2 or 3 (SH2 or SH3) domains, which mediate specific interactions with phosphotyrosine-containing sequences or proline-rich sequences, respectively (95). Instead, FAK contains consensus sequences that allow proteins with SH2 or SH3 domains to bind. Two proline-rich stretches, three tyrosine phosphorylation sites including a motif (YAEI) for the binding of Src SH2 domain, a motif (YMXM) for the binding of the SH2 domain of the p85 subunit of phosphatidylinositol 3-kinase and a motif (YENV) for binding the Grb2 SH2 domain have been identified (96).

2. FAK substrates and signaling

FAK plays a central role in integrin-mediated signaling (Fig.1-6). Integrins are receptors for extracellular matrix proteins. Presence of SH2 and SH3 binding sites in FAK ensures activation of downstream events following integrin activation. However, the mechanism of FAK activation is poorly understood. Studies have shown protein-protein interactions between the amino terminal domain of FAK and the cytoplasmic domain of the integrin β1 subunit, suggesting that the direct interaction may transduce the signal generated from integrin -ECM binding (97). Other data suggest that talin is an upsteam mediator of FAK (98). Independent of the mechanism, cell-matrix interaction results in autophosphorylation of FAK at Tyr-397 which recruits Src through the SH2 domain (99). FAK/Src interaction releases autoinhibition of Src by displacement of the C-terminal

regulatory tyrosine residue from the SH2 domain, resulting in Src enzymatic activity. This interaction is negatively regulated by C-terminal Src kinase (Csk) (100). Activated FAK/Src then phosphorylates substrates located in focal adhesions including paxillin, tensin, p130^{cas}, and cortactin (95) which regulate cytoskeleton assembly. Recently, phosphorylation of FAK Tyr-397 was shown to be required for association with PI3-

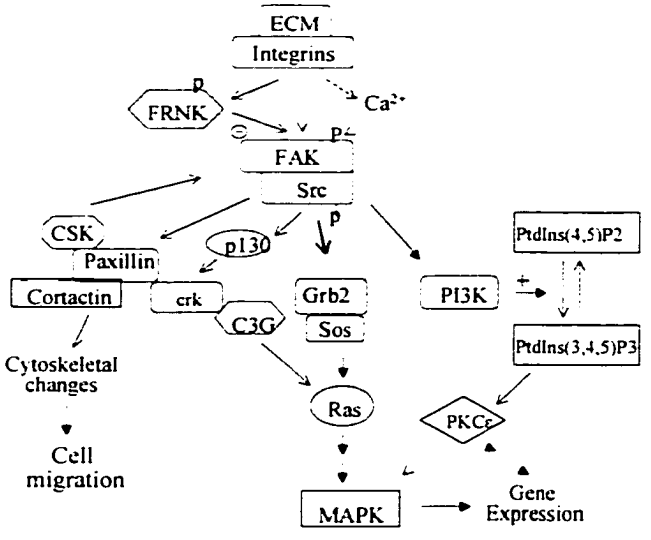

Fig. 1-6 Integrin signaling pathways through FAK. Binding of integrins to ECM leads to activation of FAK and its subsequent association with Src. The FAK/Src complex then triggers several downstream pathways, leading to altered gene expression or cytoskeletalchanges.

kinase (54). PI 3-kinase phosphorylates PtdIns(4,5)P2 to produce phosphatidylinositol 3.4.5-triphosphate [PrdIns(3,4,5)P3], which acts as a second messenger to activate PKC (101). Tyr-925 of FAK is phosphorylated by Src and serves as the binding site for the Grb2/Sos complex. FAK:Grb2/Sos association relays the signal into the nucleus via the Ras-MAP kinase pathway (102). FAK is expressed in most cell lines and tissues examined to date (98). In some cells, the carboxyl-terminal domain of FAK is expressed

autonomously as a 41,000 Mr protein, termed FAK-related nonkinase (FRNK) (103). FRNK expression is regulated via a separate promoter within an intron of the full-length protein [J.T. Parsons, personal communication]. The function of FRNK is not clear. Since FRNK contains the focal adhesion targeting sequence, it is reasonable to speculate that FRNK may play a role in localization of FAK to focal adhesion sites and in directing the binding of FAK to potential cellular substrates (91). Recently, FRNK was demonstrated to inhibit FAK phosphorylation and cell spreading following integrin activation, providing a novel mechanism for FAK regulation (104).

3. FAK and invasion

There are several indications that integrin signaling through FAK might be involved in tumor invasion and metastasis. Overexpression of FAK has been detected in a number of invasive tumors (105,106), and phosphorylated FAK has been linked to the invasive phenotype and progression of some tumors (107,108). Fibroblasts cultured from FAK knockout mice exhibited decreased motility but increased focal adhesion contacts, suggesting that FAK may play a role in focal adhesion turnover, which promotes migration (109). The regulation of cell migration by FAK was further supported by the demonstration that FAK overexpression in CHO cells resulted in increased migration towards fibronectin, whereas mutation of the autophosphorylation site in FAK (Tyr-397) abolished the migration-stimulating effects (110). In addition to cell migration, the integrin-FAK signal transduction pathway is involved in the activation of MMP secretion. Down-regulation of FAK expression by treatment with antisense oligonucleotides to FAK

attenuated MMP-9 production by ovarian tumor cells (111). **Whether FAK is involved in activation of MMP secretion and cell migration in HNSCCs has not been evaluated previously and is one aim of the present studies (Aim 5. Fig.1-7.5).**

1.6 Rationale and focus of the present study

Head and neck cancer is a common malignant disease with poor prognosis. The majority of patients die of local or regionally advanced disease. The mechanism(s) underlying the invasiveness of head and neck squamous cell carcinoma (HNSCC) are poorly understood. Previous work from our laboratory has shown that inoculation of HNSCC cells into the oral cavity of nude mice demonstrated tumor growth and invasion at the orthotopic site, which mimicked the growth and invasive behavior of human HNSCC (112). In contrast, HNSCC cells implanted subcutaneously in the scapular region of nude mice formed localized tumors that were totally encapsulated by fibrous connective tissue (112). The results suggested that the tissue microenvironment and cell-ECM interactions were likely to be important determinants in regulation of the invasive phenotype of HNSCC. How cell-matrix interactions and the microenvironment alter the invasive behavior of HNSCC remain to be determined. Studies with other cell types have shown that cell-ECM interaction not only regulates the invasive process but also plays an important role in generating and transducing signals across the cell membrane (79). Many signal transduction pathways are activated by cell-ECM interaction (81). A few studies have suggested that Ca2+ dependent signaling (88) and activation of focal adhesion kinase (82) are important in the regulation of cell motility and tumor invasion. We

hypothesize that cell adhesion to ECM proteins triggers a series of signal transduction pathways that involves Ca2+-dependent signaling, activation of focal adhesion kinase and downstream events leading to altered cell function manifested by MMP production and cell migration (Fig.1-7).

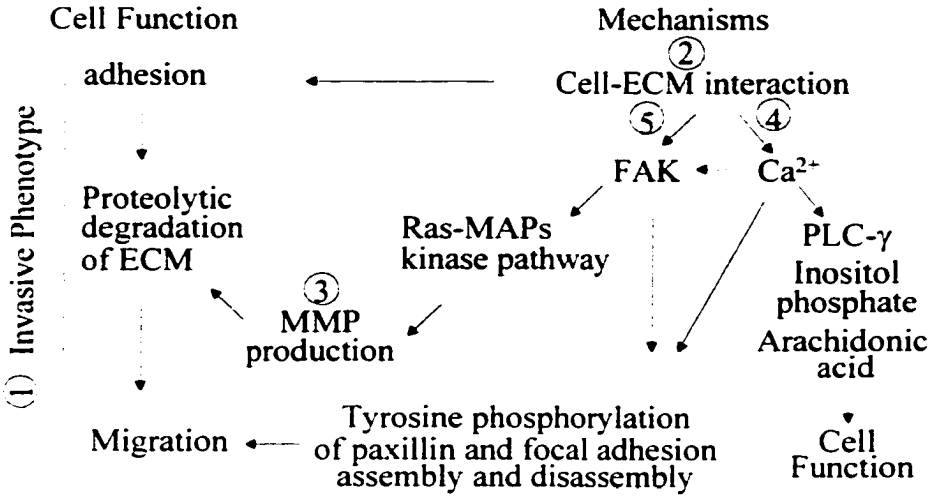

Fig. 1-7 Schematic presentation of the invasive process of HNSCC. Numbers in circles indicate the specific aims we propose in this study.

The overall aim of this thesis is to test the above hypothesis by characterizing determinants of the invasive phenotype of HNSCC and to investigate their role and involvement in regulation of the invasive behavior of HNSCC. Our long-term goal is to identify intervention targets that might be exploited to control locoregional disease progression and improve prognosis.

The specific aims are to: 1. Characterize the invasive phenotype of HNSCC. Cell lines derived from HNSCC were assessed for adhesion to ECM proteins, migration, Matrigel

invasion and production of MMPs by zymography and immunoblotting. HNSCC cell lines were further assessed for expression of selective endogenous inhibitors of MMPs, termed tissue inhibitors of metalloproteinases (TIMPs), by immunoblotting and reverse zymography.

2. Determine the degree to which MMP production and migration are regulated by cell-ECM interaction. This was achieved by measuring MMP production by ECM protein-stimulated cells and assessing the effect of cell attachment to ECM on cell migration by chemotaxis using ECM proteins as chemoattractants. The specificity of this integrin-mediated interaction was assessed with blocking peptides to adhesive recognition sequences.

3. Determine the role of MMPs in invasion of HNSCC. Antibody to MMPs was assessed for the ability to affect Matrigel invasion and MMP activity determined by zymography.

4. Determine the role of calcium signaling in the regulation of MMP secretion and generation of the invasive phenotype of HNSCCs. The role of calcium dependent signaling in generation of the invasive phenotype of HNSCC was assessed by testing the effect of carboxyamido-triazole (CAI), a novel calcium influx inhibitor, on cell migration, MMP production, and Matrigel invasion.

5. Determine the role of focal adhesion kinase (FAK) in the regulation of MMP production and generation of the invasive phenotype of HNSCCs. Specifically, a) FAK

expression and its activation by phosphorylation in HNSCC cell lines was assessed by immunoprecipitation and immunoblotting and examined for association with the invasive phenotype determined by Matrigel invasion, migration and MMP production. b) Sense FAK or the catalytically inactive isoform, FAK-related non-kinase (FRNK), was expressed by transfection of HNSCC cell lines to assess the consequences of FAK or FRNK transfection on the invasive phenotype of HNSCC cell lines. Sense FAK is expected to alter FAK expression, whereas sense FRNK expression is expected to inhibit FAK phosphorylation due to substrate competition. Manipulation of FAK expression and phosphorylation by transfection of head and neck tumor cells will assess directly the effects of FAK on the invasive phenotype of HNSCCs.

CHAPTER II

MATERIALS AND METHODS

2.1 Cell lines and cell culture

HNSCC cell lines derived from carcinoma of the tongue (EVSCC14M, EVSCC17M, EVSCC19M) and larynx (EVSCC18) were established at Eastern Virginia Medical School (113, 114). Additional HNSCC cell lines, UMSCC10A and FaDu, were derived from SCC of the larynx and hypopharynx, respectively (114). All HNSCC cell lines were cultured in EMEM supplemented with 10% fetal bovine serum (FBS) and non-essential amino acids. HaCaT, a spontaneously immortalized keratinocyte cell line (115) and RHEK-1, an SV40 T antigen immortalized human keratinocyte cell line (116) served as negative controls. HT1080, a human fibrosarcoma cell line, and A431 a wellcharacterized vulvar carcinoma cell line were used as positve controls for invasionand MMP production. NIH3T3 cells were used as a source of conditioned medium for migration and chemoinvasion assays. HaCaT, RHEK-1, HT1080, A431 and NIH3T3 cells were grown in DMEM supplemented with 10% FBS. All cells were cultured at 37°C in 5% CO_2/95% air.

2.2 Cell adhesion assay

Adhesion assays were carried out in 96-well tissue culture plates (Costar, Cambridge, MA) (117). The plates were coated overnight at 4°C with human plasma

fibronectin, mouse laminin, and type IV collagen (Gibco BRL, Gaithersburg, MD) at various concentrations. Plates were then rinsed three times in PBS and blocked by incubation in 0.1% heat inactivated bovine serum albumin (BSA) fraction V (Sigma, St. Louis, MO) for two hours at room temperature. The cells were harvested by trypsin-EDTA (Gibco BRL, Gaithersburg, MD) , washed once with growth medium containing 10% FBS and remained in suspension for 1 hour at room temperature. The cells were then resuspended in serum-free medium containing 0.1% BSA and added to ECM protein- coated 96 well culture plates at a density of $5x10^4$ cells/well. After 2 hours at 37^0C in a humidified atmosphere containing 5% CO_2, non adherent cells were removed by rinsing twice in PBS, and the adherent cells were fixed with 10% trichloroacetic acid (TCA) at 4^0C for 1 hour. The plates were then washed five times with tap water to remove TCA, air-dried, and stained for 30 minutes with 0.4% (wt/vol) sulforhodamine B (SRB) (Sigma, St. Louis, MO) dissoved in 1% acetic acid (118). At the end of the staining period, SRB was removed and the plates were quickly rinsed four times with 1% acetic acid to remove unbound dye. The bound dye was extracted in 10 mM unbuffered Tris base [tris(hydroxymethylaminomethane] (pH 10.5) for 20 minutes on a shaker. The optical density (OD) was read in a microtiter plate reader at 490 nm. To determine the effect of RGD peptides on cell adhesion to ECM, The RGD peptide, GRGDSP or the control RGE peptide, GRGESP, (Gibco BRL, Gaithersburg, MD) were added to the cell suspension at concentrations of 0.5mM, 1mM, 2mM or 4mM 30 min before the assay was initiated.

2.3 Chemoinvasion and migration assays

Chemoinvasion and cell migration assays were performed in a 48-well modified Boyden chamber. Polycarbonate filters (pore size: 8μm, Nucleopore, Cabin John, MD) were first coated with type IV collagen (5μg/filter) (Gibco BRL, Life Technologies, Grand Island, New York) and air dried. Matrigel (1mg/ml) in cold distilled water was layered onto each filter (500μg/filter). Filters were dried at room temperature and reconstituted with DMEM. Serum-free NIH 3T3 conditioned medium, obtained by incubation of cells for 24 hrs, was used as a source of chemoattractants unless otherwise noted. Cells were harvested by trypsinization and suspended in DMEM containing 0.1% BSA. The chamber was assembled using freshly prepared Matrigel-coated filters and NIH 3T3 conditioned medium was added to the lower chamber. A 56 μl sample of cell suspension ($2x10^6$/ml) was then placed in the upper compartment and incubated at 37^0C in 5% CO_2/95% air for 5 hours. At the end of the incubation, the cells on the upper surface of the filters were mechanically removed. Filters were fixed and stained with Diff-Quik (Baxter, McGaw Park, IL), and the number of invasive cells was counted using a microscope with an image analyzer. Cell migration assays in response to NIH 3T3 conditioned medium were performed similarly in modified Boyden chambers using polycarbonate filters coated with 0.01 mg/ml gelatin which is permissive for cell attachment and migration.

2.4 Collection of conditioned medium

T25 flasks seeded with $2x\ 10^6$ cells were incubated at 37^0C for 24 hrs, washed

with ITS (insulin, transferrin, selenium; Sigma, St Louis, MO) medium and incubated in 3 ml of ITS medium at 37^{0}C for 24 hours. The conditioned medium (CM) was collected, centrifuged at 400xg for 10min at 4^{0}C to remove cells and cell debris, and stored at -70^{0}C. For specific experiments, conditioned medium samples were concentrated 10-fold using an Amicon concentrator 10 (Millipore Corporation, Bedford, MA) which excluded molecules of less than 10 kDa.

2.5 Zymography

Gelatin zymography was performed as described previously (119) using a mini-gel apparatus (Bio-Rad, Richmond, CA). Briefly, aliquots of CM (40µl) were loaded onto 10% acrylamide gels containing 1mg/ml of gelatin. After electrophoresis, gels were washed twice for 30 min with 2.5% Triton X-100 and incubated in 50 mM Tris-HCl pH 7.5, 150mM NaCl, 10 mM $CaCl_2$, 0.02% Brij-35 and 0.1% Triton X-100 overnight at 37°C. The gels were place in 0.5% Coomassie Blue R_{250} dissolved in 40% ethanol and microwaved at full power for one minute. The gel was further stained in the same solution for 15 minutes and subsequently destained in three changes of acetic acid-ethanol solution (10% acetic acid, 40% ethanol) at 30, 60, 60 minutes intervals. The gels were stored in 10% acetic acid solution at 4^{0}C. The gelatinolytic activity was visualized as cleared regions of the gel and quantitated by scanning densitometry of individual lanes.

2.5 Reverse Zymography

Reverse zymography was also performed on a mini-gel apparatus according to Festruccia et al (120). Aliquots (30 ul) of 10x concentrated CM were loaded onto 10% SDS-polyacrylamide gels copolymerized with 1% (wt/vol) of gelatin and 25% of EVSCC18 conditioned medium which served as a source of MMPs. Following electrophoresis of samples, SDS was removed from the gel by exchange in 2.5% Triton X-100 solution which allows MMPs in the gel to renature. The gel was incubated 72 to 96 hours in enzyme buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij-35), stained with 0.5% Coomassie Brilliant Blue R_{250}, and destained in ethanol-acetic acid as described for zymography. Areas of inhibition were visualized as blue bands on a clear background of the reverse zymogram.

2.7 Western blot analysis of MMP-2 and TIMP-2

Monolayer cell cultures (75-80% confluency) were rinsed with ice-cold PBS, and then solubilized in lysis buffer containing 150 mM NaCl, 50mM Tris, pH 7.5 1% Triton-X 100, 0.25% Na-deoxycholate, 1mM PMSF, 1mM NaVO4, 50 ug/ml leupeptin, 0.5% aprotinin for 20 minutes on ice. Cells were scraped from the culture vessels, transferred to 1.5 ml microcentrifuge tubes, and centrifuged for 15 min at 4°C at 10^5 x g. The supernants were aliquoted and stored at -80 °C. Protein concentration was measured using the BCA protein assay kit (Pierce Chemical Co., Rockford, IL).

Cell lysates (50ug) or concentrated conditioned medium were resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, gels were equilibrated in transfer buffer consisting of 25 mM Tris HCL, 190 mM glycine, 10% methanol, 0.02% SDS and then transferred to Immobilon P membranes (Millipore, Bedford, MA) using a semi-dry electrophoretic transfer cell (Biorad, Richmond, CA). The transfer was carried out at room temperature for 40 minutes at 18 volts for MMP-2 analysis, or 35 minutes at 15 volts for TIMP-2 analysis. Following transfer, the membrane was blocked for 1.5 hours in buffer containing 50 mM Tris (pH 7.5) 150 mM NaCl, 0.05% Tween 20 and 5 % non-fat dry milk. For analysis of conditioned medium, the membrane was incubated for 1.5 hours at room temperature with 0.5 ug/ml monoclonal antibody to MMP-2 or TIMP-2 (Oncogene Science, a gift from Dr. T. Bos). For analysis of cell lysates, membranes were incubated similarly with 1μg/ml polyclonal antibody to MMP-2 and TIMP-2, obtained from Dr. Stetler- Stevenson, NCI. After three washes in 50mM Tris, 150 mM NaCl, 0. 01% Tween 20, the membranes were incubated in a horseradish peroxidase conjugated secondary antibody for one hour. Proteins were visualized by enhanced chemiluminescence (ECL) (Amersham, Arlington, Heights, IL).

2.8 Assay for effect of ECM proteins on cell migration and MMP production

The migration assay was performed in modified Boyden chambers as described in section 2.3. The ECM proteins, fibronectin, laminin, or type IV collagen at concentrations of 1, 10, 20,40, or 80 μg/ml were added individually to the lower chamber as chemoattractants, with BSA used as a negative control. Chambers were incubated at 37^0C and the assays were terminated at 5 hours. Cells on the upper surface were removed

mechanically, whereas cells on the lower surface of the membrane were stained and enumerated manually under 400 x magnification. The data are represented as the number of migrated cells per high power field. To determine the specificity of cell adhesion to ECM proteins, 0.5mM to 4 mM of RGD or RGE peptides were added to the cell suspension 2 hours before addition to upper chamber, and remained in the upper chamber during the migration assay.

To assess MMP production, cells (2×10^5) were plated into each well of a 24 well tissue culture plate and allowed to attached overnight. Adherent cells were washed twice with serum-free ITS medium, and treated with ITS medium containing 10, 20, 40, or 80 μg/ml of fibronectin, laminin, or type IV collagen. Serum-free ITS medium containing 80 μg/ml of BSA served as a negative control. Plates were incubated for 24 hours at 37^0C. Conditioned medium was collected and subjected to zymography as described in section 2.5.

2.9 Assay of MMP-2 antibody treatment and Matrigel invasion

Matrigel invasion assay was performed as described in section 2.3. Different concentrations of affinity purified anti MMP-2 rabbit polyclonal antibody ranging from 12.5 to 100 μg/ml were added to the upper chamber during the 5 hour assay. Normal rabbit antiserum (200 μg/ml) was added to the upper chamber as a control. The number of invaded cells was counted in 5 high-powered fields at 400 X magnification and data presented as the number of invaded cells per high-power field.

2.10 Treatment of cells with CAI

Carboxyamido-triazole (CAI) was generously provided by Dr. Elise Kohn, NCI, Bethesda, MD. T75 flasks were seeded with 10^6 cells in growth medium and cultured at 37^0C overnight to allow cell attachment. The next day, cells were washed with PBS and incubated in growth medium containing various concentrations of CAI (1uM to 20 uM) for 24 hours. Cells were then harvested by trypsinization and chemoinvasion and migration assays performed as described in section 2.3. CAI was present throughout the procedure and in the upper and lower chamber during the five hour chemoinvasion and migration assay.

2.11 Measurement of intracellular free calcium

Nearly confluent cultures of cells grown in T75 flasks were serum-starved for 4 hr and removed by brief trypsinization. Cells were washed and resuspended in phenol red-free Hanks' balanced salt solution (HBSS) supplemented with 0.1% BSA, fraction V (Sigma, St. Louis, MO). Cells were loaded with 4 μM of the calcium indicator dye Fura-AM (Molecular Probes, Eugene, OR) for 1 hr at 37^0C as described previously (121, 122) Calcium mobilization was assesssed in Fura-2AM loaded cells using a SPEX ARCM spectrofluorimeter at 330 and 380 nm. Calcium levels in nanomolar values were calculated as described previously (122), using calibration in the presence of CAI to compensate for any influence of CAI on fluorescence values.

2.12 Immunoprecipitation and immunoblotting analysis of FAK

Immunoprecipitation was performed as described by Matsumoto et al (123). Briefly, cells (10^6) were cultured in growth medium containing 0.5% FBS for 18 hours. Serum starved cells were washed with PBS containing 0.1 mM Na3VO4, and lysed in 1 ml of modified RIPA lysis buffer consisting of 50 mM Tris HCL (pH 7.5), 4mM EDTA, 150 mM NaCL, 10 mM sodium pyrophosphate, 10 mM NaF, 1 mM NaVO4, 0.1% sodium deoxycholate, 1 mM phenylmethysulfonyl fluoride, 10 ug/ml aprotinin, and 1% Nonidet P-40. Lysates were centrifuged at (10^5 x g) 4^0C and supernatants were aliquoted and stored at -80^0C. Protein concentration was determined as described in section 2.7. and cell lysates (800 μg protein) were precleared by incubation with protein A- agarose for 1 hour at 4^0C followed by centrifugation at room temperature at 5000 x g for 5 minutes. Supernatants were then transferred to fresh tubes and incubated at 4^0C overnight with 8 μg of monoclonal antibody to FAK (2A7, Upstate Biotechnology, Lake Placid, NY). Immunocomplexes were recovered using goat anti mouse IgG- agarose, separated by SDS-PAGE, and transferred to Immobilon-P. Membranes were probed with antiphosphotyrosine antibody (RC 20, 1 μg/ml) to assess FAK phosphorylation or 2A7 antibody (1μg/ml) to assess FAK expression, followed by incubation with peroxidase-conjugated goat anti-mouse IgG and the immunoreactive bands visualized by ECL.

2. 13 Generation of FAK and FRNK eukaryotic expression vectors

FAK and FRNK cDNA in Bluescript phagemids were kindly provided by J.T.

Parsons (University of Virginia, Charlottesville, VA) and were cloned into the expression vector pSV.Sport1 in the sense and antisense orientation. pSV. Sport1 is commercially available eukaryotic expression vector containing the SV40 promoter and polyadenyaltion signal. It was chosen because of previously successful experiments in this laboratory using the vector to express the gene Ems-1/cortactin in fibroblasts (124). The strategy for construction of the sense FAK expression vector is shown in Fig. 2-1. Briefly, pSK. FAK was cut by Xho I , followed by treatment with Klenow DNA

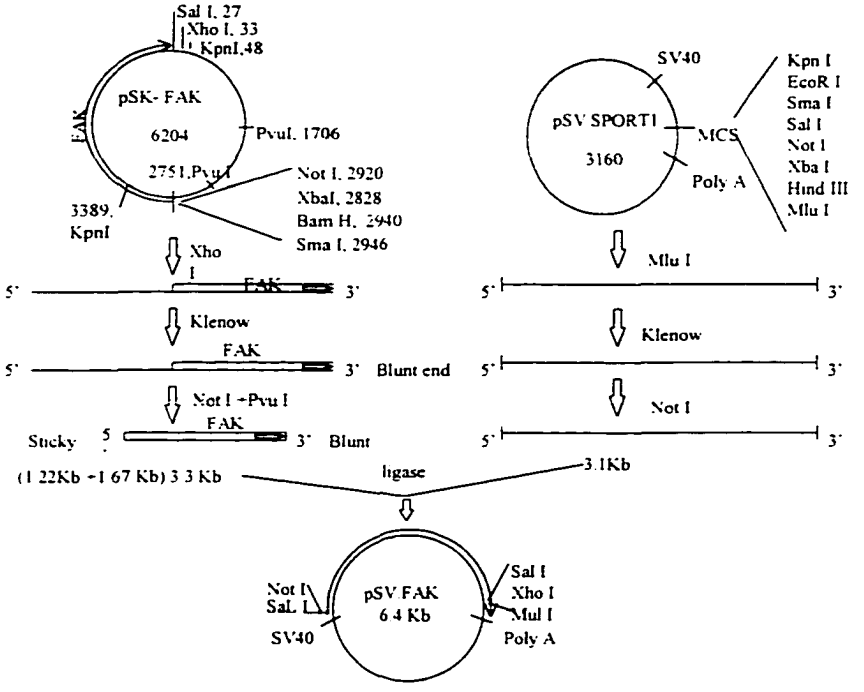

Fig. 2-1 The strategy for construction of sense FAK expression vector. pSK.FAK is cut by Xho I, followed by treatment of Klenow DNA polymerase to generate 3' blunt ends. The FAK inserts are obtained by restriction digestion with Not I which gives rise to 5' sticky ends. Pvu I is added to cut the vector backbone so that the FAK fragment is easier to separate from the vector. FAK fragments are ligated to the multiple cloning site (MCS) of pSV. Sport1 which has a blunt end near the poly A tail (generated by Mlu I digestion, followed by Klenow treatment) and a Not I sticky end near the SV40 promoter. Not I sticky ends favor ligation in the normal orientation.

polymerase to generate 3' blunt ends. The FAK insert was obtained by restriction digestion with Not I which gives rise to 5' sticky ends. Pvu I was added to cut the Bluescript vector so that the FAK fragment was easier to separate from the vector backbone. FAK fragments were then ligated to the multiple cloning site (MCS) of pSV. Sport1 which has a blunt end near the poly A tail (generated by Mlu I digestion, follow by Klenow treatment) and a Not I sticky end near the SV40 promoter. Not I sticky ends favor ligation in the normal orientation. The ligation mix was transformed into competent E. coli DH5α by electroporation. Sense FAK expression clones were screened by restriction digestion with DraI. The strategy to generate the antisense FAK expression vector. pSV.αFAK, is shown in Fig. 2-2. Briefly, pSK.FAK was cut with Sal I and XbaI.

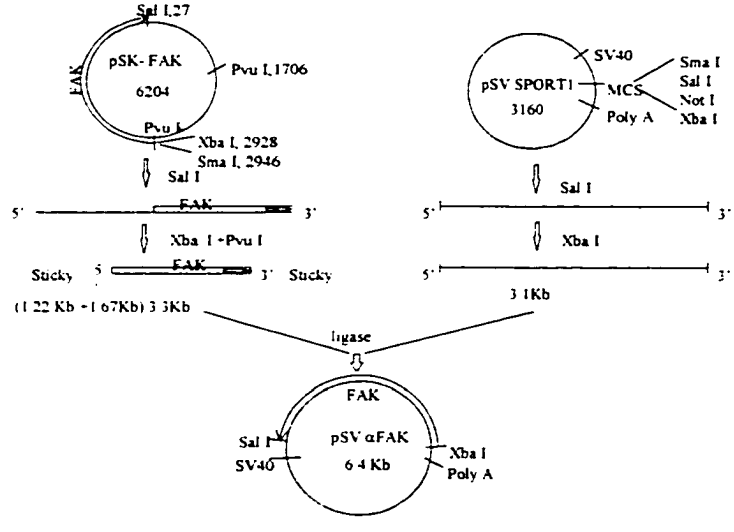

Fig.2-2 Construction of the antisense FAK expression vector. pSK.FAK is cut with Sal I and XbaI. Full length FAK with a 3' SalI sticky end and a 5' Xba I end is ligated to MCS of pSV. Sport1. Since the Sal I site is near the promoter, whereas the XbaI site is near poly A tail, the ligation favors insertion in the the reverse orientation.

Full length FAK with a 3' Sal I sticky end and a 5' Xba I end was ligated to the MCS of pSV.Sport1. Since the Sal I site is near the promoter, whereas the Xba I site is near the

poly A tail, the ligation favors insertion in the reverse orientation. The antisense FAK clone was screened by digestion with Dra I (Fig. 2-2).

Sense and antisense FRNK expression vectors were cloned by EcoR I digestion of pSK FRNK followed by ligation to the EcoR I site in the MCS of pSV.Sport1. pSV. Sport1 was treated with alkaline phosphatase before ligation to prevent self-religation and to improve efficiency. EcoR I released FRNK can be ligated to the EcoR I site in the MCS of pSV.Sport1 in either orientation. The FRNK clones were screened by Hind III

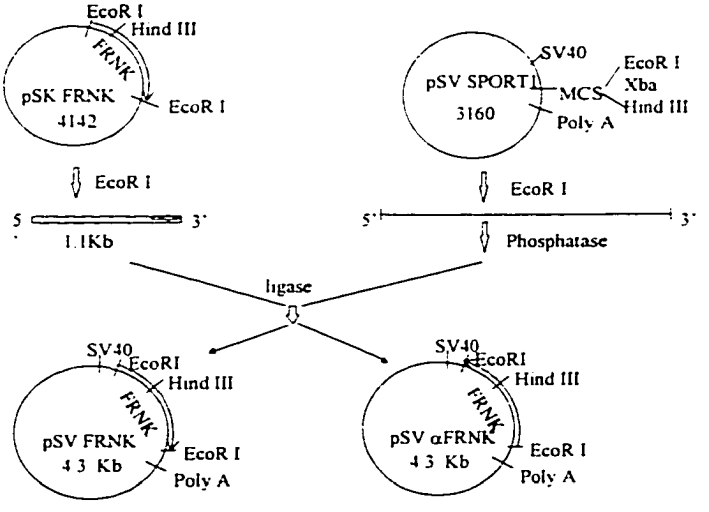

Fig.2-3 Construction of sense FRNK expression vector. Sense FRNK expression vector is cloned by EcoR I digestion of pSK.FRNK followed by ligation to the EcoR I site in MCS of pSV.Sport1. pSV.Sport1 is treated with alkaline phosphatase before ligation to prevent self-ligation and to improve efficiency. EcoR I released FRNK can be ligated to EcoRI site in MCS of pSV.Sport1 in either orientation.

digestion (Fig. 2-3).

2.14 Transfection

Cell transfection was performed with Fugene 6 reagents ((Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. Briefly, 5 x 10^4 cells were plated in six well tissue culture plates and grown overnight in growth medium. On the second day, the medium was replaced with 3 ml of fresh medium and transfection was accomplished by dropwise addition of the transfection mixture onto the cells. Briefly, 10-15 μg of FAK or FRNK plasmid DNA and 0.5-1 μg of pSV2neo plasmid DNA or pSV2neo DNA alone was transfected, using Fugene 6, into recipient cells. Stable transfectants were selected by growing cells in 400 μg/ml of G418 (Gibco BRL, Grand Island, NY). Drug-resistant colonies were picked 15-20 days after transfection and clones expanded in G418 containing medium for subsequent analysis. Cells transfected with pSV2neo alone were selected similarly, single cell clones were expanded and used as controls. Candidate clones were screened to verify FAK or FRNK expression by immunoblotting with BC3 polyclonal antibody which recognizes epitopes in the C-terminus of FAK. Selected clones were assessed for the invasive phenotype including Matrigel invasion, MMP production and cell migration.

CHAPTER III

RESULTS

3.11 Matrigel invasion, cell migration, and secretion of MMP define the invasive phenotype of HNSCC cell lines

a. Matrigel invasion and cell migration.

The mechanisms underlying the invasiveness of head and neck squamous cell carcinoma are poorly understood (3). Previous studies from our laboratory suggested that cell-matrix interactions play an important role in regulation of the invasive phenotype of HNSCCs (4). To further dissect the molecular mechanisms that regulate the invasiveness of HNSCC, we have developed a series of HNSCC cell lines. Our initial goal was to characterize the invasive phenotype of HNSCCs. To do that, we measured Matrigel invasion of HNSCC cell lines by a chemoinvasion assay which measures the capacity of cells to penetrate an in vitro reconstituted basement membrane of Matrigel in response to chemoattractants.

Six HNSCC cell lines were evaluated. HNSCC cell lines derived from carcinoma of tongue (EVSCC14M, EVSCC17M, EVSCC19M) and larynx (EVSCC18) were established at Eastern Virginia Medical School. Additional HNSCC cell lines, UMSCC10A and FaDu, were derived from SCC of the larynx and hypopharynx, respectively. HT1080, a human fibrosarcoma cell line, and A431, a well-characterized

vulvar carcinoma cell line, served as positive controls for invasion. RHEK-1, an SV40 T-antigen immortalized human keratinocyte cell line, and HaCaT, a spontaneousimmortalized human keratinocyte cell line, served as negative controls. HT1080 and A431 were highly invasive in the chemoinvasion assay, whereas, HaCaT and RHEK-1 cells were weakly and moderately invasive, respectively (Fig. 3.1). HNSCC cell lines exhibited a range of invasive potential and were classified as highly invasive (EVSCC14 M, EVSCC17M) or weakly invasive (EVSCC18, EVSCC19M, UMSCC10A, FaDu) (Fig.3-1).

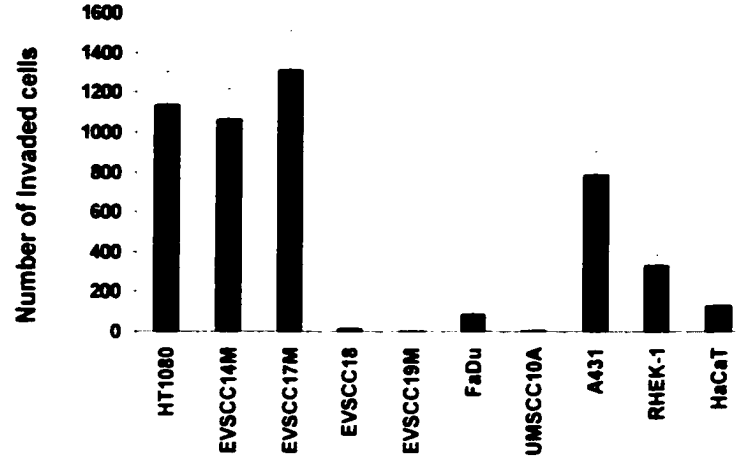

Fig.3-1 Matrigel invasion by HNSCC lines. Invasion assays were conducted with Matrigel-coated 8 μM- pore size polycarbonate membranes in a modified Boyden chamber and was measured after 5 hours of exposure to 3T3 conditioned medium. Cells on the underside of the membrane were stained and counted by a image analyzer. Results are means (Bars, SE) of two independent experiments, with a total of six replicates.

To assess if cell migration contributes to the invasive phenotype of HNSCCs, we examined cell motility of HNSCCs by a chemotaxis assay. Similar to Matrigel invasion, HNSCCs also exhibited different motilities. EVSCC14M and EVSCC17M were highly

motile, whereas EVSCC18, EVSCC19M, UMSCC10A and FaDu were less motile (Fig.3-2).

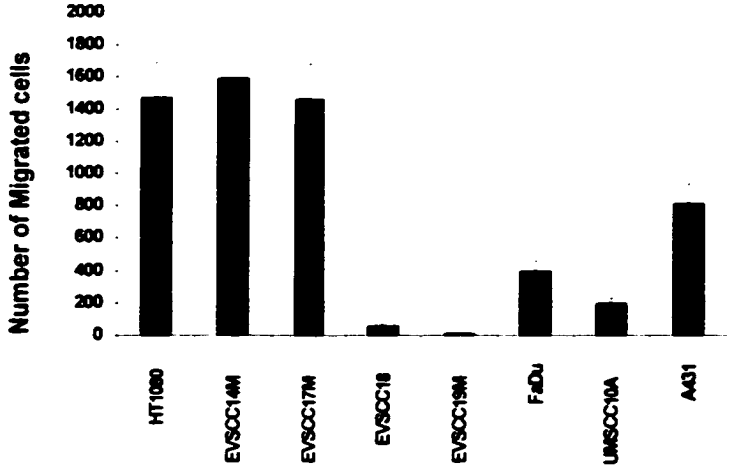

Fig.3-2 Cell migration of HNSCC lines. Migration assay were performed as described for the chemoinvasion assay, except that polycarbonate membranes were coated with diluted gelatin. The results are the means (bars,SE) of two independent experiments.

b. MMP secretion.

Because MMPs play an important role in cancer cell invasion and MMP expression is correlated with the metastatic capacity of various tumors (reviewed in 19), we screened HNSCC cell lines for MMP secretion by gelatin zymography, and quantitated levels of gelatinolytic activity by densitometry as described previously (78). All HNSCC cell lines secreted MMP-2 and/or MMP-9 (Fig3-3). EVSCC19M and UMSCC10A cells secreted increased MMP-9 relative to MMP-2, whereas EVSCC14M and EVSCC18 cells secreted MMP-2 at higher levels than MMP-9; FaDu cells secreted minimal levels of both MMP-2 and MMP-9 (Fig.3-3 and Fig.3-4).

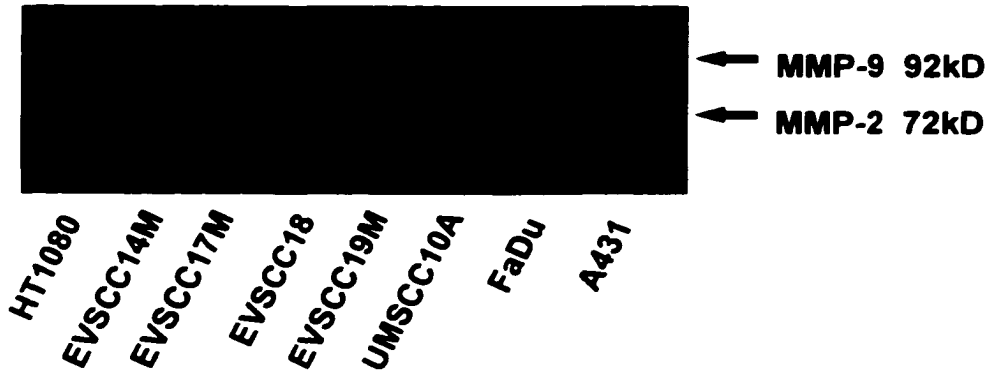

Fig.3-3 MMP production by head and neck cancer cell lines. Cells were incubated in serum free medium for 24 hrs and the conditioned medium (CM) collected. The MMP activity in CM was assessed by gelatin zymography. Shown is a representative zymogram of CM from HNSCC cell lines. Arrows indicate the position of 92 kD MMP-9 and 72kD MMP-2.

Taken together with Matrigel invasion and cell migration, the results indicate an

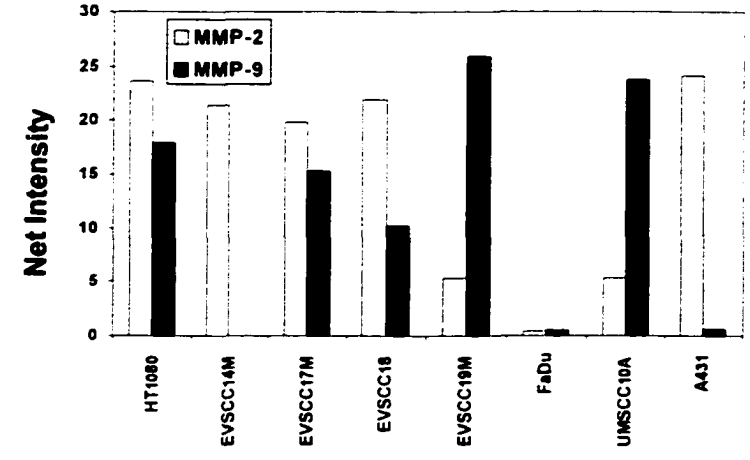

Fig.3-4 Quantitative densitometry of MMP production by HNSCC cell lines. MMP activity of CM was assessed by gelatin zymography and quantitated by densitometric scanning of individual lanes and expressed as net intensity.

association between MMP-2 secretion and invasion (See Summary of HNSCC Phenotypes Table 1, p.54). The correlation was seen most dramatically with highly invasive EVSCC14M and control A431 cell lines which secreted abundant amounts of MMP-2 but barely detectable MMP-9. Conversely, weakly invasive EVSCC19M and UMSCC10A cells secreted enhanced levels of MMP-9 but reduced levels of MMP-2 suggesting that a threshold level of MMP-2 activity may be required for invasion in a 5 hour in vitro invasion assay. EVSCC18 cells were a notable exception to the association between MMP-2 secretion and invasion. However, EVSCC18 cells exhibited limited migration potential despite enhanced MMP-2 secretion (see Fig. 3-4) emphasizing the interdependence of MMP secretion and cell migration in the fully invasive phenotype. Finally, the weakly invasive and migratory phenotype of FaDu cells which secrete barely detectable MMPs support the notion that MMPs play an important role in the invasive potential of HNSCCs.

To confirm the results of MMP production detected by zymography, cell lysates and conditioned media (CM) from the HNSCC lines were analyzed for MMP expression by immunoblotting with MMP-specific antibodies. Since zymography demonstrated that MMP-2 was associated with the invasive phenotype of HNSCCs, we focused on MMP-2 expression in subsequent experiments. In preliminary experiments, we detected minimal MMP-2 by immunobloting of non-concentrated conditioned media from MMP-2 over-expressors, HT1080 and EVSCC14M. Therefore, we used 10-fold concentrated conditioned media for further analyses. We found that MMP levels in the conditioned media detected by immunoblotting (Fig. 3-5, middle panel) correlated in part, but not

exactly, with MMP activity as assessed by zymography (Fig.3-5, upper panel). For example, EVSCC14M, EVSCC17M, EVSCC18, and A431 which exhibited the higher levels of MMP-2 activity by zymography, also showed elevated levels of MMP–2 production detected by western blot analysis. Similarly, EVSCC19M, UMSCC10A and FaDu, which had lower levels of MMP-2 detected by zymography, consistently exhibited reduced MMP-2 protein detected by immunoblotting. In western blot analysis, we also detected lower molecular weight bands (see lane 1 and lane 2, Fig. 3-5) which may

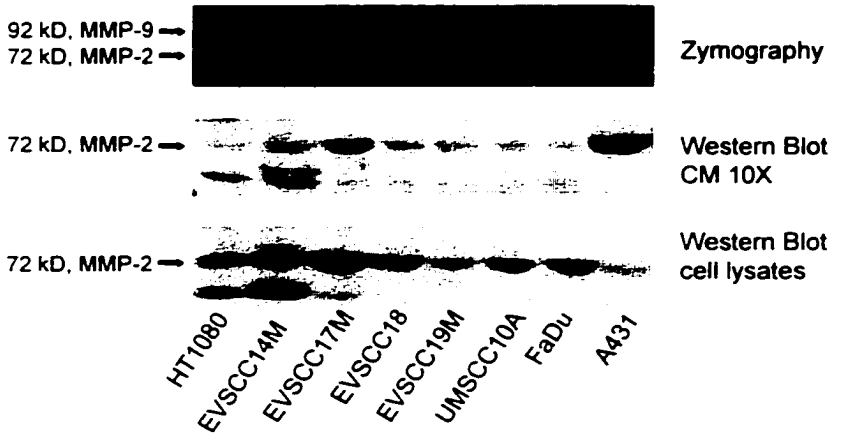

Fig. 3-5 MMP production by HNSCC cell lines: comparison of zymography and immunoblotting. Each experiment has been repeated 2-3 times. Upper panel shows a representative zymogram of CM from HNSCC cell lines. The middle panel is a representative anti MMP-2 western blot of 10x CM from HNSCC cell lines. The lower panel represents the anti-MMP-2 western blot analysis of cell lysates (50 μg protein/lane) of HNSCC cell lines. Arrows indicate the position of 92 kD MMP-9 and 72 kD MMP-2.

represent activated forms of MMP-2. We also analyzed MMP-2 expression in cell lysates of HNSCC cells by western blotting. MMP-2 expression in cell lysates was correlated generally with MMP-2 secretion in most cell lines except FaDu and A431. FaDu exhibited a high level of MMP-2 protein in cell lysates but barely detectable

enzyme activity in CM. In contrast, A431, exhibited minimal MMP-2 protein in cell lysates but showed high levels of MMP-2 enzyme activity in CM (compare Fig.3-5, middle and lower panels). Data presented in Fig 3-5 shows minimal MMP-2 detected by western blotting in CM of HT1080 cells compared to a strong bound of enzymatic activity detected by gelatin zymography. In repeated experiments, MMP-2 levels detected by western blot in CM of HT1080 were comparable to levels detected by zymography.

b. TIMP-2 secretion

We have characterized MMP secretion by HNSCC cell lines and demonstrated that MMP-2 is associated with cells of high invasive potential. Because TIMP-2 forms a specific 1:1 complex with MMP-2 (62), we assessed TIMP-2 expression and secretion by HNSCC cell lines.

To assess TIMP-2 secretion, concentrated conditioned media collected from HNSCC cell lines, was subjected to reverse zymography using EVSCC18 conditioned medium as the source of MMP-2. Conditioned medium from HT-1080 fibrosarcoma cells, known to secrete TIMP-1 and TIMP-2, was used as a positive control.

As expected, two undigested bands (the 27 kD, TIMP-1 and 21 kD, TIMP-2) were detected in HT1080 conditioned media in original gel, but are barely visible in the photographic reproduction of the gel (Fig. 3-6, upper panel, lane 1). CM from HNSCC cell lines exhibited different levels of TIMP-2 activity. EVSCC14M and EVSCC18 showed detectable levels of TIMP-2 activity, whereas the other cell lines showed

minimal to no TIMP-2 activity (Fig.3-6, upper panel). HNSCC cell lines also exhibited different levels of TIMP-1 activity detected as a 27 kD band by reverse zymography. In addition to TIMP-1 and TIMP-2, CM from EVSCC14M and EVSCC18 cells displayed a third band with a molecular weight between TIMP-1 and TIMP-2.

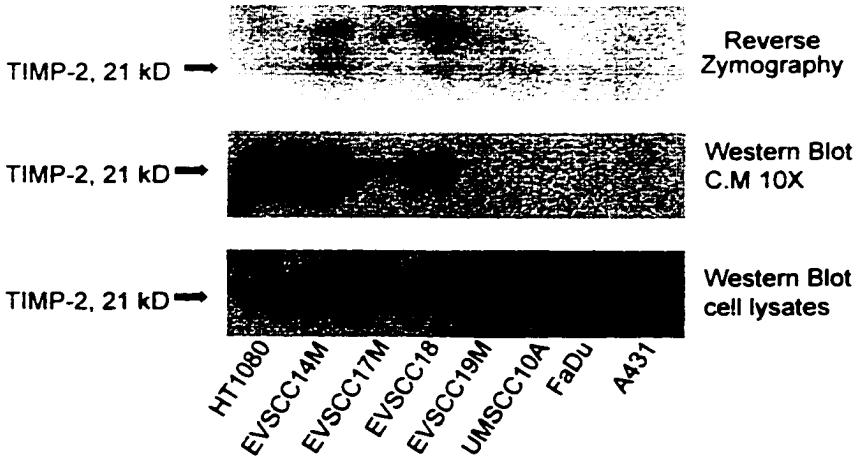

Fig. 3-6 TIMP-2 expression and secretion by HNSCC cell lines. TIMP-2 secretion in CM was analyzed by reverse zymography (upper panel) and confirmed by immunoblotting (middle panel) as described in Materials and methods). TIMp-2 expression in HNSCC cell lines was analyzed by immuno-blotting using cell lysates from HNSCC cell lines. Each is experiment has been repaeted 2-3 times. Shown is a representative data from each experient.

To confirm TIMP-2 detection by reverse zymography, concentrated conditioned media were analyzed by immunoblotting using a monoclonal antibody to TIMP-2. Levels of TIMP-2 detected by immunoblotting correlated closely with levels detected by reverse zymography (Fig.3-6, middle panel) with the exception of HT1080 (lane 1) in this experiment as noted above.

We further assessed TIMP-2 protein expression in HNSCC cell lines by

immunoblotting analysis of cell lysates. TIMP-2 expression was correlated with TIMP-2 secretion in 4 out of 6 cell lines. TIMP-2 detected in cell lysates of FaDu and A431 was not detected by immunoblotting or reverse zymography in CM (compare Fig.3-6, lower panel with middle and upper panels).

d. Cellular adhesion of HNSCC cells to ECM proteins.

Cell adhesion to the ECM, mediated by integrins, is a critical step in the process of invasion. To further characterize the invasive phenotype of HNSCC cells, we assessed the ability of cells to adhere to ECM proteins by an in vitro cell adhesion assay as described in the Materials and Methods section. In this assay, cell adhesion is measured by the protein-binding dye, SRB. In a preliminary experiment, we demonstrated a linear relationship between the number of cells and optical density (490 nm) of SRB staining (Fig.3-7) with a regression coefficient of 0.994 ($p<0.001$). The limit of the SRB assay is

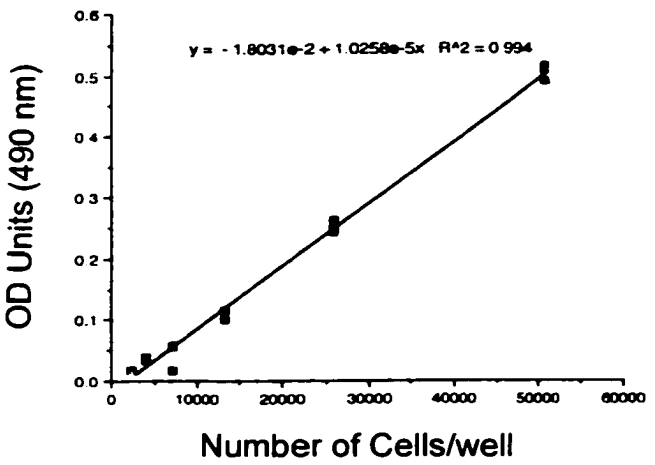

Fig. 3-7 Relationship between number of cells and SRB staining. Cells were plated at densities from 2500 to $5x10^4$ cells per well in a 96 well tissue culture plate, allowed to attach overnight and the SRB assay performed as described in Materials and Methods. Shown is a regression analysis of the data. The regression coefficient is 0.994. $P<0.001$.

between 5000 and 5 x10^4 cells/well (Fig. 3-7). Therefore, the SRB assay was used in subsequent experiments to enumerate the number of adherent cells in adhesion assays.

To determine the optimal dose of ECM proteins for adhesion assays, EVSCC14M and EVSCC17M cells were tested for their ability to attach to 96 well plates coated overnight with 0.1 μg – 100 μg/ml of fibronectin, laminin, or type IV collagen. Plates coated with 100 μg/ml of BSA were used as negative controls. EVSCC14M cell adhesion was potentiated by laminin or fibronectin in a dose dependent manner, whereas adhesion to type IV collagen was not dose-dependent (Fig. 3-8). Fibronectin or laminin (100 μg/ml each) increased EVSCC14M cell attachment approximately 20-fold (Fig. 3-8). Likewise, laminin or fibronectin, but not type IV collagen, potentiated the dose-

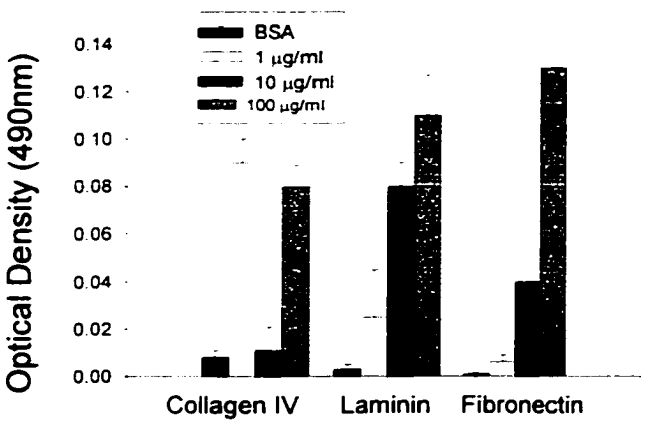

Fig. 3-8 EVSCC14M cell adhesion to ECM proteins. Cell adhesion assay was performed as described in Materials and Methods. The results are the means (bars, SE) of two independent experiments, with a total of six replicates.Cell adhesion to ECM proteins was significantly increased as compared to BSA ($P<0.001$).

dependent adhesion of EVSCC17M cells (Fig.3-9). We noted that EVSCC17M cells exhibited a higher background attachment to wells coated with BSA alone than

EVSCC14M cells. Nevertheless, fibronectin or laminin (100 μg/ml each) increased EVSCC17M cell attachment 5 fold and 3 fold, respectively (Fig. 3-9).

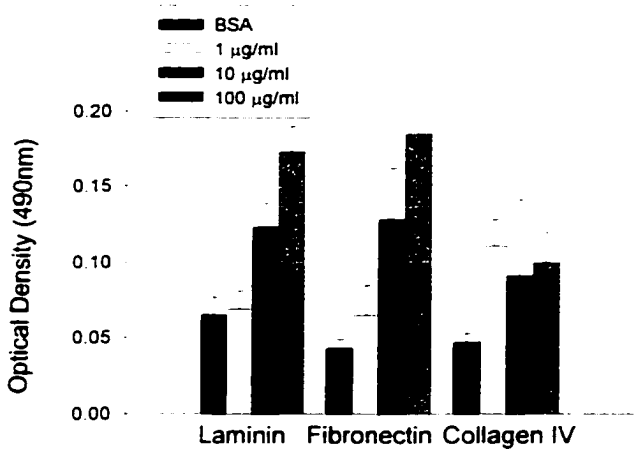

Fig. 3-9 EVSCC17M cell adhesion to ECM proteins. Cell adhesion assay was performed as described in Materials and Methods. The results are the means (bars, SE) of two independent experiments, with a total of six replicates.Cell adhesion to ECM proteins was significantly increased as compared to BSA ($P<0.001$).

Since EVSCC14M and EVSCC17M cell attachment was highest at 100 μg/ml ECM protein doses, we assessed the ability of the other HNSCC cell lines to adhere to plates coated with 100 μg/ml of collagen type IV, fibronectin, laminin, or Matrigel (a combination of type IV collagen, laminin, and fibronectin). HNSCC cell lines adhered poorly to BSA except EVSCC17M (data not shown). HNSCC cells exhibited a differential ability to adhere to ECM proteins. EVSCC14M, 17M 19M, and UMSCC10A were significantly more adhesive than weakly adhesive EVSCC18 and FaDu cells (Fig. 3-10). Cell ahesion to ECM proteins was correlated with Matrigel invasion in 4 of the 6

cell lines (See Table 1 Summary of HNSCC Phenotypes, p.54). Interestingly, cell adhesion to fibronectin was correlated with Matrigel invasion in 5 of 6 cell lines.

Cell adhesion to ECM proteins such as type IV collagen, fibronectin, and laminin is mediated by cell surface integrin receptors. Integrins bind ECM proteins by recognizing specific peptide sequences (30). For example, fibronectin and other ECM

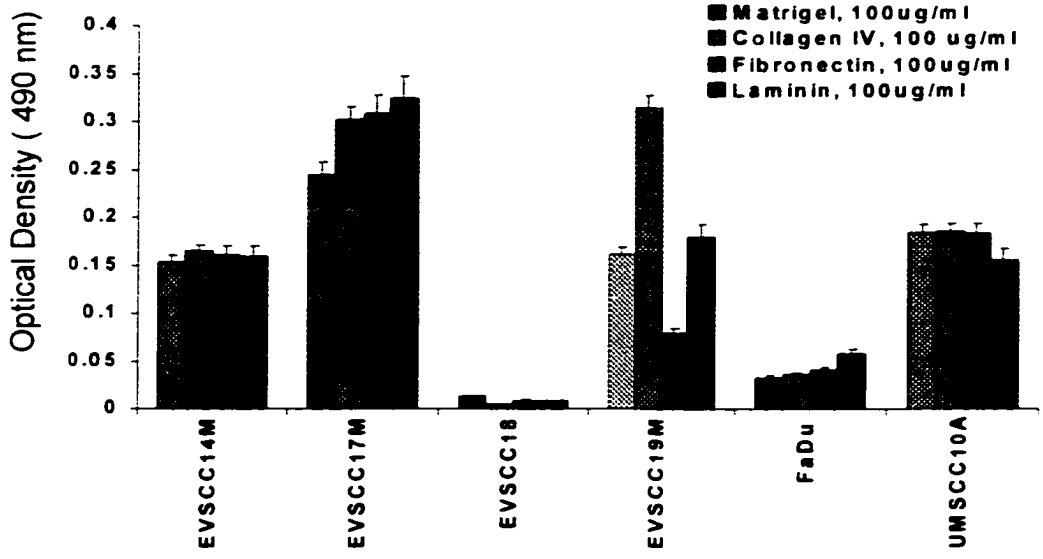

Fig. 3-10 HNSCC cell adhesion to ECM proteins. Cell adhesion assay was performed as described in Materials and Methods. The results are the means (bars, SE) of two independent experiments, with a total of six replicates. EVSCC14M, 17M, 19M and UMSCC10A are more adhesive to ECM proteins than EVSCC18 and FaDu. The difference between two groups was significant ($P<0.001$).

proteins contain arginine-glycine-aspartic acid (RGD) sequences that serve as binding sites for integrin receptors (30). To demonstrate the specificity and biological relevance

of cell adhesion to ECM proteins, cells were treated with a synthetic RGD peptide at various concentrations to test for the capacity to competitively inhibit cell adhesion.

In initial experiments, we tested the efficiency of a synthetic RGD tripeptide to inhibit EVSCC17M cell adhesion to collagen type IV, fibronectin, or laminin (Fig. 3-11). A RGE containing nucleoprotein (NP) peptide derived from influenza virus served as a

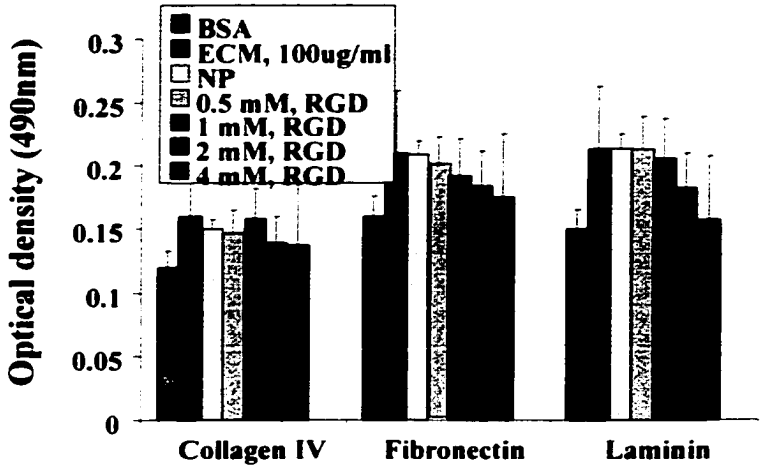

Fig. 3-11 Inhibition of EVSCC17M adhesion to ECM proteins by RGD tripeptide. Cells were preincubated with RGD peptide 30 before the adhesion assay. The adhesion assay was performed in 96 well tissue culture plates coated with 100 μg/ml ECM proteins as described in Material and Methods section. The data represents the mean (bar, SE) of two independent experiments. Statistical analysis showed that the difference between the 4 mM NP peptide and 4mM RGD tripeptide treatment was significant ($p<0.05$).

negative control. NP peptide (4mM) had no effect on EVSCC17M adhesion to ECM proteins. RGD tripeptide inhibited EVSCC17M cell adhesion to fibronectin and laminin in a dose dependent manner. However, the inhibition was not very potent and required high concentrations of RGD (4mM) (Fig.3-11). The RGD tripeptide had no effect on EVSCC17M cell adhesion to Type IV collagen (Fig.3-11). Since cell adhesion to

fibronectin is correlated with the invasive phenotype in most of HNSCC cell lines, we then tested the capacity of a fibronectin-specific recognition RGD peptide (GRGDS) to inhibit EVSCC17M cell adhesion to fibronectin. A RGE peptide (GRGES) was used as a negative control. Peptide GRGDS inhibited EVSCC17M adhesion to fibronectin in a dose-dependent manner, whereas the control RGE peptide had no effect (Fig.3-12). GRGDSP peptides were more potent competitive inhibitors of adhesion to fibronectin than RGDESP tripeptides (compare Fig.3-12 and Fig. 3-11).

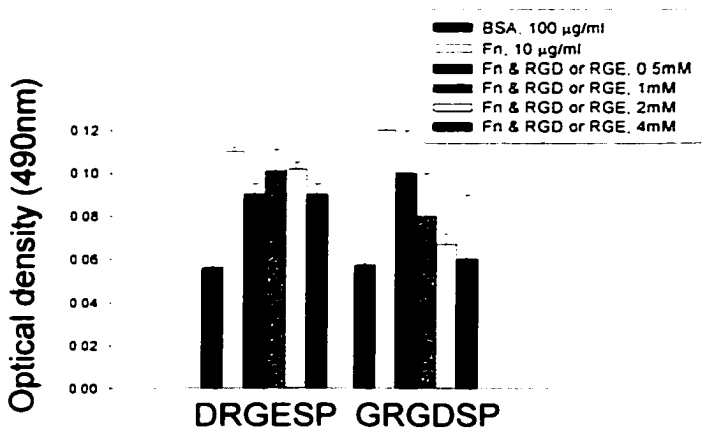

Fig. 3-12 Effect of peptides GRGDSP and GRGESPon EVSCC17M adhesion to fibronectin. Cells were preincubated with peptide 30 minutes before the adhesion assay. The adhesion assay was performed in 96 well tissue culture plates coated with100 µg/ml fibronectin (Fn) as decribed in Material and Methods section. The data represents the mean (bar, SE) of two independent experiments. Statistical analysis showed that difference between RGD and RGE peptide treatment was significant ($p<0.001$).

In summary, we have characterized the invasive phenotype of HNSCC cell lines by evaluating Matrigel invasion, cell migration, cell adhesion, and MMP/TIMP production. (Table 1). HNSCC cell lines showing high invasion and motility (EVSCC14M, EVSCC17M) or weak invasion and reduced motility (EVSCC18,

EVSCC19M, UMSCC10A, FaDu) were identified. In vitro adhesion assays showed a differential adhesion of HNSCC cells to ECM proteins and HNSCC cell adhesion to fibronectin was RGD dependent. Gelatin zymography and western blot analysis

Table 1. Summary of HNSCC Phenotypes

Cell line	Enhanced Matrigel invasion	Enhanced migration	Enhanced MMP-2	Enhanced MMP-9	Enhanced TIMP-2	Enhanced adhesion to ECM proteins	Invasive potential
EVSCC14M	+	+	+	-	+	+	high
EVSCC17M	+	+	+	+	+/-	+	high
EVSCC18	-	-	+	+	+	-	weak
EVSCC19M	-	-	-	+	-	+	weak
UMSCC10A	-	-	-	+	-	+	weak
FaDu	+/-	+/-	-	-	-	-	weak

Key findings: 1) Matrigel invasion and enhanced migration are closely linked.
2) Enhanced MMP-2 secretion, but not MMP-9, is associated with invasive potential.
3) Cell adhesion to ECM proteins may be necessary but is not sufficient for in vitro invasion.
4) TIMP-2 secretion is not associated with Matrigel invasion.

demonstrated a differential secretion of 72kD and/or 92 kD type IV collagenase (MMP-2 and/or MMP-9) by HNSCC cell lines. Matrigel invasion was associated with cell adhesion to ECM proteins in 4 of 6 cell lines and was correlated with MMP-2 secretion in 5 of 6 cell lines. TIMP-2 secretion did not correlate with Matrigel invasion, suggesting that alterations of MMP-2 rather than TIMP-2 are important in generating the invasive phenotype of HNSCCs.

3-2 Determine the degree to which MMP production and migration may be regulated by cell- ECM interactions

a. Determine the role of cell-ECM interaction in regulation of MMP production by HNSCC cell lines.

Cell adhesion to the ECM represents the initial step in the process of tumor invasion. Tumor cell-ECM interaction is hypothesized to stimulate MMP secretion and/or activation resulting in proteolysis of the basement membrane barrier and escape of tumor

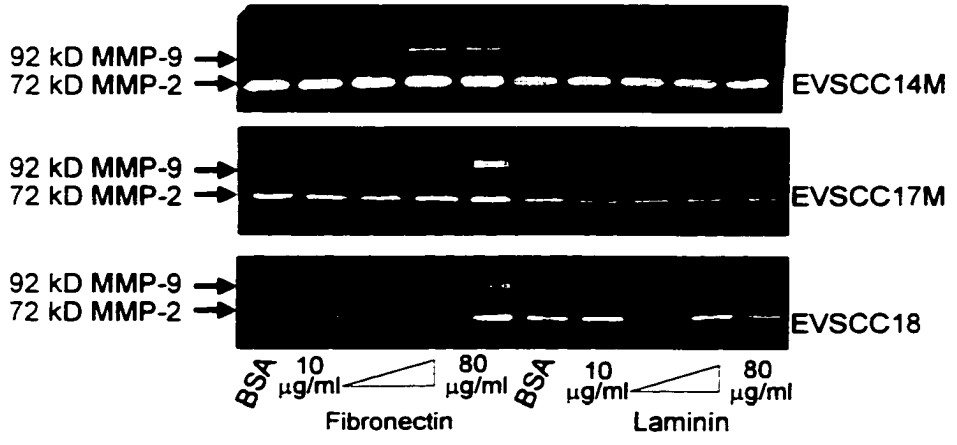

Fig. 3-13 Effects of ECM protein on MMP production by HNSCC cell lines. Cells were treated with 10, 20, 40,or 80 µg/ml of fibronectin or laminin for 24 hours, and MMP activity in CM was assessed by gelatin zymography as described in Materials and Methods. Shown is a representative zymogram of CM from EVSCC17M, EVSCC14M and EVSCC18. The Mr 92,000 and 72,000 MMP activities are indicated (kD).

cells from the primary tumor site. We have shown previously that highly invasive HNSCC cells constitutively produced MMP-2 at high levels (Section 3-1b). However, MMP-2 secretion may be required but not sufficient for acquisition of the invasive phenotype. We hypothesize that cell binding to ECM proteins promotes secretion and activation of latent MMP-2 thus facilitating degradation of the basement membrane.

Highly invasive (EVSCC14M, EVSCC17M) and weakly invasive EVSCC18 cells were used to examine the effect of cell adhesion to ECM proteins on MMP secretion and/or activation. Cells were harvested, resuspended in serum-free growth medium with ITS, plated in 24 well tissue culture plates and adherent cells treated with serum-free media containing 10, 20, 40, or 80 μg/ml of either type IV collagen, fibronectin or laminin for 24 hours. Cells treated with 80 μg/ml of BSA for 24 hours were used as negative

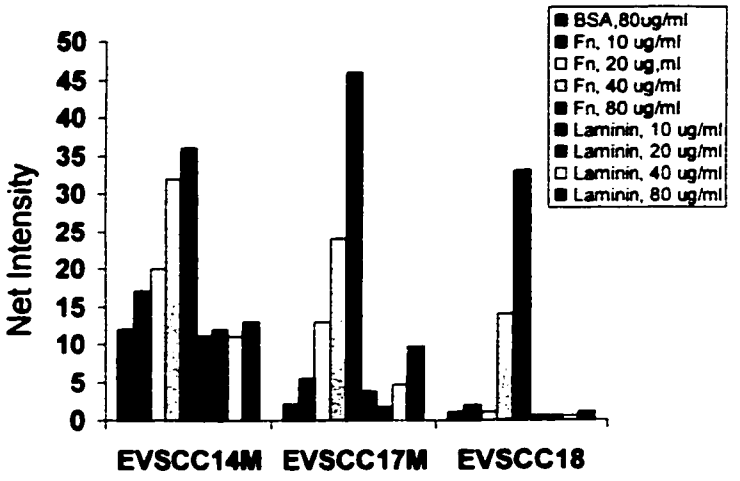

Fig. 3-14 Quantitative densitometry of MMP-9 production following treatment with fibronectin and laminin. Cells were treated with fibronectin (Fn) and laminin at various concentrations for 24 hours, and MMP activity was measured by gelatin zymography. MMP-9 activity was quantitated by Kodak Digital Science 1D™ software program.

controls. CM was collected for gelatin zymography and analyzed for secretion of latent and activated forms of MMP-2/ MMP-9. The results are shown in Fig.3-13 and displayed quantitatively in Fig. 3-14. BSA had no effect on MMP production (data not shown). Fibronectin stimulated MMP-9 production by EVSCC14M and EVSCC17M in a dose-dependent manner. However, MMP-9 secretion by EVSCC18 cell was only seen at the higher doses of fibronectin (Fig. 3-13 and Fig.3-14). Furthermore, higher doses of of

fibronectin (40 μg/ml and 80 μg/ml) resulted in the secretion of activated forms of MMP-9 visualized as a more rapidly migrating band of 84 kD (Fig. 3-13). In contrast to the stimulation of MMP-9 secretion, fibronectin had minimal effects on MMP-2 secretion by all cell lines assessed (Fig. 3-13). Laminin had no effect on MMP-2 production by any of the cell lines but did appear to weakly stimulate MMP-9 secretion by EVSCC17M (Fig. 3-14). Low doses of collagen type IV had no effect on MMP production by HNSCC cell lines (data not shown); however, higher doses of type IV collagen (40 μg/ml) inhibited MMP production. (Fig. 3-15).

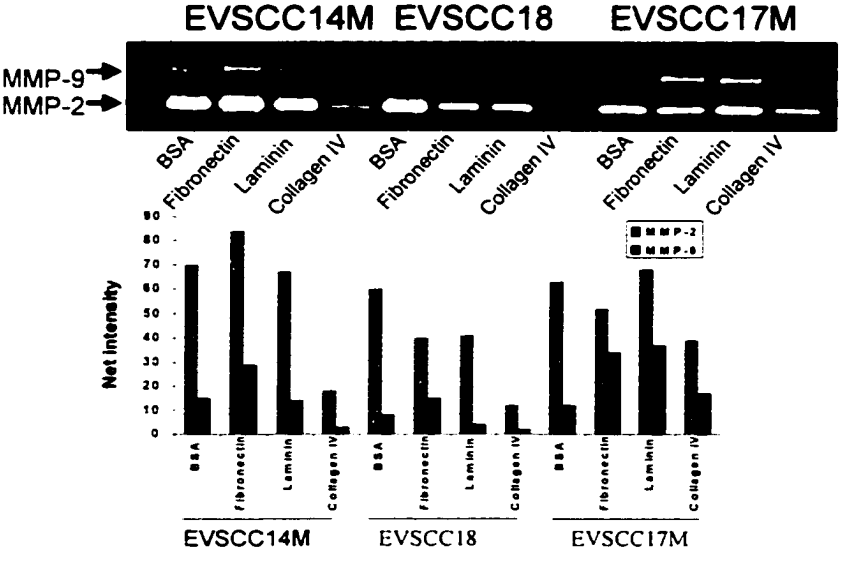

Fig. 3-15 Effect of ECM proteins on MMP production by HNSCC cell lines. Cells were treated with 40 μg/ml of ECM proteins for 24 hours, and MMP activity in CM was assessed by gelatin zymography as described in section 2.2. The upper panel is a representative zymogram. The lower panel is the quantitative densitometric analysis of the zymogram by Kodak digital science program.

b. Regulation of HNSCCs migration by cell-ECM interaction

Cell-ECM interactions not only modulate MMP production but also regulate cell

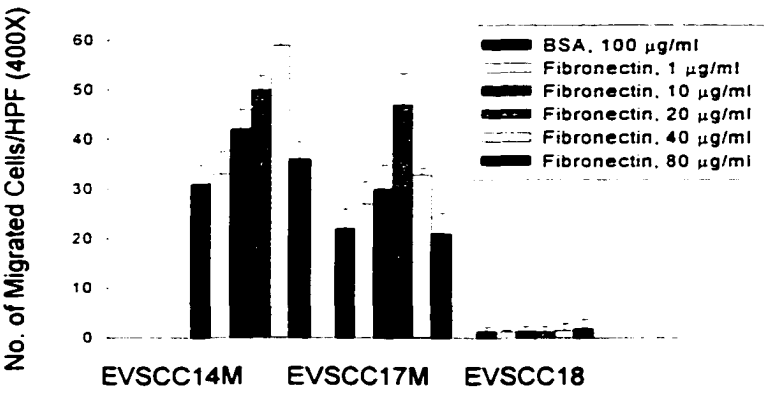

Fig. 3-16 Effect of fibronectin on cell migration of HNSCC cell lines. The migration assays were performed in modified Boyden chambers using various concentrations of fibronectin as chemoattractant as descibed in section 2. The results are mean (bar, SE) number of migrated cells per high power field (400X) from two independent experiments. The difference between cells treated with 10-40 µg/ml of fibronectin versus BSA treatment was significant for EVSCC14M and EVSCC17M ($P<0.001$) but not for EVSCC18 ($P>0.05$).

migration. Several ECM glycoproteins, including vitronectin, fibronectin, laminin, and type IV collagen have been found to stimulate locomotion in different types of tumor

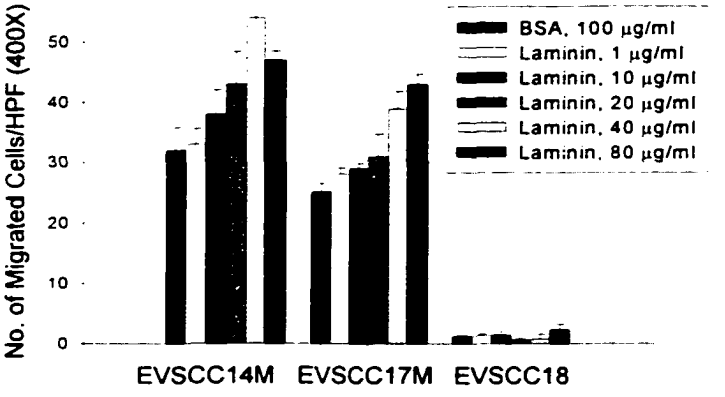

Fig. 3-17 Effect of laminin on cell migration of HNSCC cell lines. The migration assays were performed in modified Boyden chambers using various concentrations of laminin as chemoattractant as descibed insection 2. The results are mean (bar, SE) number of migrated cells per highpower field (400X) from two independent experiments. The difference between cells treated with 20-80 µg/ml laminin versus he BSA treated group was significant for EVSCC14M and EVSCC17M cells ($P<0.001$) but not for EVSCC18 ($P>0.05$).

cells (14-17). To test the hypothesis that ECM proteins might regulate migration of HNSCC cells, we performed cell migration (chemotaxis) assays in the presence or absence of different concentrations of soluble ECM proteins as chemoattractants. The effects of fibronectin, laminin, or collagen type IV on migration of EVSCC14M, EVSCC17M and EVSCC18 cells are shown in Fig. 3-16, 3-17, and 3-18 respectively . Without any chemoattractant (BSA control), EVSCC14M and EVSCC17M cells were highly motile, whereas EVSCC18 cells were poorly motile (P<0.0001). Fibronectin and

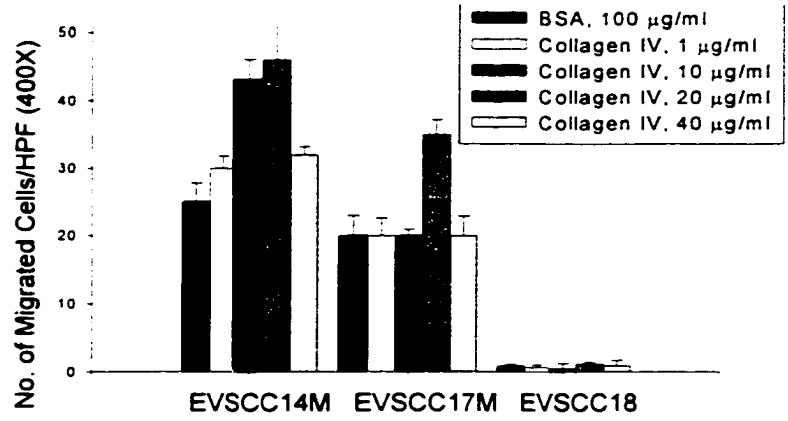

Fig. 3-18 Effect of collagen type IV on cell migration of HNSCC cell lines. The migration assays were performed in modified Boyden chambers using various concentrations of type IV collagen as chemoattractant as descibed in section 2. The results are mean (bar, SE) number of migrated cells per high power field (400X) from two independent experiments. The difference between cells treated with 20 µg/ml collagen type IV versus the BSA control was significant for EVSCC14M and EVSCC17M (P<0.001) but not for EVSCC18 (P>0.05).

laminin stimulated EVSCC14M and EVSCC17M cell migration dose- dependently (P<0.0001). whereas neither protein stimulated EVSCC18 chemotaxis, Low to intermediate doses of fibronectin (1µg/ml to 40µg/ml) stimulated cell migration of

EVSCC17M and EVSCC14M cells, whereas a higher dose (80 μg/ml) was ineffective in stimulating cell migration (Fig.3-16). This biphasic stimulation of cell migration was not observed with laminin (Fig.3-17). Collagen type IV also stimulated cell migration of EVSCC14M and EVSCC17M cells but not EVSCC18 (Fig.3-18). However, although cell migration of EVSCC14M by collagen type IV exhibited a dose-dependent effect, stimulation of EVSCC17M cell migration by collagen type IV was restricted to a dose of 20 μg/ml.

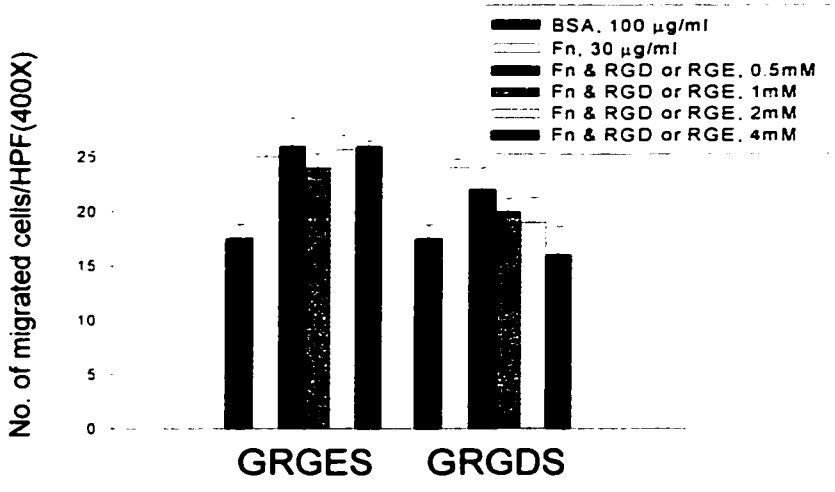

Fig.3-19 Effect of RGD or RGE peptide on EVSCC14M migration in response to fibronectin. Migration assays were performed as described in section 2.1 using 30 μg/ml fibronectin (Fn) as chemoattractant. Cells were pretreated with RGD or RGE peptide at concentrations indicated in figure legend. The results are mean (bar, SE) number of migrated cells per high power field from at least two independent experiments. GRGDS inhibited cell migration dose-dependently. The difference between RGD treatment and RGE treatment to inhibit Fn-stimulated migration was statistically significant ($P<0.001$).

To determine the specificity of cell adhesion to ECM proteins on cell migration, EVSCC14M and EVSCC17M cells were pretreated with RGD peptide, GRGDS, which is predicted to block cell surface integrin binding sites to fibronectin and thus inhibit the signals that promote migration. Pretreatment of cells with the RGE peptide, GRGES,

served as a negative control. The migration assays were performed using 30 μg/ml of fibronectin as the chemoattractant. Cell migration without chemoattractant was used as baseline. As expected, EVSCC14M and EVSCC17M were highly motile in the absence of chemoattractant (Fig. 3-19 and Fig.3-20). Treatment of cells with 30 μg/ml of fibronectin increased EVSCC14M and EVSCC17M cell migration by 1.5-2 fold ($P<0.01$). GRGDS peptide inhibited EVSCC14M and EVSCC17M cell migration dose-dependently ($P<0.001$), whereas GRGES had no inhibitory effect (Fig 3-19 and Fig. 3-20).

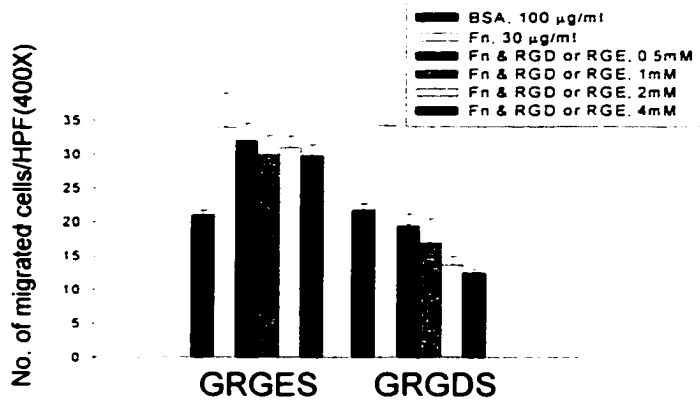

Fig.3-20 Effect of RGD or RGE peptide on EVSCC17M migration in response to fibronectin. Migration assays were performed as described in section 2.1 using 30 μg/ml fibronectin (Fn) as chemoattractant. Cells were pretreated with RGD or RGE peptide at concentrations indicated in figure legend. The results are mean (bar, SE) number of migrated cells per high power filed (400X) from at least two independent experiments. GRGDS inhibited cell migration dose-dependently. The difference between RGD treatment and RGE treatment to inhibit Fn-stimulated migration was statistically significant ($P<0.001$).

3-3. Determine the role of MMPs in invasion of HNSCC

Proteolytic degradation of the ECM is an important step in tumor invasion. The data presented in section 3.1 showed that MMP-2 production was associated with

Matrigel invasion by EVSCC14M cells. Because increased expression of MMPs by malignant tumor cells is believed to play an essential role in invasion, we hypothesized that MMP-2 production was necessary for the invasion of HNSCC cells. To test this hypothesis, we assessed the effect of antibody specific for MMP-2 on the invasive potential of EVSCC14M cells. We reasoned that if MMP-2 production was necessary for, and not simply correlated with, the invasive potential of head and neck tumor cells, then neutralization of MMP-2 activity should block in vitro invasion of EVSCC14M cells.

EVSCC14M Cells were treated with polyclonal antibody to MMP-2 or normal rabbit serum and assessed for Matrigel invasion. Normal rabbit serum had no effect on Matrigel invasion by EVSCC14M. Neutralizing antibody to MMP-2 inhibited EVSCC14M cell invasion dose-dependently. The maximum inhibition was greater than 50% ($P<0.001$) at a dose of 100μg/ml (Fig.3-21).

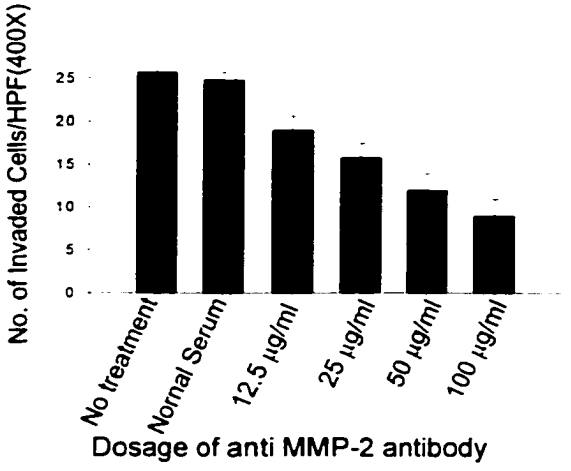

Fig. 3-21 Dose dependent inhibition of Matrigel invasion of EVSCC14M by antibody to MMP-2. Invasion assays were conducted with Matrigel coated 8 μm-pore size polycarbonate membranes in a modified Boyden chamber. Various concentrations of anti-MMP-2 antibody were added to the upper chamber and invasion was measured after 5 hours exposure to 3T3 CM. The results are mean number (bar, SE) of invaded cells per high power field (HPF) with 400X magnification. Antibody significantly inhibited matrigel invasion by EVSCC14M ($P<0.001$).

3-4. Calcium-dependent signaling and the invasive phenotype of HNSCCs

Accumulating data have demonstrated that integrin-mediated cell-ECM interactions are important in the regulation of a variety of cell functions including cell migration and MMP production (49,82). A common regulatory point in several integrin signaling pathways is intracellular calcium homeostasis. Calcium represents a signaling molecule essential in many signal transduction pathways and is known to regulate a number of biochemical processes, including cell proliferation, invasion, angiogenesis,

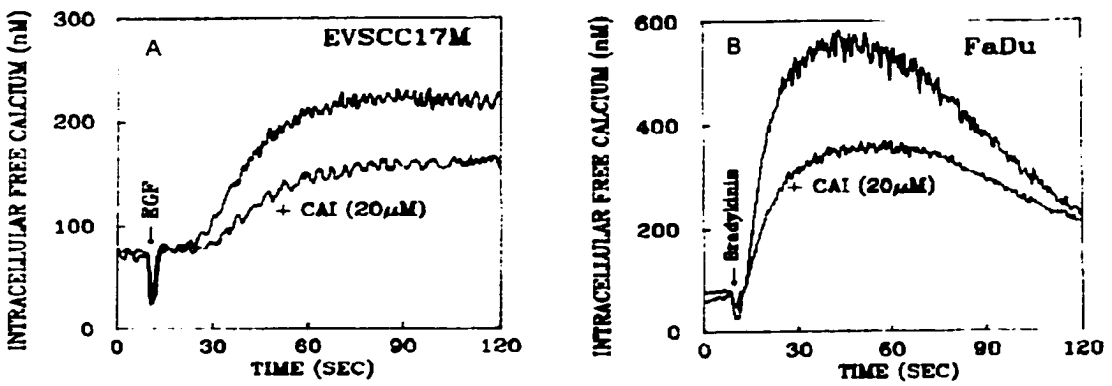

Fig. 3-22 Effect of CAI on agonist-induced elevation of $[Ca^{2+}]$ in EVSCC17M and FaDu cells. EVSCC17M and FaDu cells were loaded with Fura-2 and preincubated with 20 uM CAI for 30 min before agonist [EGF(200ng/ml) and bradykinin (1 μM)] addition, indicated by arrow. Untreated cells were incubated with an equivalent amount of DMSO vehicle.

and secretion (125). To investigate if calcium-dependent signaling pathways were important in the biology of HNSCC, we made use of a novel inhibitor of calcium influx,

carboxyamido-triazole (CAI), that selectively blocks calcium-mediated signal transduction events (126). Previous reports have shown that CAI inhibited the proliferation and invasive behavior of malignant cells in vitro and in animal tumor models (119,121,127). However, the effect of CAI on the proliferation and invasiveness of head and neck tumor cells was not known. We first evaluated the effect of CAI on calcium influx in EVSCC17M and FaDu cells treated with two general calcium agonists, epidermal growth factor (EGF) and bradykinin. Using cells loaded with the calcium fluoroprobe Fura-2, we observed a rapid elevation of intracellular calcium levels in

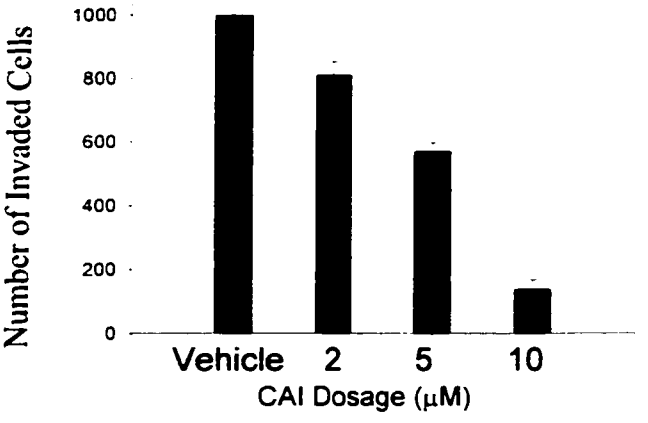

Fig. 3-23 Dose-dependent effect of CAI on Matrigel invasion by EVSCC14M. Cells were treated with different dosages of CAI and Matrigel invasion was performed as described in "Materials and Methods". The results are the mean (bar, SE) of two independent experiments with six replicas. The difference between CAI treated and untreated group was statistically significant ($P<0.001$).

EVSCC17M and FaDu cells treated with EGF and bradykinin, respectively (Fig.3-22). The EGF effect on calcium in EVSCC17M cells was dose-dependent and was attenuated >70% by EGTA in the assay buffer, indicating a significant calcium influx component involved with calcium mobilization by EGF in HNSCC cells (data not shown).

Preincubation with 20 μM CAI markedly attenuated calcium elevation in both cell lines (Fig. 3-22 A and B). Similar results were obtained with other agonists (data not shown). confirming that CAI blocks agonist-induced calcium influx in HNSCC cells. We also examined the dose dependency of CAI to attenuate bradykinin induced elevations of intracellular free calcium. CAI at a concentration of 1 μM produced a 10% inhibition. whereas 10 μM CAI produced a maximum 50% inhibition (data not shown). Increasing the concentration of CAI to 20 μM and 50 μM did not produce any further inhibition.

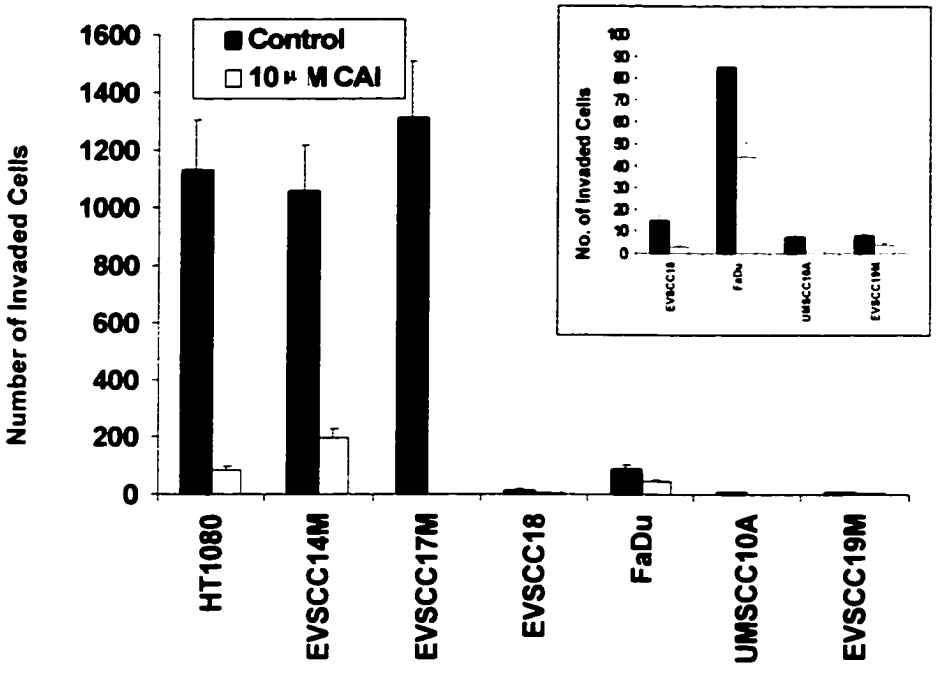

Fig.3-24 Effect of CAI on invasive potential of HNSCC cell lines. Invasion assays were conducted with Matrigel-coated 8 um pore size polycarbonate membranes in a modified Boyden chamber. Cells were exposed to medium without or with 10 uM CAI for 18 hours prior to and during the assay. Invasion was measured after 5 hr exposure to 3T3 conditioned medium. Cells on the underside of the membrane were stained and counted by an image analyzer. Results are the mean +/- SE of 2 independent experiments with total of 6 replicates. *Inset*: expanded scale of HNSCC cells with weak invasive potential. Mixed model of Nested ANOVA was performed with assistance of Instat2 software. Differences between the high and low invasive cell lines are significant ($p<0.05$). Treatment of CAI significantly reduced the invasive potential of all cell lines ($P<0.05$) except EVSCC19M.

We then tested the effects of CAI on Matrigel invasion, cell migration, and MMP production by HNSCC cell lines. In preliminary experiments, we determined that the effects of CAI on parameters of invasion were dose-dependent with half-maximal inhibitory concentrations (IC50s) between 3 and 10 uM (Fig.3-23) and used 10 uM CAI

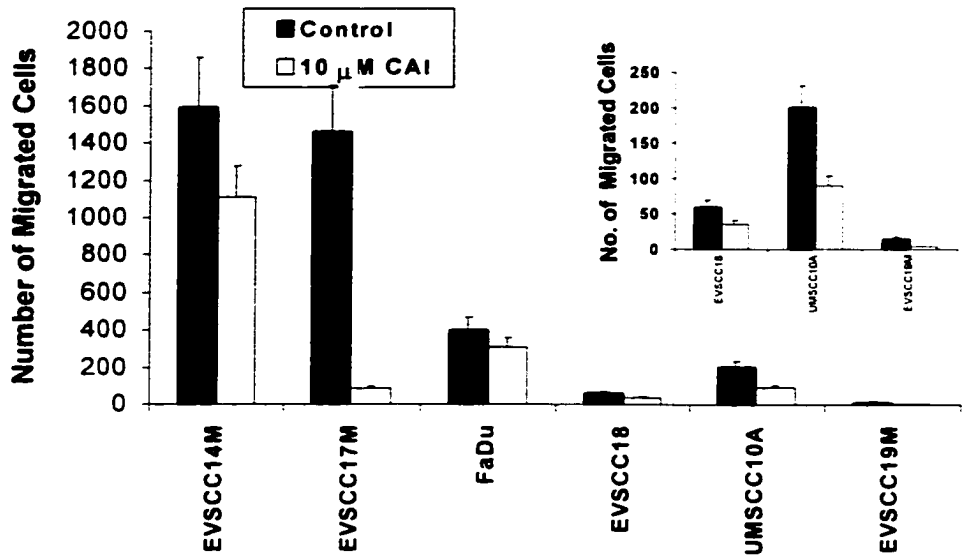

Fig.3-25 Effect of CAI on migration of HNSCC cell lines. Migration assays were performed as described for the chemoinvasive assay, except that polycarbonate membranes were coated with gelatin. The results are the means (bars, SE) of two independent experiments. Inset, expanded scale of HNSCC cells with reduced motility. Statistical analysis demonstrated that CAI significantly inhibited cell migration of cell lines ($P<0.05$).

in subsequent experiments. Treatment of HNSCC cells with CAI for 24 hours blocked Matrigel invasion and cell migration 2-14 fold (Fig. 3-24 & 3-25) and inhibited MMP-2 production 3-7 fold (Fig. 3-26 & 3-27) relative to untreated control cells. CAI inhibited

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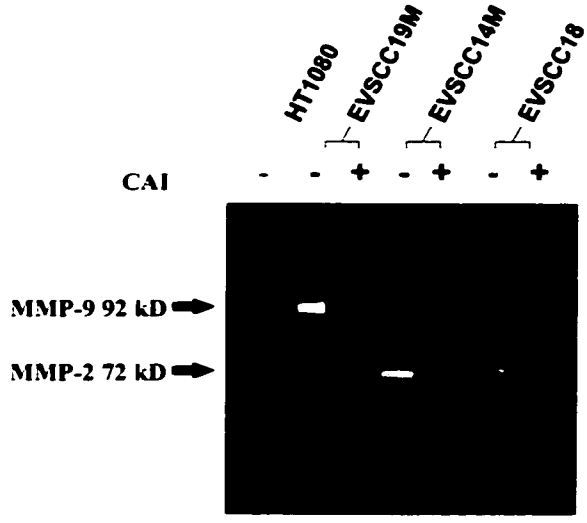

Fig.3-26 Effect of CAI on MMP production by HNSCC cells. Cells were treated without(-) or with (+) 10 μM CAI for 24 h, and MMP activity in CM was assessed by gelatin zymography. Shown is a representative zymogram of CM from EVSCC19M,EVSCC14M and EVSCC18. CM from HT1080 was used as a positive control for MMP-9 and MMP-2 activity. The M_r 92,000 and 72,000 MMP activities are indicated (kD).

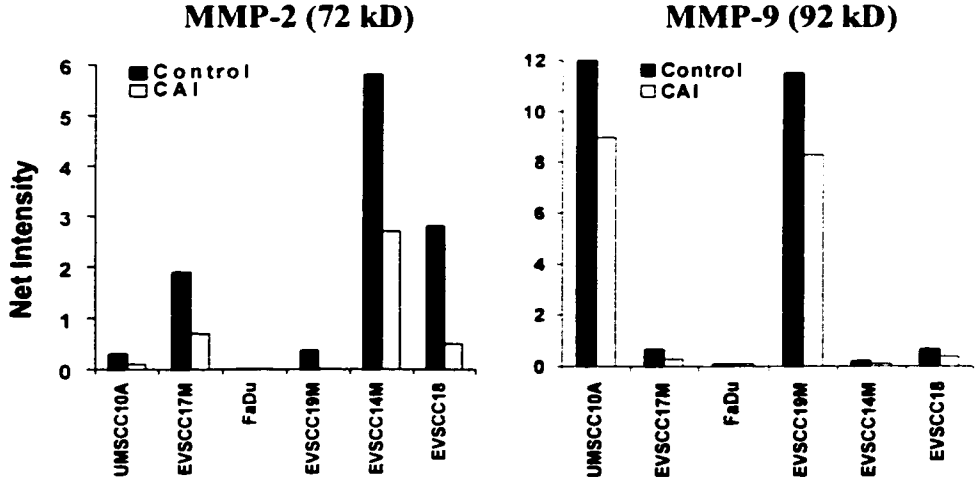

Fig. 3-27 Quantitative densitometry of MMP production by HNSCC cell lines. Cells were treated without or with 10uM CAI for 24 hr and MMP activity of conditioned medium assessed by gelatin zymography as described in " Materials and Methods". MMP activity was quantitated by densitometric scanning of individual lanes and expressed as net intensity.

MMP-9 secretion less effectively (~1.5-fold reduction). Collectively, the data demonstrate that calcium signaling is an important determinant of the invasive phenotype.

3-5 The role of focal adhesion kinase in the regulation of MMP production and expression of the invasive phenotype of HNSCCs

We have demonstrated that inhibition of calcium influx inhibited the invasive potential of HNSCCs in vitro (78). Work of others has shown that inhibition of calcium influx blocked FAK phosphorylation and kinase activity (89). Since activation of FAK has been functionally linked to the focal adhesion site and generation of signals for motility and invasion (80), we assessed the role of FAK in regulation of MMP production and expression of the invasive phenotype of HNSCCs. To achieve this goal, we first assessed expression and phosphorylation of FAK in HNSCC cell lines and then further dissected the role of FAK in generation of the invasive phenotype by transfection studies.

a. Expression and phosphorylation of FAK and invasiveness of HNSCCs

To assess FAK expression and phosphorylation, lysates from three moderately to highly invasive HNSCC lines (EVSCC14M, EVSCC17M, A431) and two weakly invasive cell lines (EVSCC18, FaDu) were immunoprecipitated with anti-FAK antibody. Since FAK is a natural substrate of src tyrosine kinase, lysates from Rat1 transfectants overexpressing src (Rat1 src cells) served as a positive control. FAK immunoprecipitates

were blotted with anti FAK antibody to assess FAK protein levels or antiphosphotyrosine antibody (RC20) to assess FAK phosphorylation. Rat1 Src cells constitutively expressed low levels of FAK, which was highly phosphorylated (Fig.3-28).

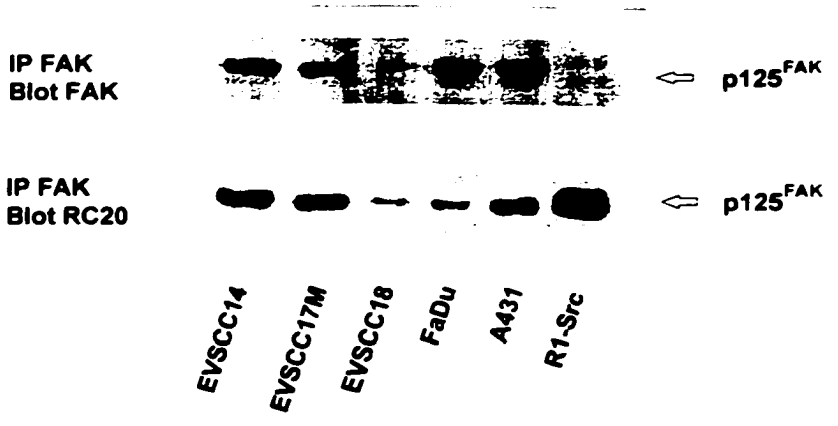

Fig.3-28 Immunodetection of $p125^{FAK}$ and phosphorylation of $p125^{FAK}$ in HNSCC cell lines. Cell lysates (800 μg protein) from near confluent HNSCC and control cell lines were immunoprecipitated (IP) with anti-FAK antibody and western blots probed with anti-FAK antibody or anti-phosphotyrosine antibody RC20). Shown is an example of an immunoblot demonstrating expression of $p125^{FAK}$ (upper panel) and the basal level of activated FAK (lower panel) in HNSCC cell lines. R1-Src are rat1 cells transformed by v-src.

Basal levels of FAK expression varied ~3-fold between the cell lines (Fig. 3-28) with no apparent correlation with the invasive phenotype. Interestingly, levels of phosphorylated FAK were correlated with invasion and cell migration. By densitometric scanning, the moderately to highly invasive cell lines (EVSCC14M, EVSCC17M, A431) exhibited 2 to 4-fold more phosphorylated FAK than the weakly invasive EVSCC18 or FaDu cells (Fig. 3-29). The findings support a role for FAK activation in regulation of cell migration and invasion.

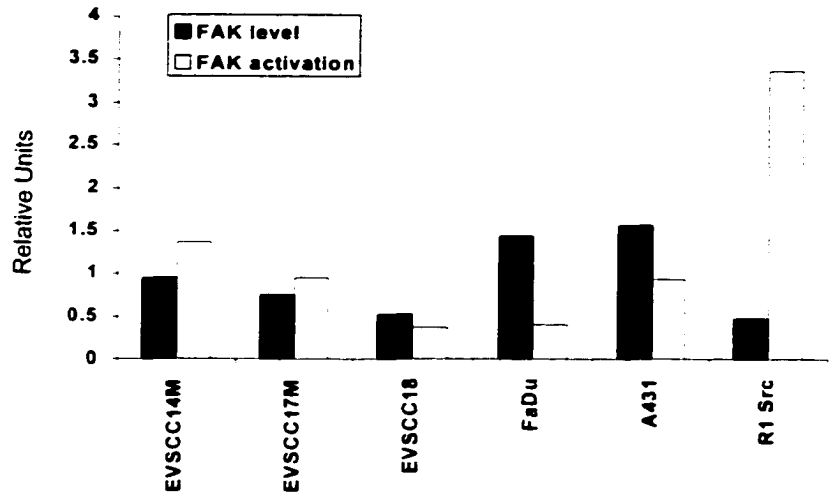

Fig.3-29 Quantitative densitometry of FAK expression and basal levels of phosphorylation of FAK in HNSCC cell lines.

b. Role of FAK in expression of the invasive phenotype of HNSCCs by modulation of FAK expression and phosphorylation

We demonstrated that levels of phosphorylated FAK were correlated with cell migration and Matrigel invasion suggesting that FAK signaling may be involved in expression of the invasive phenotype of HNSCCs. To further dissect the role of FAK signaling in generating the invasive phenotype of HNSCC, sense or antisense FAK cDNA as well as the carboxy-terminal isoform, FAK-related non-kinase (FRNK) were expressed by stable transfection of selected HNSCC cell lines. Modulation of FAK or FRNK expression was confirmed by immunoblotting and transfectants assessed for invasive potential.

1. Generation of FAK and FRNK expression plasmids

FAK and FRNK cDNA from Bluescript phagemids were cloned in pSV.Sport1 in both the sense and antisense orientation. Sense and antisense FAK expression vectors were verified by restriction digestion with DraI. As predicted, restriction analysis of sense

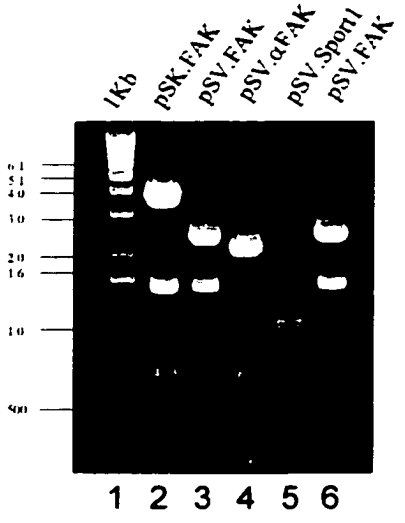

Fig. 3-30 Restriction analysis of sense and antisense FAK expression vectors. Sense and antisense expression vectors were digested with Dra I. pSK.FAK and pSV. Sport1 were used as controls. Lane1 is 1 Kb DNA marker Lane 2 is pSK.FAK, Lane 3 and Lane 6 is pSV. FAK. Lane 4 is the antisense FAK expression vector pSV αFAK. Lane 5 is pSV Sport1.

FAK expression vector with Dra I generated 2899, 1430, 1420, and 692 bp fragments, whereas restriction digestion of the antisense FAK with the same enzyme resulted in 2634, 962, and 247 bp fragments (Fig. 3-30). Sense and antisense FRNK expression plasmids were verified by restriction digestion with Hind III. Restriction digestion of

sense FRNK with Hind III generated 3.98 kb and 280 bp fragments, whereas restriction digestion of antisense FRNK resulted in 3.3 kb and 904 bp fragments (Fig.3-31).

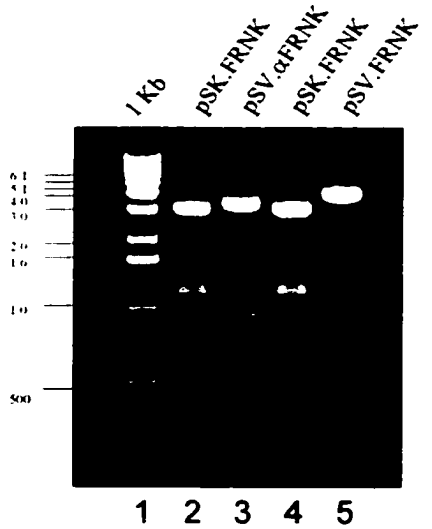

Fig. 3-31 Restriction analysis of sense and antisense FRNK constructs. pSV. FRNK and pSV.α FRNK were digested with Hind III. PSK.FRNK was used as control. Lane1 is 1 Kb DNA molecular marker. Lane 2 and Lane 4 is pSK. FRNK cDNA. Lane 3 is the antisense FRNK expression vector. Lane 5 is sense FRNK expression vector.

II. *Transfection, selection and verification of FAK or FRNK expression and phosphorylation by immunoblotting and immunoprecipitation*

Weakly invasive cell lines with low levels of FAK expression (EVSCC18) or highly invasive lines with elevated levels of FAK expression (EVSCC14M, EVSCC17M) were used for DNA transfection (Table 2). Because polybrene had been used successfully in our laboratory to introduce the EMS1 gene into 3T3 cells, initial experiments concentrated on cotransfection of sense FRNK with pSV2.neo into EVSCC14M cells with polybrene. After three attempts with this method using various ratios of pSV2.neo

Table 2: Invasive and FAK phenoype of cell lines used for transfection

	EVSCC14M	EVSCC17M	EVSCC18
Matrigel invasion	High	High	Weak
Enhanced motility	+	+	-
Elevated FAK level	+	+	-
Enhanced FAK phosphorylation	+	+	-

to pSV.FRNK. 38 colonies were obtained following G418 selection and clonal expansion. None of the G418 resistant clones overexpressed FRNK protein assessed in cell extracts by western blot analysis with FAK antibody BC3, a polyclonal antibody recognizing a carboxyl terminal FAK epitope (Table 3). Because the transfection efficiency by polybrene was low (<1%) for HNSCC cells, we evaluated a number of different methods and reagents in an effort to achieve optimal transfection efficiency. Techniques assessed for transfection efficiency included polybrene, calcium phosphate precipitation, liposome-mediated transfection (Tfx^{TM}, Promega, Madison, WI), Superfect (Qiagen, Valencia, CA), and FuGene 6 (Boehringer Mannheim, Indianapolis, IN). Using these transfection reagents and protocols provided by each manufacturer, we transfected a β−gal plasmid or pSV2.neo plasmid into HNSCC cell lines to assess transient and stable transfection efficiency, respectively. By this comparative analysis, we found that FuGene 6 reagent gave the highest transfection efficiency for most cells tested (data not shown). Therefore, we used the FuGene 6 reagent for subsequent DNA transfections. Cotransfection of sense FRNK with pSV2.neo into EVSCC14M or EVSCC17M cells

resulted in 18 and 13 stable transfectants, respectively. Immunoblotting analysis identified 3 of 18 EVSCC14M transfectants that overexpressed FRNK protein (Table3;

Table 3: Summary of transfection experiments

Cell lines expressor	Ratio of Gene/neo	Transfection Reagent	No. of Neo^R Colonies	Positive Clones *
EVSCC14M	FRNK/neo (10-30:1)	Polybrene/ DMSO	38	0
	FRNK/neo (10:1)	FuGene 6	18	3
	αFAK[2]/neo (10:1)	FuGene 6	8	2
EVSCC17M	FRNK/neo (20:1)	FuGene 6	13	6
	αFAK[2]/neo (20:1)	FuGene 6	18	2
EVSCC18	FAK/neo (20:1)	FuGene 6	11	5

* Assessed by immunoblotting with FAK antibody BC3.
2 αFAK: antisense FAK

Fig. 3-32) and 6 of 13 EVSCC17M transfectants that overexpressed FRNK (Table 3). Sense FAK and pSV2.neo cotransfection of EVSCC18 cells generated 11 stable transfectants, of which 5 over-expressed FAK protein assessed by immunoblotting with FAK antibody (Table 3, Fig. 3-39). A similar approach was used to introduce antisense FAK into EVSCC14M and/or EVSCC17M, two HNSCC cell lines that exhibit high levels of FAK expression and phosphorylation and are highly invasive (Table 2). Two clones in each group were identified that expressed decreased levels of FAK (30% to 50% reduction as compared to neo control (Table 3). EVSCC14M (antisense FAK)

and EVSCC17M (antisense FAK) transfectants were not studied further.

III. Assess the invasive phenotype of FAK and FRNK transfectants.

1. Invasive phenotype of FRNK transfectants

Data presented in section 3.5a demonstrated that FAK phosphorylation was associated with the invasive phenotype of HNSCC cell lines, suggesting that FAK activation may contribute to the invasive potential of HNSCCs. To further investigate the role of FAK activation in regulation of tumor invasion, we overexpressed FRNK in

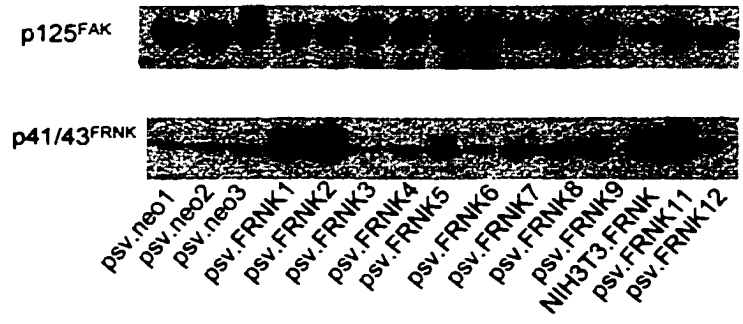

Fig.3-32 Overexpression of FRNK in EVSCC14M cells by transfection. FRNK expression plasmids were transfected into EVSCC14M as described in the Materials and Methods section. pSV.neo alone was transfected into EVSCC14M cells as a negative control. NIH3T3 cells transiently transfected with FRNK served as a positive control. Cell lysates were analyzed by immunoblotting with FAK antibody BC3 for FAK and FRNK expression. 125 kD FAK and 41/43 kD FRNK are indicated.

EVSCC14M by transfection in an effort to inhibit FAK phosphorylation. Western blot analysis showed that three FRNK clones (FRNK1, FRNK2, FRNK11) overexpressed

FRNK by 5-7 fold as compared to neo control clones, whereas FAK level was not altered by FRNK transfection (Fig.3- 32).

To assess the effect of FRNK expression on FAK phosphorylation in EVSCC14M FRNK transfectants, lysates from two clones pSV.FRNK1 and pSV.FRNK2 were immunoprecipitated with BC3 antibody and probed with BC3 for FAK and FRNK

protein expression, and RC20 antibody for FAK phosphorylation. EVSCC14M pSV.neo1 and pSVneo2 clones served as controls. As expected, FRNK levels were 3-4 times higher

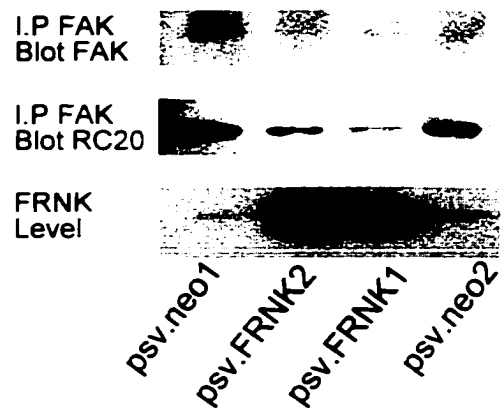

Fig. 3-33 Effect of FRNK expression on FAK phosphorylation in EVSCC14M FRNK transfectants and controls. Cell lysates from FRNK or neo control clones were immunoprecipitated with BC3 antibody. The immunocomplexes were probed with BC3 for FAK and FRNK expression and probed with RC20 antiphosphotyrosine antibody for FAK phosphorylation.

in pSV.FRNK1 and FRNK2 transfectants as compared to neo1 and neo2 controls (Fig. 3-33, lower panel). Expression of FRNK slightly reduced FAK expression but inhibited

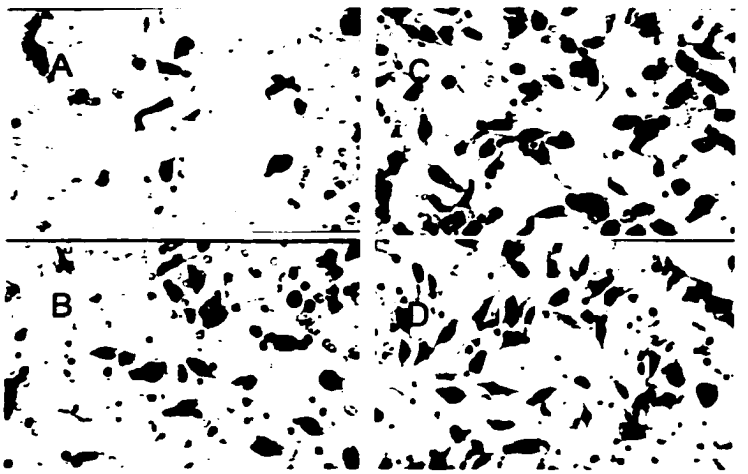

Fig. 3-34. Photomicrography of Matrigel invasion by EVSCC14M FRNK transfectants and controls. Chemoinvasion assays were performed in modified Boyden chambers as described in section2.1. Photograph was taken under 400 X magnification. A. pSV2.neo1 14M. B. pSV2.neo2.14M. C. pSV.FRNK1.14M. D. pSV.FRNK2.14M

FAK phosphorylation greater than two fold (Fig3-33, middle panel). To characterize the

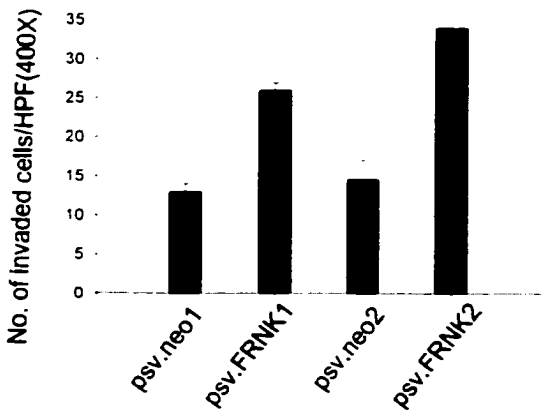

Fig.3-35 Matrigel invasion by EVSCC14M FRNK transfectants and controls. Invasion assays were conducted with Matrigel-coated 8 μM- pore size polycarbonate membranes in a modified Boyden chamber and was measured after 5 hours of exposure to 3T3 conditioned medium.Cells on the underside of the membrane were stained and counted manually with 400X magnification. Data are represented as the number of invaded cells per high power field (HPF). Results are means (Bars, SE) of two independent experiments, with a total of six replicates. Statistical analysis showed a significant difference in invasion between neo controls and FRNK transfectants ($P<0.05$).

invasive phenotype of EVSCC14M FRNK transfectants, Matrigel invasion assays were performed using 3T3 CM as chemoattractant. Unexpectedly, FRNK overexpression increased the invasive potential of EVSCC14M approximately 50% as compared to neo controls ($p<0.05$) (Fig. 3-34 and Fig.3-35) but did not change overall cell morphology. Since MMP production is an important step in tumor invasion, we assessed MMP production by EVSCC14M FRNK transfectants by gelatin zymography. FRNK overexpression had very little effect on MMP-2 secretion, but promoted MMP-9 production by 20-40% (Fig.3-36).

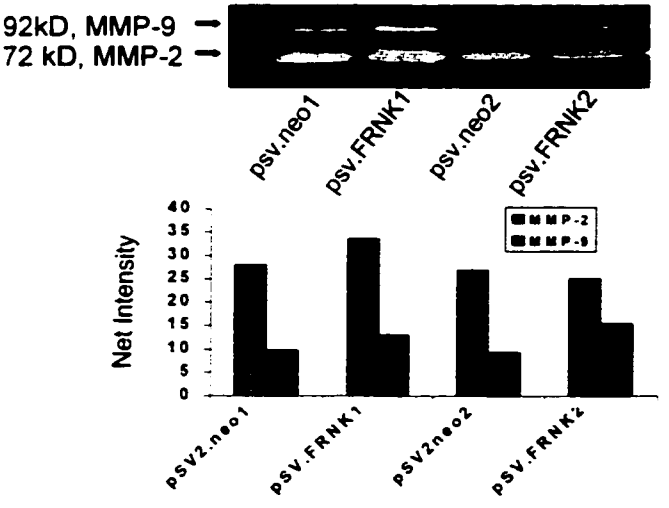

Fig. 3-36 MMP production by EVSCC14M FRNK transfectants and controls. Cells were incubated with serum-free ITS medium for 24 hours, CM were collected and MMP activity in CM was assessed by gelatin zymography as described in section 2.2. The upper panel is a representative zymogram. The lower panel is the quantitative densitometric analysis of the zymogram by Kodak digital science 1D software program.

To further investigate the invasive phenotype of EVSCC14M FRNK transfectants, we assessed the motility of the transfectants by the chemotaxis assay using 3T3 CM or fibronectin as chemoattractants. FRNK transfectants were more motile than

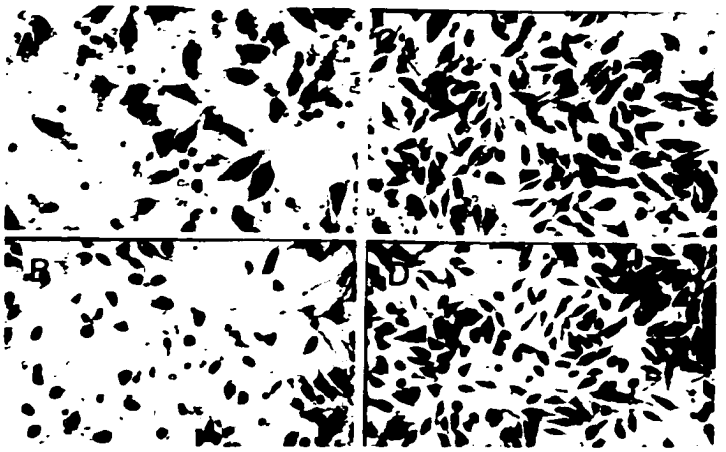

Fig. 3-37. Photomicrography of cell migration by EVSCC14M FRNK transfectants. Cell migration assays were performed in modified Boyden chambers with 30 μg/ml Fn as chemoattractant as described in section 2.1. Photograph was taken under 200 X magnification. A. pSV2.neo1 14M. B. pSV2.neo2.14M. C. pSV.FRNK1.14M. D. pSV.FRNK2.14M.

neo controls without chemoattractant. Similar to the parental cell line and neo control, migration of FRNK transfectants was stimulated by fibronectin dose dependently. In

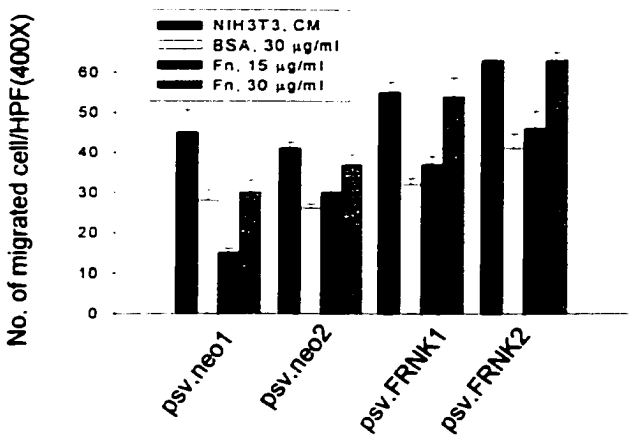

Fig.3-38 Cell migration of EVSCC14M FRNK transfectants. Migration assays were performed as described for the chemoinvasive assay, except that the polycarbonate membranes were coated with gelatin. Both 3T3 CM and fibronectin (Fn) were used as chemoattractants. Data are presented as the number of migrated cells per high power (HPF 400X). The results are the means (bars, SE) of two independent experiments.Statistical analysis demonstrated that FRNK significantly enhanced cell migration as compared to neo controls ($P<0.05$).

response to the same dose of fibronectin or 3T3 CM, FRNK transfectants were more migrative than neo controls CM ($P<0.05$) (Fig.3-37 and Fig.3-38).

2. Invasive phenotype of EVSCC18 FAK transfectants

To assess directly the effect of FAK overexpresion on the invasive phenotype of HNSCC, we transfected FAK into the low FAK expressor and weakly invasive cell line, EVSCC18. FAK overexpression in EVSCC 18 FAK transfectants was verified by western blot analysis using antibody to FAK. The blot was reprobed with antibody to

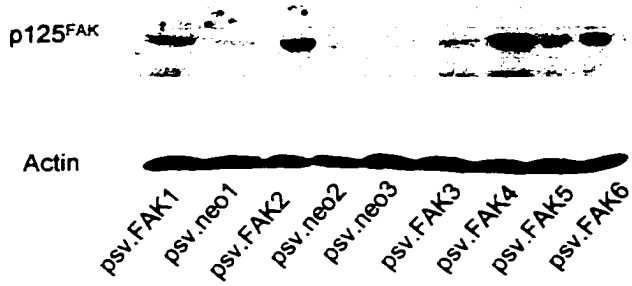

Fig.3-39 Overexpression of FAK in EVSCC18 cells by transfection. Sense FAK and pSV2neo or pSV2.neo alone were transfected into EVSCC18 cells as described in Material and Methods. G418 resistant clones were expanded and cell lysates analyzed by western blotting with FAK antibody for FAK expression and reprobed with actin as an internal control for loading.

actin as an internal control for equal loading. Figure 3-39 illustrates FAK overexpression (2-4 fold) in EVSCC18 FAK transfectants relative to the neo transfectant controls (Fig.3-39). EVSCC18 FAK expression clones, pSV.FAK.4/18 and pSV.FAK.6/18 were selected

as candidates for characterization of the invasive phenotype assessed by Matrigel invasion, cell migration, and MMP secretion. Two neo clones, pSV.neo1/18 and pSV.neo2/18 served as negative controls. In the absence of chemoattractants (BSA alone) EVSCC18 FAK transfectants and neo controls were poorly invasive, similar to parental EVSCC18 cells (Fig. 3-41). In the presence of CM as the source of chemoattractants, EVSCC18 FAK transfectant clones exhibited substantially greater Matrigel invasion relative to the neo controls (Fig 3-40 and Fig. 3-41). This difference in Matrigel invasion

Fig. 3-40 Photomicrography of Matrigel invasion by EVSCC18 FAK transfectants. Matrigel invasion assays were performed with 3T3 CM as chemoattractant as described in section2.1. Photograph was taken under 200 X magnification. A. pSV2.neo1/18. B. pSV2.neo2/18. C. pSV.FAK4/18. D. pSV.FAK6/18.

between the FAK transfectants and neo controls was most pronounced in the presence of fibronectin, which stimulated invasion in a dose-dependent manner (Fig. 3-41). Although enforced FAK expression significantly increased the invasive potential of EVSCC18 cells, the extent of Matrigel invasivion never reached the level of highly invasive

EVSCC14M and EVSCC17M cells (compare Fig3-41 with Fig 3-1). In addition, unlike EVSCC14M and EVSCC17M cells, we observed that EVSCC18 FAK transfectants appeared to clump together (Fig.3-40).

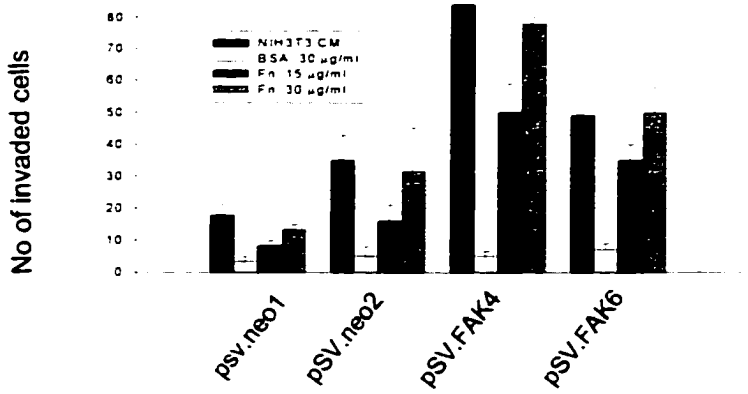

Fig.3-41 Matrigel invasion by EVSCC18 FAK transfectants. Invasion assays were conducted with Matrigel-coated 8 µM- pore size polycarbonate membranes in a modified Boyden chamber and were measured after 5 hours of exposure to either 3T3 CM, BSA or fibronectin at 15 µg/ml or 30µg/ml. Cells on the underside of the membrane were stained and counted manually with 200X magnification. Data are represented as no. of invaded cells per well. Results are means (Bars, SE) of two independent experiments, with a total of six replicates. Statistical analysis showed that the difference between neo and FRNK transfectants was significant ($P<0.01$).

To study the contribution of MMP2/MMP-9 to the increased invasive phenotype of FAK transfectants, we assessed MMP-2/MMP-9 secretion in CM from EVSCC18 transfectants and neo controls by gelatin zymography. Both FAK transfectants and neo controls exhibited variable amounts of MMP-2 secretion. Of interest, the more invasive pSV.FAK4/18 clone secreted 2-fold more MMP-2 that the less invasive pSV.FAK6/18 clone or neo controls (Fig. 3-42). MMP-9 secretion was increased by 3-4 fold in the CM of the two FAK transfectants compared to the extent of MMP-9 secretion in neo control clone (pSVneo1) with the higher level of secretion (Fig.3-42). Cell motility of

EVSCC18 FAK transfectants was assessed by chemotaxis using CM or fibronectin as chemoattractants. Cell motility of either EVSCC18 FAK transfectants or neo controls was low without chemoattractants (BSA alone).

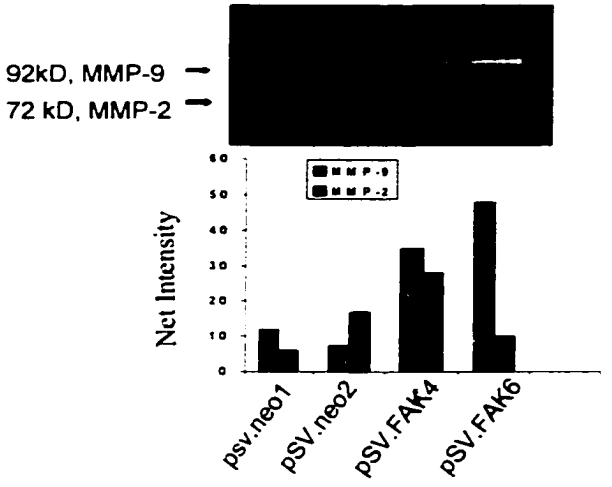

Fig. 3-42 MMP production by FAK transfectants. Cells were incubated in serum-free ITS medium for 24 hours, CM collected and MMP activity assessed by gelatin zymography as described in section 2.2. The upper panel is a representative zymogram of two experiments. The lower panel is the quantitative densitometric analysis of the zymogram by Kodak digital science 1D software program.

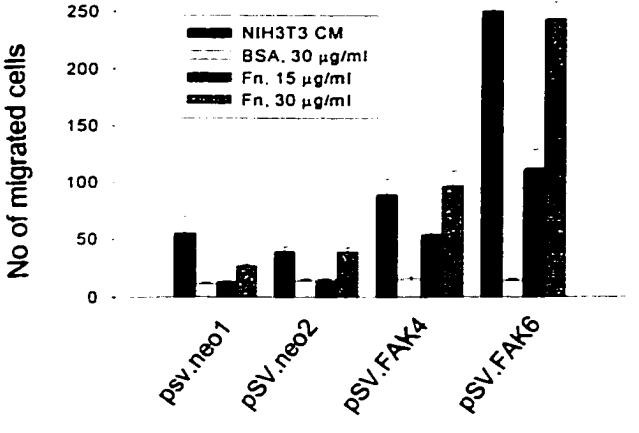

Fig.3-43 Cell migration of EVSCC18 FAK transfectants. Migration assays were performed as described in Materials and Methods. Either 3T3 CM or fibronectin were used as chemoattractants. Data are presented as number of migrated cells per well. The results are the means (bars, SE) of two independent experiments.Statistical analysis demonstrated that FAK overexpression significantly enhanced cell migration in response to CM or fibronectin as compared with neo controls ($P<0.01$).

Conditioned medium or increasing doses of fibronectin stimulated motility of EVSCC18 neo controls to a limited extent, whereas CM or fibronectin potently stimulated motility of both EVSCC18 FAK clones (Fig.3-43). The pSV.FAK 6/18 clone, in particular, exhibited a robust increase in cell motility when stimulated with CM or increasing doses of fibronectin (Fig. 3-43).

CHAPTER IV

DISCUSSION

In the present study, we sought to identify and characterize determinants of invasion by head and neck tumor cells. Utilizing a panel of HNSCC cell lines previously established in our lab, we tested the application and relevance of the three–step hypothesis of tumor invasion to HNSCC and investigated the mechanism(s) pertaining to the regulation of each step in the invasive process. The results demonstrated that 1) tumor cell invasion by HNSCC is a complex process involving three repeated sequential steps: cell adhesion, proteolytic degradation of the basement membrane, and migration; 2) MMP-2 production is essential for Matrigel invasion by HNSCCs; 3) MMP production and cell migration are regulated by integrin mediated cell-ECM interaction; and 4) Regulation of MMP production and cell migration by cell-ECM interaction depends on calcium signaling and FAK activation.

4.1 Involvement of three sequential steps: Cell adhesion, proteolytic degradation, and cell migration in tumor invasion by head and neck tumor cells

Tumor invasion of basement membranes is a crucial step for malignant dissemination and marks the beginning of metastasis by HNSCCs. Therefore, the ability of the tumor cell to breach the basement membrane defines the invasive phenotype of a tumor (13). Experimental measurement of invasive potential is a challenging task. Previously, a variety of in vitro systems have been developed to assess the invasiveness

of tumor cells (128). Several of these assays utilized tissues containing basement membranes, such as the bladder wall (129), amnion (130), lens capsule (131) and chick chorioallantoic membranes (132). Disadvantages of these approaches included the requirement of fresh sample, uneven thickness of the membrane, and the presence of microtears in the membrane that reduced the reproducibility of the assay (133). Subsequent studies have used pressed disks composed of lyophilized collagen type IV and laminin to test the invasiveness of tumor cells in Boyden chambers (134). However, disks were fragile after reconstitution and difficult to handle. To improve the handling property, a porous membrane filter coated with Matrigel, a reconstituted mixture of basement membrane proteins, was used to assess tumor invasion in different systems (133, 135). However, the original Matrigel assays required a long incubation time (72 hours), which is complicated by the need to account for cell division and also increased the opportunity for bacterial contamination. In the present study, we used Matrigel-coated porous membrane filters in a modified Boyden chamber to test the invasive of HNSCC cells in a 5 hour assay. NIH 3T3 cell conditioned medium, which contains fibronectin, collagen (136), scatter factor (123), and other uncharacterized chemoattractants was placed in the lower well beneath the filter to stimulate chemotaxis. The 5 hour assay obviates the concerns of cell division and microbial contamination that hampered the longer assays. Six head and neck cancer cell lines were evaluated for invasive potential by this method. The results demonstrated that HNSCC cell lines spanned the spectrum from highly invasive EVSCC14M and EVSCC17M cells to weakly or poorly invasive EVSCC18, EVSCC19M, FaDu, and UMSCC10A cells. This continuum of invasive potential in vitro recapitulates the biologic phenotype of HNSCC

in vivo and emphasizes the value of cell lines as representative of the tumor of origin. It is important to point out that HNSCC cell lines are remarkably stable in vitro, both cytogenetically and genetically, thus validating their use as experimental research models (137).

Active cell locomotion is an important step in tumor invasion. To further characterize the invasive phenotype, we assessed HNSCC cell lines for cell motility by a chemotaxis assay. The results showed that highly invasive cell lines (EVSCC14M and EVSCC17M) were also highly migratory whereas weakly invasive cell lines (EVSCC18, EVSCC19M, FaDu, and UMSCC10A) were less motile. Our results are consistent with previous reports of other tumor cell types (138,139). The close relationship between cell motility and Matrigel invasion of HNSCC cell lines suggested that cell migration plays an important role in the invasive process of HNSCCs.

Proteolytic degradation of the extracellular matrix is considered the rate-limiting step in tumor invasion. MMPs are a group of zinc dependent proteases responsible for degradation of extracellular matrix proteins. Studies in the past have shown that MMPs are involved in local invasion and distant dissemination of a variety of tumors (38). Since MMP-9 and MMP-2 are two well characterized MMPs, we assessed MMP-2/MMP-9 secretion into conditioned medium by gelatin zymography. HNSCC cell lines exhibited differential levels of MMP-2 activity. Densitometric analysis revealed that MMP-2 secretion was correlated with Matrigel invasion by HNSCC cell lines. One notable exception was EVSCC18 cells, which secreted high levels MMP-2 but were poorly

invasive. Further analysis showed that EVSCC18 cells were also weakly motile, suggesting that the low invasive potential of EVSCC18 was due to impaired motility. In addition to MMP-2 secretion, HNSCC cell lines also secreted variable amounts of MMP-9. However, we found that MMP-9 secretion was not correlated with Matrigel invasion. In contrast to our findings, other studies have demonstrated that MMP-9 production was required for Matrigel invasion (57). These divergent results from different laboratories likely reflect the heterogeneous nature of HNSCCs. Although our findings underscore MMP-2 as a critical determinant of in vitro invasion, they do not exclude a role for MMP-9 in the pathogenesis of HNSCC. For example, MMP-9 may target substrates other than Matrigel or may generate products that act as chemoattractants and thus influence cell behavior.

Gelatin zymography detects the enzymatic activity that degrades gelatin incorporated into a polyacrylamide gel. Therefore, the technique per se is not specific for the measurement of MMP-9/MMP-2. Recently, calcium- and zinc-independent gelatinases were identified in human circulation that exhibited similar mobilities to MMP-9/MMP-2 in gelatin zymography (140) Thus, it is possible that gelatinase activity detected by zymography was due to proteases other than MMP-9/MMP-2. To exclude this possibility and to confirm the presence of MMP-2 in conditioned medium, samples were analyzed by immunoblotting with anti-MMP-2 antibody. MMP-9 was not further evaluated by this methodology. Interestingly, the MMP-2 specific signals were only detected in nonconcentrated conditioned medium from cell lines that secreted high levels of MMP-2 enzymatic activity; however, when CM was concentrated 10-fold, the results

of western blot analysis for MMP-2 protein correlated with results obtained by zymography. Western blot analysis confirmed that HNSCC cell lines secreted MMP-2. In addition, treatment of CM with 50 mM EDTA abolished gelatinase activity, further confirmation that the gelatinase activity detected in zymography was divalent cation-dependent MMP-9/MMP-2. Taken together, the results demonstrated that western blot analysis of MMP-2 protein was less sensitive than enzymatic detection of MMP-2 by zymography. In addition to detection of MMP-2 in conditioned medium, we also analyzed the expression of MMP-2 protein in cell lysates by western blot analysis. MMP-2 expression in cell lysates was correlated with MMP-2 secretion in most cell lines except FaDu and A431. FaDu cells exhibited a high level of MMP-2 in cell lysates, which was barely detectable in CM. In contrast, A431 cells produced minimum MMP-2 in cell lysates, yet exhibited high levels of MMP-2 activity in CM. Different levels of MMPs in cell lysates and conditioned medium from specific cell lines may reflect differences in the efficiency of intracellular transport and secretion of MMPs into the culture medium.

There is ample evidence that the basement membrane is compromised in invasive HNSCC. Using immunohistochemistry, a depletion of type IV collagen and laminin in the basement membrane has been linked to lymph node metastasis (141,142). In the present study, we demonstrated that constitutive secretion of MMP-2 was correlated with invasive potential in 5 of 6 HNSCC cell lines tested. The results were consistent with a previous report which showed the association of MMP-2 expression with invasive behavior of oral carcinoma in a limited number of cell lines (77). In support of these findings, MMP-2 expression has been detected in vivo in small nests of cells at the

advancing tumor front of oral carcinoma and the presence of MMP-2 was related to lymph node metastasis (143). However, several studies have demonstrated that MMP-2 present in carcinoma in vivo may derive from the tumor stroma rather than tumor cells. MMP-2 immunoreactivity was found in the peripheral cell layer of neoplastic islands but MMP-2 mRNA expression was confined to fibroblasts (144,145) suggesting that tumor cells may be capable of utilizing MMPs produced by stromal cells to facilitate invasion. Although a role for stromal cells in the production of MMPs in vivo cannot be ignored, the detection and identification of authentic MMP-2 in epithelial HNSCC cell lines argues strongly for MMP production by the tumor cell.

Degradation of ECM is a highly controlled process and depends on the balance of MMPs and TIMPs. Up-regulation of MMPs or down-regulation of TIMPs tilts the balance towards proteolytic degradation of the ECM and promotes tumor invasion (13). We have demonstrated that MMP-2 overexpression was associated with tumor cells of high invasive potential. Because TIMP-2 preferentially binds to and inhibits MMP-2, we assessed TIMP-2 expression and secretion by HNSCC cell lines by western blot analysis and reverse zymography. HNSCC cell lines exhibited different levels of TIMP-2 activity in CM as measured by reverse zymography which correlated with TIMP-2 protein levels in CM detected by immunoblotting. Unlike MMP-2 secretion, we found no association between TIMP-2 secretion and Matrigel invasion. TIMP-2 levels were not consistently decreased in highly invasive HNSCC cells (EVSCC14M and EVSCC17M) nor increased in weakly invasive lines (eg., EVSCC19M and UMSCC10A). Thus, our results support the concept that up-regulation of MMP-2 rather than down-regulation of TIMP-2

determines the invasive phenotype of HNSCC cells. Similar findings were reported in giant cell tumors of bone (69). In contrast, reduced TIMP levels were associated with prostate tumor cells (70). Collectively, the data emphasize an imbalance of secretion between MMPs and TIMPs, which may be tumor-type dependent.

Cell attachment to extracellular matrix proteins represents the initial and crucial step in the invasion process of tumor cells (13). To further characterize the invasive phenotype of HNSCC cells, we assessed the ability of HNSCC cells to adhere to ECM proteins by an in vitro adhesion assay. HNSCC cell lines exhibited differential adhesion to the ECM proteins fibronectin, laminin, and type IV collagen. Cell adhesion to ECM proteins was dose dependent, suggesting that binding of HNSCC cells to ECM proteins was specific. Cell adhesion was correlated with Matrigel invasion in 4 of 6 cell lines tested. Highly invasive cell lines, EVSCC14M and EVSCC 17M, were highly adhesive whereas weakly invasive cell lines, EVSCC18 and FaDu, were less adhesive. In contrast, weakly invasive EVSCC19M and UMSCC 10A cells exhibited elevated levels of adhesion to most ECM proteins, suggesting that cell adhesion to ECM proteins is necessary but not sufficient for Matrigel invasion. Interestingly, we observed that HNSCC cells were either adherent to all or none of the ECM proteins evaluated. This generalized adhesive property to ECM proteins found with HNSCC cells has also been observed in other tumor cell types (25,146, 147). Although the mechanism(s) underlying this all or none cell adhesive response to ECM proteins is currently unknown, one possibility is that integrin receptors for ECM proteins may be upregulated in a subset of HNSCC. Enhanced expression of integrin receptors on tumor cells would be expected to

increase cell adhesion to ECM proteins and facilitate tumor cell migration and invasion. In support of this hypothesis, a recent study demonstrated that over-expression of integrin subunits α2, α3, α5, α6 was correlated with tumor invasion and metastasis of squamous cell carcinoma of the oral cavity (148). Since α subunits of integrin are able to associated randomly with β subunits in vivo, overexpressed α subunits resulted in the up-regulated expression of a number of integrin receptors for ECM proteins, including fibronectin, laminin, and type IV collagen tested in the present study (148). An alternative explanation to account for the generalized increase in cell adhesion to ECM proteins by specific tumor cells is based on the state of integrin activation. Integrins exist in either high or low affinity states with respect to their extracellular ligands. We speculate that integrins are constitutively activated in specific HNSCC tumor cell lines thus enabling the high affinity binding to a number of different ECM proteins. The mechanistic basis of integrin activation is poorly understood. However, the activating process appears to be regulated by Ras-related GTPases and their downstream effectors (149). In support of this concept, a recent study has shown that the activated αIIbβ3 integrin enhanced cell adhesion and invasion of human melanoma cells (150). Clearly, further studies are required to distinguish among the above possibilities.

Integrin mediated cell adhesion to the ECM is a complex process that occurs in specialized structures termed focal contacts or focal adhesions. At these adhesions, integrins bind externally to ECM proteins and internally to cytoskeletal proteins. Studies have shown that integrins can interact with same ECM protein and that an individual integrin can recognize and bind to different ECM protein ligands (20). Another layer of

complexity for integrin mediated cell-ECM interaction is the involvement of integrins in the activation of signal transduction pathways. By interacting with ECM protein ligands, integrins can sense molecular cues in the extracellular environment and transduce these signals across the cell membrane into the nucleus via a variety of signal transduction pathways to modulate cell behavior. In addition, one integrin can regulate the biological function of another integrin family member, a process referred to as integrin cross-talk, the mechanistic basis of which is unknown (20). Regardless of the above complexities, the central theme of integrin-ECM interactions is the recognition of short peptide sequences within the large ECM protein. One well-characterized integrin recognition sequence is arginine-glycine-aspartic acid (RGD). Studies with other tumor cell types showed that many integrin regulated biological events were RGD dependent e.g. cell adhesion, spreading, migration and invasion (21). Likewise, we demonstrated that the RGD peptide inhibited HNSCC cell adhesion to fibronectin and laminin in a dose-dependent manner, suggesting that cell adhesion to fibronectin and laminin was RGD dependent. RGD peptide did not inhibit HNSCC cell adhesion to type IV collagen, indicating that integrin binding to type IV collagen was mediated by sequences other than RGD. In contrast to RGD, the control peptide, RGE, had no effect on cell adhesion to any of the ECM proteins. Thus, this single amino acid change completely abolished RGD anti-adhesive activity. The results demonstrated that inhibition of cell adhesion to fibronectin and laminin by RGD peptide was specific. In addition, we also compared the efficiency of the RGD tripeptide with the peptide GRGDSP to block cell adhesion to fibronectin. GRGDSP peptides were two-fold more efficient than RGD tripeptides, suggesting that amino acids adjacent to RGD increase the efficiency of integrin-RGD

interactions. Overall, our results are consistent with previous reports (30) and demonstrate that cell adhesion to ECM proteins plays an important role in the invasive process of HNSCC cells.

In past decades, increasing interest has focused on deciphering the molecular mechanisms underlying tumor invasion. It was proposed that tumor cell invasion consists of three sequential steps: cell adhesion, proteolytic degradation of ECM proteins, and chemoattractant directed migration. The proposed three-step hypothesis received extensive experimental support and has elucidated molecular events related to each step of tumor invasion and subsequent metastasis formation (13). However, the step-wise process of invasion has not been tested systematically in the context of HNSCCs. In the present study, we first characterized the invasive phenotype of six HNSCC cell lines by measuring tumor cell invasion through a reconstituted basement membrane, cell adhesion to ECM proteins, MMP-2/MMP-9 production and cell migration. The results demonstrated that high invasive HNSCC cell lines exhibited enhanced adhesion to ECM proteins, MMP-2 production, and directed cell migration toward chemoattractants. Defects in one or more of these properties render the cell less or non-invasive. Our results demonstrate that the three-step model of tumor invasion can be expanded to include HNSCCs.

4.2 MMP-2 production is necessary for Matrigel invasion

The role of MMPs in tumor invasion has been clearly established (151). MMP-2,

which cleaves basement membrane collagen, was the first member of the collagenase family to be closely linked with the invasive phenotype (152). Expression of MMP-2 has been associated with tumor invasion and metastasis in many types of malignancy (38). However, the role of MMP-2 in tumor cell invasion by HNSCCs was not clearly established. We have demonstrated that over-expression of MMP-2 rather than down-regulation of TIMP-2 tilted the balance towards ECM degradation and that overexpression of MMP-2 was associated with the in vitro invasive potential of HNSCC cell lines. Other studies showed that expression of MMP-2 was correlated with lymph node metastasis in oral squamous cell carcinoma (77). To provide more direct evidence that MMP-2 production was necessary for, and not simply correlated with, the invasive potential of head and neck tumors cells, we demonstrated that antibody to MMP-2 blocked in vitro invasion of HNSCC cell lines. However, inhibition of in vitro invasion by anti-MMP-2 was inefficient. Large doses of MMP-2 antibody (100 ug/ml) were required to achieve substantial inhibition, suggesting that even high concentrations of antibody were insufficient to neutralize excess MMP-2 secreted by the cell line. Alternatively, HNSCC cells may secrete other proteases involved in Matrigel invasion. In support of our findings, Albini et al (74) showed that MMP-2 specific antibody inhibited HT1080 tumor cell invasion to the same extent as TIMP-2. In addition, MMP-2 antibody inhibited HT1080 cell migration by 27% suggesting that the effect of MMP-2 antibody on HT1080 cell invasion was partially mediated by blocking cell migration. Inhibition of cell migration by MMP-2 antibody supports recent evidence that MMPs are not solely involved in the proteolytic digestion of the ECM, but can also modify cell attachment. Thus, MMP-2 antibody may inhibit cell migration by interfering with cell

attachment and detachment mediated by membrane-MMP-2 (74). The role of MMP-2 in cell migration by HNSCC remains to be determined.

4.3 MMP production and cell migration is regulated by integrin mediated cell-ECM interaction

Cell adhesion to ECM proteins is the initial step in tumor invasion. It represents the most direct cell-ECM interaction. Accumulating evidence has demonstrated the importance of ECM and the tissue microenvironment in tumor progression and metastasis (153). Utilizing the nude mouse model, we have shown previously that the site of tumor implantation, i.e., the tissue microenvironment, strongly affects tumor cell behavior and invasion by HNSCCs (132). In work described above, we demonstrated that cell adhesion to ECM proteins is correlated with tumor invasion by HNSCC cells in vitro. In this section, we discuss the evidence that cell-ECM interactions regulate MMP production and cell migration in the context of our understanding of how these interactions may regulate the invasive behavior of HNSCC.

a. Regulation of MMP production by cell-ECM interactions

MMPs are secreted as soluble, latent proenzymes that are activated by proteolytic cleavage of an amino-terminal domain. Thus, a clearer understanding of MMP activation is critical to defining the invasive tumor phenotype. In the previous section, we discussed evidence showing that the extent of proMMP-2 secretion was correlated with Matrigel

invasion in 5 of 6 HNSCC cell lines. One exception, EVSCC18 cells, produced large amounts of proMMP-2 but the cells were poorly invasive suggesting that proMMP-2 secretion may be necessary but not sufficient for acquisition of the invasive phenotype. Previous studies have shown that only activated MMPs are able to digest the ECM (13). Consistent with these findings, activated MMP-2 was linked to lymph node metastasis of oral carcinoma cells (154,155). However, in our study, non-stimulated HNSCC cells produced barely detectable activated forms of MMP-2 assessed by gelatin zymography. To be functional, proMMP-2 must somehow be activated during the process of Matrigel invasion. Because cell-ECM interactions were demonstrated previously to stimulate MMP expression (22), we reasoned that ECM proteins might stimulate MMP-2 secretion or activation by HNSCC cells. The experiments to test this hypothesis generated some unexpected results (see Fig. 3-13 & 3-14). First, MMP-2 secretion and/or activation was unaffected by fibronectin or laminin in three representative highly or weakly invasive HNSCC cell lines. Second, and unexpectedly, fibronectin stimulated secretion and activation of proMMP-9 in dose-dependent manner by each cell line. Laminin also stimulated MMP-9 production, but less potently and in only one of the cell lines suggesting that the effect of laminin on MMP-9 secretion was cell line-specific. Cell specific regulation of MMP-9 by laminin is likely to reflect laminin receptor heterogeneity in HNSCCs. We also observed that type IV collagen at low doses had no effect on MMP-9/MMP-2 secretion, whereas higher doses ($\geq$40 ug/ml) inhibited secretion of MMP-9/MMP-2. A similar example of suppression of MMP secretion by fibronectin and the $\alpha 4\beta 1$ integrin was reported in fibroblasts (156). Although the impact of negative signaling by ECM-integrins is poorly understood, it seems likely that integrin

receptors and ECM ligands collaborate in vivo to regulate MMP expression in the context of the tissue microenvironment. Because both MMP-2 and MMP-9 are known to digest basement membrane constituents, we speculate that constitutive secretion of MMP-2 may be responsible for the initial breakdown of the basement membrane in vivo and that subsequent up-regulation and activation of MMP-9 by cell-ECM interactions may potentiate ECM degradation and tumor invasion.

Consistent with our findings, recent studies have shown that integrin-mediated cell-ECM interactions influence MMP expression and/or activation. Type I collagen up-regulated MMP-1 secretion in skin keratinocytes (157) and human osteosarcoma cells (35). Similarly, fibronectin was shown to induce MMP-9 secretion in a variety of cell types including fibroblasts (158), macrophages (159), and ovarian carcinoma cell lines (34). In addition, other studies have shown that type I collagen induced MMP-2 activation in normal and malignant human fibroblastoid cells (160), and laminin was reported to stimulate MMP-2 activation in rhabdomyosarcoma cells (161).

Evidence from several laboratories has demonstrated that regulation of MMPs by ECM proteins is dependent on the expression of integrin receptors. For example, cross-linking of integrins with antibodies against β1 and α3 integrins stimulated MMP-9 production in oral keratinocytes (162) and anti-α3 integrin resulted in MMP-2 activation in rhabdomyosarcoma cells (161). Similarly, clustering of the integrin αvβ3 with anti-integrin antibody led to increased expression of MMP-2 in melanoma cells (36). In addition, upregulation of β3 integrin was associated with MMP-9 expression in

melanoma cell tumors transplanted into nude mice (163). Although characterization of the integrin receptors in HNSCC cell lines is beyond the scope of this work, it seems reasonable to conclude that specific integrin engagement with ECM proteins induces MMP-9 expression in head and neck tumor cells. Further studies are needed to uncover the mechanisms responsible for fibronectin-induced activation of MMP-9.

b. Cell-ECM interactions regulate cell motility of HNSCCs

Cell-ECM interactions not only regulate MMP secretion but also modulate cell migration. Accumulating evidence has demonstrated that ECM proteins are able to stimulate locomotion in many types of tumor cells including melanoma, glioma, mesothelioma, fibrosarcoma, osteosarcoma, lung and breast carcinoma (reviewed in 49). The motility-stimulating ECM proteins include vitronectin, fibronectin, laminin, type I collagen, type IV collagen and thrombospondin (49). In the previous section, we discussed evidence showing that cell motility is an important determinant of the invasive phenotype of HNSCCs. However, little is known about the effect of ECM proteins on cell migration by HNSCC cells. In experiments aimed to determine the effect of ECM proteins on tumor cell migration (chemotaxis), we demonstrated that fibronectin, laminin, or type IV collagen stimulated chemotaxis of two highly invasive HNSCC cell lines over a broad dose range, whereas these ECM proteins failed to stimulate chemotaxis of a weakly invasive HNSCC cell line (see Figs. 3-16 to 3-18). Two observations emerged from the results of these experiments. One, the highly invasive cell lines exhibited high motility in the absence of exogenous stimulation by ECM proteins (so called

chemokinesis). In the present study, we demonstrated high motility in highly invasive cell lines even without stimulation. The mechanism underlying the enhanced motility is not clear. Interestingly, we have found that one of the highly invasive cell lines (EVSCC17M) has high levels of EGF receptor expression by immunohistochemistry (unpublished observations). Since EGF has been shown previously to stimulate cell migration by a variety of tumor cell types, we speculate that the high endogenous motility seen in EVSCC17M might be due to activation of the EGFR signaling pathway. Indeed, we have found that EGF receptor blocking antibody A225 inhibited cell migration dose-dependently (unpublished observations) suggesting the presence of an EGF-EGFR autocrine loop in EVSCC17M cells resulting in high endogenous motility. Second, cell migration in response to fibronectin and type IV collagen was biphasic. Low and intermediate doses stimulated whereas high doses reduced cell migration. The same phenomenon has been observed in other tumor cell types without clear explanations (25). One possibility is that high doses of ECM proteins result in integrin internalization (164). Temporary down regulation of the integrin receptor may lead to reduced motility. Other studies have shown that tumor cells have the ability to assemble fibronectin and the assembled fibronectin polymer could inhibit cell migration (165). High doses of fibronectin may facilitate fibronectin assembly, thus inhibiting cell migration. Although our results are unable to explain these observations, the findings demonstrate that cell-ECM protein interactions regulate chemotaxis by HNSCC cells.

We have shown that cell contacts with ECM proteins can modulate MMP secretion and cell motility. These cell-ECM interactions are mediated by integrin

receptors. Ligand binding and activation of integrins is dependent on a peptide recognition motif (RGD) recognized by several members of the integrin family (20). To provide evidence that cell contact with fibronectin was integrin receptor dependent, we demonstrated that synthetic RGD peptides specifically inhibited cell migration in response to fibronectin. Previous studies have shown that RGD peptides can block cell migration by specific ECM proteins in a variety of tumor cells (166). Thus, our findings support and extend earlier work and demonstrate that fibronectin-stimulated HNSCC locomotion is RGD dependent and at least partially mediated by integrins. Which integrin receptors are involved in regulation of HNSCC cell motility by ECM proteins is currently unknown. Studies in the past have linked $\alpha 6\beta 4$ and $\alpha v\beta 6$ to local invasion and metastasis of HNSCCs (167, 168). Recent studies demonstrated that expression of $\alpha 2$, $\alpha 3$, $\alpha 5$, and $\alpha 6$ integrin subunits was correlated with tumor invasion and poor prognosis of HNSCCs (148). Collectively, the findings suggest that these integrins may be good candidates for motility receptors. Further investigation is needed to identify and characterize integrin receptors critical for HNSCC cell migration and their role in tumor cell invasion. In the future, it may be possible to develop and clinically evaluate short peptide sequences (eg., RGD-like) capable of blocking tumor cell invasion.

4.4 Involvement of calcium signaling in regulation of the invasive phenotype of HNSCCs

Intracellular calcium homeostasis is a common regulatory point in many signal transduction pathways and is known to be essential for several biological process including cell proliferation, angiogenesis, cell migration and invasion (88). To determine

if calcium-signaling was important in HNSCC biology, we made use of CAI, a novel inhibitor of calcium influx. CAI has been shown to inhibit cell adhesion, proliferation, and cell migration in many cell types (126).

We demonstrated that CAI inhibited the invasiveness and motility of HNSCC cell lines in vitro, consistent with previous reports showing that CAI is an anti-metastatic agent (126,169). We also demonstrated that CAI inhibited both MMP-2 and MMP-9 production by HNSCC cells, indicating that calcium homeostasis is important in regulation of MMP secretion. This supports and extends previous studies showing that CAI inhibits MMP-2 production by fibrosarcoma and endothelial cell lines (170,171). Whether CAI inhibits the production of MMPs at level of transcription or translation/secretion in HNSCC is not clear. In a recent study, CAI was found to reduce MMP-2 mRNA transcription (170). Further work is necessary to determine if CAI also inhibits transcription of MMPs in HNSCCs. The reduction of MMP activity by CAI suggests that inhibition of the invasive potential of HNSCCs also involves inhibition of MMP production.

We demonstrated that integrin mediated cell-ECM is important in regulating cell migration and MMP production. Studies by others demonstrated that integrin receptor-ligand binding and activation leads to an influx of intracellular calcium. Our results demonstrated that blocking calcium influx with CAI inhibited cell migration, MMP production, and Matrigel invasion. Thus, our findings suggest that regulation of cell migration and MMP production by cell-ECM interactions is dependent on calcium

signaling and indicate that interruption of calcium-dependent signaling pathways may represent a useful strategy for the therapeutic management of HNSCC.

4.5 FAK expression and activation is important in regulation of the invasive phenotype of HNSCCs

A substantial body of evidence has demonstrated a critical role for FAK in integrin-mediated signaling (94, 96). Moreover, increased FAK expression has been proposed as a biomarker for invasive and metastatic tumors (105). Because the role of FAK in integrin-mediated signaling in HNSCC cells and the biological consequences of FAK expression and tyrosine phosphorylation have not been fully explored, we analyzed FAK expression and phosphorylation in HNSCC cell lines. The results demonstrated that HNSCC cell lines exhibited differential levels of FAK protein and phosphorylated FAK. FAK phosphorylation was correlated with cell migration and Matrigel invasion of HNSCCs, suggesting that FAK activation contributes to the invasive phenotype of HNSCCs.

To investigate whether FAK signaling has a causal role in the invasive phenotype of HNSCC, we overexpressed FAK in a weakly invasive HNSCC cell line, EVSCC18. The results demonstrated that forced expression of FAK significantly stimulated MMP-9 production, cell migration towards chemoattractants (NIH3T3 conditioned medium or fibronectin), and Matrigel invasion. Consistent with these results, overexpression of

FAK in chinese hamster ovary (CHO) cells stimulated migration towards fibronectin (110). Thus, our results confirmed and extended earlier work and suggested that FAK contributes in some way to the acquistion of the invasive phenotype of HNSCCs.

Although forced FAK expression significantly increased the in vitro invasive potential of weakly invasive EVSCC18 cells, they never achieved the invasive potential of highly invasive cell lines, EVSCC14M and EVSCC17M. Thus, we conclude that FAK overexpression is not sufficient for acquistion of the invasive phenotype and that other factors are needed for development of the invasive and metastatic phenotype. We speculate that sub-cellular localization of FAK and FAK-associated proteins to focal contact sites are important determinants in promoting enhanced rates of cell migration. Further experiments are needed to address this possibility.

To further investigate the role of FAK in the invasive phenotype, we sought to inhibit FAK function in highly invasive cells by overexpression of FRNK, an alternatively spliced product of the FAK gene, which is proposed to act as an in vivo competitive inhibitor of FAK (104). Specifically, FRNK was transfected into highly invasive EVSCC14M cells, which expressed high levels of FAK and phosphorylated FAK. Subsequent analysis demonstrated that forced expression of FRNK in EVSCC14M cells slightly inhibited FAK expression, but significantly decreased FAK phosphorylation. Previous studies have shown that mouse fibroblasts derived from FAK -/- knockout mice exhibited decreased rates of cell migration in vitro relative to cells from wild type mice (109) and that this migration defect could be rescued by

transient FAK overexpression in FAK -/- cells (172). Moreover, transient overexpression of FRNK in Balb/3T3 fibroblasts and human endothelial cells reduced cell migration (173) and stable overexpression of FRNK blocked migration of LNCaP prostate tumor cells (174). Therefore, the augmented invasive potential of EVSCC14M-FRNK transfectants, manifested as increased migration, Matrigel invasion, and MMP-9 secretion, was unexpected and not in agreement with the above findings. In support of our findings, enhanced FAK phosphorylation was associated with decreased migration in NIH3T3 fibroblasts and bovine endothelial cells and down modulation of FAK phosphorylation by expression of a v-*src* kinase-negative mutant increased cell migration (175). Taken together with our findings, the data suggest that FAK activation is tightly regulated and that increases or decreases in FAK phosphorylation modulate cell function. Alternatively, our results support a model of a dual integrin-mediated signaling pathway in HNSCC cells that was reported previously in fibroblasts (176). In this model, both FAK-dependent and FAK-independent mechanisms for integrin-mediated MAPK activation and downstream signaling can occur. Our findings of significantly decreased FAK phosphorylation in FRNK overexpressors suggest that integrin-mediated downstream cellular functions can occur independent of FAK function.

Finally, our results are compatible with a model in which FRNK overexpression could trigger downstream events leading to altered gene expression such as cell migration, Matrigel invasion, and MMP-9 secretion, as observed in EVSCC14M-FRNK transfectants. FRNK lacks catalytic activity but contains three proline-rich domains which interact with SH3 domains in target proteins, a tyrosine phosphorylation site

(Tyr925), and a focal adhesion-targeting (FAT) domain responsible for targeting the protein to focal adhesions (91,177). We propose a model in which FRNK is phosphorylated on Tyr925 by endogenous EVSCC14M FAK; this leads to the recruitment of Grb-2 and SOS to Tyr925 via their SH2 domains and the formation of signaling complexes which would be targeted to focal adhesions by FAT sequences in FRNK and promote activation of the Ras signal transduction pathway and downstream cellular functions. Support for this model derives from previous studies showing that overexpression of a kinase-defective FAK in CHO cells stimulated migration on fibronectin (110). Although the underlying mechanisms by which FRNK overexpression leads to augmentation of the invasive phenotype of HNSCC cells remain to be investigated, our results demonstrate that the FAK signaling pathway has a critical role in the modulation of cellular determinants of the invasive tumor cell phenotype.

In summary, evidence discussed in this section support the notion that FAK-dependent signaling plays an important role in generating the invasive phenotype of HNSCCs. Further investigation is required to dissect the signal transduction pathways downstream of FAK to clarify how FAK activation affects tumor cell invasion and metastasis.

CHAPTER V

SUMMARY AND CONCLUSIONS

Local invasion and lymph node metastasis are predictive of a poor clinical outcome and the major obstacle to cure of HNSCCs. A greater understanding of the cellular and molecular mechanism(s) underlying the invasive phenotype of HNSCCs is expected to translate into new therapeutic strategies. Utilizing a panel of HNSCC cell lines previously established in our laboratory, we sought to identify and characterize the cellular and molecular determinants involved in the invasive process of HNSCCs.

The invasive phenotype of six HNSCC cell lines was analyzed by measuring tumor cell invasion though a reconstituted basement membrane (Matrigel), cell adhesion to ECM proteins, MMP-2/MMP-9 production and cell migration. The results demonstrated that highly invasive HNSCC cell lines exhibited enhanced adhesion to ECM proteins, increased MMP-2 production, and directed cell migration toward chemoattractants. Defects in one or more of these properties rendered tumor cells less or non-invasive. Our results with HNSCC cells support a model of tumor invasion that involves tumor cell adhesion, ECM proteolysis, and cell migration.

In the process of characterizing the invasive phenotype of HNSCC cell lines, we found that MMP-2 production was associated with Matrigel invasion. To provide more direct evidence that MMP-2 production was necessary for, and not simply correlated with, the invasive potential of HNSCCs, we demonstrated that antibody to MMP-2

blocked in vitro invasion of the highly invasive HNSCC cell line, EVSCC14M, in a dose dependent manner, confirming that MMP-2 was a necessary determinant of invasive potential.

Cell adhesion to ECM proteins is the initial step in tumor invasion and has previously been shown in other tumor types to be important in the regulation of other steps of the invasive process. We postulated that cell-ECM interactions might also be important in modulating the invasive phenotype of HNSCCs. To test this hypothesis, HNSCC cells were exposed to soluble ECM proteins and measured for changes in cell migration, MMP production and/or activation. ECM proteins (fibronectin, laminin, or collagen type IV) stimulated cell migration of highly invasive cell lines but failed to stimulate migration of a weakly invasive cell line. Fibronectin-stimulated cell migration was specifically blocked by RGD peptides, a known recognition motif for integrin-fibronectin binding. Fibronectin also stimulated MMP-9 secretion and activation in a dose-dependent manner by HNSCC cell lines but had minimal to no effect on MMP-2 secretion and/or activation. Laminin weakly and selectively stimulated MMP-9 secretion but was without effect on MMP-2 secretion and/or activation. Collectively, our results suggest that cell migration and MMP-9 production by HNSCC cells were regulated by integrin-mediated cell-ECM interactions.

Integrin-mediated cell-ECM interactions are not only important in regulation of a variety of biological process but are also responsible for the generation and transmission of signals across the cell membrane. Previous studies demonstrated that intracellular

calcium was an important component of the integrin-mediated signal transduction pathways. Therefore, we sought to determine the role of calcium signaling in the regulation of MMP secretion and generation of the invasive phenotype of HNSCCs. Utilizing a novel calcium influx inhibitor, carboxyamido-triazole (CAI), we demonstrated that inhibition of calcium influx blocked cell motility, MMP-9/MMP-2 secretion, and Matrigel invasion. Our findings suggest that calcium signaling plays an important role in modulating the invasive phenotype of HNSCCs.

FAK has been implicated in integrin-mediated signaling pathways and linked previously to cell spreading, migration and invasion. To explore the role of FAK in integrin-mediated signaling in HNSCC cells and the biological consequences of FAK expression and activation, we analyzed FAK expression and phosphorylation in HNSCC cell lines. The results demonstrated that FAK phosphorylation was correlated with cell migration and Matrigel invasion of HNSCCs, suggesting that FAK activation contributes to the invasive phenotype of HNSCCs. To investigate whether FAK signaling has a causal role in the invasive phenotype of HNSCC, sense FAK or its negative regulator, FAK-related non-kinase (FRNK), was expressed by stable transfection of HNSCC cell lines to assess the consequences of FAK or FRNK transfection on the invasive phenotype. Overexpression of FAK in weakly invasive EVSCC18 cells significantly increased Matrigel invasion, MMP-9 production, and cell migration. Overexpression of FRNK in highly invasive EVSCC14M cells reduced FAK phosphorylation and expression, but unexpectedly, enhanced Matrigel invasion, MMP-9 secretion, and cell migration. Taken together, our findings suggest that the FAK signaling pathway has a

critical role in the modulation of cellular determinants of the invasive tumor cell phenotype.

In conclusion, data presented in the present study demonstrated that tumor cell invasion in HNSCC is a complex process involving three repeated sequential steps: adhesion, proteolytic degradation of the basement membrane, and cell migration. These three steps are linked and interdependent. For example, cell adhesion to ECM triggers a series of signal transduction pathways that involve calcium mobilization, focal adhesion kinase activation and downstream events leading to alteration of cell function manifested by migration and MMP production. Our working model extends the previous three step hypothesis of tumor invasion by underscoreing FAK as an integrator of multiple signaling pathways. Efforts to determine the role of FAK in the integration and propagation of signal transduction pathways should lead to a better understanding of the molecular mechanisms for generating the invasive phenotype of HNSCCs, which may in turn, lead to the discovery of new targets for therapy of invasive HNSCC. Identification of cellular and molecular determinants of HNSCC may prove useful potentially as biomarkers of tumor cell behavior, prognosis, and for guiding treatment strategies.

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APPENDIX

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1993 Eastern Virginia Medical School
Department of plastic Surgery
Microsurgical Fellow
Norfolk, Virginia

1990 Tongji Medical University
Department of Orthopedic Surgery
Residency, Orthopedic surgery
Wuhan, China

Education:

1998 Old Dominion University
Eastern Virginia Medical School
Ph.D. Biomedical Science Program

1987 Tongji Medical University
M.S. in Orthopedic Oncology-
A joint program with orthopedic residency

1984 Tongji Medical University
Wuhan China
M.D.

Professional Activities:

Member of Scientific Board, Association for Orthopedic Research, Switzerland.