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Vimentin Overexpression Contributes To the Biological Properties of Metastatic Head and Neck Cancer Cells

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University School of Medicine.

by

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Abstract

VIMENTIN OVEREXPRESSION CONTRIBUTES TO THE BIOLOGICAL PROPERTIES OF METASTATIC HEAD AND NECK CANCER CELLS

Rachel Josephine Paccione
Master of Science

Epithelial to mesenchymal transition occurs in the later stages of epithelial tumor progression, with cells expressing mesenchymal markers. Of these, the intermediate filament protein vimentin is frequently upregulated in metastatic carcinomas. Previously, microarray studies showed that the gene encoding vimentin is highly upregulated in metastatic HN12 cells compared to a related primary tumor cell line. In this study, we confirmed this difference using real-time quantitative PCR, western blot analysis, and immunostaining. Furthermore, EGF and TGF-β, growth factors that induce migration and invasion of HN12 cells, produced synergistic increases in vimentin expression. To assess the contribution of vimentin to the biological properties, HN12 cells were stably transfected with a plasmid that directs synthesis of vimentin shRNA. Clones expressing

decreased amounts of vimentin were isolated and characterized. These cells showed significantly reduced proliferation compared to non-targeting controls. Moreover, downregulation of vimentin led to a decrease in cell motility, as well as reducing their ability to invade through a basement membrane substitute. Using transient transfection assays, vimentin promoter activity was determined in HN12 cells to define regulatory elements important for controlling vimentin upregulation in the absence or presence of EGF and TGF-β. Taken together, the data indicate that overexpression of vimentin is important for proliferation and invasion of metastatic HN12 cells, and suggest that EGF-dependent pathways target binding elements in the proximal vimentin promoter, while TGF-β is likely to act in an AP1-dependent manner. Furthermore, both growth factors appear to synergize by stimulating promoter activation through the ASE site, suggesting involvement of Stat-dependent pathways in regulation of vimentin expression in HN12 cells.

1. Introduction

1.1. Growth Control and Tumor Development

Cancer is caused by the failure of regulatory mechanisms that control the growth and survival of cells. During normal development, intricate genetic control systems regulate the balance between cell proliferation and cell death in response to growth promoting signals, growth-inhibitory signals, and death signals. The growth of tumor cells depends not only on the increase of cells that are proceeding through cell division, but also a decrease in the number of cells that are undergoing programmed cell death (Martin, 1996). Programmed cell death or apoptosis is a physiological process that embodies a series of characteristics and genetically controlled steps, which include chromatin condensation and fragmentation, cell shrinkage, and the engulfment of the cell by neighboring cells without an inflammatory response (Martin, 1996). Apoptosis is essential for both normal development and tissue homeostasis in the adult (Stellar, 1995). It is the failure of certain tumor cells to undergo apoptosis that appears to be one of the factors underlying the genetic instability of these particular cells, their resistance to chemotheurapeutic agents, and their increase in proliferation (Martin, 1996).

The loss of cellular regulation contributes to the genesis of malignancies and is due in large part to genetic damage. Genetic changes in proto-oncogenes and tumor suppressor genes are primarily responsible for the initiation and progression of a tumor

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(Martin, 1996). Since the majority of mutations do not increase the growth characteristics of a cell, specific genetic changes must underlie the progression to cancer (Lowy, 1996). For instance, proto-oncogenes may be activated to become oncogenes by a particular mutation, which causes the gene to become excessively active in growth promotion, or they may be expressed inappropriately. In addition, tumor suppressor genes that normally restrain the growth of cells can be inactivated, thereby de-regulating cell growth.

Proto-oncogenes are appropriately named because they are essentially progenitors of oncogenes or precursors of cancer genes. Proto-oncogenes can be converted into oncogenes by several mechanisms, including intragenic mutation and chromosomal rearrangement (Bishop and Hanafusa, 1996). In either case, the result is the abnormal uninhibited expression of a gene or the deregulation of the protein product (Bishop and Hanafusa, 1996). Proto-oncogene products participate in cell signaling that drives cell growth and usually falls into one of four categories: growth factors, growth factor receptors, intracellular signal transducers, and transcription factors (Leis and Livingston, 1996). Oncogenes may be associated with a cancer predisposition as a result of a mutational event in many types of cancers, they may gain function through a dominant allele in which only one allele is mutated in cancer, and are rarely passed on to progeny through germ line transmission (Leis and Livingston, 1996). On the other hand, tumor suppressor genes are negative regulators of cell growth that undergo loss of function through mutation of both alleles, although for some genes, loss of a single allele is enough to perturb suppressor function (haploinsufficiency). Germ line transmission of a

mutant allele results in predisposition to developing malignant disease (Leis and Livingston, 1996).

As noted previously, oncogenic mutations usually only occur in somatic cells; therefore, the mutations are not passed from one generation to another. In order for oncogenic mutations to induce cancer, the mutations must occur in the dividing cells and therefore be passed to progeny cells. When such a mutation occurs in nondividing cells, *e.g.* neurons or muscle cells, the mutation generally does not induce cancer, which is one reason why tumors of muscle and nerve cells are very rare in adults.

However, there are certain inherited mutations in tumor suppressor genes, for example mutations in *RB* and *BRCA1*, which are carried through the germ-line and increase the probability that cancer will occur during an individual's lifetime (Leis and Livingston, 1996). Mutations in the proto-typical tumor suppressor genes generally behave in a recessive manner at the molecular level and therefore it is only when both copies of the gene become inactivated that an abnormal phenotype will manifest in the cell (Leis and Livingston, 1996). It is due to this recessive genetic behavior that a single mutant allele of such a gene can be passed through the germ line which is tolerated during embryogenesis because its presence is revealed in a tissue only when the remaining wild-type allele is lost (Leis and Livingston, 1996).

Oncogenic mutation or loss of tumor suppressor function may result in cancer cells acquiring a proliferative potential that does not necessarily require an external inducing signal. In addition, these cells may fail to sense particular signals that restrict cell division and continue to grow within the tissue when they should otherwise

differentiate or die. Furthermore, cancer cells often alter their attachment to adjacent cells and to extracellular matrix in order to gain the potential to migrate and invade into surrounding tissues.

Tumor cells must develop a blood supply in order to survive in the tissue, a process known as angiogenesis. Angiogenesis is a complex phenomenon of neovascularization that facilitates provision of nutrients to sustain the continued growth and survival of tumor cells. Without this, tumor growth would be severely limited. Angiogenesis requires several discrete steps; degradation of the basal lamina surrounding a nearby capillary, migration of endothelial cells lining the capillary into the tumor, endothelial cell division, and the formation of a new basement membrane around the newly elongated capillary. During the early stages of cancer, an abundant amount of new capillaries usually exists along the basement membrane below the transformed epithelium (Weinberg and Hanahan, 1996). However, if neovascularization persists in an invasive cancer, then the capillaries have the ability to breach the basement membrane and invade through the stroma (Weinberg and Hanahan, 1996). Interestingly, it is thought that transforming growth factor β (TGF-β) may play an important role in angiogenesis because along with other angiogenic factors, cells release TGF-β during development and tissue repair as part of a mechanism that promotes the vascularization of normal tissues (Weinberg and Hanahan, 1996).

The majority of benign tumors pose little risk to the host because they remain localized and are small. The cells that compose a benign tumor resemble and may even function like normal cells while maintaining cell adhesion and thereby restricting the

tumor to the site of origin. In contrast, malignant tumor cells have the capability of dividing rapidly compared to normal cells, as well as enhanced survival. Thus, malignant cells usually exhibit the characteristics of rapidly growing cells, that is, high nucleus-to-cytoplasm ratio, prominent nucleoli, and relatively little specialized structure. The major characteristics that differentiate malignant tumors from benign lesions are their invasiveness and ability to spread to local, regional and distant body sites.

1.2. Metastasis

Metastasis occurs when a tumor invades into the surrounding tissue, gets into the circulatory or lymphatic systems, allowing the cells to spread to regional or distant sites and facilitating the establishment of secondary foci of proliferation. Invasion of the underlying connective tissue proceeds in stages and is mediated by various factors produced by the tumor cell (Weinberg and Hanahan, 1996). For metastasis to occur, tumor cells must release proteases such as collagenase IV, which dissolves the collagen in the basement membrane allowing the tumor to penetrate the subjacent stroma. These invasive cancers express membrane-localized receptors for laminin and fibronectin, which are large glycoprotein components of the basement membrane and connective tissue stroma, respectively. It is the binding of these elements that provides the tumor cells with a lattice for anchorage and attachment. Enzymes such as plasminogen activators, collagenases I, cathepsins, heparanase, and hyaluronidase are released by the tumor cells and destroy the matrix constituents, thereby enabling the cells to advance further into the connective tissue (Weinberg and Hanahan, 1996).

However, fewer than 1 in 10,000 cells that escape from the primary tumor site survive to colonize another tissue and form a secondary, metastatic tumor. In the case of hematogenous spread, cells that escape the primary tumor must adhere to the endothelial lining of a capillary and migrate across or through it to the underlying tissue in order to enter circulation. In addition, tumor cells secrete autocrine motility factors that direct their migration, as well as vascular permeability factors that allow plasma proteins to accumulate in the tumor and angiogenic factors that increase the vascularity of the tumor (Weinberg and Hanahan, 1996).

As the tumor develops, the cells become very well adapted to growth and invasion of the surrounding tissues. The invasion of tissues is nonrandom, depending on the nature of both the metastasizing cell and the invaded tissue. It is thought that the tumor cells preferentially invade along the pathways that provide the least resistance, such as the connective tissue stroma (Weinberg and Hanahan, 1996).
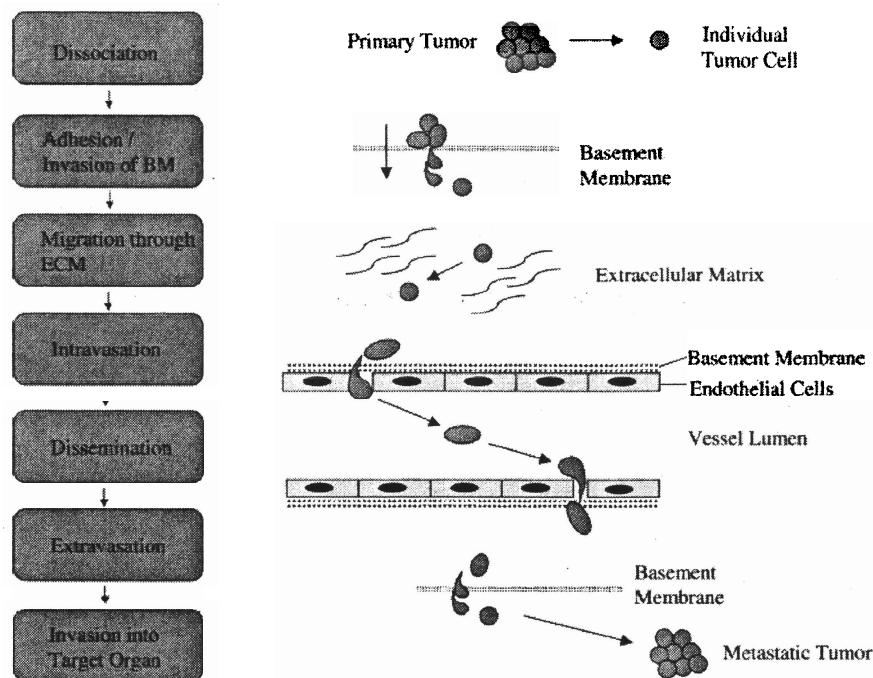
Figure 1: Sequential steps of the metastatic cascade. The process of metastasis begins when an individual tumor cells detaches from the primary tumor. The progression to metstasis requires individual tumor cells adhering and invading through the basement membrane, migrating through extracellular matrix, and intravasating into blood or lymphatic vessels, where the tumor cells can disseminate to distant sites. In addition, the tumor cells must extravasate out of the vessel and invade into the target organ, which forms a metastatic tumor. (Howell and Grandis, 2005)

1.3. Head and Neck Cancer

Squamous cell carcinoma of the head and neck region (HNSCC) is the sixth most common malignancy worldwide, representing 6% of all cancers. Each year approximately 40,000 individuals in the United States are diagnosed with HNSCC (Kim and Califano, 2004) and more than two-thirds of these cases will present with advanced stages (stage III and IV) (AJCC, 1983). The 5-year survival rate has remained largely

unchanged over the last two decades at 50% despite the significant improvements in the treatment of this disease that has decreased morbidity (Kim and Califano, 2004). Studies have shown that if the patient survives the initial lesion, a constant and continuing risk (from 2.7-4% per year) of secondary primary tumor formation exists following the initial treatment (Jovanovich *et al.,* 1994). The probability of a second malignancy forming within five years after the presentation of the initial tumor may be as high as 22% (Dhooge *et al.,* 1998).

Head and neck squamous cell carcinoma (HNSCC) is a locally aggressive malignancy that may develop after years of prolonged abuse of alcohol and tobacco products. Exposure to these particular agents results in alterations in genes that are important for the regulation of various cellular functions, which allows tumor cells to survive and grow in an uninhibited manner, as discussed above (section 1.1). Some of these important functions include the acquisition of immortality, the ability to invade tissue and/or metastasize to other sites, and acquiring the ability to induce angiogenesis (Hasina and Lingen, 2001).

HNSCC generally responds inadequately to chemotherapeutic and radiotherapeutic measures; therefore, surgery remains the primary treatment. However, patients with locally advanced, operable (HNSCC) are known to be at high risk of treatment failure, which ranges from local regrowth to lymphatic spread and systemic dissemination (Bernier and Cooper, 2005). HNSCC lesions are locally aggressive and frequently metastasize to local and regional lymph nodes. Therefore, treatment of HNSCC must address not only the initial primary lesion, but also early diagnosis of high-

risk pre-malignant lesions to prevent malignant development and progression. Poor long-term survival is due to numerous variables including delayed diagnosis as well as the development of multiple primary and secondary tumors. Thus, in addition to early detection, continued emphasis must be placed on preventing the development of secondary tumors as well as establishing more effective treatments for individuals who present with advanced disease.

1.4. Intermediate Filaments and Cancer

The intermediate filament protein family consists of at least 65 distinct proteins, in which all assemble into 10 nm wide filaments and serve as prominent structural elements both in the nucleus and the cytoplasm (Herrmann and Aebi, 2004). The family comprises both nuclear (lamins) and cytoskeletal proteins such as cytokeratins, vimentin, desmin, glial fibrillary acidic protein, neurofilaments, internexin, nestin, and peripherin (Steinert and Leim, 1990). Two other principal structural elements of the eukaryotic cell cytoskeleton are microtubules and microfilaments. Compared to both microtubules and microfilaments, which often break when subjected to shear stress, intermediate filaments become viscoelastic and are flexible (Janmey *et al*, 1998). The molecular building blocks of intermediate filaments are fibrous proteins that consist of long, uninterrupted segments of α-helices. Since single α-helical chains are unstable in aqueous solution, they often adopt a rope-like structure by forming multistranded left-handed coiled coils (Watson and Crick, 1953). With the use of electron microscopy, it has been shown that coiled-coil intermediate filaments protein dimers are 45 to 50 nm long rod-like molecules.

Intermediate filament proteins may be useful for the diagnosis of certain tumors because of their distinct expression patterns. For example, in some malignant tumors the cells lose their normal appearance and, therefore, their origin is unable to be identified purely on morphological grounds. However, the expression of particular intermediate filament proteins is one of the differentiated properties of the cell that may be retained in tumor cells. Hence, with the use of antibodies specific for the intermediate filament protein of interest, researchers can determine exactly where the tumor originated, epithelial, mesenchymal, or neuronal tissue. Identifying the intermediate filament protein in a tumor cells may allow physicians to select the most effective treatment because epithelial and mesenchymal malignancies may be sensitive to different therapeutic regimes.

1.4.1. Vimentin

The fact that intermediate filaments span from the nucleus to the cell membrane suggests that intermediate filaments have the potential to play significant roles in the structural stability of the cell and the ability to transmit and/or transduce mechanical signals into biological responses (Coulombe *et al.*, 2000). Vimentin, which is a major structural component of intermediate filaments in many different cell types, has been shown to play a significant role in essential mechanical and biological functions including cell contractility, migration, and proliferation (Wang and Stamenovic, 2002).

Live imaging of cells expressing the GFP-vimentin fusion protein revealed that the vimentin network is motile, with the filaments constantly changing the shape of the

cell (Yoon *et al.*, 1998; Helfand *et al.*, 2002). It is the motile properties of vimentin filaments that are particularly evident in the spreading cell, in which three different structural forms of vimentin are involved in the assembly of the vimentin network (Prahlad *et al.*, 1998).
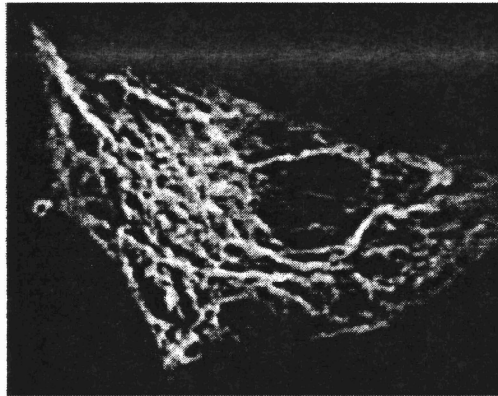
Figure 2: GFP-vimentin in a living endothelial cell. (Helmke *et al.*, 2000)

1.4.2. Vimentin and Epithelial to Mesenchymal Transition

Vimentin serves as an appropriate marker for EMT because it is an established marker for the mesenchymal cell and is not normally expressed in cells of epithelial origin. EMT is one feature that may characterize the progression of a tumor toward a highly malignant phenotype, and involves numerous changes in gene expression that result in the substitution of epithelial characteristics for those of the mesenchymal cell (Gilles and Thompson, 1996). Typically, cells that have undergone EMT exhibit a spindle-shaped morphology with organized cytoskeleton, reduced cellular adhesions, and

the expression of specific mesenchymal cell markers such as vimentin. This correlation between EMT and increased vimentin expression has been shown in a variety of tumor cells, including invasive breast cancer cells (Korsching *et al.*, 2005) and liver metastasis of a pancreatic carcinoma (Nakajima *et al.*, 2004). In addition, it has been shown that many growth factors and cytokines induce EMT (Boyer *et al.*, 2000; Thiery, 2002).

1.4.3. <u>Cytokeratin and Epithelial to Mesenchymal Transition</u>

Whereas vimentin is turned on during EMT, a normal epithelial specific intermediate filament, cytokeratin, is downregulated and/or switched to a different isoform (Wu *et al.*, 2003). In vivo studies and experimental models show that epithelial cells are polarized, display cytokeratin filaments and membrane-associated specialized junctions such as demosomes and adherens junctions (Hay, 1995; Savagner *et al.*, 1994). It is after EMT when these epithelial features are lost and the acquisition of mesenchymal characteristics occurs, which includes vimentin filaments and a flattened phenotype (Hay, 1995; Savagner *et al.*, 1994). Therefore, cytokeratins are highly regulated during EMT and progressively vanish from within the cell when the vimentin level inside the cells increases and adopts a mesenchymal phenotype (Boyer *et al.*, 1989).

It has been proposed that EMT represents a permanent switch from cytokeratin to vimentin in certain tumors (Gilles *et al.*, 1997), consistent with the findings of an androgen-independent model of prostate cancer which demonstrated an increase in vimentin expression of the metastatic cell line accompanied by a decrease in the levels of cytokeratin (Singh *et al.*, 2003) as well as mammary epithelial cells that underwent EMT

in response to transfection with the matrix metalloproteinase stromelysin 1 (Lochter *et al.*, 1997).
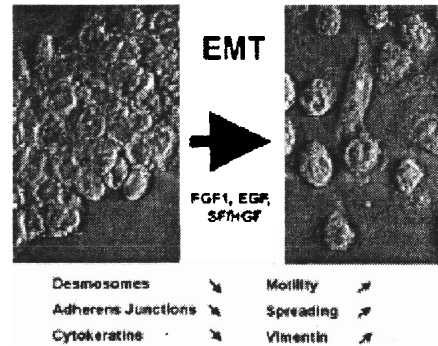
Figure 3: In vitro EMT in carcinoma cells. After EMT, the cells express mesenchymal cell markers such as vimentin, become individualized, spread, and migrate. (Savagner, 2001)

1.4.4 Vimentin Gene Expression

Vimentin expression is upregulated in some metastatic tumor cells, making it a potential marker of oncogenic progression. The promoter of the vimentin gene contains multiple elements responsible for its complex transcriptional regulation, including a TATA-box, several positive regulatory elements that include eight GC-boxes (Rittling and Baserga, 1987), a PEA3 site (Chen *et al.*, 1996), an NF-κB site (Lilienbaum and Paulin, 1993), Δ19 site (Salvetti *et al.*, 1993), a PS element (Wieczorek *et al.*, 2000), two tandem AP-1 binding sites (Rittling *et al.*, 1989), a β-catenin site (Gilles *et al.*, 2003), an antisilencer (ASE) element (Izmailova and Zehner, 1999), and a proximal silencer element (Salvetti *et al.*, 1993). Determining what controls vimentin expression could

lead to an understanding of what contributes to the changes in gene expression that occurs during transformation to the malignant state.
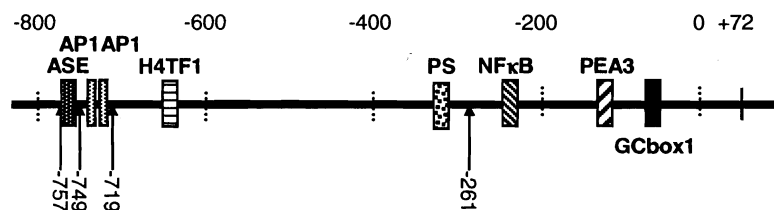
Figure 4: A diagram of the relative position of vimentin's previously reported regulatory elements; Gc-box1, PEA3, NF-κB, PS, H4TF-1, tandem AP-1 binding sites, and ASE.

1.5. Growth Factors

Growth factors interact with specific protein receptors at the cell surface, which are generally proteins that traverse the lipid bilayer of the plasma membrane. The growth factor receptors contain an external ligand-binding domain and a cytoplasmic domain, which triggers further biochemical events within the cell. The term "signal transduction" is used to characterize this process in which binding of the growth factor leads to the activation of specific intracellular pathways. The activated receptor induces a change at the cytoplasmic side of the membrane by either becoming enzymatically active or by interacting with other regulator proteins at the plasma membrane. This leads to a series of events in the cytoplasm; for example, changes in ion concentration, production of second-messengers, or activation of protein kinases, which are the enzymes that

phosphorylate other protein substrates. These cytoplasmic signals can then alter gene expression within the nucleus. (Bishop and Weinberg, 1996)

In normal tissue, adjacent cells or distant cells that have the ability to produce and release growth factors control the amount of growth factor available to the cell, which ensures normal levels of proliferation. However, certain cells have the ability to undergo autocrine stimulation in which they have the capability of expressing both the growth factor and the receptor. Therefore, once the growth factor is secreted it can be considered to be constantly in the active state because subsequent binding of the growth factor to the receptor stimulates a cellular response. Therefore, normal regulatory events that prevent prolonged interaction of the growth factor and its receptor are bypassed, thereby contributing to cellular transformation. (Pawson, 1996)

1.6. Transforming Growth Factor-Beta

1.6.1. Introduction to Transforming Growth Factor-Beta

The mammalian transforming growth factor-beta (TGF-β) family consists of five isoforms: TGF-β1, TGFβ-2, TGFβ-3, TGFβ-4 and TGFβ-5. All are secreted and activated in the extracellular environment and bind to receptors at the cell surface receptors, of which there are three types: TGF-βI, TGF-βII, and TGF-βIII. The TGF-β isoforms share 64-82% similarity at the amino acid level, largely due to a conserved cysteine knot structure that is characteristic of the TGF-β monomer (Archer *et al.*, 1993; Daopin *et al.*, 1992; Schlunegger and Grutter, 1992). Interestingly, the type III receptor

is also known as a betaglycan and can exist in a soluble form that has the ability to bind two TGF-β molecules (Bachman and Park, 2005). Therefore, it is this increased binding capacity which allows a greater concentration of TGF-β molecules to be present at the cell surface, thus maximizing the interaction of TGF-β with type I and type II receptors (Lopez-Castillas, *et al.*, 1993) which transduce the signal to the intracellular environment.

Recent advances in molecular biology reveal that tumor development requires the accumulation of a particular set of cellular characteristics. These include the ability to grow independently of exogenous growth factors, to divide indefinitely, to grow new blood vessels, and to invade surrounding tissues. The TGF-β signaling pathway regulates many of these cellular processes, and tumor cells may exploit alterations in TGF-β signaling cascade to promote malignant progression (Rich *et al.,* 2001).

1.6.2. TGF-β - Tumor Suppressor or Tumor Promoter?

Normal ➡ **Benign** ➡ **Malignant** ➡ **Metastatic**

TGF-β overexpression
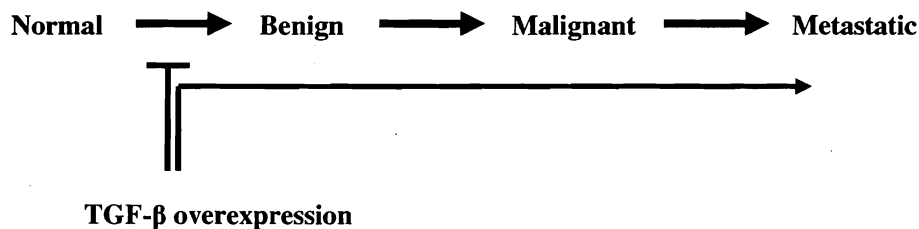
Figure 5: Model of TGF-β action in cancer.

TGF-β is a cytokine that is known for its ability to inhibit epithelial proliferation. However, many epithelial malignancies acquire a resistance to the growth-inhibitory

effects of TGF-β. Therefore, it is thought that this resistance is part of a particular signaling event is which TGF-β loses its growth inhibitory effects and is then used by the epithelial cells to promote cell growth (Bachman and Park, 2005).

It is important to note that in some model systems, mutated TGF-β receptors appear to block TGF-β signaling completely because neither growth-inhibitory nor growth-promoting responses are observed (Bachman and Park, 2005). Therefore, it is thought that the decision of the cancer cell to use TGF-β as a tumor suppressor instead of as a tumor promoter is not mediated at the level of the ligand/receptor interaction.

Over the last decade, numerous studies have demonstrated that TGF-β1 not only maintains transforming potential but can also drive malignant progression, invasion and metastasis both *in vitro* and *in vivo* (Derynck *et al.,* 2001). It has now become clear that TGF-β can act as both a tumor suppressor in which three isoforms bind to the same type II receptor (Akhurst and Derynck, 2001) and as a significant stimulator of tumor progression, invasion, and metastasis (Cui *et al.*, 1996).

At the early stages of tumorigenesis when the lesion is still benign, TGF-β acts directly on the cancer cells to suppress tumor growth. However, as the tumor progresses, genetic and/or biochemical changes allow the TGF-β to stimulate tumor progression on both the cancer cell and on the non-malignant stromal cell types on the tumor (Akhurst and Derynck, 2001). It is this stimulation and metastasis caused by TGF-β that might be of greater clinical significance because a majority of tumors retain the functional TGF-β signaling pathway.

1.6.3. TGF-β Signaling Pathway in Tumor Progression

Two general mechanisms may explain the contribution of TGF-β signaling to enhance tumor progression, invasion, and metastasis. First, there can be an early loss of signaling components, such as the type II TGF-β receptor, leading to rapid tumor cell growth. It is this increased cell division that also potentiates the probability of further cancer-causing mutations and cytogenetic changes that have the ability to drive tumor progression. Second, the TGF-β signaling pathway can remain intact, but become disrupted by other mechanisms such as deregulated Smad signaling. The altered Smad signaling leads to a direct increase in tumor cell plasticity, invasion, and metastasis. (Akhurst and Derynck, 2001)

1.6.4. TGF-β and Smad Signaling
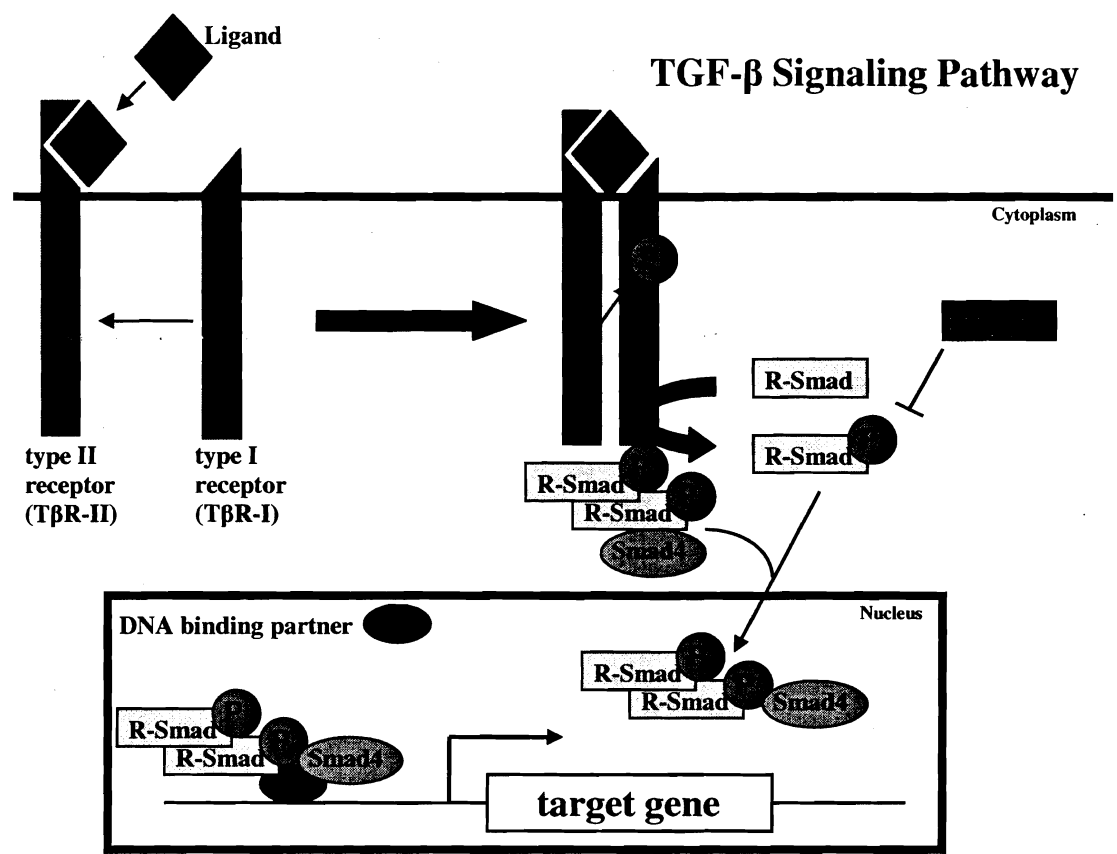
Figure 6: General mechanism of TGF-β receptor and Smad activation.

TGF-β is secreted from cells as a biologically inactive latent form that requires some processing to generate the active growth factor. At the cell surface, TGF-β binds to a complex of transmembrane receptor serine/theorine kinases (type I and II) and induces the phosphorylation of the GS segment on the type I receptor by the type II kinases. This heterodimeric transmembrane receptor complex is composed of type II (TβRII) and type I receptors (Alk5/TβRI) (Glick, 2004). The type II receptor is a constitutive kinase and capable of binding TGF-β1 on its own (Glick, 2004). However, the type I receptor

kinase is only activated after complex formation and trans-phosphorylation by the type II

receptor (Glick, 2004).

Ligand	Type II	Type I	R-Smad
TGF-β	TβRII	**ALK-5** (TβRII)	**Smad2**
		ALK-1	**Smad3**
		ALK-2	Smad1
			Smad5

Table 1: Combinatorial interactions of type II and type I receptors define the signaling responses.

The activated type I receptor phosphorylates selected Smad proteins at serine

residues within the C-terminal region. These receptor-activated Smads (R-Smads) then

form a complex with Smad4 (Derynck and Zhang, 2003). Smad2 and Smad3 are the

major substrates for the activated type I kinase (Glick, 2004). These proteins are inactive

cytoplasmic transcription factors until they become phosphorylated by the ligand-bound

receptor complex, at which point they bind to Smad4 and translocate to the nucleus

(Glick, 2004). R-Smads and Smad4 shuttle back and forth between the nucleus and

cytoplasm (Derynck and Zhang, 2003).

Upon translocating to the nucleus, Smad complexes regulate transcription of

target genes through physical interaction and functional cooperation with DNA-binding

factors or coactivators such as CREB-binding protein (CBP) and p300 (Derynck and Zhang, 2003). Positive and negative regulation of transcription occurs with the cooperation of other DNA binding proteins and transcription factors at both the Smad binding elements (SBE) in the promoter regions of target genes and at the binding sites for other transcription factors (Derynck and Zhang, 2003).
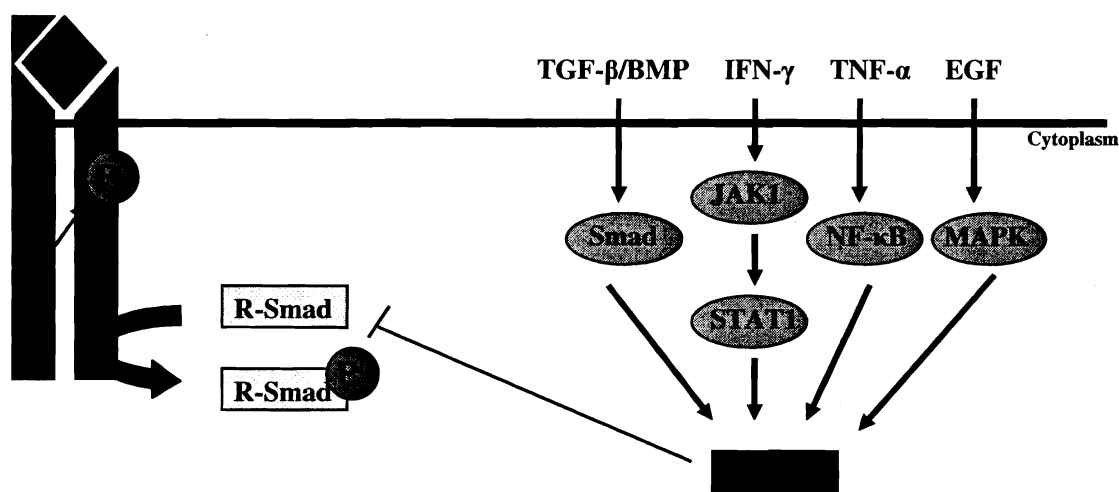
Figure 7: R-Smad activation is regulated by receptor interacting proteins and Smad6/7.

Interestingly, activation of R-Smads by type I receptor kinases is inhibited by Smad6 or Smad7 (Derynck and Zhang, 2003). In contrast to R-Smad expression, expression of the inhibitory Smad6 or Smad7 is highly regulated by extracellular signals (Derynck and Zhang, 2003). Induction of Smad6 and Smad7 expression by BMP and TGF-β respectively represents an auto-inhibitory feedback mechanism for ligand-induced signaling (Massague et al., 2000; Itoh et al., 2000; Moustakas et al., 2001). Activation of

EGF receptor and possibly other tyrosine kinase receptors, interferon-γ (INF-γ) signaling through Stat (signal transducer and activator of transcription) proteins, and activation of NF-κB by tumor-necrosis factor-α (TNF-α), also induce Smad7 expression, which leads to inhibition of TGF-β signaling (Massague *et al.*, 2000; Itoh *et al.*, 2000; Moustakas *et al.*, 2001).

1.6.5. TGF-β signaling through Smad Independent Pathways

Other proteins can associate with type I or type II receptors and regulate TGF-β ligand signaling without the direct effect of Smad activation. The activated receptor complexes activate non-Smad signaling pathways, such as TAK1/MEKK-1, Ras, RhoA, PP2A signaling pathways (Derynck and Zhang, 2003).

1.6.6. TGF-β and EMT

TGF-β1 is a ubiquitous cytokine that was originally named for its ability to transform normal fibroblasts in culture. Subsequently, TGF-β1 was found to inhibit growth of normal epithelial cells, although it was also discovered that TGF-β1 could induce tumor cells to proliferate and promote an invasive phenotype and the ability to metastasize by mediating changes in the cytoskeletal architecture, which is known as EMT (Massague *et al.*, 2000). Autocrine TGF-β expression by tumorgenic cells induces matrix degradation, downregulates basement membrane components, and cell-cell adhesion molecules; such as E-cadherin, and induces an invasive, motile phenotype (Glick, 2004).

It has been shown that TGF-β can induce morphological changes characteristic of EMT in normal and transformed mammary epithelial cells in culture (Oft *et al.*, 1996; Miettinen *et al.*, 1994). Furthermore, these experiments have also shown that this cytoskeletal reorganization is accompanied by the downregulation of adhesion and cytoskeletal proteins such as E-cadherin and keratins, and induce *de novo* expression of the mesenchymal intermediate filament protein, vimentin (Oft *et al.*, 1996; Miettinen *et al.*, 1994). Since these initial experiments, TGF-β-induced EMT has also been reported in epidermal squamous cell carcinoma (Portella *et al*, 1998), in ras-transformed mammary carcinoma cells (Oft *et al.*, 1996) and in ovarian adenosarcoma cells (Kitagawa *et al.*, 1996).

1.6.7. TGF-β Stimulates Angiogenesis

As mentioned earlier (section 1.1), tumor angiogenesis is critical for tumor growth and invasion because this process facilitates the delivery of nutrients and oxygen to the tumor cells allowing sustained, rapid growth. The mechanism of angiogenesis simulation by TGF-β1 is a combination of direct and indirect effects. Directly, TGF-β induces the expression of angiogenesis-inducing vascular endothelial cell growth factor (VEGF) (Pertovaara *et al.*, 1994). TGF-β also can directly induce capillary formation of the endothelial cells cultured on a collagen matrix (Madri *et al*, 1998). Indirectly, TGF-β induces expression of the metalloproteases MMP-2 and MMP-9, as well as downregulation of tissue inhibitor metalloproteases (TIMPs) in tumor and endothelial

cells, thus providing a protease-rich microenvironment that is conducive to the enhanced migration and invasion of active endothelial cells (Hagedorn *et al.*, 2001).

1.6. Epidermal Growth Factor

1.6.1. Epidermal Growth Factor Introduction

Epidermal Growth Factor Receptor (EGFR) is a receptor tyrosine kinase that is overexpressed in a number of human epithelial malignancies, including carcinomas of the lung, colon, ovary, bladder, and head and neck (Bishop and Weinberg, 1996). EGFR stimulates several properties that are critical for tumor progression, including proliferation, cell motility, cell adhesion, invasion, cell survival, and angiogenesis (Grandis and Sok, 2004).

EGFR is a 170-kDa plasma membrane glycoprotein containing an extracellular ligand-binding domain, a single transmembrane domain, an intracellular domain with intrinsic tyrosine activity, and a C-terminal tail that contains specific tyrosine containing sequences that become binding sites for SH2-containing signaling proteins upon phosphorylation. EGFR is one of a family of four structurally similar receptors that make up the erbB family. These receptors show homology in their kinase domains but are different in their extracellular regions and C-terminal tails. The erbB family of receptors are widely expressed in a variety of tissues and regulate different functions, including mitogenesis, differentiation, and cell survival and can undergo homo- (thick arrows) or heterodimerization (thin arrows) both constitutively and in response to the presence of

specific ligands resulting in the potential to activate many signaling pathways (Grandis and Sok, 2004).

Ligands {
EGF
TGF-α
Amphiregulin
HB-EGF
β-Cellulin
Epiregulin
}

EGFR Signaling Network

EGFR/ErbB Receptors

STATs   MAPK   PI3K   PLC
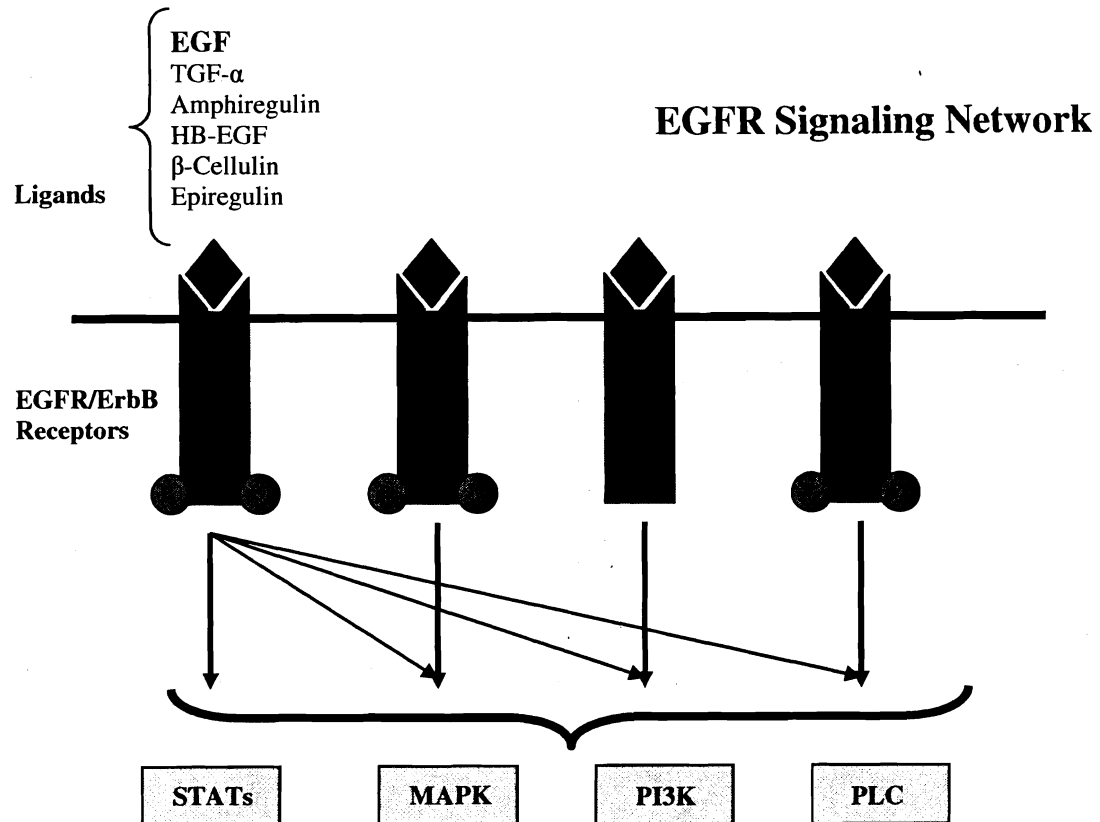
Figure 8: EGFR Signaling Pathway.

Specificity of signaling is generated, at least in part, through the range of ligands and the intracellular protein-protein interactions that occur downstream of these erbB receptors. The selective activation of the signaling transduction pathways (yellow boxes) depends on the various arrangements of the ligand-erbB engagement, tyrosine

phosphorylation, and subsequent receptor dimerization combinations beneath the cell surface (Grandis and Sok, 2004). In addition, for example, several ligands bind to EGFR/erbB1, including epidermal growth factor (EGF), transforming growth factor-alpha (TGF-α), and amphiregulin (Grandis and Sok, 2004).

1.7.2. Epidermal Growth Factor and Head and Neck Cancer

The up-regulation of EGFR expression and activity has been described in a number of epithelial tumors, including HNSCC (Howell and Grandis, 2005). 80-100% of head and neck tumors express EGFR (Grandis et al., 1998). Furthermore, EGFR/erbB1, erbB2, and erbB3 have each been independently correlated with the presence of nodal metastases and a shortened length of survival (Do et al., 2004; Xia et al., 1999). Studies have provided support for the prominent role of EGFR in controlling HNSCC invasion and metastasis and indicate EGFR as being one of the strongest predictors of survival and locoregional relapse (Ang et al., 2002; Grandis et al., 1998). Upregulation of EGFR is thought to influence important steps in tumor invasion and metastasis by enhancing cell motility, regulating cytoskeletal changes, altering cell adhesion, and directing the production of matrix-degrading proteolytic enzymes though downstream signaling effector molecules such as phospholipase C gamma-1 (PLCγ-1) and focal adhesion kinase, in addition to promoting angiogenesis (Khazaie et al., 1993).
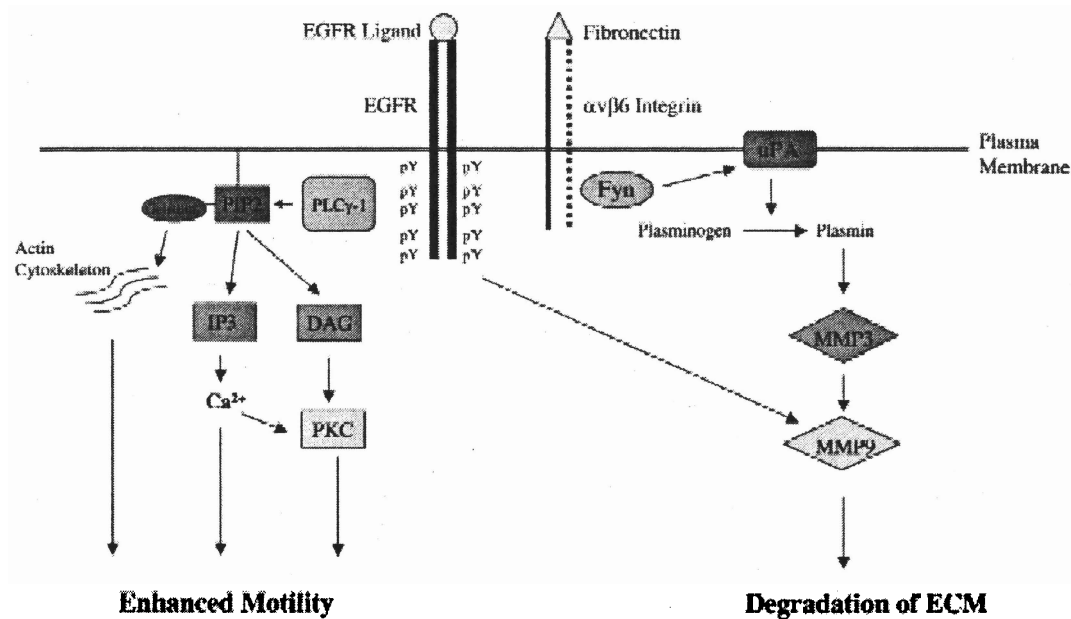
Figure 9: Signal transduction elements important to HNSCC invasion and metastasis. (Howell and Grandis, 2005)

1.7.3. Matrix Metalloproteinases in Head and Neck Cancer

EGFR activation is known to increase activation of matrix metalloproteinase (MMPs), in particular MMP-9 (Howell and Grandis, 2005). MMP-9 belongs to the MMP family of zinc dependent endopeptidases, which have been associated with tumor cell invasion, metastasis, and tumor-induced angiogenesis (Fridman *et al.*, 2003). In addition, the MMP family member pro-MMP-9, has been shown to be a key enzyme in tumor progression playing a significant role in tumor cell invasion and in angiogenesis (Fridman *et al.*, 2003). As a secreted MMP, pro-MMP-9 is released into the extracellular environment by tumor and stroma cells, degrading both the ECM matrix and non-ECM

proteins, thus participating in events that are required for tumor cell migration and tumor-induced angiogenesis (Fridman *et al.*, 2003).

An elevated EGFR level in HNSCC tumors correlates with the expression of MMP-2, MMP-3, MMP-7, MMP-9, and MMP-10 (Howell and Grandis, 2005). MMP-2 and MMP-9 overexpression in tumor cells has been shown to correlate with invasion, metastasis, and poor prognosis (Kawata *et al.*, 2002; Ikebe *et al.*, 1999; Hong *et al.*, 2000). In addition, EGF has been shown to increase HNSCC invasion through a basement membrane substitute and upregulate the expression of MMPs in HNSCC cell lines, again with a significant upregulation of MMP-9 (O-Charoenrat *et al.*, 2000). Further, studies have demonstrated a failure of MMP upregulation, migration, and invasion in HNSCC tumor cell lines on EGFR blockade (O-Charoenrat *et al.*, 2000).
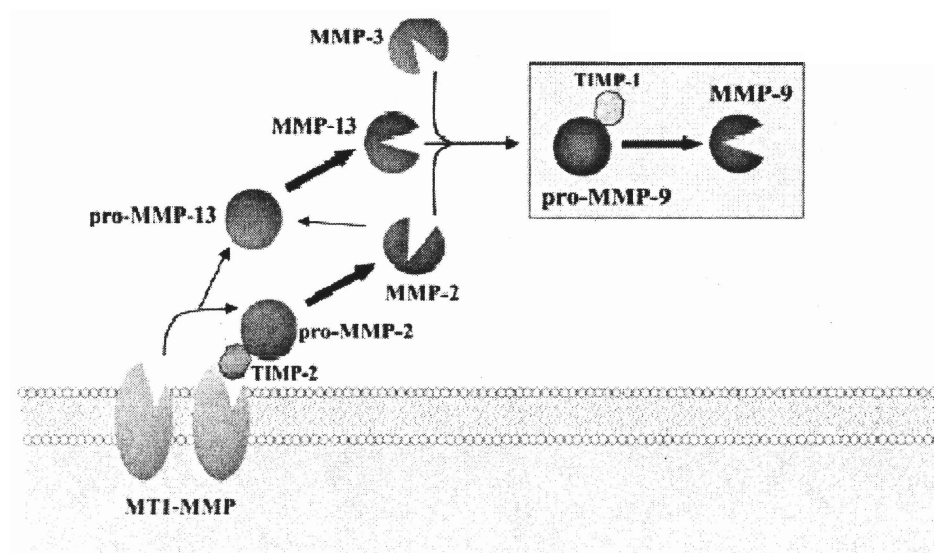
Figure 10: MMP cascade of zymogen activation involved in pro-MMP-9 activation. (Fridman *et al.*, 2003)

Pro-MMP-9 can be activated by several different MMPs, including MMP-3 (Ogata *et al.*, 1992), MMP-2 (Fridman *et al.*, 2003), and MMP13 (Knauper *et al.*, 1997. This cascade of zymogen activation is initiated by MT1-MMP located at the cell membrane and requires the activation of TIMP-2. With the generation of MMP-2 and MMP-13, it can activate pro-MMP-9 (Fridman *et al.*, 2003).

1.7.4. Phospholipase C gamma (PLCγ-1) and Head and Neck Cancer

PLCγ-1 is a ubiquitously expressed phosphoinositide-specific phospholipase that is activated downstream of EGFR (Wells and Grandis, 2003). PLCγ-1 is activated by many growth factors including EGF and is required for cell motility (Howell and Grandis, 2005). Since cell migration is critical for metastatic progression of tumor cells, it is thought that the overexpression of PLCγ-1 in HNSCC could contribute to invasion and metastasis. Interestingly, PLCγ-1 regulates cell motility in response to growth factors through the activation of actin-modifying proteins such as gelosin, and through the hydrolysis of phosphoinositide (4,5) bisphosphate (PIP2) into diacylglycerol (DAG) and inositol triphosphate (IP3), which are significant in the regulation of actin cytoskeletal alterations and adhesion/de-adhesion mechanisms (Wells and Grandis, 2003).

Increased total and phosphorylated PLCγ-1 has been reported in HNSCC tumors compared with normal adjacent mucosa (Thomas *et al.*, 2003). Furthermore, other studies have shown that inhibition of PLCγ-1 signaling significantly reduces *in vitro* invasion through matrix barriers, suggesting that PLCγ-1 plays a role in HNSCC invasion and metastasis (Thomas SM *et al.*, 2003). Therefore, the upregulation of PLCγ-1 is one downstream signal transducer that may mediate invasion and metastasis in HNSCC and other epithelial tumors.

1.7.5. EGFR and Focal Adhesion Kinase

Focal Adhesion Kinase (FAK) is upregulated in several tumor types, including HNSCC (Aronsohn *et al.*, 2003). FAK is an intracellular nonreceptor tyrosine kinase, which associates with integrins within cellular structures referred to as focal adhesions and becomes phosphorylated and activated during integrin-mediated cell adhesion to extracellular matrix (ECM) ligands (Howell and Grandis, 2005). Furthermore, FAK is an important mediator of growth factor signaling, cell proliferation, cell survival, and cell migration (McLean *et al.,* 2005).

FAK expression and its activity are frequently correlated with malignant or metastatic disease and poor patient prognosis (Recher *et al.*, 2004; Schlaepfer *et al.*, 2004). Previous studies have shown an association between the enhanced expression and phosphorylation of FAK and increased invasiveness and the metastatic ability in HNSCC cell lines (Schneider, *et al.*, 2002; Kornberg, 1998). In addition, gains in the FAK gene

copy number have been found in cells derived from head and neck cancer (Agochiya *et al.*, 1999).
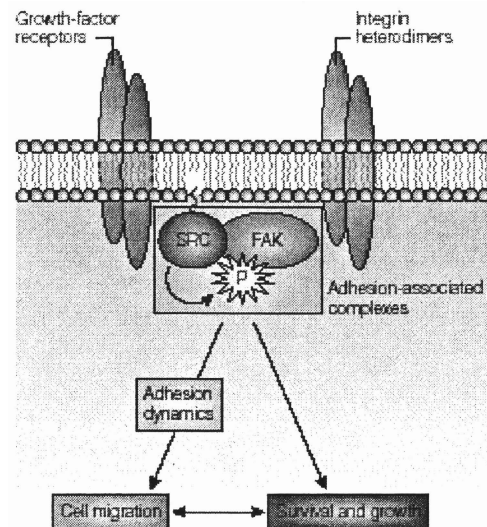
Figure 11: Focal-adhesion kinase as a signal integrator. (McLean *et al.*, 2005)

FAK carries out protein-protein interaction adaptor functions at the sites of cell attachment to the ECM, which contributes to focal-adhesion and transmits adhesion-dependent and growth factor-dependent signals into the cell interior (McLean *et al.*, 2005). FAK integrates signals from extracellular cues, including growth-factor receptors and integrins, and from the upstream SRC-family kinases, to control and coordinate adhesion dynamics/cell migration with survival signaling (McLean *et al.*, 2005).
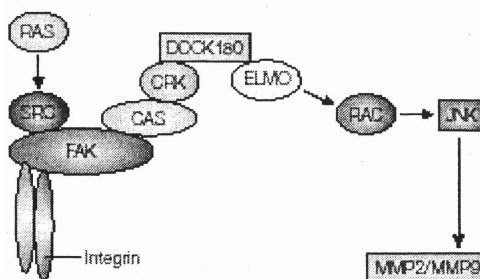
Figure 12: Focal-adhesion kinase influences cell migration through additional molecular signaling pathways. (McLean et al., 2005)

FAK mediated signaling events induce the expression of genes encoding MMPs (McLean *et al.*, 2003). As stated previously (section 1.7.3), once the MMPs are secreted by the cell, they have the ability to mediate the breakdown of surrounding ECM substrates and promote cell invasion (McLean *et al.*, 2003).

1.7.6. EGFR and Angiogenesis

EGFR activation has been linked with an increase in angiogenesis and metastasis. It has been proposed that EGFR-mediated signaling upregulates the expression of VEGF and interleukin-8 (IL-8) by inducing downstream signaling pathways, which promote the coactivation of transcription factors for IL-8 and VEGF expression, including nuclear factor kappaB (NF-κB) and activator protein-1 (AP-1) (Bancroft *et al.*, 2001). VEGF and IL-8 are proangiogenic factors that are upregulated and coexpressed in HNSCC tumors, and are associated with aggressive tumor growth and decreased survival rate (Eisma *et al.*, 1999; Bancroft *et al.*, 2001). AP-1 is thought to activate the transcription of both

VEGF and IL-8, whereas, NF-κB activates IL-8 transcription as well as being important for the expression of growth-regulated oncogene-1 (GRO-1), which is a neutrophil chemoattractant whose increased expression has also been associated with HNSCC tumor angiogenesis and metastasis (Bancroft *et al.*, 2001; Shintani *et al.*, 2004)

1.8. Aims of the Current Study

1. To determine how vimentin expression differs in primary and metastatic HNSCC.

2. To determine if vimentin expression is modulated by the invasion-inducing transforming growth factor β (TGF-β) and/or epidermal growth factor (EGF).

3. To determine if vimentin expression contributes to the invasive phenotype of metastatic HNSCC cells.

4. To determine what mechanisms regulate vimentin gene transcription in metastatic HNSCC.

2. <u>Paper Introduction</u>

In cancer progression, epithelial to mesenchymal transition (EMT) is associated with tumor invasiveness, and intravasation and extravasations of metastatic cells (reviewed in Thiery, 2002). For example, many invasive and/or metastatic tumors are characterized by partial or complete EMT, in which the epithelial phenotype of tight intercellular junctions and polarity across the epithelial layer is replaced by a more mesenchymal phenotype with reduced cell-cell adhesions, altered shape, expression of mesenchymal cellular markers, and enhanced cell motility (Ruiz and Gunthert, 1996; Boyer *et al.*, 2000; Wu *et al.*, 2004). EMT is now being recognized as a hallmark of tumor progression, characterizing invasive and metastatic carcinomas (Birchmeier *et al.*, 1996).

It has been suggested that the intermediate filament protein, vimentin, may be a potential diagnostic marker for the initial progression of cells from a localized epithelial lesion to become migratory, metastatic tumor cells (Gilles *et al.*, 1996). There are approximately fifty different types of intermediate filament protein that are categorized into five subtypes, with vimentin belonging to type III. Vimentin is normally expressed in the cells of mesenchymal origin; therefore, it serves as an appropriate marker for the transition of carcinoma cells from an epithelial to mesenchymal phenotype.

Vimentin displays a complex pattern of gene expression. Eukaryotic gene regulation studies suggest that multiple binding motifs are located within the human vimentin promoter, some of which bind factors that enhance gene expression and others that repress expression. These include: a TATA-box and several positive regulatory elements such as eight GC-boxes (Rittling and Baserga, 1987); a PEA3 site (Chen et al., 1996); an NF-κB site (Lilienbaum and Paulin, 1993); a Δ19 site (Salvetti et al., 1993); a PS element (Wieczorek et al., 2000); two tandem AP-1 binding sites (Rittling et al., 1989); a β-catenin site (Gilles et al., 2003); an antisilencer (ASE) element (Izmailova and Zehner, 1999); and a proximal silencer (PS) element (Salvetti et al., 1993).

It has been shown that binding of the GC-box (at position −64 to −55) is critical for the regulation of human vimentin gene expression (Izamailova et al., 1999). The c-Jun protein synergizes with the activator protein sp1 to enhance vimentin gene expression by binding to the GC-box1 (Wu et al., 2003). In addition to direct interaction with sp1, c-Jun also binds to tandem AP-1 sites located upstream in the vimentin promoter in order to activate vimentin gene expression (Wu et al., 2003). Interestingly, ZBP-89, a known Kruppel-like zinc finger protein, is capable of interacting with sp1 and inhibiting the activation of the vimentin promoter (Zhang et al., 2003). Furthermore, the PS silencer element located between positions −319 and −261 binds ZBP-89, which represses gene transcription (Wieczorek et al., 2000). An antisilencer element, ASE, located further upstream (position −757) binds Stat3 and can overcome ZBP-89-dependent repression (Wu Y., 2004). Interestingly, recent findings have shown that Stat3 is constitutively activated in many metastatic tumors from a range of cell lineages (Bromberg et al., 1999;

Bromberg *et al.*, 2001; Garcia *et al.*, 2001; Zajchowski *et al.*, 2001; Levy and Darnell, 2002).

EMT can be induced by a variety of growth factors, including EGF and TGF-β (Miettinen *et al.*, 1994; Bhowmick *et al.*, 2001; Piek *et al.*, 1999; Lu *et al.*, 2003), and a synergistic effect on EMT has been observed following treatment with a combination of EGF and TGF-β (Grande *et al.*, 2002). Interestingly, at the transcriptional level, EGF and TGF-β activate different binding sites on the vimetin promoter, EGF targeting the transcriptional binding protein, sp1 binding to GC-box and TGF-β targeting the tandem AP-1 sites. In addition, EGF and TGF-β synergistically activate the transcriptional binding protein, Stat3, which binds to the ASE site on the vimentin promoter.

Squamous cell carcinoma of the head and neck region (HNSCC) is the sixth most common malignancy worldwide. HNSCC lesions are locally aggressive and frequently undergo EMT, with metastasis to local and regional lymph nodes, which dramatically decreases the likelihood of a good clinical outcome. Thus, prevention, early detection, and the ability to prevent invasion and regional metastasis of the primary lesion are major goals to combat this disease. In order to develop a model system in which to investigate genetic, biochemical, and biological changes that occur during metastatic progression of head and neck squamous cell carcinoma, we established HN4 cells from a primary tongue tumor and HN12 cells from a synchronous lymph node metastasis and characterized their behavior (Yeudall *et al.*, 2005). Metastasis-derived HN12 cells are invasive *in vitro* and tumorigenic *in vivo*, whereas HN4 cells are not. In addition, invasion of HN12 cells is enhanced by epidermal growth factor (EGF; Yeudall *et al.*, 2005) and by transforming

growth factor β (TGF-β; Miyazaki *et al.*, in press). Comparing global gene expression profiles of HN4 and HN12 cells revealed that vimentin gene expression was found to be upregulated in metastasis-derived HN12 cells (Miyazaki *et al.*, 2005).

Determining the mechanisms of vimentin gene regulation will contribute to our understanding of processes involved in EMT and might, potentially, provide insight into factors controlling tumor cell invasiveness. In the present study, we have investigated regulation of vimentin expression by EGF and TGF-β in metastatic HN12 cells, and the contribution of vimentin overexpression to the cellular phenotype.

3. Materials and Methods

3.1 Cell Cultures

HN4 cells were derived from a primary squamous cell carcinoma of the head and neck region, while HN12 was derived from a nodal metastasis in the same patient from whom the HN4 cells originated. HNSCC cell lines were cultured in DMEM (Invitrogen Corporation, NY) supplemented with 10% fetal bovine serum (FBS), and penicillin - streptomycin (10ug/ml) (Mediatech, Cellgro) in the presence of puromycin (1μg/ml) at 37°C in 90% air/10% CO_2.

3.2 Plasmids

Targeting sequences for vimentin-shRNA plasmids were designed using web-based software. (http://www.ambion.com/techlib/misc/siRNA_finder.html) (Ambion, Austin, TX) and the sequences for sense/antisense oligonucleotides are as follows: vimentin-shRNA sense, 5'-GATCCGTAAGCACTAGCCACGACACTTTTCAAGAGAGTGTCGTGGCTAGTGCTTATTCTTTTTTTCTAGAG-3'; antisense, 5'-AATTCTCTAGAAAAAAAGAATAAGCACTAGCCACGACACTCTCTTGAAAAGTGTCGTGGCTAGTGCTTAG-3'. Oligonucleotides for non-targeting controls were sense, 5'-

GATCCGGCATGTACTAGCCTAAGCGTTTTCAAGAGACGCTTAGGCTAGTACA

TGCTTCTTTTTTTCTAGAG-3'; antisense, 5'-

AATTCTCTAGAAAAAAAGAAGCATGTACTAGCCTAAGCGTCTCTTGAAAACG

CTTAGGCTAGTACATGCG-3', respectively. Complimentary oligonucleotides were

diluted to 10μM, mixed together, boiled for 5 min in a water bath, and left overnight at

ambient temperature. Annealed oligonucleotides were ligated into the pSIREN-Retro-Q

retroviral vector (BD Biosciences Clontech, Palo Alto, CA) digested by BamHI and

EcoRI.

For chloramphenicol acetyltransferase (CAT) assays, 5'-deleted sequences

representing nucleotides -757/+72, -749/+72, -719/+72, or -216/+72 or point-mutated

sequences (-747/+72mAP1) of the human vimentin promoter were cloned upstream of

the CAT gene as described (Izmailova and Zehner, 1999; Izmailova *et al.*, 2000;

Wieczorek *et al.*, 2000; Wu *et al.*, 2004). Promoter sequences are indicated in Figure 17

A and B.

3.3 Generation of Cell Lines that Stably Express shRNA Plasmids

HN12 cells containing vimentin shRNA or non-targeted control plasmids were

prepared as follows. HN12 cells were plated in 10cm tissue culture dishes at 60%

confluency, transfected with 3μg of plasmid DNA using TransIT Keratinocyte Reagent

(Mirus Bio Corporation, Madison, WI). 48h later, cells were selected in the presence of

1μg/ml puromycin.   Individual puromycin resistant colonies were isolated and propagated.

3.4 Cell Transfection and CAT Assay

.HN12 cells were transfected using *Amaxa* Nucleofector Kit (Amaxa Inc., Gaithersburg, MD) according to the manufacturer's recommended protocol. Cells were plated (5×10^5) in one well of a six-well plate and transfected with 1.5μg of plasmid DNA and 0.5μg GFP in order to serve as an internal control for transfection efficiency. The amount of each plasmid was optimized by transfecting different amounts of plasmid DNA. After transfection (48h), cell lysates were prepared and CAT Elisa assays were performed according to the provided protocol by Roche Diagnostic Corporation (Indianapolis, IN). Alternately, after 24h transfection, cells were serum starved or treated with the appropriate growth factor for 24h. Western blot analysis was performed using GFP B-2 specific antibody (Santa Cruz Biotechnology, Santa Cruz, California).

3.5 Western Blot Analysis

Cell extracts were prepared from HNSCC, parental vimentin shRNA, and control transfected HN12 cell lines after 48h serum starvation or 24h serum starvation followed by treatment with growth factors, EGF, TGF-β, or TGF-β and EGF for 24h. Cells were washed and resuspended in 500μl of ice-cold Lysis buffer (25 mM HEPES, pH 7.5, 0.3M NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM DTT, 1% Triton X-100, 0.1% SDS, and 1mM PMSF, and 0.4M NaCl). Cleared lysates were combined with SDS sample buffer,

denatured for 5 min at 100 °C, and resolved by 10% SDS-PAGE. Fractionated proteins were electroblotted to PVDF membranes (Immobilon-P, Millipore Corporation, Bedford, MA) overnight. The membranes were then blocked with 4% non fat dried milk in 0.03% tween-TBS (T-TBS) for 1h at room temperature, washed with 0.03% T-TBS three times, and incubated with anti-vimentin monoclonal antibody 1:1000 diluted in 4% non fat dried milk for 1h at room temperature. Membranes were then washed with 0.03% T-TBS three times, incubated with horseradish peroxidase-conjugated (HRP) goat anti-mouse monoclonal antibody 1:10000 diluted in blocking buffer for 1h at room temperature, and washed four times in 0.03% T-TBS at room temperature. The specific antigen-antibody interactions were detected using enhanced chemiluminescence (ECL Plus, Amersham Biosciences, Piscataway, NJ).

3.6 Immunofluorescence

Cell lines (2×10^5) were plated on coverslips in twelve-well plates and allowed to attach for 24h. Cells were then serum starved for 48h, washed, and fixed with cold methanol at -20°C for 20 min. Cells were washed with PBS for 5 min, and blocked in 5% BSA, 0.1% Triton-X 100 in PBS for 1h at room temperature. Cells were incubated with monoclonal anti-vimentin antibody (Sigma, St. Louis, MO) 1:250 diluted in blocking buffer overnight at 4°C. The cells were then washed three times with PBS and incubated with a FITC-conjugated anti-mouse antibody at 1:500 dilution in 5% BSA, 0.1% Triton-X 100 in PBS for 1h at room temperature. Coverslips were mounted on a

microscope slide and viewed with a Zeiss Axiovent 200 inverted fluorescence microscope.

3.7 Immunocytochemistry

Cell lines were plated on coverslips in twelve-well plates at 2×10^5 cells/well. After 24h culture, cells were washed twice with PBS and serum starved for 48h or serum starved for 24h and treated with EGF, TGF-β, or EGF and TGF-β for 24h. Cells were washed, fixed with cold methanol at -20 °C for 20 min, washed with PBS for 5 min, and blocked in 5% BSA, 0.1% Triton-X 100 in PBS for 1h. Cells were incubated with anti-vimentin antibody as described above overnight at 4°C. Cells were then washed three times with PBS and incubated with HRP-conjugated goat anti-mouse antibody (Cappel, ICN Pharmaceuticals Inc., Aurora, OH) 1:500 diluted in blocking buffer for 1h at room temperature and washed three times in PBS. The specific antigen-antibody interactions were detected using a DAB staining kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instruction. Coverslips were mounted on microscope slides and viewed under an Olympus CK40 microscope.

3.8 Migration Assay

Subconfluent 10cm plates of cell lines were detached using HBSS / 5mM EDTA / 25mM Hepes pH 7.2 solution (Mediatech, Cellgro). The cells were then washed twice in DMEM/0.1% BSA and resuspended in DMEM/0.1% BSA at a concentration of 1×10^6 cells/ml. Cells (1×10^5) were added to the upper chamber of a 8μm pore size Transwell

(Corning Incorporated, Beverley, MA) and allowed to migrate for 6h. EGF (10ng/ml) (Austral Biological, San Roman, CA) in DMEM/0.1% BSA was added to the lower chamber of each well as a chemoattractant. The cells were then fixed in 100% methanol, stained with 0.1% crystal violet in 0.1M sodium borate pH 9, 2% EtOH overnight at ambient temperature, and destained in ddH$_2$O. The non-migratory cells on the upper surface of the membrane were removed with cotton swabs and mounted on a microscope slide. Migrated cells were counted in five randomly selected high power fields per membrane using an Olympus CK40 microscope.

3.9 Invasion Assay

Subconfluent 10cm plates of the HNSCC and vimentin stably transfected HN12 cell lines were detached using Cellstripper (Mediatech, Cellgro). The cells were then resuspended in DMEM supplemented with 0.1% Bovine Serum Albumin (BSA) and plated (2 × 10^4/400μl) in the upper chamber of Matrigel-coated Transwell inserts (pore size 12μm) (Corning Incorporated, Beverley, MA). In addition, 500μl of DMEM supplemented with 0.1% BSA with 10ng/ml EGF was added to the lower chamber of each well. The cells were allowed to invade for 16h and then fixed with 0.5ml of 5% glutaraldehyde in 1x PBS for 10 min at ambient temperature and washed three times in ddH$_2$O. The cells were stained with 0.1% crystal violet in 0.1M sodium borate pH 9, 2% EtOH for 20 min at room temperature and then washed three times with ddH20. The non-invading cells on the upper surface of the membrane were removed with cotton swabs and membranes were mounted on microscope slides. Invading cells were counted

in five randomly selected high power fields per membrane using an Olympus CK40 microscope.

3.10 Proliferation Assay

Cell lines were trypsinized using 0.25% Trypsin-EDTA (Invitrogen Corporation, NY). The cells were then resuspended in complete growth medium containing 1μg/ml puromycin, counted, and plated 2×10^3 cells/well in twenty four-well plates. After 24h of culture, triplicate wells were counted daily for eight consecutive days using a hemacytometer.

3.11 qRT-PCR

To design the oligonucleotide primers for quantitative RT-PCR, we utilized the Primerbank database

(http://pga.mgh.harvard.edu/primerbank/index.html) (Wang and Seed, 2003). We initially screened several set of primers by running PCR experiments to confirm that these produced PCR products of the predicted size. The primers used were as follows: Vimentin-qPCR sense, 5'-CTCCTCCCCCTGTCACATAC-3'; Vimentin-qPCR antisense, 5'-TGATTGGCATCAGGACCGTTG-3'. Total RNA was extracted from 10cm plates using 8ml of TriZol reagent after washing with PBS twice, and 3μg of total RNA was converted to cDNA using Superscript III (Invitrogen Corporation) according to the manufacturer's instructions. Real-time quantitative PCR was performed using a LightCycler (Roche Diagnostics, Indianapolis, IN) with fluorescence signal detection

(SYBR green) after each cycle of amplification as previously described (Deb *et al.,* 2002*)*. Product-specific amplification was confirmed by a melting curve analysis and agarose gel electrophoresis analysis. Quantification was focused on the log-linear phase of amplification above the baseline using the LightCycler software. The calculated cDNA copy number in each sample was derived from an extrapolated crossing point of a mathematically derived line extending from the exponential phase of amplification in a plot of fluorescence intensity (SYBR green) versus cycle number. For each reaction, diluted amounts of known templates provided quantitative standard curve reactions from which cDNA copy number could be determined. GAPDH was used as a housekeeping gene to normalize the initial content of total cDNA in the samples.

4. Results

4.1 Upregulation of Vimentin in HN12 cells.

The derivation of the metastatic HN12 squamous carcinoma cell line and the non-metastatic HN4 cell line has been described previously. When comparing the gene expression pattern of these two cell lines using cDNA microarrays (Miyazaki *et al.*, 2005), we discovered overexpression of vimentin in HN12 cells. In order to confirm this finding, we prepared total cellular RNA from serum-deprived HN4 and HN12 cells, reverse-transcribed this and performed quantitative real-time PCR (qRT-PCR) on the cDNA template using vimentin-specific primers. As shown in Fig. 13A (upper panel), vimentin RNA is around 4-fold more abundant in HN12 cells compared to HN4. Further, we confirmed vimentin overexpression in HN12 cells by western blot analysis of total cellular protein lysates. Whereas vimentin was readily detectable in HN12 cell lysates, no signal was obtained with HN4 lysates (Fig. 13A, lower panel). In addition, immunocytochemical staining (Fig. 13B) showed strong vimentin ubiquitous staining in HN12 cells, whereas this was absent in HN4 cells. Immunofluorescence microscopy (Fig 13C) revealed a dense network of vimentin filaments in the HN12 cells. These data confirm that expression of vimentin is upregulated in cells at a later stage of tumor progression and confirm the microarray findings.
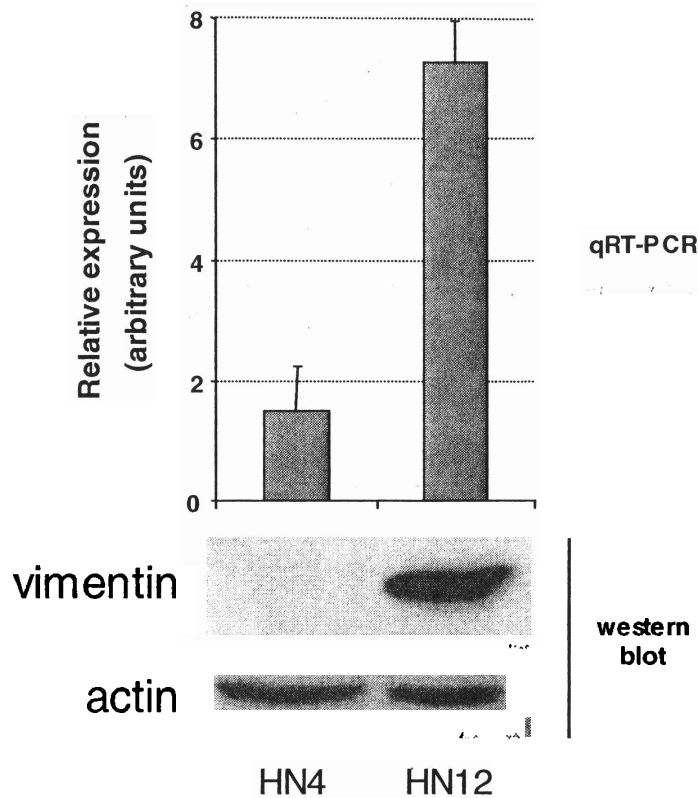
Figure 13A: Total RNA was extracted from subconfluent cultures, reverse-transcribed to generate cDNA and subjected to real-time quantitative PCR as described in 'Materials and Methods'. The relative expression ratio is defined as the expression levels of vimentin to that of an internal standard, GAPDH. Assays were carried out in triplicate and means±1SD are indicated. Parallel cultures, similarly treated, were used for western blot analysis of vimentin expression. After 48h of serum deprivation, total cell protein extracts were prepared as described in 'Materials and Methods' and analyzed for vimentin expression (top panel). Levels of β-actin were determined as a loading control (lower panel).
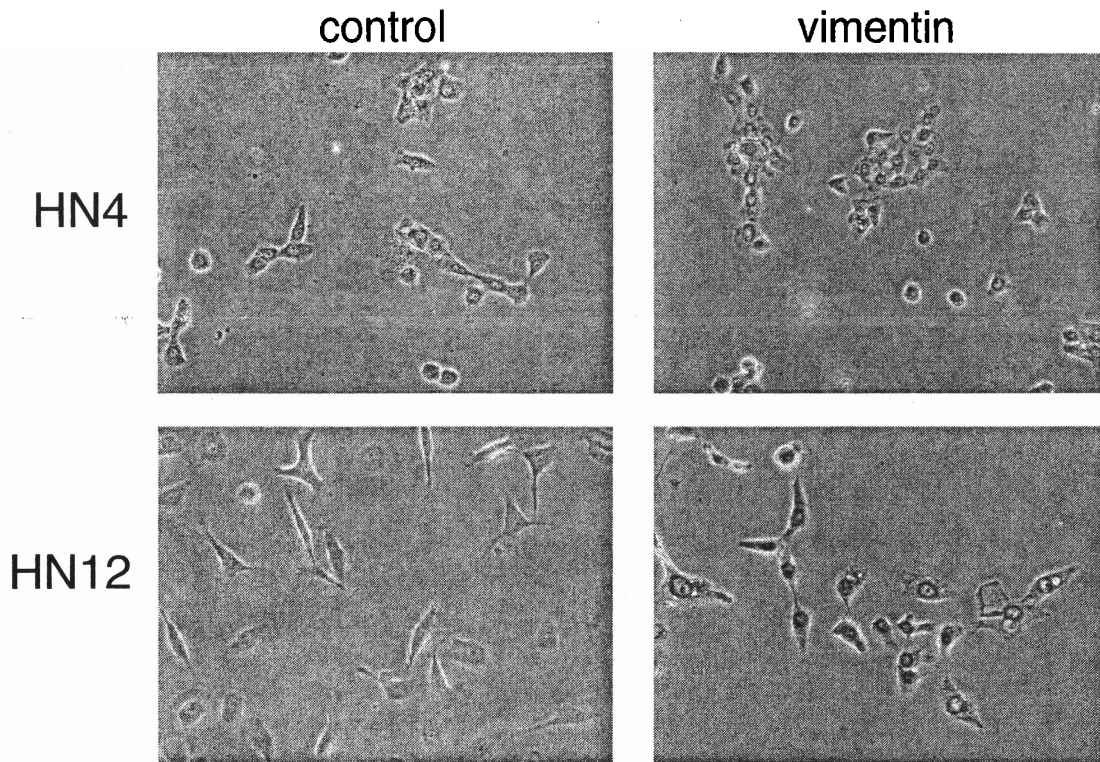
Figure 13B: Analysis of vimentin expression in HN4 and HN12 cells using immunocytochemistry. Cells were cultured on glass coverslips for 24h, serum deprived for 48h, and fixed as described in 'Materials and Methods'. Cells were incubated with anti-vimentin antibody (or mouse IgG as control) overnight and detected with HRP-conjugated goat anti-mouse antibody and DAB. Images were viewed under a light microscope.
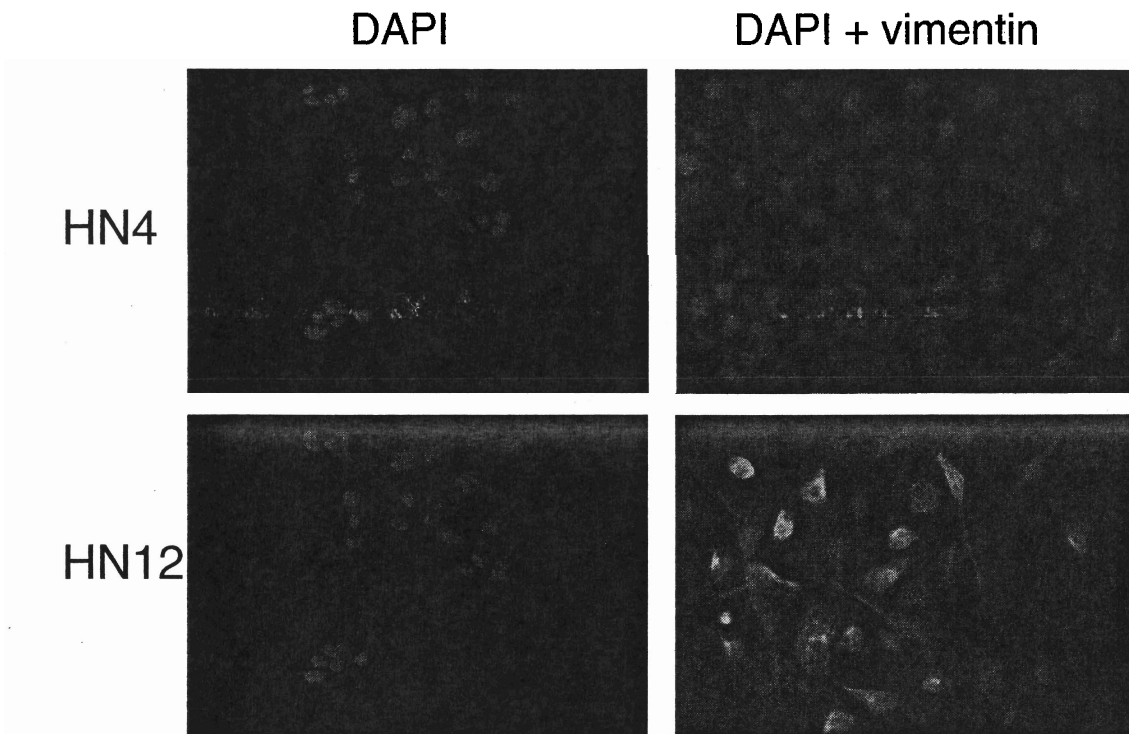
Figure 13C: Analysis of vimentin expression in HN4 and HN12 cells using immunofluorescence. Cells were cultured on glass coverslips for 24h, serum deprived for 48h, and fixed as described in 'Materials and Methods'. Cells were incubated with anti-vimentin antibody (or mouse IgG as control) overnight then incubated with a FITC-conjugated anti-mouse antibody for 1h and counterstained with DAPI. Images were viewed using a fluorescence microscope.

4.2 <u>EGF and TGF-β Synergize to Increase Vimentin Expression.</u>

Epidermal growth factor (EGF) and transforming growth factor β (TGF-β) are thought to contribute to tumor metastasis through a number of mechanisms, including enhanced cell migration and epithelial to mesenchymal transition (EMT), and have been demonstrated to enhance migration and invasion of HN12 cells (Yeudall et al., 2005; Miyazaki et al., in press). To determine whether either of these growth factors resulted in upregulation of vimentin expression, we treated HN12 cells with EGF or TGF-β, or a combination of both, then performed qRT-PCR using cDNA prepared from HN12 RNA as a template. As shown in Fig. 14A (upper panel), vimentin RNA was increased by around three-fold in TGF-β treated cells over basal expression levels, around five-fold with the addition of EGF, and a synergistic seven-fold increase when cells were treated with a combination of TGF-β and EGF. Interestingly in HN4 cells, we observed a similar trend of vimentin upregulation but, even with the addition of both EGF and TGF-β, expression was considerably lower than basal levels in HN12 cells.

As we had found that EGF and TGF-β elevated levels of vimentin RNA in HN12 cells, we sought to determine whether this was reflected by an increased abundance of vimentin protein. Therefore, we treated cells with growth factors as before, prepared total cellular protein extracts, and performed western blot analysis. Vimentin protein expression was undetectable in HN4 cells by our assay (Fig. 14A, lower panel), even with addition of growth factors. However, increased levels of vimentin protein were detected in TGF-β and EGF-treated HN12 cells, concomitant with the previously

observed rise in vimentin RNA.   Further, we analyzed vimentin expression in growth

factor-treated cells by immunocytochemical staining.   HN12 cells displayed an

ubiquitous distribution pattern of vimentin throughout the cell, which was further

enhanced, with the addition of EGF and TGF-β (Fig 14B).   Taken together, EGF and

TGF-β treatment result in increased vimentin RNA and protein levels in HN12 cells,

suggesting that both growth factors may play a role in EMT in this system. Further, EGF

and TGF-β stimulate expression of vimentin RNA in primary tumor-derived HN4 cells

with a trend similar to that seen in HN12 cells; however RNA levels are extremely low

and vimentin protein is undetectable.
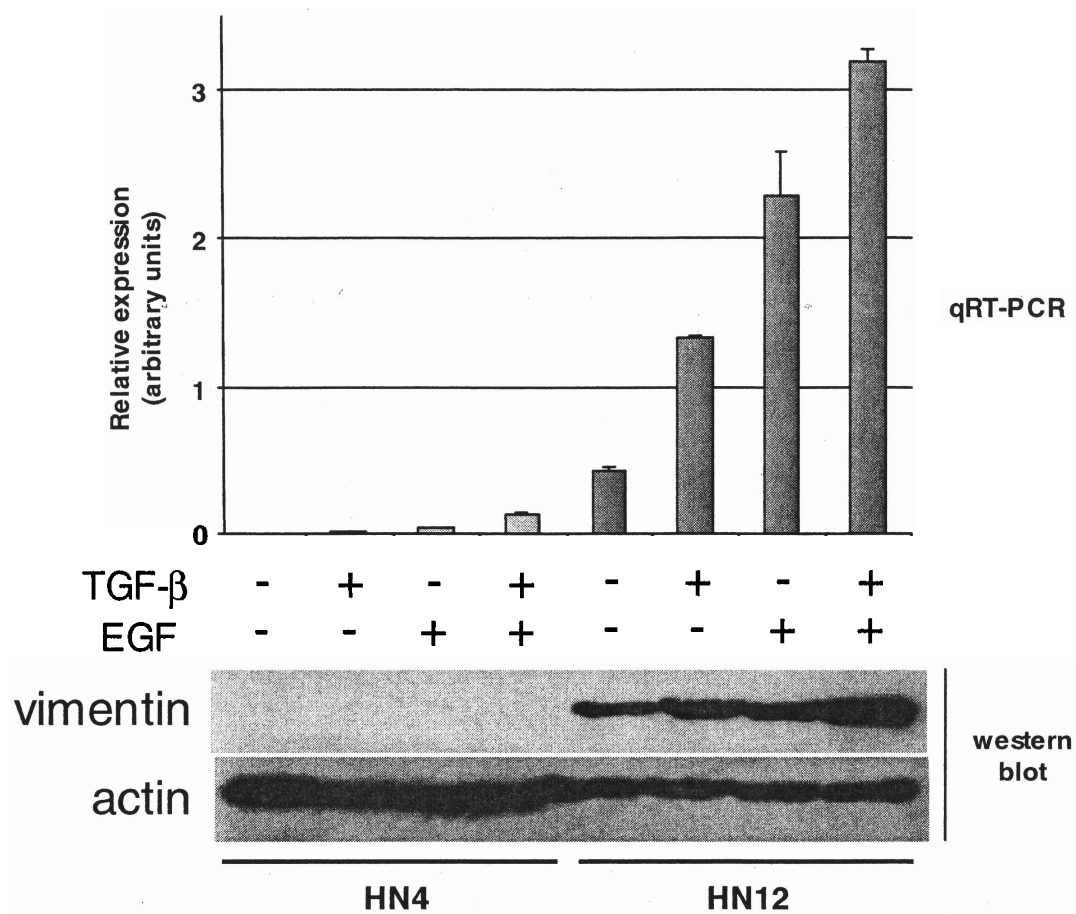
Figure 14A: Cells were cultured in the presence or absence of growth factors as indicated. Total RNA was extracted from subconfluent cultures, reverse-transcribed to generate cDNA, and subjected to quantitative real-time PCR as described in 'Materials and Methods'. The relative expression ratio is defined as level of vimentin expression to that of an internal standard, GAPDH. Assays were carried out in triplicate and means±1SD are indicated. Parallel cultures, similarly treated, were used for western blot analysis of vimentin expression as described in 'Materials and Methods' and as described in Figure 1.
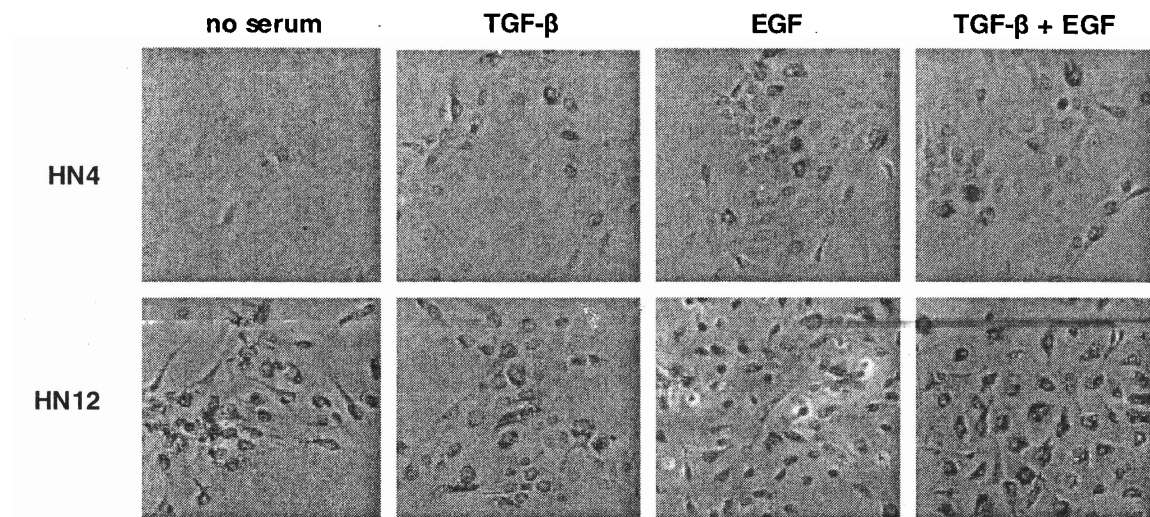
Figure 14B: Immunocytochemical analysis of vimentin expression in HN4 and HN12 cells with the addition of TGF-β and EGF. Cells were cultured on glass coverslips for 24h, then serum deprived for 48h or serum deprived for 24h and treated with EGF, TGF-β, or EGF and/or TGF-β for 24h and then fixed as described in 'Materials and Methods'. Cells were incubated with anti-vimentin antibody (or mouse IgG as control) overnight and detected with HRP-conjugated goat anti-mouse antibody and DAB. Images were viewed under a light microscope.

4.3 Targeted Suppression of Vimentin Expression.

In order to study the contribution of vimentin expression to the biological properties of HN12 cells, we used RNA interference (RNAi) technology to reduce vimentin levels. Thus, HN12 cells were stably transfected with a plasmid that directs synthesis of a vimentin shRNA, or a non-targeting control (NTC) plasmid, and colonies selected for resistance to puromycin and screened for vimentin expression by qRT PCR, western blot analysis, and immunofluorescence. As shown in Fig 15A, qRT-PCR identified a ten-fold minimum decrease in vimentin expression in HN12-shVim clones compared to the HN12-NTC control cells. In addition, this reduction of vimentin expression was confirmed by immunofluorescence microscopy, which showed a cytosolic distribution pattern of staining in the HN12-NTC clones and the absence of staining in the HN12-shVim clones (Fig 15B). Furthermore, vimentin expression was undetectable by western blot analysis in either of the two HN12-shVim clones compared to the parental HN12 cell line and HN12-NTC clones (Fig. 15A). Taken together, these data indicate that vimentin expression is substantially reduced in HN12-shVim clones compared to non-targeting controls and, thus, may be suitable for investigating the contribution of vimentin expression to the biological phenotype of metastatic HN12 cells.

Immunocytochemical staining demonstrated a significant increase of vimentin expression in HN12-NTC compared to the HN12-shVim clones (Fig 15C). With the addition of EGF and TGF-β, the expression pattern of vimentin in HN12-shVim cells (Fig. 15C) is similar to the pattern seen in HN4 cells (Fig. 14B). Interestingly, there is a

slight increase in vimentin expression with the addition of either EGF or TGF-β individually and an even greater increase with the addition of both growth factors together. However, even with the addition of both growth factors to the HN12-shVim cell line, the expression of vimentin is considerably lower then the basal expression of vimentin in HN12 and HN12-NTC cells. Further, we confirmed these results using western blot analysis of total cellular protein lysates (Fig 15C).

Figure 15A: Cells lines that stably express vimentin shRNA or non-targeting control plasmids were generated as described in 'Materials and Methods'. Total RNA was prepared from subconfluent cultures, reversed transcribed, and the resultant cDNA subjected to quantitative real-time PCR as described above. The relative expression ratio is defined as the expression level of vimentin to that of an internal standard, GAPDH. Assays were carried out in triplicate and means$\pm$1SD are indicated. Western blot analysis of vimentin expression (top panel) and actin as a control was carried out as described above (lower panel).
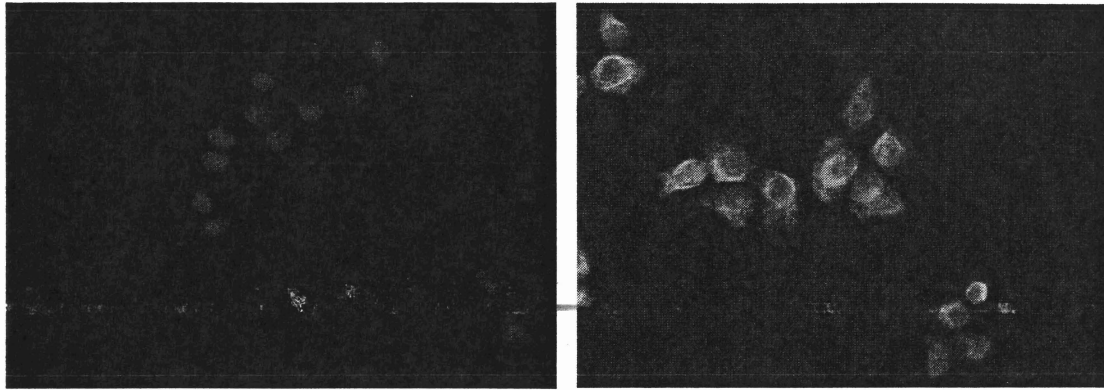
shVim　　　　　　　　　**NTC**

Figure 15B: Cells were plated on glass coverslips for 24h, serum deprived for 48h and then fixed as described in 'Materials and Methods'. Cells were incubated with anti-vimentin antibody (or mouse IgG as control) overnight followed by FITC-conjugated goat anti-mouse antibody. Cells were counterstained with DAPI and detected using a fluorescence microscope.
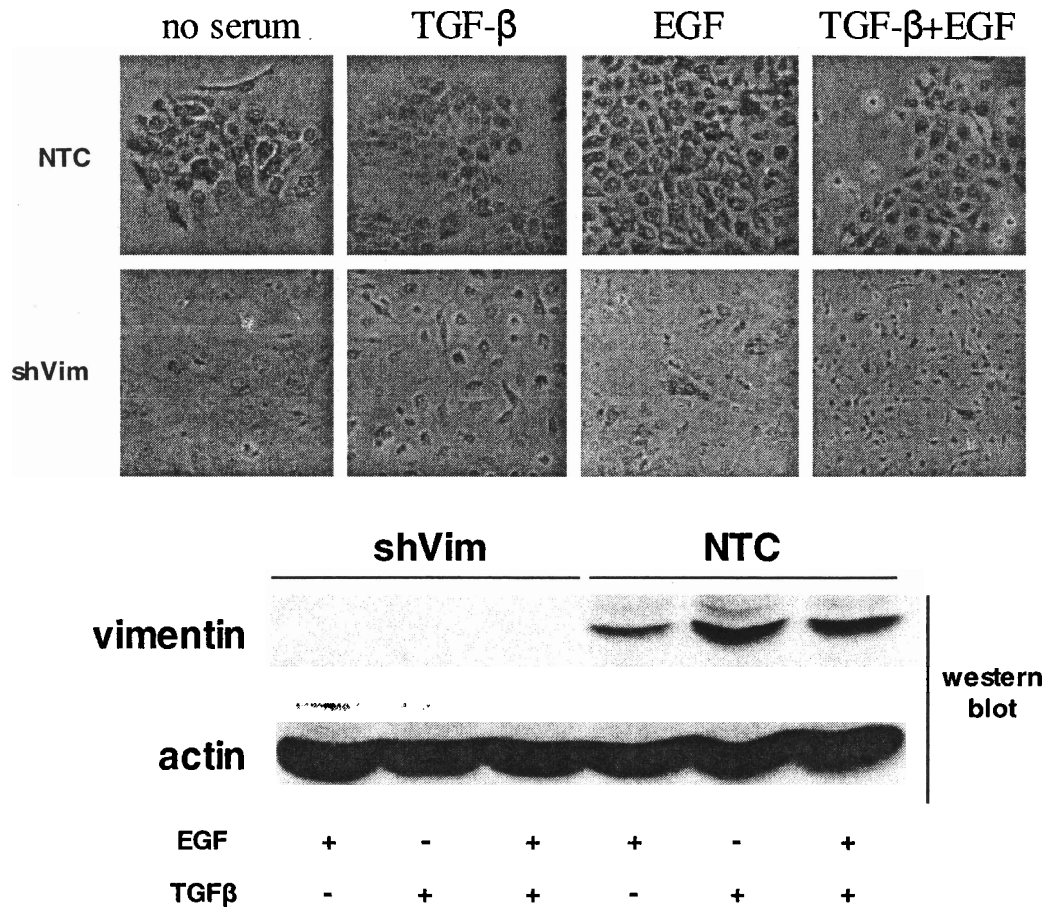
Figure 15C: Cells containing vimentin shRNA or non-targeting control plasmids were cultured on coverslips for 24h, serum starved for 48h, and fixed as described in 'Materials and Methods'. Cells were incubated with anti-vimentin antibody (or mouse IgG as control) overnight then incubated with a HRP-conjugated anti-mouse a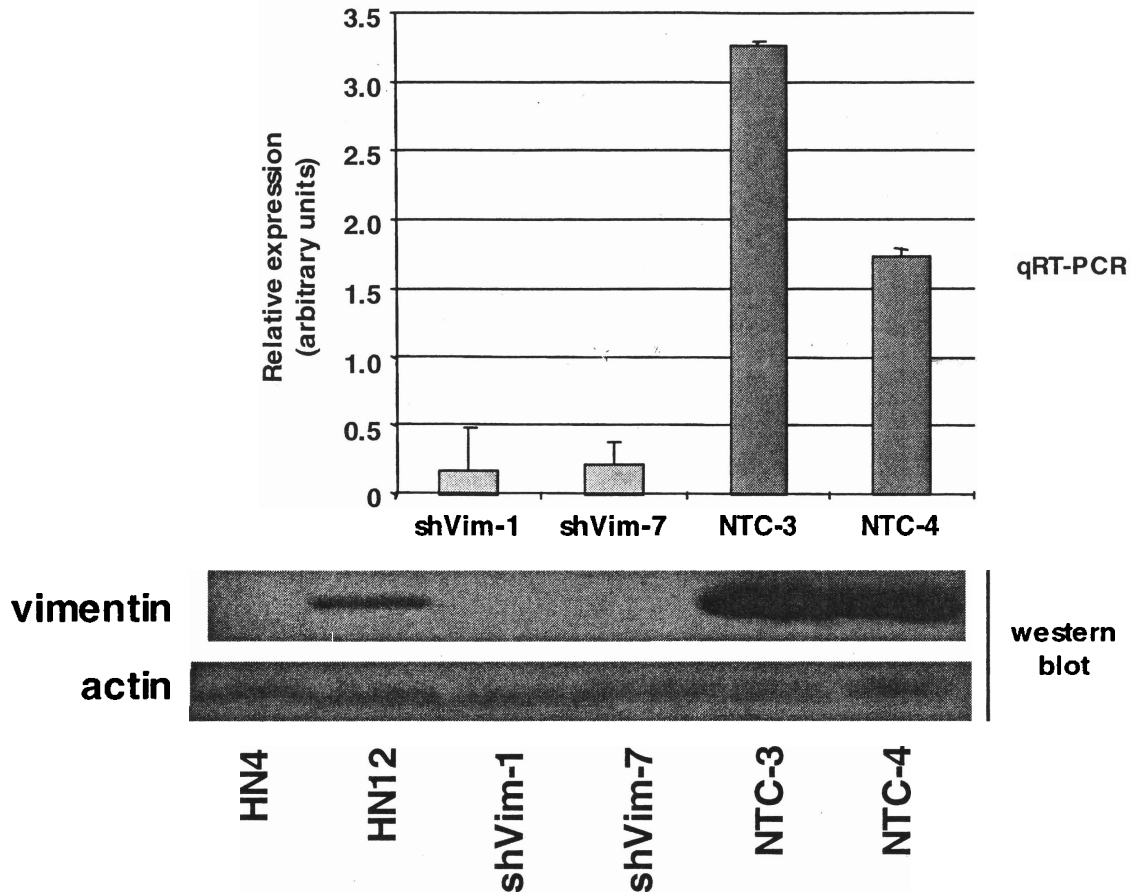ntibody for 1h. Images were viewed using a light microscope. Western blot analysis of vimentin expression (top panel) and actin as a control was carried out on parallel cultures as described above (lower panels).

4.4 <u>Vimentin Overexpression is Important for Biological Properties of HN12 Cells.</u>

Squamous carcinoma cells tend to grow more rapidly and/or fail to die at a normal rate. In addition, these cells can also invade surrounding tissue and metastasize. Many cells undergo EMT as a feature of tumor progression. Thus, in order to determine whether the mesenchymal cell marker, vimentin, contributes to the phenotype of HN12 cells, we first compared the proliferation rate in culture of HN12-shVim and HN12-NTC cells. Cells were seeded in quadruplicate in multi-well plates, then trypsinized and counted over an eight day period. As is apparent from Fig. 16A, HN12-shVim clones exhibit a marked decrease in proliferation compared to the HN12-NTC cells, suggesting that vimentin may contribute to cell growth. Next, we compared the contribution of vimentin overexpression on the motility and invasiveness of HN12 cells using *in vitro* assays. As shown in Fig. 16B, downregulation of vimentin expression in HN12-shVim cell lines decreases migration in Transwell assays by four-fold in comparison to the HN12-NTC control clones. Furthermore, *in vitro* invasion through Matrigel is markedly decreased in cells with lower expression of vimentin (Fig. 16C). Taken together, these data indicate a crucial role for vimentin in the proliferation and invasion of metastatic HN12 squamous carcinoma cells.

Proliferation
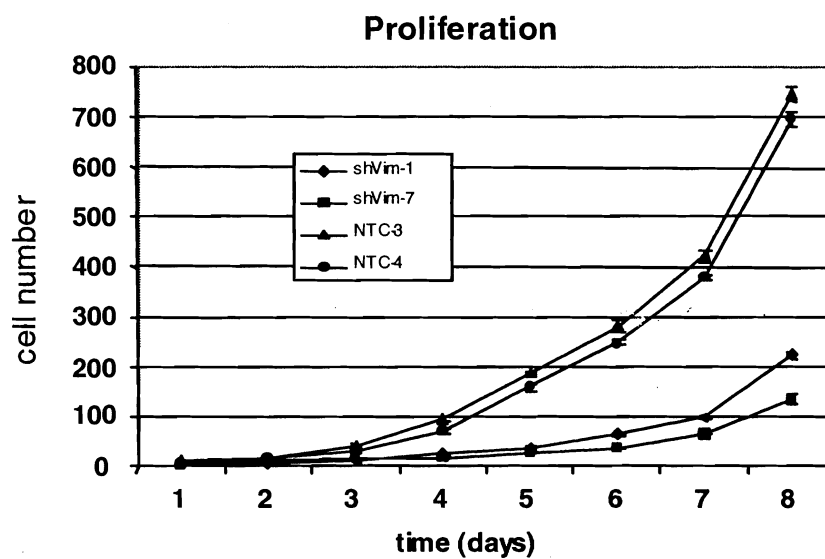
Figure 16A: Cells (2 × 10³ per well) were plated in triplicate in 24-well plates and incubated under standard culture conditions as described in 'Materials and Methods'. Cells were trypsinized and counted daily for eight consecutive days. Values shown are means±1SD.
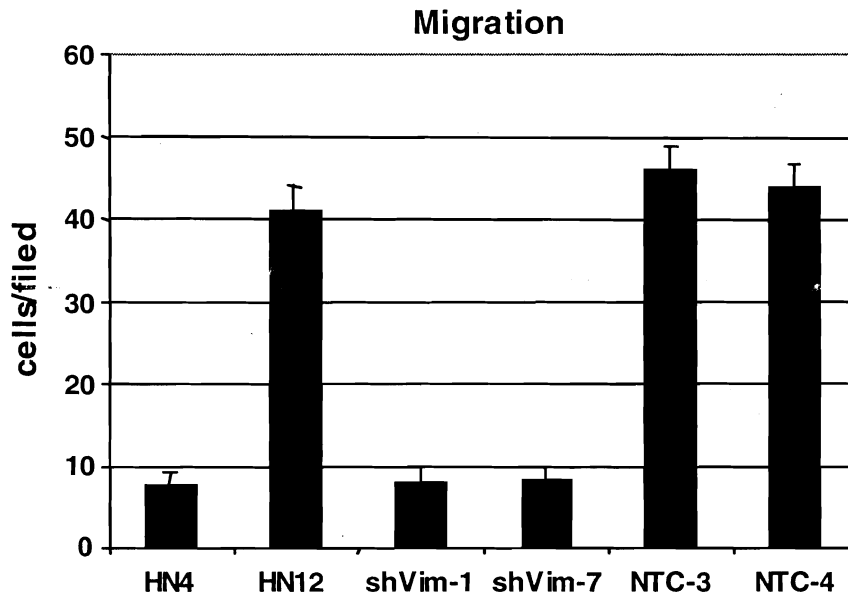
Figure 16B: Cells from subconfluent cultures were detached in the absence of trypsin, washed, and plated in Transwell chambers as described in 'Materials and Methods' with EGF in the lower chamber to act as a chemoattractant. 6h later, migrated cells were stained and counted in 10 random high power fields. Data shown represent means±1SD.
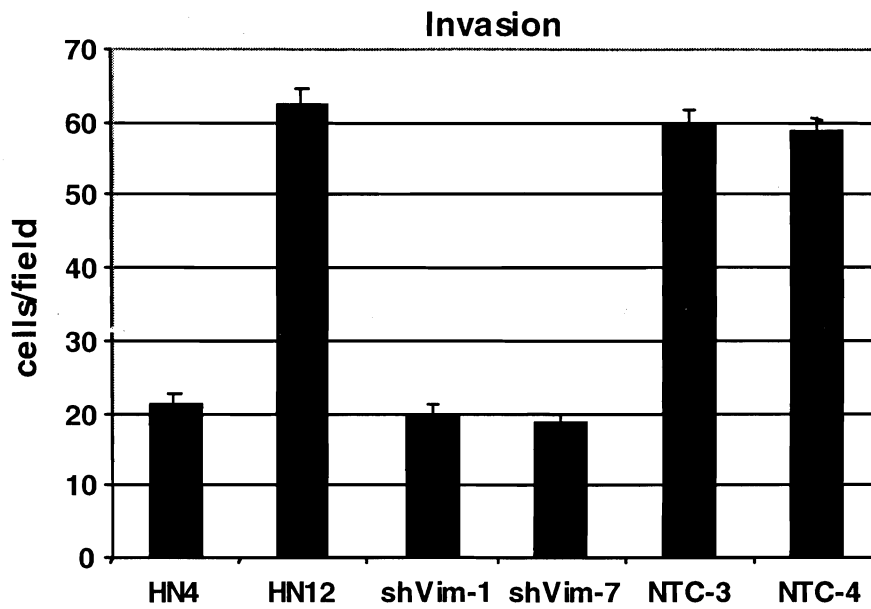
Figure 16C: Cells from subconfluent cultures were detached in the absence of trypsin, washed, and plated in Matrigel-coated Transwell chambers as described in 'Materials and Methods'. 16h later, invading cells were stained and counted in 10 random high power fields. Data shown represents means$\pm$1SD.

4.5 Regulation of Vimentin Promoter Activity in HN12 Cells by EGF and TGF-β.

How the vimentin gene is regulated is important for understanding the relationship between gene expression and tumor progression which, based on our data presented above, could ultimately contribute to controlling the invasiveness in some tumors. Therefore, we used vimentin promoter fragments, which begin at +72 and extend to −261, -719, -749, and −757 to investigate which transcription factor binding sites mediate upregulation of vimentin expression in HN12 cells. These promoter fragments were cloned 5' to a CAT reporter gene and are shown in schematic form in Fig. 17A. The vimentin promoter sequence is shown in Fig. 17B. Plasmids encoding vimentin promoter-CAT sequences were transiently transfected into HN12 cells and CAT activity measured by ELISA assay following serum withdrawal (Fig. 17C). The 261CAT sequence containing the GC-box1, PEA3 and NF-κB sites showed substantial activity under conditions of growth factor withdrawal. This activity was much reduced in cells transfected with the 719CAT plasmid, which contains two sites (PS and H4TF-1) that bind the ZBP-89 repressor protein. Moderate restoration of activity was achieved with the addition of two AP-1 binding sites in the promoter (749CAT), while further addition of the ASE site (757CAT) further increased promoter activity (Fig. 17C). Taken together, the data suggest that basal activity of the vimentin promoter in HN12 cells is mediated through ASE and AP-1 sites, as well as one or more of the GC-box1, PEA3 and NF-κB sites. Furthermore, the data indicate that ZBP-89 repressor activity is high in these cells.

Next, we examined vimentin promoter activity in the presence of EGF and TGF-β to determine the binding elements responsible for the increased vimentin expression observed by qRT-PCR, western blot analysis, immunocytochemistry, and immunofluorescence. Using the 757CAT sequence, we found that treatment of cells with either EGF or TGF-β stimulated promoter activity above that observed under conditions of serum withdrawal, while the presence of both growth factors further elevated CAT activity (Fig. 17D). In contrast, CAT activity in cells transfected with the 261CAT sequences (containing the GC-box, PEA3 and NF-κB sites) was maximal in the presence of EGF, which stimulated promoter activity by around 3-fold over basal conditions (Fig. 17E). Treatment of these cells with TGF-β did not produce any significant increase in promoter activity. Surprisingly, addition of both growth factors simultaneously resulted in lower activity than with EGF alone (Fig. 17E), suggesting that TGF-β may antagonize the action of EGF on these promoter elements. However, in cells transfected with the 749CAT plasmid, TGF-β produced an increase in promoter activity of around 2.5-fold over basal levels, whereas EGF had little effect (Fig. 17F). Similar to the results with the 261CAT sequences, addition of both growth factors failed to produce a synergistic effect, implying that EGF antagonizes the TGF-β-mediated stimulation of the AP-1 sites. Taken together, the data suggest that EGF-dependent pathways target binding elements in the proximal vimentin promoter, while TGF-β is likely to act in an AP-1-dependent manner. Furthermore, both growth factors appear to synergize by stimulating promoter activation through the ASE site, suggesting involvement of Stat-dependent pathways in regulation of vimentin expression in HN12 cells.
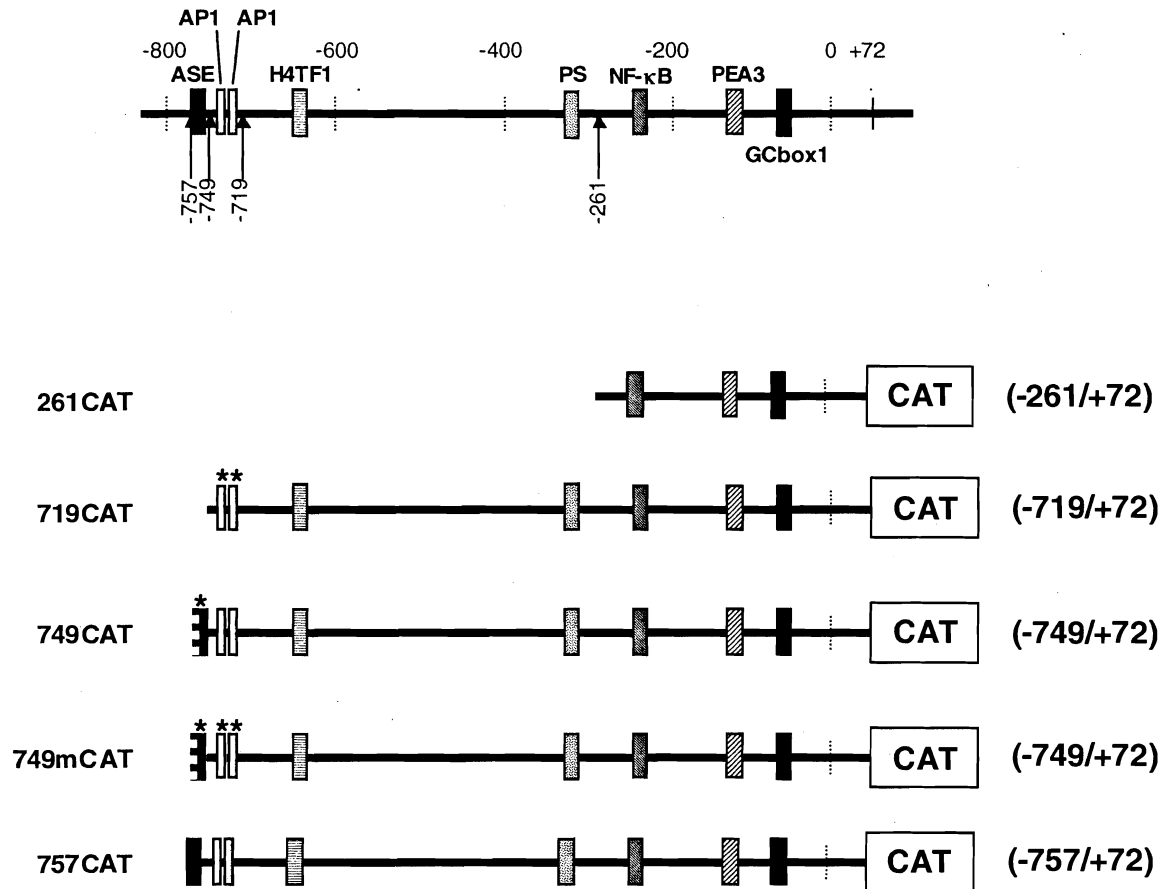
Figure 17A: Regulatory elements [transcription factor binding sites] in the vimentin promoter. The relative position of regulatory elements previously reported to be present in the vimentin promoter is shown (top panel). 5'-deleted sequences or point-mutated sequences of the human vimentin promoter were cloned upstream of the CAT reporter gene as described in 'Materials and Methods'. * indicates site of point mutation.

-900 catggcccagctgtaagttggtagcactgagaactagcagcgcgcgcggagcccgctgag

-840 acttgaatcaatctggtctaacgg*tttcccctaaaccgctaggagccctcaatcggcggg*
                                    ASE

-780 *acag*cagggcgcgg*tgagtca*ccgccgg*tgacta*agcgacccacccctctccctcgggc
                   AP1   **AP1**

-720 tttcctctgccaccgccgtctcgcaactcccgccgtccgaagctggactgagcccgttag

-660 gtccctcgacagaacctc*ccctcccccc*aacatctctccgccaaggcaagtcgatggaca
                        H4TF-1

-600 gaggcgcgggccggagcagcccccctttccaagcgggcggcgcgcgaggctgcggcgagg

-540 cctgagccctgcgttcctgcgctgtgcgcgcccccaccccgcgttccaatctcaggcgct

-480 ctttgtttctttctccgcgacttcagatctgagggattccttactctttcctcttcccgc

-420 tcctttgcccgcgggtctccccgcctgaccgcagccccgagaccgccgcgcacctcctcc

-360 cacgcccctttggcgtggtgccaccggacccctctggttca*gtcccaggcggaccccccc*
                                             PS

-300 *ctcaccgcgcg*accccgcctttttcagcaccccagggtgagcccagctcagactatcatc

-240 *cggaaagcccccaaaa*gtcccagcccagcgctgaagtaacgggaccatgcccagtcccag
      NF-κB

-180 gccccg*gagcaggaaggctcgaggcgccccc*acccacccgcccaccctccccgcttct
         PEA-3   **GCbox1**

-120 cgctaggtccctattggctggcgcgctccgcggctgggatggcagtgggaggggaccctc

-60 tttcctaacggggttataaaaacagcgccctcggcggggtccagtcctctgccactctc**g** -1

0 ctccgaggtccccgcgccagagacgcagccgcgctcccaccacccacacccaccgcgccC
   tcgttcgcctcttctccgggagccagtccgcgccaccgccgccgcccaggccatcgccAc
   cctccgcagcc**ATG**TCCACCAGGTCCGTGTCCTCGTCCTCCTACCGCAGGATGTTCGGCG
   GCCCGGGCACCGCGAGCCGGCCGA

Figure 17B: Nucleotide sequence of the vimentin promoter. Specific regulatory elements are indicated (underline). Coding sequence is indicated (capitals).
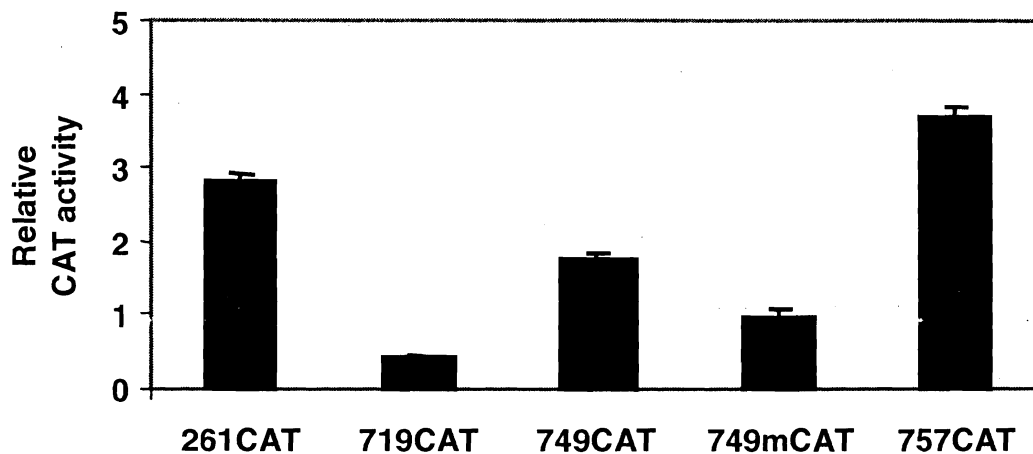
Figure 17C: HN12 cells were transfected with vimentin promoter fragments, which begin at +72 and extend to −261, -719, -749, and −757 as described in 'Materials and Methods'. 24h after transfection, cells were serum starved for 24h and then CAT activity was determined by ELISA assay. The relative expression ratio is defined as the expression level of vimentin to that of an internal standard, GFP. Values shown are means±1SD.
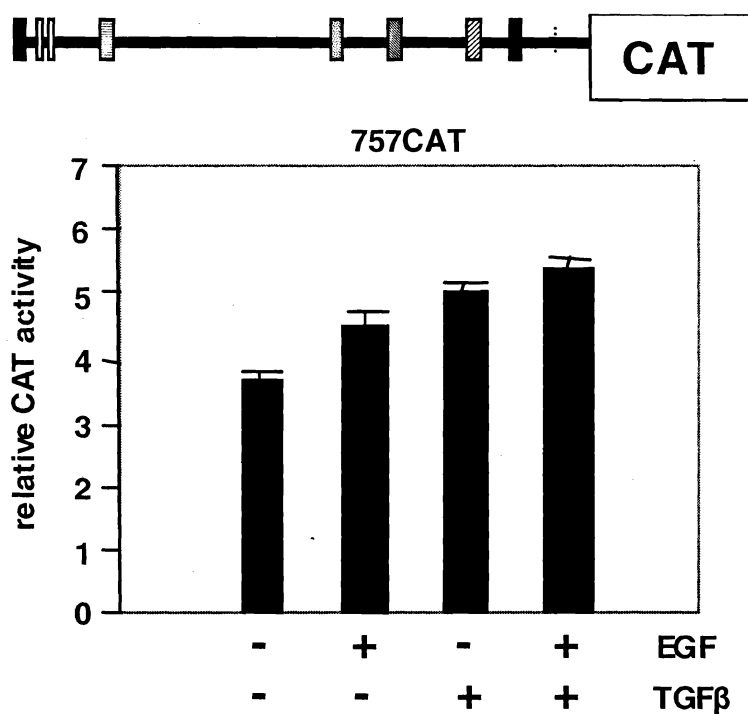
Figure 17D: Effects of EGF and TGF-β on vimentin promoter activity. HN12 cells were transfected with 757 CAT plasmid. Serum was withdrawn for 24h, then cells were treated with the indicated growth factors for 24h after which CAT activity was determined by ELISA assay. The relative expression ratio is defined as the expression level of vimentin to that of an internal standard, GFP. Values shown are means±1SD.
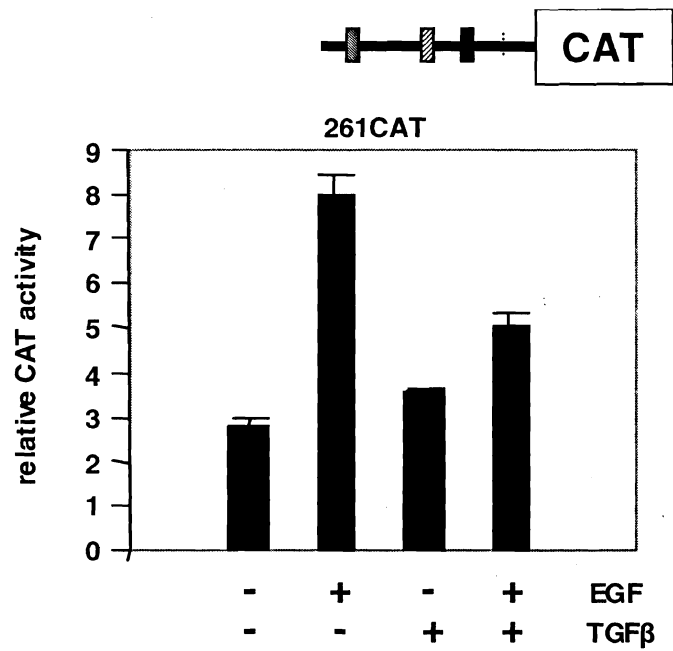
Figure 17E: Effects of EGF and TGF-β on vimentin promoter activity. HN12 cells were

transfected with 261 CAT plasmid. Serum was withdrawn for 24h, then cells were

treated with the indicated growth factors for 24h after which CAT activity was

determined by ELISA assay. The relative expression ratio is defined as the expression

level of vimentin to that of an internal standard, GFP. Values shown are means±1SD.
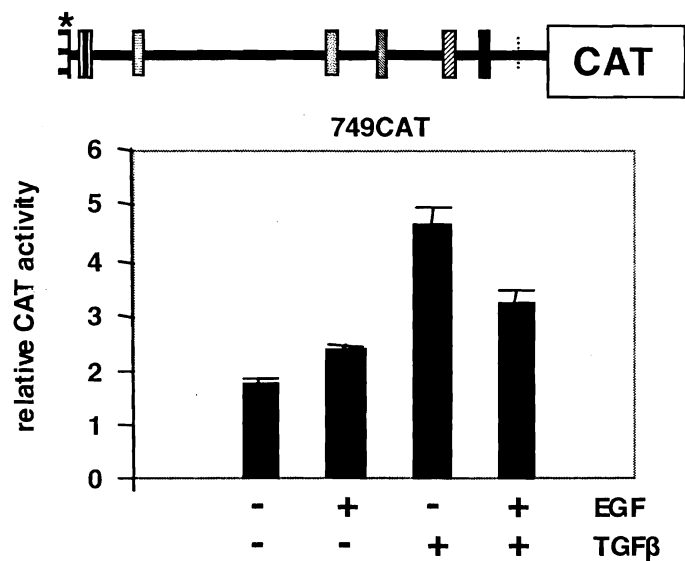
Figure 17F: Effects of EGF and TGF-β on vimentin promoter activity. HN12 cells were transfected with 749 CAT plasmid. Serum was withdrawn for 24h, then cells were treated with the indicated growth factors for 24h after which CAT activity was determined by ELISA assay. The relative expression ratio is defined as the expression level of vimentin to that of an internal standard, GFP. Values shown are means±1SD.

5. Discussion

5.1 Aims of Current Study

The aims of this study were to determine how vimentin expression differs in primary and metastatic HNSCC cells; to determine if vimentin expression is modulated by the invasion-inducing growth factors TGF-β and/or EGF; to determine if vimentin expression contributes to the invasive phenotype of metastatic HNSCC cells; and to determine what mechanisms regulate vimentin gene transcription in metastatic HNSCC.

5.2 HNSCC Model System

In order to develop a model system to study the nodal metastasis or oral squamous cell carcinoma, the HN4 cell line was generated from a primary carcinoma of the tongue and the HN12 cell line from a co-existing lymph node metastasis. Both of the cell lines were found to have undergone identical inactivation of *CDKN2A* and *P53* genes, which strongly suggests that the metastatic cells were the results of malignant progression of cells from the primary lesion (Yeudall *et al.*, 1994; Yeudall *et al.*, 1997) and that they were likely to have a similar genetic background. Previous studies have demonstrated that P53 (Burns *et al.*, 1994; Koch *et al.*, 1994) and CDKN2A (Yeudall *et al.*, 1994) mutation found in the primary tumor are maintained during metastatic progression. HN4 cells were unable to form tumors in immunodeficient mice and HN12 cells were highly tumorigenic *in vivo*, indicating that the HN12 cells are at a more advanced stage of tumor progression (Yeudall *et al.*, 2005).

5.3 Microarray Data

A high throughput analytical method, high density microarray technology, was used to identify the genetic alterations that occur during metastatic progression or primary oral squamous cell carcinoma (Miyazaki *et al.*, 2005). The gene set documented in this report, including vimentin, overlap those identified from *in vivo* studies, which suggest that the model has relevance for the study of HNSCC metastasis (Miyazaki *et al.*, 2005). The high basal levels of vimentin present in the metastatic cells, consistent with their more mesenchymal appearance as well as the expression being elevated by both EGF and TGF-β served as the foundation for the present study.

In addition, simultaneous exposure of both growth factors to HN12 cells resulted in a higher level of vimentin expression than that found with either EGF or TGF-β alone indicating some level of cooperatively between the EGF- and TGF-β-dependent signaling pathways to the upregulation of vimentin expression (Miyazaki *et al.*, in press). Here, we investigate regulation of the vimentin promoter in the presence of EGF and TGF-β to determine distinct promoter elements acted upon by these signaling pathways.

5.4 Vimentin as a Molecular Marker

Different intermediate filament proteins serve as markers for the identification of normal, highly differentiated cells (Anguelov, 2000). Furthermore, intermediate filament proteins are relied upon to help diagnosis tumor types, for example, vimentin is being used as a marker for melanomas and keratin as a marker for adenocarcinomas (Anguelov, 2000). Molecular markers for malignancy, such as vimentin, are expected to become

reliable prognostic tools that will aid oncologists in fine-tuning their cancer management strategies (Anguelov, 2000). This molecular diagnosis would allow physicians to stage the patient's cancer more effectively and specifically tailor a treatment strategy that is clinically and cost effective (Anguelov, 2000).

5.4.1 Upregulation of Vimentin in HN12 cells

In this study, vimentin RNA was found to be more abundant in HN12 cells compared to HN4. Vimentin overexpression in HN12 cells was confirmed by western blot analysis, immunocytochemical staining, and immunofluorescence microscopy. Immunocytochemical staining showed strong vimentin ubiquitous staining in HN12 cells, whereas this was absent in HN4 cells and immunofluorescence microscopy revealed a dense network of vimentin filaments in the HN12 cells. These findings confirm that expression of vimentin is upregulated in cells at a later stage of tumor progression confirming the microarray findings. Concurrent with our results, other metastatic cell lines have also been reported an upregulation in expression of the intermediate filament, vimentin.

Using an androgen-independent model of prostate cancer, among the differentially expressed genes between the two cells lines, LNCaP and CL1, vimentin was overexpressed 20-fold in the CL1 cells (Singh *et al.*, 2003). The androgen-sensitive LNCaP prostate cancer cell line is less invasive than hormone-insensitive cell lines (Singh *et al.*, 2003). CL1 is an aggressive hormone-insensitive LNCaP subline that was derived by continuous culture in hormone-depleted medium (Singh *et al.*, 2003).

Upregulation of vimentin expression was confirmed by northern and western blots and visualized by immunofluorescence microscopy (Singh *et al.*, 2003). In addition, these data confirm the findings of previous studies in which the phenotypes of prostate cancer cell line have been examined and LNCaP cells showed low levels of vimentin expression in contrast to the high expression seen in their more aggressive DU145 and PC3 counterparts (Nagle *et al.*, 1987; Mitchell *et al.*, 2000).

5.5 Vimentin Expression and Epithelial to Mesenchymal Transition

A major problem in cancer management is metastasis, the ability of the primary tumor cells to migrate by the way of the blood or lymphatic vessels and to form tumors at distant sites. It has been reported that EMT facilitates the dissemination of single carcinoma cells from the sites of primary tumors and is involved in the dedifferentiation that is typical of metastatic carcinoma (Theiry, 2002). Originally, EMT was described in the morphogenic remodelings during embryonic development (Hay, 1995; Boyer *et al.*, 1999). The acquisition of mesenchymal features by epithelial cells induced migration during embryological processes (Savagner *et al.*, 1994; Hay, 1995).

Now, we have come to appreciate the contribution of this phenomenon to both pathological and normal processes where EMT can be diagnosed by the expression of vimentin and the loss of epithelial cell-adhesion molecules (Savagner *et al.*, 1994; Hay, 1995). EMT has been shown to occur in cultured mammary epithelial cells (Stoker *et al.*, 1987) in a bladder carcinoma cell line, NBT-ll (Boyer *et al.*, 1989) and a prostate carcinoma cell line (Singh *et al.*, 2003). In culture, the definition of EMT is limited to

the escape of single cells from epithelial sheets, increased motility, and a modification of the differentiation so that the migrating cells no longer express epithelial characteristics, but require a mesenchymal phenotype (Boyer *et al.*, 1989).

Using the bladder cell line, NBT-ll, a study showed the mesenchymal phenotype defined by the acquisition of vimentin, while the loss of the epithelial phenotype is defined by the decline in keratin expression, in which a single marker defines the phenotype (Petersen *et al.*, 2001). It is this definition that has been adopted in the field of breast cancer research (Petersen *et al.*, 2001).

Thus, in a report studying EMT in human micrometastatic and primary breast carcinoma cells, findings showed that all micrometastatic cancer cell lines displayed loss of epithelial cytokeratins (CK8, CK18, and CK19) and ectopic expression of vimentin commonly present in mesenchymal cells (Willipinski-Stapelfeldt *et al.*, 2005). Immunohistochemical analysis of breast cancer samples further showed that the loss of cytokertain and ectopic vimentin expression were significantly associated with a higher tumor grade and higher mitotic index. This study indicated that micrometastatic cancer cells exhibit marked changes in the expression of cytoskeletal proteins indicative of EMT and is associated with the aggressive behavior of breast cancer cells *in vivo* (Willipinski-Stapelfeldt *et al.*, 2005), and is consistent with our observations in HNSCC cells. This increased expression of vimentin it the metastatic cell has been suggested as a critical marker to distinguish 'true complete EMT' from cell scattering' or 'partial EMT' (Grunert *et al.*, 2003).

5.6 Vimentin Expression Suppressed in HN12 Cells

In order to study the contribution of vimentin expression to the biological properties of HN12 cells, we used RNA interference (RNAi) technology to reduce vimentin levels. Colonies were screened for vimentin expression by qRT PCR, western blot analysis, and immunofluorescence, which showed a cytosolic distribution pattern of staining in the HN12-NTC clones and the absence of staining in the HN12-shVim clones. HN12-shVim clones show an efficient reduction compared to non-targeting controls and thus were suitable for investigating the contribution of vimentin expression to the biological phenotype of metastatic HN12 cells.

5.7 Vimentin Expression and Biological Properties

The ability of tumor cells to migrate and invade , which is interpreted clinically as tumor aggressiveness, has been associated with changes in intermediate filaments (Anguelov, 2000). The deregulation of intermediate filament gene expression in tumor cells results in an intermediate filament phenotype, which is conducive to a higher potential for invasion and migration (Anguelov, 2000). The observation that an altered intermediate filament phenotype is associated with the ability of tumor cells to invade adjacent tissues and organs and migrate in specific ways throughout the body could lead to new strategies for cancer management.

5.7.1 Vimentin Expression is Important for the Biological Properties of HNSCC

The presence of regional metastasis in patients with HNSCC is a common adverse event associated with poor prognosis and high mortality (Howell and Grandis, 2005). Squamous carcinoma cells tend to grow more rapidly and/or fail to die at a normal rate. In addition, these cells can also invade surrounding tissue and metastasize.

In order to determine whether the mesenchymal cell marker, vimentin, contributes to the phenotype of HN12 cells, we first compared the proliferation rate in culture of HN12-shVim and HN12-NTC cells. HN12-shVim clones exhibit a marked decrease in proliferation compared to the HN12-NTC cells, suggesting that vimentin contributes positively to cell growth. In addition, *in vitro* invasion through Matrigel and migration assays displayed markedly decrease in cells with lower expression of vimentin. These findings indicate a crucial role for vimentin in the proliferation and invasion of metastatic HN12 squamous carcinoma cells.

In accord with our HNSCC results, a study comparing the phenotype and behavior of seven head and neck squamous cell carcinoma cell lines found that the presence of the intermediate filament, vimentin coincided with the loss of anchorage dependency and a malignant phenotype (Tomson *et al.*, 1996).

5.7.2 Vimentin Expression and Biological Properties of Other Cell Lineages

After establishing that vimentin expression was only slightly detectable in LNCaP cells and highly expressed in the faster-growing, more aggressive CL1 subline, the study mentioned previously (Section 5.4.2) assessed the contribution of vimentin to an invasive phenotype (Singh *et al.*, 2003). This study found that experimentally reducing the

expression of vimentin in the CL1 cell line effectively abolished the invasive potential of CL1 in the *in vitro* Matrigel invasion assays (Singh *et al.*, 2003).

In addition, breast and cervical carcinoma models show the expression of vimentin and the loss of E-Cadherin, an epithelial cell marker, to be associated with high invasive abilities (Gilles et al., 1997) Experiments conducted with a breast cancer cell line showed that transient down-regulation of vimentin in MDA-MB-231 cells led to a decrease in their migratory ability (Hendrix *et al.*, 1997). The findings of another breast cancer study revealed adriamycin-resistant MCF-7 cells expressing vimentin with diminished keratin 19 expression to reduce desmosome and tight junction formation as determined by reduced immunodetection of their components desmoplakins I and II and zonula occludens (ZO)-1 (Sommers *et al.*, 2002). Consistent with our data in HNSCC cells, these studies confirm vimentin's central role in the proliferation and motility/invasiveness of advanced stage tumor cells.

5.8 Expression of the Vimentin Promoter in HN12 Cells

How the vimentin gene is regulated is important for understanding the relationship between gene expression and tumor progression, and, based on our data presented above, could ultimately contribute to controlling the invasiveness of some tumors. For the most part, vimentin expression coincides with cellular growth and is cell cycle regulated (Franke *et al*, 1979; Rittling and Baserga, 1987). We used vimentin promoter fragments, which begin at +72 and extend to –261, -719, -749, and –757 relative to the translational start site to investigate which transcription factor binding sites

mediate upregulation of vimentin expression in HN12 cells. The current model of eukaryotic gene regulation suggests that many positive and negative acting factors control the expression of the vimentin promoter (Wieczorek *et al.*, 2000).

The 261CAT sequence containing the GC-box, PEA3 and NF-κB sites showed substantial activity under conditions of growth factor withdrawal. It has been reported that at least eight sequences, which match the GC-box consensus site were found within the vimentin promoter (Rittling and Baserga, 1987). However, findings show that of these multiple GC-boxes, expression of the vimentin promoter is dependent on GC-box1 located at position –64 which binds Sp1 (Izmailova *et al.*, 1999). Mutation of the GC-box1 to a nonfunctional sequence results in little reporter gene expression despite the addition of considerable upstream DNA demonstrating that this element is required for regulated gene expression (Izmailova *et al.*, 1999). Here, our findings demonstrate that the transcriptional elements within 261CAT are important regulators for the activation of vimentin expression with evidence specifically pointing to GC-box and NF-κB sites.

A recent article reveals that the activation of the transcription factor, NF-κB, occurs in many human tumors and studies have shown that NF-κB can promote cell proliferation and oncogenesis, possibly by protecting cells from apoptosis (Huber *et al.*, 2004). Little is known about whether NF-κB is involved in tumor progression including EMT, which is a central process governing both morphogenesis and carcinoma progression in multicellular organisms. However, in a combined *in vitro/in vivo* model of mammary carcinogenesis, NF-κB was found to be essential in both the induction and maintenance of EMT and for *in vivo* metastasis (Huber *et al.*, 2004).

The activity of the vimentin promoter was much reduced in cells transfected with the 719CAT plasmid, which contains two sites (PS and H4TF-1) that bind the ZBP-89 repressor protein. ZBP-89 is a zinc-finger, Kruppel-like protein, which is thought to be ubiquitously expressed (Wu *et al.*, 2004). ZBP-89 is a transcriptional repressor in the case of vimentin (Wieczorek *et al.*, 2000), epithelial neutrophil-activating peptide-78 (Keates *et al.*, 2001), gastrin (Merchant *et al.*, 1996), ornithine decarboxylase (Law *et al.*, 1998b), and bovine adrenodoxin (Law *et al.*, 2000). However, for other genes such as p21 (Hasegawa *et al.*, 1999), a lymphocyte-specific protein tyrosine kinase (*lck*) (Yamada *et al.*, 2001), type 1 collagen (Hasegawa *et al.*, 1997), and stromelysin (Ye *et al.*, 1999), it appears to activate gene expression (Wu *et al.*, 2004). Furthermore, it has been suggested that ZBP-89 is a negative regulator of cell growth as overexpression in two gastric tumor cell lines (GH4 and AGS) inhibited cellular proliferation and led to cell cycle arrest of GH4 cells in G1 (Remington *et al.*, 1997).

Exactly, how ZBP-89 can function both as an activator and as a repressor of gene expression is unknown. It has been proposed that, for some genes, ZBP-89 and Sp1 can compete for binding to a GC-rich sequence (Law *et al.*, 1998a). However, in the case of the vimetin promoter, a separate PS element (at position –319 to –278) has been found, which does not directly bind Sp1 (Wieczorek *et al.*, 2000). Thus, in the vimentin gene, ZBP-89 and Sp1 (or Sp3) bind to separate DNA elements and interact with each other directly (Zhang *et al.*, 2003). It has been proposed that this interaction accounts for the dependence of vimentin gene activity on a functional GC-box1 and its associated factors (Wieczorek *et al.*, 2000). Evident by our results using 719CAT is the apparent

interaction between ZBP-89 and PS and H4TF-1, transcriptional factors within the promoter to repress the expression of vimentin.

Moderate restoration of activity was achieved with the addition of two AP 1 binding sites in the promoter (749CAT). Previous studies have implicated the activator protein (AP1) transcription factors in tumor progression (Young *et al.*, 1999). The AP1 family of basic, leucine-zipper (bZIP) proteins is composed of heterodimers of Jun (c-Jun, JunB, JunD), Fos (cFos, FosB, Fra1 and Fra2) or ATF (ATF-1, ATF-2)/CREB, or homodimers of Jun/Jun (Angel and Karin 1991; Rahmsdorf, 1996). Given the large number of interactive partners, it is evident that the AP1 family can mediate the expression of a wide variety of genes that could contribute to tumor progression (Wu *et al.*, 2003). The tandem AP1 sites with the vimentin promoter have been shown to be important for the serum and TPA inducibility of the vimentin gene (Rittling *et al.*, 1989), as well as expression in vimentin-positive breast cancer cell lines (Sommers *et al.*, 1994). Here, the impacts of the AP1 sites on the vimentin promoter is evident not only with the data demonstrating the increase of vimentin expression with the 749CAT plasmid compared to 719CAT, but also the substantial decrease seen when the AP1 sites are mutated in the 747mCAT.

It has been reported that, unlike many other proteins, c-Jun has the ability to activate vimentin gene expression both by the classical method of c-Jun binding as either a homodimer or heterodimer to its tandem AP1 sites as well as by an independent, synergistic interaction with Sp1, thereby enhancing its ability to recruit transcription factors (Wu *et al.*, 2003). In addition, it has been reported that c-Jun synergizes with Sp1

to enhance its subsequent binding to a GC-box element and only the leucine-zipper region of c-Jun is required for this (Wu *et al.*, 2003). This dual mechanism of action could greatly affect levels of gene expression.

Addition of the ASE site (757CAT) further increased promoter activity. Previously, it has been reported that the vimentin gene promoter contain an ASE site, which is unlike the usual enhancer element in that it requires the presence of the PS silencer element in *cis* to activate transcription fully (Stover and Zehner, 1992; Izmailova and Zehner, 1999). It has been reported that the upstream ASE antisilencer site binds Stat3 whereby its interaction with ZBP-89 can overcome repression and restore gene expression (Wu *et al.*, 2004). Therefore Stat3, a proven oncogene, can interact with a suggested growth suppressor, ZBP-89, to restore gene expression (Wu *et al.*, 2004). Thus, the interplay between Stat3 and ZBP-89, which could contribute to the activation of vimentin gene expression as occurs in EMT (Gilles and Thompson, 1996) and is crucial for controlling cellular growth by direct effects on gene expression.

Stats are a family of transcriptional activators, which play a central role in signaling pathways involving cytokines, growth factors, or peptide hormones (Ihle, 1996; Darnell, 1997; Horvath, 2000; Levy and Darnell, 2002). Activated receptors are protein-kinases of the JAK family, which phosphorylate Stats. Phosphorylated Stats form dimers via their SH2 domains and rapidly translocate to the nucleus where they bind regulatory DNA elements of target genes involved in cell proliferation, differentiation, apoptosis, and development (Wu *et al.*, 2004).

Stat3 is constitutively active in many tumors such as lymphomas, leukemia, multiple myeloma, brain, breast, lung, head and neck, and prostate cancers (Bowman *et al.*, 2000), and the constitutively active form of Stat3 can mediate cellular transformation (Bromberg *et al.*, 1999). In addition, vimentin has been shown to be highly expressed in these tumors (Bussemakers *et al.*, 1992; Hsieh *et al.*, 1995; Zajchowski *et al.*, 2001). Thus, a correlation between activated Stat3 and vimentin gene expression is indicated, which coincides with the results of this present study.

In conclusion, the data suggest that basal activity of the vimentin promoter in HN12 cells is mediated through ASE and AP1 sites, as well as one or more of the GC box, PEA3 and NF-κB sites. Furthermore, the data indicate that ZBP-89 repressor activity is high in these cells.

5.9 EGF and TGF-β and Vimentin Expression

Epidermal growth factor (EGF) and transforming growth factor β (TGF-β) are thought to contribute to tumor metastasis through a number of mechanisms, including enhanced cell migration and EMT, and have been demonstrated to enhance migration and invasion of HN12 cells (Yeudall et al., 2005; Miyazaki et al., in press). Vimentin RNA was increased in TGF-β treated cells over basal expression levels, as well as with the addition of EGF. In addition, a synergistic increase was evident when cells were treated with a combination of TGF-β and EGF, suggesting that both growth factors play a role in EMT in this system. In addition, western blot analysis demonstrated increased levels of

vimentin protein were detected in TGF-β and EGF-treated HN12 cells, concomitant with the previously observed rise in vimentin RNA and immunocytochemical staining, HN12 cells displayed an ubiquitous distribution pattern of vimentin throughout the cell, which was further enhanced with the addition of EGF and TGF-β.

5.10 EGF and TGF-β and the Vimentin Promoter

Next, we examined vimentin promoter activity in the presence of EGF and TGF-β to determine the binding elements responsible for the increased vimentin expression observed by qRT-PCR, western blot analysis, immunocytochemistry, and immunofluorescence. Using the 757CAT sequence, we found that treatment of cells with either EGF or TGF-β stimulated promoter activity above that observed under conditions of serum withdrawal, while the presence of both growth factors further elevated CAT activity. In contrast, CAT activity in cells transfected with the 261CAT sequences (containing the GC box, PEA3 and NFκB sites) was maximal in the presence of EGF, which stimulated promoter activity. However, in cells transfected with the 749CAT plasmid, TGF-β produced an increase in promoter activity over basal levels, whereas EGF had little effect.

Taken together, the data suggest that EGF-dependent pathways target binding elements in the proximal vimentin promoter, while TGF-β is likely to act in an AP1-dependent manner. Furthermore, both growth factors appear to synergize by stimulating promoter activation through the ASE site, suggesting involvement of Stat-dependent pathways in regulation of vimentin expression in HN12 cells. Here, our findings

demonstrate that the induction of vimentin expression with the addition of growth factors, EGF and TGF-β occurs at the transcriptional level of the promoter targeting specific transcriptional sites.

5 11 TGF-β and Tumorigenesis

TGF-β is a multipotent cytokine that regulates a variety of cellular activities, such as cell proliferation, differentiation, and extracellular matrix (ECM) formation (Liberati et al., 1999). TGF-β plays a complex role in tumorigenesis since it has both tumor suppressor and oncogenic activities (Akhurst and Derynck, 2001; Derynck et al., 2001; Wakefield and Roberts, 2002). During the early stages of tumorigenesis, TGF-β, acts as a tumor suppressor. It is suggested to be predominantly through its ability to induce growth factor arrest and apoptosis in epithelial cells for which the majority of human cancers derive (Nicolas and Hill, 2003). It is at the late stages when the pro-oncogenic activities of TGF-β dominate. TGF-β acts directly on the tumor cells to induce EMT, and to increase motility, invasiveness and metastasis, and on the surrounding stroma to suppress immune surveillance and increase angiogenesis (Akhurst and Derynck, 2001; Derynck et al., 2001; Wakefield and Roberts, 2002).

TGF-β profoundly influences the differentiation of many cell types of mesechymal origin, including preadipocytes (Ignotz and Masague, 1985; Choy et al., 2000) osteoblasts (Centrella et al., 1994) and myoblasts (Olson, 1992). The identification of genes transcriptionally regulated by TGF-β and the elucidation of the molecular mechanisms responsible for this transcriptional regulation will help define how TGF-β

exerts its cellular effects and its role in resulting physiological processes (Liberati *et al.*, 1999).

Interestingly, research studies have shown that it is the association of the Smad complexes with transcription factors and transcriptional co-activators/co-repressors in the nucleus that further regulate transcriptional control by TGF-β (Davies *et al.*, 2005). The receptor-associated Smads (R-Smads) interact directly with, and are phosphorylated by, activated type I receptors of the TGF-β superfamily (Verrecchia *et al.*, 2001).

5.12 TGF-β and AP1 Binding Sites

Numerous studies have characterized the differential expression of specific genes in response to TGF-β, revealing a common link in the ability of TGF-β to regulate many of these genes through the functions of the AP-1 family of transcription factors (Liberati *et al*, 1999). The ability of TGF-β to induce the expression of several genes, including PAI-1, clusterin, monocyte chemoattractant protein-1 (JE/MCP-1), type I collagen, and TGF-β itself depends on specific AP1 DNA-binding sites in the promoter regions of these genes (Keeton *et al.*, 1991; Jin and Howe, 1997; Armendaruz-Borunda *et al.*, 1994; Kim *et al.*, 1990; Takeshita *et al.*, 1995; Mauviel *et al.*, 1996). It has also been shown that expression of many AP1 proteins themselves is induced as an early response to TGF-β in a cell type-specific manner (Beauchamp *et al.*, 1996; Blatti *et al.*, 1992; Pertovaara *et al.*, 1989; Wong *et al.*, 1999). These studies demonstrate a link between TGF-β signaling and AP1 in TGF-β regulated expression of various genes (Liberati *et al.*, 1999). Interestingly, components of the AP1 transcriptional complex were recognized early as

transcriptional targets of TGF-β signaling before the mechanisms of TGF-β transduction were known (Pertovaara *et al.*, 1989; Mauviel *et al.*, 1998; Kim *et al.*, 1990)

5.13 TGF-β Induction via Smad Dependent and Independent Pathways

It has been suggested that several genes, such as those encoding the plasminogen activator inhibitor-1 (PAI-1), the α2 chain of type I collagen (COL1A2), or the interstitial collagnease (MMP-1), in which TGF-β responsiveness was originally described as AP-1 dependent (Keeton *et al.*, 1991; Chung *et al.*, 1996; Mauviel *et al.*, 1996) can also be regulated by Smads, via either Smad-specific *cis* elements. It is known that Smad3 activation occurs in response to TGF-β, in which Smad3/DNA interaction is detectable as early as 10 minutes after TGF-β addition (Vindevoghel *et al.*, 1998a). Recent findings have demonstrated transcriptional responses depend on the structure of the target promoter, whether they contain AP1 or Smad specific cis-elements (Verrecchia *et al.*, 2001).

It is now recognized that TGF-β1 can activate mitogen-activated protein kinases (MAPKs) (Hartsough and Mulder, 1995; Yamaguchi *et al.*, 1995; Atfi *et al.*, 1997; Bhowmick *et al.*, 2001b). The AP1 transcriptional complex is a primary target of a number of MAPK pathways and it has been shown that AP1 components can interact directly with Smad3 (Zhang *et al.*, 1998, Peron *et al.*, 2001; Verrechia *et al.*, 2001). This suggests that AP1 may be central to crosstalk between Smad and MAPK pathways (Davies *et al.*, 2005).

There is convincing evidence for the involvement of MAPK signaling in TGF-β1-induced EMT (Ellenrieder *et al.*, 2001; Zavadil *et al.*, 2001; Bakin *et al*, 2002). A recent study showed that TGF-β1-induced EMT involves Smad-dependent pathways (Davies *et al.*, 2005), which is in agreement with the limited number of studies using cells of human origin (Ellenrieder *et al.*, 2001; Tian *et al.*, 2003). MAPKs function to enhance Smad 2/3-dependent transcription TGF-β1-induced EMT (Davies *et al.*, 2005). In conclusion, MAPK and Smads pathways, together with the AP-1 complex mediate TGF-β1-induced EMT (Davies *et al.*, 2005).

These research findings confirm our results, in which cells transfected with the 749CAT plasmid, TGF-β produced a substantial increase in promoter activity over basal levels. This upregulation of the 749CAT vimentin promoter can be explained through TGF-β interaction with the promoter tandem AP1 sites as there are a number of research findings that provide strong evidence to support the role of Ap-1 in TGF-β1-induced EMT.

Interestingly, research studies also show that the transcriptional factor NF-κB functionally cooperates with Smad3 as well as the transcriptional factor protein, Sp1, which is found to functionally cooperate with Smad 2/3/4 (Miyazono *et al.*, 2001) These research findings can be used to explain the upregulation of vimentin expression with the addition of TGF-β to the 261CAT plasmid since both Smad 2 and 3 are activated by the growth factor TGF-β.

5.14 EGFR Induction

The precise mechanisms by which growth factor-induced cell surface signals are transmitted to the nucleus and ultimately result in cell division are partially understood. In the response to ligands, EGFR dimerizes and becomes phosphorylated on multiple tyrosine residues. These phosphotyrosines, in turn, allow the activated receptor to associate with other signaling proteins. Several EGFR signaling intermediates have been described including ras/MAP kinase, PI-3-kinase, and PLCγ.

5.15 EGF and sp1 Binding Protein

Despite the proven ability of sp1 to regulate the transcriptional activity of a variety of genes involved in cell differentiation and proliferation (Merchant et al., 1995, Li et al., 1995), little is known about its role in tumor growth and progression. High levels of sp1 have been reported in gastric carcinomas (Kitadai *et al.*, 1992) and a coordinate overexpression of sp1 and lamin-γ1 was found in human hepatocellular carcinomas (Lietard *et al.*, 1997).

In a study using breast cancer models, western blot analysis with anti-Sp1 antibody on nuclear extracts from seven malignant specimens and three breast lesions conformed the presence of varying nuclear levels of sp1 proteins in the malignant tumors and the absence of a detectable signal in benign breast lesions (Zannettii *et al.*, 2000). Promoter assays were studied and the binding data reflect Sp1 protein relative to the levels assessed by western blot analysis of the same samples (Zannettii *et al.*, 2000).

In addition , it has been previously shown for other genes, such as, gastrin (Porchet *et al.*, 1999) or apolipoprotein A-I (Zheng *et al.*, 2001), that sp1 may participate to EGF-mediated up-regulation of the target gene. Using a lung cancer cell line, researchers were able to show in MUC2 and MUC5AC, two target genes of EGFR ligands in lung cancer cells, that sp1 not only transactivated MUC2 and MUC5AC transcription but also participates in their up-regulation by EGF and TGF-α (Perrais *et al.*, 2002).

These studies confirm the substantial activation of 261CAT by EGF, indicating that EGF targets the sp1 transcriptional binding protein within the vimentin promoter. Interestingly, further studies might investigate the amount of sp1 protein in metastatic HN12 cells to determine that not only is sp1 activated by EGF signaling pathway, but there might also be an increased present amount of sp1 protein in the metastatic cell similar to the breast cancer mentioned previously. In conclusion, these studies help define new therapeutic strategies in carcinomas in which one may want to efficiently and selectively inhibit EGFR-signaling pathway to reduce or prevent metastasis.

5.16 Stat3 Protein and Metastasis

It has been shown that the Stat3 protein plays an important role in the growth and survival of breast cancers in culture and *in vivo* (Gracia *et al.*, 2001; Zajchowski *et al.*, 2001). Maximal Stat3 activation is thought to result from the cooperation of different growth factors receptors, cytokine receptors, and nonreceptor tyrosine kinases (Wu et al., 2004). Thus, constitutive activation of Stat3 is more predominant in highly aggressive,

metastatic breast cancers, and inhibiting Stat3 activation can block the malignant progression of breast tumors (Bowman et al., 2000; Gracia et al., 2001). Established breast cancer cell lines demonstrate constitutively active Stat3 homodimers (Sartor et al., 1997).

EGF has been shown to be capable of activating Stats 1 and 3 in EGFR-overexpressing cells (Zhong et al., 1994; Leaman et al., 1996; Chin et al., 1996; Nakamura et al., 1996), Stat activation in these cells has not been linked to proliferation or transformation. However, recent studies show that Src-induced transformation of fibroblasts results in Stat3-mediated gene expression (Turkson et al., 1998) and that Stat3 is constitutively activated in breast carcinoma cell lines but not in normal breast epithelial cells (Gracia et al., 1997). The results presented in these studies are the first demonstration of growth inhibition after selective downmodulation of a Stat protein in EGFR-overexpressiong cells.

These research findings confer with our results in which the EGF induced activation of the 757CAT vimentin promoter, which contains the ASE transcriptional site that is activated by the transcriptional protein, Stat3. In addition, 757CAT also displayed induction with the addition of growth factor, TGF-β.

It has been proposed in other cell models the formation of a complex between Stat3 and Smad1 indicating that these two binding motifs may physically interact via an adaptor molecule. The CREB-binding protein (CBP)/p300 family of transcriptional coactivators interact with various transcription factors, such as, AP1, Myb, and nuclear receptors altering there activity (Kamei et al., 1998). Interestingly, Smad1, 2, 3, and 4

associate with CBP/p300 family members (Nishihara *et al.*, 1998; Janknecht *et al.*, 1998). This study showed the formation of a complex between Stat3 and Smad1, bridged by p300, is involved in the cooperative signaling of LIF and BMP2 and the subsequent induction of astrocytes from neural progenitors (Nakashima et al., 1999). In conclusion, the transcriptional coactivator p300 physically interacts with Stat3 and Smad1, in which the formation of a complex between Stat3 and Smad1, bridged by p300, is involved in the cooperative signaling of the pathway (Nakashima et al., 1999).

With Smad1 being a downstream target of TGF-β and CBP/p300 family members also associating with Smad2 and 3 provides strong evidence for the further increase in induction of the 757CAT plasmid with the addition of TGF-β compared to EGF stimulation alone. Furthermore, this concept provides increasing evidence for the synergistic effect found with the addition of both growth factors confirming Stat3 as the target for induction by both EGF and TGF-β.

5.17 Conclusion

In conclusion, HNSCC remains the sixth most common malignancy worldwide (Parkin *et al.*, 1999; Landis *et al.*, 1999). While primary lesion undergo hematogenous spread only rarely, they are locally aggressive, and frequently metastasize to local and regional lymph nodes, with lethal consequences (Yeudall *et al.*, 2005). Therefore, it is the prevention and early detection that are of great importance to reduce the incidence of, and consequent morbidity from, HNSCC (Yeudall *et al.*, 2005). One of the major goals to combat this disease involves the ability to prevent invasion and regional metastasis of

the primary lesions. This requires a detailed understanding of the basic cellular biology and biochemistry of invasion and metastasis in this cell system (Yeudall *et al.*, 2005).

With our findings, it can be suggested that particular transcription factors and the signaling pathways involved in their activation should receive attention as potential targets for the developments of novel anti-metastatic cancer treatments. Future studies involve using a CHIP assay to confirm the findings of the transcriptional data. As well as further evaluating our preliminary finding, which demonstrate a decrease of cytokertain in the metastatic, HN12 cell line.

6. Future Experiments

6.1 Downregulation of Cytokeratin in HN12 cells
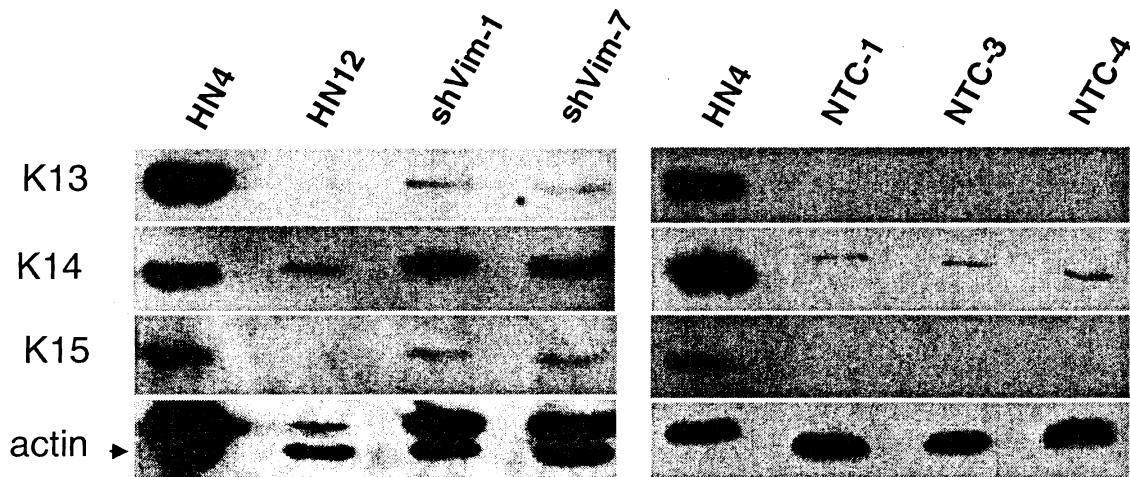
Whereas vimentin is overexpressed in metastatic HN12 cells, an epithelial specific intermediate filament, cytokeratin, is downregulated. Here, we show that HN12 cells demonstrate a downregulation of cytokeratin 13, 14, and 15 by western blot analysis of total cellular protein lysates. After 48h of serum starvation, total cell protein extracts were prepared as described in 'Materials and Methods' and analyzed for cytokeratin expression (top panels). Levels of β-actin were determined as a loading control (lower panel). Anti-cytokeratin 13, 14, and 15 monoclonal antibodies were used (Waseem *et al.*, 1998; Waseem *et al.*, 1999).

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Whereas each cytokeratin was readily detectable in HN4 cell lysates, no signal was obtained in HN12 lysates with cytokeratins 13 and 15 and diminished signal with cytokeratin 14. Furthermore, increased cytokeratin 13, 14, and 15 expression was detectable by western blot analysis in two HN12-shVim clones compared to the parental HN12 cell line and HN12-NTC clones. These data indicate that cytokeratin 13, 14, and 15 expression is downregulated at a later stage of tumor progression.

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