RAS SIGNALING IN TUMOR INITIATION AND MAINTENANCE

by

Brooke B. Ancrile

University Program in Genetics and Genomics
Duke University

Date: April 4, 2008

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Joseph R. Nevins, Ph.D.

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Christopher D. Kontos, M.D.

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University Program in Genetics and Genomics in the Graduate School of Duke University

2008
ABSTRACT

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Abstract

The Ras proteins, composed of H, N, and KRas, are a family of small GTPases that normally transmit extracellular cues to the cell in a regulated manner. However, Ras is commonly mutated to be inappropriately activated in human cancers, promoting a vast array of tumor phenotypes. Activation of the Raf, PI3K, and RaGEF Ras effector pathways is required to promote Ras-mediated tumorigenesis, leading not only to cell autonomous tumor phenotypes, but also the establishment of a tumor microenvironment. However, following tumor initiation, the requirement upon oncogenic Ras signaling is reduced to activation of PI3K, most likely due to a contribution of the tumor microenvironment. In order to further delineate the requirements for oncogenic Ras signaling pathways during tumorigenesis, I sought to 1) identify PI3K-independent factors necessary for tumor initiation, and 2) determine how PI3K activation maintains tumor growth in the absence of oncogenic Ras. Using cell-based assays and tumorigenesis assays in mice, I have shown that interleukin-6 (IL-6) is secreted upon induction of Ras expression, is required for Ras-mediated tumor initiation, and promotes tumorigenesis in a paracrine manner by fostering angiogenesis. Additionally, I have shown that eNOS, a downstream target of the PI3K pathway, is required for Ras-induced tumor initiation and maintenance, and, moreover, that eNOS-mediated S-nitrosylation and activation of wildtype Ras proteins is required throughout
tumorigenesis. Pancreatic cancer is the cancer most highly associated with oncogenic Ras mutations, and I have shown that both IL-6 and eNOS are required for the tumorigenic growth of pancreatic cancer cell lines in mice. I therefore suggest that these proteins, perhaps in combination with other Ras inhibitors, may provide potential anti-cancer targets for oncogenic-Ras driven cancers in the clinic.
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<table>
<thead>
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<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-OHT</td>
<td>4-hydroxytamoxifen</td>
</tr>
<tr>
<td>AKT</td>
<td>protein kinase B (PKB), serine/threonine specific kinase</td>
</tr>
<tr>
<td>BH₄</td>
<td>tetrahydrobiopterin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DMBA</td>
<td>7,12-Dimethylbenzanthracene</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen response element</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>FMN</td>
<td>flavin mononucleotide</td>
</tr>
<tr>
<td>GADPH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
</tbody>
</table>
GDP: guanosine diphosphate
GEF: guanine nucleotide exchange factor
GNE: guanine nucleotide exchange
GRO-1: growth-regulated oncogene
GTP: guanosine triphosphate
GTPase: guanosine triphosphatase
H & E: hematoxylin and eosin stain
HEK: human embryonic kidney cells
HMEC: human mammary epithelial cells
hTERT: human catalytic subunit of telomerase
HMVEC: human microvascular endothelial cells
IgG: Immunoglobulin G
IL-6: interleukin 6
IL-6R: interleukin 6 receptor
IL-8: interleukin 8
iNOS: inducible nitric oxide synthase
KC: keratinocyte chemoattractant
MAPK: mitogen-activated protein kinase
MEK: MAPK/ERK kinase
MIP-2: macrophage inflammatory protein
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NO$_2$</td>
<td>nitrogen dioxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>nNOS</td>
<td>neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>O$_2$</td>
<td>oxygen</td>
</tr>
<tr>
<td>PanIN</td>
<td>pancreatic intraepithelial neoplasia</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>phosphatidylinositol-3,4,5-triphosphate</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PP2A</td>
<td>protein phosphatase 2A</td>
</tr>
<tr>
<td>pRb (Rb)</td>
<td>retinoblastoma protein</td>
</tr>
<tr>
<td>RalGEFs</td>
<td>Ral guanine nucleotide exchange factors</td>
</tr>
<tr>
<td>Ras</td>
<td>H/K/N-isoforms, small GTPase, protooncogene</td>
</tr>
<tr>
<td>Ras$^{G12V}$</td>
<td>oncogenic form of Ras</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>sGC</td>
<td>soluble guanylyl cyclase</td>
</tr>
<tr>
<td>shRNA</td>
<td>small hairpin ribonucleic acid</td>
</tr>
<tr>
<td>SNO</td>
<td>S-nitrosothiol</td>
</tr>
<tr>
<td>STC-1</td>
<td>stanniocalcin-1</td>
</tr>
<tr>
<td>SV40</td>
<td>simian virus 40</td>
</tr>
<tr>
<td>t-Ag</td>
<td>small t antigen</td>
</tr>
<tr>
<td>T-Ag</td>
<td>large T antigen</td>
</tr>
<tr>
<td>TERT</td>
<td>catalytic subunit of telomerase</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol-13-acetate</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase mediated dUTP nick end labeling</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
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Thank you for leaving your job to move to North Carolina with me, without ever having been to the state before you drove into it in the moving truck. I love you very, very much, and am so thankful that I have you to take care of me. Thank you to the rest of the Ancrile family, for all of the love and support you have provided Jordy and me; both before and during our time in North Carolina.

Most of all, I’d like to thank God, for blessing me with everything I have in my life. My faith in you has kept me going and provided the inspiration to reach my goals.
1. Introduction

1.1 Ras: Signaling and Role in Tumorigenesis

The development of human cancer is a multi-step process during which the accumulation of genetic alterations converts normal cells into malignant tumors. Such changes include acquired limitless replicative potential, insensitivity to anti-growth signals, and self-sufficiency in growth signals (Hanahan and Weinberg 2000). In human cancers, commonly found genetic alterations include activation of the telomerase catalytic subunit hTERT, disruption of the p53 and Rb tumor suppressor pathways, and inappropriate activation of the Ras pathway. Similarly, a wide variety of cell types can be driven to a tumorigenic state via the expression of hTERT, simian virus 40 large T- and small t-antigens (SV40 T/t), which disrupt the p53 and Rb pathways and inhibit the function of PP2A, and HRasG12V, a constitutively active mutant form of the Ras oncogene (Hahn et al. 1999). This model system provides a simple, genetically malleable system for studying Ras oncogenesis.

The small GTPase family of Ras proteins, composed of N, K, and HRas, transmit extracellular cues from surface receptors to the cell. Simulation of cell surface receptors leads to the activation of guanine exchange factors, which in turn convert Ras from an inactive GDP-bound state to an active GTP-bound state. In this active state, Ras adopts a conformation that permits effector proteins such as PI3Ks, Rafs, and RalGEFs to bind
Ras, which in turn leads to their activation and propagation of signaling. This signal is, in turn, terminated by GTPase activity proteins (GAPs) that stimulate the GTPase activity of Ras, returning Ras to its inactive GDP-bound state (Downward 2003). This signaling pathway is summarized in Figure 1.

![Figure 1: Ras upstream and downstream signaling](image)

Extracellular stimuli signal through cell surface plasma membrane receptors, for example, RTKs. Through a variety of adaptor proteins, these signals cause guanine nucleotide exchange factors (GEFs) to replace the GDP-bound to inactive Ras with GTP. GAPs trigger the hydrolysis of GTP back to the inactive GDP-bound form. GTP-bound Ras binds to a plethora of downstream effector molecules to stimulate intracellular signaling of several pathways. Those with established roles in Ras oncogenesis include the Raf serine/threonine kinases, the PI3K lipid kinases, and RapGEFs. Activation of these pathways and others has been shown to cause changes in many mechanisms leading to transformation, invasion and metastasis (Campbell and Der 2004). Adapted with permission of the publisher.
While the Ras family of proteins normally plays an important role in several cell processes, these proteins are mutated to yield a constitutively active oncogenic protein in one third of all human cancers (Bos 1989). Common point mutations at G12, G13, or Q61 impair intrinsic and GAP-stimulated GTP hydrolysis, leaving oncogenic Ras in a constitutively active GTP-bound state (Bos 1989). In fact, in some cancers such as pancreatic, 90% of tumors carry an activating Ras mutation (Bos 1989). The most commonly mutated Ras family member is KRas, representing about 85% of total Ras mutations, while NRas and HRas mutations represent about 15% and <1%, respectively (Downward 2003). A summary of the rates of Ras mutations in different cancers and the most commonly mutated Ras family member(s) in each tumor type are shown in Table 1.

If Ras itself is not mutated, often Ras signaling components are inappropriately activated or repressed to promote Ras signaling. Upstream cell surface receptors that activate Ras, such as EGFR and Her2/Neu, are often amplified or mutated to remain active, resulting in chronic Ras activation (Downward 2003). In fewer cases, there is a loss of a RasGAP that renders Ras active (Downward 2003). On the other hand, activating mutations to Raf and PI3K or loss of PTEN, an inhibitor of the PI3K signaling pathway, can similarly lead to activation of downstream effector pathways Raf and PI3K (Downward 2003). Thus, the Ras pathway is typically activated in human cancers.
Table 1: Activation of Ras signaling pathways in different tumors

<table>
<thead>
<tr>
<th>Defect or mutation</th>
<th>Tumour type</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAS mutation</td>
<td>Pancreas</td>
<td>90 (K)</td>
</tr>
<tr>
<td></td>
<td>Lung adenocarcinoma (non-small-cell)</td>
<td>35 (K)</td>
</tr>
<tr>
<td></td>
<td>Colorectal</td>
<td>45 (K)</td>
</tr>
<tr>
<td></td>
<td>Thyroid (Follicular)</td>
<td>55 (H, K, N)</td>
</tr>
<tr>
<td></td>
<td>Thyroid (Undifferentiated papillary)</td>
<td>60 (H, K, N)</td>
</tr>
<tr>
<td></td>
<td>Seminoma</td>
<td>45 (K, N)</td>
</tr>
<tr>
<td></td>
<td>Melanoma</td>
<td>15 (N)</td>
</tr>
<tr>
<td></td>
<td>Bladder</td>
<td>10 (H)</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>30 (N)</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>10 (H)</td>
</tr>
<tr>
<td></td>
<td>Myelodysplastic syndrome</td>
<td>40 (N, K)</td>
</tr>
<tr>
<td></td>
<td>Acute myelogenous leukaemia</td>
<td>30 (N)</td>
</tr>
<tr>
<td>BRAF mutation</td>
<td>Melanoma</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>Colorectal</td>
<td>12</td>
</tr>
<tr>
<td>EGFR overexpression</td>
<td>Most carcinomas</td>
<td>&gt;50</td>
</tr>
<tr>
<td>ERBB2 amplification</td>
<td>Breast</td>
<td>30</td>
</tr>
<tr>
<td>PTEN loss</td>
<td>Glioblastoma multiforme</td>
<td>20–30</td>
</tr>
<tr>
<td></td>
<td>Prostate</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Pancreas</td>
<td>40</td>
</tr>
<tr>
<td>AKT2 amplification</td>
<td>Ovarian</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Pancreas</td>
<td>10</td>
</tr>
<tr>
<td>PI3K amplification</td>
<td>Ovarian</td>
<td>40</td>
</tr>
</tbody>
</table>

EGFR, epidermal-growth-factor receptor; PI3K, phosphatidylinositol 3-kinase. H, K, and N refer to HRAS, KRAS, and NRAS, respectively (Downward 2003). Used with permission from the publisher.
Constitutive activation of Ras promotes a variety of tumor phenotypes. Gain of function mutations to Ras that occur in human cancers, such as those at position 12, 13, and 61, endow the protein with the ability to promote cell proliferation, cell survival, cell migration, tumor growth, and metastasis in cell culture and animal models (Shields et al. 2000). In contrast, extinguishing oncogenic Ras expression in established tumors leads to tumor regression (Chin et al. 1999; Fisher et al. 2001; Felsher 2004). Thus, inappropriate activation of Ras is oncogenic, and hence understanding how Ras promotes tumorigenesis could have therapeutic value in the treatment of many types of cancers.

Active Ras stimulates multiple downstream effector pathways. The Raf, Ral guanine nucleotide exchange factor (RalGEF), and phosphoinositide 3-kinase (PI3K) pathways have been shown to play an important role in the tumorigenic process (Figure 1). Activation of Raf leads to phosphorylation and activation of mitogen-activated protein kinase kinases (MEKs), which then phosphorylate the mitogen-activated protein kinases (MAPKs), ERK1 and ERK2, and the activation of this pathway is thought to stimulate transcription and cell-cycle progression, contribute to changes in cell morphology, and promote anchorage-independent growth and angiogenesis (Morrison and Cutler 1997; Shields et al. 2000; Downward 2003). Stimulation of RalGEFs by GTP-Ras leads to activation of the small GTPases RalA and RalB, and this pathway is also thought to contribute to cell-cycle progression (Downward 2003; Feig 2003). Despite a
high degree of sequence similarity, RalA and RalB seem to play different roles in tumorigenesis. While RalA is required for transformation of human cells, RalB may be important for cell survival and tumor metastasis (Lim et al. 2005; Chien et al. 2006; Lim et al. 2006).

Ras activates class IA PI3Ks, which are a family of lipid kinases made up a p85 regulatory subunit and p110 catalytic subunit. These proteins are activated upon stimulation of a RTK, resulting in recruitment to the membrane either by binding of the p85 subunit to an adaptor protein or by binding of the p110 subunit to active, GTP-bound Ras (Engelman et al. 2006). Following recruitment to the membrane, PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to generate phosphatidylinositol-3,4,5-triphosphate (PIP3), which then recruits several proteins, including the kinases AKT (PKB) and PDK-1. PDK-1 phosphorylates and activates AKT, which in turn phosphorylates many proteins, resulting in increased cell-survival, as well as changing cell morphology and promoting angiogenesis (Downward 2003; Luo et al. 2003). PTEN, a tumor suppressor, acts as an inhibitor of the pathway by blocking conversion of PIP2 to PIP3. The AKT signaling pathway is commonly inappropriately activated in cancers, either by an activating mutation in Ras, RTK overexpression, amplification of AKT or PI3K, or loss of PTEN (Table 1) (Downward 2003).

Activation of effector pathways by oncogenic Ras is thought to provide self-sufficiency in growth signals, leading increased cell proliferation and cell survival of the
tumor cells. However, in addition to autocrine growth signals, Ras also communicates with the surrounding stroma during tumor initiation to establish the tumor microenvironment. The tumor microenvironment plays a critical role in tumorigenesis (Coussens and Werb 2002; Bergers and Benjamin 2003; Mueller and Fusenig 2004). Modification of the tumor stroma, which is composed of fibroblasts, immune and inflammatory cells, fat cells, and blood-vessel cells, is important for tumor growth and development. During tumor initiation, paracrine signaling from cancer cells modulates the surrounding stroma to establish a microenvironment conducive for tumor growth (Mueller and Fusenig 2004). Important modulations of the stroma include activation of fibroblasts and recruitment of inflammatory cells, which secrete proteases that release growth factors (Coussens and Werb 2002; Mueller and Fusenig 2004). The recruitment of endothelial cells for the formation of a vascular system, which provides oxygen and nutrients to the tumor cells (Bergers and Benjamin 2003), is also an important step in the establishment of a microenvironment, and infiltrating inflammatory cells in the tumor stroma have been shown to play an important role in the angiogenic process (Coussens et al. 1999). An increasing number of examples demonstrate that activation of Ras in tumor cells positively acts to foster changes in the tumor microenvironment. For example, oncogenic Ras has been shown to promote tumor cell invasion, in part due to the induction of matrix metalloproteinases, which are proteases involved in degradation of the extracellular matrix, an event necessary for tumor invasion and metastasis.
Oncogenic Ras has also been linked to the induction of tumor angiogenesis. Loss of oncogenic Ras expression in tumor cells results in apoptosis of CD31-positive (endothelial) cells, and a subsequent collapse in tumor vasculature and regression of the tumor, suggesting that oncogenic Ras paracrine signaling promotes survival of endothelial cells (Tang et al. 2005). Additionally, Ras has been shown to transcriptionally upregulate VEGF, which acts upon endothelial cells to promote angiogenesis (Okada et al. 1998; Rak et al. 2000).

Because the development of the tumor microenvironment following tumor initiation is known to foster tumorigenic growth, Lim and Counter (Lim and Counter 2005) sought to determine the requirement for oncogenic Ras effectors for tumor initiation versus during tumor maintenance. Human embryonic kidney (HEK) cells engineered to express molecules that preferentially activate the MAPK, PI3K, or RalGEF pathway, either alone or in combination, were tested for their ability to initiate tumor growth. Only cells expressing all three pathways were able to initiate tumor growth, suggesting that each of these effector pathways is required for tumor initiation. In order to determine the requirement for these Ras effector pathways during tumor maintenance, HEK cells carrying an inducible ER:RasG12V protein were injected into mice. These mice were treated with 4-hydroxytamoxifen (4-OHT), resulting in expression of the oncogenic Ras protein, to initiate tumor growth. Following tumor initiation, 4-OHT treatments were stopped, leading to tumor regression due to a continued requirement
for oncogenic Ras during tumor maintenance. To determine which Ras effector pathway(s) were required to maintain tumor growth, cells expressing ER:RasG12V in combination with an effector mutant to preferentially activate PI3K, MAPK or RalGEF were injected into mice, and the mice were treated with 4-OHT until a tumor mass was visible. 4-OHT treatments were then ceased and tumor growth was monitored. Interestingly, tumor growth was maintained in the absence of oncogenic Ras if tumor cells expressed activated PI3K, while tumors regressed in cells with activated MAPK or RalGEF. These results indicate that in an established tumor, the requirement for oncogenic Ras signaling in tumor cells is reduced to activation of the PI3K pathway alone, potentially due to the contribution of the tumor microenvironment during tumor maintenance.

In order to further delineate the requirements for oncogenic Ras signaling pathways during tumorigenesis, I sought to 1) identify PI3K-independent factors necessary for tumor initiation, and 2) determine how PI3K activation maintains tumor growth in the absence of oncogenic Ras. These questions were the topic of my graduate work, and I will address my findings to these matters in this thesis.
1.2 Ras and Tumor Initiation

1.2.1 Identification of Factors Necessary for Tumor Initiation

In an attempt to identify PI3K-independent factors required for tumor initiation, I focused on secreted proteins, which play an important role in communication with the tumor stroma and development of the tumor microenvironment, and are desirable anti-cancer targets. Because they are located in the interstitial space, these “druggable” molecules are more accessible to pharmacological inhibitors than intracellular targets, and can be inhibited with neutralizing antibodies. Specifically, I focused on secreted proteins required for tumor angiogenesis, as Ras is known to promote the establishment of tumor vasculature (Rak et al. 1995; Arbiser et al. 1997), an important step in the formation of a tumor microenvironment. Therefore, the profiles of secreted angiogenic proteins were compared in Ras-expressing cells versus non-tumorigenic cells with activated PI3K, and I found that several cytokines, including interleukin-6, were highly induced in cells with a constitutively activated Ras pathway. These results are supported by several recent studies showing that cytokines may mediate oncogenic Ras-induced paracrine signaling.
1.2.2 Ras and Cytokines

Recent data suggest that cytokines, a class of secreted proteins, may mediate oncogenic Ras paracrine signaling. Cytokines are small, secreted proteins which act in a paracrine manner to facilitate the interaction and communication of other cells, often eliciting an immune response. Recent studies have documented that oncogenic Ras upregulates the expression of the cytokines interleukin-8 (CXCL-8/IL-8), and chemokine growth-regulated oncogene (GRO-1).

Sparmann and Bar-Sagi (Sparmann and Bar-Sagi 2004) reported that activation of a tetracycline-inducible oncogenic HRas transgene in HeLa cells led to elevated mRNA and secreted protein levels of IL-8. Overexpression of oncogenic HRas similarly increased expression and secretion of IL-8 in lung carcinoma (H125) and breast epithelial (MCF10A) cell lines, suggesting that this effect was not specific to one cell type. Increased IL-8 expression was critical for Ras-driven tumor growth in the HeLa cells, as antibody-mediated neutralization of IL-8 activity dramatically reduced subcutaneous tumor growth in immunocompromised mice. Mechanistically, the tumor-promoting activity of secreted IL-8 was attributed to enhancing angiogenesis through paracrine signaling, a supposition that is supported by several observations. First, the tested HeLa cells lacked expression of the IL-8 receptors CXCR-1 and -2. Second, inhibition of IL-8 by injection of the neutralizing antibody greatly decreased the number of inflammatory cells within the tumor. Consistent with the link between inflammatory cells and
angiogenesis, inhibition of IL-8 effectively halved the number of endothelial cells in early stage tumors without any detectable change in rates of cell proliferation or apoptosis. In addition, end stage tumors were clearly necrotic, consistent with the diminished tumor vasculature. Taken together, these data suggest that oncogenic HRas–induced IL-8 expression in tumor cells plays an important role in tumor development by promoting the inflammatory response and tumor angiogenesis. It is worth noting that IL-8 concentrations are elevated in the serum of cancer patients, including those with pancreatic (Wigmore et al. 2002), lung (Tas et al. 2006), melanoma (Brennecke et al. 2005), breast (Kozlowski et al. 2003; Benoy et al. 2004), prostate (Pfitzenmaier et al. 2003; Lehrer et al. 2004), and ovarian cancers (Lokshin et al. 2006; Lambeck et al. 2007). Moreover, in breast cancer, elevated levels of circulating IL-8 correlate with advanced disease and diminished survival rate (Benoy et al. 2004).

To further elaborate the role of Ras-induced IL-8 secretion in tumorigenesis, Wislez et al. (Wislez et al. 2006) utilized transgenic mice with an oncogenic KRas allele, which consequently develop alveolar neoplastic lesions. In this model of lung cancer, mice that bear the oncogenic KRas allele express elevated levels of two functional homologs of IL-8 [i.e., macrophage inflammatory protein 2 (MIP-2) and keratinocyte chemoattractant (KC)] relative to their wildtype littermates. Moreover, antibody raised against the CXCR2 receptor for the IL-8 homologs significantly reduced the number of lung lesions and malignancies. Conversely, when isolated and grown in vitro,
adenocarcinoma cells derived from the engineered mice were unaffected by the CXCR2-neutralizing antibody. The growth medium collected from these cells, however, elicits a positive response from alveolar inflammatory cells in a cell migration assay, and this cell migration is inhibited by the CXCR2-neutralizing antibody. Finally, progression of lung tumorigenesis in vivo correlated with the recruitment of CXCR2-expressing neutrophils and endothelial cells. Overall, these findings support a model whereby oncogenic KRas–mediated secretion of IL-8 acts in a paracrine fashion to promote tumorigenesis.

In addition, GRO-1, belonging to the same cytokine family as IL-8, has been linked to Ras-mediated modulation of the tumor microenvironment. Specifically, immortalized human ovarian epithelial cells can be transformed by oncogenic HRas or KRas, which concomitantly results in the expression of GRO-1 and other cytokines (Liu et al. 2004). Yang et al. (Yang et al. 2006) found that short hairpin (sh)RNA-mediated knockdown of GRO-1 expression in such oncogenic HRas–expressing (transformed) ovarian cells greatly reduced their tumorigenicity. In this case, however, the paracrine effect was linked not to inflammatory cells, but rather to the fibroblast component of the tumor stroma. Specifically, treatment of cultured fibroblasts with recombinant GRO-1 promoted the growth arrest state of senescence. Moreover, the mixing of GRO-1–treated fibroblasts with immortalized but otherwise non-transformed ovarian epithelial cells prior to injection elicited tumors in immunocompromised mice. Lastly, cancerous human ovarian tissue samples contain greater numbers of growth-arrested fibroblasts
than do normal control tissues, and the fibroblast cell lines isolated from these tumor tissues express higher levels of GRO-1. These results suggest that GRO-1 participates in Ras-induced tumorigenesis via the modulation of tumor-associated fibroblasts. Together, these studies that cytokines may play an important role in Ras-mediated tumorigenesis, by acting in a paracrine manner to foster the development of the tumor microenvironment (Figure 2).
Oncogenic Ras (Ras-GTP) promotes the transcription of cytokine genes (double helix), leading to elevated levels of secreted cytokines (blue spheres) that act to modulate the immune system (yellow cells), promote angiogenesis (purple capillaries), and activate tumor stroma (brown cells) (Ancrile et al. 2008). Used with permission of the publisher.
1.2.3 Interleukin-6

IL-6, a pleiotropic cytokine that plays a role in various biological functions, including inflammation, immunity, reproduction, hematopoiesis, neural development, and bone metabolism, has also been implicated in various physiological disorders, including aging, autoimmune disease, chronic inflammation, osteoporosis, Alzheimer’s, and cancer (Keller et al. 1996; Barton 1997; Trikha et al. 2003). In fact, IL-6 has previously been shown to contribute to various cancer processes, including cell survival, tumor angiogenesis, and tumor metastasis (Jee et al. 2001; Shariat et al. 2001; Wei et al. 2003).

IL-6 activity is dependent upon binding of the IL-6 receptor (IL-6R or gp80). The IL-6/IL-6R complex then homodimerizes and binds two molecules of gp130, resulting in a hexameric complex (Kishimoto et al. 1992; Ward et al. 1994). gp130 is receptor that is expressed on many different cell types and binds several different cytokines, unlike IL-6R, which specifically binds IL-6 and is expressed only on hepatocytes and monocytes, neutrophils, T cells, and B cells (Jones et al. 2001). Formation of the hexameric complex results in autophosphorylation of the cytosolic domain of gp130, initiating a signaling cascade (Kishimoto et al. 1992).

Previously, IL-6 had typically been considered an activator of the Ras pathway (Rowley and Van Ness 2002). However, in this thesis I show that IL-6 is induced by oncogenic Ras, plays an essential role in Ras-induced tumor initiation, and is required for the establishment of tumor vasculature, an important part in the development of the
tumor microenvironment (Ancrile et al. 2007). Identification of this cytokine as a mediator of Ras oncogenic function provides a potential therapeutic target for the treatment of human cancers. This finding contributes to recent findings that suggest that cytokines may play an important role in oncogenic Ras-induced tumor initiation.

1.3 Ras and Tumor Maintenance

1.3.1 Determining how PI3K Activation Fosters Tumor Maintenance

In order to determine the role of the PI3K-AKT pathway in tumor maintenance, I sought to determine which direct targets of AKT phosphorylation are required to maintain tumor growth initiated by oncogenic Ras. This was accomplished by inhibiting these target proteins and determining if their inhibition prevents AKT from promoting tumor growth in an established tumor. Specifically, eNOS, a downstream target of AKT, was found to be required for tumor maintenance. Furthermore, I have shown that nitrosylation of wildtype Ras proteins by eNOS is required for tumorigenesis, most likely due to diversification of the Ras signaling pathway (Lim et al. 2008).
1.3.2 eNOS

The family of nitric oxide (NO) synthases, composed of eNOS (NOS3), iNOS (NOS2), and nNOS (NOS1), convert L-arginine and oxygen into L-citrulline and NO, a free radical (Knowles and Moncada 1994; Griffith and Stuehr 1995). These enzymes function as dimers, and their activity requires the binding of several cofactors (Vallance and Leiper 2002). Unlike iNOS, which is transcriptionally induced, eNOS and nNOS are regulated post-translationally by Ca\(^{2+}\)/calmodulin-dependent pathways (Nathan 1992). N-myristoylation and palmitoylation of eNOS anchor the protein to the plasma membrane and golgi (Sessa et al. 1993; Robinson and Michel 1995; Sessa et al. 1995; Sowa et al. 1999), while phosphorylation is thought to regulate catalytic activity of the enzyme (Dudzinski et al. 2006). For example, when inactive, eNOS is phosphorylated at T\(^{497}\), and this phosphorylation prevents the binding of calmodulin. However, upon increased Ca\(^{2+}\) levels, the phosphatase PP1 dephosphorylates T\(^{497}\), which allows calmodulin to bind, thereby activating the protein (Fleming and Busse 2003). When active, eNOS enzymes dimerize and bind several cofactors, including tetrahydrobiopterin (BH\(_4\), haem, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD), to catalyze oxidation of L-arginine to citrulline, using oxygen and NADPH as co-substrates (Figure 3) (Vallance and Leiper 2002).
Figure 3: The NOS pathway

For enzymatic activity, nitric oxide synthase (NOS) enzymes must dimerize and bind the cofactors tetrahydrobiopterin (BH4), haem, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). On binding calmodulin (CAL), the active enzyme catalyses the oxidation of L-arginine to citrulline and nitric oxide (NO) and requires molecular oxygen and NADPH as co-substrates. Each NOS dimer coordinates a single zinc (Zn) atom (Vallance and Leiper 2002). Used with permission of the publisher.
In addition to Ca\(^{2+}\)/calmodulin-mediated regulation, eNOS has also been shown to be a direct target of AKT, which phosphorylates the enzyme primarily at S\(^{1177}\), resulting in activation of eNOS and, consequently, production of NO (Dimmeler et al. 1999; Fulton et al. 1999; Michell et al. 1999). Mutation of this catalytic site to either S\(^{1177}\)A or S\(^{1177}\)D will inhibit or enhance phosphorylation, respectively, leading to altered enzyme activity (Dimmeler et al. 1999; Fulton et al. 1999). Moreover, phosphorylation and activation of eNOS is inhibited by the PI3K inhibitor wortmannin (Dimmeler et al. 1999; Michell et al. 1999).

eNOS-generated NO can alter cell signaling via two distinct mechanisms, which include activation of the soluble guanylyl cyclase (sGC) pathway and protein nitrosylation. NO binds to and activates sGC, which then converts GTP to cGMP, and increased cGMP levels can lead to activation of proteins (Davis et al. 2001). For example, eNOS-generated NO is able to diffuse into vascular smooth muscle, leading to elevated levels of cGMP, thereby activating cGMP-dependent protein kinase, or protein kinase G (PKG), which regulates platelet aggregation and vasodilation/vasoconstriction (Dudzinski et al. 2006). Indeed, while eNOS knockout mice are viable and fertile, these mice display elevated systemic and pulmonary blood pressure (Shesely et al. 1996).

In addition to activation of the sGC pathway, another signaling mechanism carried out by eNOS-generated NO is the modification of proteins via S-nitrosylation, a post-translational modification which involves the coupling of NO to a cysteine thiol,
generating an S-nitrosothiol (SNO) (Stamler et al. 1992; Hess et al. 2005). Because eNOS produces a localized burst of NO, it most likely nitrosylates proteins in its vicinity (Hess et al. 2005). Many proteins are known to be S-nitrosylated by NO, and such nitrosylation can either activate or inhibit protein function (Hess et al. 2005). One such protein is HRas, which is S-nitrosylated by NO at the thiol group on C\textsubscript{118}. This cysteine residue is located in the NKXD guanine nucleotide-binding motif of Ras, and nitrosylation at this site leads to activation of the protein due to enhanced dissociation of guanine nucleotides (Lander et al. 1996). Moreover, mutation of this cysteine site to serine (C\textsubscript{118}S) prevents S-nitrosylation and activation of Ras (Lander et al. 1997).

In an attempt to identify the mechanism by which Ras S-nitrosylation at C\textsubscript{118} by NO enhances guanine nucleotide exchange (GNE) to increase Ras-GTP levels, Williams et al. (Williams et al. 2003) sought to determine if Ras S-nitrosylation results in any structural changes in the protein. However, NMR spectroscopy studies showed that Ras S-nitrosylation did not alter protein structure. Moreover, Ras guanine nucleotide exchange assays, which measure radioactive GDP incorporation, showed that GNE did not differ between stably S-nitrosylated Ras and unmodified Ras, indicating that it is not the presence of the NO moiety on Ras that facilitates dissociation of guanine nucleotides. However, addition of NO reactive species enhanced the rate of GNE on wildtype Ras but not Ras C\textsubscript{118}S, suggesting that it is the process rather than the end product of nitrosylation that results in increased GNE on Ras (Williams et al. 2003). In a follow up
study, Heo and Campbell (Heo and Campbell 2004) found that nitrogen dioxide (NO₂),
generated via reaction of NO with oxygen (O₂), mediates Ras S-nitrosylation at C₁₁₈ via
the formation of an intermediate product, a Ras thiol-radical intermediate. This
intermediate product, rather than the end product of Ras S-nitrosylation, enhanced GNE
on Ras.

Heo et al. (Heo et al. 2005) sought to determine how the Ras thiol-radical
intermediate enhanced Ras GNE. Biochemical, mutagenesis, and spectroscopic analyses
indicated that treatment of Ras with NO and O₂ results in the production 5-nitro-GDP,
derived from GDP bound to Ras. Specifically, the authors suggest that the Ras thiol-
radical intermediate generated by NO₂ withdraws an electron from Ras-bound GDP,
generating a GDP radical. This radical then reacts with another molecule of NO₂ to
produce 5-nitro-GDP. The alteration of GDP to 5-nitro-GDP inhibits its binding to Ras,
and it is released from the protein, thereby resulting in increased guanine nucleotide
dissociation and increasing levels of GTP-bound Ras.

While there is evidence to suggest that NO may play a role in cancer, there is
conflicting data as to what that role may be. For example, NOS levels have been shown
to both positively and negatively correlate with tumor progression (Fukumura et al.
2006). eNOS, which is predominately expressed in endothelial cells but is expressed in
many cell types and tissues (Michel and Feron 1997), has been detected in the tumor
vasculature of several different cancers, often at elevated levels compared to normal
tissue (Fukumura et al. 2006). Similarly, there is evidence to suggest that eNOS plays a role in angiogenesis. For example, inhibition of eNOS with the peptide cavtratin blocks angiogenesis in tumor xenografts generated by Lewis lung carcinoma and human hepatocarcinoma cell lines (Gratton et al. 2003). However, eNOS has also been found to be expressed in tumor cells, including those of brain, lung, breast, stomach, bladder, Kaposi sarcoma, and melanoma tumors (Fukumura et al. 2006).

In this thesis, I demonstrate an important role for eNOS in Ras-driven tumor cells. Specifically, I show that AKT-mediated phosphorylation and activation of eNOS is required for tumor initiation and maintenance. Furthermore, I find that eNOS promotes tumor growth via S-nitrosylation and activation of wildtype Ras proteins, which are required throughout tumorigenesis.
1.4 Summary

In this thesis I investigate the requirement for oncogenic Ras and Ras effectors during both tumor initiation and tumor maintenance. In Chapter 3, I demonstrate the requirement for IL-6, which is induced upon oncogenic Ras expression, during tumor initiation. Furthermore, I show that IL-6 fosters tumor angiogenesis, an important step in the development of the tumor microenvironment. I worked on this project with Kian-Huat Lim, who performed initial profiling experiments to identify secreted proteins upregulated in response to oncogenic Ras expression.

In Chapter 4, I demonstrate the requirement for eNOS, a target of AKT phosphorylation, during tumor maintenance. Additionally, I propose a potential mechanism by which eNOS contributes to the tumorigenic process. I worked on this project with Kian-Huat Lim and Dave Kashatus. Kian-Huat Lim performed all cell mixing assays and also performed some Western blots, and Dave Kashatus performed nitrosylation assays and some Western blots shown in Chapter 4. Specifically, Kian – Huat Lim performed experiments shown in Figures 12B-C, 13A,C,D, 14E, and 16A-B, and Dave Kashatus performed experiments shown in Figure 13B, 14A-D, 15A, and 16C, as denoted in Figure legends.
2. Materials and Methods

2.1 IL-6 is Required for Ras-induced Tumorigenesis

2.1.1 Retroviral vectors

pBabepuro, pBabepuro-HRas\textsuperscript{G12V}, and pBabepuro-ER:Ras\textsuperscript{G12V} were previously described (Lim and Counter 2005). IL-6 shRNA-1, shRNA-2, and shRNA-3, and scramble control sequences (5ʹ-AGATGGATGCTTCCAATCTGG-3ʹ, 5ʹ-AAGGCAAAGAATCTAGATGCA-3ʹ, 5ʹ-AGACATGTAACAAGAGTAA-3ʹ, and 5ʹ-AGACGGAGGCTTACAGTCTGG-3ʹ, respectively) were cloned into pSUPER-RETRO-PURO.

2.1.2 Cell lines

Human (embryonic) kidney cells, BJ fibroblasts, mammary epithelial cells, and skeletal muscle myoblasts stably expressing the early region of SV40 (which produce the proteins T-Ag and t-Ag); hTERT; and either HRas\textsuperscript{G12V} or no transgene (O’Hayer K and Counter 2005) were stably infected with retroviruses generated from the indicated vectors to generate polyclonal populations as previously described (O’Hayer K and Counter 2005). The ER:Ras\textsuperscript{G12V}-expressing tumor kidney cells also expressed p110-CAAX for unrelated reasons (Lim and Counter 2005). B9 cells (Aarden et al. 1987) were a kind gift of Peter Lansdorp (University of British Columbia, Vancouver, BC, Canada). U266
cells were obtained from American Type Culture Collection. HMVECs were a kind gift of Xiao-Fan Wang (Duke University Medical Center, Durham, NC).

2.1.3 RT–PCR

Total RNA was isolated, reverse-transcribed with an oligoT primer, and PCR-amplified with the primers 5'-ATGTAGCCGCCACACAGA-3' and 5'-CATCCATCTTTTCAGCCAT-3' to detect IL-6, and 5'-GAAGGTGTCGGAGACAA-3' and 5'-GCAGAGGGGCAGAGATGA T-3' to detect GAPDH, using a previously described protocol (Hamad et al. 2002). Cycle number varied between 25 and 40 cycles, depending on cell type and transcript.

2.1.4 Immunoblot

Lysates from the described cell lines were immunoblotted with the primary antibody α-pan-Ras (Oncogene) or α-actin C-2 (Santa Cruz Biotechnology) using standard methods.

2.1.5 IL-6R Detection

Cells (2 x 10⁶) were incubated with 20 μL of IL-6R FITC-conjugated antibody (Abcam) in 3% BSA-PBS for 1 h at 4°C. Cells were then washed three times in PBS and IL-6R-positive cells were detected by flow cytometry.
2.1.6 ELISA

Cells were plated at ~80% confluency. Twenty-four hours later, cells were washed three times with PBS and cultured in serum-free medium, and 48 h later, cells were collected and analyzed in duplicate with a human IL-6 or IL-8 ELISA Kit (R&D Systems). Results are reported as means ± standard deviation.

2.1.7 Cell Proliferation

Cells (1 x 10^4 per 6-cm dish) were seeded in triplicate, and viable trypan blue-negative cells were counted daily for 5 d. To measure cell proliferation rate under stress conditions, cells were plated at varying densities in a 96-well plate. Twenty-four hours later, medium was replaced with serum-free medium. Four days later, 50 μL of 5 mg/mL 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma) was added to each well, and 4 h later, medium was aspirated and cells were resuspended in 200 μL of DMSO. Absorbencies were recorded at 540 nm. IL-6 antibody neutralization was assayed by seeding 3 x 10^4 B9 cells per well in a 96-well plate in medium supplemented with 10 pg/mL human IL-6 and 0.04 μg/mL monoclonal anti-human IL-6 antibody (R&D Systems), which was preincubated to allow binding of the antibody to the cytokine. Forty-eight hours later, 10 μL of 5 mg/mL MTT was added per well. Four hours later, 50 μL of 20% SDS/50% DMF was added per well, and 24 h later, the absorbencies at 570–650 nm were measured.
2.1.8 Soft Agar

Of the indicated cells, \(5 \times 10^4\) cells were suspended in soft agar in triplicate three times independently, and colonies >30 cells were scored after 3 wk, as previously described (Cifone and Fidler 1980; Hamad et al. 2002).

2.1.9 Tumor Growth

Cells \((1 \times 10^7)\) mixed with Matrigel were injected subcutaneously into one or both flanks of SCID/beige mice for a total of four injections per cell line, after which tumor volumes were determined at regular intervals as described previously (Hamad et al. 2002). In cases in which tumor-derived cells were retested for IL-6 levels, tumors were established in culture under hygromycin selection to enrich for tumor cells as previously described (Lim and Counter 2005). For antibody neutralization of IL-6, four animals were pretreated with an injection of 100 μg per mouse IL-6-neutralizing antibody (MAB206; R&D Systems) or isotype control antibody (R&D Systems) 2 d prior to tumor cell injection. Starting 1 d after tumor cell injection, mice were treated with neutralizing IL-6 antibody or control antibody every 3 d at the tumor site. For chemical carcinogenesis, the backs of 15 control (\(IL-6^{+/+}\)) C57BL/6J mice and 15 experimental (\(IL-6^{-/-}\)) C57BL/6J mice, in which both alleles of \(IL-6\) were disrupted (B6.129S2-IL6tm1Kop/J) (Kopf et al. 1994) (Jackson Laboratory) were shaved, and the following day, 150 μL of 125 μg/mL DMBA (Sigma) in DMSO were applied topically, followed 1 wk later by twice-weekly topical applications of 150 μL of \(10^{-4}\) M TPA (Sigma) in DMSO for 20 wk. One \(IL-\)
6\textsuperscript{th} mouse died at week 11 for unrelated reasons and was excluded from analysis. Tumor number and size were recorded weekly. Student’s $t$-test was used to compare tumor growth in the various models. The differences between means were considered significant if $P < 0.05$. All procedures with mice were done under an Institutional Animal Care and Use Committee-approved protocol.

### 2.1.10 Immunohistochemistry

Excised tumors were fixed in formalin and sectioned. H&E staining, Ki67 ($\alpha$Ki-67 Ab, Zymed), phospho-histone H3 ($\alpha$ phospho-Histone H3 [Ser10] Ab; Upstate Biotechnology), and CD31 ($\alpha$-PECAM-1 Ab; Santa Cruz Biotechnology) immunohistochemistry, and TUNEL assay (Apoptag Plus Peroxidase Apoptosis Detection Kit; Chemicon) were performed using standard protocols.

### 2.2 The Role of eNOS in Ras-mediated Tumorigenesis

#### 2.2.1 Plasmids

pBabepuro, neo, bleo, and hygro were used as control vectors (Lim and Counter 2005). HA-IKK$\alpha^{544A}$ (Woronicz et al. 1997), FOXO3a-A3 cDNA3 engineered with an N-terminal HA tag, FLAG-TSC2$^{S393A,T1462A}$ cDNA4, eNOS cDNA engineered with a C-terminal HA tag and to be resistant to shRNA by introducing the three silent mutations $G_{1821} \rightarrow A$, $T_{1827} \rightarrow C$ and $G_{1830} \rightarrow A$ alone (eNOS$^R$) or in conjunction with the mutation $
A359GC→GCC that altered S1177 to A (S1177A eNOSR), and wildtype FLAG-epitope tagged HRas or NRas cDNAs engineered to be resistant to shRNA by introducing the silent mutations in the region targeted by RNAi (FLAG-HRasR; FLAG-NRasR) alone or in conjunction with the mutation T342GT→TCT (C118S FLAG-HRasR, C118S FLAG-NRasR) that altered C118 to S, were subcloned into one of the aforementioned pBabe vectors. Bcl-XL shRNA (5'-AGCGTAGACAAGGAGATGC), eNOS shRNA (5'-AAGAGTTATAAGATCCGCTTC), HRas shRNA (5'-GGCAAGAGTGCGCTGACCATC), NRas shRNA (5'-CAAGAAGAGTACAGTGCCATG) or an eNOS scramble control (5'-AAGCGTTAAAGATCCGCTTC) sequences were cloned into pSUPER-PURO-RETRO (Oligoengine). The plasmid system for dox-inducible shRNA (Lim and Counter 2005) was adapted to encode eNOS shRNA.

2.2.2 Cell Lines

TtH and the pancreatic cancer cell lines were previously described (Lim et al. 2005). Derived lines were generated by stable infection with the indicated combinations of amphotrophic retroviruses generated from the aforementioned pBabe plasmids, as previously described (O’Hayer and Counter 2006).
2.2.3 Cell Treatments

Cells were treated with LY294002 (Cell Signaling Technologies) or wortmannin (Sigma) at a final concentration of 20 mM or 10 nM, respectively, for 1 hr prior to analysis.

2.2.4 Immunoblot

HA-IKKα<sup>K44A</sup>, FOXO3a-A3-HA, HA-eNOS or variants thereof, endogenous Bcl-X<sub>L</sub>, p70 S6 kinase, T<sub>389</sub> phosphorylated p70 S6 kinase, HRas, KRas or NRas, S<sub>1177</sub> phosphorylated eNOS (both to detect activated eNOS and assess eNOS expression), S<sub>573</sub> phosphorylated AKT, actin, p65, and tubulin were detected by immunoblotting with αHA (Roche), αBcl-x<sub>L</sub>, αp70 S6 Kinase, αThr389 Phospho-p70 S6 Kinase, αSer1177 Phospho-eNOS, αSer473 Phospho-AKT (Cell Signalling Technology), αHRas, αKRas, αNRas, actin (Santa Cruz), αp65 (Rockland), and αtubulin (Sigma) antibodies, respectively, using standard protocols.

2.2.5 RT-PCR

eNOS and GAPDH mRNA was RT-PCR amplified with the primers 5′-

CAGTGTCACCATGCTGCTGGAAATTG and 5′-

TAAAGGTCTTCTTCCTGGTGATGC, and the primers 5′-

ACCACAGTCCATGCCATCAC and 5′-TCCACCACCTGGTGCTGA, respectively.
2.2.6 GTP and Nitrosylated Ras

GTP-bound (de Rooij and Bos 1997) or nitrosylated Ras (Jaffrey et al. 2001) were captured as previously described and immunoblotted with either an αFLAG (Sigma) or an αHRas, αKRas or αNRas (Santa Cruz) antibody to detect FLAG-HRas or endogenous H, N, or KRas proteins, respectively.

2.2.7 Soft Agar

Soft agar assays were done in triplicate and twice independently as previously described (Lim et al. 2005).

2.2.8 Tumor Growth

As previously described (Lim and Counter 2005), the tested cell line (tumor initiation) or a mixture of two cell lines (cell mixing assay for tumor maintenance) were injected subcutaneously into four flanks of SCID/Beige mice. For tumor initiation experiments, tumors were removed and photographed when control tumors reached maximum volume. For cell mixing assays, tumors were removed when they reached maximum volume, and tumors or human cells derived from the tumors by re-culture in selective media (G418), were treated with X-gal to stain LacZ⁺ cells blue and photographed. CFPac-1 and MIAPaCa-2 cells engineered to contain a dox-inducible eNOS shRNA (Lim and Counter 2005) were injected into both flanks of 5 SCID/Beige mice, tumors were permitted to reach a diameter of 0.6 cm, after which 3 mice were
provided with doxycycline in their diet and 2 mice left untreated for 11 days (CFPac-1 cells) or 13 days (MIAPaCa-2 cells), after which tumors were removed and photographed. DMBA/TPA treatments were performed as previously described (Ancrile et al. 2007) on 15 eNOS/+/C57BL/6J and 15 eNOS/-/C57BL/6J (B6.129P2-Nos3tm1Unc/J) mice (Jackson Laboratory) (Shesely et al. 1996). All animal work was approved by DUMC IACUC.

2.2.9 Tumor and Normal Human Specimens

Flash frozen tissue samples were provided devoid of all identifying information under a DUMC approved IRB protocol.
3. Oncogenic Ras-induced Secretion of IL-6 is Required for Tumorigenesis

3.1 Introduction

The small GTPase family of Ras proteins function as GDP/GTP-regulated binary switches that normally relay signals from extracellular stimulus-activated cell surface receptors to diverse cytoplasmic signaling networks in a regulated fashion. Ras is mutated to remain in a stimulus-independent, constitutively active GTP-bound state in one third of tumors, or if Ras itself is not mutated, the Ras pathway is often inappropriately activated through mutations to upstream receptor tyrosine kinases or downstream components. Such inappropriate activation of Ras provides self-sufficiency in growth signals, leading to increased cell proliferation and survival, among other phenotypes characteristic of cancer cells. As such, Ras is a desirable target for cancer therapy, although attempts to inhibit Ras have not yet borne out clinically (Downward 2003). On the other hand, secreted proteins are druggable, typically with neutralizing antibodies (Adams and Weiner 2005). We therefore speculated that inhibiting secreted proteins induced by Ras might block Ras oncogenesis.

The cancer most associated with oncogenic Ras mutations is pancreatic (Downward 2003). Proteins elevated in the serum of pancreatic cancer patients may correspondingly be the product of oncogenic Ras activity. One such protein elevated in pancreatic cancer patients is IL-6 (Barber et al. 1999; Wigmore et al. 2002; Ebrahimi et al.)
IL-6 is a pleiotropic cytokine functioning in inflammation, immunity, bone metabolism, neural development, reproduction, and hematopoiesis (Keller et al. 1996), but it has also been implicated in the etiology of some cancers (Trikha et al. 2003). Given the alluring link of elevated IL-6 levels in patients with a cancer characterized by Ras mutations, and the observation that Ras-induced secretion of another cytokine, IL-8, is important for tumor growth of HeLa cells over-expressing oncogenic Ras (Sparmann and Bar-Sagi 2004), we examined whether IL-6 plays any role in Ras-mediated cancers.

### 3.2 Results

#### 3.2.1 Oncogenic Ras$^{G12V}$ Induces IL-6 Expression

Normal human embryonic kidney (HEK) cells genetically transformed with SV40 T/t-Ags, the telomerase catalytic subunit hTERT, and a 4-hydroxytamoxifen (4-OHT) inducible ER:Ras$^{G12V}$ fusion protein were assayed for secreted IL-6 levels in the absence of 4-OHT, and hence no ER:Ras$^{G12V}$ protein, and presence of 4-OHT, and hence the presence of ER:Ras$^{G12V}$ which converts these cells to a tumorigenic state (Lim and Counter 2005). Upon induction of ER:Ras$^{G12V}$ by 4-OHT the levels of secreted IL-6 was elevated almost 40 fold (Figure 4A). Thus, induction of a tumorigenic state by oncogenic Ras is associated with an increase in IL-6 secretion.
Figure 4: Oncogenic Ras induces secretion of IL-6 independent of cell type

(A) IL-6 secreted protein levels increase upon activation of Ras. HEK cells expressing T/t-Ag, hTERT, and ER:RasG12V (and p110-CAAX) were confirmed by immunoblot to upregulate ER:RasG12V in the presence of 4-OHT (top), which led to elevated levels of IL-6 present in cell media as detected by ELISA (bottom). Actin: loading control. (B) Oncogenic Ras increases secreted IL-6 independent of cell type. The indicated four primary human cell types expressing T/t-Ag and hTERT and, where indicated, also RasG12V as assessed by immunoblot (top), were shown to increase the levels of IL-6 mRNA, as assessed by RT-PCR (middle), and secreted IL-6 protein, as assessed by ELISA (bottom). Actin: loading control. GAPDH: RT-PCR control. IL-6 protein levels are reported as the percent mean ± standard error of duplicate experiments relative the IL-6 secreted by RasG12V expressing cells.
Ras can elicit very different effects in different cell backgrounds (Shields et al. 2000). To address whether induction of IL-6 secretion occurred in other cell types, IL-6 secretion was compared to the Ras status and tumorigenic phenotype of four different cell types that retained a constant genetic background (O'Hayer and Counter 2006). Both mRNA and protein levels of IL-6 were greatly increased in normal primary human kidney embryonic cells, fibroblasts, myoblasts, and mammary epithelial cells expressing T/t-Ag and hTERT that were driven to be tumorigenic by ectopic expression of RasG12V compared to the non-tumorigenic cells lacking RasG12V (Figure 4B). Thus, an oncogenic Ras-mediated tumorigenic state is associated with elevated levels of IL-6 in multiple cell types.

### 3.2.2 Ras-induced Secretion of IL-6 is Required for Human Tumor Cell Growth In Vivo

To test whether the Ras-induced secretion of IL-6 is required for Ras oncogenic function, IL-6 levels were stably reduced >90% (Figure 5A) by IL-6 shRNA in the tumorigenic HEK cells expressing T/t-Ag, hTERT and RasG12V. The resultant cells or the scramble control counterpart were then tested for tumor growth in immunocompromised mice. While scramble control cells rapidly formed tumors, reaching maximum tumor volume within ~20 days, IL-6 shRNA-treated cells barely generated palpable masses in this time span (p < 0.001, Figures 5B,C). Even though tumors eventually did arise, IL-6 was re-expressed in these tumors (Figure 5D), strongly suggesting that a loss of IL-6 must be overcome for tumor growth.
We validated these results with two more independent IL-6 shRNA sequences. Identical to the first IL-6 shRNA, knockdown of IL-6 by these two new sequences (Figure 5A) blocked tumor growth of the aforementioned Ras\textsuperscript{G12V}-transformed tumorigenic HEK cells (Figure 5C). To address whether IL-6 is required for Ras-driven growth independent of cell type, IL-6 was knocked down by shRNA in the described human fibroblasts (Figure 5E) and myoblasts (Figure 5H) engineered to be tumorigenic by the expression of T/t-Ags, hTERT and Ras\textsuperscript{G12V} (O'Hayer and Counter 2006) and again found to reduce tumor size by at least 97% compared to scramble control cells (Figure 5F,5G,5I,5J). Knockdown of IL-6 thus presents a formidable barrier to Ras-induced human tumor growth of cells derived from very different lineages.
Figure 5: IL-6 is required for oncogenic Ras-mediated tumorigenesis

Primary HEK cells (a-d), fibroblasts (e-g), and myoblasts (h-j) expressing T/t-Ags, hTERT, RasG12V and IL-6 shRNA-1 or a scramble (scram) control sequence exhibited a decrease in secreted IL-6 as assessed by ELISA (a,e,h), decreased tumor growth when injected into one or both flanks of mice, as visualized in a representative mouse or resected tumors (b,f,i), and decreased tumor kinetics in a plot of tumor volume (cm3) ± standard error versus time (c,g,j, scram control: □, all IL-6 shRNAs: ✷) when IL-6 was knocked down. Furthermore, knocking down IL-6 in the HEK cells using two other IL-6 shRNA sequences (a, IL-6 shRNA-2,3) resulted in identical tumor growth kinetics as IL-6 shRNA-1. Two tumors (tumor 1, tumor 2) eventually arising from HEK cells expressing IL-6 shRNA-1 were isolated, cultured, and assayed for secreted IL-6 by ELISA, with the finding that these tumors exhibited increased IL-6 compared to the original IL-6 shRNA-1 donor cells. (*p < 0.05, **p < 0.01, ***p < 0.001)
3.2.3 IL-6−/− Mice are highly Resistant to Spontaneous Induced Ras-driven Tumors

To address whether IL-6 is also required for Ras-mediated tumor growth in a system that recapitulates the spontaneous process of tumorigenesis, we tested whether IL-6+/− knockout mice are resistant to carcinogen-induced tumors. Premalignant papillomas with a high level of Ras mutations are induced by topical application of the carcinogen 7,12-Dimethylbenzantracene (DMBA) followed by repetitive application of 12-O-tetradecanoylphorbol-13-acetate (TPA) (Quintanilla et al. 1986). Thus, IL-6+/− and IL-6−/− mice were treated topically with DMBA and then TPA for 20 weeks, and tumor growth monitored. Tumors appeared within 12 weeks of initiation in IL-6+/− mice, and by termination of the experiment 93% of the mice had tumors, with an average of 2.4 tumors per mouse (Figure 6A-C). In sharp contrast, tumors appeared two weeks later in the IL-6−/− mice (Figure 6A,B), and by the termination of the experiment only 27% of the mice had tumors (Figure 6B), and the average number of tumors dropped to 0.5 per mouse (p < 0.01, Figure 6C). Perhaps most telling, the total tumor volume per mouse at week 20, a measurement of both the size and number of tumors dropped almost 30 fold in IL-6−/− mice (p < 0.05, Figure 6D). In agreement, a one week delay in tumor onset and a 20% reduction in mice with tumors had been described for DMBA/TPA-treated IL-6−/− mice, although in this case a more aggressive carcinogen protocol was used (Suganuma et al. 2002). Thus, loss of IL-6 inhibits spontaneous formation of Ras-driven tumors.
Figure 6: IL-6<sup>−/−</sup> mice are resistant to carcinogen-induced skin tumors

14 IL-6<sup>+/+</sup> and 15 IL-6<sup>−/−</sup> mice were treated with a single topical application of DMBA followed one week later by twice weekly topical applications of TPA for 20 weeks to induce skin tumors. (A) Representative mice of the indicated genotype at 20 weeks. Arrows: tumors. (B) Percentage of IL-6<sup>+/+</sup> (□) and IL-6<sup>−/−</sup> (■) mice with tumors versus time after initial application of DMBA (weeks). (C) Mean number of tumors per IL-6<sup>+/+</sup> (□) and IL-6<sup>−/−</sup> (■) mouse versus time after initial application of DMBA (weeks). **p < 0.01 (D) Mean tumor volume per IL-6<sup>+/+</sup> (□) and IL-6<sup>−/−</sup> (■) mouse versus time after initial application of DMBA (weeks). *p < 0.05
3.2.4 Ras-induced IL-6 Secretion Acts in a Paracrine Fashion to Promote Tumor Growth

To address how Ras-induced secretion of IL-6 promotes tumor growth in vivo, we tested whether the tumor cells were themselves the target of IL-6. However, we did not find any evidence for autocrine signaling. RasG12V-transformed tumorigenic kidney cells, which require IL-6 for tumor growth, do not express detectable IL-6 receptor (IL-6R, Figure 7A), and knockdown of IL-6 in these cells did not, when compared to vector control cells, have any effect on cell proliferation under normal serum (Figure 7B, left panel) or in the stress condition of low serum (Figure 7B, middle panel) or growth in soft agar (Figure 7C), nor did addition of exogenous IL-6 foster the growth of the same cells in the absence of Ras (Figure 7B, right panel). We thus explored the possibility that Ras-induced secretion of IL-6 acts in a paracrine fashion to promote Ras tumorigenesis. Immunohistological staining revealed that tumors eventually arising from IL-6 shRNA-treated RasG12V-transformed kidney cells did not exhibit any gross histological differences, or changes in cell proliferation, as assessed by Ki67 and phospho-histone H3 staining, or apoptosis, as detected by the TUNEL assay, compared to scramble control tumors (p > 0.05, Figure 8). This could mean that either loss of IL-6 has no effect on apoptosis or proliferation, or that by the time we are able to study these tumors, the observed re-activation of IL-6 (Fig 5D) masked these effects. Nevertheless, we did find that CD31-positive (endothelial) cells were reduced 18 fold in IL-6 shRNA-treated
Figure 7: IL-6 does not act in an autocrine manner in Ras-transformed HEK cells

(A) Detection of IL-6R (pink profile) by flow-cytometric analysis in positive control U266 cells (top graph), but not RasG12V-transformed human kidney cells (bottom graph). No antibody (blue profile) serves as a negative control. (B) IL-6 does not alter the growth of RasG12V-transformed human kidney cells. Absorbance ± standard error versus time (days) as measured by the MTT assay of RasG12V-transformed human kidney cells stably expressing IL-6 shRNA-1 (black box) or the appropriate scramble sequence (open box) cultured in 10% serum (top) or serum-free (middle) medium, or the same kidney cells lacking RasG12V plated at the indicated densities in serum free medium (black box) or serum-free medium containing 100 pg/mL IL-6 (open box). (C) Similar anchorage-independent growth of RasG12V-transformed human kidney cells stably expressing IL-6 shRNA-1 versus the appropriate scramble sequence. Average number of colonies ± standard error calculated from three independent experiments conducted in triplicate.
tumors (p < 0.01, **Figure 8**). In agreement, IL-6 has been shown to promote angiogenesis (Cohen et al. 1996; Wei et al. 2003; Huang et al. 2004; Loeffler et al. 2005; Nilsson et al. 2005). However, endothelial cells typically do not express detectable levels of IL-6R (**Figure 9** and Romano et al., 1997) or respond to IL-6 (Podor et al., 1989; Sironi et al., 1989), arguing that the reduction of CD31-positive cells by knocking down IL-6 in tumorigenic cells is indirect. This paracrine effect must also be local, as tumors arising from scramble control cells on one flank of the mice failed to promote tumorigenic growth of IL-6 shRNA treated cells on the opposite flank (**Fig 5B**).
Figure 8: IL-6 acts in a paracrine manner to foster angiogenesis

(A) Loss of IL-6 inhibits angiogenesis. Tumors from Ras\textsuperscript{G12V}-transformed HEK cells stably expressing IL-6 shRNA-1 or the appropriate scramble sequence were excised, formalin-fixed, and stained for H&E or assayed for Ki67, phospho-histone H3 (P-H3), TUNEL, or CD31-positive (dark brown) cells. (Bottom) Average number of marker-positive cells ± standard deviation from five independent fields of two to four different tumors. *P < 0.01

Figure 9: IL-6R is not detected in human microvascular endothelial cells (HMVEC)

Flow cytometric analysis of IL-6R (pink profile) or no antibody negative control (blue profile) in primary HMVECs.
3.2.5 IL-6 as a Target for Cancer Therapy

To expand our studies into more clinically relevant settings, we tested whether stable knockdown of IL-6 (Figure 10A, top panels) inhibited the tumorigenic growth of in the human pancreatic cancer cell lines CFPac-1, Capan-1 and HPAC containing an oncogenic KRas allele (Kita et al. 1999; Moore et al. 2001; Lim et al. 2006), as such cell lines better model the genetic chaos of human cancers that underlies much of the variability in the effectiveness of cancer therapies. While knockdown of IL-6 had no obvious effect on CFPac-1 cells, it did cause massive necrosis in the tumors arising from Capan-1 cells, and retarded the tumor growth of HPAC cells compared to scramble control treated cells (Figure 10A, bottom panels). Thus, IL-6 is important for tumorigenic growth in two of the three tested human mutant KRas-positive cancer cell lines, suggesting that an anti-IL-6 therapy could be effective in human cancers.

We next tested if a more clinically relevant mode of inhibiting IL-6 function could impede Ras-driven tumor growth. As IL-6 is a secreted protein, it is amendable to targeting by neutralizing antibodies. Moreover, such an antibody is well tolerated in humans (Trikha et al. 2003). Thus, we tested whether a neutralizing IL-6 antibody could inhibit the tumor growth of the RasG12V-transformed kidney tumor cells. We first confirmed that such an antibody effectively neutralized IL-6 in a biological assay. Addition of the neutralizing IL-6 antibody to culture media reduced the IL-6-dependant growth of B9 cells to the same level as cells that were not induced by IL-6 (Figure 10B).
Next, mice pretreated with either the IL-6 neutralizing antibody or a control IgG antibody were injected with Ras\textsuperscript{G12V}-transformed kidney tumor cells, and then again treated every three days by intra-tumor injections of either the anti-IL-6 or control antibody. Tumors in mice treated with the control IgG antibody first appeared at week 2, and reached maximum tumor volume by day 23. This growth was retarded in the IL-6 neutralizing antibody treatment group, and tumors were approximately half the size (p < 0.01) of control tumors (Figure 10C,D). Consistent with these data, anti-IL-6 antibodies have been reported to reduce the tumor growth of prostate cancer cells (Smith and Keller, 2001; Wallner et al., 2006). These experiments suggest the intriguing possibility that IL-6 neutralizing antibodies may have therapeutic value in the treatment of Ras-driven cancers.
Figure 10: IL-6 as a therapeutic target

(A) Knockdown of IL-6 inhibits tumor growth. (Top panel) Reduction of IL-6, as detected by ELISA from the conditioned medium of the indicated three human pancreatic cancer cell lines expressing IL-6 shRNA-1, but not the scramble (scram) control counterpart. (Bottom panel) Representative tumors from the indicated three human pancreatic cancer cell lines stably expressing an IL-6 shRNA-1 or scramble sequence after injection subcutaneously into the flanks of mice. (Arrow) Region of necrosis. (B) Neutralization of IL-6 activity by an anti-IL-6 antibody (αIL-6 Ab). Cell numbers as measured by average absorbance ± standard error using the MTT assay 48 h after B9 cells were plated in serum-free medium (untreated) in the presence of IL-6 to stimulate cell proliferation (IL-6) or in the presence of IL-6 and αIL-6 Ab (IL-6 + αIL-6 Ab). (C) An IL-6-neutralizing antibody inhibits Ras-driven tumor growth. Tumor volume (cubic centimeters) ± standard error versus time (days) of RasG12V-transformed human kidney cells growing in mice injected at the tumor site every 3 d with 100 μg of either the IgG1 control antibody (■) or the IL-6-neutralizing antibody (▲). *P < 0.01. (D) Representative tumors of RasG12V-transformed human kidney cells growing in mice injected every 3 d with 100 μg of either the IgG1 control antibody (control Ab) or the IL-6-neutralizing antibody αIL-6 Ab.)
3.3 Discussion

We show that oncogenic Ras induces the secretion of the cytokine IL-6 from a variety of cell types, and that knockdown or genetic ablation of IL-6 thwarts Ras-mediated tumor growth of human tumor cell lines and chemical carcinogen-induced tumors. IL-6 is thus a bona fide downstream effector of oncogenic Ras that promotes the tumorigenic effects of Ras. IL-6 can also act upstream of Ras (Rowley and Van Ness 2002), and correspondingly the tumor-promoting effects of IL-6 can be context dependant. Nevertheless, in the case of the tested oncogenic Ras-driven tumor cells, IL-6 acts downstream of Ras in a paracrine fashion to promote angiogenesis. Over-expression of oncogenic Ras in the tumorigenic HeLa cancer cell line also induced the related, but distinct cytokine IL-8, and inhibiting IL-8 reduced tumor growth of these cells and the number of CD31-positive cells in a tumor (Sparmann and Bar-Sagi 2004). Like IL-6, IL-8 was secreted upon expression of Ras in human cells (Figure 11). Thus, Ras-induced secretion of multiple cytokines may underlie the ability of this oncogene to potently induce angiogenesis. Secreted proteins that promote angiogenesis have been successfully inhibited with neutralizing antibodies with desirable clinical outcomes in the treatment of many different human cancers (Adams and Weiner 2005). Consistent with this promising therapeutic strategy to target angiogenesis, we demonstrate that an IL-6 neutralizing antibody inhibits Ras-driven tumor growth. IL-6 is thus a druggable protein that holds promise as a target for the treatment of Ras-driven cancers.
Figure 11: Ras induces secretion of IL-8

The indicated four primary human cells types expressing T/t-Ag and hTERT were shown to increase the levels of secreted IL-8 protein, as assessed by ELISA, upon expression (+) of RasG12V. IL-8 protein levels are reported as the percent mean ± standard deviation of duplicate experiments relative to IL-8 secreted by RasG12V-expressing cells.
4. Tumor Maintenance is Mediated by eNOS

4.1 Introduction

Tumor cells become addicted to the expression of their initiating oncogenes, such that loss of oncogene expression in established tumors leads to tumor regression. Indeed, oncogene addiction is the basis for a number of cancer therapies (Giuriato et al. 2004). The Ras family, composed of HRas, NRas, and KRas, is one of the most commonly activated oncogenes, being mutated to remain in the active GTP-bound state in a third of human cancers (Downward 2003). GTP-bound Ras binds to and activates three major effector proteins: RalGEFs, Rafs, and PI3K (Downward 2003). While multiple effectors initiate human tumor growth, only PI3K, through activation of the kinase AKT (PKB), must still remain activated by oncogenic Ras to maintain this growth (Lim and Counter 2005). Why cancer cells remain dependent upon the PI3K-AKT arm of oncogenic Ras signaling throughout tumorigenesis was unknown. Here we show that of the common substrates of AKT, blocking phosphorylation of eNOS inhibits tumor maintenance to the largest degree. Moreover, eNOS enhances the nitrosylation and activation of endogenous wildtype Ras proteins, which is required for both tumor initiation and maintenance. We thus suggest that activation of the PI3K-AKT-eNOS-(wildtype)Ras pathway by oncogenic Ras in cancer cells is required to initiate and maintain tumor growth.
4.2 Results

4.2.1 AKT Phosphorylation of eNOS is Required for Tumor Maintenance

Human tumor cells maintain growth following loss of oncogenic Ras expression only if the PI3K-AKT Ras effector pathway remains activated. This reduction in oncogene dependence appears to be a consequence of redundant signaling provided once a tumor microenvironment is established. Indeed, activation of AKT fosters tumorigenic growth of otherwise non-tumorigenic cells, provided such cells are mixed with tumor cells to establish the tumor microenvironment (Lim and Counter 2005). We exploited this cell-mixing assay to interrogate the signaling pathway downstream of AKT required for tumor maintenance. AKT is known to phosphorylate the proteins MDM2, GSK3, p21, p27, BAD, FOXO, IKKα, TSC2, and endothelial nitric oxide synthase (eNOS or NOS3) (Luo et al. 2003). We chose to focus on the latter five proteins, as the consequence of AKT phosphorylation of MDM2, GSK3, p21, and p27 is redundant with the functions of the viral SV40 oncoproteins T-Ag and t-Ag (Ali and DeCaprio 2001; Luo et al. 2003; Yeh et al. 2004) expressed in the cells (Lim and Counter 2005) used for these studies (Figure 12A).

Non-tumorigenic PI3K-TtH^{LacZ} cells, derived from primary normal human kidney (HEK) cells transformed by T-Ag and t-Ag and immortalized by hTERT (hereafter termed TtH cells), expressing p110-CAAX (to activate the PI3K-AKT pathway) and LacZ
(to demark the cells in the tumor), were stably infected with retroviruses encoding Bcl-X\textsubscript{i} shRNA, eNOS shRNA, or as reported by others, dominant-acting FOXO3a-A3, TSC2\textsuperscript{S939A,T1462A} (mutated at AKT phosphorylation sites) (Brunet et al. 1999; Manning et al. 2002) or IKK\textalpha\textsuperscript{K44A} (kinase-inactive) (Regnier et al. 1997) proteins (Figure 12A) to suppress the effects of AKT on these individual pathways. We validated knockdown of Bcl-X\textsubscript{i} and eNOS, ectopic expression and nuclear localization of FOXO3a-A3 (Brunet et al. 1999), ectopic expression of TSC2\textsuperscript{S939A,T1462A} leading to mTOR repression, as assessed by decreased S6k phosphorylation (Manning et al. 2002), and ectopic expression of IKK\textalpha\textsuperscript{K44A} leading to repression of NF-kB, as assessed by nuclear exclusion of p65 (Regnier et al. 1997) (Figure 12B and Figure 12C). The five cell lines were then mixed with the tumorigenic HRas\textsuperscript{G12V} transformed TtH cells (termed Ras\textsuperscript{G12V}-TtH cells) to establish a tumor microenvironment, injected into mice, and assayed for their contribution to the resultant tumor mass.

Upon treating tumors or resulting cells with X-gal to stain LacZ\textsuperscript{+} cells blue, PI3K-TtH\textsuperscript{LacZ} cells infected with a vector control retrovirus, when mixed with Ras\textsuperscript{G12V}-TtH cells, extensively populated tumors, as assessed by the prominent blue staining of the tumors and tumor-derived cell cultures. Conversely, in the absence of PI3K-AKT signaling, vector control TtH\textsuperscript{LacZ} cells failed to contribute to the tumor mass, as evidenced by the lack of blue staining. Expression of IKK\textalpha\textsuperscript{K44A} had little effect on the
Figure 12: Expression or knockdown of proteins downstream of AKT

(A) Pathways explored downstream of AKT (Luo et al. 2003). Light boxes, pathways altered by T/t-Ag. Black boxes, pathways altered using the indicated (red) molecules. (B) Expression or knockdown of the indicated transgenes or proteins in PI3K-TtH LacZ cells, as assessed by immunoblot. *non-specific band. (C) Confirmation of disruption of FOXO3a and NF-κB pathways through mutant transgene expression. Subcellular localization and protein analysis by immunofluorescence. The indicated transgenes were expressed in PI3K-TtH LacZ cells. Mutant HA-FOXO-3a-WT demonstrates appropriate localization to the nucleus, and expression of mutant IKKαK44A results in exclusion of p65 from the nucleus. Kian-Huat Lim performed experiments shown in (B) and (C).
ability of PI3K-AKT to promote tumor growth, given the prominent blue staining in tumors and tumor-derived cell cultures, whereas Bcl-X\textsubscript{i} shRNA, FOXO3a-A3, and TSC2\textsuperscript{2939A,T1462A} reduced the contribution of PI3K-TtH\textsuperscript{LacZ} cells in tumors by about half. Notably, the greatest inhibition of tumor maintenance was observed upon knocking down eNOS, as evidenced by a near complete lack of LacZ\textsuperscript{+} blue cells (Figure 13A). Given that knockdown of eNOS had the greatest effect on tumor maintenance, we focused on elucidating the role of eNOS in tumorigenesis.

To confirm that the loss of tumor maintenance upon knockdown of eNOS was indeed due to blocking eNOS activation by AKT, and not just due to an absence of eNOS protein, we first confirmed that pharmacological inhibition of AKT signaling with LY294002, as detected by a decrease in AKT phosphorylation, in PI3K-TtH\textsuperscript{LacZ} cells led to a reduction in the phosphorylation of the AKT site S\textsubscript{1177} on endogenous eNOS (Dimmeler et al. 1999; Fulton et al. 1999; Michell et al. 1999). We then demonstrated that phosphorylation at this AKT site was lost upon mutating S\textsubscript{1177} of eNOS to alanine (Figure 13B). Next, we tested whether expression of an RNAi-resistant version of human eNOS (eNOS\textsuperscript{R}), in which the S\textsubscript{1177} was mutated to alanine, would fail to rescue the loss of tumor maintenance following knockdown of eNOS. Specifically, PI3K-TtH\textsuperscript{LacZ} cells, in which eNOS was knocked down below the level detected in scramble control or the parental line, were engineered to express eNOS\textsuperscript{R} in the wildtype or S\textsubscript{1177}A mutant
Figure 13: AKT promotes tumor maintenance by phosphorylation of eNOS

(A) PI3K-TtHlacZ cells or (D) TtHlacZ cells expressing the indicated constructs were mixed with RasG12V-TtH cells, injected into mice, and tumors or recultured tumor cells were stained with X-gal to visualize the LacZ-expressing cells. (B) PI3K-TtHlacZ cells expressing wildtype (WT) or S1177A mutant eNOS were treated with either vehicle (DMSO) or the PI3K inhibitor LY294002 and levels of expression or phosphorylation (p) of AKT and eNOS (at S1177) were assayed by immunoblot. ect: ectopic, end: endogenous. (C) mRNA expression of eNOS in PI3K-TtHlacZ treated with scramble control or eNOS shRNA and complemented with either vector or shRNA-resistant eNOS (eNOSR) in the wildtype (WT) or S1177A mutant configuration. GAPDH: RT-PCR control. Kian-Huat Lim performed experiments shown in (A), (C), and (D), and Dave Kashatus performed experiments shown in (B).
configuration (Figure 13C). These two cell lines, and the appropriate controls, were individually mixed with RasG12V-TtH cells, injected into mice, and the resultant tumors assayed for blue LacZ+ cells. Control PI3K-TtHlacZ cells populated tumors, as evidenced by the dark blue staining, which upon knockdown of eNOS, was markedly reduced to a level similar to cells in which AKT was not activated. This loss of blue staining due to eNOS shRNA was rescued by expressing wildtype eNOSR, but not eNOSR harboring the S1177A mutation (Figure 13D). Thus, the AKT phosphorylation site in eNOS is required for tumor maintenance, suggesting that activation of the PI3K-AKT-eNOS pathway promotes tumor maintenance.

4.2.2 eNOS Promotes Nitrosylation of Wildtype Ras

eNOS has been detected in tumor cells (Fukumura et al. 2006), and catalyzes the synthesis of nitric oxide (NO). NO facilitates S-nitrosylation of the thiol group on C118 of HRas, which enhances the dissociation of guanine nucleotides thereby increasing GTP-bound HRas (Lander et al. 1996). Wildtype Ras proteins have been reported to be required for activation of the MAPK pathway by oncogenic Ras (Hamilton and Wolfman 1998), and membrane-targeting of RasGAP, which inhibits wildtype but not oncogenic Ras, reverts oncogenic Ras transformation of NIH3T3 cells (Huang et al. 1993), suggesting that wildtype Ras proteins may facilitate oncogenic signaling. Collectively, we speculated that AKT activation of eNOS maintains tumor growth in the absence of oncogenic Ras by activating wildtype Ras through S-nitrosylation at C118. To test this
hypothesis, we first demonstrated activated AKT in PI3K-TtH cells, as evidenced by elevated AKT phosphorylation, resulted in nitrosylation of HRas, which was abrogated by treatment with the PI3K inhibitor wortmannin (Figure 14A). The majority of this nitrosylation was lost upon mutating C118 in HRas to Serine, a minor change that exchanges the sulphur atom for oxygen but nevertheless blocks nitrosylation (Lander et al. 1996) (Fig 14B). This effect also depended upon eNOS expression, as knockdown of eNOS by shRNA greatly diminished the nitrosylation of endogenous HRas induced by activation of AKT in PI3K-TtH cells (Figure 14C). This reduction in HRas nitrosylation was, in turn, associated with a lower amount of active GTP-bound HRas, as evidenced by the fact that eNOS shRNA expressing cells exhibit a lower level of GTP-bound HRas compared to vector control cells (Figure 14C). Since TtH cells express HRas and NRas, but not KRas (Figure 14C), and C118 is conserved amongst all Ras proteins, we tested if NRas would similarly be nitrosylated and activated by AKT stimulation in TtH cells. Indeed, activated AKT in PI3K-Tth cells led to elevated levels of nitrosylated and GTP-bound endogenous NRas, and this depended upon eNOS, as knockdown of eNOS reduced both the nitrosylation and GTP-loading of NRas (Figure 14C). Thus, AKT activation of eNOS promotes nitrosylation and activation of wildtype Ras proteins.

To assess the biological consequence of S-nitrosylation of wildtype HRas in tumor maintenance, we tested whether replacing endogenous wildtype HRas with the nitrosylation-resistant C118S mutant version reduced tumor maintenance. HRas shRNA
Figure 14: eNOS activates wildtype HRas to promote tumor maintenance

(A) Levels of activated AKT (p-AKT) and S-nitrosylated HRas (nitroso-HRas) detected by immunoblot in PI3K-TtHlacZ cells treated with vehicle (DMSO) or the PI3K inhibitor wortmannin. HRas and tubulin: loading controls. (B) Levels of HRas S-nitrosylation detected in PI3K-TtHlacZ cells transiently transfected with a wildtype or C11S mutant version of HRas. HRas and tubulin: loading controls. (C) Levels of S-nitrosylated, GTP-bound, or total HRas and NRas, as assessed by immunoblot, in TtHlacZ cells expressing the indicated transgenes or shRNA. (D) Immunoblot analysis of Ras proteins, and total and GTP-bound wildtype (WT) or C11S mutant versions of shRNA-resistant FLAG-HRasR in PI3K-TtHlacZ cells expressing the indicated transgenes or shRNA. (E) RasG12V-TtH cells were mixed with PI3K-TtHlacZ cells expressing the indicated constructs, injected into mice, and tumors or re-cultured tumor cells were stained with X-gal to visualize PI3K-TtHlacZ cells n=5, mean ± s.e.m.). Kian-Huat Lim performed experiments shown in (E), and Dave Kashatus performed experiments shown in (A-D).
was expressed in PI3K-TtH\[^{LacZ}\] cells and confirmed to knockdown expression of endogenous HRas, but not NRas compared to scramble control cells (Figure 14D) as detected by immunoblot. Again, KRas was undetectable in these cells. The resultant cell line was then complemented with vector encoding HRas resistant to RNAi (HRas\[^{R}\]) in the wildtype or C\[^{118S}\] mutant configuration, or as a control, no transgene. Ectopic HRas expression was validated by immunoblot analysis, and most importantly, wildtype HRas protein was shown to have much higher levels of GTP loading compared to the C\[^{118S}\] mutant (Figure 14D). These two cell lines, or as controls cells expressing either a scramble control sequence or HRas shRNA, were individually mixed with Ras\[^{G12V}\]-TtH cells, injected into mice, and the resultant tumors assayed for the presence of blue LacZ\[^{+}\] cells. Knockdown of wildtype HRas reduced the ability of PI3K-AKT to foster tumor maintenance, as evidenced by a sixfold reduction of LacZ\[^{+}\] (blue) cells in the tumors. While wildtype HRas\[^{R}\] completely rescued this effect, as evidenced by the abundant LacZ\[^{+}\] cells, introducing the single C\[^{118S}\] mutation reduced this rescue threefold (Figure 14E). Taken together, these data support a model whereby activation of the PI3K-AKT-eNOS pathway leads to S-nitrosylation and activation of wildtype Ras, providing a critical signal for tumor maintenance (Figure 18D).
4.2.3 eNOS is Required for Ras-induced Tumor Initiation

Given that oncogenic Ras must activate the PI3K-AKT pathway to both initiate and maintain tumor growth of human cells (Lim and Counter 2005), we tested whether activation of eNOS was also required for the establishment of tumors. Aggressively tumorigenic Ras$^{G12V}$-TtH cells were stably infected with retroviruses encoding a scramble control or eNOS shRNA, after which the knockdown of eNOS was complemented by wildtype or S$^{1177A}$ mutant versions of RNAi-resistant eNOS (eNOS$^{R}$), or as a control, vector alone. Immunoblot analysis confirmed that wildtype eNOS$^{R}$ was phosphorylated on S$^{1177}$ in these cells, and that this phosphorylation was undetectable in the S$^{1177A}$ mutant (Figure 15A). These four cell lines were then each injected into mice and tumor growth monitored over time. Scramble control cells rapidly formed tumors, whereas tumor growth was nearly abolished upon knockdown of eNOS. This loss of tumor growth was rescued by expressing the wildtype, but not the S$^{1177A}$ version of eNOS$^{R}$ (Figure 15B,C). Consistent with the requirement for PI3K-AKT activation throughout tumorigenesis (Lim and Counter 2005), S$^{1177}$ phosphorylation and, consequently, activation of eNOS is required for tumor initiation and maintenance. PI3K-AKT-eNOS signaling thus appears to be so critical that it must be continually activated during oncogenic Ras-mediated tumorigenesis. We validated these results in a model that better reflects the spontaneous nature of cancer. Specifically, chemical carcinogens DMBA followed by TPA were topically applied to eNOS$^{+/+}$ and eNOS$^{-/-}$ mice
Figure 15: eNOS activation is required for tumor growth

(A) Detection of S1177-phosphorylated eNOS by immunoblot in RasG12V-TtH cells
(B) Representative mice and/or tumors after injection with RasG12V-TtH cells expressing the indicated constructs. (C) Tumor volume versus time of RasG12V-TtH cells expressing a scramble control sequence (□) or eNOS shRNA in conjunction with a vector control (■) or RNAi-resistant eNOS in either the wildtype (▲) or S1177A mutant (●) configurations when injected into mice. *p<0.001: difference in tumor size between scramble and eNOS shRNA complemented with S1177A mutant eNOSR. (D) DMBA/TPA-induced skin tumors (arrows) on representative mice at week 20. (E) Mean number of tumors per eNOS+/+ (□) and eNOS−/− (■) mouse versus time after initial application of DMBA. *p<0.01. Dave Kashatus performed experiments shown in (A).
to induce skin papillomas characterized by Ras oncogenic mutations (Quintanilla et al. 1986), with the end result being an approximate threefold drop in the number of tumors per eNOS\(^{-/-}\) mouse (Figure 15D,E). Thus, independent models of cancer demonstrate a critical requirement for eNOS.

4.2.4 Nitrosylation of Wildtype H and NRas is required for KRas-driven Pancreatic Cancer

We next addressed whether eNOS similarly mediates oncogenic Ras signaling in human pancreatic cancer, as this is the cancer most associated with the most commonly mutated Ras family member, KRas (Downward 2003). First, we assayed the amount of activated (S\(^{1177}\) phosphorylated) eNOS in cancer cell lines and tumor specimens isolated from patients diagnosed with pancreatic cancer. When compared to normal tissue specimens, CFPac-1, MIAPaCa-2, and Capan-1 cells exhibited the highest level of S\(^{1177}\) phosphorylation of eNOS (Figure 16A, top panels). Activated KRas and S\(^{1177}\) phosphorylated eNOS were also both elevated in the tumor specimens compared to matched and unmatched normal tissue controls (Figure 16B and (Lim et al. 2006)), with the caveat that unlike tumor cell lines, cancer biopsies contain stromal tissue that could contribute to the eNOS phosphorylation detected. Second, we tested whether the knockdown of eNOS reduced the tumor growth of CFPac-1 and MIAPaCa-2 cancer cell lines. Specifically, endogenous eNOS expression was reduced by shRNA, as assessed by immunoblot, in both cell lines compared to scramble control cells (Figure 16C), after
Figure 16: eNOS activation fosters pancreatic cancer cell growth

(A, B) Levels of phosphorylated eNOS (p-eNOS) and tubulin in pancreatic cancer lines (A), tumor, and normal tissue (B). (C) Detection by immunoblot of GTP-bound and total endogenous H, N, and KRas, as well as p-eNOS and tubulin in CFPac-1 and MIAPaCa-2 cells expressing eNOS shRNA or scramble control. (D, E) Excised tumors or tumor growth versus time of (D) CFPac-1 or (E) MIAPaCa-2 cancer cells expressing a scramble control (□) or eNOS shRNA (■) after injection into mice. *p<0.01 (CFPac-1) and p<0.05 (MIAPaCa-2). (F) Levels of p-eNOS and tubulin as assessed by immunoblot and excised tumors of CFPac-1 and MIAPaCa-2 cells expressing dox-inducible eNOS shRNA ± dox. In MIAPaCa-2 tumors, black regions are areas of necrosis. Experiments shown in (A-B) and (C) performed by Kian-Huat Lim and Dave Kashatus, respectively.
which the four cell lines were injected into mice and monitored for tumor growth. At the termination of the experiment, knockdown of eNOS had reduced tumor growth by 50 fold in CFPac-1 cells (Figure 16D), and reduced tumor size of MIAPaCa-2 cells by 80% (Figure 16E). Third, using a more direct assay for tumor maintenance, we tested whether knockdown of eNOS after tumors were established from pancreatic cancer cells reduced continued tumor growth. Specifically, CFPac-1 and MIAPaCa-2 cells were engineered to express a doxycycline (dox)-inducible eNOS shRNA, and dox treatments confirmed by immunoblot to reduce the level of endogenous activated eNOS (Figure 16F) (Lim and Counter 2005). These cells were each injected into mice to establish tumors, after which mice were either left untreated, thereby retaining eNOS, or provided with dox in their diet to repress eNOS. While control tumors continued to grow unabated, upon treating mice with dox to knockdown endogenous eNOS, tumor growth was inhibited in both cancer cell lines, as evidenced by reduced size and/or gross necrosis of tumors excised at the termination of the experiment, and reduced tumor growth kinetics (Figure 16F). In some mice, this eventually led to tumor regression. Thus, eNOS is required to both initiate and maintain tumor growth of oncogenic KRas-driven human pancreatic cancer cells.

Lastly, to test if the reliance on eNOS for tumor growth was due to nitrosylation of Ras in human pancreatic cancer cell lines, again because of the relevance of KRas mutations in the etiology of this cancer (Downward 2003), we explored which Ras
family members were required for tumor growth of CFPac-1 and MIAPaCa-2 cells. First, we tested whether eNOS fostered activation of endogenous HRas, NRas, or KRas by assaying the level of GTP-bound Ras proteins in eNOS knockdown versus scramble control CFPac-1 and MIAPaCa-2 cells (Figure 16C). Not surprising, GTP-bound levels of endogenous KRas are unchanged in both eNOS knockdown cells (Figure 16C), as KRas is mutated to remain active in these cell lines (Kita et al. 1999; Moore et al. 2001). Consistent with this, oncogenic KRas harboring the C11S mutation remained tumorigenic (Figure 17), pointing towards endogenous wildtype Ras proteins as the target of eNOS signaling.

As the wildtype allele of KRas has been commonly found deleted in tumors (Li et al. 2003; Wan et al. 2006) and cell lines (Kita et al. 1999) encoding a mutant KRas oncogene allele, and indeed is deleted in the MIAPaCa-2 cells (Kita et al. 1999) that depend upon eNOS for tumor growth (Figure 16E, F), wildtype HRas and NRas, and not wildtype KRas, are presumably the targets of eNOS signaling. In agreement, GTP-bound levels of endogenous wildtype HRas and NRas were reduced in the eNOS knockdown cells compared to scramble control cells (Figure 16C). Thus, endogenous eNOS promotes activation of endogenous wildtype HRas and NRas in oncogenic KRas-driven human pancreatic cancer cells. To test whether this activation of HRas by eNOS is required for pancreatic tumor growth, HRas was knocked down by shRNA in CFPac-
Figure 17: Oncogenic KRas harboring a C118S mutation remains tumorigenic

TtH cells harboring oncogenic KRas$^{G12V}$ or KRas$^{G12V,C118S}$ were injected into mice and assayed for tumor growth. Excised tumors are shown.
and MIAPaCa-2 cells, as assessed by immunoblot, and then complemented with an HRas engineered to be resistant to RNAi (HRasR) in the wildtype or the C118S mutant configuration (Figure 18A, B). The resultant CFPac-1 and MIAPaCa-2 cell lines were then each injected into mice and tumor growth monitored. Positive control scramble treated CFPac-1 and MIAPaCa-2 cells readily formed tumors in mice, whereas this growth was reduced 3 and 13 fold, respectively, when endogenous HRas was knocked down. This loss of tumor growth was rescued to some extent by expressing wildtype HRas, but not the C118S nitrosylation mutant (Figure 18A, B). We repeated these experiments by complementing the shRNA knockdown of endogenous NRas in MIAPaCa-2 cells with a vector control or one encoding an RNAi-resistant version of NRas (NRasR) in the wildtype or C118S mutant configuration (Figure 18C). Again, knockdown of NRas reduced tumor growth of this pancreatic cancer cell line, although not quite to the extent of HRas shRNA, and this loss of tumor growth was rescued by the wildtype, but less so by the C118S mutant version of NRas (Figure 18C). Thus, oncogenic KRas-driven pancreatic cancer tumor growth is mediated by eNOS nitrosylation of endogenous wildtype HRas and NRas (Figure 18D).
Figure 18: Activation of Wildtype H and N Ras by eNOS is required for pancreatic cancer cell growth

Immunoblot analysis, excised tumors, and tumor growth versus time of CFPac-1 and MIAPaCa-2 cells expressing a scramble control (scram, □), HRas (A, B) or NRas (C) shRNA in conjunction with vector control (vector, ■) or RNAi-resistant HRas (HRas\textsuperscript{R}) or NRas (NRas\textsuperscript{R}) in the wildtype (WT, □) or C\textsubscript{118S} mutant (C\textsubscript{118S}, ●) configuration. *p<0.001 and p<0.05: difference in tumor size for CFPac-1 and MIAPaCa-2 cells, respectively, expressing scramble versus eNOS shRNA and HRas\textsuperscript{R}-C\textsubscript{118S}. *p<0.01: difference in tumor size for CFPAC-1 cells expressing scramble versus eNOS shRNA and NRas\textsuperscript{R}-C\textsubscript{118S}. (D) Proposed model of eNOS signaling to wildtype HRas and NRas.
4.3 Discussion

In summary, we demonstrate that the continual need for PI3K-AKT signaling during initiation and maintenance of oncogenic Ras-driven tumor growth is due, at least in part, to activation of eNOS through phosphorylation of S1177. Effects of eNOS on tumorigenesis have been largely attributed to its activity in endothelial cells (Fukumura et al. 2006). Our results now suggest a key role for tumor-expressed eNOS in the tumorigenic process. As eNOS plays multiple roles in tumorigenesis (Fukumura et al. 2006), and delivery of a peptide fragment of the protein cavtratin, which can inhibit eNOS, displays anti-tumor activity (Gratton et al. 2003), we speculate that inhibition of eNOS could have therapeutic value in the treatment of oncogenic Ras-driven human cancers such as pancreatic. Mechanistically, we suggest that activation of eNOS by oncogenic Ras leads to S-nitrosylation at C118 and correspondingly, activation of wildtype Ras proteins, and that this activation is required for tumor growth. We favor a model whereby oncogenic Ras, for example KRas in the case of pancreatic cancer, must activate the other wildtype Ras proteins HRas and NRas, and not the wildtype KRas protein, to promote tumor initiation and maintenance (Figure 18D). This feedback signaling serves to diversify the Ras signal beyond that of oncogenic KRas, as opposed to simply amplifying the amount of Ras activity. In support of the notion that the wildtype counterpart of oncogenic Ras does not mediate this signaling, wildtype KRas is not required for oncogenic KRas-driven tumor growth (Figure 18A, B, C). In fact, the
wildtype allele of KRas has been found to be actually deleted in some KRas mutation-positive tumors (Li et al. 2003; Wan et al. 2006), and knockdown of the wildtype counterpart of a Ras oncoprotein has no effect on tumor growth (Figure 19). On the other hand, both HRas and NRas are required for oncogenic KRas-driven tumor growth, and both gain- and loss-of-function analysis in mice or derived fibroblasts demonstrate HRas, NRas, and KRas specific phenotypes (Johnson et al. 1997; Esteban et al. 2001; Fotiadou et al. 2007; Parikh et al. 2007), suggestive of non-redundant signaling by the three family members that, in turn, could be important for tumor growth. To conclude, our studies suggest that the dependence upon AKT for tumor initiation and maintenance in oncogenic Ras-driven cancers such as pancreatic is due, in part, to activation of eNOS, which in turn promotes tumor growth through nitrosylation and activation of the other wildtype Ras family members (Figure 18D).
Figure 19: Loss of wildtype HRas does not inhibit oncogenic HRasG12V-mediated tumorigenesis

(A) Detection of HRas by immunoblot in TtH cells treated with either scramble or HRas shRNA in addition to RNAi-resistant oncogenic HRasG12V. (B) Representative excised tumors from mice injected with TtH cells treated with either scramble or HRas shRNA and engineered to express oncogenic HRasG12V.
5. Future Directions

5.1 Future Directions: Examining the Role of Cytokines in Ras-mediated Tumor Initiation

As described in the previous sections, I have shown that IL-6 is induced upon expression of oncogenic Ras, is required for Ras-induced tumor initiation, and is required for the growth of pancreatic cancer cell lines. These findings indicate that IL-6 may be a valid therapeutic target for pancreatic cancer, a cancer associated with a high rate of Ras mutations (Bos 1989). Therefore, it is of considerable interest to further evaluate the role of IL-6 in a more clinically relevant model of pancreatic cancer, in order to lay the groundwork for future exploration of this cytokine as a therapeutic target.

Similarly, recent data has shown that other cytokines, such as IL-8 and GRO-1, may also be important modulators of Ras tumorigenesis (Sparmann and Bar-Sagi 2004; Wislez et al. 2006; Yang et al. 2006). The identification of secreted proteins required for Ras tumorigenesis in addition to those mentioned above may lead to an increased number of candidates for Ras-targeted therapy. This is of particular interest, as the identification of additional targets increases the options for combination treatments in Ras-driven tumors, which may provide more success in the clinic than treatment with a single agent.
5.1.1 Determining the Role of IL-6 in a Mouse Model of Spontaneous Pancreatic Cancer Driven by Ras Mutation

In Chapter 3, I have shown that IL-6 is activated by oncogenic Ras, and this activation is required for Ras-induced tumor growth of human cells. Pancreatic cancers are characterized by a high rate of Ras mutations (Bos 1989), and IL-6 levels have been found to be elevated in the serum of pancreatic cancer patients (Barber et al. 1999; Wigmore et al. 2002; Trikha et al. 2003; Ebrahimi et al. 2004). Pancreatic cancer is highly resistant to current treatments, and thus new therapies are highly desirable. As IL-6 is a secreted protein, it serves as a potential target for therapeutic intervention of pancreatic cancer. Our previous experiments have shown that knockdown of IL-6 by shRNA resulted in diminished tumor growth in two of three tested human pancreatic cancer cell lines in a xenograft model. These are valuable experiments that provide the advantage of using human cells with a defined genetic background. However, xenograft models also have some disadvantages, including using mice without an intact immune system and producing non-spontaneous tumors that are in an artificial environment. Therefore, it is of considerable interest to validate our findings in an orthotopic model of spontaneous pancreatic tumorigenesis driven by oncogenic Ras, which recapitulates the features of human pancreatic cancer, in immunocompetent mice.

In order to further evaluate the effect of IL-6 on pancreatic tumor growth, a more physiologically relevant mouse model of pancreatic cancer driven by Ras mutations may be utilized to determine the effect of loss of IL-6 in this system. Mice heterozygous for
the mutant lox-stop-lox-KRas\textsuperscript{G12D} (LSL- KRas\textsuperscript{G12D/+}) allele, in which a silencing cassette prohibits expression of the mutant KRas\textsuperscript{G12D} (Jackson et al. 2001), are crossed to PDX-1-Cre transgenic mice (Hingorani et al. 2003), which express Cre recombinase from a pancreatic-specific promoter. This cross results in Cre-mediated excision of the silencing cassette, causing expression of the mutant KRas allele in cells of the pancreas. These mice develop pancreatic lesions characteristic of human pancreatic intraepithelial neoplasias (PanINs), which spontaneously progress to invasive and metastatic adenocarcinoma at a low rate (Hingorani et al. 2003).

Generating a mouse that is homozygous null for IL-6 (IL-6\textsuperscript{−/−}) and has pancreas-specific Cre-mediated activation of a mutant KRas allele (KRas\textsuperscript{G12D}), involves first crossing the heterozygous LSL-KRas\textsuperscript{G12D/+} mouse to IL-6\textsuperscript{−/−} mice (Kopf et al. 1994), to generate LSL-KRas\textsuperscript{G12D/+}; IL-6\textsuperscript{−/−} mice, heterozygous for both alleles, at a rate of 1:2. These progeny mice can then be backcrossed to IL-6\textsuperscript{−/−} mice to produce LSL-KRas\textsuperscript{G12D/+}; IL-6\textsuperscript{−/−} mice (1:4), which are heterozygous for the mutant KRas allele and homozygous null for IL-6 (Figure 20A). Concurrently, in order to generate PDX-1-Cre; IL-6\textsuperscript{−/−} mice, PDX-1-Cre transgenic mice will be crossed with IL-6\textsuperscript{−/−} mice to generate PDX-1-Cre; IL-6\textsuperscript{−/−} mice (1:2). These progeny mice are then backcrossed to IL-6\textsuperscript{−/−} mice to produce PDX-1-Cre; IL-6\textsuperscript{−/−} mice at a rate of 1:4 (Figure 20B).
Figure 20: Mouse crossing strategy to generate PDX-1-Cre; LSL-KRas<sup>G12D</sup>/+; IL-6<sup>−/−</sup> mice
Next, LSL-KRas\(^{G12D/+}\); IL-6\(^{-/-}\) and PDX-1-Cre; IL-6\(^{-/-}\) mice can be intercrossed to produce mice that are homozygous null for IL-6 and heterozygous for the LSL-KRas\(^{G12D}\) and PDX-1-Cre alleles at a rate of 1:4 (Figure 20C). Expression of Cre-recombinase from the pancreatic-specific PDX-1 promoter will drive excision of the KRas\(^{G12D}\) silencing cassette in the pancreatic cells of the mouse. As a control, PDX-1-Cre; IL-6\(^{+/+}\) mice will be crossed to LSL-KRas\(^{G12D/+}\); IL-6\(^{+/+}\) mice, to generate PDX-1-Cre; LSL-KRas\(^{G12D/+}\); IL-6\(^{+/+}\) mice. The rate of PanIN development as well as progression to adenocarcinoma in these two groups of mice (IL-6\(^{+/+}\) and IL-6\(^{-/-}\)) can then be compared to determine if a loss of IL-6 affects the development of pancreatic lesions.

Based on my previous results, in which I have shown that IL-6 is required for tumorigenic growth of pancreatic cancer cell lines, I hypothesize that inhibition of IL-6 in a spontaneous model of pancreatic cancer driven by Ras mutation will result in impaired tumorigenesis. However, two there are two potential outcomes, 1) loss of IL-6 decreases the frequency of pancreatic lesions and slows their development into invasive and metastatic adenocarcinomas, or 2) there is no difference in the occurrence of pancreatic lesions or the rate of development into adenocarcinomas with or without IL-6.

A decreased frequency of pancreatic lesion formation in IL-6\(^{-/-}\) mice compared to controls would support the hypothesis that IL-6 plays an important role in the formation and development of pancreatic cancer, suggesting that inhibition of IL-6 may be an
adequate target for pancreatic cancer inhibition. In this scenario, I would extend my studies to a therapeutic approach for inhibiting IL-6 in pancreatic cancer. I would investigate the effect of a humanized IL-6 neutralizing antibody on the growth of a panel of pancreatic cancer cell lines in vivo. Such an antibody has shown promise in the treatment of several cancers in clinical trials and is well tolerated in patients (Trikha et al. 2003). Targeting IL-6 in this preclinical model may lay the groundwork for the extension into a Phase I clinical trial for treatment of pancreatic cancer.

If there is no difference in the development of pancreatic lesions in the absence of IL-6, it could mean 1) either IL-6 is not important for the development of pancreatic cancer, although my preliminary data utilizing an IL-6 shRNA in pancreatic cancer cell lines argues against this possibility, or 2) loss of IL-6 is important but not sufficient to decrease pancreatic tumor growth. For instance, Ras leads to the secretion of a set of cytokines and growth factors, and these other secreted factors may function redundantly to IL-6. In the case that this outcome is observed, I would further investigate the role of IL-6 as a combination therapeutic. For instance, I would treat the control mice and the mice lacking IL-6 with a current pancreatic cancer treatment drug, such as Gemcitabine, or with neutralizing antibodies against other secreted proteins known to be induced by Ras, such as IL-8 or VEGF. I could then compare the rate of tumor development in these mice, to determine if loss of IL-6 accentuates the effect of these drugs.
5.1.2 Identification of Secreted Proteins Required for Ras Tumorigenesis

I have shown that IL-6 is required for Ras-mediated tumorigenesis and may be a potential anti-cancer target (Ancrile et al. 2007). Similarly, other secreted proteins, including IL-8 and GRO-1, have also been implicated in Ras oncogenesis (Sparmann and Bar-Sagi 2004; Wislez et al. 2006; Yang et al. 2006), suggesting that cytokines may play an important role in the tumorigenic process, and that Ras may activate a set of secreted proteins that are required for tumor initiation. Moreover, such secreted proteins may be targetable with neutralizing antibodies (Ancrile et al. 2008). While these proteins present valid anti-cancer targets, combination treatments may produce the most success in the clinic. Therefore, it is of considerable interest to identify additional secreted proteins that may be required for Ras tumorigenesis and may therefore be valid cancer therapeutic targets. I have begun to look for candidates in two ways: 1) profile comparison of secreted proteins known to be required for angiogenesis in tumorigenic Ras-expressing cells versus non-tumorigenic cells, and 2) profile comparison of secreted proteins in a large mass spectrometry screen in tumorigenic Ras-expressing cells versus non-tumorigenic cells without oncogenic Ras expression.

In a small screen of secreted, angiogenic factors, I found that RANTES, a cytokine, was upregulated in Ras-expressing cells. I further evaluated RANTES expression in 4 different cell types, to determine if RANTES upregulation upon Ras expression was cell type specific. Using an ELISA assay, I found that RANTES was
preferentially upregulated in fibroblasts and myoblasts, as compared to kidney cells and
mammary epithelial cells, suggesting that RANTES expression in response to Ras
activation may be specific to cells of a mesenchymal origin (Figure 21). Further analysis
to elucidate the importance of RANTES in Ras-driven tumors of this type, such as
rhabdomyosarcoma, may determine if RANTES plays a role in such cancers.
Specifically, I plan to create an shRNA against the protein, and stably express it in
human skeletal muscle myoblast (HSMM) and BJ fibroblast cells, which show the largest
induction of the protein upon expression of oncogenic Ras. Loss of function
experiments can then be performed, both in vitro and in vivo, to determine the effect of
RANTES expression in tumorigenic cells. Cell-based assays, such as growth curve
experiments and soft agar assays may help delineate whether RANTES is playing an
autocrine or paracrine role in Ras tumorigenesis. Tumorigenic assays, in which
RANTES knockdown cells and scramble control-expressing cells are injected into the
flanks of immunocompromised mice and tumor growth is monitored, will further
evaluate the importance of RANTES in Ras tumorigenesis in these cell types. If
RANTES knockdown is shown to impede tumorigenesis in these cell lines, its role could
be further evaluated in cancer cell lines of mesenchymal origin via shRNA knockdown,
and, moreover, it’s potential as an anti-cancer target further examined by determining
the effects of a RANTES neutralizing monoclonal antibody on tumor growth.
Figure 21: RANTES levels in four different cell types upon Ras expression
In an additional attempt to identify secreted proteins that may be required for Ras tumorigenesis and candidates as anti-cancer targets, I sought to expand upon my previously generated profile of secreted proteins by utilizing a mass spectrometry screen, as my previous screen only allowed my to examine the levels of a limited number of secreted proteins thought to play a role in angiogenesis. I therefore collected media from Ras-expressing, tumorigenic cells and non-Ras expressing, non-tumorigenic cells, and used acetone precipitation to concentrate the proteins. I then had mass spectrometry performed in order to analyze the profile of secreted proteins from each cell type. While this method has the advantage of recognizing a very large number of proteins, the disadvantages include the inability to pick up very small, very large, very basic, or very acidic proteins. One protein, stanniocalcin-1 (STC-1), which was upregulated in the screen, had not previously been linked to oncogenic Ras expression. STC-1 is an endocrine hormone that is expressed in a wide variety of tissues, and may play a role in human cancer (Chang et al. 2003). STC-1 has been shown to be upregulated in several cancers, including breast, ovary, colon, thyroid, and hepatocellular carcinomas (Chang et al. 2003). There is some evidence that STC-1 can be upregulated by VEGF (Bell et al. 2001; Liu et al. 2003; Wary et al. 2003) and is thought to play some role in the establishment of the tumor vasculature (Kahn et al. 2000; Gerritsen et al. 2002), although the exact role of this protein in cancer processes has yet to be determined.
In order to validate the upregulation of STC-1 in response to oncogenic Ras expression, I examined STC-1 levels by RT-PCR in four different cell types upon expression of Ras\(^{G12V}\). RT-PCR revealed that STC-1 levels were induced upon expression of Ras\(^{G12V}\) in kidney cells, fibroblasts, myoblasts, and mammary epithelial cells (Figure 22), suggesting that this upregulation of STC-1 by oncogenic Ras is not a cell type specific effect. Thus, this protein may provide an exciting new target in Ras-driven tumors. In order to evaluate the importance of STC-1, I plan to create an shRNA against the protein, stably express it in HEK cells, and evaluate whether knockdown of STC-1 results in diminished tumor growth compared to scramble control cells when injected into the flanks of immunocompromised mice. If such a phenotype is seen, these results can be confirmed using additional shRNAs against STC-1 and in other cell types expressing oncogenic Ras, such as HSMM or BJ cells, to determine if loss of tumorigenic potential is a cell-specific event. Cell-based assays, such as growth curve experiments and soft agar assays can be used to help delineate whether STC-1 is playing an autocrine or paracrine role in Ras tumorigenesis. Furthermore, if STC-1 is shown to be important for Ras-induced tumorigenesis, the development and evaluation of a neutralizing antibody may further evaluate the potential of STC-1 as an anti-cancer target.
Figure 22: STC-1 levels, as measured by RT-PCR, in 4 different cell types upon expression of Ras$^{G12V}$
5.2 Future Directions:Examining the Role of eNOS in Ras-mediated Tumorigenesis

As described in Chapter 4, we have demonstrated that eNOS is phosphorylated and activated by ATK, leading to eNOS-mediated nitrosylation and activation of wildtype Ras proteins. Additionally, I have shown that these proteins are required throughout tumorigenesis. Therefore, inhibition of eNOS, perhaps in combination with inhibition of wildtype Ras protein function or processing, may have therapeutic value in the treatment of Ras-driven tumors. Such therapeutic targets may be of particular use in pancreatic cancer, as it is associated with approximately a 90% rate of Ras mutation (Bos 1989) and is highly resistant to current treatments. Utilizing shRNAs against eNOS, HRas, and NRas, I have shown that these proteins are required for tumorigenic growth of pancreatic cancer cell lines. Therefore, I aim to validate my previous findings in a more clinically relevant model of pancreatic cancer, a model of mouse spontaneous pancreatic cancer driven by oncogenic KRas.

5.2.1 Determining the Role of eNOS and Wildtype Ras Proteins in a Mouse Model of Spontaneous Pancreatic Cancer Driven by Ras Mutation

I have previously shown that eNOS and, furthermore, wildtype Ras proteins, are required for pancreatic cancer cell growth in a xenograft model, a system which provides the advantages of utilizing human cells with a defined genetic background. However, it would be useful to validate these results in a more physiologically-relevant,
orthotopic model of spontaneous pancreatic tumorigenesis driven by oncogenic KRas, which recapitulates the features of human pancreatic cancer in immunocompetent mice. Using this system, I could compare the development of pancreatic tumors in control mice to mice either deficient in eNOS (eNOS^{-/-}) or H and NRas (HRas^{+/+}; NRas^{+/+}).

The mouse model of spontaneous pancreatic cancer driven by KRas^{G12D} under control of a pancreatic specific promoter provides a means by which to study the importance of eNOS or H and NRas in a more clinically relevant setting. This system was mentioned in a previous section. Briefly, mice heterozygous for the mutant lox-stop-lox-KRas^{G12D} (LSL- KRas^{G12D/+}) allele are crossed to PDX-1-Cre transgenic mice, resulting in Cre-mediated excision of the silencing cassette in the cells of the pancreas. Consequential expression of the mutant KRas allele leads to the development of pancreatic lesions characteristic of human PanINs, which spontaneously progress to invasive and metastatic adenocarcinoma (Hingorani et al. 2003).

In order to evaluate the importance of eNOS in this system, the development of pancreatic lesions can be compared in PDX-1-Cre; LSL-KRas^{G12D/+}; eNOS^{+/+} mice versus PDX-1-Cre; LSL-KRas^{G12D/+}; eNOS^{-/-} mice. The generation of these mince can be achieved using the breeding strategy shown in Figure 23. The rate of PanIN development as well as progression to adenocarcinoma in these two groups of mice (eNOS^{+/+} versus eNOS^{-/-}) can be compared to determine if a loss of eNOS affects the development of pancreatic
Figure 23: Mouse crossing strategy to generate PDX-1-Cre; LSL-KRasG12D/+; eNOS−/− mice
lesions. I have acquired LSL-KRas^{G12D}, PDX-1-Cre, and eNOS^{+/} mice, and am currently in the process of performing the above crosses.

Similarly, this system of spontaneous pancreatic tumorigenesis can be used to validate my findings that wildtype Ras proteins play an important role in pancreatic tumorigenesis (Chapter 4). Specifically, in this model of tumorigenesis driven by oncogenic KRas, it would be of use to determine the result of a loss of wildtype HRas and NRas. This could be accomplished by comparing the rate of pancreatic tumor development in control mice compared to HRas^{+/};NRas^{+/} mice. We currently have LSL-KRas^{G12D/+}, PDX-1-Cre, and HRas^{+/}; NRas^{+/} in our lab, and I have begun crosses using the breeding strategy shown in Figure 24, in order to generate mice deficient for H and NRas expressing KRas^{G12D} only in cells of the pancreas.

Based on my previous results in which I have shown that both eNOS and eNOS-mediated activation of wildtype Ras proteins are required for tumorigenic growth of pancreatic cancer cell lines, I hypothesize that loss of these proteins in a spontaneous model of pancreatic cancer driven by Ras mutation will result in impaired tumorigenesis. However, there are two potential outcomes, 1) loss of eNOS or wildtype Ras proteins decreases the frequency of pancreatic lesions and slows their development into invasive and metastatic adenocarcinomas, or 2) there is no difference in the occurrence of pancreatic lesions or the rate of development into adenocarcinomas in the absence of eNOS or wildtype Ras.
Figure 24: Mouse crossing strategy to generate PDX-1-Cre, LSL-KRas^G12D/+; HRas^+/−; NRas^−/− mice
A decreased frequency of pancreatic lesion formation in either eNOS\textsuperscript{\textminus/\textminus} or HRas\textsuperscript{\textminus/\textminus}; NRas\textsuperscript{\textminus/\textminus} mice compared to controls would agree with my hypothesis that these proteins play an important role in the formation and development of pancreatic cancer, suggesting that eNOS inhibitors, potentially in combination with Ras inhibitors, may be a suitable target for pancreatic cancer inhibition. In this scenario, I would first attempt to determine a mechanism of tumorigenesis by comparing the tumor histology in from different groups of mice to determine if there is a difference in cell proliferation, apoptosis, or tumor vasculature. I would also extend my studies to a therapeutic approach for inhibiting eNOS in pancreatic cancer. Specifically, I would investigate the effect of a NOS inhibitor on the growth of a panel of pancreatic cancer cell lines in vivo. Development of an eNOS-specific inhibitor would be of most use, as it would be less likely to generate undesired side effects than a general NOS inhibitor.

If there is no difference in the development of pancreatic lesions in the absence of eNOS or HRas and NRas, it could mean 1) either these proteins are not important for the development of pancreatic cancer, although my preliminary data utilizing shRNAs in pancreatic cancer cell lines argues against this possibility, or 2) loss of these proteins is important but not sufficient to decrease pancreatic tumor growth. For instance, other members of the NOS family may compensate for a loss in eNOS. In the case that this outcome is observed, I would both determine the levels of the other NOS isoforms in the pancreatic lesions and repeat the experiment using mice deficient in both eNOS and
another NOS isoform. This outcome may also suggest that global disruption of eNOS in the mouse inhibits the anti-tumor effects caused by loss of eNOS only in tumor cells. In order to address this, a mouse in which eNOS is flanked by lox sites could be generated, such that crossing to a PDX-1-Cre mouse would result in conditional loss of eNOS only in the pancreas.
6. Conclusions

6.1 Secreted Proteins: A Paracrine Model of Tumor Promotion Mediated by Oncogenic Ras

In an attempt to identify secreted proteins that may be essential for Ras-induced tumorigenesis, potentially by promoting the development of a tumor microenvironment, we used a small array to determine the levels of angiogenic proteins in the presence and absence of oncogenic Ras. One protein significantly upregulated in this screen was IL-6. As described in this thesis, I confirmed that IL-6 levels are induced upon expression of Ras, and showed that this effect is not cell type specific. Using a xenograft system, I demonstrated that loss of IL-6 prevented the growth of multiple Ras-transformed cell types in mice, and validated these results in a spontaneous mouse model of skin carcinogenesis characterized by Ras mutation. Furthermore, I showed that IL-6 was required for the growth of pancreatic cancer cell lines and a neutralizing IL-6 antibody inhibited the growth of xenograft tumors. These results suggest that IL-6 may be a valid anti-cancer target in Ras-driven tumors. Therefore, it is of considerable interest to further validate the role of IL-6 in a more clinically-relevant model of tumorigenesis, such as the mouse model of spontaneous pancreatic cancer described in Chapter 5. Such experiments in turn may lay the groundwork for future exploration of anti-IL-6 therapy to treat Ras-driven cancers.
While I have shown that IL-6 is important for Ras-driven tumors, the mechanism of action by which IL-6 mediates tumorigenesis is still not completely understood. Using cell-based assays, I demonstrated that IL-6 does not exert cell-autonomous effects in Ras-expressing tumor cells, and, moreover, found that loss of IL-6 in Ras-driven tumors resulted in diminished endothelial cell recruitment. Together, these experiments suggest that IL-6 acts in a paracrine manner to foster angiogenesis (Figure 25). Similarly, others have found that cytokines such as IL-8 and GRO-1 mediate Ras-driven tumorigenesis in a paracrine manner (Sparmann and Bar-Sagi 2004; Wislez et al. 2006; Yang et al. 2006; Ancrile et al. 2007). However, the exact mode of action seems to vary between molecules. For instance, Ras induced IL-8 has been shown to act in a paracrine manner to foster angiogenesis via the recruitment of inflammatory cells (Sparmann and Bar-Sagi 2004). On the other hand, Ras-induced expression of GRO-1, although also shown to act in a paracrine manner, altered the stromal environment by modulation of fibroblasts (Yang et al. 2006).

The mechanism by which IL-6 mediates tumor angiogenesis has yet to be determined. It is unlikely that IL-6 is communicating with endothelial cells directly, as these cells do not express the IL-6 receptor (Figure 9). Cell types that do express the IL-6 receptor include T cells and B cells, neutrophils, and macrophages. However, the immunodeficient SCID/beige mice used for our previous experiments lack functional T
Figure 25: Model for the effects of IL-6 production by Ras-expressing tumor cells and effects on the microenvironment

The tumor (green cells) produces IL-6 (yellow), which diffuses into the surrounding stroma and activates receptors expressed on infiltrating cells (pink). The identity of these intermediary cells is uncertain, but they could be epithelial cells, T lymphocytes, myeloid cells, and/or dendritic cells. This IL-6-responsive population might produce soluble factors that directly stimulate tumor growth (blue arrow). These cells might also express cell surface proteins (green bar) and/or produce soluble factors (green circles) that recruit endothelial cells (orange) into the microenvironment. Endothelial cells, in turn, could contribute to tumor growth either by feedback activation of tumor cells (orange arrow) and/or by releasing angiogenic factors (short red arrows) that promote blood vessel formation around the tumor (Diaz-Flores and Shannon 2007). Used with permission of the publisher.
cells and B cells (Bosma et al. 1983), and, therefore, these cells can be excluded as potential candidates. Macrophages are commonly found in the tumor microenvironment, and have been demonstrated to possess both anti-tumor and tumor-promoting properties (Coussens and Werb 2002). Macrophages have been shown to be recruited to the tumor site by tumor-secreted cytokines, including IL-6 (Elgert et al. 1998), and are therefore a potential cell type to be mediating the response to IL-6 signaling.

Immunohistochemical analysis can be used to help determine which cell type in the tumor microenvironment IL-6 is communicating with to facilitate angiogenesis. Tumor sections generated by cells expressing oncogenic Ras and either IL-6 shRNA or scramble control can be stained with a macrophage marker, for example, and the number of each infiltrating cell type quantified. If a difference in the number of positively-stained cells is seen, it would suggest that IL-6 is necessary for recruitment of these cells to the tumor site. In order to further evaluate the importance of IL-6 communication with a particular cell type, it would be of use to conditionally knockout the IL-6 receptor (IL-6R) in specific cell types, and determine the resulting effect on tumor growth. This would require the generation of a mouse in which IL-6R is flanked by lox sites and then crossing to a mouse in which Cre is expressed under a cell type specific promoter. For example, lysM-Cre mice (Clausen et al. 1999) express Cre recombinase under the control of the lysosome M promoter, which is specifically
expressed in macrophages and granulocytes. The DMBA/TPA chemical carcinogen system could then be used to determine if loss of IL-6R on macrophages impaired tumorigenesis. If this was found to be the case, tumors could further be analyzed by immunohistochemical analysis to determine if loss of IL-6 recruitment results in diminished endothelial cell recruitment.

Overall, I have demonstrated an important role for IL-6 in Ras-mediated tumors, and, similar to the findings of others (Sparmann and Bar-Sagi 2004; Wislez et al. 2006; Yang et al. 2006), suggesting that IL-6 promoting tumorigenesis via paracrine signaling to the tumor microenvironment. While the exact mechanism of this signaling has yet to be determined, IL-6 may represent an important anti-Ras target in the clinic.

6.2 eNOS Signaling in Ras Tumorigenesis: a Feedback Mechanism to Activate Wildtype Ras proteins

Following tumor initiation, redundant signaling from the tumor microenvironment reduces the signaling requirement of oncogenic Ras to activation of the PI3K-AKT pathway. We sought to elucidate the signaling pathway downstream of AKT required for tumor maintenance. In Chapter 4, I have shown that stimulation of the PI3K pathway leads to activation of eNOS and demonstrated that this activation is achieved by phosphorylation of eNOS at S1177 by AKT. Using a xenograft system, I have shown that knockdown of eNOS by shRNA in Ras-expressing cells abrogates both
tumor initiation and tumor maintenance. This phenotype could be rescued using an RNAi-resistant eNOS protein, except when that protein was carrying a S1177A mutation, suggesting that AKT-mediated phosphorylation of eNOS is required throughout tumorigenesis. Furthermore, we have demonstrated that eNOS mediates S-nitrosylation of H and NRas, thereby increasing the GTP-bound, activated levels of these proteins. Using shRNA, I have shown that loss of wildtype H and NRas in pancreatic cancer cell lines driven by oncogenic KRas, impairs xenograft tumor growth. Importantly, this tumor phenotype can be rescued by RNAi resistant H or NRas, but not if the proteins carry a mutation that alters the nitrosylation site, suggesting that it is the activation of these proteins by eNOS that is required for tumor growth. Based on these results, we propose a model in which eNOS-mediated nitrosylation of wildtype Ras proteins serves to diversify signal beyond that of oncogenic Ras. These results suggest that eNOS may be a valid anti-cancer target in Ras-driven tumors, perhaps in combination with Ras inhibitors. Therefore, it is of considerable interest to further validate the role of eNOS in a more clinically-relevant model of tumorigenesis, such as the mouse model of spontaneous pancreatic cancer described in Chapter 5.

While pancreatic cancer is the cancer most associated with oncogenic Ras mutations (Bos 1989), and may be a valid target for eNOS anti-cancer therapies, the importance of eNOS may extend to many other cancers. In addition to the many different cancer types associated with oncogenic Ras mutations, several other cancers
are characterized by inappropriate activation of the AKT pathway (Table 1). For example, amplification of AKT, amplification of PI3K, or loss of the tumor suppressor PTEN are common mutations that lead to inappropriate activation of the AKT pathway (Downward 2003). As eNOS is a downstream target of AKT, it may promote tumor growth in these cancers with inappropriate activation of the pathway. To determine the requirement of eNOS in these tumors, it can be knocked down in cancer cell lines with genetic alterations that activate AKT. For example, PTEN expression is commonly lost in human glioblastoma. If loss of eNOS via shRNA results in diminished tumor growth in glioblastoma cancer cell lines with PTEN mutations, these results could be verified utilizing an independent system of tumorigenesis, such as the DMBA/TPA chemical carcinogenesis. In this system, PTEN+/− mice, which display accelerated skin tumorigenesis compared to PTEN+/+ controls (Mao et al. 2004), could be crossed to eNOS−/− mice, and the rate of tumorigenesis compared in PTEN+/−;eNOS+/− versus PTEN+/−;eNOS−/− mice. These experiments may demonstrate an importance for eNOS in additional tumor types, further validating its potential as an anti-cancer target.

In addition to determining the role of eNOS in various cancers driven by inappropriate activation of the AKT pathway, it is also important to investigate the mechanism by which eNOS promotes tumor growth. eNOS-mediated generation of NO results in S-nitrosylation of proteins (Hess et al. 2005), which is a potential signaling mechanism. I have shown in Chapter 4 that eNOS mediates nitrosylation of wildtype
Ras proteins, thereby promoting tumorigenesis. However, eNOS-generated NO may nitrosylate other proteins localized in the vicinity of activated eNOS, thereby altering activation of these proteins and contributing to the tumor-promoting phenotype. This could be accomplished by performing a biotin-switch assay on lysates from cells with an activated AKT pathway expressing an eNOS shRNA or scramble control sequence, and then blotting for specific candidate proteins to determine if nitrosylation levels vary when eNOS is knocked down. Alternatively, blotting for biotin would pick up all nitrosylated proteins, and then proteomics could be used to identify the candidates. To then determine if nitrosylation of such identified proteins are required for tumor growth, they could be knocked down via shRNA and then rescued with RNAi resistant protein engineered to be defective in nitrosylation. Such identification of these proteins would further elucidate the mechanism by which eNOS promotes tumor growth.

In summary, I have demonstrated an important role for eNOS in Ras-mediated tumors, and suggest that eNOS promotes tumor growth via protein nitrosylation. We have specifically demonstrated that eNOS is required for nitrosylation and activation of H and NRas, and these proteins are required throughout pancreatic tumorigenesis. However, the AKT signaling pathway is altered in several types of cancer, and, therefore, eNOS may be required for the growth of multiple different tumor types. Therefore, eNOS may be a valid anti-cancer target in the clinic.
6.3 Targeting Oncogenic-Ras in the Clinic

Multiple models demonstrate that the inhibition of chronic Ras activation greatly impedes the growth of tumors that arise either from activating mutations in Ras or illegitimate activation of cell surface receptors. Translating these observations to the clinic, however, has been difficult. For example, oncogenic activity of Ras is dependent on the posttranslational addition of lipid moieties (Konstantinopoulos et al. 2007), yet small-molecule inhibitors that specifically interfere with farnesyl transferase, the enzyme that adds one such lipid moiety, have failed to counteract oncogenic Ras and thereby provide anti-tumor activity. Moreover, these enzyme inhibitors fail to prevent KRas and NRas membrane targeting, because both of these Ras family members can acquire an alternative membrane-anchoring lipid moiety through the activity of geranylgeranyl transferase (Whyte et al. 1997). Unfortunately, the use of inhibitors against geranylgeranyl transferase or both geranylgeranyl and farnesyl transferases is hampered in the clinic by toxicity (Friday and Adjei 2005).

Given the difficulty of directly inhibiting Ras, the inhibition of signals downstream of Ras has been pursued. The RAF inhibitor BAY 43-9006 has some efficacy in colon, breast, and non-small cell lung carcinomas (Friday and Adjei 2005), and small-molecule inhibitors of MEK1 and MEK2, downstream targets of RAF, have yielded positive results in the clinic in the treatment of renal cell carcinoma (Friday and Adjei 2005). Inhibition of the PI3K pathway by mTOR inhibitors has also yielded limited
results (Friday and Adjei 2005). These data indicate that the concept of targeting Ras downstream signaling components has merit as a means to inhibit the growth of Ras-driven tumors. Therefore, the development of anti-Ras targets are of great therapeutic interest, either by inhibiting molecules that are bioavailable or targeting different components of the Ras signaling pathway.

6.3.1 Cytokines as Targets of Ras Oncogenesis

Because their secretion is induced by oncogenic Ras, cytokines represent an attractive target for the development of therapeutics that might interfere with Ras signaling. As discussed in previous chapters, IL-6 and IL-8, as well as other cytokines, are elevated in the serum of patients diagnosed with cancers characterized by oncogenic Ras mutations (Wigmore et al. 2002; Kozlowski et al. 2003; Pfitzenmaier et al. 2003; Trikha et al. 2003; Benoy et al. 2004; Lehrer et al. 2004; Brennecke et al. 2005; Lokshin et al. 2006; Tas et al. 2006; Lambeck et al. 2007). Moreover, oncogenic Ras–mediated tumorigenesis is significantly abated in the presence of neutralizing antibodies against such cytokines and by shRNA knockdown or ablation of cytokine-encoding genes (Sparmann and Bar-Sagi 2004; Wislez et al. 2006; Yang et al. 2006; Ancrile et al. 2007).

The targeting of cytokines may provide several clinical advantages. The inhibition of cytokines may be tolerated in patients, at least in the case of IL-6, as genetic ablation of IL-6 in mice is not lethal (Kopf et al. 1994). In addition, cytokines are located in the interstitial space are thus much more bioavailable than intracellular targets.
Indeed, antibodies that are already available or readily generated can be used to inhibit cytokine function, and antibody therapy is generally well tolerated in patients (Imai and Takaoka 2006), and preliminary studies already demonstrate anti-tumor activity of neutralizing antibodies against IL-8 and IL-6 (Sparmann and Bar-Sagi 2004; Ancrile et al. 2007).

Humanized antibodies against cytokines like IL-8, GRO-1, and IL-6 may have utility in the treatment of Ras-driven cancers. Indeed, inhibition of proteins by humanized antibodies has proven successful in the clinic, as exemplified by bevacizumab (Avastin®) as well as a variety of other anti-VEGF drugs (Ho and Kuo 2007). Several completed and ongoing clinical trials have investigated the ability of monoclonal antibodies to inhibit IL-6 and thereby function as cancer therapeutics. Multiple myeloma in particular depends upon IL-6 and has been the subject of relevant antibody-based therapy (Trikha et al. 2003), and additional trials are focusing on the treatment of prostate cancer with anti-IL-6 monoclonal antibody (Health 2007; Health 2007; Health 2007). The discovery that oncogenic Ras increases the secretion of cytokines that are important for tumor growth will indeed provide impetus for a number of strategies to target a central pathway that is activated in the vast majority of cancers.
6.3.2 Targeting the PI3K-AKT-eNOS-(wildtype) Ras Pathway

The requirement of oncogenic Ras is reduced to activation of the PI3K/AKT pathway during tumor maintenance (Lim and Counter 2005). Tumor maintenance may be the most clinically relevant stage of tumorigenesis, as the majority of patients are diagnosed and treated following the initiation and establishment of a tumor. Therefore, the PI3K/AKT pathway may be an important anti-cancer target.

The data in Chapter 4 of this thesis demonstrates that blocking phosphorylation of eNOS, a substrate of AKT, inhibits both tumor initiation and maintenance. Furthermore, the nitrosylation of wildtype Ras proteins by eNOS is required for tumorigenesis, most likely due to diversification of the Ras signal. Importantly, eNOS knockout mice are viable and fertile (Shesely et al. 1996), suggesting that inhibition of the protein would be tolerated in patients. Therefore, eNOS may be a clinically relevant target for Ras-driven cancers such as pancreatic, perhaps in combination with Ras inhibitors.

Several aspects of NOS catalysis represent valid inhibitory targets. For instance, NOS enzymes only function as dimers, and must bind to several cofactors (Vallance and Leiper 2002; Dudzinski et al. 2006). NOS activity is mediated by the binding of calmodulin, and, in the case of eNOS, the binding of calmodulin is dependent upon calcium (Vallance and Leiper 2002). Generation of NO by NOS requires substrates, such as oxygen, NADPH, and L-arginine (Vallance 2003). Post-translational modifications,
such as activation of eNOS by phosphorylation by AKT at S1177, and protein-protein interactions, such as the inhibitory binding of caveolin-1 to eNOS, also regulate NOS activity (Vallance and Leiper 2002). Any of these cofactors, substrates, or modifiers represent valid drug targets for NOS inhibition, as do substrate analogues and dimerization inhibitors. There are several NOS inhibitors currently available, some of which have been used in clinical trials, although inhibitors with a higher degree of isoform specificity are desired (Vallance and Leiper 2002). An eNOS-specific inhibitor may have fewer deleterious side effects than a general NOS inhibitor, as the three NOS isoforms perform physiologically distinct roles in the body. This is evidenced by genetic knockouts of each of the three enzymes in mice, which lead to very different phenotypes (Table 2).

Preclinical animal models using NOS inhibitors also provide evidence that a specific eNOS inhibitor may be more beneficial than a general NOS inhibitor. For example, iNOS-induced production of NO is important for host immune defense (Bogdan 2001), evidenced by the fact that iNOS knockout mice display an increased susceptibility to pathogens (Table 2 and (MacMicking et al. 1995; Wei et al. 1995)). Treating mice with latent tuberculosis infection with L-NIL, the partially-specific iNOS inhibitor, resulted in at 50% mortality rate following 30 days of treatment, compared to no mortality in untreated control mice (MacMicking et al. 1997). Similarly, inhibition of
Table 2: Some phenotypic features of NOS knockout mice

<table>
<thead>
<tr>
<th>Knockout mouse</th>
<th>Phenotype</th>
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| eNos<sup>−/−</sup> | Elevated systemic and pulmonary blood pressure  
Increased neointimal thickening after vessel injury  
Aneurysm formation  
Increased leukocyte adhesion  
Increased cardiac inotropic response  
Increased tissue damage in experimental models of stroke and global cerebral ischaemia  
Reduced neurotransmitter release |
| nNos<sup>−/−</sup> | Reduced tissue damage in experimental models of stroke and global cerebral ischaemia  
Reduced neurotransmitter release  
Altered airway responsiveness  
Increased leukocyte adhesion  
Increased stomach size (pyloric stenosis)  
Gastroparesis, abnormal IJP and NANC relaxation in GI smooth muscle  
Urinary bladder urethral sphincter dysfunction  
Hypogonadism and infertility |
| iNos<sup>−/−</sup> | Increased susceptibility to pathogens  
Resistance to sepsis-induced hypotension  
Increased susceptibility to tumours  
Impaired osteoclastic bone resorption  
Impaired wound healing  
Increased tissue damage in models of lung injury |

eNos, endothelial nitric oxide synthase; GI, gastrointestinal; IJP, inhibitory-junction potential; iNos, inducible nitric oxide synthase; NANC, non-adrenergic, non-cholinergic; nNos, neuronal nitric oxide synthase (Vallance and Leiper 2002). Used with permission of the publisher.
nNOS, which is highly expressed in the central nervous system (CNS) (Vallance and Leiper 2002), has led to altered animal behavior in mouse models. Treating mice with 7-NI, a compound which is partially specific for nNOS, acted as an anti-depressant and also led to increased aggression in mice (Demas et al. 1997; Trainor et al. 2007). These findings suggest that an eNOS-specific NOS inhibitor may have fewer side effects than a general NOS inhibitor.

Several clinical trials have been performed using NOS inhibitors, primarily in patients with cardiovascular compromise. Following promising results from a Phase II clinical trial (Grover et al. 1999), a Phase III trial was conducted using the general NOS inhibitor NMA to treat hypotension in patients with septic shock (Lopez et al. 2004). However, an increase in mortality in the NMA treatment group caused the trial to be stopped prematurely. A Phase III clinical trial using the nonspecific NOS inhibitor L-NAME to treat patients with cardiogenic shock found that the drug effectively increased systolic blood pressure, but did not alter mortality rates at 30 days (Alexander et al. 2007).

While most clinical trials using NOS inhibitors have focused on cardiovascular pathology, a limited number of trials have investigated the use of these drugs in other diseases. A Phase I trial examined the ability of L-NNA to alter tumor blood flow in patients with non-small cell lung cancer, prostate cancer, and cervical cancer (Ng et al. 2007). Following a single dose of L-NNA, 8 of 18 patients displayed decreased tumor
blood flow for up to 24 hours after treatment. Side effects were limited, with three patients exhibiting grade I hypertension, two patients with grade I sinus bradycardia, and one patient with grade I palpitations.

While NOS inhibitors hold potential as drug treatment options, specific enzyme inhibitors would be advantageous. One potential inhibitor of eNOS function is cavtratin, a cell-permeable peptide containing the eNOS binding domain of caveolin-1 (Bucci et al. 2000). Gratton et al. (Gratton et al. 2003) demonstrated that administration of this peptide, which selectively inhibits eNOS, reduced tumor growth and increased apoptosis and necrosis of tumors generated by the subcutaneous injection of lung carcinoma or hepatocarcinoma cells into the flanks of mice. Although peptides generally are not good drug candidates, this study suggests that specific inhibition of eNOS may hold promise in the treatment of tumors.
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Biography

Brooke B. Ancrile

BORN: January 1, 1982 in Jackson, MI

EDUCATION

Duke University
Ph.D. in Genetics and Genomics
Durham, NC

Michigan State University
B.S. in Zoology; Certificate in Genetics (GPA: 3.9)
East Lansing, MI
August 2000 – May 2004

PUBLICATIONS

Lim, K.H.*, Ancrile, B.B.*, Kashatus, D.F.* (*these authors contributed equally to this work), and Counter, C.M (2008). Tumour maintenance is mediated by eNOS. *Nature*. 452:646-649.


HONORS AND AWARDS

Breast Cancer Research Program Predoctoral Award, US Department of Defense, 2007-present
2007 Young Investigator Award, Duke Comprehensive Cancer Center
2008 Young Investigator Award, Duke Comprehensive Cancer Center Presenter: 2007 Duke Comprehensive Cancer Center Annual Meeting Presenter: 2008 Duke Comprehensive Cancer Center Annual Meeting
$5000 Citizen’s Advisory Council Research Award, 2008