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By

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ABSTRACT

RAC1 AND CDC42 ARE REQUIRED IN HRAS^{V12}-TANSFORMATION OF HUMAN FIBROBLASTS AND IN VEGF AND UPA EXPRESSION

By

DANIEL M. APPLEDORN

To determine whether the activity of Rac1, Cdc42, or both proteins is required to mediate HRas^{V12}-induced malignant transformation of human fibroblasts, and to identify any Rac1- and Cdc42-regulated genes whose expression plays a role in such transformation, we inhibited Rac1 or Cdc42 activity, or the activities of both proteins, by expressing dominant-negative Rac1^{N17} and/or Cdc42^{N17} in an HRas^{V12}-malignantly transformed human fibroblast cell line, PH3MT. Inhibition of Rac1 significantly suppressed tumor formation. The results of experiments designed to inhibit expression of Cdc42 were not as consistent. Nevertheless, in every instance when tumors formed, analysis of the cells from the tumors revealed that dominant-negative Rac1^{N17} and Cdc42^{N17} were no longer expressed. These results indicate that for HRas^{V12}-induced malignant transformation of these human fibroblasts, Rac1 and Cdc42 activity is required. We also demonstrated that expression of constitutively-active Rac1^{V12} or Cdc42^{V12}, in the absence of HRas^{V12}, failed to malignantly transform the parental infinite life span cell strain, MSU-1.1, from which the PH3MT cell line was derived. These results indicate that activation of parallel HRas^{V12}-induced pathways is required to induce malignant transformation. To identify genes whose expression is controlled by Rac1 and/or Cdc42, we carried out microarray

analysis. Fourteen of the 29 genes that we identified, such as *uPA* and *VEGF*, have a known role in the development of cancer. Using ELISA assays to determine if inhibition of Rac1 alone, Cdc42 alone, or both proteins results in decreased levels of secreted uPA or VEGF proteins, we found that in the $HRas^{V12}$-transformed human fibroblast cell line, PH3MT, Rac1 and Cdc42 independently regulate secreted levels of uPA and VEGF under non-hypoxic and hypoxic conditions. We also found that expression of $Cdc42^{V12}$, but not $Rac1^{V12}$ was able to induce high levels of secreted VEGF protein in the MSU-1.1 parental cell strain. Furthermore, our results suggest that *Sprouty-2*, whose expression is up-regulated upon $HRas^{V12}$-induced transformation, mediates oncogenic Ras-induced uPA expression, VEGF expression, and anchorage independent growth, by regulating the activity of Rac1.

Dedicated to my father

Robert Appledorn
October 6, 1942 – December 29, 2004

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all, I thank my wife, Annalee. She is strong, inspiring, conscientious, supportive, and beautiful. But most importantly, she loves me.

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SUMMARY

ABBREVIATIONS

AKT	protein kinase B
ALT	Alternative Lengthening of Telomeres
AP	Activator Protein
APC	Adenomatous Polyposis Coli
BAD	Bcl-2/Bcl-X_L-Antagonist, causing cell Death
BPDE	Benzo-A-Pyrene-Diol-Epoxide
CDK	Cyclin Dependent Kinase
C/EBPβ	CCAAT-Enhancer Binding Protein-Beta
CRD	Cysteine-Rich Domain
DH	Dbl Homology
ECM	ExtraCellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EIF	Elongation Factor
ELISA	Enzyme-Linked Immunosorbent Assay
ELK	ETS-Like
ENU	EthylNitrosoUrea
ERK	Extracellular signal-Regulated Kinase
FAP	Familial Adenomatous Polyposis
FGF	Fibroblast Growth Factor
GAP	GTPase Activating Protein
GDP	Guanine Diphosphate

GDS	Guanine Dissociation Stimulator
GEF	Guanine nucleotide Exchange Factor
GFP	Green Fluorescent Protein
GSK3	Glycogen Synthase Kinase-3
GTP	Guanine Triphosphate
HGF/SF	Hepatocyte Growth Factor/Scatter Factor
HIF	Hypoxia Inducible Factor
HNPCC	Hereditary NonPolyposis Colorectal Cancer
HRE	Hypoxia Response Element
HSC	Hematopoietic Stem Cell
IGF	Insulin Growth Factor
IL	InterLeukin
IPTG	IsoPropylThio-β-d-Galactoside
JNK	c-Jun N-terminal Kinase
LMW-PTP	Low Molecular Weight-Protein Tyrosine Phosphatase
LPA	LysoPhosphatidic Acid
LPS	LipoPolySaccharide
MAPK	Mitogen Activated Protein Kinase
MDM2	Murine Double Mutant-2
MEK	MAPK/ERK Kinase
MKK3	Mitogen-Activated Protein Kinase Kinase-3
MMP	Matrix Metalloproteinase
MMR	MisMatch Repair

MNU	MethylNitrosoUrea
MSU	Michigan State University
NF1	NeuroFibromatosis-1
NFκB	Nuclear Factor kappa B
NSAID	NonSteroidal Anti-Inflammatory
ODDD	Oxygen Dependent Degradation Domain
PAI	Plasminogen Activator Inhibitor
PAK	P21-Activated serine/threonine Kinase
PDGF	Platelet Derived Growth Factor
PEA3	Polyoma Enhancer Activator-3
PH	Pleckstrin Homology
PHD	Prolyl HyDroxylase
PI3K	Phosphatidyl Inositol 3-Kinase
PIP	Phosphatidyl Inositol Phosphate
PKC	Protein Kinase C
PLD	PhosphoLipase D
PTEN	Phosphatase and TENsin homolog deleted on chrom. 10
Rb	Retinoblastoma
RBD	Ras Binding Domain
RFLP	Restriction Fragment Length Polymorphism
ROS	Reactive Oxygen Species
RTK	Receptor Tyrosine Kinase
SAPK	Stress Activated Protein Kinase

SH	Src Homology
SOS	Son Of Sevenless
STAT	Signal Transducer and Activator of Transcription
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor
UPA	Urokinase Plasminogen Activator
UPAR	Urokinase Plasminogen Activator Receptor
UTR	UnTranslated Region
UV	Ultra-Violet
VEGF	Vascular Endothelial Growth Factor
VHL	VonHippel-Lindau
WASP	Wiskott-Aldrich Syndrome Protein

INTRODUCTION

It is commonly accepted that most human cancers arise from a single cell, which over a period of time acquires the necessary mutations that confer on the cells the ability to form a tumor. In the first section of chapter one, I present evidence that supports the multistep hypothesis of cancer development. I also describe an isogenic human fibroblast cell lineage, developed by the Carcinogenesis Laboratory, where each successive clonal population of cells has acquired genetic changes required for malignancy. Using this model system, I have focused my efforts on identifying what these changes are.

In the second section of chapter one, I use a review published in 2000 by Hanahan and Weinberg (1) as a guide to provide a broad overview of common characteristics of a cancer cell. These characteristics include a cell's ability to: 1) provide self-sufficient mitogenic signals, 2) evade anti-proliferative signals and apoptosis, 3) replicate limitlessly, 4) sustain angiogenesis, and 5) invade surrounding tissues and metastasize.

Genes involved in the development of cancer can be separated into two categories. The first class consists of tumor suppressor genes. Mutation of both wild-type alleles is required to completely eliminate tumor suppressive function. The tumor suppressor gene p53 is the most commonly mutated gene in all tumor types (2).

The second class consists of oncogenes which act in a dominant fashion to facilitate the transformation from normal cells to tumor cells. Ras is a proto-oncogene that when mutated in specific codons, can lead to malignant

transformation of both mouse and human fibroblast cell strains in culture (3, 4). In the third section of chapter one, I discuss both the normal and pathological functions of Ras. Ras regulates multiple effector pathways that are involved in malignant transformation, including the Raf-MEK-ERK1/2 signaling cascade, the PI3K/Akt survival pathway, and the RalGDS signaling sequence. The importance of each of these pathways in Ras-transformation is dependent on the cell type and species from which it is derived. For example, $HRas^{V12}$-induced transformation of rodent fibroblasts depends heavily upon Raf-MEK-ERK1/2 signaling, whereas recent evidence suggests that transformation of human fibroblast cell strains may require a different combination of Ras-induced effectors (5).

In the last section of chapter one, I describe the cellular functions of Rho-GTPases Rac1 and Cdc42. These two Ras-homologous G-proteins regulate cytoskeletal organization, cell motility, and signaling networks (6). Their activity is required to mediate the malignant transformation of rodent fibroblasts induced by the expression of oncogenic HRas (7, 8). Effectors regulated by Rac1 and Cdc42 include PAK, JNK, and SAPK/p38 MAPKs. However, it is not known whether the activity of Rac1 and Cdc42 are required to mediate $HRas^{V12}$-induced transformation of human fibroblasts. Furthermore, there is little known about the expression of Ras^{V12}-induced genes that require the activity of Rac1 and Cdc42, and that may play a significant role in $HRas^{V12}$-mediated transformation.

Chapter two consists of a manuscript that will be submitted to the AACR journal *Cancer Research*. Dr. Kim-Hien T. Dao, a previous graduate student in

the Carcinogenesis Laboratory, derived a $HRas^{V12}$-transformed cell strain that expresses dominant-negative $Rac1^{N17}$ and/or $Cdc42^{N17}$ in order to inhibit the activities of Rac1 and Cdc42 proteins. I used these cells to carry out experiments designed to determine the importance of these proteins in malignant transformation. Our data are consistent with studies conducted in rodent fibroblasts, which indicate that activities of both Rac1 and Cdc42 are required to mediate $HRas^{V12}$-induced transformation. I found that expression of constitutively-active $Rac1^{V12}$ protein alone, or $Cdc42^{V12}$ protein alone results in transformation, but does not cause the cells to be malignantly transformed, i.e. form sarcomas when injected into athymic mice. Using Affymetrix GeneChip technology, I identified 29 genes whose expression was significantly changed ($p<0.001$) upon Rac1 and Cdc42 inhibition in the $HRas^{V12}$-transformed human fibroblast cell strain PH3MT. Of these genes, fourteen have been reported to have a role in cancer development. Using ELISA analyses, I verified that secreted levels of both uPA and VEGF are regulated by both Rac1 and Cdc42 through independent signaling pathways. Moreover, I found that Rac1 and Cdc42 regulate levels of secreted VEGF in non-hypoxic and hypoxic conditions. In summary, the data presented in chapter two supports a role for Rac1 and Cdc42 in $HRas^{V12}$-induced transformation of human fibroblasts, and identifies genes that may mediate this process. These genes are potential targets for pharmaceutical intervention in the treatment of cancer patients.

Chapter three consists of data collected in collaboration with Dr. Piro Lito, who recently defended his dissertation. Dr. Lito identified an essential role for

Sprouty-2 (*Spry2*) in $HRas^{V12}$-induced transformation of human fibroblasts (9). Using shRNA to reduce Spry2 expression in PH3MT cells, he observed that reduction of Spry2 resulted in cells, that no longer have the ability to form large colonies in agarose, and that fail to form tumors in athymic mice. He also found that reduction of Spry2 resulted in decreased Rac1 activity (10). For this reason, I expressed constitutively-active $Rac1^{V12}$ in these cells to determine if its expression could recover the malignantly transformed phenotype. My data indicate that Spry2 regulates $HRas^{V12}$-induced anchorage independent growth by regulating the activity of Rac1, but multiple Spry2 regulated pathways are required to mediate malignant transformation induced by $HRas^{V12}$ expression. Furthermore, my data indicate that Spry2 regulates the expression of both uPA and VEGF in $HRas^{V12}$-transformed fibroblasts.

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CHAPTER I

LITERATURE REVIEW

I. Cancer is a Genetic and Epigenetic Disorder

The most recent statistics collected by the American Cancer Society indicate that there will be 1.4 million new cases of cancer, and over 560,000 cancer related deaths in 2006 (1). The 5-year relative survival rate of patients diagnosed with cancer between 1995 and 2001 is 65%, up from 50% in 1974-1976. Advancements in research and technology have lead to earlier diagnoses, better treatments, and improved prognoses.

It is now accepted that cancer is, in essence, a genetic disorder. However there are two critical differences between cancer and most other genetic disorders. First, in order for cancer to occur, somatic mutations must occur. In contrast, most other genetic disorders are caused by germ-line mutations. Second, cancers are not caused by a single mutation, but from the accumulation of mutations that confer the phenotypic changes necessary for cancer formation.

In addition to mutations, epigenetic events, such as methylation and acetylation can also affect gene expression (2). Methylation of important tumor related gene loci may prevent the expression of genes that prevent aberrant growth of cells (3). CpG palindromic sequences, called CpG islands, are found within promoter regions of various genes. Within CpG islands, cytosines are targets of methylation. Methylation can inhibit transcription by allowing increased binding of methylation-dependent sequence-specific DNA binding proteins, such

as MDBP, that can repress transcriptional activity. Methylation can also interfere with DNA-binding transcription factors (2). Important cancer related gene promoters that contain CpG islands include p53, VHL, and APC and will be discussed in detail below (4-6).

A. The Multistep Process of Carcinogenesis

The correlation between cancer incidence and age is the most profound piece of evidence that suggests cancer is a multistep process (7). As reviewed by Vogelstein and Kinzler (8), the cancer incidence rate increases 10^3 - 10^7 fold when plotted against age. This indicates the necessity for multiple, at least 3 – 7 "hits", or mutations in a single cell are necessary to develop into a malignancy. These mutations are located in proto-oncogenes or tumor suppressor genes that provide "gain-of-function", or "loss-of-function" phenotypes respectively. Consequently, these mutations often result in cell immortality, increased cell proliferation, evasion of apoptotic signals, the ability to induce angiogenesis, and the ability to invade and metastasize surrounding tissue. In this way, cancer is considered a microevolutionary process occurring within an organism, where each mutation may provide a selective advantage over previous populations and results in malignancy.

The evolution of cancer continues past primary tumor formation. For example, cells of a malignant tumor typically continue to acquire mutations that culminate in a heterogeneous tumor cell population. This phenomenon complicates the clinical aspects of treatment. For instance, radiation therapy

effectively kills the majority of tumor cells of various cancer types. However, a small population of radio-resistant cells is commonly not eliminated (8). These cells proliferate and form a population of cells that must be attacked using alternate modalities.

Epidemiological analyses support the multistep hypothesis of carcinogensis. Patients exposed to radiation, either in cases of X-ray therapy to treat tuberculosis or breast cancers, often develop cancers. However, their cancers are not manifest until 15 – 30 years after exposure. Likewise, it has long been known that tobacco and smoking can cause cancer of the mouth and/or lung. However, decades of heavy smoking are required for these individuals to develop lung cancers, and many never do. The lag in time from carcinogen exposure to cancer development suggests that multiple mutations, in specific cancer related genes, are required for cancer formation (9).

Most colon cancers develop sporadically. However, studies of inherited versions of colon cancers have provided evidence for the multi-step nature of cancer formation. Individuals that inherit either Familial Adenomatous Polyposis (FAP), or Hereditary Nonpolyposis Colorectal Cancer (HNPCC) have a predisposition to colorectal tumor formation (10). The progression of cancer development in these individuals is marked by distinct morphological changes and can be used as a model system for studying tumor formation.

1. Familial Adenomatous Polyposis

FAP is an autosomal dominant disease that affects 1 in 7000 individuals. These individuals develop thousands of benign colorectal tumors, called

adenomatous polyps, during their 20s and 30s. This large number of lesions virtually guarantees that one such lesion will accrue the necessary changes to become an adenocarcinoma, a malignant tumor. Using positional cloning techniques, it was found that mutation of the adenomatous polyposis coli (APC) gene results in this disease (11, 12). Although individuals with FAP are born with one mutant APC allele, it takes decades to develop a malignant colorectal tumor. Therefore, it is assumed that the rate-limiting step in the development of a tumor is somatic mutation of the wild type allele (13, 14). This is an example of a "two hit" system, where biallelic mutation of one gene predisposes an individual to tumor formation.

By way of its interaction with β-catenin, APC regulates two critical pathways in the development of cancer (15). First, there is a direct interaction between β-catenin and cadherins, a family of adhesion molecules determined to be involved in tumor invasion (16). Second, β-catenin plays a critical role in the Wnt signal transduction pathway (17). Wnt signaling regulates many cancer related processes including proliferation, cell motility, and apoptosis.

2. Hereditary Nonpolyposis Colorectal Cancer

HNPCC is also a hereditary syndrome that results in colorectal tumor formation. HNPCC derived tumors account for 2% of the total incidence of colorectal cancers. However, the study of colon cancer development in individuals with HNPCC also provides insight into the mechanism of sporadic colon cancer development. Strand et al. (18) reported that microsatellite instability observed in tumors of HNPCC patients was similar to that observed in

bacteria with mutations in mismatch repair (MMR) genes. Therefore, they hypothesized that patients with HNPCC also had mutations in genes necessary for efficient mismatch repair. Several laboratories found that mutations in one of three MMR genes are found in HNPCC kindreds. These genes include hMSH2, hMLH1, and hPMS2 (19, 20). The loss of these MMR proteins results in cells with a mutation rate two to three times higher than the rate in normal cells (21, 22). For this reason, patients that inherit mutant MMR genes have a higher incidence of colorectal tumors. (23). These data indicate that acquisition of multiple mutations are required to transform a normal cell into a tumor cell, and that increased mutation rate results in a higher rate of tumor formation.

B. The MSU1 Lineage as a Model System for the Multistep Process of Carcinogenesis

Vogelstein and Kinzler (8) estimate that at least 7 genetic changes must occur to derive malignant cells from normal cells. Identification of these genetic changes provides potential therapeutic targets. To identify genetic changes that occur in the progression from normal cells to tumor cells, McCormick, Maher and colleagues have developed the MSU1 lineage of cell strains (Fig. 1). The MSU1 lineage is a family of human fibroblast cell strains, derived clonally, one-from-another, with each new strain exhibiting more transformed characteristics. Most studies that attempt to identify cancer related genetic changes do not compare cancer cells to their immediate precursor cells. In contrast, the MSU1 lineage of cells provides a unique opportunity to study an isogenic cell system, where each successive clonally derived strain is one step closer to becoming a cancer.

Figure 1. The MSU1 fibroblast lineage was developed in the Carcinogenesis Laboratory by McCormick, Maher and colleagues in order to identify genetic changes that occur from a normal cell to a tumor cell. This diagram provides a description of this lineage. Each triangle represents the expansion of a clone derived from the previous population. Arrows indicate significant changes or events that resulted in the development of the subsequent population. The original fibroblast cell line, designated LG1, was derived from the foreskin of a normal human neonate. This cell line was transfected with the *v-Myc* oncogene, indicated by the first arrow in the diagram. The transfected cell strains were cultured to the end of their lifespan when most entered senescence and eventually died. At this time, one cell strain designated MSU-1.0, maintained replicating potential and was expanded. Characterization of this immortalized cell strain indicated activation of telomerase and increased Sp1 expression. While culturing MSU-1.0 cells, a highly proliferating, clonal population of cells overgrew the culture. These cells were isolated and designated MSU-1.1. Genomic characterization revealed two marker chromosomes. Whereas MSU-1.0 cells can not be transformed, MSU-1.1 cells are able to be transformed using multiple strategies. Transfection of the $HRas^{V12}$ oncogene into the MSU-1.1 cell strain resulted in the ability for these cells to form foci. Cells from one focus were isolated and injected subcutaneously into athymic mice. Injection of these cells resulted in sarcoma formation with a short latency. The PH3MT cell line was derived from one such tumor.

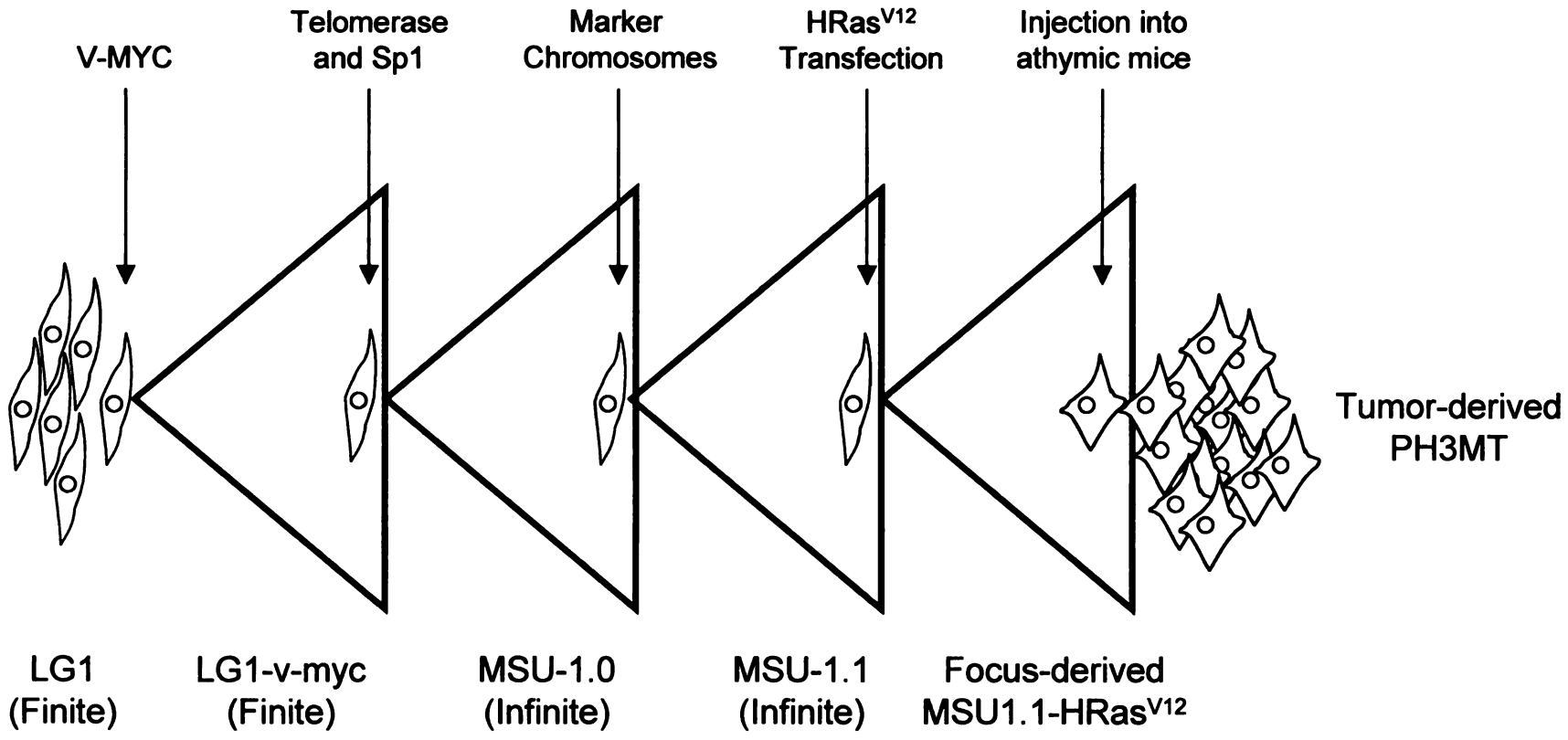
V-MYC
Telomerase and Sp1
Marker Chromosomes
HRas^{V12} Transfection
Injection into athymic mice
Tumor-derived PH3MT
LG1 (Finite)
LG1-v-myc (Finite)
MSU-1.0 (Infinite)
MSU-1.1 (Infinite)
Focus-derived MSU1.1-HRas^{V12}

Figure 1

The cell strain that marks the starting point is called LG1. LG1 was derived from the foreskin of a normal human neonate. Data reported by Land et al. (24), and Schwab and Bishop (25), indicated that in rat embryo fibroblasts, expression of various Myc isoforms caused immortalization. In an attempt to immortalize human fibroblasts, a member of the Carcinogenesis Laboratory transfected LG1 cells with a vector that contained the *v-Myc* oncogene and a selectable marker. Selected vector control strains, as well as a v-Myc protein expressing strain, were grown to the end of their lifespan and they senesced and died. However, a few live cells remained in the v-Myc expressing population and they gave rise to a clonally derived, chromosomally stable, diploid cell strain which was designated MSU-1.0. MSU-1.0 cells were cultured for over 100 doublings and it was clear that they were immortal. Because all but a few v-Myc expressing cells senesced and died, it was assumed that this subpopulation of cells spontaneously acquired a unique genetic change that resulted in immortalization. A closer analysis of MSU-1.0 cells reveals up-regulation of both telomerase and the transcription factor Sp1 (26). Studies in hamster cells indicate that Sp1 can cooperate with Myc in the induction of telomerase expression (27, 28). This suggests that high levels of Myc and Sp1 protein levels in MSU-1.0 cells results in their immortalized phenotype.

A faster growing, clonally derived cell strain arose out of MSU-1.0 cells. This variant cell strain was designated MSU-1.1 (29). Genotypic analyses of this cell strain revealed two chromosomal translocations. These cells consist of 45 chromosomes including two marker chromosomes, M1 and M2, monosomy of

chromosomes 11, 12 and 15, and partial trisomy of chromosome 1. The marker chromosome M1 is a result of the translocation of chromosome 11 (11p15→qter) to chromosome 1 at p11. This translocation not only results in a marker chromosome, but a trisomy of the q arm of chromosome 1. The marker chromosome M2 is a result of the translocation of chromosome 12 (12qter→12q11) to chromosome 15 (15p11→15qter). Southern blot analyses of the *v-Myc* integration site indicate that MSU-1.1 cells were clonally derived from MSU-1.0 cells, and that MSU-1.0 cells were clonally derived from the parental LG1 cells.

Various terms are used to describe cells with cancer-like properties. I will use the term transformation to represent cells that have acquired such properties. The phenotypes usually exhibited by transformed cells include changes in cell morphology, immortality, the ability to proliferate in the absence of growth factors, and to proliferate in an anchorage independent way. Anchorage independence is the ability for cells to proliferate in an environment where they are not attached to a substratum. Experimentally, malignant transformation is defined as having the ability to form a cancer in a suitable animal host.

The MSU-1.1 cell strain exhibits partial growth factor independence, and therefore is assumed to be one step closer to malignant transformation compared to the MSU-1.0 cell strain. However, MSU-1.1 cells do not exhibit other characteristics typical of transformed cells, i.e. anchorage independent growth, or the ability to form tumors. MSU-1.1 cells were transfected with

oncogenes, or exposed to carcinogens to determine if this human fibroblast cell strain could be transformed. MSU-1.1 cells can be transformed into tumorigenic cells by transfection of a highly expressed HRas (30) or NRas (31) oncogene or by a single exposure to BPDE (32), ionizing radiation (33, 34), or MNU (35). Similar attempts to transform MSU-1.0 cells were unsuccessful.

In 1989, Hurlin et al. (30) used a vector containing enhancer regions and the T24 *HRas* oncogene sequence to transfect MSU-1.1 cells. The T24 *HRas* oncogene contains a V12 activating mutation ($HRas^{V12}$) that results in constitutive activation. Cells that formed foci were selected and used to complete further experiments. Many of the cell strains derived from such foci exhibited anchorage independent growth, and formed tumors with a when injected subcutaneously into athymic mice. These tumors were found to be fibrosarcomas. The tumors were isolated and cell lines were derived from them. One such cell line is designated PH3MT. The tumorigenicity of the PH3MT cell line was validated by reinjecting them into athymic mice. Sarcomas formed at all sites with a three to six week latency. We estimate that these malignant cells are the result of approximately five to six genetic changes. Cell strains from the MSU1 lineage can be used to study critical differences between different stages in the process of tumorigenicity. The Carcinogenesis Laboratory is focused on elucidating these important differences.

II. Characteristics of Cancer

In 2000, Hanahan and Weinberg (36) described specific phenotypic changes typically involved in cancer. They include: 1) self-sufficiency in growth signaling, 2) evading apoptosis and insensitivity to antigrowth signals, 3) limitless replicative potential, 4) sustained angiogenesis, and 5) tissue invasion and metastasis.

A. Self-Sufficiency in Growth Signaling

Cell proliferation requires initiation of mitogenic signal-transduction pathways. These pathways typically commence with the binding of growth factors such as PDGF, or TGF-α to their appropriate receptor (37-39). Often, these growth factors are secreted by neighboring cells that bind to their appropriate receptors on target cells. To illustrate this, Fukumura et al. (40) implanted solid tumors in transgenic mice, whose cells contain a vector carrying a green fluorescent protein (GFP) under the control of the vascular endothelial growth factor (VEGF) promoter. Shortly after implantation, induction of VEGF promoter activity was observed in cells of the stroma. This suggested that cells within the tumor were secreting proteins that interacted with cells in the tumor microenvironment. This indicates that cells making up this environment are key regulators of growth stimulatory pathways *in vivo*.

Stimulation of growth factor signal-transduction pathways can also be initiated by an interaction between integrins and the extracellular matrix (ECM)

(41, 42). For example, integrin interactions can stimulate the SOS-Grb2-Ras-Raf-MEK-ERK1/2 pathway (43).

Most cancer cells, however, do not depend on exogenous growth factor secretion. They provide their own mitogenic growth signals. Genes that mediate this effect are referred to as oncogenes. Oncogenes are gain-of-function mutant forms of normal genes (proto-oncogenes) that regulate cell proliferation. Mutations in such genes can result in aberrant cell proliferation. Ras is an example of a proto-oncogene that normally regulates cell proliferation and apoptosis. Activating mutations of Ras, most notably the point mutations V12 and L61 (discussed in greater detail below) result in continuous growth signals independent of associated receptor activity. Mutations in Ras genes are found in approximately 30% of human tumors (44, 45). The highest incidence is found in colon carcinomas and pancreatic cancers. Constitutive Ras activity regulates almost all aspects of tumor biology including stimulation of growth factor pathways, apoptosis, tissue invasion, metastasis and angiogenesis (46).

The best characterized Ras regulated pathway is the Raf mediated MAPK pathway, in which Raf binds directly to activated Ras through two distinct sites. Once anchored in the membrane, Raf acts as a kinase to phosphorylate and activate MEK ,which activates ERK, which then activates numerous transcription factors such as ELK-1 (47). An in depth discussion of the molecular structure and function of Ras is to follow.

Changes in the tissue environment can also stimulate growth factor pathways and proliferation. Chronic inflammation is commonly associated with

cell proliferation and various types of cancer (48-50). In light of this, nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, have been shown to reduce the risk for developing colon cancer by 40-50% (51). Most NSAIDs inhibit cyclooxygenases-1 and -2, Cox-1 and Cox-2 respectively. Cyclooxygenases convert arachadonic acid into inflammatory mediators called prostaglandins (52). Cox-1 is ubiquitously expressed. Cox-2 expression is inducible by growth factors and oncogenes (52, 53). High levels of Cox-2 expression are found in common cancers such as colon, prostate, breast, pancreas, non-small-cell lung, bladder, endometrium and basal and squamous cell skin carcinomas (54-57). Tumor relevant processes associated with elevated Cox-2 levels include increased cell survival, invasion, generation of mutagenic reactive oxygen species (ROS), and angiogenesis (58). Specifically, Cox-2 expression stimulates growth stimulatory, and angiogenic factors bFGF, PDGF and VEGF (59).

Cox-2 expression is regulated by oncogenes, which elicit a mitogenic response. In 1998, Sheng et al. (60) showed that $HRas^{V12}$-mediated transformation of rat-1 fibroblasts induces Cox-2 expression. Briefly, *$HRas^{V12}$* cDNA was transfected into rat-1 cells under the control of an IPTG inducible Lac operon. They found that Cox-2 mRNA and protein expression were greatly increased in parallel with the induction of $HRas^{V12}$ expression. They found that this effect is mediated by both ERK1/2 activity and Cox-2 mRNA stability (60). Whereas $HRas^{V12}$-mediated transformation induces Cox-2 expression, research conducted by Zhang et al. (61) using mouse fibroblasts derived from cyclooxygenase deficient embryos, indicates that Cox-2 expression is

dispensable for $HRas^{V12}$ mediated transformation. However, the ability for these cells to form tumors was not investigated. These data support the hypothesis that Cox-2 expression is important in malignant transformation.

Phosphorylation of C/EBPβ and/or the transcription factor PEA3 by ERK1/2 is required for HRas induced Cox-2 up-regulation (62, 63). The activity of these transcription factors can be regulated by the Ras homologous G-proteins Rac1 and Cdc42. Downstream of Ras, Rac1 and Cdc42 can also regulate Cox-2 expression by inducing NFκB transcriptional activity (64). Rac1 and Cdc42 GEFs Dbl, Ost and Vav also induce NFκB activity (65). Rac1 also regulates the Cox-2 promoter through a mechanism independent of NFκB. To illustrate this, Slice et al. (66) transfected NIH3T3 mouse fibroblasts with a reporter gene construct containing the Cox-2 promoter which does not contain κB elements. They observed that Cox-2 transcription was mediated by activated Rac1, but not activated Cdc42.

Cox-2 protein levels are also regulated postranscriptionaly. The stability of Cox-2 mRNA is increased by expression of activated KRas in intestinal epithelial cells. The 3' UTR of Cox-2 mRNA contains adenine and uridine rich sequence elements that are targets of a p38 mediated stability pathway (67). This same adenosine/uridine-rich sequence is also found in the 3' UTR of both VEGF and uPA, discussed in detail below (68, 69).

Self-sufficiency in growth signaling is an important acquired characteristic in the progression towards cancer development. However, antigrowth signals and programmed cell death pathways, in addition to their normal function, serve

as checkpoints that prevent highly proliferating cells from developing into a malignancy. For this reason, cells must also acquire the ability to evade antigrowth signals and apoptosis in order to form a tumor.

B. Evasion of Antigrowth Signals and Apoptosis

Apoptotic programming and antigrowth signals are present in nearly all cell types throughout the body. Once the apoptotic program is initiated, a sequence of events occurs in which cell membranes are disrupted, the cytoskeleton is broken down, and DNA is fragmented.

Mutations in genes that regulate apoptosis can have deleterious effects. Inactivating or activating mutations may disrupt normal programmed cell death. For example, mutation of the p53 gene abolishes its pro-apoptotic activities and increases the propensity for a cell to develop into cancer. For this reason, p53 is considered a tumor suppressor gene, as wild type expression helps to prevent aberrant cell growth.

Tumor suppressor genes are generally recessive. Expression of wild-type protein from one allele is usually sufficient to prevent tumor susceptibility. Therefore, mutation of both alleles is required to disrupt tumor suppressor activity. Somatic mutation of the second allele, referred to as "Loss-of-Heterozygosity" (LOH), results in an increased susceptibility for that cell to form cancer. However, in cases of haploinsufficiency, loss of a single allele may result in loss of tumor suppressor activity (70, 71).

Pro-apoptotic and antigrowth signaling genes are often categorized as tumor suppressor genes and are commonly mutated or otherwise inactivated in

cancers. Ligands and receptors that are known to initiate apoptotic pathways include: IGF-1 and IGF-2, ligands that bind to the receptor IGF-1R; IL-3 and its receptor IL-3R; FAS ligand that binds to the FAS receptor; and TNF-α that binds to TNF-R1 (72-74).

Sensors that monitor the extracellular- and intracellular-environments can also trigger apoptotic pathways. For example, prolyl-hydroxylases (PHDs) serve as intracellular-sensors that monitor oxygen tension. In an environment with physiologically normal oxygen tension, PHDs hydroxylate proline residues found in the oxygen dependent degradation domain (ODDD) of hypoxia inducible factors (HIFs). Proline modification targets HIFs for proteolytic degradation mediated by the E3 ubiquitin ligase von-Hippel Lindau protein (pVHL). In the absence of oxygen, PHDs are inactive, HIFs are not targeted for degradation, and protein levels increase. Activated HIFs regulate numerous pathways including pro-apoptotic machinery (75). Therefore, disruption of this pathway can lead to deregulation of the apoptotic program. For example, pVHL is mutated in a high percentage of renal cell carcinomas and is considered a tumor suppressor gene.

Once an apoptotic signal has been initiated, effectors carry out the apoptotic program to completion. For example, p53 is a tetrameric transcription factor responsible for initiating transcription of over 150 different genes. Most of which impact the cell cycle. Levels of p53 are tightly regulated in the cell. The p53 protein has a short half-life, and is quickly ubiquitinated by the E3 ubiquitin ligase MDM2. This keeps levels of p53 low (76). When the cell encounters

stress, such as DNA damage, ubiquitination is suppressed and increased p53 protein levels suppress anti-apoptotic proteins such as Bcl-2 and Bcl-X_L. Levels of p53 accumulate in the nucleus and drive the transcription of apoptotic genes. Bax is one such gene. When Bax expression is induced, mitochondria release cytochrome-c. Cytochrome-c is a critical member of the "apoptosome" that is made up of cytochrome-c, Apaf-1 and procaspase-9. Complex formation activates caspase-9, which processes and activates other caspases to carry out destruction of subcellular structures and fragmentation of the DNA (77, 78). A more detailed description of p53 is given below.

Mutations in two classical tumor suppressor genes, *p53* and *Rb*, are found in various cancers (79). They function in regulating growth, differentiation, and apoptosis. A detailed description of p53 and Rb indicates a critical role for tumor suppressors in cancer development.

1. The Tumor Suppressor Gene *Rb*

Alfred Knudson's characterization of retinoblastoma was the first significant evidence indicating that more than one mutation is required for a cell to develop into a tumor. Pedigree analyses of patients with retinoblastoma indicated a hereditary component. Retinoblastoma is a cancer derived from an embryonal cell in the developing eye. There is an exponential decline in embryonal cells with age. Therefore, all cases of retinoblastoma are diagnosed in early childhood.

Knudson hypothesized that, in familial retinoblastoma patients, one mutated allele was inherited, and the other mutated by random somatic mutation

(80). This would result in the growth of tumors in both eyes (bilateral disease). In cases of nonhereditary disease, Knudson hypothesized that two random somatic mutations were needed, onset would be later, and tumors would develop in only one eye (unilateral disease). Knudson's data indicate that in 23 cases of hereditary (bilateral) retinoblastoma, 50% of cases had been diagnosed by 10 months. In 25 cases of nonhereditary (unilateral) disease, time to diagnosis of 50% of cases was increased three fold, to 30 months. This suggests the necessity of two mutations in cases where one germline-mutation was not inherited. This hypothesis is supported by statistical analyses comparing mutation rate and age of diagnosis. These analyses revealed that in individuals with non-familial retinoblastoma, the somatic mutation rate for each mutation was equal and onset was later. This mutation rate was similar to the mutation rate found in patients with hereditary disease. However, only the remaining wild-type gene needs to be mutated in order to develop into a tumor. In this case, tumor growth occurs earlier. These data conclude that in hereditary disease, one mutation is inherited and the other mutated by random mutation, whereas in non-hereditary disease, two random somatic mutations are required for a cell to develop into a tumor.

In 1976, several research laboratories identified deletions of the chromosome locus 13q14 in retinoblastomas. This suggested a potential location of the retinoblastoma (*Rb*) gene locus (81, 82). Using RFLP analyses, the location of the *Rb* gene was further characterized and in 1986, the *Rb* gene was cloned (83, 84).

The Rb family consists of Rb itself and two related proteins–p107, and p130. This gene family regulates cell cycle progression, apoptosis and cellular differentiation. In its active form, Rb is unphosphorylated. Activated Rb blocks proliferation by sequestering the E2F family transcription factors that are required for G_1 to S progression (85, 86). Rb recruits histone deacetylases and chromatin remodeling factors to E2F promoters. Modification of such promoters represses expression of E2F regulated genes (87). Among other regulatory proteins, cyclin dependent kinases (CDKs) inactivate Rb by phosphorylation (88). There are multiple regulators of Rb activity. One such regulator is TGF-β. TGF-β prevents Rb inactivation by increasing the expression of $p15^{INK4B}$ and p21 (89). These proteins inhibit the CDK4:cyclinD complex that is responsible for inactivation of Rb (90). In this way, TGF-β signaling maintains the cell cycle inhibitory function of Rb.

Disruptions in Rb signaling pathways may result in the ability of cells to circumvent normal antigrowth signals. These alterations have been found in various human tumors (91). For example, deregulation and/or mutation of the TGF-β receptor is found in various tumor types (92). Other tumors exhibit a deletion in $p15^{INK4B}$ locus, which is an inhibitor of the CDK4:cyclinD complex. Various cancer types, including melanomas, express mutant CDK4 protein that no longer interacts with $p15^{INK4B}$ protein. This liberates the CDK4:cyclinD complex which inactivates Rb (93, 94). Yet other tumors have a mutation in Rb itself or express viral oncoproteins that sequester Rb and eliminate its function

(95). These data support the hypothesis that Rb acts as a tumor suppressor gene by controlling progression of the cell cycle.

2. The Tumor Suppressor Gene p53

Two laboratories reported that p53 collaborates with mutant Ras to transform rodent fibroblasts (96, 97). In contrast, a murine p53 cDNA derived from F9 embryonal carcinoma cells, failed to collaborate with Ras to induce transformation. Rather, expression of mutant p53 resulted in a transformed phenotype (98). It was later discovered that mutant p53 inactivated the function of wild-type p53 by forming heterotetrameric complexes (98, 99). These led to the hypothesis that wild-type p53 represses tumor development (100). This is supported by evidence indicating that one allele of p53 is deleted, and the other mutated in a high percentage of human colorectal cancers (101, 102). Mutations of p53 were also discovered in many other cancers, and in individuals with familial Li-Fraumeni cancer susceptibility syndrome (103). These patients have an increased susceptibility to cancer development.

The p53 protein is a DNA binding, homotetrameric transcription factor. Expression of p53 is induced by various stresses, including DNA damage, hypoxia and oncogene activation (104, 105). Upon stimulation, increased expression of p53 results in G_1 phase arrest (105). Global transcriptional responses, which repress cellular proliferation, or induce apoptosis are also regulated by p53.

Regulation of p53 is similar to that of Rb. Oncogenic signals induce the INK4a-ARF locus and results in increased $p14^{ARF}$ expression (106, 107). This

protein binds directly to MDM2 and prevents its interaction with p53 (108, 109). MDM2 negatively regulates p53 levels by ubiquitination which leads proteosomal degradation (104). Interaction between $p14^{ARF}$ and MDM2 stabilizes p53 protein and leads to apoptotic signaling.

The INK4a-ARF locus also encodes the protein $p16^{INK4a}$ (107). However, the $p16^{INK4a}$ gene and the $p14^{ARF}$ gene utilize a different reading from. Therefore the functions of these proteins are distinct. The $p16^{INK4a}$ protein is inactivated in cases of familial melanoma, and various other cancer types (110, 111). Oncogenic signaling induces $p16^{INK4a}$ expression. This blocks the inactivation of Rb by protecting from the cyclinD/Cdk4,6 complex (88). This leads to Rb activation, which stops G_1 – S phase progression. In summary, using different reading frames the INK4a-ARF locus encodes two proteins, $p14^{ARF}$ and $p16^{INK4a}$, that mediate the inhibitory roles of Rb and p53.

Increased expression of p53 can also induce the activation of Rb. For example, DNA damage, or enhanced oncogenic signaling results in an increase in p53 expression. This increase leads to activation of $p21^{CIP1}$. This protein maintains Rb activity by inhibiting the cyclinE/Cdk2 complex (112). For this reason, a mutation in p53 allows the cell to evade apoptosis, and prevents the ability for Rb to inhibit cell cycle progression. Disruption of either p53 or Rb genes results in expansion of multiplication potential. However, cells with mutations in these genes alone eventually enter crisis and die (113). Therefore, it is assumed that further genetic alterations are required to attain the ability to proliferate limitlessly.

C. Limitless Replicative Potential

In culture, after 50 – 60 doublings, normal cells typically enter a state of senescence – a G_0-like state that renders a cell unable to divide any further (114). However, in most cases tumor derived cell lines grown in culture are immortal. In 85-90% of all tumor derived lines, this is a result of increased telomerase expression (115). Telomeres, sequences found at the end of chromosomes, are progressively shortened by approximately 65 bp with each round of DNA replication (116). This shortening is a result of the inability of DNA polymerase to replicate the 3' end of a chromosome during S-phase of the cell cycle (116). Eventually unprotected chromosome ends participate in chromosomal end joining and karyotypic disarray. This leads to cell crisis and eventual death. Telomerase maintains chromosomal ends by adding hexanucleotide repeats to telomeric sequences. This allows more space for DNA polymerase binding (116). However, Bryan et al. (117) tested 35 immortalized cell lines and found that 15 of them did not have up-regulated telomerase. In these cases, an alternate pathway (ALT) is responsible for immortalization. The ALT pathway relies on recombination-based interchromosomal exchanges of sequence information to maintain telomeric DNA and confer the infinite lifespan phenotype. In combination with self-sufficient mitogenic growth signaling, evasion of apoptosis, and evasion of antigrowth signals, immortalization can lead to the growth of a small tumor. However, cells require oxygen and nutrients in order to form a malignant tumor. For this reason, the growth of new vasculature, or angiogenesis, is required for a malignant tumor to develop.

D. Sustained Angiogenesis

Angiogenesis refers to the sprouting of new vasculature from existing vasculature (118). In a tissue, cells must be within 100 μm of a capillary blood vessel in order to receive oxygen and nutrients required for metabolism. A good example of this is shown in the studies of Gimbrone et al. (119) who found that angiogenesis was required for tumor growth. Tumor fragments, or cultured tumor cells were placed in an avascularized site in the cornea of a rabbit's eye. As angiogenesis proceeded, new vasculature sprouted from the limbus. If angiogenesis was physically blocked, tumor growth was dramatically inhibited, as the neoplastic sites grew to a diameter of approximately 0.4 mm, but not larger. These data suggest that as a tumor continues to grow, angiogenesis is required for a small malignant tumor to become a large tumor. If the tumor lacks access to vasculature, small tumors become necrotic or apoptotic (120, 121).

The process of angiogenesis is carefully regulated by balancing anti-angiogenic factors with pro-angiogenic factors. Like most other mitogenic processes, both pro- and anti-angiogenic signals are mediated by soluble factors that bind to and activate transmembrane receptors. This stimulates signal transduction pathways and alters gene expression. The best studied pro-angiogenic factor is vascular endothelial growth factor.

1. Vascular Endothelial Growth Factor (VEGF)

VEGF-A is the best characterized member of the VEGF family, and most often implicated in tumor angiogenesis (reviewed in (122)). Other members of the VEGF family include placenta growth factor (PlGF), VEGF-B, VEGF-C and

VEGF-D (123-126). Whereas other angiogenic growth factors are known, it is widely recognized that VEGF signaling is the rate limiting step in both normal and pathological blood vessel growth (127). VEGF can be secreted by endothelial cells as well as other cells in the tumor microenvironment, including fibroblasts (40). VEGF-C and VEGF-D are not implicated in vascular angiogenesis, but play a role in the growth of lymphatic vessels or lymphangiogenesis (128).

The *VEGF-A* gene includes eight exons and seven introns (129, 130). Alternative splicing of the gene product results in four distinct VEGF isoforms: $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$ (131). The 165 amino acid isoform, which lacks exon 6, is predominant, followed by the 121 amino acid isoform, which lacks exons 6 and 7. There are also four less common isoforms including 145, 183, 162 and, a distinct 165 amino acid isoform called 165b. Current data indicates that alternative splicing of VEGF provides one level of VEGF regulation (reviewed in (127)).

Interactions between VEGF and the extracellular matrix (ECM) can dictate levels of VEGF protein. $VEGF_{121}$ fails to bind heparin and therefore is freely diffusible. $VEGF_{165}$ is secreted at high levels, but its heparin binding domains cause much of this isoform to bind to the external surface of the cell membrane and the ECM (132, 133). For various reasons, it is thought that $VEGF_{165}$ is the most physiologically relevant isoform (134, 135). $VEGF_{189}$ is highly basic and is almost completely bound to the ECM, limiting its bioavailability (132, 133). For this reason, proteolysis of the ECM can have a major impact in regulating the bioavailability of such VEGF proteins. Plasmin, the active form of plasminogen

(discussed in detail below), is able to cleave $VEGF_{165}$ or $VEGF_{189}$ and release a biologically active VEGF protein consisting of the first 110 amino-terminal amino acids (136). This indicates an important role of matrix remodeling proteins in sustaining angiogenesis.

VEGF protein activity promotes the growth of vascular endothelial cells from arteries, veins, and lymphatics (137), and induces potent angiogenic responses in a variety of *in vivo* models (131, 138). *In vitro*, VEGF prevents endothelial cell apoptosis induced by activating the PI3K/Akt pathway (139), and inducing the expression of the anti-apoptotic protein Bcl2 (140).

There are three known VEGF receptors: VEGFR1 (Flt-1), VEGFR2 (Flk-1 or KDR) and VEGFR3 (Flt-4) (141-146). Mutated VEGFR1 and/or VEGFR2 expression in mouse embryos is lethal. This suggests that VEGF plays an important role in early development (147). VEGFR2 is the major mediator of VEGF induced mitogenic, angiogenic and vascular permeability signals. For example, activation of VEGFR2 in aortic endothelial cells induces the phosphorylation of PLCγ, PI3K, Ras GTPase activating protein (RasGAP) and Src (148, 149). Activation of the PI3K/Akt pathway is required for the anti-apoptotic effects of VEGF in human umbilical vein endothelial cells, and also activates integrins known to be involved in angiogenesis, including $\alpha v\beta 5$, $\alpha 5\beta 1$ and $\alpha 2\beta 1$ (139, 140, 150). VEGFR2 can also activate the Raf-MEK-ERK1/2 kinase cascade through PLC, a Ras-independent mechanism (151, 152).

Signaling from VEGFR1 is much more complex, and is only indirectly implicated in mitogenesis or angiogenesis. VEGFR1 may serve as a decoy to

sequester VEGF from binding to VEGFR2, which could prevent mitogenic signal induction (153). VEGFR1 may play a role in hematopoiesis by regulating hematopoietic stem cell (HSC) survival (154). VEGFR1 activation also stimulates the migration of monocyte and bone marrow derived cells, which may be incorporated into vasculature (154-157). Activation of VEGFR1 induces matrix-metalloprotienase (MMP) expression (158). By knocking out VEGFR1 tyrosine kinase activity, Hiratsuka et al. (158) showed a significant suppression of MMP9 expression in premetastatic lung endothelial cells and macrophages. These data suggest a positive feedback mechanism where activation of VEGFR1 induces MMP9 secretion that then cleaves ECM bound VEGF making it more bioavailable. VEGFR1 has also been implicated in the paracrine release of growth factors from endothelial cells (159). Collectively, these data indicate the importance of VEGFR1 in tumor growth and metastasis.

VEGF expression is regulated by a number of transcription factors including AP-2, Sp1 and HIFs (130). As previously described, HIF1 protein is regulated primarily by oxygen tension. In response to hypoxia there is an increase in HIF1 protein levels. HIF1 binds to sequence specific hypoxia response elements (HREs) in the VEGF promoter, and drives the expression of VEGF protein (160). Small GTPases Rac1 and Cdc42 mediate this effect by regulating HIF1 protein levels and transactivation (161, 162). Rac1 modulates HIF1 activity by regulating the phosphorylation of its transactivating domain. Furthermore, both Rac1 and Cdc42 modulate HIF1 protein levels by reducing p53 and VHL protein levels in response to hypoxia (161). A recent study

indicates that Rac1 and Cdc42 mediate VEGF expression in non-hypoxic conditions as well (163). Both Rac1 and Cdc42 can regulate the activity of c-jun N-terminal kinase (JNK). Activation of c-jun results in increased expression of VEGF. Therefore, inhibition of Rac1 and Cdc42 prevents c-jun activation and VEGF expression (163).

Mutations in oncogenes, or amplification of oncogenes can result in increased VEGF expression. Thompson et al. (164) first described the angiogenic contribution of various oncogenes, including N- and HRas, in a reconstituted mouse prostate gland that expressed these oncoproteins. These researchers showed that activated Ras caused hypervascularization of the gland. These data were supported by Grugel et al. (165) who showed that transformation of NIH3T3 mouse fibroblasts with v-HRas or v-Raf resulted in increased VEGF mRNA expression. However, data presented by Okada et al. (166) indicate that whereas mutant Ras up-regulation of VEGF in human colorectal carcinoma cells was necessary for tumorigenicity, its expression alone could not induce malignant transformation of fibroblasts (167).

Oncogenic Ras modulates numerous mitogenic pathways that regulate VEGF expression. Ras-regulated transcription factors that bind to the VEGF promoter include HIF1, Sp1, Sp3 and AP-2 (130). Within the promoter region, HIF1 binds to HREs whereas Sp1, Sp3 and AP-2 bind GC-rich sequences. In hamster fibroblasts, Milanini et al. (168) showed that ectopic expression of oncogenic Ras, Raf, or MEK induced VEGF expression through activation of ERK1/2. This effect may be mediated by direct phosphorylation of HIF1 or Sp1

that increases transcriptional activation or DNA binding affinity respectively (169, 170). However, recent data from Lou et al. (26) showed that down regulation of Sp1 and Sp3 protein in $HRas^{V12}$ transformed human fibroblasts did not affect the level of VEGF present in conditioned media. These data suggest there may be cell type and/or species type differences in VEGF regulation.

VEGF expression levels are not solely determined by transcriptional regulation. VEGF mRNA stability and VEGF protein translation are also modulated by Ras-dependent pathways. Through the Ras-Rac1-MEKK1-JNK pathway, VEGF mRNA stability is modulated via adenosine-uridine regions in the 3' UTR of the VEGF transcript (171, 172). For example, in $HRas^{V12}$-transformed EJ bladder carcinoma cells and in HT1080, a human fibrosarcoma derived cell line expressing oncogenic NRas, VEGF mRNA is three to five times more stable (173).

Ras also regulates VEGF translation. Eukaryotic translation initiation factors (eIFs), a component of the translation initiation complex, scans the 5' end of mRNAs for AUG start sequences (reviewed in (174)). Once an appropriate start sequence is found, the complex falls off and translation begins. The 4E-binding protein 1 (4E-BP1) is a negative regulator of eIF-4E. Phosphorylation of 4E-BP1 decreases binding of eIF-4E resulting in enhanced translation initiation. Several studies indicate that increased eIF-4E activity results in increased VEGF expression. For instance, Kevil et al. (175) expressed eIF-4E protein in CHO cells and observed a 120 fold increase in secreted levels of VEGF protein. Furthermore, experiments by Crew et al. (176) show VEGF protein levels in

bladder cancers correlated with high levels of eIF-4E protein expression. This indicates that eIF-4E may play a role in VEGF translational regulation. Finally, both the PI3K pathway and the Raf-MEK-ERK pathway that are activated by Ras, have been shown to phosphorylate 4E-BP1 leading to a general increase in translation (177, 178).

In summary, VEGF is a critical factor associated with tumor angiogenesis. Regulation of VEGF is mediated by a number of transcription factors that modulate its expression. Likewise, post-transcriptional, such as mRNA stability, and translational mechanisms regulate VEGF protein levels. Clearly, Ras signal transduction pathways play a significant role in angiogenesis and VEGF expression. However, stimulation of angiogenesis is not accomplished by expression of pro-angiogenic factors alone. Down-regulation of anti-angiogenic factors is equally important. Thrombospondin-1 is one such factor.

2. Thrombospondin 1 (TSP-1)

The thrombospondin family consists of five members. TSP-1 is a 450 kD extracellular matrix glycoprotein secreted as a homotrimer. TSP-1 modulates cell motility, adhesion and proliferation (179, 180). It has been shown to inhibit proliferation and migration of endothelial cells in culture. TSP-1 expression also induces apoptosis (181-183). In 1990, Good et al. (184) showed that TSP-1 could inhibit angiogenesis *in vivo*. Briefly, implantation of a pellet containing the pro-angiogenic factor basic fibroblast growth factor (bFGF) into a rat cornea prompted a positive neovascular response, while implantation of a pellet

containing both bFGF and TSP-1 did not elicit the same response. This was the first indication *in vivo* that TSP-1 played a significant role in angiogenesis.

TSP-1 expression limits neovascularization of tumors (185, 186). There are at least 8 membrane receptors on endothelial cells that respond to TSP-1 expression. These include CD36, heparin sulfate proteoglycans, and $\alpha 3\beta 1$ and $\alpha 4\beta 1$ integrins (183, 187-191). Each of these receptors have been shown to modulate an anti-angiogenic signal, however, TSP-1 interaction with the CD36 receptor is the best characterized. Activation of CD36 initiates a signal transduction pathway through c-jun N-terminal kinase (JNK) to p38 MAPK, which are necessary to regulate TSP-1 mediated inhibition of angiogenesis (188). Active p38 is an inhibitor of FAS dependent apoptosis. This suggests a potential mechanism for the anti-angiogenic activity of TSP-1 (192).

The pro-angiogenic factor VEGF is up-regulated upon activation of Ras. Likewise, Ras activity negatively regulates both TSP-1 and TSP-2 expression. For example, NIH3T3 mouse fibroblasts transformed with Polyoma middle-T (mT) antigen typically exhibit activated Ras and decreased levels of TSP-1 as a result of Ras activation. In these cells, activated Ras enhances c-jun phosphorylation and subsequent AP-1 transcription factor activity (193). In a previous report, Mettouchi et al. (194) showed that transformation of Rat1 embryonal fibroblasts with c-jun results in repression of TSP-1 expression. These studies indicate that Ras activation induces c-jun phosphorylation, and AP-1 activation, which results in repression of TSP-1 expression.

Recruitment of vasculature to the tumor environment is necessary in order to carry away waste and deliver oxygen and nutrients to a developing tumor. Innervating vasculature and lymphatics also provide a path by which tumor cells can metastasize to different areas of the body.

E. Tissue Invasion and Metastasis

The major cause of cancer related deaths is metastatic disease (195). Developing a metastasis is a complicated process. In order to metastasize, a cell must detach from the original tumor, infiltrate surrounding connective tissue, cross the basal lamina of vasculature or lymphatics, evade host immune and non-immune responses such as blood turbulence, monocytes, lymphocytes and natural killer cells, exit the vasculature, and adhere to distant tissue. New colony growth depends on the ability of a cell to recruit vasculature and proliferate. Fidler et al. (196) injected radiolabeled murine B16 melanoma cells directly into the venous circulation of mice. After counting the number of secondary sites, they found that less than 0.1 percent of the original population survived to proliferate into secondary growths. This suggests that metastasis is a highly selective process.

In 1991, Frixen et al. (197) reported that E-cadherin mediated cell-cell adhesion prevents the invasiveness of human carcinoma cells. Using immunofluorescence microscopy and Western blotting these researchers observed that carcinoma cells with a fibroblastoid phenotype had lost E-cadherin expression as opposed to non-invasive carcinomas exhibiting an epitheloid phenotype. This study suggested that interruption of cell-cell adhesion molecule

(CAM) is necessary for the ability of a cell to break away from the original tumor. E-cadherin is ubiquitously expressed on epithelial cells and serves to induce antigrowth signals via interaction with β-catenin. Loss of E-cadherin, or inactivating mutations in the β-catenin gene is present in a variety of primary tumors including melanoma, colon cancer, gastric cancer and prostate cancer (reviewed by (198)).

Integrin expression and signaling also play a major role in the motility of tumor cells through the ECM. They also mediate invasion of cells through the basil lamina of lymphatics or vasculature. Integrins consist of a large family of both α and β subunits. Most often, α and β subunits heterodimerize to form a functional integrin molecule. Integrins play a prominent role in cell motility as well as cell cycle progression, differentiation and apoptosis (199). In 1991, Chan et al. (200) reported that expression of α2β1 integrin, a receptor for laminin and collagen, potentiated a metastatic phenotype in a rhabdomyosarcoma cell line. Furthermore, the expression of the most predominant integrin, αvβ3 mediates cellular motility of metastatic melanoma cells via its interactions with an array of extracellular components including laminin, vitronectin, fibronectin, fibrinogen, collagen, von Willibrand factor and osteopontin among others (201-203).

Tumor cells must be able to degrade the ECM in order to infiltrate tissue surrounding a tumor. In 1996, a seminal paper in the journal *Cell* published by Brooks et al. (204), reported a direct interaction between MMP-2 and the integrin αvβ3 on both angiogenic blood vessels and melanoma cells *in vivo.* This study suggested a coordinated activity of extracellular degradation and cellular motility

that may facilitate the process of tumor cell invasion. MMPs not only play an important role in invasion and metastasis, but also in bioavailability of the pro-angiogenic factor VEGF. By a mechanism similar to that of MMPs, the urokinase plasminogen activator system also plays an important role in the process of tumor invasion and metastasis.

Urokinase Plasminogen Activator (uPA)

Early stages in tumor metastasis require the degradation of the ECM to allow invasion of a tumor cell into host tissue via the vasculature or lymphatic systems. This degradation is mediated by a growing number of factors including matrix metalloproteinases (MMPs) and the urokinase-type plasminogen activator (uPA) system (205). The uPA system includes the serine protease uPA, its receptor uPAR and its inhibitors, plasminogen activator inhibitor 1 (PAI1) and plasminogen activator inhibitor 2 (PAI2). uPA converts plasminogen to plasmin which, in turn, degrades the ECM and activates MMPs and growth factors (205). uPA and its inhibitors can either be expressed by tumor cells, and/or by adjacent stromal cells resulting in a coordinated proteolysis of the matrix (206).

Expression of uPA is commonly found in breast tumors. Patients with breast carcinomas that express high levels of uPA have a shorter disease-free interval than patients with low levels of expression (207-209). Both uPA and its inhibitor PAI1 are breast cancer markers that have prognostic use for patients (210).

Signaling molecules implicated in uPA protein up-regulation include hepatocyte growth factor/scatter factor (HGF/SF), epidermal growth factor

(EGF), insulin-like growth factors I and II (IGF-I and IGF-II), bFGF, lysophosphatidic acid (LPA), colony stimulating factor-1 (CSF-1), thrombin, and VEGF (211-218). Most of these factors presumably activate the uPA system through activation of their corresponding receptors. This leads to activation of PLC, PKC, PLD, Ral, Ras, Raf, MEK and ERK1/2 (219-221). Ras is a modulator of uPA expression. Jankun et al. (222) observed a constitutive up-regulation of uPA expression in both patient derived sarcoma cell lines and human fibroblast cell lines malignantly transformed with either K-, N- or $HRas^{V12}$. Through the Raf-MEK-ERK1/2 pathway, Ras activates the transcription factor PEA3. Ras also activates the AP-1 transcription factor by inducing phosphorylation of c-jun. This pathway is mediated by the activities of Rac1 and JNK. Both AP-1 and PEA3 binding sites are located within the uPA promoter (223, 224). Ras also regulates the expression of uPA through activation of the RalGDS pathway. Aguirre-Ghiso et al. (225) showed that dominant negative Ral expression repressed Ras induced uPA over-production in v-Ras transformed NIH3T3 cells.

Regulation of uPA expression is also regulated post-transcriptionally through adenosine/uridine-rich elements (AREs), a well characterized AUUUA sequence found in 3' UTRs of genes that are regulated by mRNA degradation (226). These data indicate a connection between the Ras pathway and that of small GTPases Rac1 and Cdc42. Briefly, Ras regulates Rac1 and Cdc42 activity, which in turn play a role in vitronectin induced regulation of the Rac1-MKK3-p38 MAPK cascade that results in uPA mRNA stabilization and protein up-regulation (227).

In summary, cancer is a genetic and epigenetic disorder. Multiple mutations are required in order for a cell to acquire the characteristics necessary to develop into a malignancy. The activation of the Ras oncogene is commonly found in tumors of the colon and pancreas and is a good example of a gene that when mutated, contributes to multiple tumor phenotypes. For example, a cell with an activating Ras mutation typically exhibits self-sufficiency in growth signaling through activation of mitogenic pathways such as Raf-MEK-ERK1/2 or Cox-2 expression. Likewise, activation of Ras induces the expression of the angiogenic factor VEGF and suppresses the expression of TSP-1, an anti-angiogenic protein. The ability to sustain angiogenesis is a critical characteristic acquired by cancer cells that is necessary to develop into a sizable tumor. Recruitment of vasculature to a tumor also provides an avenue for tumor cell invasion and metastasis. Ras expression induces the expression of MMPs and the uPA system that breaks down ECM components, and facilitates invasion. Clearly, Ras plays a crucial role in cellular transformation.

III. The Oncogene Ras

In 1964, Jennifer Harvey observed that a murine leukemia viral preparation taken from a leukemic rat, induced sarcomas in newly born rats. This marked the beginning of *HRas* research. *HRas* was then identified as the cellular homologue of an oncogene carried by this virus. Since then, two more Ras isoforms have been identified, *KRas* and *NRas*. In 1982 the nucleotide sequences of *v-HRas* and *v-KRas* oncogenes were published. Three laboratories reported the molecular cloning of a human transforming gene from the T24 bladder carcinoma cell line (228-230). By 1983 a number of labs determined that the transforming factors in the T24 bladder carcinoma cell line was homologous to sequences found in the v-HRas and v-KRas genes (231-236).

The biological function of Ras has been the subject of intense research as a result of its role in human cancer development. A point mutation in the *Ras* gene, resulting in a valine substitution at codon 12, was found to be responsible for aberrant Ras activity in T24 bladder cancer cell lines (237). Since then, a lysine substitution at codon 61 was also found to lead to constitutive Ras activity (238). Activating mutations in *Ras* genes have been detected in approximately 30% of all human cancers, with 50-90% observed in some human carcinomas (44, 45). These findings clearly indicate that the activating mutations found in the *Ras* genes are not a laboratory artifact, but a genetic change important in cancer development found in human tumors.

Ras proteins function as a molecular switch by cycling between an inactive GDP-bound state and an active GTP-bound conformation (Fig.2). Their

Figure 2. Ras functions as a molecular switch at the cell surface by cycling between inactive and active conformations. Guanine-nucleotide exchange factors (GEFs) facilitate the release of GDP from the nucleotide-binding pocket of the G-protein. This opens the pocket for binding of a GTP nucleotide. Once bound, Ras undergoes a conformational change, which exposes its effector-binding domain. This results in an increase in mitogenic signaling. GTPase activating proteins (GAPs) increase the activity of Ras' intrinsic GTPase domain. This induces GTP hydrolysis and results in a conformational shift that prevents effector binding. Ras is normally tightly regulated. However mutation of specific residues within the GTP-hydrolysis domain results in constitutive-activation. This results in continuous mitogenic signaling. Mutations in Ras are found in 30% of all cancer types including 90% of pancreatic tumors. Once activated, Ras affects numerous signaling pathways including the Raf-Mek-ERK MAPK cascade, the RalGDS signaling pathway, and the PI3K-Akt survival pathway. Activation of PI3K results in activation of the small GTPases Rac1 and Cdc42. Ras also activates exchange factors specific for Rac1, Tiam1, and Rho, p120-p190.

intrinsic GTPase activity is responsible for hydrolysis of GTP to GDP and results in inactivation. Two types of proteins regulate the activity of these GTPases. Guanine nucleotide exchange factors (GEFs) facilitate the release of GDP and allow binding of GTP. In this way GEFs induce Ras activity. GTPase activating proteins (GAPs) induce intrinsic GTPase activity resulting in Ras inactivation. The molecular structure, regulation and Ras mediated effector pathways are discussed in further detail below.

A. Molecular Structure

In 1988, two separate laboratories (239, 240) reported the crystal structure of the Ras protein both with and without activating mutations. The three-dimensional structural analysis revealed that six stranded β-sheets, four α-helices, and nine connecting loops make up the structure of Ras. Four of the nine loops (L1, L2, L7, and L9) form a pocket that constitutes the nucleotide binding site. Residues 10 to 16 of L1 are in proximity to the phosphates. Residue 30, found within L2 interacts with the ribose sugar. The majority of activating mutations found both *in vitro* and *in vivo* are found within three loops L1, L4, and L7. Specifically, Val12 is located in L1 near the phosphates and interferes with GTP hydrolysis. Krengel et al. (241) and Scheffzek et al. (242) revealed that Glycine 12 is also close to the binding site of GAPs. They suggest that any other amino acid in this position would interfere with GAP binding and hydrolysis would be affected. Gln61 is found in L4, which is not in contact with the phosphate groups of the nucleotide, but comes in direct contact with L1, and also interferes with GTP hydrolysis. Gln61 plays a significant role in catalysis. Specifically,

Gln61 forms a hydrogen bond with Arg789 of GAP120, a GAP specific for Ras, in order to allow nucleophilic attack of a water molecule that catalyzes the hydrolysis of GTP. Mutation at this site impairs GTPase activity resulting in constitutive activation and continuous transmission of proliferative signals. Mutations in the same region can also result in ineffective nucleotide release. Asn17 is a critical residue found in the nucleotide binding pocket and is necessary for efficient release of GDP. Therefore, Ras proteins with Asn17 mutations are inactivated as a result of their inability to release GDP in favor of the activating GTP nucleotide (243).

A more generalized view of Ras structure reveals three critical domains important in Ras function. First, regions involved in GTP hydrolysis surround residues found in L1 and L4 that fold into proximity to each other (240). Second, post-translational modification of Ras at a cysteine residue (cys186) near the C-terminus is required for Ras activity (244). Briefly, a farnesyl group is added via the enzyme farnesyltransferase, and Ras becomes membrane localized. Mutations in this region result in incorrect cellular localization and render the protein inactive. For this reason, great efforts have been made to develop drugs that target farnesyltransferase. Initial studies using farnesyltransferase inhibitors (FTIs) have shown anti-proliferative, pro-apoptotic and anti-angiogenic activities (245). However, high concentrations are needed in order to inhibit oncogenic KRas (246), the most often mutated isoform of Ras found in tumors. Recent reports indicate in cases of farnesyltransferase inhibition, KRas and NRas, but not HRas can be geranylgeranylated (247). This is an alternative modification

that is mediated by geranylgeranyltransferase and results in the addition of two palmitoyl long-chain fatty acid groups added to a cysteine residue just upstream of the farnesylated cysteine. This rescues Ras activity by re-localizing it back to the membrane.

Third, when Ras transitions from the GDP to the GTP-bound state, a conformational shift occurs between switch I and switch II regions. The switch I region overlaps the effector binding domain (residues 32-40) (240). Mutational analyses of residues within and immediately surrounding this region indicate that this domain is a critical site for effector binding upon activation.

B. Ras Activation

Activation of the epidermal growth factor receptor (EGFR) was the first growth factor pathway known to stimulate nucleotide exchange and activity of Ras (248). More than 20 extracellular signals encompassing growth factors, cytokines and hormones are known to stimulate RTKs, non-receptor tyrosine kinase-associated receptors and G-protein coupled receptors that either directly or indirectly regulate the activity of Ras. Briefly, the association of EGF with EGFR results in auto-phosphorylation of its cytoplasmic tail exposing binding sites for adapter proteins and other EGFR effectors. Using the auto-phosphorylated cytoplasmic tail of as bait, Lowenstein et al. (249) found an adapter protein, Grb2, that mediated the connection between activated EGFR and factors that regulate Ras activity. Grb2 consists of one src-homology region (SH2) domain surrounded on either side by two SH3 domains. Auto-phosphorylation of the EGFR provides a unique binding site for the SH2 domain

while the two SH3 domains, implicated in protein-protein interactions through proline-rich regions, provide a docking site for factors directly involved in stimulating Ras activity such as GEFs. The human homologue of the *Drosophila* Son of sevenless (SOS) gene was identified as a result of its high sequence homology to a known Ras stimulatory gene in yeast (*Cdc25*) (250). SOS is a Ras-specific GEF that binds to Grb2. This results in recruitment of Ras to the cell membrane and activation.

GAP120 is a prototypical GTPase activating protein. GAP120 interacts with the GTPase hydrolysis domain of Ras and facilitates the hydrolysis of GTP (251). Neurofibromatosis type 1 (NF1) is a hereditary cancer syndrome in which patients develop both benign and malignant tumors of the central (CNS) and peripheral nervous systems (PNS). Individuals with NF1 are predisposed to developing astrocytomas. Pedigree analyses, cDNA walking and sequencing completed by Xu et al. in 1990, identified biallelic mutations of NF1 gene (252). Characterization of the NF1 sequence revealed high sequence homology to GAP120 indicating GTPase activating properties. These data suggest that mutation of a negative regulator of Ras predisposes patients to CNS and PNS tumors.

C. Ras Effectors

There are three general signal transduction pathways associated with Ras activity (Fig. 2). First, the best characterized Ras effector is Raf. Activation of Raf causes transmission of a signal through MEK, ERK, and various transcription factors that carry out Ras regulated gene transcription. Second, the p110

catalytic subunit of PI3K has been shown to directly interact with the activated confirmation of Ras. PI3K regulates the activities of apoptotic pathways through Akt and PTEN. Third, activated Ras regulates the activity of RalGDS a GEF specific for the small G-protein Ral. Ral activity has been implicated in various processes including angiogenesis and cell motility.

1. Raf

The Raf family is made up of ARaf, BRaf and c-Raf1. All three family members have an auto-inhibitory NH_2-terminal domain. Raf is activated by Ras through direct interaction of the effector binding domain of Ras and two domains within Raf, the Ras Binding Domain (RBD) found within the auto-inhibitory NH_2-terminal domain and a cysteine-rich domain (CRD). These interactions localize Raf to the membrane where it is subsequently activated (253, 254). Deletion of the NH_2 terminal domain results in constitutive activation, and ectopic expression can transform multiple cell types including NIH3T3 mouse fibroblasts (255).

Activation of Raf results in phosphorylation of MEK (256). MEK proteins are dual-specificity kinases that contain two motifs responsible for phosphorylation of serine/threonine and tyrosine residues. Both MEK1 and MEK2 are expressed ubiquitously in human cells and are responsible for the phosphorylation of ERK at two sites, Tyr185 and Thr183 (257). ERK translocates to the nucleus where it phosphorylates members of the ETS transcription factor family. Studies using Chinese hamster lung fibroblasts (CCL39) that expressed either dominant negative versions of ERK1/2 or antisense DNA oligomers

illustrate that suppression of ERK activity blocks thrombin-induced proliferation (258).

Raf proteins are mediators of tumor formation. *In vitro*, expression of constitutively-active Raf gives rise to a transformed phenotype in cells, which is similar to that formed by an activated Ras. Activated B-Raf mutations are found in almost 70% of all human melanomas (259, 260). Raf also has anti-apoptotic properties (261). Briefly, activated ERK increases expression levels of the 90 kD ribosomal S6 (RSK) protein. RSK activates CREB by phosphorylation, a transcription factor that promotes cell survival (262). Through phosphorylation, RSK also inactivates the pro-apoptotic protein BAD (263).

Raf can also impact cell survival by activating NFκB independently of ERK activity (264). Furthermore, independent of its kinase activity, Raf directly binds to and blocks the pro-apoptotic function of apoptosis signal-regulated kinase 1 (265). In these ways, Raf acts as both a stimulator of cell proliferation and an inhibitor of apoptosis.

Developing inhibitors of the Raf-MAPK pathway has been a major thrust in the past 5 years. However, clinical trials of such agents have been only mildly successful. The most successful Raf inhibitor, BAY 43-9006 was found using a combinatorial chemistry approach (266). BAY 43-9006 has shown promise in treating renal-cell carcinoma. (267) However, BAY 43-9006 is not only an inhibitor of Raf activity, but also that of VEGFR (268). It is not known whether the anti-tumor effect of BAY 43-9006 is due to inhibition of Raf, and/or VEGFR. Selective MEK inhibitors are also currently being used in clinical trials.

PD0325901 and ARRY-142886 have been shown to have anticancer activity against a broad spectrum of human xenografts, significantly inhibiting their growth in numerous human models tested (269).

2. Phosphatidyl Inositol 3-Kinase (PI3K)

PI3K is a heterodimeric protein consisting of an 85 kD subunit containing one SH3 and two SH2 domains that support activated RTK binding, and a 110 kD catalytic subunit. PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) creating phosphatidylinositol-3,4,5-triphosphate ($PI(3,4,5)P_3$). These lipids then bind to numerous proteins through pleckstrin homology (PH) domains, and have been shown to impact many enzymatic pathways (270).

In 1991, Sjölander et al. (271), using IGF-1 stimulated Ras^{V12}-transformed rat liver epithelial cells found an interaction between activated Ras and the 110 kD catalytic subunit of PI3K using co-immunoprecipitation techniques. In 1994, research conducted by Rodriguez-Viciana confirmed the importance of PI3K as an effector of Ras activity (272). They showed that dominant-negative Ras (Ras^{N17}) expression in a rat epithelial cell line prevents growth factor induced up-regulation of phosphatidyl-inositides, which is a result of increased PI3K activity. They also report that transfection of Ras, but not Raf, into monkey-derived epithelial cells results in increased levels of these lipids. These results indicate a divergence in Ras signaling at the level of PI3K and Raf.

Effectors of PI3K are involved in pro-survival pathways. For example, PI3K activity induces Akt activity (273). Akt prevents apoptosis by phosphorylating numerous targets including GSK3 and Forkhead (274, 275).

Over-expression of GSK3 in Rat1 fibroblasts and rat epithelial cells induces apoptosis in the absence or presence of growth factors. Conversely, expression of kinase dead GSK3 prevents apoptotic events (274). Therefore, inactivation of GSK3 by Akt inhibits its pro-apoptotic function. Activation of the Forkhead transcription factor results in increased expression of the Fas ligand. Fas ligand expression and secretion activates the apoptotic pathway by binding to the FAS receptor (275). This interaction triggers a signaling cascade that leads to caspase activation and apoptosis. Akt inactivates Forkhead activity, confirming a role for PI3K in preventing Forkhead induced apoptosis.

Importantly, PI3K activity induces the activation Ras related G-proteins Rac1 and Cdc42. This occurs through a direct interaction between p85 subunit of PI3K and the G-protein. PI3K can also induce Rac1 and Cdc42 indirectly through activation of phosphoinositides. Microinjection of activated Ras protein into endothelial cells induces actin rearrangement and membrane ruffling. To determine if PI3K and Rac1 mediated this effect, endothelial cells were microinjected with Ras^{V12}, activated PI3K or activated Rac1 ($Rac1^{V12}$) (276). Each was treated with the PI3K inhibitor LY294002. The researchers found that the ability of Ras^{V12} and activated PI3K to induce actin cytoskeleton reorganization was completely blocked. However, LY294002 did not block $Rac1^{V12}$-induced membrane ruffling. This indicates a direct role for PI3K activity upstream of Rac1.

Rac1 is also activated in a PI3K independent pathway. Sharing much homology with Ras, members of the Rho-family of GTPases are also regulated

by GEFs and GAPs. They cycle between an inactive GDP-bound form and an active GTP-bound form. Therefore, control of GEFs and GAPs are critical points of Rac1 and Cdc42 regulation. Tiam1 is a GEF specific for Rac1. Activation of Ras results in the direct binding of Tiam1 to Ras through the RBD on Tiam1. This interaction results in activation of the GEF activity of Tiam1 and direct activation of Rac1 (276). Activation of Rho GTPases by Ras plays a critical role in transformation. Rho GTPases play a role in cell motility, actin cytoskeleton reorganization, and signal transduction. In this way, Rho GTPases serve as moderators of key phenotypes acquired by cancerous cells including invasion, metastasis and angiogenesis.

Very few attempts at inhibiting PI3K regulated pathways in patients have been attempted. Identifying inhibitors of the pro-survival activities of Akt is currently a focus of intense research. PI3K activity is an enticing target for inhibition, however its high level in the hierarchical pathway makes it less attractive.

3. RalGDS

In 1994, several research laboratories identified RalGDS (Ral-GDP dissociation stimulator) as a direct effector of activated Ras using a yeast two-hybrid approach (277). To date, four human RalGDS' have been identified. RalGDS is a GEF that activates the small G-proteins RalA and RalB. Rals are small G-proteins with homology to Ras. Both active RalA and RalB interact with many effectors including Sec5 and Exo84, subunits of the exocyst complex (278, 279). Furthermore, both RalA and RalB bind constitutively to phospholipase D1

(PLD1) and stimulate the activation of c-src, c-jun, STAT, AFX, NFκB and cyclinD through yet unidentified effectors (280-283). The best characterized effector of Ral is Ral Binding Protein-1 (RALBP1) (284). RALBP1 is a GAP specific for the Rho-family GTPases, Rac1 and Cdc42.

The activity of RalA is essential for Ras^{V12}-induced transformation of rodent fibroblasts. Originally, the contribution of RalA activity in Ras^{V12}-induced transformation was considered minor. Inhibition of RalA activity, by expression of dominant-negative mutants, prevented Ras^{V12}-induced transformation (285). However, expression of constitutively active RalA mutants did not induce transformation in either rodent fibroblasts or epithelial cells (286-289). Current research, however, indicates that the role of Ral activity in human cell transformation is more important (290). RalA potently induces transformation of both telomerase immortalized human embryonic kidney cells, and human fibroblasts. Using siRNA, Lim et al. (290) also found that reduction in RalA expression in the human sarcoma derived cell line HT1080 reduced tumor latency.

The transforming ability of RalA is dependent on its interactions with its effectors i.e. Sec5, Exo84, and RALBP1. Recent studies indicate that RalB competes for effector binding with RalA (290). In this way, RalB antagonizes the activation of RalA and prevents transformation. These data indicate an important role for RalA and RalB effectors in mediating transformation induced by RalA activation.

Research by Lim et al. (290) suggests that binding of RALBP1 to activated RalA may sequester RALBP1 from exerting its inhibitory effects on Rac1 and Cdc42. This would result in constitutive activation of the Rac1 and Cdc42 regulated pathways. This suggests that RalA induced transformation may be dependent on its ability to activate Rac1 and Cdc42. These studies have yet to be completed. Under my direction, an undergraduate student in the Carcinogenesis Laboratory is currently investigating the role of RalA in the HRas transformed cell line PH3MT. Briefly, we are attempting to reduce RalA using an shRNA construct. We hypothesize that reduction in RalA will result in reduced tumor formation. Furthermore, we expect that reduction in RalA protein expression will result in altered Rac1 and Cdc42 mediated gene expression. Consistent with this hypothesis, Aguirre-Ghiso et al. (225) showed that dominant negative Ral expression repressed Ras induced uPA over-production in v-Ras transformed NIH3T3 cells. Our data, presented in chapter II, indicate that both Rac1 and Cdc42 regulate Ras induced uPA expression in a $HRas^{V12}$-transformed cell line (PH3MT). This suggests that Ral activity may mediate Ras induced uPA expression by regulating the activities of Rac1 and Cdc42. The data collected in this study will solidify Ral as a target for pharmaceutical inhibition. Furthermore, it will identify downstream targets of Ral activity that could be targets of pharmaceutical agents used to treat cancer patients.

IV. Rho-GTPases and their Cellular Function

The Rho-family (Ras homologous) GTPases currently consists of 23 candidate members that can be subdivided according to their sequence and functions. These subdivisions include proteins most similar to RhoA, most similar to Rac1 and Cdc42, and those that lack GTPase activity. Early studies of Rho family members focused on their role in actin cytoskeleton regulation (291). However, members of this family are also important in several other cellular activities including gene transcription, lipid metabolism, and vesicle trafficking that make up complex networks. These networks impact proliferation, motile behavior, malignant transformation, and tumor metastasis.

A. Regulation of Rho GTPases

Like Ras, Rho-GTPases are targeted to the cell membrane and act as a molecular switch by cycling between the inactive GDP-bound form and the active GTP-bound form. Activity of the Rho-family GTPases is regulated by Rho-guanine nucleotide exchange factors (Rho-GEFs) that facilitate the dissociation of GDP, allowing GTP, which is present at a high concentration, to bind to the GTPase. About 80 known Rho-GAPs (Rho-GTPase-activating proteins) catalyze GTP hydrolysis resulting in inactivation (292). An additional level of regulation is provided by members of the Rho-GDP dissociation inhibitor (Rho-GDI) family that prevent release of GDP, thus rendering the protein inactive (293). C-terminal cysteine residues within Rho-GTPase proteins are targets of lipid-modification and are required for membrane localization and efficient regulation.

More than 60 distinct Rho-GEFs have been identified. Many of them exhibit tissue specificity. Their expression and activation are also tightly regulated (294). Rho-GEFs share a common Dbl homology (DH) domain, responsible for the catalytic activity, and a pleckstrin homology (PH) domain that serves as a site for protein and lipid interaction (295). The PH domain is invariably found directly C-terminal to the DH domain. The PH domain does not directly interact with the G-protein. However, evidence obtained from the crystal structure of Rac1 and its interaction with the Rac1-GEF Tiam1 indicates that the proximal PH domain is important in stabilizing the interaction of the DH domain with the G-protein (296). Deletions of the PH domain in Rho-GEFs result in proteins that still maintain the ability to induce exchange on G-proteins, but with a 100 fold reduction in activity (297). Interaction of Rac1 and Tiam1 results in a conformational shift of two important switch regions within the G-protein. This conformational change exposes the nucleotide binding site allowing for nucleotide binding and activation (296). Similar interactions have been identified between the specific Cdc42-GEF fgd1 and Cdc42 protein (298). Promiscuous Rho-GEFs such as Vav that support nucleotide exchange on RhoA, RhoG, Rac1 and Cdc42 are thought to induce similar conformational changes within each respective G-protein (299). Specificity of the GEF for a specific G-protein is thought to be determined by the conformational organization of the DH domain (296).

A new family of Rho-GEFs, lacking the DH-PH tandem domains has recently been identified. About 10 members have been found including DOCK180, the founding member of this family (300, 301). In many but not all

cases, members of the DOCK180 family require an accessory protein (ELMO) in order to exchange nucleotides on Rac1 (302).

B. Signaling from Growth Factors

In 1992 Ridley and Hall (303) described signaling from cell surface receptors to Rho proteins. They identified lipopolysaccharide (LPS) and bombesin as activators of Rho and Rac1 respectively. Growth factors such as PDGF, EGF and insulin were also found to activate Rac1 and lead to subsequent Rho-activation (304). PKC agonists such as PMA were found to activate Rac1 but, activation of Rho was not observed. PKC agonists activate the Rho-GAP p190RHO-GAP through c-Src thereby antagonizing the activity of Rho (305). These differences in signal transduction are a result of differential activation of GEFs and GAPs specific to Rho and/or Rac1.

Nobes et al. (306) found that growth factor induced membrane ruffling, mediated by Rac1, was blocked by PI3K inhibitors. They found that expression of $Rac1^{V12}$ could induce membrane ruffles in the presence of PI3K inhibitor. This supports the role of PI3K acting upstream of Rac1. Further studies on PIP_2 and PIP_3 indicate that phosphoinositides play an integral role in regulation of Rac1 activity (294, 307). For instance, Tiam1 is recruited to the cell membrane and is activated by PIP_3. This results in activation of Rac1. Furthermore, a Rac1-GEF complex containing SOS, EPS8, and ABI1/E3B1 binds directly to the p85 subunit of PI3K. This indicates a direct role for PI3K in Rac1 activation (308). Both Rac1 and Cdc42 also bind to and activate PI 4,5-kinase as well as PI3K. This suggests a potential role for these GTPases in a positive feedback mechanism. (309).

Activation of GEFs in PI3K independent pathways include a direct interaction of the Rac1-GEF Tiam1 to Ras (276). Rac1 activity can also be induced in a PI3K and Ras independent pathway. For example, in Swiss 3T3 fibroblasts, PDGF stimulation leads to Tiam1 phosphorylation and subsequent Rac1 activation (307).

C. Actin Cytoskeleton Reorganization

The three best characterized Rho-GTPase family members Rho, Rac1 and Cdc42 have each been shown to play a crucial role in the reorganization of the actin cytoskeleton. For instance, LPS induced stress fiber formation is regulated by Rho. Growth factor and RTK induced lamellapodial extensions are regulated by Rac1, and activity of filipodia is regulated by Cdc42 (310).

The identification of the Rho effector ROCK (Rho-kinase) indicates a potential mechanism by which Rho regulates stress fiber formation. ROCK is a serine/threonine kinase that phosphorylates the myosin-binding subunit (MBS) of myosin light chain (MLC). This leads to myosin contractile activity and stress fiber formation. Active Rho protein can also induce stress fiber formation through the formin family of proteins. Formin family members interact with both actin and profilin and directly affect the regulation of cell structure and polarity (311, 312).

Lamellipodial extensions are thin protrusive structures generated at the leading edge of migrating cells. When these protrusions fail to adhere to a substrate, a membrane ruffling effect is observed. Ridley, Hall and colleagues (304) found that expression of constitutively activated Rac1 induces membrane ruffling. They also found that inhibition of Rac1, by dominant-negative Rac1

expression, inhibits the ruffling phenotype. They hypothesized that Rac1-regulation of PIP_2 exposes barbed ends of actin filaments at the leading edge, which could lead to ruffling. However, in 1998 Miki et al. identified an interaction between Rac1 and the Arp2/3 complex. This observation shifted the focus from Rac1 controlled phospholipid regulation to Rac1 induced activation of the Arp2/3 complex mediated by WAVE/Scar protein. WAVE/Scar protein is a member of the WASP family (313, 314). Cdc42 binds WASP and N-WASP inducing a conformational change that stimulates Arp2/3 to nucleate actin polymerization. Rac1 also stimulates Arp2/3 through its interaction with WAVE/Scar via an IRSp53 linkage (315).

The p21-activated kinase (PAK) is the best characterized effector of both Rac1 and Cdc42. It is implicated in both cytoskeletal reorganization and cell signaling. However, its role in various signaling pathways is complex and often times cell type specific (316). PAK is activated by both Rac1 and Cdc42 and its expression can promote the formation of lamellipodia and lead to the loss of stress fibers and focal adhesions (317, 318). Filamin and LIM kinase (LIMK) are substrates for PAK and are implicated in actin cytoskeletal reorganization (319, 320). Filamin binds directly to PAK through an interaction with its C-terminal domain, and the Rac1 and Cdc42 binding domain on PAK. The binding of PAK to filamin induces the organization of actin into lamellapodia (321). Activated PAK also phosphorylates LIMK. This leads to inactivation of cofilin. Cofilin is a protein that promotes depolymerization of F-actin (320).

Rho GTPases also make up the complexity of focal adhesion contacts. Recently, many cell adhesions molecules have been shown to affect Rho protein activity. Adhesion molecules implicated in regulating Rho-GTPases include integrins (322), cadherins (323) and Ig superfamily members (324). The involvement of cadherins in Rho signaling implicates a role for Rho proteins in invasion and metastasis.

D. Crosstalk

The most complicated aspect of Rho-GTPase family signaling involves their interactions with other Rho-family members and their respective pathways. Ridley and Hall (303) first observed that membrane ruffling induced by Ras was meditated by Rac1. Preliminary evidence indicated that this effect occurred in a Rho-dependent fashion. These data suggested Ras, Rac1 and Rho participate in a signaling pathway where Ras activates Rac1, and Rac1 activates Rho. Sander et al. (325) criticized this pathway because they found evidence of an inverse relationship between Rac1 and Rho. Results from their studies indicate that activation of Rac1 leads to the inactivation of Rho and vice versa. In 1995, two separate groups showed that Cdc42 also fed into the same pathway by activating Rac1 (326, 327). Rac1's role in the NADPH oxidase complex could explain how the activation of Rac1 can inhibit the activation of Rho. Activation of Rac1 can lead to the release of reactive oxygen species (ROS), which in turn can inhibit the low molecular weight protein tyrosine phosphatase (LMW-PTP). LMW-PTP is an inhibitor of p190RhoGAP. ROS inhibition of LMW-PTP permits p190RhoGAP inhibition of Rho activity (328).

Crosstalk between Rho family members can occur multiple ways. For example, one family member may suppress the activity of another by stimulating GAP activity. Conversely, one family member may activate a GEF that in turn activates another Rho family member. Furthermore, there are proteins such as Bcr and Abr that contain both GAP and GEF domains for different Rho members. This is an example of crosstalk mediated by a single protein (294).

E. Cell Proliferation

Rho-GTPases also contribute to mitogenic signal-transduction pathways. Rho-GTPases regulate G1 cell cycle progression by affecting cyclin D1 expression. The expression of cyclin D1 increases as cells enter the cell cycle and is crucial for cell proliferation (329). Through Rho-GTPases, cyclin D1 transcription is controlled by ETS, AP-1 and NFκB transcription factors (330, 331). Dimers of fos, jun and ATF transcription factor families make up the heterodimeric transcription factor AP-1. Activation of Rac1 and Cdc42 leads to increased phosphorylation and enhanced activity of the AP-1 components jun and ATF. This occurs as a result of Rac1 and Cdc42 induced activation of JNK and p38 MAPK cascades (331, 332). Effectors of Rac1 and Cdc42 implicated in activation of JNK and p38 include PAKs, mixed lineage kinases (MLKs), the MAPK kinase kinases (MEKKs) and the scaffold protein plenty-of-SH3-domains (POSH) (reviewed in (333).

Rho-GTPases can also induce cyclin D1 expression through the activation of ERK1/2 (334, 335). Signal initiation by Ras promotes the transcription of cyclin D1 through activation of ETS and AP-1 via ERK1/2 (336). In this pathway,

Rho-proteins are thought to determine the magnitude or duration of ERK1/2 activation, but do not serve as a direct regulator of ERK1/2 activity (335). Rac1 and/or Cdc42 can regulate ERK1/2 activity through regulation of PAK. Activated PAK can directly phosphorylate and promote the activity of Raf and MEK leading to increased ERK1/2 activity (335). Integrin signaling can also affect the activity of ERK1/2. Briefly, the activities of both Rho and ROCK are required for integrin assembly into focal adhesions (337). These complexes promote the activation of ERK1/2 through activation of the Src or focal adhesion kinase (FAK) tyrosine kinases, which leads to Ras activation (338). In this case, Ras activity is downstream of Rho activity which results in increased ERK1/2 activity.

NFκB is a transcription factor that also regulates cyclin D1 expression. Rac1 induces the activity of inhibitor-of-κB-kinase (IKK). IKK phosphorylates and inactivates inhibitor-of-κB (IκB) resulting in increased NFκB activity (339). Rac1 and Cdc42 can also activate NFκB through an IKK independent mechanism involving PAKs (339). Furthermore, a complex containing Rac1, Cdc42, the cell polarity gene PAR6 and atypical protein kinase c isoform (PKC) can also activate NFκB (340, 341). This complex is required for Rac1-mediatated transformation of rodent fibroblasts (342). In conjunction with cyclin D1 regulation, NFκB might also promote tumorigenesis by increasing transcription of Cox-2, MMPs, anti-apoptotic and pro-angiogenic genes (66, 343, 344).

F. Rac1 and Cdc42 in Cancer

Interest in the role of Rac1 and Cdc42 in cancer arose based on an observation that Ras^{V12}-transformation of NIH3T3 fibroblasts resulted in

extensive membrane ruffling. Ridley and Hall later attributed this effect to the activity of Rac1 (304). Since this discovery, both Rac1 and Cdc42 have been the subject of intense research that attempts to describe their role in malignant transformation.

1. Rac1 and Cdc42 Mutants

Like Ras, mutations of Rac1 or Cdc42 can result in constitutive activation or inactivation of the G-protein (Fig. 3). Substitution of a valine at codon 12 ($Rac1^{V12}$ or $Cdc42^{V12}$) results in a constitutively-active mutant while substitution of a serine by an asparagine at position 17 ($Rac1^{N17}$ or $Cdc42^{N17}$) is an inactivating mutation. The V12 mutation disrupts activity of the intrinsic GTPase-domain. As a result, the G-protein is unable to hydrolize GTP to GDP allowing sustained GTP binding, and signal transduction. Substituting either amino acid 61 or 28 with a lysine also activates these proteins. However, whereas L61 also renders the GTPase domain inactive, the L28 mutation induces increased GDP-GTP cycling that increases the amount of active protein at any one time.

The N17 mutant works by competing with the normal G-protein for exchange factors. N17 mutants are inefficient at releasing GDP and therefore cannot bind with downstream effectors. When expressed in cells, N17 mutants sequester GEFs from endogenously expressed proteins thereby inactivating their respective pathways (243).

Unlike Ras^{V12}-mutations found in various types of cancers, there are no reported cases of activating Rho-GTPase mutations in tumors. However, increased activity of Rho-GTPases has been observed in various tumor types.

Figure 3. The Rho-family GTPases share common domains that are required for normal function. The nucleotide binding and GTPase domains are important in the regulation of G-protein activity. Mutations within these domains can result in constitutive-activation, or inactivation of the G-protein. Mutations used in this report include a G12V mutation that results in constitutive activation, and a T17N mutation that renders the protein GDP-bound and inactive. When over-expressed in cells, Rho-GTPases with the T17N mutation can be used as dominant-negative mutants to inhibit the activity of their endogenous counterpart. Other activating mutations found within the GTP-binding and hydrolysis domains, include Q61L, and F28L. These mutants are also used in studies to determine their respective function. The effector-binding domain is exposed upon activation and is required to mediate G-protein signaling pathways. All Rho-family members contain a Rho-insert domain, an α-helical domain required for binding guanine dissociation inhibitors. The membrane localization domain is modified with lipids that are required for cell membrane localization. Mutations in the membrane localization domain prevent normal function.

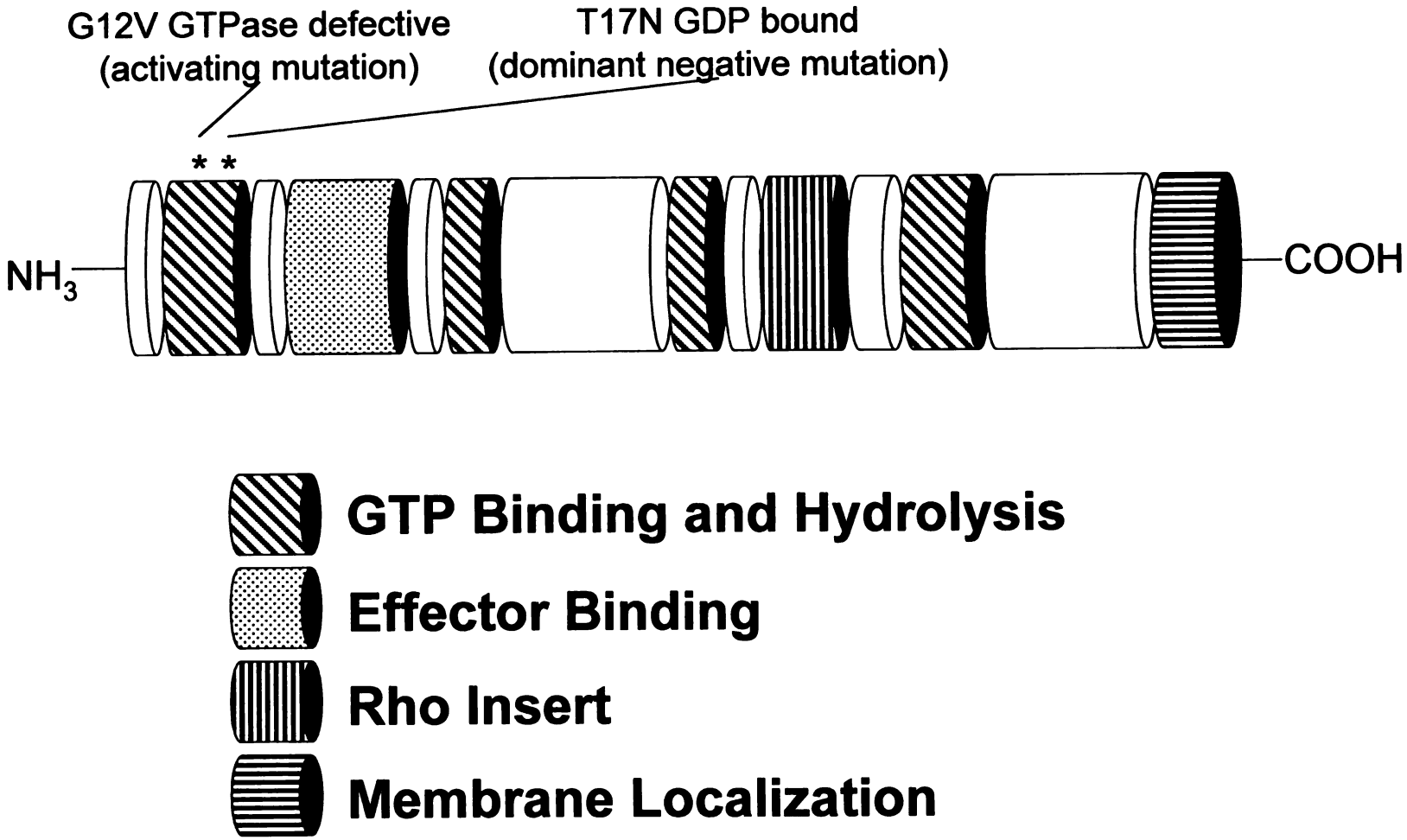
G12V GTPase defective
(activating mutation)
T17N GDP bound
(dominant negative mutation)
* *
NH3
COOH
GTP Binding and Hydrolysis
Effector Binding
Rho Insert
Membrane Localization

Figure 3

For example, over-expression and activation of both Rac1 and Cdc42 has been observed in breast cancer (345). This observation implicates GEFs and GAPs, proteins that regulate Rac1 and Cdc42 activity, as critical factors the transformation of cells.

2. Transformation Induced by GEFs and GAPs of Rac1 and Cdc42

GEFs and GAPs specific to Rho-family proteins were originally identified as transforming proteins and only later identified as regulators of Rho activation. These proteins are characterized by the presence of DH and immediately adjacent PH domains. However, most Rho-family GEFs were identified by having the ability to induce foci when over-expressed in NIH3T3 fibroblasts. Some GEFs exhibit Rho-GTPase specificity, while others activate more than one Rho protein. For instance, Lfc is specific for Rho, Fgd1 is specific for Cdc42 and Tiam1 is specific for Rac1 (346-348). However, the GEF Vav activates RhoA, Rac1 and Cdc42 (299, 349-351).

There are three members of the Vav GEF family including Vav, Vav2 and Vav3 (Reviewed in (294)). Activation of Vav is induced by activated RTK's including EGFR (352). Up-regulation and activation of EGFR is observed in various cancer types (353). For this reason, through Rho GTPases, Vav may be involved in modulating EGFR mediated oncogenesis. Fernandez-Zapico et al. (354) were the first to describe a human malignancy with increased Vav expression. Their data indicates that Vav is over-expressed in pancreatic adenocarcinomas and is associated with decreased survival of these patients.

Crespo et al. (355) found that Vav induces transformation of NIH3T3 fibroblasts through activation of Rac1. Briefly, activated Vav was expressed alone or along with the $Rac1^{N17}$ dominant negative mutant. Expressing Vav alone induced foci. Co-expression of $Rac1^{N17}$ and Vav drastically reduced this effect. Recently Palmby et al. showed that expression of $Cdc42^{N17}$ or dominant-negative Rho ($RhoA^{N19}$) also inhibits transformation induced by Vav. Furthermore, they determined that Vav activation of NFκB and c-jun are important mediators of Vav induced transformation. In summary, this research indicates that Vav acts through Rac1, Cdc42 and RhoA to regulate cell proliferation and transformation.

Although very rare, deregulated Rho-GTPase activation can also be a result of GAP inactivation. Various studies have found deletions or mutations of Rho-GAPs in cancers such as juvenile myelomonocytic leukemia, acute myeloid leukemia and hepatocellular carcinoma (356-358).

3. Rac1 and Cdc42 in $HRas^{V12}$-Induced Transformation

Several reports (255, 359-362) indicate that expression $Rac1^{V12}$ and/or $Cdc42^{V12}$ is sufficient for cellular and malignant transformation of rodent fibroblasts. Furthermore, in rodent fibroblasts, Rac1 and Cdc42 activity is required to mediate cellular and malignant transformation induced by Ras^{V12} (255, 362).

Over-expression of GEFs induces cellular transformation of NIH3T3 mouse fibroblasts. Therefore, it was hypothesized that expression of $Rac1^{V12}$ and/or $Cdc42^{V12}$ may induce transformation as well. Qiu et al. found that expression of $Rac1^{V12}$ or $Cdc42^{V12}$ in mouse and rat fibroblasts was weakly

transforming compared to $HRas^{V12}$ expression i.e. they formed small foci and were able to proliferate in media with reduced serum (255, 360-363). These data indicate that while Rac1 and Cdc42 may play a role in $HRas^{V12}$-meditated transformation, other pathways, perhaps the Raf-MEK-ERK, or RalGDS pathways, are necessary to induce complete transformation. However, individual expression of activated Rac1 or Cdc42 in rodent fibroblasts induces malignant transformation i.e. they form sarcomas when injected s.c. into athymic mice (255, 359, 362). This suggests that Rac1 and Cdc42 play an oncogenic role in malignant transformation. To my knowledge, the ability for activated Rac1 or Cdc42 to induce transformation in human fibroblasts has not been studied.

Rac1 and Cdc42 also play a supporting role in $HRas^{V12}$-mediated transformation. Inhibition of Rac1 or Cdc42 by expression of $Rac1^{N17}$ or $Cdc42^{N17}$ dominant-negative proteins inhibits $HRas^{V12}$ induced focus formation (255, 362). Furthermore, several studies indicate that Rac1 and Cdc42 play distinct roles in mediating $HRas^{V12}$-transformation. For example, expression of $Cdc42^{L28}$ induces anchorage independent growth to a much greater level than expression of $Rac1^{L28}$ (359). However, $Rac1^{N17}$, but not $Cdc42^{N17}$, inhibits the growth of $HRas^{V12}$-transformed cells in reduced serum (359). This indicates that Cdc42 plays a more dominant role in the cells ability to form colonies in agarose, but Rac1 plays a predominant role in inducing growth in reduced serum. Rangarajan et al. (364) found that Ras effector pathways, i.e. Raf-MEK-ERK, PI3K, and RalGDS, play very different roles in $HRas^{V12}$-induced transformation based on the species and cell types studied. For this reason, research that focuses on the

contribution of Rac1 and Cdc42 in human derived fibroblasts would provide a greater understanding of their importance in $HRas^{V12}$-induced transformation.

Currently, very little is known about the effector pathways mediated by aberrant Rac1 and/or Cdc42 activity that contribute to transformation. However it seems probable that these G-proteins elicit their transforming effect through mitogenic signaling pathways. Recent evidence suggests that regulation of HIF and JNK pathways by Rac1 and Cdc42 plays a role in angiogenesis. Hirota et al. (162) reported that Rac1 activity is required for the activation of HIF. Briefly, cells were co-transfected with $Rac1^{N17}$ and a reporter gene under the control of the VEGF promoter. When exposed to hypoxia, inhibition of Rac1 suppressed hypoxia-induced transcription. This data is supported by Xue et al. (161), who show that both Rac1 and Cdc42 activities are induced upon hypoxia, and their induction leads to increased activation of HIF. Furthermore, a recent report from Saniger et al. (163) indicates that in non-hypoxic conditions, Rac1 and Cdc42 mediate the expression of VEGF at a transcriptional level through c-jun. It is not known whether Rac1 and Cdc42 mediate the expression of VEGF in the context of oncogene signaling.

In an attempt to identify genes that are regulated by the activation of Rac1 and Cdc42, Teramoto et al. (365) used a mouse cDNA microarray chip which contained sequence representation of 19,117 unique genes to determine the profile of Rac1 or Cdc42 regulated genes in NIH3T3 fibroblasts. They observed up-regulation of numerous ECM proteins including fibronectin, vinculin and collagen. Unexpectedly, mitogenic signaling pathways were not elucidated.

However, these experiments were not conducted in the context of $HRas^{V12}$-activation. This may be the reason Rac1 and Cdc42 regulated signaling pathway were not identified. Experiments of this kind in human cells transformed by $HRas^{V12}$ are needed to determine what genes, downstream of Rac1 and Cdc42, are required for Ras^{V12}-transformation.

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CHAPTER II

RAC1 AND CDC42 ARE REQUIRED IN HRAS^{V12}-MALIGNANT TRANSFORMATION OF HUMAN FIBROBLASTS AND IN VEGF AND UPA EXPRESSION

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Abstract

To determine whether the activity of Rac1, Cdc42, or both proteins is required to mediate $HRas^{V12}$-induced malignant transformation of human fibroblasts, and to identify any Rac1- and Cdc42-regulated genes whose expression plays a role in such transformation, we inhibited Rac1 or Cdc42 activity, or the activities of both proteins, by expressing dominant-negative $Rac1^{N17}$ and/or $Cdc42^{N17}$ in an $HRas^{V12}$-malignantly transformed human fibroblast cell line, PH3MT. Inhibition of Rac1 significantly suppressed tumor formation. The results of experiments designed to inhibit expression of Cdc42 were not as consistent. Nevertheless, in every instance when tumors formed, analysis of the cells from the tumors revealed that dominant-negative $Rac1^{N17}$ and $Cdc42^{N17}$ were no longer expressed. These results indicate that for $HRas^{V12}$-induced malignant transformation of these human fibroblasts, Rac1 and Cdc42 activity is required. We also demonstrated that expression of constitutively-active $Rac1^{V12}$ or $Cdc42^{V12}$, in the absence of $HRas^{V12}$, failed to malignantly transform the parental infinite life span cell strain, MSU-1.1, from which the PH3MT cell line was derived. These results indicate that activation of parallel $HRas^{V12}$-induced pathways is required to induce malignant-transformation. To identify genes whose expression is controlled by Rac1 and/or Cdc42, we carried out microarray analysis. The results identified 29 genes, such as *uPA* and *VEGF*, whose mRNA expression is affected by the activity of Rac1 and/or Cdc42. Using ELISA assays to determine if inhibition of Rac1 alone, Cdc42 alone, or both proteins results in decreased levels of secreted uPA or VEGF proteins, we found that in the

HRas^{V12}-transformed human fibroblast cell line, PH3MT, Rac1 and Cdc42 independently regulate secreted levels of uPA and VEGF under non-hypoxic and hypoxic conditions. We also found that expression of Cdc42^{V12}, but not Rac1^{V12} was able to induce high levels of secreted VEGF protein in the MSU-1.1 parental cell strain.

Introduction

The Ras-family GTPases regulate multiple cell processes, including cellular proliferation, differentiation, and actin-cytoskeletal organization. Altered expression or activation (mutated form) of *Ras* oncogenes has been found in 30% of human cancers (1, 2). Ras acts as a molecular switch by cycling between an inactive GDP-bound state and an active GTP-bound conformation. In its active form, Ras initiates mitogenic signals through various pathways, including the well-studied Raf-MEK-ERK1/2, PI3K/Akt, and RalGDS cascades.

Transformation of NIH3T3 mouse fibroblasts and Rat1 fibroblasts, by oncogenic Ras protein expression, requires the activities of small Rho-GTPases Rac1 and Cdc42 (3, 4). Traditionally, constitutively-active (V12) mutants, or dominant-negative (N17) mutants of Rac1 and Cdc42 have been used to elucidate their unique oncogenic roles. Rat1 fibroblasts, and NIH3T3 and Swiss-3T3 mouse fibroblasts, that express activated forms of either Rac1 or Cdc42 are able to form sarcomas following subcutaneous injection into athymic mice. In NIH3T3 mouse fibroblasts, and Rat1 fibroblasts, $Rac1^{V12}$ expression confers growth factor independence, whereas $Cdc42^{V12}$ expression confers anchorage independent growth (3). However, in Swiss-3T3 mouse fibroblasts, both constitutively-activated proteins confer growth in medium with reduced serum (5). This indicates that Rac1 and Cdc42 may have distinct roles in transformation depending on the cell line and/or species from which the cells are derived. Although it is clear that Rac1 and Cdc42 play a role in $HRas^{V12}$-induced

transformation of rodent fibroblasts, it is thought that $HRas^{V12}$-induced transformation of human fibroblasts is mechanistically distinct (6).

The functions of Rac1 and Cdc42 were originally investigated in Swiss-3T3 mouse fibroblasts, and were found to be regulators of the actin cytoskeleton (7). Rac1 controls lamellipodia and ruffling behavior, whereas Cdc42 is involved in the extension of filipodia. However, Rac1 and Cdc42 also have signaling functions. For example, they contribute to the regulation of several signal transduction proteins, including p21-activated kinase (PAK), p38/stress-activated protein kinases (SAPK), c-jun N-terminal kinases (JNK), nuclear factor κB (NFκB), and serum-responsive factor (SRF) (8). To elucidate the complex molecular mechanisms by which these proteins regulate cellular transformation, Teramoto et al. (9) found that a diverse set of downstream targets, including extracellular-matrix components and signaling molecules are regulated by Rac1 and Cdc42 activity in NIH3T3 mouse fibroblasts.

In the present study, we wished to determine whether the activity of Rac1 or Cdc42, or both is required for $HRas^{V12}$-induced transformation of human fibroblasts. Moreover, we sought to identify Rac1- and/or Cdc42-mediated gene expression differences in the context of oncogenic HRas signaling. The data presented in this study confirm that both Rac1 and Cdc42 activity is required for such $HRas^{V12}$-induced transformation. In addition, using a genomic array approach, we identified 29 genes whose expression in $HRas^{V12}$-transformed cells is regulated by Rac1 and Cdc42. Many of these genes, e.g. vascular endothelial

growth factor (*VEGF*) and urokinase plasminogen activator (*uPA*), are known to play a significant role in cancer.

Materials and Methods

Cell Strains and Culture Conditions

Unless otherwise indicated, the growth medium for human foreskin-derived fibroblasts, i.e. MSU-1.1 and PH3MT strains and their derivatives, consisted of Eagle's minimal essential medium supplemented with 0.2 mM L-aspartic acid, 0.2 mM L-serine, 1.0 mM sodium pyruvate, 10% supplemented calf serum (SCS) (Hyclone Laboratories, Logan, UT), 100 units/ml penicillin, and 100 μg/ml streptomycin. All cell lines were grown at 37°C in a humidified incubator containing 5% CO_2, 95% ambient air.

In an effort to determine the genetic changes required for malignant transformation of human fibroblasts, McCormick, Maher, and their colleagues developed the MSU-1 lineage of human fibroblasts, beginning with finite life span skin fibroblasts from a normal neonate (10). The MSU-1.1 cell strain in that lineage is a chromosomally-stable, near-diploid, telomerase-positive, infinite life span cell strain that is capable of being transformed into malignant cells by various approaches (11-15). For example, over-expression of oncogenic T24 HRas protein, which contains an activating V12 mutation ($HRas^{V12}$), in MSU-1.1 cells resulted in malignant transformation i.e., the cells were able to form sarcomas when injected subcutaneously into athymic mice (11). One cell line derived from such a tumor, designated PH3MT, was used in the present study.

Construction of Human Cell Strains Expressing Dominant-negative Mutant Genes Under the Control of Tetracycline

The pTet-tTAk and pTet-splice vectors were obtained from Invitrogen (Carlsbad, CA). This Tet-off system allows tetracycline-regulated expression of genes in mammalian cells. To facilitate selection, we constructed a pTet-tTAk vector that contains a gene coding for histidinol resistance, which we designate pTet-tTak^{HisR}. We also constructed a pTet-splice vector containing a gene coding for puromycin resistance, which we designate pTet^{PuroR}. The Flag-Cdc42^{N17} and myc-Rac1^{N17} mutant cDNAs were derived by PCR amplification using PFU polymerase (Stratagene, La Jolla, CA), and separately subcloned into the pTet^{PuroR} vector. The cDNA templates used for PCR amplification of Cdc42^{N17} and Rac1^{N17} were kindly provided by Dr. K. Gallo (Michigan State University, East Lansing, MI) and Dr. G. Bokoch (Scripps Institute, La Jolla, CA), respectively. We verified the resulting pTet-Flag-Cdc42^{N17} and pTet-myc-Rac1^{N17} vector constructs by automated DNA sequencing (Visible Genetics, Bayer, Toronto, ON, Canada). Lipofectamine (Invitrogen, Carlsbad, CA) was used to transfect the plasmids into human cells.

PH3MT cells were transfected with the pTet-tTak^{HisR} vector, selected using histidinol (1mM) (Sigma, St. Louis, MO), and designated PH3MT-tTak-C1. Such cells were then transfected with the empty vector pTet^{PuroR}, or with the pTet^{PuroR} vector containing myc-Rac1^{N17} cDNA, or the pTet^{PuroR} vector containing FLAG-Cdc42^{N17} cDNA, and selected using puromcyin (0.5 μg/ml) (Sigma, St. Louis, MO). A puromycin resistant strain resulting from transfection of the empty

vector was designated PH3MT-VC-C2. Two independent clones exhibiting tetracycline-regulated expression of myc-Rac1^{N17} were chosen for study and designated PH3MT-Rac1^{N17} (-C1 and -C2). Two independent clones with regulatable FLAG-Cdc42^{N17} expression were similarly selected and designated PH3MT-Cdc42^{N17} (-C1, and C2). A sixth cell strain, designated PH3MT-Rac1^{N17}/Cdc42^{N17}, expresses both myc-Rac1^{N17} and FLAG-Cdc42^{N17} dominant-negative proteins.

Construction of Cell Strains Expressing Constitutively-activated Mutants

To generate MSU-1.1 cell strains expressing GFP-Rac1^{V12} or GFP-Cdc42^{V12}, the GFP nucleotide sequence from the pCRUZ-GFP vector (Santa Cruz Biotechnology, Santa Cruz, CA) was isolated and ligated into the pcDNA6-V5-HisA vector (Invitrogen, Carlsbad, CA), which confers blasticidin resistance. Rac1^{V12} and Cdc42^{V12} cDNA template sequences were purchased from University of Missouri-Rolla Research Center (UMR) (Rolla, MO), PCR-amplified, and ligated downstream of the GFP nucleotide sequence to enable the transfectants to express N-terminally labeled proteins. The resulting constructs were transfected into the parental, non-transformed MSU-1.1 cell strain. Clones were selected for with blasticidin (1 μg/ml) (Invitrogen, Carlsbad, CA) and screened by fluorescence microscopy for expression of the GFP-fusion protein. Identified clones were isolated, expanded and screened for GFP-tagged Rac1^{V12} or Cdc42^{V12} protein expression by Western blotting.

Cell Lysates and Western Blot Analysis

Whole cell protein extracts were prepared using a lysis buffer consisting of 50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 50 mM NaF, 0.5% NP-40, 1 mM Na_3V0_4, 200 mM benzamidine, 1 mM PMSF, 25 μg/ml aprotinin, and 25 μg/ml leupeptin. Total protein concentration was quantified using the Coomassie protein assay reagent (Pierce Biotechnology, Rockford, IL). Lysates were denatured in 5X Laemelli sample buffer, separated by either 10% or 12% SDS-PAGE, and transferred to PVDF membrane. The membrane was blocked for 2 h with Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% (w/v) non-fat milk. For the majority of the studies, the membrane was probed with the primary antibody at 4°C overnight, then probed with the appropriate horseradish peroxidase-linked secondary antibody (Sigma and Santa Cruz Biotechnology), for 1 hr at room temperature. Both antibodies were diluted in TBST containing 5% milk. The membrane was incubated with the Supersignal West Pico chemiluminescent horseradish peroxidase substrate (Pierce Biotechnology, Rockford, IL) and then exposed to film. The primary antibodies used were anti-FLAG, 1:1000 dilution (Sigma, St. Louis, MO), anti-myc 9E10, 1:500 dilution, and anti-GFP, 1:1000 dilution (Santa Cruz Biotechnology, Santa Cruz, CA).

Assay for Growth Factor Independence

To determine if MSU-1.1 cells expressing constitutively-active Rac1 or Cdc42 had acquired the ability to proliferate in 0.5% serum, cells expressing these proteins were plated at a density of 1 x 10^4 cells per 60-mm diameter dish in growth medium containing 10% serum and incubated for 48 hrs. The cells

were then washed twice and medium containing 0.5% SCS was added. Cells from three replicate dishes were counted at regular intervals and plotted using Microsoft Excel. An equation derived from a best-fit exponential curve was used to determine the doubling time of cells in log-phase growth. This experiment was complete three times.

Assay for Anchorage-independence

The ability for cells to form large colonies in agarose is indicative of the ability of these cells to form tumors when injected into athymic mice. To determine if MSU-1.1 cells expressing constitutively-active Rac1 or Cdc42 had acquired the ability to form large colonies in agar, 5,000 cells were plated in 0.33% top agarose per 60-mm-diameter culture dish and covered with 2 mL of growth medium. The growth medium was replaced weekly. After 3 wks, the cells were fixed with 2.5% glutaraldehyde. This experiment was completed three times.

Assay for Tumorigenicity

To determine if PH3MT derivative cell strains expressing dominant-negative proteins were able to form tumors in athymic mice, 1 X 10^6 cells were injected subcutaneously into the right and left flank of athymic BALB/c mice. To suppress dominant-negative protein expression, drinking water of such mice was supplemented with 1 mg/ml tetracycline. All mice were given drinking water containing 5% sucrose to mask the flavor of tetracycline. Tumor measurements were made weekly, and mice were sacrificed when a tumor on either flank

reached a volume of 1 cm^3. Kaplan-Meier survival plots were constructed using MedCalc Version 8.2 (http://www.medcalc.be). A log-rank test was used to determine statistical significance.

To determine if the MSU1.1 derivative cell strains expressing GFP-Rac^{V12} or GFP-$Cdc42^{V12}$ were able to form tumors in athymic mice, 1 X 10^7 cells were injected directly into an absorbable gelatin sponge (Gelfoam size 50, Pharmacia) that was cut into 1 cm cubes and inserted into each injection site 1 wk prior to injection. Sponges were used to ensure that a high number of injected cells were contained at the injection site.

Affymetrix GeneChip Expression Analysis

PolyA+RNA was extracted using the Micropure PolyA+RNA extraction kit following the instructions provided by the manufacturer (Ambion, Applied Biosystems, Austin, TX). The detailed steps to synthesize cRNA products from 3 μg of polyA+RNA were followed as described in the Affymetrix expression analysis technical manual. Personnel at the Genomics Technology Sequence Facility at Michigan State University carried out the hybridization to the Affymetrix HU95A human genome chip and probed, washed and scanned arrays as described in the manual. This experiment was carried out in duplicate.

Total mRNA expression was calculated for each gene represented on the Affymetrix chip. Expression differences were calculated within and between experiments, resulting in four separate data sets, i.e. both data sets collected from cells grown in the presence of tetracycline were compared to both data sets collected from cells grown in the absence of tetracycline. Comparisons were

made using MAS5.0 software (Affymetrix, Santa Clara, CA). All significant changes in each of the four comparisons, as determined by MAS5.0, were identified using the Microsoft Access query tool and are shown in Table 1.

ELISA Analyses

To detect levels of secreted uPA or VEGF protein in PH3MT cells expressing myc-Rac1^{N17} or FLAG-Cdc42^{N17}, cells expressing either of these proteins, or both were plated in 100 mm dishes in growth medium containing tetracycline (1 mg/ml). After 24 hrs, cells were washed twice, and tetracycline-free medium was added. Cells were incubated for another 24 hrs to allow for dominant-negative expression. At this time, cells were washed twice, serum starved 24 hrs, and then stimulated with growth medium, cobalt chloride ($CoCl_2$) (100 μM), desferrioxamine (DFO) (100 μM) or incubated in a hypoxic chamber (1% O_2) for 24 hrs. The media were collected, and centrifuged at 5000 x g for 5 min. To detect secreted VEGF protein, a sandwich ELISA using the human VEGF DuoSet (R and D Systems, Minneapolis, MN) ELISA kit was used following the manufacturer's protocol. To detect secreted levels of uPA protein, a sandwich ELISA using the uPA ELISA kit (Oncogene Science, Bayer, Cambridge, MA) was used following the manufacturer's protocol.

To determine the level of secreted VEGF and uPA protein from MSU-1.1 derivative cell strains expressing GFP-Rac1^{V12} or GFP-Cdc42^{V12}, 1.5 X 10^5 cells were plated in a 100 mm tissue culture dish, and incubated for 24 hrs. At this time, cells were washed twice with serum-free medium, and serum-starved for another 24 hrs. Cells were then stimulated with growth medium containing 10%

SCS. Using the previously described procedure, levels of both uPA and VEGF proteins were detected. For all ELISA analyses, after the removal of conditioned medium, whole cell protein extracts were made, quantitated and used to normalize detected uPA and VEGF protein levels by dividing the concentration of uPA or VEGF protein in the conditioned medium by the total amount of protein in the whole cell extract. The results are expressed as either percent of vector control, or fold change. Each graph represents one experiment containing replicate dishes. Each experiment was completed three times, and similar results were observed in each. Error bars indicate the standard deviation from the mean. The student's *t*-test was used to determine statistical significance.

Results

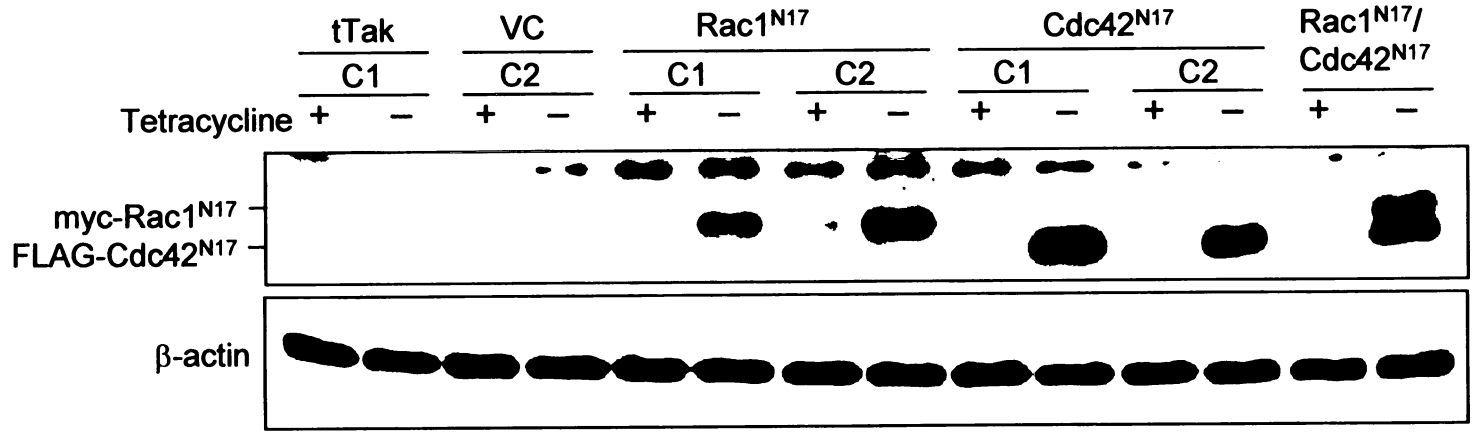
Role of Rac1 and Cdc42 Proteins in $HRas^{V12}$-transformation of Human Fibroblasts

To investigate whether the activity of Rac1, Cdc42, or both proteins is required for $HRas^{V12}$-induced malignant transformation of human fibroblasts, we transfected the $HRas^{V12}$-malignantly transformed human cell line, PH3MT (11), with a gene coding for a dominant-negative form of Rac1, a dominant negative form of Cdc42, or with both genes, whose expression was negatively regulated by tetracycline (Fig. 1). These derivative strains were then examined for evidence that Rac1 and/or Cdc42 play a role in the transformation of PH3MT cells.

To determine if inhibition of Rac1, Cdc42, or both alters the ability of these cells to form tumors in athymic mice, the transfected cells expressing dominant-negative $Rac1^{N17}$ or dominant negative $Cdc42^{N17}$, or both mutant genes, were injected subcutaneously into athymic mice and tumor growth was monitored. Mice were also injected with the parental cell strain, PH3MT-tTAk-C1, and a vector control cell strain PH3MT-VC-C2, as controls. As shown in Fig. 2A and 2B, by 15 weeks, all mice that had been injected with the latter two cell strains, in the presence or absence of tetracycline, developed tumors and were sacrificed ($p>0.1$).

In contrast, dominant-negative interference of Rac1 activity in PH3MT cells designated PH3MT-$Rac1^{N17}$-C1 resulted in decreased tumor forming ability, and mice injected with these cells showed a significant prolongation of a tumor-free lifespan (Fig. 2C). Not all the mice escaped tumor-formation, and we

Figure 1. Tetracycline-regulatable expression of myc-Rac1^{N17} and/or FLAG-Cdc42^{N17} in HRas^{V12}-transformed human fibroblasts (PH3MT). Cells were grown in the presence or absence of tetracycline (Tet) (1 μg/ml). PH3MT-tTak-C1 is the parental cell strain. PH3MT-VC-C2 is transfected with empty vector. Whole cell extracts were made and Western blots were probed with either myc or FLAG antibody. β-actin levels were used to indicate loading.


tTak
VC
Rac1N17
Cdc42N17
Rac1N17/
Cdc42N17
C1
C2
C1
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C1
C2
Tetracycline + − + − + − + − + − + − + −
myc-Rac1N17
FLAG-Cdc42N17
β-actin

Figure 1

Figure 2. Evidence that Rac1 activity is required for $HRas^{V12}$-induced tumor formation. Solid lines represent mice that were not administered tetracycline (Tet). Dotted lines represent mice injected with the same cell strain, but mice were given Tet in order to suppress dominant-negative expression. Tumors were measured weekly. When tumors reached a volume of approximately 0.5 cm^3, mice were sacrificed and the tumors were removed for study. The data are plotted using Kaplan-Meier analyses. *A*, PH3MT-tTak-C1 (parent); N=4 (– Tet), N=4 (+ Tet) $p>0.1$. *B*, PH3MT-VC-C2 (vector control); N=5 (– Tet), N=6 (+ Tet) $p>0.1$. *C*, PH3MT-$Rac1^{N17}$-C1; N=7 (– Tet), N=9 (+ Tet), $p<0.001$. *D*, PH3MT-$Rac1^{N17}$-C2; N=4 (– Tet), N=6 (+ Tet), $p>0.1$. Directly below their respective Kaplan-Meier plots, are Western blots probed with myc (9E10) antibody to detect dominant-negative protein expression in two tumor-derived cell lines (Tumor 1 and Tumor 2) Both blots were probed with β-actin to verify loading.

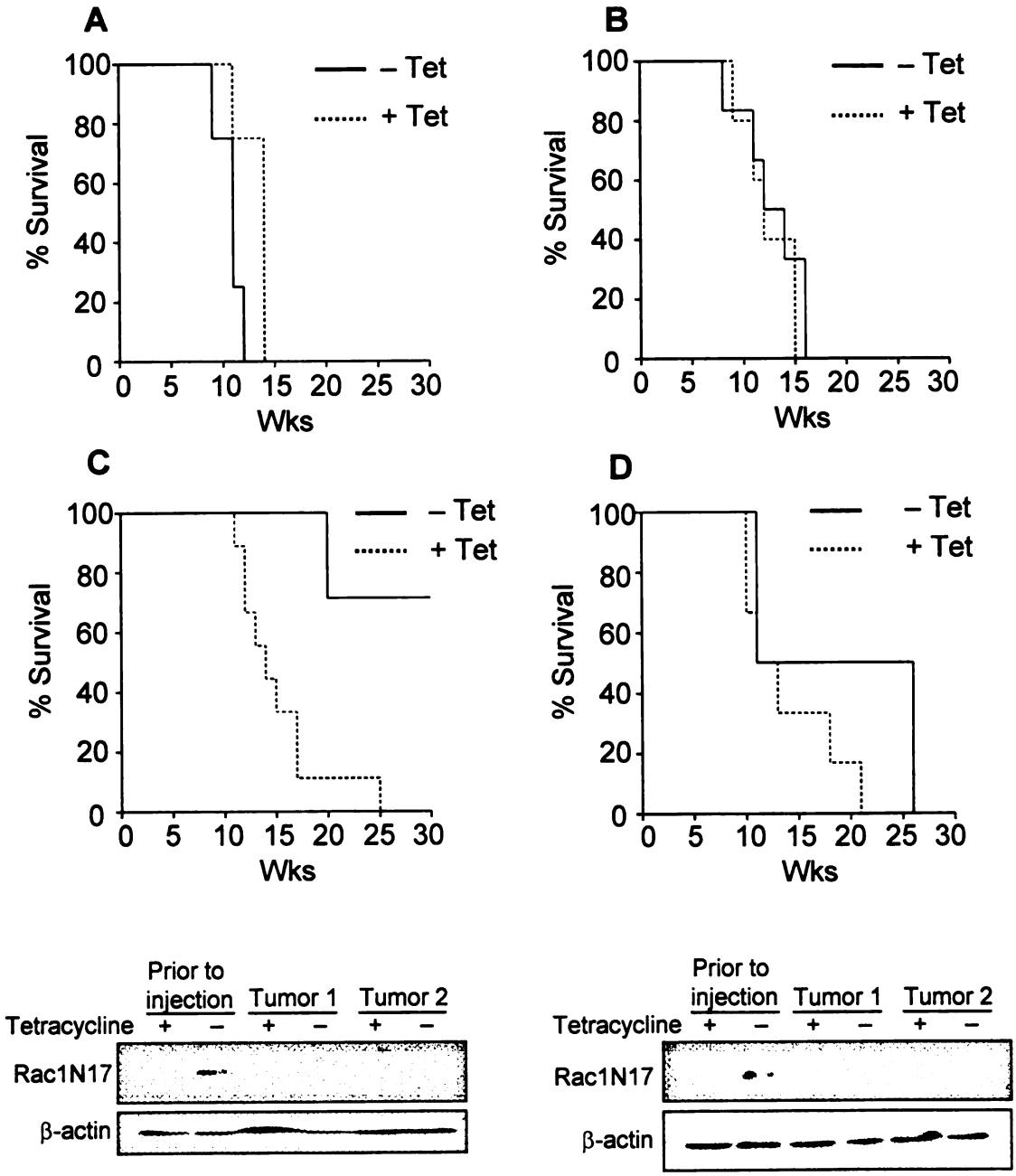

A
B
C
D
100
80
60
40
20
0
% Survival
0 5 10 15 20 25 30
Wks
– Tet
+ Tet
Prior to injection
Tumor 1
Tumor 2
Tetracycline
+ – + – + –
Rac1N17
β-actin

Figure 2

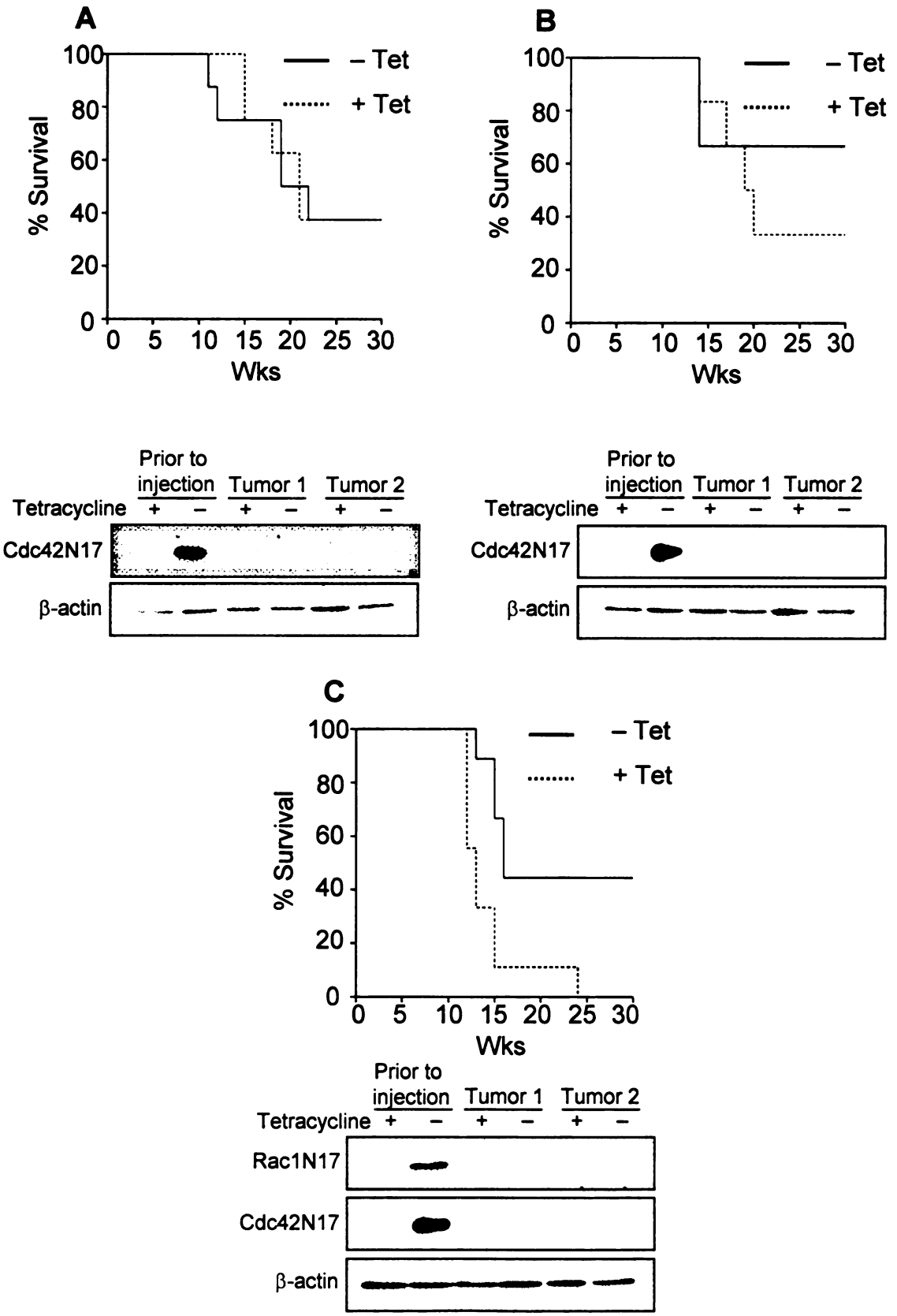
hypothesized that cells making up these tumors had lost expression of the myc-Rac1^{N17} dominant-negative protein. To test this, using Western blot analysis, we compared the level of myc-Rac1^{N17} expression in cells derived from two such tumors to that which was expressed in the original cell strain used for injection. As shown in Fig. 2C, the former had lost all expression of dominant negative myc-Rac1^{N17} protein. These results indicate that Rac1 expression plays a critical role in malignant transformation of human fibroblasts by the HRas^{V12} oncogene.

A similar situation was found when such a study was carried out with malignant PH3MT-cells transfected with dominant-negative FLAG-Cdc42^{N17}. The results are shown in Figs. 3A and 3B.

Whereas the Kaplan-Meier survival analyses do not indicate a statistically-significant difference in the number of mice that had to be sacrificed because they developed tumors following injection of the cell strains PH3MT-Rac1^{V12}-C2 (Fig. 2*D*), PH3MT-Cdc42^{V12}-C1 (Fig. 3*A*), and PH3MT-Cdc42^{V12}-C2 (Fig. 3*B*), analyses of dominant-negative protein expression following tumor resection indicate that only cells that no longer express detectable levels of myc-Rac1^{N17} or FLAG-Cdc42^{N17} are able to form tumors. As shown in Fig. 3C, when we inhibited both Rac1 and Cdc42, the tumor-free survival of mice injected with the PH3MT-Rac1^{N17}/Cdc42^{N17} cell strain was significantly prolonged ($p<0.0001$). Western blot analysis of two tumor derived cell lines indicated results similar to those described above. This suggests that the activity of both Rac1 and Cdc42 is essential for HRas^{V12}-induced transformation of human fibroblasts.

Figure 3. Evidence that Rac1 and/or Cdc42 activity is required for $HRas^{V12}$-induced tumor formation. Tumors were measured and volumes were calculated weekly. When tumors reached a volume of approximately 0.5 cm^3, mice were sacrificed and the tumors were removed for further study. Kaplan-Meier survival analyses were carried out. Mice were given Tet to suppress the expression of dominant-negative protein. Solid lines indicate mice injected with a cell strain transfected with a vector which codes for $Cdc42^{N17}$ or with two vectors, one that codes for $Rac1^{N17}$ and another that codes for $Cdc42^{N17}$. Dotted lines represent mice injected with the same cell strains, but Tet was administered in order to suppress mutant protein expression. Directly below their respective Kaplan-Meier plots are Western blots probed with myc (9E10) or FLAG antibody to detect dominant-negative protein expression in two tumor derived cell lines (Tumor 1 and Tumor 2) *A*, PH3MT-$Cdc42^{N17}$-C1; N=8 (– Tet), N=8 (+ Tet), $p>0.1$. *B*, PH3MT-$Cdc42^{N17}$-C2; N=6 (– Tet), N=6 (+ Tet), $p>0.1$. *C*, PH3MT-$Rac1^{N17}$/$Cdc42^{N17}$; N=9 (– Tet), N=9 (+ Tet) $p<0.001$. All blots were probed with β-actin to verify loading.

Figure 3


A
100
80
60
40
20
0
% Survival
0 5 10 15 20 25 30
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B
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% Survival
0 5 10 15 20 25 30
Wks
– Tet
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Prior to injection
Tumor 1
Tumor 2
Tetracycline + – + – + –
Cdc42N17
β-actin
Prior to injection
Tumor 1
Tumor 2
Tetracycline + – + – + –
Cdc42N17
β-actin
C
100
80
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% Survival
0 5 10 15 20 25 30
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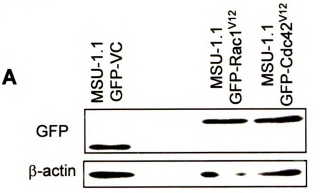
Prior to injection
Tumor 1
Tumor 2
Tetracycline + – + – + –
Rac1N17
Cdc42N17
β-actin

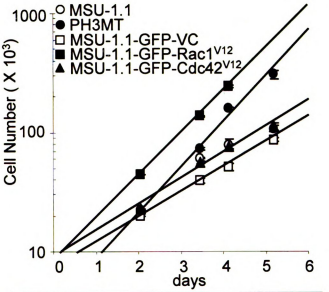
Role of $Rac1^{V12}$ or $Cdc42^{V12}$ Protein in the Transformation of Human Fibroblasts

In mouse and rat fibroblasts, expression of constitutively-active $Rac1^{V12}$ or $Cdc42^{V12}$ induces malignant transformation (3, 4). Therefore, we hypothesized that expression of GFP-$Rac1^{V12}$ or GFP-$Cdc42^{V12}$ in human fibroblasts would induce malignant transformation. To test this hypothesis, we stably transfected the parental MSU-1.1 cell strain, the same strain that was transformed by $HRas^{V12}$ expression, with a vector coding for GFP-$Rac1^{V12}$ or GFP-$Cdc42^{V12}$ (Fig. 4*A*), and assayed them for the ability to grow in medium with reduced serum, form large colonies in agarose, and develop into sarcomas in athymic mice. Expression of GFP-$Rac1^{V12}$, but not GFP-$Cdc42^{V12}$ permits human MSU-1.1 fibroblasts to grow in medium containing 0.5% serum (Fig. 4*B*). The doubling time of the parental MSU-1.1 cell strain, and the vector control cell strain MSU-1.1-GFP-VC, in such medium is 33 and 36 hrs respectively. Expression of GFP-$Cdc42^{V12}$ did not affect that doubling time. However, expression of GFP-$Rac1^{V12}$ reduces the doubling time to 21 hrs, which is similar to the 19 hr doubling time exhibited by $HRas^{V12}$-transformed MSU-1.1 derivative cell line PH3MT. To determine if expression of $Rac1^{V12}$ or $Cdc42^{V12}$ in human fibroblasts confers the ability for cells to form large colonies in agarose, MSU-1.1 cell strains expressing either activated protein were suspended in agarose and monitored for colony formation (Fig. 4*C*). In agreement with studies in mouse and rat fibroblasts (3), expression of $Rac1^{V12}$ in MSU-1.1 cells allows formation of small colonies,

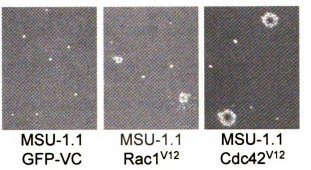
Figure 4. Evidence that expression of $Rac1^{V12}$ or $Cdc42^{V12}$ in human fibroblasts does not induce malignant transformation. *A*, MSU-1.1 fibroblasts expressing either GFP alone (MSU-1.1-GFP-VC), or GFP-tagged constitutively-activated proteins. *B*, the indicated cell strains were grown in medium with reduced serum (0.5% SCS). Growth curves were plotted. Doubling time was calculated based on an equation derived from a best-fit exponential curve when cells were in log-phase growth. Error bars represent the SD of triplicate experiments. *C*, the indicated cell strains were plated in 0.33% agarose and grown for three weeks. Each picture represents one representative field from each cell line. This experiment was completed in triplicate, each with similar results.

Figure 4





Cell Strain	Doubling Time (hrs)
MSU-1.1	32.8
PH3MT	19.2
MSU-1.1-GFP-VC	35.8
MSU-1.1-GFP-Rac1^{V12}	20.5
MSU-1.1-GFP-CDC42^{V12}	32.9



whereas Cdc42^{V12} expression confers the ability for these cells to form large anchorage independent colonies.

To determine if constitutively-active Rac1^{V12} or Cdc42^{V12} expression in MSU-1.1 human fibroblasts results in malignant transformation, we injected MSU-1.1-GFP-Rac1^{V12} and MSU-1.1-GFP-Cdc42^{V12} cell stains into athymic mice. Neither GFP-Rac1^{V12} nor GFP-Cdc42^{V12} expression resulted in the ability for these cells to form tumors after 28 weeks (data not shown). It appears, therefore, that in contrast to studies conducted in rodent fibroblasts, expression of constitutively-active Rac1^{V12} alone, or Cdc42^{V12} alone, does not induce malignant transformation of human fibroblasts.

Identification of Rac1 and Cdc42 Regulated Genes in HRas^{V12}-transformed Human Fibroblasts

Because the activities of both Rac1 and Cdc42 are required in mediating HRas^{V12}-induced transformation, we hypothesized that the activities of these two G-proteins regulate the expression of genes whose expression is also required to mediate oncogenic HRas induced transformation. To identify these genes, mRNA was harvested from the PH3MT-Rac1^{N17}/Cdc42^{N17} cell strain grown either in the presence, or absence of tetracycline. This allowed us to use the same cell strain as both the control, i.e. normal Rac1 and Cdc42 signaling, and experimental, i.e. inhibited Rac1 and Cdc42 signaling, groups. Using Affymetrix GeneChip technology, a total of 29 significant expression differences were identified (Tbl. 1). Genes with a known role in cancer, such as *uPA* (16) and

Table 1. Summary of 29 Rac1 and Cdc42 regulated gene changes identified using Affymetrix GeneChip analyses.

Genes up-regulated		
Name	Fold Change	Reference
Ubiquitin Conjugating enzyme 12 (UBC12) *	6.4	(48)
Elongation factor-1 alpha-2 *	2.3	(49)
Guanine nucleotide-binding regulatory protein (G-y-alpha)	2.2	
mSin3A associated polypeptide p30 *	2.1	(50)
Rac protein kinase alpha	1.8	
Coupling protein G(s) alpha-subunit (alpha-S1)	1.8	
Histone H1x	1.8	
80K-L protein *	1.6	(51)
Killer cell lectin-like receptor, Subfam. C, member 2 (NKG2C) *	1.6	(52)
Alpha subunit of GsGTP binding protein	1.5	
Lipoprotein-associated coagulation inhibitor (LACI)	1.5	
ATP synthase aspha subunit	1.3	
Heat shock protein 70 (hsp70) *	1.3	(53)

Genes down-regulated		
Name	Fold Change	
Insulin-like growth factor-binding protein-3 *	-1.9	(54)
28s Ribosomal RNA gene	-1.6	
Cyclooxygenase-2 (hCox-2) *	-1.6	(47)
Vascular endothelial growth factor (VEGF) *	-1.6	(17)
Urokinase plasminogen activator (uPA) *	-1.5	(16)
Human KIAA0628	-1.5	
Asparagine synthetase	-1.5	
Axl tyrosine kinase receptor *	-1.5	(26)
High mobility group isoform C (HMGI-C) *	-1.5	(24)
Lnk adaptor protein	-1.4	
GTPase-activating protein ras p21 (RASA)	-1.4	
Human KIAA0728 protein	-1.3	
Axl tyrosine kinase receptor splice 2 *	-1.3	(26)
N-myristoyltransferase 2	-1.3	
Caveolin 2 *	-1.3	(55)
Glycosylphosphatidylinositol-H (GPI-H)	-1.3	

NOTE: The cell line PH3MT-Rac1^{N17}/Cdc42^{N17} was used to determine Rac1 and Cdc42 regulated gene expression differences in the HRas^{V12}-transformed human fibroblast cell strain (PH3MT). Inhibition of Rac1 and Cdc42 activity was regulated using tetracycline. The genes listed changed expression when both Rac1 and Cdc42 activities were inhibited.

* Denotes genes with a known role in cancer. References describing these roles are also provided.

VEGF (17), were of particular interest. We detected >1.5 fold decrease in both VEGF and uPA mRNA expression when both Rac1 and Cdc42 were inhibited. These data suggest that Rac1 and/or Cdc42 play a role in uPA and VEGF expression induced by oncogenic HRas.

Rac1 and Cdc42 Independently Regulate Secreted Levels of uPA Protein in the Context of Activated HRas

To determine if the difference in uPA mRNA levels corresponded to alterations in secreted levels of uPA protein, ELISA analyses were conducted (Fig. 5*A*). Inhibition of either Rac1 or Cdc42 in malignant PH3MT cells resulted in a 60% and 70% reduction in secreted uPA protein levels respectively. Inhibition of both proteins resulted in a greater reduction in uPA levels. These data indicate that both Rac1 and Cdc42 play an important role in $HRas^{V12}$-regulated uPA expression. The additive reduction of uPA levels upon inhibition of both Rac1 and Cdc42 indicates that both proteins independently regulate converging pathways that affect uPA expression in $HRas^{V12}$ transformed human fibroblasts.

To determine whether the expression of constitutively-active GFP-$Rac1^{V12}$ or GFP-$Cdc42^{V12}$ could increase secreted uPA protein levels in the parental, non-transformed MSU-1.1 cell strain, ELISA analyses were completed using both MSU1.1-GFP-$Rac1^{V12}$ and MSU-1.1-GFP-$Cdc42^{V12}$ cell strains. Expression of neither GFP-$Cdc42^{V12}$ nor GFP-$Rac1^{V12}$ resulted in an increase in levels of secreted uPA protein (Fig. 5B). Surprisingly, activation of Rac1 resulted in a small, but reproducible decrease in levels of secreted uPA protein. Therefore, Rac1 and Cdc42 are mediators of induced secreted uPA levels only in the

Figure 5. Evidence that Rac1 and Cdc42 independently regulate uPA expression. The indicated cell strains were serum starved for 24 hours then stimulated with medium containing 10% SCS. *A*, grown in the absence of tetracycline, PH3MT cell strains expressing a vector control (PH3MT-VC-C2), $Rac1^{N17}$ (PH3MT- $Rac1^{N17}$-C1), $Cdc42^{N17}$ (PH3MT-$Cdc42^{N17}$-C2) or both $Rac1^{N17}$ and $Cdc42^{N17}$ (PH3MT-$Rac1^{N17}$/$Cdc42^{N17}$) were tested for uPA expression levels using ELISA. Data is presented as percent of control. Error bars indicate the SD from triplicate experiments. * indicates significant difference, $p<0.05$. ** indicates a significant difference compared to PH3MT-$Rac1^{N17}$-C1 and PH3MT-$Cdc42^{N17}$-C2, $p<0.05$ *B*, conditioned medium was collected from MSU-1.1 cells expressing GFP alone (MSU-1.1-GFP-VC), GFP-tagged $Rac1^{V12}$ (MSU-1.1-GFP-$Rac1^{V12}$) or GFP-tagged $Cdc42^{V12}$ (MSU-1.1-GFP-$Cdc42^{V12}$), and uPA expression was analyzed. Data is presented as fold-induction of uPA expression. Error bars represent the SD from triplicate experiments. * indicates significant difference, $p<0.05$.

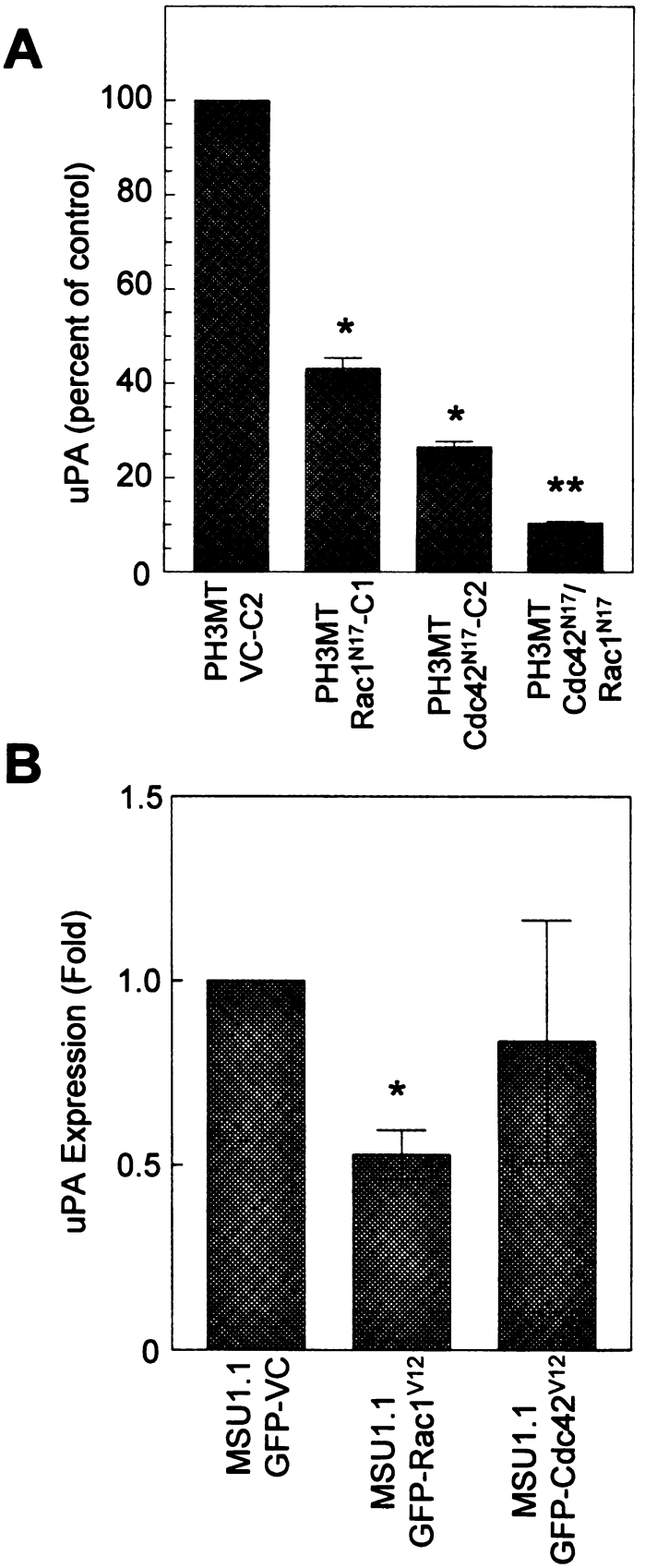

A
uPA (percent of control)
100
80
60
40
20
0
*
*
**
PH3MT VC-C2
PH3MT Rac1N17-C1
PH3MT Cdc42N17-C2
PH3MT Cdc42N17/ Rac1N17
B
uPA Expression (Fold)
1.5
1.0
0.5
0
*
MSU1.1 GFP-VC
MSU1.1 GFP-Rac1V12
MSU1.1 GFP-Cdc42V12

Figure 5

context of activated Ras. However, in the absence of activated Ras, activation of Rac1 may negatively impact the secretion of uPA protein.

Rac1 and Cdc42 Independently Regulate Secreted Levels of VEGF Protein

To determine if Rac1 and/or Cdc42 regulate the secreted levels of VEGF protein in PH3MT fibroblasts, we measured the amount of VEGF protein secreted from PH3MT cells strains expressing either, or both dominant-negative proteins. As shown in Fig. 6*A*, inhibition of Rac1 alone, or both Rac1 and Cdc42 completely abrogates $HRas^{V12}$-induced secreted VEGF levels. However, inhibition of Cdc42 alone reduces VEGF levels by approximately 40%. These data indicate that Cdc42 plays only a minor role, whereas Rac1 is required to mediate $HRas^{V12}$-induced VEGF protein levels in non-hypoxic conditions.

Recent studies indicate that both Rac1 and Cdc42 regulate hypoxia inducible factor (HIF) induced VEGF expression in response to hypoxia (18, 19). For this reason, we hypothesized that both Rac1 and Cdc42 regulate secreted levels of VEGF protein from PH3MT cells grown in hypoxic conditions. To address this hypothesis, we grew PH3MT derivative cell strains that express myc-$Rac1^{N17}$, FLAG-$Cdc42^{N17}$, or both in an incubator where oxygen levels were held to 1% O_2. We carried out parallel studies using two hypoxia mimetics $CoCl_2$ or DFO. As shown in Fig. 6A, under each condition, inhibition of either protein resulted in a 50% - 60% reduction in secreted VEGF protein levels, whereas inhibition of both Rac1 and Cdc42 completely eliminated a detectable level of VEGF protein when these cells were exposed to $CoCl_2$ or DFO. This indicates that independently regulated Rac1 and Cdc42 pathways are required to regulate

Figure 6. Evidence that Rac1 and Cdc42 regulate VEGF expression in both non-hypoxic and hypoxic conditions. All cell strains were serum starved for 24 hours and then the indicated agent. Conditioned media were collected after 24 hrs and ELISA analyses were completed. The data is presented as either percent of control, or fold induction as indicated in the figure. Error bars represent the SD from triplicate experiments *A*, PH3MT cell strains expressing a vector control (PH3MT-VC-C2), $Rac1^{N17}$ (PH3MT- $Rac1^{N17}$-C1), $Cdc42^{N17}$ (PH3MT-$Cdc42^{N17}$-C2), or both (PH3MT-$Rac1^{N17}$/$Cdc42^{N17}$), were stimulated with either medium containing 10% SCS, 100 μM DFO, 100 μM $CoCl_2$ or Hypoxia (1% O_2). *B*, MSU-1.1 cells expressing GFP alone (MSU-1.1-GFP-VC-C2), or GFP-tagged $Rac1^{V12}$ (MSU1.1-GFP-$Rac1^{V12}$), or GFP-tagged $Cdc42^{V12}$ (MSU-1.1-GFP-$Cdc42^{V12}$). Cells were stimulated with medium containing 10% SCS. * denotes a significant difference ($p<0.01$).

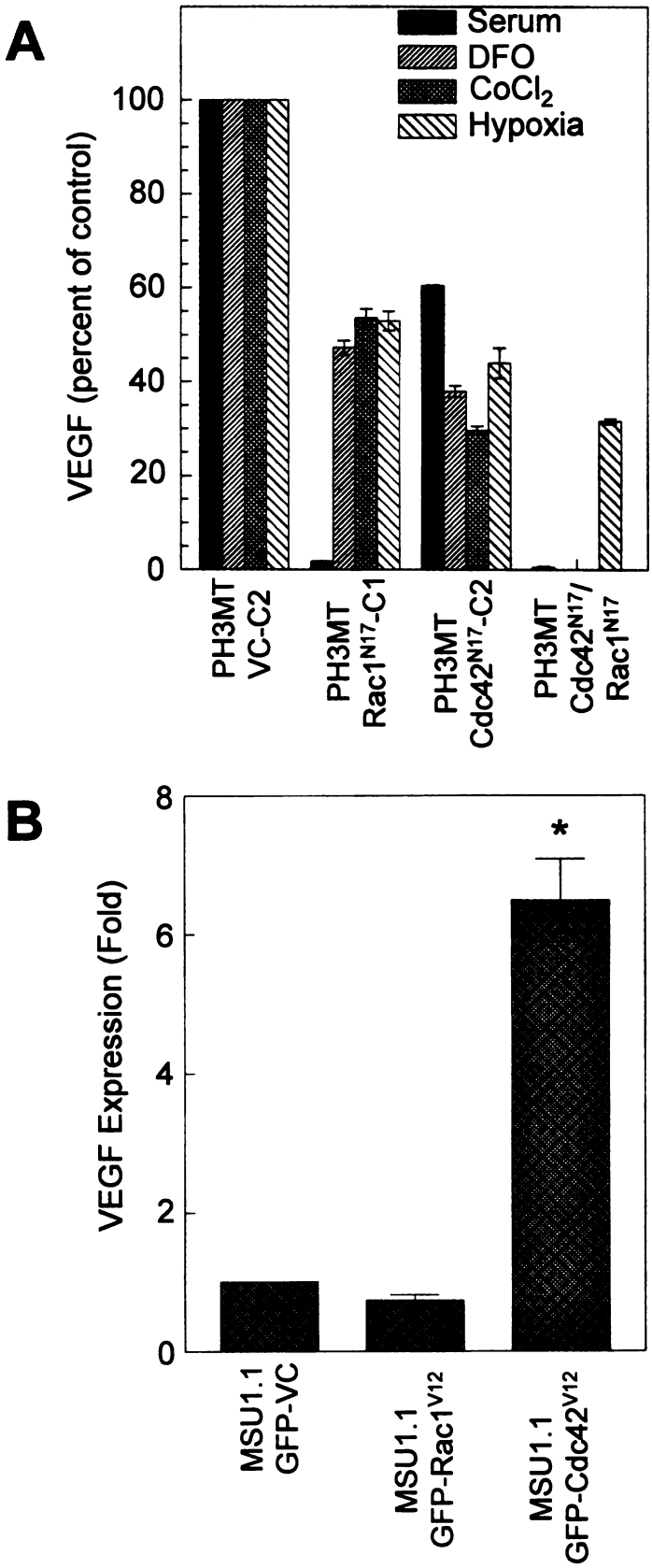

A
Serum
DFO
CoCl2
Hypoxia
VEGF (percent of control)
100
80
60
40
20
0
PH3MT VC-C2
PH3MT Rac1N17-C1
PH3MT Cdc42N17-C2
PH3MT Cdc42N17/ Rac1N17
B
VEGF Expression (Fold)
8
6
4
2
0
*
MSU1.1 GFP-VC
MSU1.1 GFP-Rac1V12
MSU1.1 GFP-Cdc42V12

Figure 6

VEGF expression in $HRas^{V12}$-transformed human fibroblasts exposed to the hypoxia mimetics $CoCl_2$ or DFO. Inhibition of both Rac1 and Cdc42 in the same cell strain, grown under hypoxic conditions, resulted in a 70% reduction of secreted VEGF protein levels. These data indicate that when $HRas^{V12}$-transformed human fibroblasts are proliferating in a hypoxic environment, the activities both Rac1 and Cdc42 are required to maintain high levels of secreted VEGF protein.

Because we found that VEGF expression requires Rac1 and Cdc42 activity, we hypothesized that introduction of constitutively-activated Rac1 or Cdc42 protein in the non-transformed parent cell strain MSU-1.1, would result in an increased level of VEGF protein secretion (Fig. 6*B*). Our data indicate that expression of GFP-$Rac1^{V12}$ does not induce VEGF expression in human fibroblasts, whereas expression of $Cdc42^{V12}$ induced a six-fold increase in VEGF protein levels. These data indicate that, in human fibroblasts, Rac1 regulates secreted VEGF levels only in the context of $HRas^{V12}$-induced mitogenic signaling, whereas expression of constitutively-active Cdc42 can increase levels of secreted VEGF independent of other oncogenic HRas mediated effector pathways.

Discussion

The data presented in this study show that both Rac1 and Cdc42 activity are required for $HRas^{V12}$-induced malignant transformation of human fibroblasts. We show that inhibition of Rac1 activity in $HRas^{V12}$-transformed human fibroblasts results in decreased tumor formation. However, the tumor-forming ability of cells with reduced Cdc42 activity was not significantly different from those with normal Cdc42 function. Cell strains derived from these tumors had undetectable levels of dominant-negative Rac1 or Cdc42 protein expression. These results indicate that functional Rac1 and Cdc42 are both required for malignant-transformation of human fibroblasts by $HRas^{V12}$-expression. This agrees with results in rodent fibroblasts (3, 4, 20).

We show that expression of $Rac1^{V12}$ in human fibroblasts results in the ability of these cells to grow in medium with reduced serum, and expression of $Cdc42^{V12}$ results in the ability for these cells to form large colonies in agarose. Rodent fibroblasts exhibit these same properties when constitutively-active mutants of Rac1 and Cdc42 are expressed (3, 5). Self-sufficient mitogenic growth (21) and anchorage independent growth are characteristics acquired by cancer cells. However, because MSU-1.1 cells expressing either constitutively-active protein were unable to form sarcomas in athymic mice, it seems that either expression levels of these proteins was insufficient, or malignant transformation of MSU-1.1 cells requires the simultaneous expression of activated Rac1 and Cdc42, or activated Rac1, Cdc42 and an activated form of RalGDS (6, 22) or other downstream effectors of HRas.

Because Rac1 and Cdc42 signaling are essential mediators of $HRas^{V12}$-induced transformation, we carried out Affymetrix GeneChip analyses of $HRas^{V12}$-transformed cells expressing both $Rac1^{N17}$ and $Cdc42^{N17}$ dominant-negative proteins which were regulated by tetracycline. This allowed us to use the same cell strain as the control and experimental cell populations. We identified a group of 29 significant gene changes. When we carried out a literature search on these 29 genes, we found that fourteen had been previously reported to play a role in cancer.

Teramota et al. (9) used a cDNA microarray representing 19,117 unique reading frames to identify the expression of genes that are regulated by expression of constitutively-active Ras, RhoA, Rac1 and Cdc42 in NIH3T3 cells. Among differences in expression, their work indicated that high mobility group isoform-C (HMGI-C) expression is induced by oncogenic Ras expression, which confirmed previous reports (23). Expression of an activated form of the HMGI-C is sufficient to malignantly transform NIH3T3 cells (24). Our Affymetrix results indicate that inhibition of Rac1 and/or Cdc42 mitigates this up-regulation. This implies a Ras-Rac1/Cdc42-HMGI-C signaling pathway that may contribute to Ras-induced transformation. The majority of expression differences that we observed, however, differ from those found by Teramoto et al. This may be a result of species differences. Our study differs from that of Teramoto et al. because we sought to identify expression differences in a human cell strain transformed with $HRas^{V12}$ in which Rac1 and Cdc42 are inactivated.

Many cancer-related genes that we identified have pro-angiogenic properties. For example, our data indicate that in $HRas^{V12}$-transformed fibroblasts, Rac1 and/or Cdc42 regulate the expression of Axl, a receptor tyrosine kinase stimulated by Gas6 (25). Axl is an important modulator of neovascularization *in vitro* and angiogenesis *in vivo* (26).

In agreement with recent reports (27-29), our array analysis also showed that cyclooxygenase-2 (Cox-2), a protein involved in chronic inflammation and angiogenesis (30), is regulated by Rac1 and Cdc42. The Ras-regulated Raf-MEK-ERK1/2 pathway and the JNK pathway are mediators of HRas-induced Cox-2 up-regulation (29). Rac1 and Cdc42 may regulate Cox-2 expression by altering JNK activity. Activation of the JNK pathway induces the Cox-2 transcription factors AP-1 (31), and PEA3 (32), and promotes Cox-2 mRNA stability via A/U rich sequences in the 3'UTR (33). JNK-mediated regulation of mRNA stability modulates expression of Cox-2, uPA and VEGF (33-35). Therefore, it is likely that Rac1 and Cdc42 mediate the expression of these genes by modulating both transcriptional and post-transcriptional mechanisms.

Similar transcriptional mechanisms regulate expression of the urokinase plasminogen activator (uPA) protein. Cooperation of two PEA3/AP-1 binding sites in the uPA promoter are required to mediate TPA induced uPA expression in NIH3T3 fibroblasts (36). Expression of uPA is strongly correlated with an invasive phenotype (16). Research from this laboratory reported that N-,K-, or $HRas^{V12}$ expression in human fibroblasts induces activated uPA expression (37). The results in the present study indicate that both Rac1 and Cdc42 regulate

$HRas^{V12}$-induced uPA expression in parallel pathways, perhaps through JNK and AP-1 regulation. However, expression of activated Rac1 or Cdc42 did not induce uPA expression in MSU-1.1 human fibroblasts. Recently, Aguirre-Ghiso et al. (38) found that expression of dominant negative RalA protein in v-Ras transformed NIH3T3 cells completely abrogated v-Ras induced uPA expression, as well as blocked tumor formation. This may explain why expression of activated Rac1 or Cdc42 alone did not induce uPA expression, and suggests that Ras induced RalA, Rac1 and Cdc42 activities are required to coordinately regulate secreted levels of uPA protein in human fibroblasts.

VEGF is the best studied mediator of angiogenesis (39). Our results show that in non-hypoxic conditions, $HRas^{V12}$ induces VEGF expression in a Rac1-dependent manner, whereas Cdc42 plays only a minor role. Ras-induced VEGF expression is regulated by multiple mechanisms, including increased transcription, translation and mRNA stabilization (18, 35, 40-42). For example, activation of ERK1/2, through the Raf-mediated Ras pathway, increases the activity of HIF1 and Sp1 transcription factors (18, 41). Furthermore, Saniger et al. (42) recently showed that activation of either Rac1 or Cdc42 can induce transcription of VEGF through activation of JNK. Activation of these pathways results in increased transcription of the VEGF gene product. However, recent data from our laboratory indicates that an Sp1-targeted ribozyme that markedly reduced Sp1 expression in $HRas^{V12}$-transformed human fibroblasts, does not affect VEGF expression (43). Therefore in $HRas^{V12}$-transformed human

fibroblasts, Rac1 may induce HIF1 and/or JNK activity and increase levels of secreted VEGF protein.

In MSU-1.1 cells, expression of activated Cdc42, but not activated Rac1 results in high levels of secreted VEGF protein. This indicates that Cdc42 can regulate the expression of VEGF in the absence of other Ras-mediated effector pathways, whereas Rac1 cannot. Activation of the RhoA and Cdc42 specific GEF, Ost, can potently induce JNK transcriptional pathways (44). Furthermore, recent evidence indicates that Cdc42 can regulate EGFR signaling in an autocrine fashion (45). These are mechanisms by which Cdc42 activity may induce VEGF expression in human fibroblasts independent of Ras activity.

Rac1 and Cdc42 have also been implicated in hypoxic-induction of VEGF expression through regulation of p53 and VHL protein levels, as well as HIF1, and JNK activation (18, 19, 42). In agreement with these studies, we find that both Rac1 and Cdc42 play a role in hypoxia-induced VEGF expression in $HRas^{V12}$-transformed fibroblasts. We also show that both Rac1 and Cdc42 mediate parallel pathways that are each required to regulate VEGF expression under hypoxia. However, there is not a significant additive increase in suppression of VEGF expression when Rac1 and Cdc42 are both inhibited in cells exposed to hypoxia. For this reason, the additive suppression of VEGF observed may not be physiologically relevant.

In conclusion, our research indicates that although constitutively activated expression of Rac1 and Cdc42 does not induce malignant-transformation of human fibroblasts. However, both Rac1 and Cdc42 are essential mediators of

$HRas^{V12}$-induced transformation of such cells. We also identified Ras-induced Rac1- and Cdc42-regulated genes including uPA and VEGF. Treating cancer patients with pharmaceuticals that target VEGF (46) and Cox-2 (47) show promise in clinical trials. Furthermore, uPA is currently used as a prognostic indicator in breast cancer patients (16). Identification of more cancer-related downstream effectors of Rac1 and Cdc42, such as Axl and HMGI-C, may provide additional targets for cancer therapy.

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CHAPTER III

THROUGH RAC1, SPROUTY-2 REGULATES SECRETED LEVELS OF BOTH VEGF AND UPA, AND ANCHORAGE INDEPENDENT GROWTH IN HRAS^{V12}-TRANSFORMED FIBROBLASTS.

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Abstract

Multiple genetic changes are required for normal cells to become malignant cells. We previously examined genetic differences between cells in an isogenic human fibroblast cell lineage where each successive clonally derived cell strain brings the cells one step closer to becoming malignantly transformed. Research in this laboratory recently showed that Sprouty-2 (Spry2) is a gene whose expression increases stepwise in the lineage, and is further increased following $HRas^{V12}$-transformation. Furthermore, when Spry2 protein levels were reduced, the cells exhibited a loss of anchorage-independent growth, failed to form tumors in athymic mice, and exhibited a coordinated decrease in Rac1 activity. The activity of Rac1 is required for $HRas^{V12}$-induced transformation of human fibroblasts. In this study, we report that Spry2 mediates $HRas^{V12}$-induced anchorage independent growth by regulating the activity of Rac1. However, when we restored Rac1 activity in $HRas^{V12}$-transformed human fibroblasts with reduced Spry2, the cells did not acquire the ability to form sarcomas in athymic mice. Our data also indicate that $HRas^{V12}$-induced expression of uPA is dependent on Spry2 protein expression, and that Spry2 regulates expression of uPA by regulating Rac1 activity. Although we found that Spry2 also regulates the expression of $HRas^{V12}$-induced VEGF expression, our data suggest that multiple Spry2 regulated pathways are required to mediate this induction.

Introduction

It is commonly accepted that cancer is caused by multiple genetic changes. McCormick, Maher and colleagues have developed an isogenic human fibroblast cell lineage to identify these changes. The MSU1 lineage is a family of human fibroblast cell strains derived clonally, one-from-another, where each successive strain is closer to being malignantly transformed. The LG1 cell line, derived from the foreskin of a normal human neonate, marks the starting point in this lineage. LG1 fibroblasts were transfected with a vector containing the *v-Myc* oncogene and a selectable marker. Transfected cells expressing the v-Myc protein were grown to the end of their lifespan when the vast majority senesced. However, a few live cells remained and gave rise to a telomerase positive, immortalized, chromosomally stable, diploid cell strain which was designated MSU-1.0. Such cells cannot be transformed by carcinogen treatment or oncogene expression. A rapidly proliferating variant cell strain spontaneously arose in a population of MSU-1.0 cells and was designated MSU-1.1 (1). The MSU-1.1 cell strain has been malignantly transformed using various methods including treatment with carcinogens (2-5) and expression of oncogenes (6, 7) followed by suitable selection. For example, over-expression of the T24 $HRas^{V12}$ oncoprotein in MSU-1.1 cells resulted in cells that formed sarcomas when injected into athymic mice (7). One cell line derived from such a tumor, designated PH3MT, was used in the present study.

Using microarray analyses, we have identified multiple gene expression differences between cell strains along the MSU1 lineage (unpublished data).

These data show that expression of Spry2 was increased in MSU-1.1 cells and again from MSU-1.1 to PH3MT. The Sprouty family consists of four members. *Sprouty-2* shares the most homology to the ancestral gene originally identified as an inhibitor of FGFR signaling in *Drosophila Melanogaster* (8). Increased levels of Spry2 protein is found in multiple human fibrosarcoma and pancreatic carcinoma-derived cell lines, but not in normal fibroblast or pancreatic cells (9).

In a recent study from our laboratory, it was reported that reduction of Spry2 protein levels in the PH3MT cell strain, results in cells that are no longer able to form large colonies in agarose, and these cells fail to form tumors in athymic mice (9). A separate report also showed similar results following inhibition of either Rac1 or Cdc42. This suggests that Spry2, Rac1, and Cdc42 play essential roles in $HRas^{V12}$-induced transformation of human fibroblasts (10). In a third report, data collected in our laboratory indicate that a reduction in Spry2 levels results in decreased Rac1 activity (11). Furthermore, in PH3MT cells, Rac1 and Cdc42 regulate independent signaling pathways that can increase uPA and VEGF expression (10).

The present study was conducted to determine if PH3MT cells with reduced Spry2 (PH3MT-2A3) fail to form colonies in agarose, or fail to form sarcomas in athymic mice as a result of decreased Rac1 or Cdc42 activity. Expression of either $Rac1^{V12}$ or $Cdc42^{V12}$ restored the ability for PH3MT-2A3 cells to form large colonies in agarose, but did not restore the ability for these cells to form tumors in athymic mice. Because Rac1 and Cdc42 regulate the expression of uPA and VEGF levels in $HRas^{V12}$ transformed fibroblasts we also

analyzed the ability for Spry2 to regulate the expression of these two proteins. Our results indicate that induction of secreted uPA and secreted VEGF levels by $HRas^{V12}$ is dependent on Spry2 protein expression.

Materials and Methods

Cell Strains and Culture Conditions

Unless otherwise indicated, the growth medium for human foreskin derived fibroblasts, i.e. MSU-1.1 and PH3MT strains and their derivatives, consisted of Eagle's minimal essential medium supplemented with 0.2 mM L-aspartic acid, 0.2 mM L-serine, 1.0 mM sodium pyruvate, 10% supplemented calf serum (SCS) (Hyclone Laboratories, Logan, UT), 100 units/ml penicillin, and 100 μg/ml streptomycin. All cell lines were grown at 37°C in a humidified incubator containing 5% CO_2 in air.

Generation of GFP-Rac1^{V12} and GFP-Cdc42^{V12} expression vectors has been described previously (10). pcDNA6-GFP-Rac1^{V12} and pcDNA6-GFP-Cdc42^{V12} expression vectors were transfected into PH3MT-2A3 cells using lipofectamine reagent following the manufacturer's protocol. Cell culture dishes were scanned using fluorescence microscopy and fluorescing clones were identified, isolated and screened for transgene expression. Two clones expressing GFP-Rac1^{V12} and one clone expressing GFP-Cdc42^{V12} were isolated and used to complete further experiments.

Cell Lysates and Western Blot Analysis

Whole cell protein extracts were prepared using a lysis buffer consisting of 50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 50 mM NaF, 0.5% NP-40, 1 mM Na_3V0_4, 200 mM benzamidine, 1 mM PMSF, 25 μg/ml aprotinin, and 25 μg/ml leupeptin. Total protein concentration was quantified using the Coomassie protein assay

reagent (Pierce Biotechnology, Rockford, IL). Lysates were denatured in 5X Laemelli sample buffer, separated by either 10% or 12% SDS-PAGE, and transferred to PVDF membrane. The membrane was blocked for 2 h with Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% (w/v) non-fat milk. For the majority of the studies, the membrane was probed with the primary antibody at 4°C overnight, then probed with the appropriate horseradish peroxidase-linked secondary antibody (Sigma and Santa Cruz Biotechnology), for 1 hr at room temperature. Both antibodies were diluted in TBST containing 5% milk. The membrane was incubated with the Supersignal West Pico chemiluminescent horseradish peroxidase substrate (Pierce Biotechnology, Rockford, IL) and then exposed to film. The primary antibodies used were anti-GFP (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Spry2 (Calbiochem, EMD Biosciences, San Diego, CA) and anti-Ku80 (Serotec, Oxford, UK). This experiment was completed three times.

Assay for Anchorage-Independence

To determine the ability of cells to form large colonies in agar, 5,000 cells were plated in 0.33% top agarose per 60-mm-diameter culture dish and covered with 2 mL of growth medium. The growth medium was replaced weekly. After 3 wks, the cells were fixed with 2.5% glutaraldehyde. One representative field is presented. This experiment was completed three times with similar results.

Assay for Tumorigenicity

PH3MT-2A3 derivatives cell strains expressing GFP-Rac1^{V12} or GFP-Cdc42^{V12} protein were injected subcutaneously into the right and left rear flanks of athymic BALB/c mice at a concentration of 1 X 10^6 cells per site and monitored weekly for tumor growth.

ELISA Analyses

1.5 X 10^5 cells were plated in a 100 mm tissue culture dish, and incubated for 24 hrs. At this time, cells were washed twice with serum-free medium, and serum-starved for another 24 hrs. Cells were then stimulated with growth medium containing 10% SCS. The media were collected, and centrifuged at 5000 x g for 5 min. To detect secreted VEGF protein, a sandwich ELISA using the human VEGF DuoSet (R and D Systems, Minneapolis, MN) ELISA kit was used following the manufacturer's protocol. To detect secreted levels of uPA protein, a sandwich ELISA using the uPA ELISA kit (Oncogene Science, Bayer, Cambridge, MA) was used following the manufacturer's protocol. After the removal of conditioned medium, whole cell protein extracts were made, quantitated and used to normalize detected uPA and VEGF protein levels by dividing the concentration of uPA or VEGF protein in the conditioned medium by the total amount of protein in the whole cell extract. The results are expressed as either percent of vector control, or fold change. Each graph represents one experiment containing replicate dishes. Each experiment was completed three times, and similar results were observed in each. Error bars indicate the standard

deviation from the mean. The student's *t*-test was used to determine statistical significance.

Results

Spry2 mediates HRas^{V12}-induced anchorage independent growth through Rac1.

To determine if expression of constitutively-activate Rac1^{V12} or Cdc42^{V12} could rescue the transformed phenotype in PH3MT-2A3 cells, we expressed GFP-Rac1^{V12}, or GFP-Cdc42^{V12} in these cells (Fig. 1) and measured their ability to form tumors in athymic mice and large colonies in agarose. Two independent clones expressing GFP-Rac1^{V12}, (PH3MT-2A3-GFP-Rac1^{V12}-C1, -C2), and one strain expressing GFP-Cdc42^{V12}, (PH3MT-2A3-GFP-Cdc42^{V12}) were tested for their ability to form sarcomas in athymic mice. Neither of the GFP-Rac1^{V12} expressing clones nor the GFP-Cdc42^{V12} expressing cell strain produced tumors (data not shown). This indicates that Spry2 exerts its oncogenic effect through multiple pathways, one of which may be regulation of Rac1 and/or Cdc42 activity.

To determine if expression of Rac1^{V12} and/or Cdc42^{V12} could recover anchorage independent growth of PH3MT-2A3 cells, we tested both GFP-Rac1^{V12} expressing clones, and the clone expressing GFP-Cdc42^{V12} for their ability to form colonies in agarose (Fig. 2). Each cell strain was able to form large colonies. This suggests that Spry2 mediates HRas^{V12}-induced anchorage independent growth by regulating the activities of Rac1 and Cdc42.

Figure 1..Expression of $Rac1^{V12}$ or $Cdc42^{V12}$ in PH3MT-2A3 cells. Cells strains expressed either GFP-$Rac1^{V12}$ (PH3MT-2A3-GFP-$Rac1^{V12}$-C1,-C2), or GFP-$Cdc42^{V12}$ PH3MT-2A3-GFP-$Cdc42^{V12}$) proteins. The scrambled control, PH3MT-SC, and parental PH3MT-2A3 are also shown. The blots were probed with Ku80 antibody to verify loading.

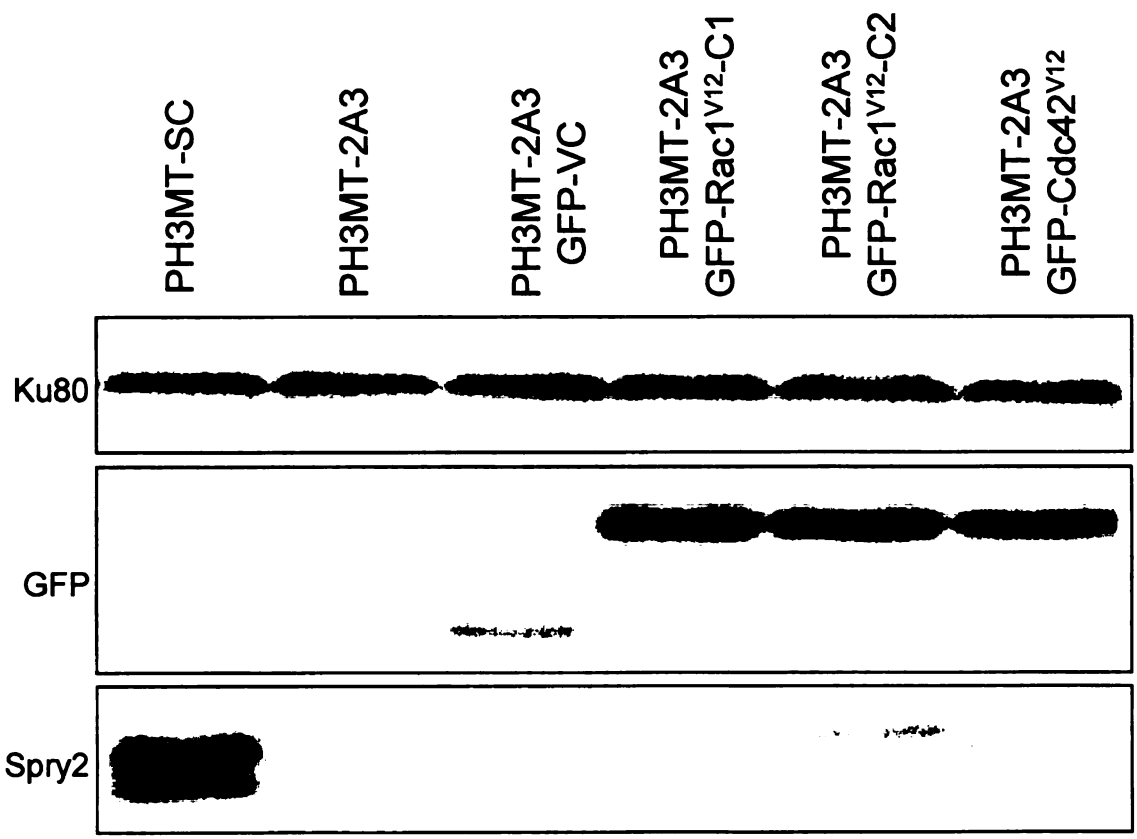
PH3MT-SC
PH3MT-2A3
PH3MT-2A3 GFP-VC
PH3MT-2A3 GFP-Rac1V12-C1
PH3MT-2A3 GFP-Rac1V12-C2
PH3MT-2A3 GFP-Cdc42V12
Ku80
GFP
Spry2

Figure 1

Figure 2. Expression of $Rac1^{V12}$ or $Cdc42^{V12}$ in PH3MT-2A3 cells rescues anchorage independent growth. The cell strains were plated in 0.33% agarose and grown for three weeks. Each picture represents one representative field from each cell line. This experiment was completed in triplicate, each with similar results.

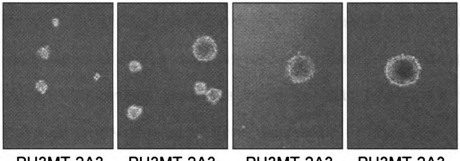
PH3MT-2A3 VC
PH3MT-2A3 Rac1V12-C1
PH3MT-2A3 Rac1V12-C2
PH3MT-2A3 Cdc42V12

Figure 2

Spry2 protein is required to mediate $HRas^{V12}$-induced uPA and VEGF expression.

To determine if Spry2 mediates $HRas^{V12}$-induced secreted VEGF and uPA levels, we measured the amount of uPA and VEGF in the medium from PH3MT-2A3 cells, and from PH3MT cells transfected with a scrambled shRNA control (PH3MT-SC) (Fig. 3*A*, *B*). The PH3MT-SC cell strain retains a high level of Spry2 expression (Fig. 1). We found that cells with reduced Spry2 levels also displayed significantly reduced (<10% of control cells) levels of secreted uPA and VEGF. These data indicate that Spry2 expression mediates $HRas^{V12}$-induced expression of uPA and VEGF. Moreover, these results are consistent with the hypothesis that Rac1 activity is downstream of Spry2 expression in $HRas^{V12}$-transformed human fibroblasts, and that down-regulated Rac1 activity prevents $HRas^{V12}$-induced VEGF and uPA expression.

Spry2 regulates $HRas^{V12}$-induced uPA expression by regulating Rac1 and Cdc42 activity.

To determine if expression of either GFP-$Rac1^{V12}$ or GFP-$Cdc42^{V12}$ could rescue reduced uPA expression in PH3MT-2A3 cells, ELISA analyses were completed using the PH3MT-2A3 cell strains expressing constitutively-activate $Rac1^{V12}$ or $Cdc42^{V12}$. Expression of GFP-$Rac1^{V12}$ resulted in a three to five fold increase in secreted uPA levels in both clones tested (Fig. 4*A*). These results indicate that there is a linear Ras-Spry2-Tiam1-Rac1-uPA signaling sequence. It should be noted, however, that Rac1 does not completely restore uPA

Figure 3. Evidence that Spry protein is required for $HRas^{V12}$-induced expression of uPA and VEGF. The two cell strains were serum starved for 24 hrs and then stimulated with medium containing 10% SCS. Media was collected and analyzed. The PH3MT cell strain transfected with a scrambled control, PH3MT-SC and the PH3MT-2A3 cell line with reduced Spry2 levels were tested for levels of secreted (*A*) uPA and (*B*) VEGF protein. Data is presented as percent of control. Error bars represent SD from triplicate experiments. * statistical significance, $p<0.001$

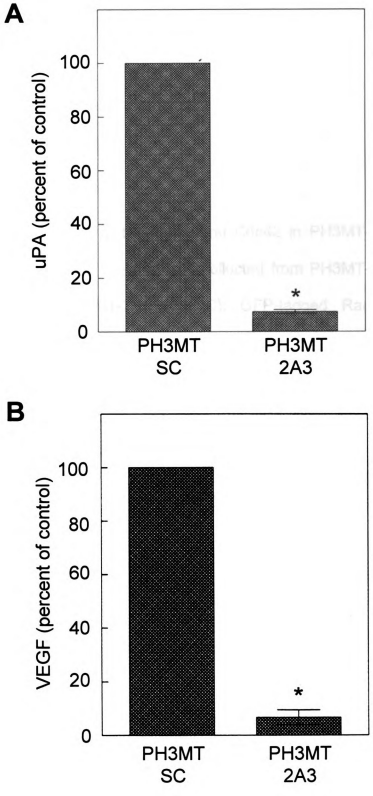
A
uPA (percent of control)
100
80
60
40
20
0
*
PH3MT SC
PH3MT 2A3
B
VEGF (percent of control)
100
80
60
40
20
0
*
PH3MT SC
PH3MT 2A3

Figure 3

Figure 4. Expression of constitutively-active Rac1 and Cdc42 in PH3MT-2A3 cells with reduced Spry2. Conditioned medium was collected from PH3MT-2A3 cells expressing GFP alone (PH3MT-2A3-GFP-VC), GFP-tagged $Rac1^{V12}$ (PH3MT-2A3-GFP-$Rac1^{V12}$-C1,-C2) or GFP-tagged $Cdc42^{V12}$ (PH3MT-2A3-GFP-$Cdc42^{V12}$), and uPA and VEGF protein levels were analyzed by ELISA. Data is presented as fold-induction. Error bars represent the SD from triplicate experiments. * statistical significance, $p<0.05$.

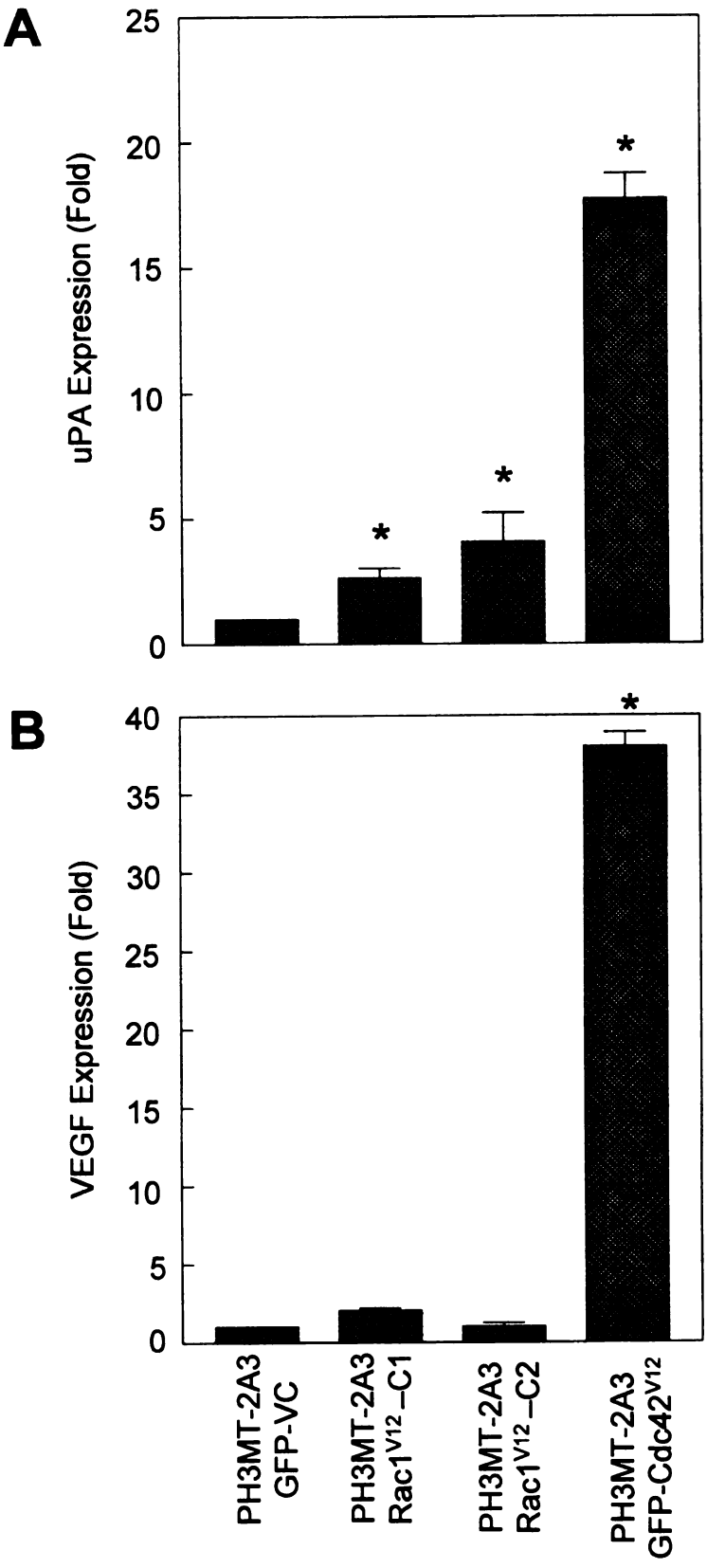
A
uPA Expression (Fold)
25
20
15
10
5
0
*
*
*
B
VEGF Expression (Fold)
40
35
30
25
20
15
10
5
0
*
PH3MT-2A3 GFP-VC
PH3MT-2A3 Rac1V12–C1
PH3MT-2A3 Rac1V12–C2
PH3MT-2A3 GFP-Cdc42V12

Figure 4

expression. This suggests that parallel Spry2 regulated pathways, independent of Rac1, also contribute to the regulation of secreted uPA levels. Expression of $Cdc42^{V12}$ resulted in a 15-fold increase in secreted uPA levels. This also suggests a possible a linear signaling pathway, e.g. Ras-Spry2-Cdc42-uPA.

$HRas^{V12}$-induced VEGF expression requires multiple Spry2 regulated pathways.

Because reduction of Spry2 protein results in a coordinate reduction of both Rac1 activity and secreted VEGF levels, we determined if expression of $Rac1^{V12}$ or $Cdc42^{V12}$ could rescue VEGF expression (Fig. 4*B*). We analyzed levels of VEGF secretion from both GFP-$Rac1^{V12}$ PH3MT-2A3 cell strains and the PH3MT-2A3-$Cdc42^{V12}$ cell strain. Our results show that GFP-$Rac1^{V12}$ expression, in cells with reduced Spry2, does not restore the high levels of VEGF expression. This indicates that Spry2 regulates the activity of multiple pathways, including the Rac1 mediated signaling pathway, that are required to regulate VEGF expression. However, introduction of $Cdc42^{V12}$ induced high levels of VEGF secretion. Our laboratory recently reported that expression of $Cdc42^{V12}$ in MSU-1.1 fibroblasts also induced VEGF expression (10). Taken together, these data indicate that Cdc42 regulates VEGF expression independent of Spry2 protein.

Discussion

$HRas^{V12}$-induced transformation of human fibroblasts requires both Spry2 expression and Rac1 activity. Research in our laboratory has previously shown that reducing Spry2 protein levels in PH3MT cells results in decreased Rac1 activity. Disruption of a Ras-Spry2-Tiam1-Rac1 complex may be the cause of this phenotype (11). Reducing Spry2 in PH3MT cells also results in decreased anchorage independent growth, and complete inhibition of tumor formation (9). This is consistent with results from our laboratory that shows inhibition of Rac1 in PH3MT cells also inhibits tumor growth (10). In the present study, we determined that expression of activated Rac1 in cells with reduced Spry2 restored the ability to form colonies in agarose, but did not restore the ability to form tumors. Downstream of Ras, Spry2 also regulates the activity of PI3K and ERK1/2 (9, 11). Introduction of activated Rac1 probably complements only one pathway necessary for malignant transformation.

Data collected in our laboratory indicated that inhibition of Rac1 results in decreased levels of secreted uPA (10). This suggests that Spry2 may regulate uPA levels via regulation of Rac1 activity. Re-introduction of activated Rac1 into cells with reduced Spry2 partially restored uPA expression. This suggests that Ras induces uPA expression via a Ras-Spry2-Rac1-uPA signal transduction pathway. A recent publication indicates that in NIH3T3 mouse fibroblasts, activation of RalA is required for oncogenic Ras induced uPA expression (12). This is consistent with our finding that activated Rac1 and Cdc42 did not increase uPA levels in MSU-1.1 fibroblasts. Spry2 is not implicated in the regulation of the

RalGDS pathway. Therefore, RalA is expected to be activated in the $HRas^{V12}$-transformed PH3MT-2A3 cell strain. For this reason, re-introduction of Rac1 into cells with reduced Spry2 was able complement reduced levels of secreted uPA. However, activated Rac1 expression did not induce secreted uPA levels comparable to such levels in PH3MT cells with highly expressed Spry2. This suggests that Spry2 also affects uPA expression through regulation of alternate mitogenic pathways e.g. PI3K or ERK1/2.

Experiments in our laboratory have also shown that inhibition of Rac1 drastically reduces the secreted levels of VEGF protein in non-hypoxic conditions (10). In this study, we show that cells with reduced Spry2 expression also exhibit a drastic reduction in the levels of secreted VEGF. Therefore, we hypothesized that reduction in VEGF levels is a direct result of down-regulated Spry2 protein that results in reduced Rac1 activity. However, expression of $Rac1^{V12}$ did not restore increased VEGF expression. Therefore, it must be assumed that not only does Spry2 regulate Rac1 activity, but it also regulates mitogenic pathways that control VEGF expression, e.g. ERK1/2 and PI3K. This is consistent with our recent observation that over-expression of activated Rac1 in MSU-1.1 fibroblasts, that have low Spry2 levels, did not induce VEGF expression (10). Furthermore, Spry2 over-expression in MSU-1.1 cells induces ERK1/2 activity. However, it is not known if over-expression of Spry2 in MSU-1.1 cells induces VEGF expression. It is known that reduction of Spry2 in PH3MT cells results in decreased ERK1/2 activity as well as decreased PI3K activity, which correlates with a decrease in secreted VEGF levels. This suggests that ERK1/2 and PI3K

pathways are candidates for parallel Spry2-regulated pathways that function along with Rac1 and Cdc42 to regulate VEGF expression. It seems probable that these parallel pathways are required to mediate the up-regulation of VEGF in response to Ras activation.

Our data also indicate a role for Cdc42 in regulation of VEGF expression downstream of Spry2, or in a Spry2-independent pathway. In PH3MT cells, inhibition of Cdc42 activity results in a moderate decrease in secreted VEGF and in the non-transformed parent cell line, MSU-1.1, expression of Cdc42 increases the levels of secreted VEGF protein (10). In cells with reduced Spry2 expression, we observe a similar induction. These results do not differentiate between Spry2-dependent or Spry2-independent regulation of VEGF by Cdc42. Because activated Cdc42 can induce VEGF expression in both MSU-1.1 cells and in PH3MT cells with reduced Spry2, Cdc42 probably induces VEGF in a Spry2-independent pathway.

Recently, Wu et al. (13) found that activated Cdc42 can bind with p85Cool-1/beta-Pix, a protein that directly associates with c-Cbl. c-Cbl is an E3 ubiquitin ligase that ubiquitinates EGFR and targets it for degradation. Spry2 can sustain EGFR signaling by forming a complex with c-Cbl and preventing it from ubiquitinating EGFR (14). In a similar way, activated Cdc42 can prevent EGFR degradation by binding with p85Cool-1/beta-Pix and c-Cbl. Interruption of the binding of c-Cbl to Cdc42 reduces Cdc42-induced growth factor independence and anchorage independence. Furthermore, the interaction between c-Cbl and activated Cdc42 is critical to induce transformation of NIH3T3 mouse fibroblasts.

These data indicate that activated Cdc42 may function similar to Spry2 i.e. it sequesters c-Cbl, prevents EGFR degradation, and induces mitogenic pathways that lead to transformation. This suggests a potential mechanism by which expression of activated Cdc42 in MSU-1.1 cells induces VEGF expression. These data support the hypothesis that Spry2 expression is essential for anchorage independent growth by sequestering c-Cbl, and protecting EGFR protein levels.

It is clear that Spry2 is required for the malignant transformation of human fibroblasts by $HRas^{V12}$ expression. However, we are only beginning to understand the role Spry2 plays to this process. In this study, we show that Spry2 mediates $HRas^{V12}$-induced anchorage independent growth by regulating the activity of Rac1. We also show that reduction of Spry2 in $HRas^{V12}$ transformed fibroblasts results in decreased levels of the cancer-related proteins VEGF and uPA. It is likely that this effect is mediated by the activation state of Rac1 and Cdc42. However, the complete mechanism by which Spry2 regulates VEGF and uPA is not clear, and will be the focus of future research. Elucidating this mechanism will provide insight into the oncogenic role of Spry2 and suggests that pharmaceuticals that target Spry2 function may be useful as a treatment for cancer.

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SUMMARY

Epigenetic and/or genetic alterations in oncogenes or tumor-suppressor genes result in changes in gene-expression or protein-activation that bring about phenotypic properties commonly found in cancer cells. These characteristics include the ability for cells to provide self-sufficient mitogenic signals, evade anti-proliferative signals, evade apoptosis, replicate limitlessly, sustain angiogenesis, invade surrounding tissues, and metastasize (1).

A constitutively-activating mutation in the Ras gene results in a Ras protein that causes sustained signaling. Major pathways affected by Ras-signaling include the Raf-Mek-ERK MAPK pathway, the RalGDS signaling pathway, and the PI3K-Akt survival pathway. Activation of PI3K induces the activity of the small Rho-GTPases Rac1 and Cdc42 (2). In addition, a direct interaction between Ras and the Rac1-specific exchange factor Tiam1 results in the activation of Rac1 (3).

Rac1 and Cdc42 were originally identified as regulators of the actin-cytoskeleton (4). However, over the last ten years, their involvement in major signaling pathways has been the subject of intense research. Rac1 and Cdc42 have been shown to regulate the JNK, p38 and SRF/SAPK signal transduction pathways (5). Through these pathways, both Rac1 and Cdc42 affect cell-cycle progression, proliferation and apoptosis.

Research in the mid- to late-1990s indicates that Rac1 and Cdc42 are required for $HRas^{V12}$-induced transformation of Rat1 fibroblasts, NIH3T3 mouse fibroblasts, and Swiss-3T3 mouse fibroblasts (6-10). Using dominant-negative

mutants, $Rac1^{N17}$ and $Cdc42^{N17}$, to inhibit the function of each protein individually as well as constitutively-active mutants, $Rac1^{V12}$ and $Cdc42^{V12}$, investigators found that Rac1 activity is required for proliferation under reduced serum conditions, and that Cdc42 activity is required for the ability for cells to form large anchorage-independent colonies. Furthermore, they showed that Rat1 fibroblasts and Swiss-3T3 mouse fibroblasts that express activated Rac1 or Cdc42 proteins formed tumors when injected into athymic mice. This research indicated that Rac1 and Cdc42 are potential therapeutic targets for patients with tumors carrying activated Ras genes.

However, it is not known if the activities of Rac1 and Cdc42 are required for $HRas^{V12}$-induced transformation of human fibroblasts. The present study was designed to determine the roles of Rac1 and/or Cdc42 in $HRas^{V12}$-induced transformation of the human fibroblast cell line, PH3MT, and to determine whether expression of activated mutants of Rac1 and Cdc42 in the parental cell strain of PH3MT is able to induce phenotypes characteristic of cancer cells, namely, the ability y to proliferate in reduced serum, to form large anchorage independent colonies in agarose, and to form malignant tumors. Affymetrix analyses were also completed to identify genes whose expression are controlled by Rac1 and/or Cdc42, and may be important in Ras-induced malignant transformation. These genes may provide insight into the mechanism by which Rac1 and Cdc42 mediate Ras-induced transformation, and as a consequence, identify potential therapeutic targets.

Inhibition of Rac1 significantly suppressed tumor formation. The results of experiments designed to inhibit the activity of Cdc42 were not as consistent. Nevertheless, in every instance when tumors formed, analysis of the cells from the tumors revealed that dominant-negative $Rac1^{N17}$ and $Cdc42^{N17}$ were no longer expressed. These results indicate that for $HRas^{V12}$-induced malignant transformation of these human fibroblasts, Rac1 and Cdc42 activity is required.

To identify HRas-regulated genes that require the activities of Rac1 and Cdc42, I analyzed cells with tetracycline-regulatable expression of $Rac1^{N17}$ and $Cdc42^{N17}$ proteins. These cells were grown in the presence or absence of tetracycline, mRNA was harvested, and Affymetrix analyses were completed. Using MAS5.0 software to identify changes in gene expression, I identified 29 statistically significant gene expression changes, 14 of which have been found in various cancers, to have a role in transformation. I chose to verify the expression of two such proteins, VEGF, an angiogenic factor, and uPA, a factor involved in cancer cell invasion and metastasis, uPA.

I completed ELISA analyses to verify that Rac1 and Cdc42 play a role in regulating the expression of VEGF and uPA in the $HRas^{V12}$-transformed cell line, PH3MT. I also completed a parallel study to identify the individual roles of Rac1 and Cdc42 in regulating the expression of these two proteins. The results of these experiments indicate that Rac1 and Cdc42 are members of two independent signaling pathways that are required to regulate the expression of uPA in the $HRas^{V12}$-transformed cell line, PH3MT. I also found that whereas

Cdc42 plays a minor role in regulating VEGF expression, Rac1 plays a major role in regulating secreted levels of VEGF protein.

Recent evidence suggests that Rac1 plays a role in regulating VEGF expression in cells exposed to hypoxia, and that both Rac1 and Cdc42 activities are required for VEGF expression in non-hypoxic conditions (11-12). Therefore I carried out parallel studies using a hypoxia chamber (1% O_2) as well as DFO and $CoCl_2$ to mimic hypoxia. Results from this study indicate that the activities of both Rac1 and Cdc42 are required for hypoxia-, DFO- and $CoCl_2$-induced expression of VEGF. Reports indicate that Rac1 and Cdc42 probably regulate the levels of both uPA and VEGF through transcriptional and post-transcriptional pathways. However, these mechanisms are not addressed in this dissertation. My results indicate that uPA and VEGF play a role in malignant transformation of fibroblasts induced by activated HRas.

To determine if expression of constitutively-active $Rac1^{V12}$ or $Cdc42^{V12}$ in human fibroblasts results in properties characteristic of cancer cells, I expressed each these genes in the MSU-1.1 cell strain, the parent strain of the $HRas^{V12}$-transformed cell strain, PH3MT. I found that expression of $Rac1^{V12}$ allowed cells to grow in reduced serum (Fig. 1A), whereas expression of $Cdc42^{V12}$ resulted in the ability for cells to form anchorage independent colonies (Fig. 1B). These results are similar to those found with rodent fibroblasts. However, unlike the results in rodent fibroblasts, these cells did not form tumors.. Interruption of activity caused by the N-terminal GFP tag, or low levels of $Rac1^{V12}$, or $Cdc42^{V12}$ expression could explain this result. However, because expression of either

Figure 1. Schematic diagrams representing the contribution of Rac1, Cdc42, and Spry2 in anchorage independent growth, tumor formation, uPA expression, and VEGF expression. *A.* Expression of $Rac1^{V12}$ in MSU-1.1 cells results in their ability to proliferate in reduced serum. Expression of $Cdc42^{V12}$ does not. Inhibition of Rac1 in PH3MT cells does not affect growth in reduced serum. Therefore, more than one Ras-induced pathway "Y" regulates mitogenic signals that allow growth in reduced serum. *B.* Expression of $Cdc42^{V12}$ in MSU-1.1 cells results in the ability to form large anchorage independent colonies. Expression of $Rac1^{V12}$ does not. Reduction of Spry2 results in a reduction in anchorage independent growth. Expression of $Rac1^{V12}$ or $Cdc42^{V12}$ in PH3MT-2A3 cells restores this ability indicating that Rac1 plays a role in oncogenic HRas-induced anchorage independence downstream of Spry2. However, other pathways regulated by either Ras "Y", or Spry2 "X" must be present for Rac1 to have an effect. *C.* Inhibition of Rac1 or Cdc42, or reduction in Spry2 expression in PH3MT cells results in their inability to form tumors. This indicates that Rac1, Cdc42, and Spry2 are required for $HRas^{V12}$-induced malignant transformation. Expression of $Rac1^{V12}$, $Cdc42^{V12}$, or Spry2 (Lito and McCormick, unpublished) in MSU-1.1 cells does not result in the ability for these cells to form tumors. This indicates that pathway "Y" in the schematic diagram must be present for tumor formation. Pathway "X" is probably also necessary. However, this dissertation research does not identify these pathways. *D.* In PH3MT cells, inhibition of Rac1 or Cdc42, or reduction of Spry2 reduces the expression of uPA. Inhibition of both Rac1 and Cdc42 results in an additive suppression of secreted uPA levels. This indicates that Rac1 and Cdc42 function in parallel pathways to regulate uPA secretion, and that Spry2 also regulates the secretion of uPA. Because Rac1 activity is downstream of Spry2 in PH3MT cells, it seems probable that Spry2 regulates uPA expression through Rac1. Expression of $Rac1^{V12}$ or $Cdc42^{V12}$ in MSU-1.1 cells does not induce uPA expression, however, expression of these proteins in PH3MT-2A3 cells restores high levels of uPA expression. This indicates that another Ras-regulated effector pathway "Y", present in PH3MT-2A3 cells, is required for Rac1 and Cdc42 to affect the expression of uPA. *E.* In PH3MT cells grown in non-hypoxic conditions, inhibition of Rac1, or reduction in Spry2 expression results in decreased VEGF protein secretion. Inhibition of Cdc42 results in a minor reduction of VEGF expression. Expression of $Rac1^{V12}$ in MSU-1.1 cells or in PH3MT-2A3 cells does not recover high levels of VEGF expression. Therefore, alternate Ras-regulated effector pathways "Y" or alternate Spry2-regulated effector pathways "X" are required for Rac1 activity to affect VEGF expression. In contrast, expression of Cdc42 in either MSU-1.1 cells or PH3MT-2A3 cells restores high levels of VEGF expression. This indicates that activation of Cdc42 can regulate VEGF expression independent of alternate Ras-regulated effector pathways.

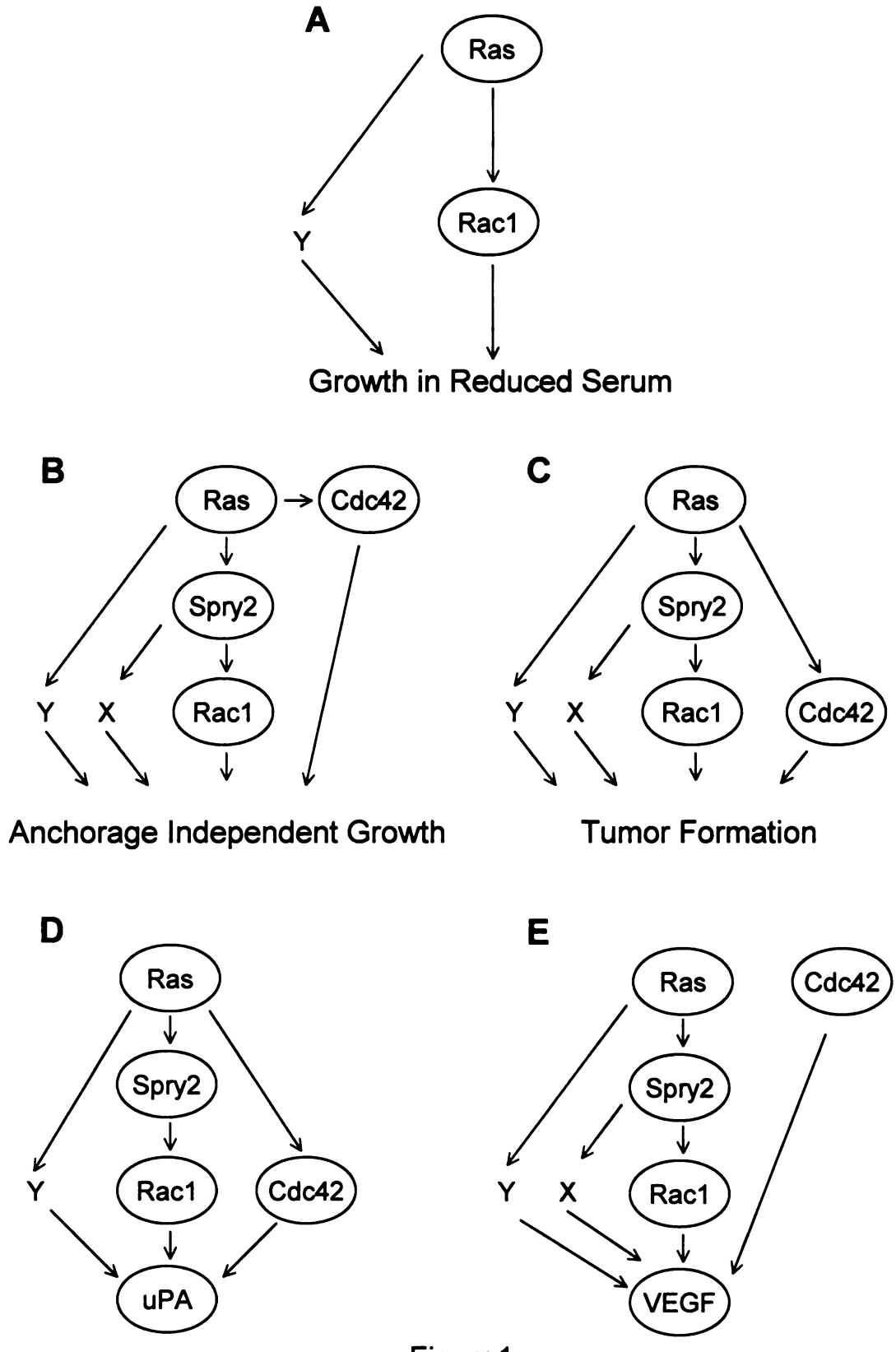
A
Ras
Rac1
Y
Growth in Reduced Serum
B
Ras
Cdc42
Spry2
Rac1
Y
X
Anchorage Independent Growth
C
Ras
Spry2
Rac1
Cdc42
Y
X
Tumor Formation
D
Ras
Spry2
Rac1
Cdc42
Y
uPA
E
Ras
Cdc42
Spry2
Rac1
Y
X
VEGF

Figure1

activated form resulted in the cells acquiring transformed characteristics in culture, it seems likely that these proteins are unable to induce malignant transformation acting alone. This result suggests that Ras-effector pathways, such as the RalGDS pathway, must be activated with Rac1 and Cdc42 for malignant transformation to occur (Fig. 1C).

I also found that neither activated Rac1 nor activated Cdc42 were able to induce the expression of uPA. These data indicate that in human fibroblasts, other Ras-effector pathways, parallel to the Rac1 and Cdc42 regulated pathways, are required for the expression of uPA (Fig. 1D). Expression of $Rac1^{V12}$ did not induce the expression of VEGF. In contrast, expression of $Cdc42^{V12}$ induced VEGF secretion by approximately 6 fold. These data indicate that Rac1 requires parallel Ras-regulated pathways to mediate VEGF secretion, whereas activated Cdc42 can induce VEGF secretion independent of HRas activation (Fig. 1E).

In a separate study, Dr. Piro Lito and I investigated whether Spry2 plays a role in regulating $HRas^{V12}$-induced transformation of human fibroblasts, as well as uPA and VEGF expression via regulation of Rac1 activity. Dr. Lito recently found that Spry2 is a regulator of Rac1 activity and is required for $HRas^{V12}$-induced transformation of the fibroblast cell line, PH3MT. He found that reduction of Spry2 protein levels results in decreased Rac1 activity. Therefore I hypothesized that PH3MT cells with reduced Spry2, designated PH3MT-2A3, would also have reduced uPA and VEGF levels, and that reintroduction of activated $Rac1^{V12}$ or activated $Cdc42^{V12}$ would restore uPA and VEGF

expression, as well as their ability to form large anchorage-independent colonies and tumors in athymic mice.

These results show that expression of activated Rac1 or Cdc42 restores the ability for these cells to form large anchorage independent colonies indicating that Spry2 regulates $HRas^{V12}$-induced anchorage independent growth by regulating the activity of Rac1 (Fig. 1*B*). However, expression of either mutant failed to restore the ability for these cells to form malignant tumors (Fig. 1*C*). These data indicate that Spry2 regulates multiple pathways that are collectively required for $HRas^{V12}$ oncogene expression to induce transformation.

Results from ELISA analyses show that in contrast to studies in MSU-1.1 fibroblasts, expression of either $Rac1^{V12}$ or $Cdc42^{V12}$ in PH3MT-2A3 cells restores high levels of uPA secretion. This suggests that Ras-effector pathways present in PH3MT-2A3 cells, along with activated Rac1 and Cdc42 are required to regulate the levels of secreted uPA protein (Fig 1*D*). Expression of activated Rac1 does not induce the secretion of VEGF, whereas expression of activated Cdc42 does. These data suggest that activation of Cdc42 can induce VEGF expression independent of activated Ras, whereas Rac1 requires alternate Ras, or alternate Spry2 regulated pathways to regulate the expression of VEGF (Fig. 1*E*).

This dissertation provides evidence that the activities of Rac1 and Cdc42 are required for the malignant transformation of human fibroblast cells by the HRas oncogene and identifies the transformed phenotype each confers. Furthermore, I identified 29 genes, including *VEGF* and *uPA*, which are regulated

by Rac1, Cdc42 and Spry2 that may play an important role in $HRas^{V12}$-induced transformation of human fibroblasts.

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