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THE ROLE OF SPROUTY-2 IN THE MALIGNANT TRANSFORMATION OF HUMAN FIBROBLASTS BY *HRAS* ONCOGENE

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Piro Lito

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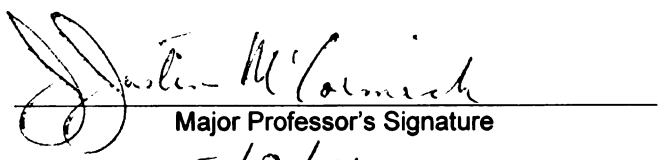

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THE ROLE OF SPROUTY-2 IN THE MALIGNANT TRANSFORMATION OF HUMAN FIBROBLASTS BY *HRAS* ONCOGENE

By

Piro Lito

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Abstract

THE ROLE OF SPROUTY-2 IN THE MALIGNANT TRANSFORMATION OF HUMAN FIBROBLASTS BY *HRAS* ONCOGENE

By

Piro Lito

Sprouty-2 (Spry2) plays a regulatory role in the signaling pathways induced by a number of growth factor receptors. One aspect of the function of Spry2 is to prevent the c-Cbl-induced degradation of epidermal growth factor receptor (EGFR). I found that a human fibroblast cell strain, malignantly transformed by $HRas^{V12}$, exhibited an increase in the expression of Spry2, compared to its parental cell strain. This correlated with an increase in the level of EGFR protein in HRas-transformed cells compared to their parental cells. EGFR activity was required if HRas-transformed cells were to exhibit growth factor independence. To determine whether Spry2 plays a role in *HRas*-transformation, I down-regulated the expression of Spry2 using *Spry2*-specific shRNA. The cell strains with down-regulated Spry2 exhibited not only decreased levels of EGFR, but also decreased levels of ERK activation. I also demonstrated that HRas and Spry2 interact in HRas-transformed fibroblasts, and that HRas interacts with c-Cbl and CIN85 in a Spry2-dependent fashion, suggesting that HRas regulates the turnover of EGFR through Spry2. HRas-transformed cells with down-regulated levels of Spry2 failed to form tumors when injected into athymic mice. Expression of Spry2 in immortalized human fibroblasts independently of $HRas^{V12}$, was not sufficient to induce the malignant transformation of these cells, suggesting that

the role of Spry2 in cancer formation is dependent on *HRas* oncogene. Ras is reported to reduce the sensitivity of cells to DNA damage-induced apoptosis. In the HRas-transformed cell strain that expresses high levels of Spry2, the ability of HRas to prevent UV-induced apoptosis was dependent on intact PI3K- and Rac1-activity. By comparing HRas-transformed cells with endogenous levels of Spry2 to HRas-transformed cells with down-regulated Spry2, I found that Spry2 sustained the activation of PI3K and Akt. Furthermore, I demonstrated that Spry2 sustained the activation of Rac1 by HRas, in part by modulating the interaction between HRas and Tiam1, a GDP-releasing factor for Rac1. Consistent with these findings, the down-regulation of Spry2 in HRas-transformed cells resulted in increased levels of UV-induced apoptosis. The down-regulation of Spry2, also, resulted in an increase in the level of p53, paralleled by a decrease in the level of MDM2 phosphorylated at Ser166, an Akt specific site. Expression of Spry2 in immortalized human fibroblasts resulted in a decrease in the level of UV-induced apoptosis, and in a decrease in the level of p53 protein. Taken together these results indicate that Spry2 facilitates the regulation of EGFR by HRas, and is necessary for the malignant transformation of human fibroblasts by *HRas* oncogene. The data also suggest that Spry2 is an important mediator of survival signals in HRas-transformed cells, because it inhibits DNA-damage induced-apoptosis through the regulation of the p53/MDM2 pathway.

Dedicated to Alex, Zana and Melina

"Science and everyday life cannot and should not be separated. Science, for me, gives a partial explanation to life. In so far as it goes, it is based on fact, experience and experiment". Rosalind Franklin, in a letter to her father, 1940

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Abbreviations

Rb ..... Retinoblastoma

APC ..... Adanomatus Polyposis Coli

EGF ..... Epidermal Growth Factor

PDGF ..... Platelet-derived Growth Factor

EGFR ..... EGF-receptor

PDGFR ..... PDGF-receptor

RTK ..... Receptor Tyrosine Kinases

SOS ..... Son of Sevenless

GTP ..... Guanine Triphosphate

GDP ..... Guanine Diphosphate

ERK ..... Extracellular signal-Regulated Kinase

TGFα ..... Transforming Growth Factor a

NFκB ..... Nuclear Factor kappa Beta

STAT ..... *Signal Transducer Activator of Transcription*

eIF ..... Elongation Factor

CDK ..... Cyclin Ddependent Kinase

ATM ..... Ataxia telangectasia mutant

MDM2 ..... Murine double mutant-2

ALT ..... Alternative lengthening of telomeres

TNF ..... Tumor necrosis facotr

IAP ..... Inhibitor of Apoptosis Proteins

XP ..... Xeroderma Pigmentosum

VEGF ..... Vascular endothelial growth factor

HIF1 ..... Hypoxia inducible factor-1

vHL ..... Von-Hippel Lindau

FAK ..... Focal adhesion kinase

ECM ..... Extracellular Matrix

MMP ..... Matrix Metalloprotease

BPDE ..... Benzo-A-pyrene-diol-epoxide

ENU ..... Ethyl-nitrosourea a

HGF ..... Hepatocyte growth factor

GEF ..... Guanine nucleotide exchange factors

GDI ..... Guanine nucleotide dissociation inhibitor

GAP ..... GTPase activating proteins

DH ..... Dbl homology

PH ..... Pleckstrin homology

IGFR ..... Insulin growth factor receptor

PIP ..... Phosphatidyl inositol phosphate

RBD ..... Ras binding domain

RA ..... Ras association

CR ..... conserved region

MAPK ..... mitogen-activated protein kinase

MAPKK ..... mitogen-activated protein kinase Kinase

MAPKKK ..... mitogen-activated protein kinase Kinase Kinase

PI3K ..... Phosphatidyl inositol-3-kinase

mTOR ..... Mammalian inhibitior of rapamyocin

Spry ..... Sprouty

SH2 .....Src homology-2

SH3 .....Src homology-3

WT1 .....Wilm's tumor-1

FGF ..... Fibroblasts growth factor

NGF ..... Nerve growth factor

PTP1B .....Protien tyrosine phosphatase-1B

GDNF .....Glial derived growth factor

Introduction

Cancer is a genetic disorder that results from the accumulation of genetic and/or epigenetic changes. Such changes uncouple cellular functions form their regulatory mechanisms, and lead to the emergence of a population of cells that has acquired the necessary characteristics to form a cancer. Although distinct types of cancers exhibit specific characteristics, there are several traits that are commonly observed in various types of cancers. These traits include uncontrolled proliferation, deregulated cell cycle, limitless replicative potential, evasion of apoptosis, inefficient DNA repair mechanisms, genetic instability, angiogenesis and invasion and metastasis [1].

As cancer results from changes at the genomic level, a great effort has been placed to determine which genes play a role in cancer formation. In a broad sense, cancer-related genes are classified as oncogenes and tumor suppressor genes, which are altered through gain-of-function or loss-of-function genetic events, respectively [2-4]. These events include mutations, epigenetic regulation, chromosomal translocations and expressional changes [5].

The process of carcinogenesis progresses through distinct intermediate clonal populations of cels, which may have accumulated some of the characteristics of cancer cells, even though such populations of cells are not malignant. The isolation of such intermediate populations from human tumors *in vivo* has been

successful for colorectal cancer [6], but has proven difficult other types of cancer. To overcome this problem, a number of systems have been generated in order to mimic the process of cancer formation. One such system is the MSU-1 lineage of human fibroblasts [7, 8]. This system consists of isogenic cell strains, which have acquired specific genetic changes in a sequential order, and display a progressive accumulation of traits related to cancer. This lineage originates form a normal fibroblast cell line, and culminates in a cell strain capable of forming tumors in athymic mice. Within these extremes, there are a series of isogenic cell strains with an intermediate status. These characteristics make this lineage an efficient model system to the study genetic elements that play a role at the various stages of carcinogenesis.

The Ras GTPase binds to and hydrolyzes GTP to GDP. Ras functions as a critical molecular switch that regulates a number of cellular signaling pathways important for cellular proliferation, survival, and organization of the actin cytoskeleton [9, 10]. Binding to GTP induces a conformational change within Ras that results in Ras activation [11, 12]. Active Ras binds to, and activates a number of effector proteins, including Raf, PI3K and Ral. When GTP is dissociated to GDP, Ras assumes an inactive conformation. This results in the dissociation of effector proteins from Ras and the activation of effector-mediated pathways by Ras is attenuated. *Ras*, a proto-oncogene, is activated to its oncogenic form in approximately 30% of human tumors [13]. The oncogenic activation of *Ras* is the result of mutations in several codons, including codons 12 and 59. These

mutations abrogate the ability of Ras to hydrolyze GTP, stabilizing the active conformation of Ras [14]. The role of *Ras* oncogenes in cancer formation is mediated by the same effectors that mediate the effect of *Ras* proto-oncogenes under normal cellular conditions (i.e. Raf, PI3K, Ral etc.) [15-17]. The difference with oncogenic *Ras*, however, is that the activation of effector pathways by Ras is constitutive, resulting in unrestrained proliferation and in the inactivation of apoptotic programs.

Sprouty (Spry) was identified in *Drosophila melanogaster*, as an inhibitor of receptor tyrosine kinase (RTK) signaling [18-20]. This function of Spry is important for normal development of several organs including the tracheal system and the eye. Mammalian cells express four Spry proteins, which, like the *Drosophila* homolog, retain the ability to suppress RTK signaling [18].

The expression of Spry is prominent in locations where fibroblast growth factor (FGF) and epidermal growth factor (EGF) are prominent. In mammals, Spry acts in a negative feedback fashion to repress signaling form these growth factors. This function of Spry is important in several developmental processes including the development of the kidney, the vestibular apparatus and the bone [21-23].

In *Drosophila* Spry is a general inhibitor of RTK signaling [19]. In mammalian cells, however, Spry proteins, particularly Spry2, sustain RTK signaling induced

by EGF [24-26]. The cellular functions that are mediated by this ability of Spry remain uncharacterized.

Spry proteins may play a role in cancer formation, as several recent studies found that the expression of Spry proteins is altered in some types of cancer. Spry1 and Spry2 are expressed at lower levels in breast and pancreatic tumors [27, 28]. Moreover, Spry2 suppresses tumor formation upon expression in breast cancer cells [29]. This ability is consistent with the function of Spry as an inhibitor of RTK signaling. Spry2 is also expressed at higher levels in melanomas [30, 31]. Although this finding correlates with the ability of Spry2 to sustain epidermal growth factor receptor signaling, the role of Spry2 in the formation of these tumors remains unknown.

The interest of our laboratory in the study of Spry was sparked by a gene expression analysis comparing the expression profiles of cells in the MSU 1 lineage. In particular, this study compared the expression profiles of MSU-1.0, an immortalized diploid human fibroblast cell strain, MSU-1.1, a cell strain derived from MSU-1.0 cells, and PH3MT, a tumor derived cell strain originating from the malignant transformation of MSU-1.1 cell with the $HRas^{V12}$-oncogene. Spry2 was identified as one of the genes that were increased in expression in MSU-1.1 and PH3MT cells, when compared to MSU-1.0 cells. This finding led to the hypothesis that Spry2 promotes tumor formation in HRas-transformed cells, the proof of which is the scope of this dissertation.

Chapter I will provide a broad review of the literature in the field of cancer research, then proceed with more depth to the review of the function of Ras, and finally address in detail the functions of Spry proteins, with an emphasis placed on Spry2.

Chapter II will describe the role of Spry2 in the transformation of immortalized human fibroblasts by oncogenic *HRas*. This study found that Spry2 is necessary for the ability of HRas transformed fibroblasts to form tumors in athymic mice. Furthermore, this study demonstrated that HRas interacts with Spry2 and two Spry2-binding partners c-Cbl and CIN85.

Chapter III will describe the role of Spry2 in the ability of HRas to induce survival pathways that desensitize human fibroblasts to DNA damage-induced apoptosis. This study found that Spry2 is necessary to protect Ras-transformed cells from UV-induced apoptosis. In this context, Spry2 sustained the activation of enzymes involved in survival pathways, including phosphatidyl inositol-3 kinase (PI3K), Akt, and Rac1, while maintaining a low level of the pro-apoptotic tumor suppressor p53.

Appendix A will describe the gene expression study that compared the expression profiles of MSU-1.0, MSU-1.1 and PH3MT cells. In addition to the identification of Spry2 with a possible role in cancer formation, this study also

found a number of other genes differentially expressed between malignant and pre-malignant cells.

Appendix B will describe research in progress to determine the role of Spry2 in the ability of human patient-derived fibrosarcoma cell lines to form tumors in athymic mice. Two cell lines, HT1080 and VIP:FT, which contain oncogenic $NRas^{Q59}$ and wild type *Ras* respectively, express high levels of Spry2. This study found that Spry2 contributes to the malignant phenotype of VIP:FT cells, and, at a lesser extent, to the malignant phenotype of HT1080 cells, suggesting that Spry2 contributes to tumor formation in a context-specific fashion.

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Chapter I: Review of Literature

Cancer as a genetic disorder

The American cancer society estimates that cancer caused approximately 556,000 deaths in 2003, accounting for nearly 23% of deaths in the United States [1]. Worldwide, 10 million new cases were reported in 2000, and 6 million people died from cancer [2]. Cancer is a complex pathologic disorder that is a result of multiple changes in normal physiology. Initially, change(s) within a cell result in unregulated cellular growth, leading to an abnormal accumulation of cells and the formation of a tumor at a particular location, which represents the primary tumor site or tumor origin. In some cases, tumors infiltrate the tissue surrounding the primary site and invade lymph and/or blood vessels, a process known as invasion. In addition, tumors that invade can implant to a secondary location, a process known as metastasis. Tumors that invade and metastasize are defined as malignant tumors, and constitute a cancer. In contrast tumors that are restricted to the site of origin and exhibit no evidence of invasion are defined as benign tumors. These are often precursors of malignant tumors, but benign tumors themselves do not constitute a cancer [3].

Cancer-related genes

Cancers arise by the sequential acquisition of genetic and/or epigenetic changes, which ultimately modify the activity of proteins encoded by the affected genes [4].

Many of these changes confer upon a cell some selective advantage that allows the expansion of a clonal population. By reiteration of this pattern over a period of years, a single cell emerges that has acquired all the necessary changes to form a cancer [5, 6]. Based on epidemiological evidence on the frequency of cancer incidence, Renan predicted that a normal cell requires between four to seven changes to form a cancer [7].

A great deal of effort has been made to determine which cellular genes are involved in cancer formation. Such genes can be classified into two groups: oncogenes, which are activated by gain-of-function genetic events and tumor suppressor genes, which inactivated by loss-of-function genetic events [8-10].

In an attempt to elucidate the origins of cancer, early studies found that injection of several RNA viruses in some animals resulted in cancer formation [8, 11]. These viruses were also found to induce cellular transformation in culture. Cellular transformation refers to the acquisition of some of the characteristics of tumor cells (e.g. morphological change or limitless replicative potential), without including malignant tumor formation. The transforming ability such viruses is mediated by single genetic elements (e.g. *v-Src* and *v-Myc*), which are homologous to genes found in normal cells [8, 12, 13]. The cellular counterparts of these genes (e.g. *c-Src* and *c-Myc*) are important for maintaining normal cell growth and differentiation, and play a causal role in cancer when they are activated [14, 15].

Genes that lead to cancer formation upon their activation are defined as oncogenes, while the cellular genes form which they are derived are defined as proto-oncogenes. Oncogenes act in a dominant fashion, implying that the activation of a single genetic allele suffices for their activation. Oncogene activation, results from point mutations (e.g. Ras^{V12} in pancreatic carcinomas [16], [17]), amplified expression (e.g. *Her2/Neu* in breast cancers [18, 19]), and chromosomal translocations (e.g. *c-Myc* in Burkitts lymphoma and *c-Abl* in chronic myelogenous leukemia [20, 21]).

Tumor suppressor genes also play an important role in cancer formation. The cancer in which the role of tumor suppressors became evident is Retinoblastoma. This condition is characterized by unilateral or bilateral retinal tumors that afflict young children [22, 23]. Knudson [24] hypothesized that Retinoblastoma resulted from inactivating mutations in a gene encoding a growth inhibitory protein. In light of the fact that some children develop unilateral lesions, whereas others develop bilateral lesions, Knudson postulated that when tumors were found in both eyes, this was a consequence of a single inactivating mutation, which was inherited, and a second mutation acquired independently as retinal cells divided to form the retina. The gene responsible for this condition, designated *retinoblastoma* (*Rb*), was the first tumor suppressor gene to be identified [25-27]. Normally, Rb serves to repress cellular proliferation by inhibiting cell cycle progression (discussed below), thus suppressing uncontrolled proliferation of cells. In Retinoblastoma,

this gene is inactivated by loss-of-function mutations, and therefore the ability of Rb to inhibit cell cycle progression is lost [28]. Since the discovery of Rb, a number of other tumor suppressor genes have been identified, which regulate diverse cellular functions and protect cells form malignant transformation [29].

Inactivation of a single allele of a tumor suppressor gene is not sufficient for tumor formation, as the other allele of the gene remains intact and can provide enough wild type protein to maintain a normal phenotype. Therefore, both copies of the gene must be inactivated in order for the function of the tumor suppressor gene to be lost. This is commonly known as the "two hit hypothesis" [30].

Tumor suppressor genes have been further classified as gatekeepers or as caretakers [31]. Vogelstein and colleagues discovered that *APC*, a tumor suppressor gene, is inactivated in a type of colon cancer known as Familial Adenomatus Coli [32, 33]. Biallelic inactivation of *APC* is the rate limiting step for the formation of these tumors. Genes with this property in cancer formation are designated gatekeepers. Another subset of colon cancers, known as Hereditary Nonpolyposis Colorectal Cancer, arises form mutations in DNA mismatch repair genes [34, 35]. Because these genes are important in repairing damaged DNA, their inactivation increases the mutation rate and chromosomal instability, which facilitate cancer formation [36]. Tumor suppressor genes that act in this fashion are designated as caretaker genes, i.e. genes whose inactivation accelerates malignant transformation.

Mutations are the most important cause for the alteration of normal cellular genes in the process of tumor formation. Nevertheless, epigenetic events, such as imprinting and hypermethylation have also proven to be important in inducing the necessary changes for cancer formation. Epigenetic regulation refers to the control of gene expression through modifications of chromatin structure in the gene promoter region [37]. These modifications facilitate (e.g. acetylation), or impede (e.g. methylation) transcription from a particular gene promoter, resulting in alterations in gene expression. Disruption of epigenetic regulation can also contribute to cancer formation. For example, loss of imprinting facilitates the formation of Wilm's tumor [18] and hypermethylation of the *p21* promoter results in the loss of expression of *p21* in various tumors [38].

Characteristics of cancer cells

The family of cancer consists of a large number of distinct types, each displaying some unique characteristics, particularly in regards to the cell of origin. Nevertheless, there are also characteristics that are commonly found in various cancer subtypes [39]. Such common traits include: (1) enhanced or uncontrolled proliferation, (2) aberrant cell cycle control, (3) limitless replication potential, (4) evasion of apoptosis, (5) inefficient DNA repair mechanisms, (6) genomic instability, (7) angiogenesis and (8) invasion and metastasis.

Uncontrolled proliferation

Under normal conditions, a cell requires mitogenic growth signals for proliferation. Cancer cells, however, have acquired the ability of autonomous proliferation, i.e. they can replicate in the absence of exogenous growth factors. This autonomy is acquired as a result of changes that enable cancer cells to uncouple normal proliferation pathways from their regulatory mechanisms.

With this in mind, it is important to describe the process responsible for the regulation of proliferation in normal cells, before describing how cancer cells acquire their proliferative autonomy. Of the several pathways that regulate cellular proliferation within a cell, the pathways induced by growth factors are the most important. Extracellular protein growth factors, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), bind to and activate transmembrane receptors, such as the EGF-receptor (EGFR) and the PDGF-receptor (PDGFR), respectively. These receptors belong to a wider family, known as receptor tyrosine kinases (RTK).

Growth factors bind to these receptors and induced their dimerization. This process leads to autophosphorylation of the receptors on their cytoplasmic domains, and this leads to receptor activation [40]. The phosphorylation sites on RTKs, serve as docking sites for numerous effectors, including the adaptor protein Grb2 [41]. Grb2 contains a Src homology 2 (SH2) domain, which binds to phospho-tyrosine residues and guides the migration of Grb2 to the

phosphotyrosine residues on the cytoplasmic tail of the RTK [42]. In this fashion, growth factors stimulate the translocation of Grb2 from its cytosolic localization to the plasma membrane. Grb2 forms a complex with son of sevenless (SOS), leading to the translocation of SOS to the plasma membrane alongside Grb2 [43, 44]. At the plasma membrane SOS activates Ras GTPases, which are embedded in the plasma membrane by farnesylation [45]. In this fashion, the extracellular ligand-induced activation of receptor tyrosine kinases results in the activation of Ras GTPases. These GTPases catalyze the hydrolysis of GTP into GDP, and serve as a molecular switch for the activation of many intracellular pathways [46, 47]. In normal cells, Ras oscillates between an active, GTP-bound state and an inactive, GDP-bound state [48]. The Grb2:SOS-mediated activation of Ras in response to growth factor stimulation is a critical event in the regulation of proliferation. This is mainly because active Ras regulates the Raf/MEK/ERK pathway [49]. Activation of this pathway directly regulates the expression of many genes that are important for cell cycle progression and proliferation (e.g. cyclin D) [50-52].

One mechanism by which cancer cells hijack this regulatory pathway is the production of endogenous growth factors. For example, glioblastomas and sarcomas have been frequently found to produce PDGF and TGFα respectively [39, 53, 54]. Upon secretion, these growth factors act in an autocrine fashion to stimulate RTK signaling and proliferation of cancer cells in the absence of exogenous growth factors.

Cancer cells may also exhibit an increased level of RTK activity, because of mutations that result in constitutive activation of the receptor, or amplification of the receptor. For example, c-Kit receptor tyrosine kinase, is activated in gastrointestinal stromal tumors by a point mutation, which results in the constitutive dimerization of this receptor [55]. Growth factors activate RTKs by inducing their dimerization. Upon growth factor withdrawal, the receptors revert to a monomeric state. Thus, constitutively dimerized receptors are active even in the absence of growth factor stimulation, which results in uncontrolled signaling activity. Alternatively, and more commonly, growth factor receptors are overexpressed in cancer cells, as is the case in brain, breast and stomach tumors, which overexpress the tyrosine kinase receptor EGFR [56]. This also results in sustained activity from the receptor, and a sustained drive for cellular proliferation.

Proliferative autonomy in cancer is most often acquired via the constitutive activation of proto-oncogenes, such as *Ras* in colon and pancreatic cancers [57] and *Abl* in chronic myelogenous leukemia [58]. Activation of the *Ras* proto-oncogene is a result of point mutations in codons 12, 13, 59 and 61 [59-61]. These mutations diminish the GTPase activity of Ras, which results in higher levels of active, GTP-bound Ras, and lead to sustained proliferative signaling from Ras. Abl is a non receptor tyrosine kinase that also functions in signaling pathways that regulate cell growth [62]. In chronic myelogenous leukemia *Abl* is

activated through reciprocal translocation between chromosomes 9 and 22, resulting in the formation of the "Philadelphia chromosome", which contains a fusion of *Abl* with *BCR* [63]. Although the exact mechanism by which BCR-Abl affects cancer formation remains under investigation, one mechanism appears to be an increase in the kinase activity of Abl [64, 65]. This increase results in part because an inhibitory region on the N-terminus of Abl is lost upon fusion of Abl with BCR [66].

The modulation of transcription factor activity also contributes to the higher rate of proliferation observed in most cancer cells. The transcription factors that play a role in cancer can be generalized into three groups [67, 68], including steroid receptors, such as estrogen and androgen receptors, nuclear transcription factors, such as c-Myc and c-Jun, and cytoplasmic factors, such as NFκB and STATS. Expressional amplification, or constitutive activation of these transcription factors not only contributes to the unrestrained growth of cancer cells, but is also important for the acquisition of other characteristics of cancer cells, such as evasion of apoptosis and deregulation of the cell cycle. The c-Myc transcription factor is of particular importance, given that *c-Myc* proto-oncogene is involved in the formation of many cancers, including those of the prostate, breast and skin [69–71]. *c-Myc* encodes a basic helix-loop-helix-zipper transcription factor, which specifically binds to E-box sequences in the DNA, upon heterodimerization with its binding partner MAX [12]. As part of this complex, c-Myc activates a diverse group of genes, including cell cycle regulators (e.g. *cyclin D2* and *CDK2*) and

translation initiation factors (e.g. *eIF2* and *eIF4*). Functionally, c-Myc drives cellular proliferation, inhibits differentiation, and induces apoptosis [72-74].

Aberrant cell cycle regulation

In somatic cells, the normal cell cycle consists of a resting stage, Go, in which the cell is not dividing, and four stages, G1, S, G2 and M, which are present in dividing cells. S is the stage during which DNA synthesis take places, while M is the stage of mitosis, were cell division occurs. G1 is a gap phase between M and S, whereas G2 is a gap phase between S and M. These gaps allow the cell to prepare for the ensuing stage, as well as to repair DNA damage [75].

The cell cycle is regulated by an array of proteins, including cyclins (A, B and D), cyclin-dependent kinases (CDK1-4) and CDK inhibitors (CKI, e.g. p21 and p27) [76, 77]. In broad terms, a specific cyclin binds to a specific CDK, and leads to CDK activation. This CDK, in turn, phosphorylates and regulates enzymes that are responsible for progression through the different stages of the cells cycle. In this fashion, cyclin/CDK complexes promote cell cycle progression. Importantly, the progression through a particular phase of the cell cycle is regulated by a specific cyclin/CDK complex. CDK inhibitors bind to cyclin/CDK complexes and inhibit their activity. Consequently, these inhibitors arrest progression through the cell cycle [76, 78, 79].

The transition through G1 to S is of particular importance, because it is the part of the cell cycle where many intracellular signaling pathways exert their control on cell cycle progression. This transition is mainly regulated by the E2F family of transcription factors. E2F regulates a number of genes that are involved in maintaining S phase, such as DNA polymerases and cyclin E [80]. In quiescent cells, E2F is repressed by the Rb tumor suppressor, when the latter is in a hypophosphorylated form. Growth stimulation by mitogenic signals activates the Ras/MAPK cascade, resulting in transcriptional activation of cyclin D. Cyclin D interacts with CDK4, and the cyclinD/CDK4 complex hyperphosphorylates Rb, promoting the release of E2F from RB. Active E2F transcriptionally activates genes that are necessary for progression from G1 to S [81, 82].

Aberrations in the mechanisms that regulate this transition through the cell cycle occur frequently in human cancers, and include inactivation of Rb (e.g. Retinoblastoma), as well as overexpression of cyclin D1, which is commonly observed in breast, lung and colon cancers [83, 84].

The S phase is maintained predominantly by the cyclin E/CDK2 complex [85, 86]. The cyclin E/CDK2 complex also activates E2F [87]. Activation of E2F, at this period of the cell cycle, induces cyclin A expression. Cyclin A, is responsible for the transition from G2 to M, which leads the cells into the stage of division [88]. Cyclin A also regulates the first half of mitosis, whereas cyclin B, in complex with CDK1, is responsible for regulation of the second half of mitosis [3].

CDK inhibitors include members of the CIP/KIP family (p21, p27 and p57) and the *INK4a* locus ($p16^{INK4a}$ and $p14^{ARF}$). p21 and p27 inhibit the function of cyclin/CDK complexes, and therefore, they prevent progression through S and G2 phases [89]. $p16^{INK4a}$ inhibits the cyclin D/CDK interaction, thereby inactivating E2F, whereas $p14^{ARF}$ prevents cell cycle progression by inhibiting the ubiquitinylation and degradation of p53 by MDM2 [90].

In addition to the regulatory programs described above, there exists another level of control, termed cell cycle checkpoint control, which is responsible for ensuring the integrity of the genome as the cell progresses through the different phases of the cell cycle [91]. Regulation at this level involves sensor, transducer, and effector proteins, and is divided into the G1/S checkpoint, the S-phase checkpoint, and the G2/M checkpoint. Such checkpoints prevent cells with damaged DNA from entering S-phase (G1/S), prevent the start of replication in the case of genotoxic insults (S-phase), or prevent cells with aberrant DNA replication from entering mitosis (G2/M) [92-94]. In the absence, or in the inactivation of these checkpoints, damaged DNA is replicated, leading to a higher frequency of mutations, thus increasing the risk of cancer formation.

The sensors of DNA damage, i.e. the factors that initiate checkpoint control involve mainly ATM and ATR. The former is instantaneously activated by DNA lesions, whereas the latter is activated by stalling of the replication fork. ATM and

ATR activate signaling transducers, such as CHK proteins, which then recruit and activate effectors including p53 [91].

The tumor suppressor gene *p53* plays an important role in cell cycle regulation and cancer formation. *p53* is found to be mutated in approximately 50% of human cancers. *p53* encodes for a transcription factor that acts as a homotetramer to regulate cell cycle arrest, apoptosis and DNA repair [95-97]. Under physiological conditions, the levels of p53 are maintained at a low level by MDM2, which is an E3 ubiquitin ligase enzyme [98]. MDM2 ubiquitinates p53 and promotes its degradation by the proteasome [99].

The transcriptional activity of p53 is activated in response to cellular stresses that induce DNA damage. Upon DNA damage, the ubiquitination of p53 by MDM2 is abolished, and p53 translocates to the nucleus, where it induces the transcription of genes such as *p21*, *Bax*, and *Gadd5* [100]. This results in the activation of the p21 CKI, which, as described above, arrests the cell cycle by inhibiting cyclin/CDK complexes [101]. The consequence of this arrest is to allow more time for the repair of DNA damage. If the damage is not repaired, p53 may induce apoptosis via the transcriptional activation of BAX, a pro-apoptotic factor that inhibits the anti-apoptotic factor Bcl-2 [102].

*Lim*itless replication potential

In 1961, Hayflick suggested that human cells in culture have a limited lifespan [103]. Fibroblasts, for example, can replicate for 60-80 cell doublings, after which they stop the progression through the cell cycle and enter a metabolically active state termed senescence. Senescent fibroblasts are enlarged and exhibit a flat morphology, and can persist for many years in this state. The cell cycle arrest during senescence is dependent on the activities of the p53 and Rb tumor suppressors [39]. Upon inhibition of these pathways, cells can propagate beyond their normal life span in culture. Nevertheless, within a limited number of population doublings, such cells enter a state termed crisis that is characterized by massive apoptosis. In some instances, cells survive this state and emerge with a limitless replicative potential [104].

Cell replication in culture is limited by the shortening of telomeric ends of chromosomes. Telomeres consist of tandem repeats of the sequence *TTAGGG* [105]. These repeats are limited in number, and they are consecutively shortened during the replication of chromosome [106]. In this way, the length of telomeres serves as a molecular timer that counts down with every cell replication. When the telomeric repeats fall below a critical number, terminal parts of chromosomal DNA are lost during DNA synthesis. This process triggers the cellular programs that bring on senescence, although the exact mechanism is not fully understood [107]. If the cell continues to divide the progressive loss of chromosomal ends leads to genomic instability, which is responsible for the induction of crisis.

Cancer cells in culture typically exhibit limitless replicative potential, suggesting that this characteristic is acquired during tumor progression [108]. In some malignant cells, proliferation is paralleled by widespread apoptosis, suggesting that the limited lifespan of somatic cells needs to be surpassed for cancers to form [39]. Telomere maintenance is present in the majority of cancer cells [109]. This is mainly attributed to the up regulation of telomerase expression, an enzyme responsible for the synthesis of telomeres [110]. Not surprisingly, expression of telomerase is sufficient to bypass senescence and crisis, conferring limitless replicative potential to cells [111]. It should be noted that there is a telomerase-independent mechanism for telomere maintenance. This mechanism involves recombination and is referred to as alternative lengthening of telomeres [112].

Evasion of apoptosis

Apoptosis is regulated by two distinct pathways. One is mediated by death receptors in response to extracellular signals and is termed the 'extrinsic pathway'. The other is mediated by the mitochondria in response to internal cues, including DNA damage, and is referred to as the 'intrinsic pathway' [113, 114].

In the extrinsic pathway, death receptors, such as CD95 and TRAIL-R1, are activated through their interaction with various ligands that belong to the TNF family of secreted proteins [115]. This interaction induces receptor clustering, and

leads to the recruitment of caspase-8 and caspase-10 to the receptor, via the adaptor protein FADD [116]. Caspases are cysteine proteases that are synthesized as inactive zymogens. Most caspases are activated by proteolytic cleavage, usually induced by another active caspase [117]. After their recruitment to the death receptor, both caspase-8 and caspase-10 are cleaved into their active forms [118]. c-FLIP negatively regulates the extrinsic apoptotic pathway by inhibiting caspase-8 activation by the death receptor-FADD complex [119]. Activation of caspase-8 leads to the step wise activation of a cascade of caspases culminating in the activation of caspase-3.

In the intrinsic pathway, DNA damage results in the secretion of cytochrome c from the mitochondria [120]. Cytochrome c release from mitochondria is regulated by pro-apoptotic (BAX, BID, BAD) and anti-apoptotic (BCL2, BCL-X_L) factors, and the net outcome results from an imbalance between the two types of factors. In the cytosol, cytochrome c interacts with Apaf-1 and caspase-9, resulting in the formation of the "apoptosome" [121]. The 'apoptosome' activates caspase-3, a process that is antagonized by inhibitor of apoptosis proteins (IAP), which in turns are inactivated by Smac/DIABLO [122]. Therefore the intrinsic and extrinsic pathways converge at the activation of caspase-3, an important effector caspase that targets critical cellular enzymes resulting in apoptosis.

The cellular phenotype associated with apoptosis results form the proteolysis of various cellular substrates. Proteolysis of substrates such as nuclear lamins

results in nuclear condensation, proteolysis of DNase inhibitor ICAD, activates an endonuclease that fragmentates DNA, whereas proteolysis of cytoskeletal proteins results in cell fragmentation [114].

Apoptosis acts as a potent control to prevent malignant transformation. As noted above, cancers arise as a result of genetic alterations (e.g. oncogene activation), and subsequent clonal expansion of the cells. Activation of apoptotic pathways acts to prevent clonal expansion, and thus limit the chance for full blown neoplasm formation. Interestingly, the activation of some oncogenes (e.g. overexpression of *c-Myc* and *E2F*), in itself sensitizes cells to apoptosis, thus preventing malignant transformation [73].

Apoptosis can also be initiated in response to extensive DNA damage. This is mediated by the function of p53, which "senses" DNA damage and activates the intrinsic apoptotic pathway through the induction of BAX [102].

The treatment of cancers by radiation or chemotherapy also relies on the induction of apoptosis. Inactivation of pro-apoptotic pathways in cancer cells compromises the efficacy of such treatments. For example, malignant melanomas that have lost expression of APAF1 become resistant to chemotherapy [123].

The importance of apoptosis in slowing cancer progression is also apparent in that almost all of the factors involved in the regulation of apoptosis are affected in human cancers. For example, the anti-apoptotic factors BCL2 and BCL-X_L are overexpressed in myeloid leukemia and in acute lymphoblastic leukemia, respectively. Also, the pro-apoptotic factor BAX has been found to be down-regulated in colon cancer. Furthermore, additional apoptotic regulators, such as FLIP, soluble death ligands, IAPs, p53, PI3K, AKT and PTEN are reported to be deregulated in tumors [114].

Inefficient DNA repair mechanisms

DNA damage is especially important for cancer formation, because it causes mutations in replicating cells. As indicated above, mutations may result in the loss of function of tumor suppressor genes, as well as in the activation of oncogenes. These events are sufficient to initiate and maintain the process of malignant transformation [124].

DNA damage is as a result of exogenous or endogenous chemicals that form adducts with DNA bases directly, or indirectly, through their metabolites. Furthermore, DNA damage is a result of physical agents such as UV and ionizing irradiation. In order to maintain the information encoded in the DNA unaltered, cells have developed mechanisms to repair such damage, which results in a low frequency of mutation. The repertoire of the cell's DNA repair machinery includes base excision repair, nucleotide excision repair and mismatch repair, as well as

homologous recombination and nonhomologous end joining [125]. What is more, there exists a "damage tolerance" pathway, in which specialized polymerases (Y-Family polymerases) bypass lesions that stall the major replication polymerase (Pol δ) [126].

Furthermore, as cells progress through the cell cycle, the cells must pass through several checkpoints, which are regulated by distinct cellular enzymes, such as p53 and ATM. These enzymes are responsible for arresting cell cycle progression, and inducing programmed cell death, thus preventing cells with damaged DNA from propagating.

Deficiencies in the DNA-repair machinery enhance mutation frequency and are detrimental for normal cell function. Paradigmatically, mismatch repair genes are inactivated in human hereditary colorectal cancer, a common malignancy of the colon [31, 34, 35]. Mismatch repair genes (e.g. *MSH2*) act as caretaker genes and their inactivation enhances the mutation frequency, which can lead to cancer formation. In this setting, oncogenes and tumor suppressor genes are more likely to be activated and suppressed, respectively.

Xeroderma Pigmentosum (XP) is a syndrome that renders patients susceptible to skin cancer [127]. The cells of these patients are deficient in nucleotide excision repair, resulting in a higher frequency of sunlight-induced mutations in these cells [128, 129]. A subset of XP patients has a normal nucleotide excision repair

mechanism, yet the frequency of UV-induced mutations in cells derived from these patients is also high. This condition, designated Xeroderma Pigmentosum Variant is characterized by defects in polymerase eta, an error-free specialized polymerase involved in translesion synthesis [130].

Genetic instability

Genetic instability, a feature of many cancer cells, refers to the consistent failure to transmit an accurate copy of a complete genome from one cell to its two daughter cells. This can be subdivided into microsatellite and chromosomal instability [131]. Microsatellite instability is a result of mutations or inactivation of DNA-mismatch-repair genes, such as *MSH2*. Chromosomal instability can be further subdivided into instability in chromosome structure and instability in chromosome number. Instability in chromosome structure involves deletions, inversions, translocations and insertions of small sequences of DNA. In tumor cells, this type of instability often results from the inactivation of DNA-damage checkpoint genes, such as *ATM* and *p53*, as well as from deficiencies in genes involved in double strand break repair like *DNA-PK*. Instability in chromosome number is a product of abnormal centrosome duplication with multipolar mitoses, and arises form deficiencies in *BRCA1* and spindle checkpoint genes (*MAD1*). Chromosomal instability leads to an enhanced rate of loss of heterozygosity, which is an important mechanism of inactivating tumor suppressor genes [132].

Angiogenesis

Cells within a tissue require the delivery of oxygen and nutrients for their growth and proliferation. To achieve this, cells are generally located within 100-200 μm from blood vessels [3]. With this in mind, organismal growth requires that new blood vessels are formed, so that the new cells are constantly being perfused. The process by which new vessels are formed is defined as angiogenesis. This process is strictly regulated by a plethora of factors, which either stimulate or inhibit the formation of new blood vessels. In normal tissue, the tendency of the pro-angiogenic factor to stimulate angiogenesis is balanced by that of the anti-angiogenic factors [133, 134].

In solid tumors, growth is frequently limited by the hypoxic condition at the center of the tumor, and by the sparsity of blood vessels to deliver oxygen and nutrients to the tumor site. Therefore, a tumor cannot grow beyond a critical size (estimated at 200 μm) unless the tumor receives sufficient blood perfusion to support its own metabolic needs. Tumors can remain in this stage for a period of months to years [3]. When the balance between pro- and anti- angiogenic factors is shifted to promote the formation of new vessels, a process referred to as "angiogenic switch", tumor growth resumes, as oxygen and nutrients are being delivered to the tumor site [135, 136].

Typically, angiogenesis is initiated and carried out by endothelial cells lining up existing blood vessels [137, 138]. Endothelial cells express receptors for pro-

angiogenic factors that are secreted in the interstitial fluid, or that are incorporated in the extracellular matrix. Upon binding to their ligands, these receptors become active and they turn on endothelial angiogenic programs [139]. In addition, upon their stimulation to neovascularize, endothelial cells secrete matrix proteases that degrade the extracellular matrix and allow the endothelial cells to proliferate towards the source of their stimulus [140]. Furthermore, angiogenesis also involves the arrangement of endothelial cells into tubular structures, their canalization and their intussusceptions into existing vessels [138, 141, 142].

Vascular endothelium growth factor (VEGF) is the best characterized regulator of angiogenesis. VEGF is secreted by the tumor cells or by the tumor stroma, and is critically important for the 'de novo' formation of angiogenesis [143-145]. The secretion of VEGF is regulated by the transcription factor HIF1, as a response to hypoxic stress in the tumor microenvironment [146]. When oxygen is abundant in the microenvironment, non-heme iron dependent oxygeneases hydroxylate HIF1 on specific residues, mediating an interaction between HIF1 and the vHL E3 ubiquitin ligase. This process results in the ubiquitinylation and subsequent degradation of HIF1. Upon hypoxic conditions, the oxygenases responsible for HIF hydroxylation remain in an inactive state, thereby failing to induce the post-translational down regulation of HIF1 [147]. This failure results in a transcriptional activation of HIF1 inducible genes, which include the angiogenic stimulator VEGF. The VEGF receptor, belongs to the class of RTKs, and transduces the

VEGF angiogenic signal through a number of effector proteins including FAK, PI3K and Ras-dependent pathways [148, 149].

Invasion and metastasis

Malignant tumors are characterized by their ability to invade surrounding tissues and as a result to metastasize into other locations in the body. The metastatic process involves detachment of the tumor from its primary site, degradation of its extracellular matrix (ECM), invasion into the blood vessel, and transplantation to a secondary site [3].

The initial stages in the metastatic process are regulated by cell adhesion molecules and integrins [150, 151]. E-cadherin, a transmembrane glycoprotein, is an important factor for epithelial cell adhesion. Some carcinomas express reduced levels of E-cadherin, resulting in loose attachment between the epithelial cells, thereby enhancing their potential for metastasis. Alternatively, carcinomas with normal levels of E-cadherin, contain inactivated catenin, which is an intracellular effector of E-cadherin [152].

To infiltrate through the ECM, tumor cells first bind to the components of the ECM via transmembrane proteins of the integrin family and laminin family [151, 153]. These proteins, which are expressed in normal cells as well, are frequently amplified in cancer cells. In particular, cancer cells express integrins that are not specific for the type of tissue were the cancer originated from. This enables

invading cells to attach to new locations giving rise to new foci of tumor growth [151]. Once bound to ECM, the tumor cell secretes specific proteases, such as matrix metalloproteases (MMP2 and 9), which degrade ECM to facilitate the infiltration of tumor cells [154, 155].

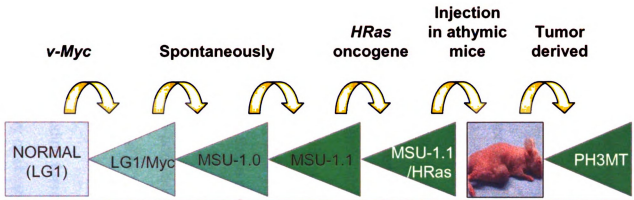
In the process of invading adjacent tissue, cancer cells may invade into blood vessels. Once in the blood vessel, the tumor cells evade the immune system by homotypic adhesion (i.e. aggregation of tumor cells with each other) or heterotypic adhesions (aggregation between tumor and blood cells) [156]. The site of extravasation is dependent in part on the anatomical location of the primary tumor. Extravasation and transplantation of the tumor cells involves the attachment of tumor cells into a new tissue type, a process that is facilitated by laminin receptors and integrins, which bind to ligands embedded in the ECM of the metastatic site [157]. In addition, chemokines and their receptors also play a role in determining the target site for metastasis, particularly in breast cancer cells [158].

MSU-1 lineage as model system to study malignant transformation

To study the process of malignant transformation, McCormick and Maher developed the MSU-1 lineage of human fibroblasts (Fig. 1), as a model system that mimics the process by which normal cells become malignant [159-163]. This lineage originates from the transfection of normal foreskin-derived human fibroblasts with a vector encoding the *v-Myc* oncogene and a neomycin resistance marker [164]. As neomycin resistant clones were being propagated in culture, they underwent senescence, and the majority of the clones succumbed to crisis. Nevertheless, it became apparent that several clones had survived this process. Because cells that escape senescence and crisis spontaneously acquire an immortal life span [104, 165], the *v-Myc*-expressing clones were propagated in culture for many cell doublings to determine if exhibited extended lifespan. It was found that the cells from these clones were indeed immortal, and they were designated MSU-1.0.

Experiments conducted later showed that MSU-1.0 cells express telomerase, a gene known to immortalize to cells (McCormick, unpublished data). Myc confers immortality to cells by inducing the expression of telomerase [166-169], yet in our system it is unlikely that Myc alone is responsible for the immortalization of MSU-1.0 cells. This is because all but one of the Myc expressing clones succumbed to crisis, just like the mock transfected clones. What is more, MSU-1.0 cells also express elevated levels of the transcription factor Sp1, when compared to their

Figure 1. MSU 1 lineage of human fibroblasts. The MSU 1 lineage consists of isogenic cell strains which have been derived from the same normal cell line, and have progressively increasing malignant characteristics. Some characteristics of each cell line, as well as the known genetic modification(s) that are responsible for its formation are indicated.



Chromosomally stable	Yes	Yes	Yes	Yes	Yes	Yes
Diploid	Yes	Yes	Yes	Nearly	Nearly	Nearly
Marker chromosomes	No	No	No	Yes	Yes	Yes
Immortal	No	No	Yes	Yes	Yes	Yes
Transformable	No	No	No	Yes	Transformed	Transformed
Growth factor independence	No	No	No	Partial	Yes	Yes

Figure 1

precursor cells. Sp1 has been shown to cooperate with Myc in the induction of telomerase expression [170, 171], suggesting that Myc and Sp1 are most likely responsible for telomerase expression in MSU-1.0 cells (McCormick, unpublished data).

Apart from being immortal, MSU-1.0 cells exhibit the usual characteristics of normal fibroblasts, i.e., they are diploid, chromosomally stable, require normal levels of growth factors for proliferation, do not form foci or colonies in agar, and they fail to form tumors upon injection in athymic mice. Furthermore, expression of oncogenes such as, and v-sis in these cells, as well as treatment with benzo-A-pyrene-diol-epoxide (BPDE) and focus selection alone, or treatment with BPDE, focus selection and subsequent expression of the $HRas^{V12}$ oncogene, did not malignantly transformed these cells.

A cell in the MSU-1.0 population spontaneously underwent two chromosomal translocations, giving rise to a variant clonal population, designated MSU-1.1. MSU-1.1 cells are chromosomally stable, and nearly diploid, i.e., they consist of 45 chromosomes, including two characteristic chromosome markers, M1 and M2 along with a monosomy of chromosomes 11, 12 and 15 [164]. M1 resulted from the translocation of chromosome 11 (11p15→qter) to chromosome 1 at p11, whereas M2 resulted from translocation of chromosome 12 (12qter→12q11) to chromosome 15 (15p11→15qter). Fingerprint analysis, and analysis of the v-myc integration site by southern blotting, showed that MSU-1.0 and MSU-1.1 cells

were both derived from LG1 cells, and MSU-1.1 cells must have been derived form MSU-1.0 cells, respectively.

MSU-1.1 cells exhibit an immortal life span in culture, but they do not form foci, colonies in agar, or tumors upon injection in athymic mice. Nevertheless, unlike their precursor cells, MSU-1.1 cells exhibit partial growth factor independence, and can be malignantly transformed by the expression of various oncogenes, or by carcinogen treatment followed by focus selection.

Expression of oncogenes, such as $HRas^{V12}$, $NRas^{Q59}$ and *v-sis* at expression levels similar to the expression of the respective proto-oncogenes, results in transformation of MSU-1.1 cells (Fig. 2A) [172-174]. MSU-1.1 cells expressing these oncogenes form foci and colonies in agarose at higher rates that the parental MSU-1.1 cells. These cells, however, are not malignant, i.e., they do not form tumors when injected in athymic mice. However, overexpression of the $HRas^{V12}$, $NRas^{V12}$, *v-KRas* and $KRas^{V12}$ oncogenes, results in malignant transformation of these cells, i.e., MSU-1.1 cells expressing these oncogenes form malignant tumors in athymic mice [173-177]. These results suggest that more than one genetic change is required for the malignant transformation of MSU-1.1 cells. Consistently, sequential expression of two oncogenes, each of which is expressed at levels similar to its endogenous level, followed by clonal selection after each expression, results in the malignant transformation of MSU-1.1 cells. In this way, the coexpression of either $HRas^{V12}$ or $NRas^{Q59}$ with *v-fes*, or

Figure 2. MSU 1 lineage as a tool for the study of malignant transformation. The MSU 1 lineage has been used to study the role of many genes in malignant transformation. (A) Unlike their precursor cells, MSU-1.1 cells are malignantly transformed by the overexpression of a single oncogene, or consecutive expression of two cooperative oncogenes. Expression of *Ras* oncogenes at a high level is sufficient for malignant transformation of MSU-1.1 cells. Instead, expression of *Ras* or *sis* (PDGF) oncogenes at a low level is insufficient to malignantly transform MSU-1.1 cells. Nevertheless, consecutive expression of a cooperative oncogene in cells expressing *Ras* or *sis* oncogenes results in malignant transformation of MSU-1.1 cells, suggesting that MSU-1.1 cells are at least two genetic changes short of being malignant. Interestingly, application of the same genetic changes that malignantly transform MSU-1.1 cells in MSU-1.0 cells fails to malignantly transform these cells. (B) Cells derived from tumors resulting form the malignant transformation of MSU-1.1 cells (MSU-1.1 derivative) have also been used to study genes that are involved in cancer. When the expression of the indicated genes is altered, the malignant MSU-1 derivatives exhibit a reduction in their ability to form tumors in athymic mice.

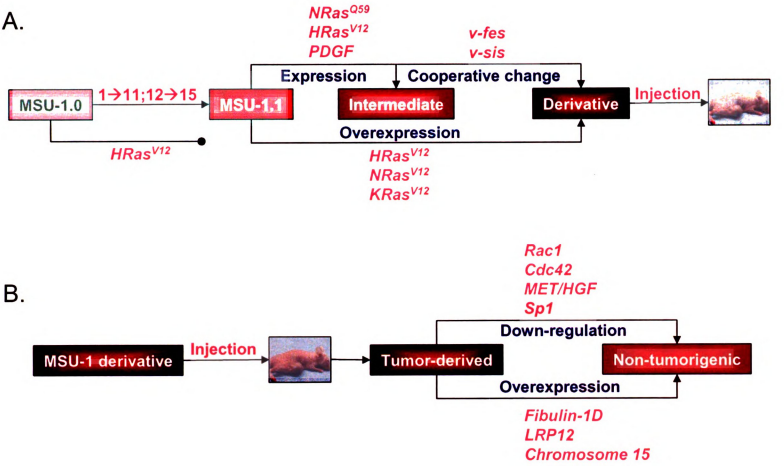

A.
NRasQ59
HRasV12
PDGF
v-fes
v-sis
Expression
Cooperative change
MSU-1.0
1→11;12→15
MSU-1.1
Intermediate
Derivative
Injection
Overexpression
HRasV12
HRasV12
NRasV12
KRasV12
B.
Rac1
Cdc42
MET/HGF
Sp1
Down-regulation
MSU-1 derivative
Injection
Tumor-derived
Non-tumorigenic
Overexpression
Fibulin-1D
LRP12
Chromosome 15

Figure 2

coexpression of *v-sis* with *v-fes*, in MSU-1.1 cells, results in their malignant transformation (Lin et al., 1995; Lin et al., 1994). The viral oncogene *v-sis* encodes for platelet derived growth factor (PDGF), while *v-fes* encodes an active form of the soluble tyrosine kinase c-fes. The cooperativity between *v-sis*, *v-fes* and *Ras* oncogenes in the malignant transformation of MSU-1.1 cells is evident in the fact that these enzymes are involved in receptor tyrosine signaling. Growth factors (such as PDGF) induce the activation of RTK signaling, which results in the activation of intracellular effectors such as Ras and Src [178, 179]. Soluble tyrosine kinases (such as c-fes) partially mediate the functions of activated RTKs. In addition, soluble tyrosine kinases, especially Src and c-fes, can also activate Ras [180-182].

As indicated above, MSU-1.1 can be malignantly transformed by carcinogen treatment followed by focus selection. Treatment of MSU-1.1 cells with a number of carcinogens, including BPDE, ethyl-nitrosourea (ENU) and gamma-irradiation, results in the malignant transformation of these cells [183-185]. In these cases, the resulting malignant cells exhibit inactivation of the p53 tumor suppressor pathway and are chromosomally unstable (McCormick, unpublished data).

The cells derived from the tumors formed by the malignantly transformed MSU-1.1 cells have been studied to determine genes that are important for this process (Fig. 2B). Expression of dominant negative forms of Rho GTPases Rac1 and Cdc42 in $HRas^{V12}$-transformed MSU-1.1 cells results in a decrease in the ability

of HRas-transformed cells to form tumors in athymic mice, indicating that Rac1 and Cdc42 are necessary for Ras-transformation in human cells (Appledorn and McCormick, unpublished data). Also, down-regulation of Sp1 in $HRas^{V12}$-, and in gamma irradiation-transformed MSU-1.1 cells, abrogates tumor-forming ability, suggesting an oncogenic function for Sp1 in these contexts[186]. Furthermore, c-Met and HGF, are both necessary for the malignant phenotype of gamma irradiation-transformed MSU-1.1 cells [187]. The activation of the HGF/c-Met pathway results in the induction of Sp1 expression [187], suggesting that Sp1 mediates the effect of HGF and c-Met in malignant transformation. Finally, expression of Fibulin-1D, an extracellular matrix protein, in BPDE-transformed MSU-1.1 cells abrogates the ability of these cells to form tumors in athymic mice, indicating the importance of extracellular matrix in the transformation of immortalized human fibroblasts [188].

The fact that the cells of the MSU-1 lineage are isogenic, i.e. they are derived from the same precursor, has allowed the study of genetic changes that occur early in the transformation process and predispose the cells to malignant transformation. Understanding the reason behind the ability of MSU-1.1 cells to be malignantly transformed by certain oncogenes, while their precursor MSU-1.0 cells fail to do so, is important in fully understanding the mechanism by which malignant transformation occurs.

The marker chromosomes that are present in MSU-1.1 cells may play a role in predisposing MSU-1.1 cells to malignant transformation by the oncogenes indicated above. Insertion of chromosome 15 in MSU-1.1 cells prevented their transformability by overexpression of $HRas^{V12}$, and also the expression of the same chromosome in cells derived from tumors formed by $HRas^{V12}$-transformed MSU-1.1 cells, abrogated their tumor forming potential. Chromosomes 1, 11 and 12 did not have an effect, when studied similarly, suggesting that alteration of chromosome 15 is important for predisposing cells to HRas-transformation (Kaplan et al. manuscript in preparation).

Ras GTPase

Ras genes, including *HRas*, *KRas* and *NRas*, are prototypical examples of oncogenes [189]. Approximately 30% of human tumors, particularly pancreatic carcinomas, adenocarcinomas of the lung, myeloblastomas and colorectal carcinomas contain activated *Ras* genes [57, 190, 191]. HRas and KRas were discovered thirty years ago, when mouse leukemia viruses were injected in rats giving rise to soft tissue sarcomas [192, 193]. These viruses encode for oncogenic forms of *HRas* and *KRas* cellular genes. The same oncogenic forms of *Ras* genes, encoded by the transforming viruses, were also found to be expressed in human tumors. [194, 195] The expression of *Ras* oncogenes is a causal factor for the ability of leukemia viruses to transform cells, as well as for the malignant phenotype of human tumors. The other member of the Ras family, NRas, was discovered in a human tumor of neural origin and plays an oncogenic role in tumor formation as well [194-197].

The oncogenic form of *Ras* differs by a single point mutation, when compared to the respective *Ras* proto-oncogene [198]. Although only one amino acid is affected, this mutation is critical for the function of Ras. This is because the protein that is encoded by the oncogenic form of Ras has the ability to evade normal regulation and exhibits increased activity [198-200]. This increased activity mediates a number of cellular functions, including enhanced proliferation and survival, which are necessary for cancer formation.

Catalytic function

The members of the Ras family of genes encode for small G proteins. Unlike the classic heterotrimeric G proteins, small G proteins lack the regulatory β and γ subunits, and consist only of the catalytic α subunit [201]. Small G proteins, including Ras family members, are GTPases, i.e., they can bind to, and hydrolyze GTP to GDP [46, 47, 202].

This activity is mediated by six structural components of Ras that are conserved among the family members and can be categorized into "G box" sequences[189, 203]. The G1 box, [aaaaGxxxxGK(S/T); a: L/I/V/M, x: any amino acid] mediates purine nucleotide binding, whereas the G3 box, [bIbbDxxGI; b: hydrophobic, l: hydrophilic] binds to Mg^{2+}. The G4 box, [bbbb(N/T)(K/Q)xD], forms hydrogen bonds with the guanine ring, thus conferring specificity over binding to adenine. The G5 box, [bbE(A/C/S/T)SA(K/L)], interacts indirectly with guanine nucleotides and is less conserved among the family members.

The catalytic activity of Ras is cyclic over time, i.e., the hydrolysis of a GTP molecule to GDP, is followed by the dissociation of GDP and the association of a new GTP molecule, leading to a new cycle of hydrolysis [204]. Consequently, Ras exists in two states (conformations): in one state, Ras is bound to GTP and in the other, Ras is bound to GDP [205]. When Ras is bound to GTP, it interacts with its effector proteins, a process that results in the activation of these effectors, which then mediate the cellular functions of Ras [203, 206]. Structurally, this is mediated

by the G2 box, [YDPTIEDSY], which adopts an orientation that has a high affinity for Ras effectors, when Ras is in a GTP-bound state. This is due to conformational changes on two loop regions on Ras, referred to as switch 1 and switch 2 [205]. These changes are induced when GTP binds Ras. Upon hydrolysis of GTP to GDP, the G2 box adopts an inactive conformation, and the activation of Ras effectors is attenuated. The fact that Ras oscillates between an active, GTP-bound conformation and an inactive, GDP-bound conformation, enables this protein to function as a molecular switch to regulate cellular activity.

Regulation of Ras activity

Because Ras functions as a molecular switch, it is important that Ras is strictly regulated in order to maintain normal cellular function (Fig. 3). The regulation of Ras is based on two kinetic parameters that characterize Ras-mediated catalysis: (i) the dissociation of GDP from Ras is rate limiting, and (ii) the intrinsic GTPase activity is low [207]. The dissociation of GDP is catalyzed by guanine nucleotide exchange factors (GEFs) [204]. This process enables Ras to bind another GTP molecule, which enhances its ability to activate its effectors. Furthermore, guanine nucleotide dissociation inhibitors (GDIs) bind specifically to GDP-bound Ras and inhibit the dissociation of GDP, thus prolonging the inactive state[45, 208-210]. The hydrolysis of GTP to GDP is catalyzed by GTPase activating proteins (GAPs). These enhance the rate of GTP-hydrolysis by Ras, leading to the inactivation of Ras [211-213]. The point mutations present in the oncogenic

Figure 3. Ras GTPase as a molecular switch. The ability of Ras GTPases to hydrolyze GTP to GDP is associated with two distinct Ras conformations: an active conformation, when Ras is GTP-bound, and an inactive conformation, when Ras is GDP-bound. In normal cells, Ras oscillates between the inactive to the active conformation and back. This is regulated by guanine nucleotide exchange factors, including SOS and RasGEF, which catalyze the release of GDP, and enhance Ras activation. Instead, GTPase activating proteins, such as $p120^{GAP}$ enhance the intrinsic ability of Ras to hydrolyze GTP to GDP, a process that leads to Ras inactivation.

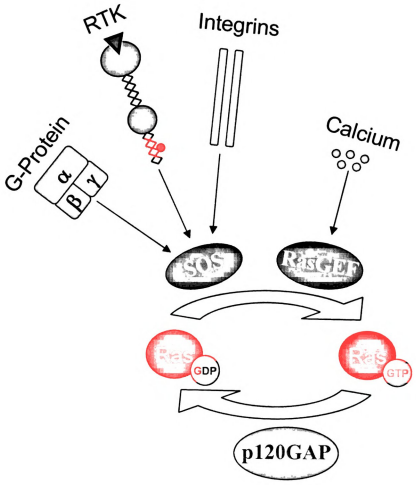

RTK
Integrins
G-Protein
α
β
γ
Calcium
SOS
RASGEF
Ras
GDP
Ras
GTP
p120GAP

Figure 3

forms of *Ras*, e.g., at codons 12 or 61, render Ras resistant to GAP activity, thereby prolonging the active state of Ras, and leading to tumor formation [191, 214].

GEFs and activation of Ras

GEFs, including SOS, RasGEF and RasGRP, mediate the activation of small GTPases. These GEFs are activated in a signal-specific manner, and each displays specificity for the type of Ras-family GTPase that it activates.

SOS is the best studied Ras specific GEF. Structurally, SOS comprises of a Cdc25 domain, a Dbl homology (DH), and a pleckstrin homology (PH) domain [215]. The Cdc25 domain catalyzes the dissociation of GDP from Ras, and is conserved in Ras-specific GEFs. In addition, the C-terminus of SOS contains a proline-rich region, which binds to the SH3 domain of the Grb2 adaptor protein. This adaptor also contains an SH2 domain that is recruited to phospho-tyrosine residues of proteins localized at the plasma membrane (e.g. transmembrane receptors, or other adaptors). As Grb2 is bound to SOS, the recruitment of Grb2 to the plasma membrane facilitates the translocation of SOS to the plasma membrane, where it activates Ras [216].

This mechanism of SOS activation is responsible for mediating Ras activation by several upstream receptors including RTKs, Integrins and G-protein coupled receptors (GPCR). Upon binding to their ligands, tyrosine kinase receptors, like

the epidermal growth factor receptor (EGFR), dimerize and autophosphorylate their own intracellular domains on tyrosine residues. These residues act as docking sites for the SH2 domain of Grb2 and recruit the Grb2:SOS complex to the plasma membrane [40, 217]. Other RTKs, notably the insulin growth factor receptor (IGFR), recruit and phosphorylate adaptors, such as Shc, which then become docking sites for Grb2:SOS [218]. In a similar fashion, Shc acts as a docking platform for Grb2:SOS during the activation of Ras by integrins or by heterotrimeric G-proteins. Upon binding to the extracellular matrix, integrins dimerize and activate focal adhesion kinase (FAK), which in turn phosphorylates Shc, thus potentiating the recruitment of Grb2:SOS to Shc [151]. Heterotrimeric G proteins can activate Ras through their $\beta\gamma$ subunit, in a process involving the activation of Src, and Shp-mediated recruitment of Grb2:SOS to the plasma membrane [219, 220].

In addition to the interaction with Grb2, the activity of SOS is also regulated by phosphorylation. Extracellular-signal related kinase (ERK) and its effector p90RSK, both phosphorylate and inactivate SOS in a negative feedback fashion [221]. Furthermore, phosphatidyl inositols (i.e. PIP_2) interact with the PH domain of SOS to inhibit its activity [222].

The RasGEF family consists of the same structural domains (i.e., DH, PH and cdc25) as the SOS family [223]. Their signal specificity, however, differs from that of the SOS family. RasGEF are mediators of calcium induced-Ras activation

[204]. These GEFs also contain an IQ motif, which binds to the calcium-calmodulin complex leading to the activation of RasGEF [224]. The RasGRP family is not only regulated by calcium, but its function also depends on diaglycerol [223].

GAPs and inactivation of Ras

The ability of GAPs to inactivate Ras lies on their ability to interact with Ras and enhance the rate of GTP hydrolysis by Ras [213]This activity is crucial for the cycling of Ras between "on" and "off" states. The study of GTPase activating proteins (including $p120^{GAP}$ and Neurofibromatosis-1 (NF1)) has been focused mainly on $p120^{GAP}$, which consists of a C-teminal catalytic domain, two N-terminal SH2 domains flanking and SH3 domain, a PH domain, as well as a lipid binding domain[212]. The exact mechanism by which the activity of $p120^{GAP}$ is regulated is still under investigation, but it seem that maximal activity is dependent not only on the catalytic domain, but also on the SH2 and SH3 domains of $p120^{GAP}$ [225].

Posttranslational regulation

Although synthesized as a cytosolic protein, Ras is subjected to posttranslational processing. This results in the dynamic interaction of Ras with cellular membranes, including the plasma membrane, endosomes, and other intracellular membranes [226-228]. This process is directed by the CAAX motif at the carboxy-terminal of Ras. Initially, the cysteine residue of this motif is farnesylated by a

farnesyl transferase [229]. Subsequently, an endopeptidase cleaves off the AAX tripeptide [230], followed by methylation of the α-carboxy group of the farnesylated cysteine [231]. At this point, the processing of Ras proteins can follow two distinct pathways, in an isoform specific fashion. HRas and NRas become palmitoylated on a cysteine residue on the amino terminal side of the farnesylated residue, and are trafficked through the Golgi system to reach the plasma membrane. In contrast, KRas lacks this cysteine residue and reaches the plasma membrane in Golgi-independent mechanism[232].

Ras Effectors

Ras plays a central role in intracellular signal transduction, not only because signaling pathways induced by various transmembrane receptors converge at the level of Ras GTPase, but also, because, Ras regulates a number of diverging signaling pathways that regulate many cellular functions. In this fashion, Ras acts as a gearbox that guides cellular function in a particular environment. This function of Ras relies on its ability to activate a number of effectors, including Raf, PI3K, Tiam1, RalGDS, Rin1, AF-6, RASSF1 and others. Based on their catalytic functions such effectors can be classified into distinct groups consisting of: 1) serine/threonine kinases (Raf), 2) phosphoinositide-3-kinases (PI3K), 3) GEFs (RalGDS, Tiam1 and Rin1), 4) lipases (PLCγ), 5) adaptors (AF-6 and RASSF1) and 6) RasGAPs ($p120^{GAP}$ and NF1) [233].

A Ras effector is defined as a protein that interacts specifically with the effector domain of Ras, when Ras is in its GTP-bound form [233]. Effector proteins are

typically characterized by a domain that interacts with Ras. Three such domains have been identified to date; the RBD domain of Raf and Tiam1, the RBD domain found in the isoforms of the p110 subunit of PI(3)K family and the Ras association domain, such as the one present in RalGDS, Rin1, AF6 and others [233, 234]. Even though these Ras-binding regions are different in their amino acid sequences, their three dimensional conformations are similar. This is not surprising, since all these regions interact with the effector-binding domain of Ras (G2 box), and therefore, they need to have a structural conformation that fits that of the G2 box of Ras. This review will focus on effectors of Ras, which have critical and established functions in Ras signaling, as well as on a few additional effectors that have been studied to a lesser extent, but may have a significant role in Ras function (Fig. 4).

Raf

The Raf serine/threonine kinase is the best characterized effector of Ras. From a structural perspective, Raf consists of three conserved regions CR1, CR2 and CR3, which contains the catalytic domain [235]. Although the exact mechanism by which Raf is activated is still under investigation, Ras interacts with two domains, i.e., RBD and CRD in the CR1 region of Raf [236, 237]. This association results in the recruitment of Raf to the plasma membrane, and represents the initial step in the activation of Raf [238]. The activation of Raf is also potentiated

Figure 4. Ras effector pathways. The conformational change that is associated with Ras activation enables Ras to interact with a number of effector proteins, leading to the activation of many intracellular pathways and the regulation of diverse cellular functions.

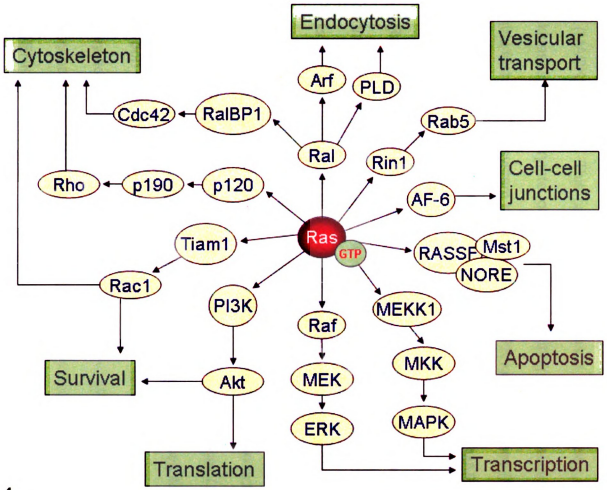

Endocytosis
Cytoskeleton
Vesicular transport
Arf
PLD
Cdc42
RalBP1
Rab5
Ral
Rin1
Rho
p190
p120
AF-6
Cell-cell junctions
Tiam1
Ras
GTP
RASSF
Mst1
NORE
Rac1
PI3K
Raf
MEKK1
Apoptosis
Survival
Akt
MEK
MKK
ERK
MAPK
Translation
Transcription

Figure 4

by additional factors such as 14-3-3, phosphatidyl serine, Hsp90 and serine/threonine kinases [239].

Through the activation of Raf, Ras can activate the mitogen-activated protein kinase (MAPK)-cascade. This cascade functions as a phosphorelay system, in which kinases phosphorylate other kinases resulting in their activation in a sequential fashion. This system is organized in MAPK kinase kinases (MAPKKK), which activate MAPK kinases (MAPKK), and these in turn activate MAPKs [240].

Active Raf acts as a MAPKKK to activate MEK proteins by phosphorylation on two serine residues (S217 and S221 in MEK1). MEK proteins are dual-specificity MAPKKs that phosphorylate ERK1 and ERK2 on threonine and tyrosine residues (T202 and Y204 in ERK1), resulting in ERK activation [235, 241]. ERK proteins, which are serine/threonine MAPKs, translocate to the nucleus when activated. In the nucleus, ERKs phosphorylate and activate transcription factors (e.g. Elk1) [242]. ERKs also activate cytoplasmic substrates, including kinases MSK1 and $p90^{Rsk}$. Upon ERK phsohorylation, MSK1 and $p90^{Rsk}$ translocate to the nucleus where they activate other transcription factors (e.g. Fos, SRF), histones (e.g. H3) and transcriptional regulators (e.g. CREB) [242]. In this fashion, Ras, through the Raf/MEK/ERK pathway regulates gene expression at the transcription factor level.

PI3K

PI3Ks are lipid kinases that phosphorylate phosphoinositides on the 3' position of the inositol ring [243, 244]. PI3K consists of two subunits; a regulatory p85 subunit and a catalytic p110 subunit. The regulatory subunit consists of two SH2 domains which interact with phosphorylated tyrosine residues on activated growth factor receptors [245, 246]. Since the region between the two SH2 domains is tightly bound to the p110 catalytic subunit, the association of p85 with activated growth factor receptors recruits the catalytic subunit to the plasma membrane, were it can phosphorylate phosphoinositides. Furthermore, the p110 catalytic subunit of PI3K interacts with Ras, when Ras is GTP-bound [247]. This interaction is synergistic with the function of the p85 subunit to yield optimal activation of PI3K in response to growth factors.

Activated PI3K converts $PI(4,5)P_2$ into $PI(3,4,5)P_3$, an important secondary messenger that mediates its effects through its binding to plextrin homology (PH) and FYVE domains [248, 249]. Therefore, proteins containing the PH domain, including Akt, PDK, and Tiam1 are critical mediators of PI3K signaling. Akt, a serine/threonine kinase, is the best studied effector of PI3K signaling. Through its PH domain, Akt translocates to the plasma membrane and binds to $PI(3,4,5)P_3$. This results in a conformational change within Akt, which exposes two main phosphorylation sites (T308 and S473) [250]. Subsequently, PDK phosphorylates Akt, leading to the stabilization of its active conformation, resulting in enhanced signaling activity by Akt.

The ability of Ras to promote cellular survival is mediated in part by the activation of the PI3K/Akt pathway. Akt, in particular, is important because it exerts a direct effect on many pathways that regulate survival [251]. One of the substrates of Akt, BAD, is a pro-apoptotic protein that binds to Bcl-2 and Bcl-X and inhibits their anti-apoptotic potential. Phosphorylation of BAD on Ser136 by Akt results in loss of inhibition of Bcl-2 and Bcl-X [252].

IKK, a serine/threonine kinase, is activated upon phosphorylation by Akt. Subsequently, IKK phosphorylates the NFκB inhibitor IκB, which is then ubiquitinated and targeted for degradation by the proteasome pathway. This results in the activation of the NFκB transcription factor, which translocates to the nucleus and transactivates a number of pro-survival genes[253].

Another important Akt substrate is the Forkhead family of transcription factors (FoxO), which regulate the expression of apoptosis inducing factors, such as Fas, TRAIL and TRADD [254]. Akt phosphorylation inhibits the function of FoxO transcription factors, thus enhancing the survival potential of the cell.

MDM2 is an ubiquitin ligase that targets p53 for proteosomal degradation. Akt phosphorylates MDM2 on two serine residues, which sustains its ubiquitin ligase activity. This decreases the ability of p53 to induce cell cycle arrest and apoptosis [255, 256].

Finally, GSK3 is primarily involved in regulating the conversion of glucose to glycogen, although it has additional functions, including a role in β-catenin signaling, as well as in cell cycle regulation[257]. GSK3 phosphorylation by Akt results in the inhibition of GSK3 function[258].

Tiam1

Like Ras, Rho GTPases are molecular switches that regulate a number of cellular functions [259]. The Rho family, which includes Rac1, Cdc42 and Rho, plays an important role in mediating the functions of active Ras, particularly in the regulation of the actin cytoskeleton and cellular migration. Nevertheless, the mechanism by which Ras recruits this family has not been fully elucidated. One possible mechanism involves PI3K and the production of the secondary messenger PIP_3. As noted above, PI3K interacts with proteins containing PH domains and recruits them to the plasma membrane. Rho GREs have of PH domains, and thus can be recruited to the plasma membrane by PIP_3, a fact that enhances the activation of Rho GTPases. Alternatively, RhoGAPs can be regulated by Ral and p120GAP (see below).

An important step in determining the mechanism of Rho activation by Ras was made by the discovery that Tiam1, a GEF for Rac1, is a direct effector of Ras [260]. Tiam1 contains a Raf-like RBD domain through which it interacts with Ras, when the latter is in a GTP-bound state. In addition, Tiam1 consists of a catalytic

DH/PH domain, characteristic of RhoGEFs, as well as a N-terminal PH domain, which can bind to PIP_3. The presence of a PH domain on Tiam1 indicates that, in addition to Ras, Tiam1 can be activated by PI3K [261]. Tiam1 requires membrane localization and threonine phosphorylation for maximal function [262, 263], suggesting that these are necessary regulatory steps in Tiam1 activation. In addition, Tiam1 is regulated by an autoinhibitory region and a PEST domain, both of which are located on its N-terminus. The PEST domain targets Tiam1 for degradation, a process that results in the cleavage of the autoinhibitory region and increased activity of Tiam1 [264]. Although the interaction of Ras with Tiam1 enhances the translocation of Tiam1 to the plasma membrane, whether Ras has an effect on the other aspects of Tiam1 activation remains unknown.

RalGEFs

The RalGEF family members, including RalGEF, Rlf and Rgl, catalyze guanine nucleotide exchange for Ral GTPases. RalGEFs interact directly with Ras, in a GTP-dependent manner [265]. This interaction is dependent on the RA region of RalGEFs, which is very similar in structure to the RBD of Raf1 [266]. The interaction with Ras causes the redistribution of RalGEFs to the plasma membrane, where they can activate Ral GTPase [267].

Ral has a number of targets that appear to mediate its activity. Ral interacts with PLD1 and Arf, suggesting that Ral plays a role in vesicular tansport [268]. RalBP1 is a putative effector of Ral that interacts with tyrosine phosphorylated proteins

(Pob1 and Reps1), which complex with EGFR and are homologous to proteins that regulate receptor endocytosis [269]. Furthermore, RalBP1 has GAP activity, which is specific for Rac1 and Cdc42 GTPases, an ability that implicates Ral in the regulation of Rho GTPases [270].

MEKK1

MEKK1 interacts with GTP-bound Ras through its C-teminal region, [271]. MEKK1 is a serine/threonine kinase MAPKKK, which regulates a similar MAPK-cascade to that regulated by Raf. MEKK1 activates MKKs, which then activate p38 or JNK [272]. Active p38 and JNK translocate to the nucleus where they activate transcription factors and regulate gene expression. It is important to note that although these MAPKs function primarily in the nucleus, they also regulate cytoplasmic enzymes.

Rin1

Ras interacts with Rin1 in a GTP-dependent manner, through its effector-binding domain. Rin1 is a GEF for Rab5 GTPase, which is involved in receptor-mediated endocytosis [273]. This process may be facilitated by the ability of Rin1 to interact with tyrosine phosphorylated receptors through its SH2 domain [274]. In addition to Rab5, Rin1 interacts with Abl tyrosine kinase to enhance its catalytic activity [275]. The interaction with Ras enhances the ability of Rin1 to activate the Rab5 GTPase, as well as the ability of Rin1 to activate Abl [273].

AF-6

AF-6 is another protein that interacts with Ras in its GTP-bound state [276]. Ras interacts with the RA region of AF-6, an interaction that interferes with the binding of Raf to Ras. Although the function of AF-6 in the cell remains unknown, this protein interacts with the Rap1 GTPase, profilin, and with Eph receptors [277-279] and may be involved in signal transduction at cell-cell junctions [279].

RASSF

RASSF proteins (including NORE1 and RASSF1), interact with GTP-bound Ras through their RA region [280]. Interestingly, the expression of these proteins is lost in several tumors [281, 282]. In addition, *in vitro* and *in vivo* studies propose that these protein have anti-proliferative functions, thereby acting to suppress Ras-transformation. Importantly, RASSF1 and NORE heterodimerize, and form a complex with Mst1, a serine/threonine kinase that enhances caspase-3 activation. Ras associates with this complex, and induces apoptosis by means of caspase-3 activation [283].

Cellular functions mediated by Ras

Regulation of cell cycle and proliferation

One of the most important cellular effects induced by active Ras is the progression through cell cycle, leading to cellular replication. Expression of a

dominant negative form of Ras inhibits G1→S cell cycle progression, whereas progression is enhanced upon expression of oncogenic Ras [284].

G1→S progression is regulated mainly by the activity of the Rb protein, which is normally in a hypophosphorylated form. In this form Rb sequesters and inhibits the function of E2F transcription factor. Rb phosphorylation is regulated by cyclin:CDK complexes. The cyclin D:CDK4 complex, in particular, hyperphosphorylates Rb, a process that attenuates the inhibition of E2F by Rb. This results in the transcriptional activation a number of E2F-dependent genes that are important for cellular proliferation.

The primary regulatory function of Ras at this stage of the cell cycle is to inactivate the suppression of E2F by Rb [285]. The activation of Raf/MEK/ERK pathway by Ras, induces the expression of cyclin D1, which as described leads to the activation of E2F [286]. The activation of additional Ras-effector mediated pathways, including those mediated by PI3K and Rac1/Cdc42 are also important in mediating the inactivation of Rb by Ras [287, 288]. PI3K regulates the expression of cyclin D1 through GSK3. GSK3 phosphorylates cyclin D1 and this initiates the degradation of cyclin D1 through the proteasomal pathway. PI3K, by means of an Akt-dependent function, inhibits the activity of GSK3, thus sustaining the levels and activity of cyclin D [289]. Rac1 is also important in inducing cyclin D accumulation, in a process that is dependent on the activation of NFκB [290].

In addition to the regulation of Rb, Ras can also regulate cell cycle inhibitors, such as p21 and p27, to promote G1→S transition. Through the Raf/MEK/ERK pathway, Ras can decrease p27 levels both transcriptionally and through proteosomal degradation [50, 291]. What is more, activation of PI3K by Ras results in the inactivation of FoxO-dependent transcription, including the transcription of *p27* [292]. Also, the activation of Rho induces the formation of cyclin E:CDK2 complex, which phosphorylates p27, and provides a signal for the ubiquitination and degradation of p27 [293].

Ras may also inhibit cell cycle progression depending on the context of its activation. Expression of Ras oncogene in primary fibroblasts leads to *INK4a* and p53-dependent cellular senescence, whereas inactivation of these proteins results in evasion of the senescent phenotype [294]. Downstream of Ras, even Raf can have different outcomes on cell fate. Whereas moderate levels of Raf activation result in enhanced proliferation, through the cyclin D pathway, high levels of Raf activation result in p21-mediated cell cycle arrest [295].

Regulation of protein synthesis

Ras can regulate protein synthesis through the PI3K/Akt pathway. In addition to its other functions, Akt also exerts control over the Rheb/mTOR pathway by phosphorylating and inactivating TSC1/2 proteins [296]. TSC1/2 proteins act as GAPs for Rheb GTPase, which activates the mammalian target of rapamycin protein (mTOR) [297], and this in turn activates $p70^{S6K}$ and 4EBP1 [298, 299].

$p70^{S6K}$ phosphorylates the S6 ribosomal protein. This results in the translation of enzymes that make up the protein synthesis apparatus. Normally, 4EBP1 inhibits translation by sequestering the translation elongation factor eIF4E. mTOR phosphorylates 4EBP1 and induces the release of eIF4E, facilitating the initiation of translation [300].

Regulation of apoptosis

In addition to its role in cell proliferation and cell cycle progression, Ras can also regulate cellular programs that control apoptosis. Several of the effectors that are activated by Ras are important in mediating this function of Ras, and their outcomes, with respect to apoptosis, depend on the particular effector that is activated. For example, activation of PI3K by Ras activates survival pathways, whereas activation of RASSF1 leads to activation of cell death pathways. In some cases the activation of the same effector may lead to distinct apoptotic fates within the same cells, as is the case with the activation of Raf.

The activation of survival pathways by the Ras effector PI3K is mediated by the activation of Akt. As indicated above, the ability of Akt to prevent programmed cell death is in turn mediated by a number of Akt effector substrates. Akt can inhibit BAD, thus preventing Bcl-2 and Bcl-X induced cytochrome c release from mitochondria [301]. In addition, Akt can regulate apoptosis by regulating transcription factors as NFκB and FoxO, which regulate cellular fates via the transactivation of anti- and pro- apoptotic genes, respectively. Akt can intercept

the p53 apoptotic pathway at the level of MDM2, thus protecting the cell from DNA-damage induced apoptosis [302]. What is more, Ras can promote survival via Rac1-dependent activation of NFκB [303]. Thus, it appears that Ras activation of NFκB requires synergistic signaling from both Rac1 and PI3K/Akt pathways.

Whereas PI3K and Rac1 activation promotes survival pathways, the Raf/MEK/ERK pathway can either enhance or inhibit apoptosis, in a context-specific manner. In fibroblasts deprived of serum, Ras induces apoptosis in a Raf/MEK/ERK-dependent fashion [304]. In other systems, Raf can induce apoptosis in a MEK/ERK-independent fashion, suggesting that other effectors may mediate the pro-apoptotic function of Raf [305, 306]. Despite this pro-apoptotic function, Raf can enhance cellular resistance to p53-mediated apoptosis. In Ras-transformed fibroblasts, activation of Raf/MEK/ERK elevates the expression of MDM2, which acts to degrade p53, thus facilitating cellular survival [307].

Regulation of the actin cytoskeleton

The actin cytoskeleton plays a crucial role in cellular morphology and function. The cytoskeleton is regulated in a dynamic manner through the synergistic function of Ras and Rho GTPases (Rho, Rac1 and Cdc42). In particular, Rho GTPase is important for stress fiber formation and focal adhesion formation, Rac1 mediates cellular ruffling and lamelipodial protrusion, and Cdc42 is involved in filopodial formation and actin microspike formation [308]. Ras can activate Rac1

directly, through the interaction of Ras with Tiam1, or indirectly, through PI3K and the elevation of PIP_3, which then binds to the PH domain of Rho GEFs leading to their membrane localization and their subsequent activation [309]. How Ras regulates Rho and Cdc42 function is not clearly understood, but it may involve PI3K/PIP3-dependent, $p120^{GAP}$-dependent or Ral/RalBP1-dependent mechanisms.

Regulation of cellular migration

Rho GTPases play an important role in the regulation of cellular migration. Rac1 is necessary for the initiation of cellular movement in fibroblasts and epithelial cells [310, 311]. Cdc42 mediates the polarity signals needed for unidirectional movement toward a stimulus [312]. In addition, Rho is required in order to maintain cell adhesion during movement [310-313]. Ras itself is involved in the regulation of cellular migration, in a cell type-specific mechanism [314]. In endothelial cells, the effect of Ras in migration is mediated by ERK. Through the phosphorylation of MLCK, ERK increases the level of phosphorylated myosin, therefore increasing cellular contractility [315]. In fibroblasts, the effect of Ras is mediated by decreasing the activity of Rho, though $p120^{GAP}$, which, in addition to serving a GAP activity for Ras, acts as an effector of Ras function as well. In particular, $p120^{GAP}$ interacts with $p190^{RhoGAP}$, which regulates the activity of Rho proteins [316, 317].

Regulation of invasion and metastasis

The processes of invasion and metastasis are in part regulated by matrix metalloproteinases (MMP). These enzymes are involved in the turnover of extracellular matrix through proteolytic degradation, and are commonly expressed by tumor cells resulting in the degradation of the surrounding matrix, and facilitating tumor invasion and metastasis. Constitutively active Ras signaling induces the expression of MMP proteins in a cell type specific manner [318, 319]. This induction is mediated in part by Ets and AP1 transcription factors that can be activated by Ras in a MAPK-dependent manner[320], and also by the NFκB transcription factor [321]. Ras has also been implicated in tumor metastasis, a property that may be dependent on the induction of Met expression by oncogenic Ras [322, 323].

Sprouty

Sprouty was identified as a repressor of tracheal morphogenesis in *Drosophila melanogaster* (D.m.) [324]. This process is under the regulation of fibroblast growth factor (FGF) signaling, which acts through the FGF receptor (FGFR) to induce secondary branches near the tips of the primary branches of the Drosophila's tracheal tube [325, 326]. Spry mutants exhibit overactive FGF signaling and develop ectopic branches on the stalks of primary branches[324]. In addition, D. m. Spry plays a role in eye development, a process that is regulated by epidermal growth factor receptor (EGFR) signaling. Loss-of-function Spry mutants have excess photoreceptors, cone cells and pigment cells [327]. Further studies showed that Spry is a general inhibitor of RTK signaling in D. m. [328].

Mammals express four Spry isoforms, which also repress RTK signaling [329-332]. Mammalian Spry proteins regulate additional developmental programs, including bone development [333], oogenesis [334] and angiogenesis [335].

Spry has emerged as an important repressor of RTK signaling and a great deal of effort has been placed in determining the specific functions of Spry, as well as the regulatory machinery by which these functions of Spry are controlled. Understanding the function of Spry, has been complicated recently, given that some mammalian Sprys can also potentate RTK signaling, particularly in response to EGF stimulation. This review will provide a summary of the function and regulation of Spry in mammalian cells, focusing on Spry2, which, in addition

to being the best characterized Spry isoform, exhibits a dual activity, both as an activator, as well as a repressor of RTK signaling.

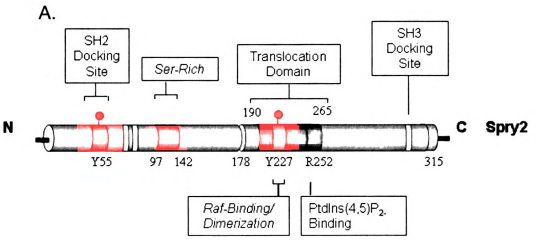
Sprouty structure

The first member of the Spry family to be identified, i.e., the D. m. Spry, encodes a 63 kDa protein containing a unique 143 cysteine-rich region on its C-terminus [324, 327]. Mammalian cells express four Spry isoforms (Spry1-4) that are considerably smaller than the Drosophila ortholog (approximately 30-40 kDa). These isoforms exhibit a significant homology on the C-terminal half of the protein, including the cys-rich region, which spans almost the entire C-terminus of mammalian Spry proteins. The N-teminal region of mammalian Sprys differs significantly among isoforms [334, 336, 337].

Studies have identified several structural elements that are necessary for Spry function (Fig. 5). Although exhibiting differences among Spry isoforms, the N-terminus of Spry proteins contain a small conserved amino acid region with a tyrosine residue (Y55 on Spry2). This residue becomes phosphorylated upon growth factor stimulation [338-340], and serves as a docking site for the SH2 domains of Grb2 [340] or c-Cbl [338, 339]. Despite relying on the same structural element, these interactions have distinct consequences on cellular function as it will be described below.

Figure 5. Structure of Spry. (A) Spry proteins contain a conserved cysteine-rich C-terminus and a variable N-terminus. Several important Spry2 structural elements are shown in this figure. The tyrosine residue on position 55 becomes phosphorylated upon growth factor stimulation, creating docking site for the SH2 domains of Grb2 and c-Cbl. Spry2 contains three proline rich motifs (PxxPR) that act as docking sites for the SH3 domains of CIN85. Upon growth factor stimulation, Spry2 translocates to the plasma membrane, a process mediated by a unique translocation domain on Spry2. This domain targets Spry2 to phosphatidyl inositol (4,5) bisphosphate (PtdIns(4,5)P_2). R252 is indispensable for this process. Finally, the region spanning amino acids 208-239 on Spry2 has been implicated in the interaction between Spry2 and Raf, as well as in the ability of Spry proteins to form dimmers. (B) The alignment of several structural elements between Spry members is shown. The numbers in bold represent positions on the Spry2 sequence.

A.



B.

SH2 Docking Site

```
Spry1 GSNEYTE R
Spry2 NTNEYTE R
Spry4 VENDYLDN R
Spry3 ASNDYWER R
      51  *  *  :  *  59
```

Ser-rich Region

```
Spry1 STSTGSAASSG-----SNSSASSEQGLLGRSPPTRPVPGHRSERAIRTQPK
Spry2 SISTVSSGSRSSTRTSTSSSSSSEQRLLGSSFSSGPVADG-----IIRVQPK
Spry4 SVS-------------SSSSTSSDQRLLDHMAPP-PVADQASPRAVRIQPK
Spry3 SIASS----------MSHSTTASDQRLLASITPS-PSGQS----IIRTQPG
    110 *  :              *  *:::*:*  **         *          :*  **  155
```

Translocation Domain; Raf-Binding Region

```
Spry1 GECTAPRTLPSCLACNRQCLCSAESMVEVGTCMCLVKGIFVHCSN
Spry2 KECTYPRPLPSDWICDKQCLCSAQNVIDVGTCVCCVKGLFVHCSN
Spry4 KECASPRTLPSCWVCNQECLCSAQTLVNYGTCMCLVQGIFVHCTN
Spry3 VPCTAARPLPSCWLCNQRCLCSAESLLDYGTCLCCVKGLFVHCST
    185   *:   * ***    *:: ***** : :::****:* *:*:*:**:

Spry1 DDEGDSYSDNPCSCSQSHCCSFVLCMGAMSIFLPCLLCYPPAKGCLKL
Spry2 DDE-DNCADNPCSCSQSHCCTFVSAMGVMSLFLPCLWCYLPAKGCLKL
Spry4 EDDEGSCADHPCSCSRSNCCAFVSFMGALSVVLPCLLCYLPATGCVKL
Spry3 DDE-DNCADEPCSCGPSSCFVHVAAMSLISLFLPCLCCYLPTRGCLHL
      :*:     :* ****  * *   *:   *  :*: **** ** *: **:: * 278
```

Figure 5

Additional regulatory elements, which have been studied to a lesser extent than the N-terminal phosphorylation site of Spry, reside within the conserved cys-rich region. The region encompassed by amino acids 178-315 on Spry2 has been defined as the Spry translocation domain[341] and regulates the localization of Spry to plasma membrane upon growth factor stimulation.

Spry proteins form homo- and hetero-dimmers with other isoforms. The structural element important for this function is located in a region spanning position 209-238 on mSpry1 [340].

Spry proteins interact with Raf kinase through a Raf binding domain spanning residues 209-240 on Spry2. Surprisingly, the region important in Raf binding spans the same residues as does the region important for Spry dimerization.

Recently a novel growth factor-specific tyrosine phosphorylation site was discovered on the C-terminus (Y227) of Spry2 [342]. Phosphorylation at this site appears to regulate if Spry inhibits or potentates RTK signaling.

Finally, Spry2 contains three PxxPR motifs on positions 64, 72 and 309, which act as biding sites for the SH3 domains of CIN85 [343].

Sprouty expression

In *Drosophila*, *Spry* is expressed during tracheal [324] and eye development [327], as well as in midline glia [344], in wing veins and in ovarian follicle cells [344, 345]. These developmental processes are under the control of FGF or EGF [328], and loss-of-function mutations in *Spry* mimic the loss of expression of these growth factors in these tissues.

In vertebrate embryos, such as the mouse and chick embryo, Spry protein expression is induced by FGF, and not surprisingly, it is more prominent in the locations where FGF signaling predominates [346]. These include the primitive streak, the forebrain and the hindbrain. It is important to note, however, that the expression of *Spry* genes in these tissues occurs in an isoform-specific fashion. For example, Spry2 and Spry4 are expressed in the primitive streak, whereas Spry1 is not, and Spry1 and Spry2 are expressed in the midbrain region, whereas Spry4 is not [333].

In the mouse, *Spry* genes are also expressed during organogenesis of the cochlea and semicircular canals, the teeth, the lungs, the digestive track, and the kidneys [347]. In the semicircular canal and the teeth, Spry1 and Spry2 are expressed in the epithelium, whereas Spry4 is expressed in mesenchymal or neuronal tissue. In the lung, Spry1, Spry2 and Spry4 are all expressed in epithelial tissue. In the kidney, Spry1 is expressed in the ureteric bud, whereas

Spry2 and Spry4 are expressed in the ureteric bud, mesenchyme and glomerulus.

In the adult tissue, Spry1 is expressed in the heart, lung and kidney [348], Spry2 is expressed in the brain, heart, lung and kidney [349] and Spry4 is expressed in liver, skeletal muscle, heart, lung, kidney, spleen, placenta and small intestine [350].

Consistent with the observations described above, the expression of Spry in cultured fibroblasts or endothelial cells is induced by growth factor stimulation [351]. At a molecular level, this is in part dependent on ERK activation, as selective inhibitors of MEK abrogates FGF-induced Spry2 and Spry4 expression [351]. Alternatively, FGF may induce the expression of Spry1 and Spry2, in an ERK-independent fashion, through the activation of PLCγ and calcium-dependent signaling, as demonstrated in a study were calcium chelation and PLCγ inhibition abrogated the induction of Spry by FGF in a mouse chondogenic cell line [352].

At the transcriptional regulation level, the expression of Spry genes, particularly the expression of Spry1, is regulated by the WT1 transcription factor. WT1 directly associates with, and activates the Spry1 promoter. Also, expression of wild type, but not of catalytically inactive mutant WT1, induces the expression of Spry1 in osteosarcoma cells [348]. Furthermore, analysis of the promoter region of Spry2 revealed the presence of several cis-activng elements for AP2, CREB,

ETS and Sp1, leading to the hypothesis that the expression of Spry2 may be regulated by these transcription factors/regulators [353].

Sprouty localization

Initial studies on the localization of Spry in *Drosophila m.* showed that Spry is associated with the inner leaf of the plasma membrane [327]. In mammalian cells, however, the attempt to determine the exact localization of Spry proteins has lead to several findings. In human embryonic kidney cells, Spry2 localizes to membrane ruffles upon stimulation with EGF [341, 354]. In the absence of stimulation, Spry proteins attain a diffusely cytoplasmic localization, while Spry2 co-localizes with microtubules [341]. Other studies have found that in Chinese hamster ovary cells, Spry2 is associated with vesicular structures resembling endosomes upon EGF-stimulation. These same structures become loaded with EGFR, under the same stimulation [338, 339]. It may be that Spry2 localizes in both of these structures, in a cell-type specific matter, given that in mouse fibroblasts, Spry2 translocates both to vesicular structures, as well as to the plasma membrane, upon EGF stimulation [355].

In endothelial cells, Spry1 and Spry2 are located in the perinuclear region and in vesicular structures, under serum starvation conditions. Upon growth factor stimulation, they translocate to the lamelipodia at the leading edge of the plasma membrane [356]. In this system, Spry proteins directly interact and co-localize with calveolin-1, but do not localize in lipid rafts [356].

The translocation of Spry protein to the plasma membrane is necessary for their activity, because it facilitates the phosphorylation and subsequent activation of the proteins [340]. One possible mechanism employed by Spry proteins in order to attain their membrane localization is the use of a unique translocation domain (SpryTD) located in the C-terminal region [341]. Deletion of the region spanning residues 178-221 in Spry2 abolishes the EGF-induced translocation of Spry2 to the plasma membrane [341]. The translocation of SpryTD to the plasma membrane is downstream of active Rac1. $Rac1^{N17}$ (a dominant negative mutant) inhibits the translocation of SpryTD [341, 354]. A later study found that the SpryTD specifically interacts with PIP_2 [354]. This was demonstrated by the co-localization of SpryTD with the PH domain of PLCδ, which interacts with PIP_2 at the plasma membrane, as well as by the direct association of SpryTD with PIP_2-bound lipid vesicles. The importance of the association between SpryTD and PIP_2 was evaluated with a Spry2 mutant ($Spry2^{R252D}$), which fails to interact with PIP_2. This mutant Spry2 is incapable of repressing MAPK activation in response to FGF stimulation, unlike wild type Spry2, which localizes to PIP^2 and inhibits MAPK activation [354].

Alternatively, Spry proteins are targeted to the plasma membrane through palmitoylation. Evidence for this modification comes from a study were radiolabeled palmitate was incorporated in Spry, which was ectopically expressed in endothelial cells [356]. Nevertheless, the palmitoylating enzyme, the position

on Spry, and whether it takes place in additional cell types, are questions that remain to be answered.

Regulation of Sprouty

Spry proteins function in a negative feedback fashion to repress RTK-dependent MAPK activation induced by FGF, PDGF, VEGF and HGF/Met. Surprisingly, in EGF-induced signaling, Spry is involved in a positive feedback loop, sustaining EGFR and MAPK activity. The involvement of Spry proteins in both negative and feedback loops, in a signal signal-specific fashion, suggest an important role for Spry in the regulation of RTK signaling. With this in mind, it is important to consider several mechanisms that regulate the function of Spry.

Phosphorylation of the N-terminus of Sprouty

The activity of Spry proteins is mainly regulated by the phosphorylation of a conserved tyrosine residue on the N-terminus of Spry (Y53 and Y55 in the case of Spry1 and Spry2, respectively) [338-340]. This phosphorylation has been observed in various cellular contexts, including fibroblasts and endothelial cells of both human and murine origin. The phosphorylation of Spry is induced by stimulation of the cells with growth factors such as EGF, FGF and PDGF [334, 336, 337, 357]. Spry isoforms exhibit different propensities for phosphorylation in response to different growth factors [355]. Spry1 is more likely to become phosphorylated upon stimulation of fibroblasts by FGF and PDGF whereas Spry2

is more likely to be phosphorylated in response to EGF and FGF. Furthermore, the kinetics of Spry phosphorylation may differ in a growth factor-specific manner. For example, Spry2 exhibits a relatively transient tyrosine phosphorylation in response to EGF stimulation, whereas in response to FGF signaling, the tyrosine phosphorylation of Spry2 exhibits a sustained profile [355].

The phosphorylation of Spry is necessary for the ability of Spry to regulate MAPK activation in response to growth factor stimulation. Spry mutants that are incapable of becoming phosphorylated on this conserved tyrosine residue (e.g. Spry2^{Y55A}), fail to repress ERK activation in response to FGF, contrary to the ability of wild type Spry proteins [340]. Given that these mutants can still translocate to the plasma membrane, it has been proposed that they have a dominant negative function [340, 358].

Unlike the wild type protein, Spry2^{Y55A} fails to sustain EGF signaling as well, suggesting that phosphorylation of Y55 is also important for the ability of Spry2 to sustain EGFR signaling [338]. These findings suggest that the ability of Spry2 to repress RTK signaling, as well as the ability of Spry2 to sustain EGFR signaling, rely, in part, on the same molecular mechanism, i.e. the phosphorylation of Y55.

The phosphorylation of Spry on Y53/55 (Spry1/2) has several implications, which attest dependence of Spry2 function on phosphorylation. One of the first studies to identify the phosphorylation of Spry1 and Spry2, demonstrated that in mouse

myoblast cells this phsophotyrosine residue serves as a docking site for the SH2 domain of Grb2. Grb2 mutants with a defective SH2-domain fail to interact with Spry1 or Spry2 [340]. In addition, this interaction is abrogated by mutations in the amino acids adjacent to the phosphotyrosine residue (T56I and E57Q in Spry2), indicating that these residues are also necessary for the interaction between Spry2 and Grb2.

The tyrosine phosphorylation of Spry2 on residue 55 is important for the interaction between Spry2 and the E3 ubiquitin ligase c-Cbl [359, 360]. Normally, c-Cbl ubiquitinates EGFR and targets this receptor for degradation. c-Cbl contains an atypical SH2 domain through which it interacts with phosphotyrosine residues on its substrates, such as EGFR and Zap70 [361]. Upon growth factor stimulation, Spry2 becomes phosphorylated on Y55, a site that then acts as a docking site for c-Cbl's SH2 domain [338, 339]. In this fashion, Spry2 competes with EGFR for binding to c-Cbl, a process that prevents the degradation of EGFR. The region flanking Y55 on Spry2 is highly conserved among Spry isoforms, and several amino acids in this region (i.e. N53, P59 and G58) are also important for the Spry2-c-Cbl interaction. Consistently, mutations in these residues abrogate the Spry2-c-Cbl complex, regardless of the fact that Y55 phosphorylation is unaffected [362]. It should be noted that Spry2 interacts with c-Cbl even in the absence of growth factor stimulation, albeit this occurs at low levels [338, 339, 362]. This interaction may be attributed to the ability of Spry2 to interact with the RING finger of c-Cbl [363].

Sprouty-2 kinase

In view of the importance of the phosphorylation of Spry2 on Y55, efforts have been made to identify the kinase that phosphorylates Spry2. A recent study proposes that Src, activated by FGFR in a FRS2-dependent fashion, phosphorylates Spry2 on Y55. The same study demonstrated that Spry2 is a direct substrate for Src *in vivo* and *in vitro* [364].

Another study suggests that EGFR phosphorylates Spry2 on Y55, in response to EGF stimulation [338]. Although a direct complex formation was not shown, EGFR was found to coimmunoprecipitate with Spry2, and immunoprecipitated EGFR was found to phosphorylate recombinant Spry2.

Sprouty-2 phosphatase

Phosphorylation frequently serves as a molecular switch to regulate protein activity. In many cases, including Spry2, phosphorylation leads to the activation of the protein function. In order to maintain normal cellular function, proteins that are activated by phosphorylation must be inactivated. This process is accomplished, in part, via the de-phosphorylation of proteins by phosphatases. In the case of Spry2, recent evidence implicates Shp2 as the phosphatase responsible for the inactivation of Spry2 [365]. Shp2 is a widely expressed protein-tyrosine phosphatase that mediates MAPK activation in response to growth factor

stimulation [366]. Shp2, through its SH2 domain is recruited to phosphotyrosine residues on RTK, where it dephosphorylates the receptor. Alternatively, Shp2 can function as a molecular adaptor for cellular proteins such as Grb2 [367]. Expression of Shp2 in murine myogenic cells decreases the phosphorylation of Spry2, and recombinant Shp2 reduces the level of phosphorylation on immunoprecipitated Spry2. Dephosphorylation of Spry2 by Shp2 results in the dissociation of Spry2 from Grb2, and decreases the inhibition of FGF signaling by Spry2.

Sprouty-2 ubiquitination

Another regulatory mechanism for Spry2 involves ubiquitination and proteosomal degradation. Stimulation with either EGF or FGF stimulation results in the ubiquitination of Spry2, followed by a decrease in the levels of Spry2 protein. The ubiquitination of Spry2 is attributed to its interaction with c-Cbl. Co-expression of Spry2 with c-Cbl results in ubiquitination of Spry2, while co-expression of Spry2 with c-Cbl mutants that are defective in their ability to ubiquitinate does not lead to ubiquitination of Spry2 [338, 339]. Also, expression of a Spry2 mutant that cannot interact with c-Cbl ($Spry2^{Y55A}$) in cells with high levels of endogenous c-Cbl, does not result in ubiquitination and degradation of Spry2 following EGF-stimulation.

Phosphorylation of the C-terminus of Sprouty-2

An important characteristic of Spry2 is that it can sustain EGF-induced signaling, while also inhibiting FGF- induced signaling. A recent study by Rubin et al. [342] has made an important effort in explaining the molecular mechanism underlying this function. As noted above, Spry2 is phosphorylated on its N-terminus (Y55) in response to EGF or FGF stimulation. Nevertheless, Spry2 also becomes phosphorylated on several tyrosine residues located on its C-terminus. These sites are specifically phosphorylated in response to FGF stimulation, and they are necessary for maximal inhibitory function of Spry2 in FGF-induced signaling. Among the C-terminal tyrosine phosphorylation sites, residue Y227 is the most important for the efficient inhibition of FGFR signaling by Spry2 [342]. Thus, the inhibitory function Spry2 in RTK signaling appears to be a response to signals that stimulate phosphorylation of both the N-terminal Y55 and the C-terminal Y227 residues, whereas the function of Spry2 as an activator of RTK signaling may be a response to phosphorylation of Y55 alone.

Cellular functions of Sprouty

Inhibition of receptor tyrosine kinase signaling

The cellular function of Spry proteins that is conserved among different species, is the ability of Spry proteins to inhibit receptor tyrosine kinase signaling (Fig. 6A) [334]. To date, Spry proteins repress RTK signals induced by growth factors, including FGF, PDGF, VEGF and HGF/Met, and therefore play an important role in regulating the biological processes mediated by these signals [329, 340, 356, 368].

RTK signaling is initiated by an extracellular ligand that activates a transmembrane receptor, which undergoes dimerization and autophosphorylation, leading to the recruitment of adaptor and effector proteins, which engage a number of diverse intracellular pathways [40]. Nonetheless, Spry proteins appear to inhibit specific RTK-induced intracellular pathways, which results mainly in the activation of the MAPK-cascade leading to ERK. It does not, however, appear to regulate JNK and p38 MAPKs, at least in response to FGFR activation [369]. In some cases, Spry2 inhibits Akt activation [368, 370], although the mechanism and the consequence of this function have not been fully elucidated.

The exact location along the RTK-Ras-Raf/MEK/ERK cascade where Spry exerts its inhibitory role remains under investigation. It appears that Spry acts in a signal

Figure 6. Regulation of RTK signaling by Spry. (A) Spry inhibits FGFR-mediated signaling. In the absence of Spry, binding of FGF to its receptor leads to tyrosine phosphorylation of the receptor and the scaffold protein FRS2, producing docking sites for the Grb2:SOS complex. Recruitment of this complex to the localization of the receptor leads to the activation of Ras, which in turn activates the Raf/MEK/ERK cascade through the direct activation of Raf. When Spry is present, it interacts with Grb2 and prevents Grb2:SOS from being recruited to FGFR, leading to diminished activation of ERK in response to FGF. (B) Spry inhibits VEGFR-mediated signaling, through a different mechanism than that involved in the regulation of FGFR. In this context, Spry is involved in the regulation of Ras-independent Raf activation. In the absence of Spry, the activation of VEGFR results in the activation of PKC_{δ} in a PLC_{γ}-mediated pathway. PKC_{δ} activates the Raf/MEK/ERK pathway through the phosphorylation and activation of Raf. Spry interacts with Raf and prevents the activation of Raf by PKC_{δ}, inhibiting ERK activation in response to VEGF stimulation. (C) The activation of EGFR induces a signaling pathway that is similar to that induced by FGFR. EGFR is regulated by a number of mechanisms, one of which involves c-Cbl and CIN85. c-Cbl, an E3 ubiquitin ligase, ubiquitinates EGFR and targets it for degradation. CIN85 is involved in receptor endocytosis and facilitates the function of c-Cbl. These functions result in the silencing of receptor mediated signaling. Spry2 interacts with c-Cbl and CIN85, and prevents the degradation of EGFR. This results in sustained signaling activity from this receptor, culminating in enhanced activation of ERK.

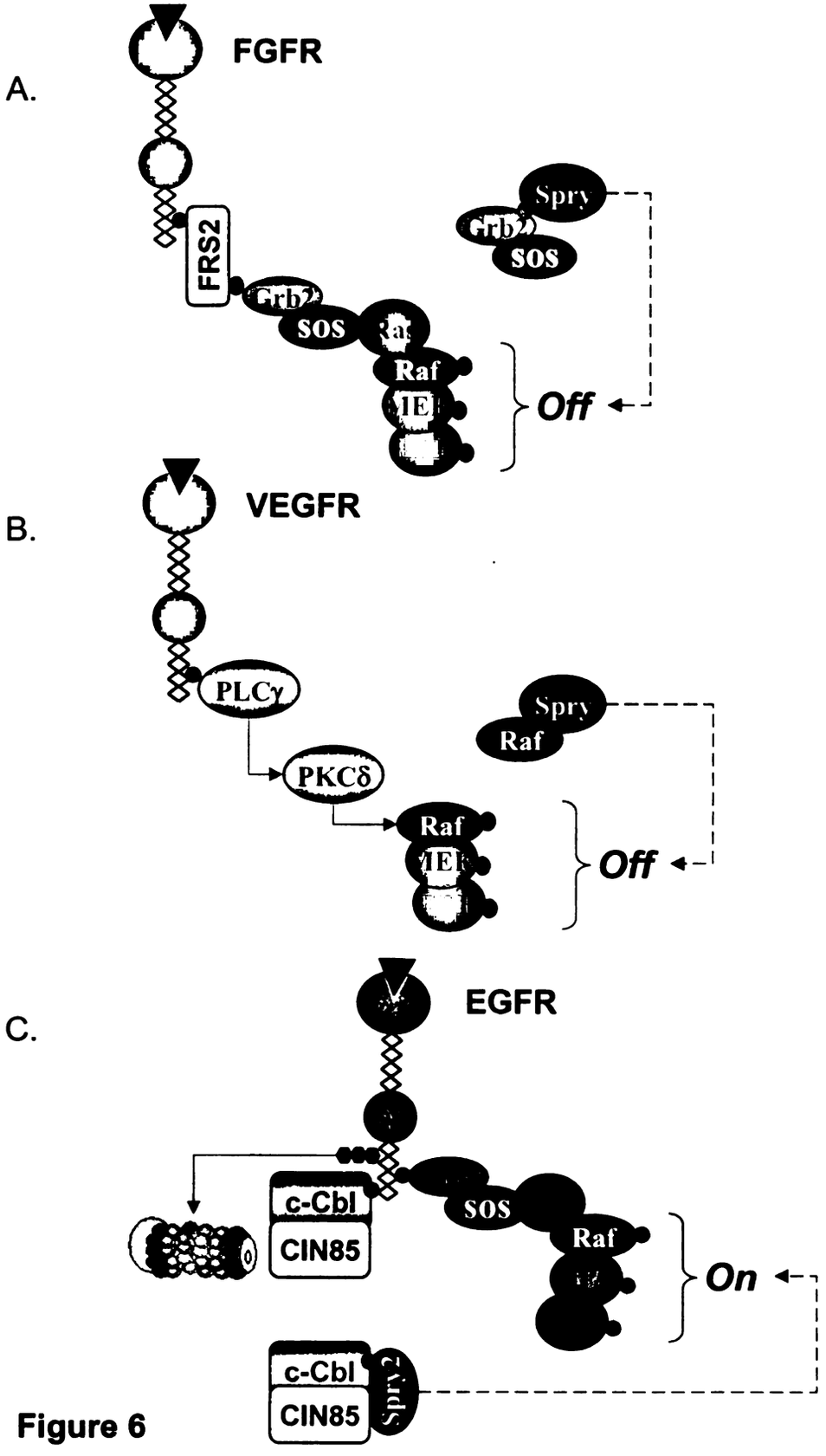

A.
FGFR
FRS2
Grb2
SOS
Ras
Raf
MEK
Spry
Grb2
SOS
Off
B.
VEGFR
PLCγ
PKCδ
Raf
MEK
Spry
Raf
Off
C.
EGFR
c-Cbl
CIN85
SOS
Raf
On
c-Cbl
CIN85
Spry2

Figure 6

specific and/or in a cellular context-specific manner. The conclusion that emerges from studies in different cellular systems is that Spry's inhibition on RTK signaling occurs somewhere between the level of the receptor and Ras/Raf activation. The evidence leading to this conclusion is presented in the following paragraphs.

One mechanism to explain the repression of FGF signaling by Spry focuses on the interaction of Spry with Grb2. In mouse myoblast cells (C2C12) this interaction is induced by growth factor stimulation, and results in the juxtaposition of the tyrosine phosphorylated Spry with the SH2 domain of Grb2. The consequence of this interaction in FGF signaling is to diminish the association of Grb2:SOS with FGFR adaptors like FRS2 and Shp2, which in turn diminishes the activation of Ras and Raf/MEK/ERK pathway [340]. It should be noted, however, that inducible expression of Spry in mouse fibroblasts (NIH3T3) showed that Spry had no effect on the formation of the FRS2-Grb2:SOS complex, yet Spry still diminished the activation of Ras. Taken together, these results suggest that Spry may have cellular context-specific mechanism for preventing Ras activation in FGFR signaling [329].

Coexpression of Spry with constitutively active forms of Ras, Raf and MEK, in human embryonic kidney (HEK293) cells, found that Spry inhibits FGF signaling at the level of Raf activation [369].

The ability of Spry to intercept RTK signaling at the level of Raf activation is also observed in human endothelial cells, where VEGF activates ERK in a Ras-independent fashion (Fig. 6B) [371]. In this system VEGFR, through its effector PLCγ, activates PKCδ and this in turn activates the Raf/MAPK cascade. Expression of Spry4 in this system prevents the Ras-independent activation of ERK induced by VEGF, without affecting Ras-dependent ERK activation induced by EGF. Spry4 interacts with Raf, though its carboxy-teminal region. This interaction is necessary for its inhibitory function. Spry2 can also interact with Raf [371], suggesting that Spry2 has a similar effect in this pathway.

The interaction between Spry and Raf has is also evident in melanoma cell lines [372]. In melanomas expressing wild type BRaf, both Spry2 and Spry4 interact with BRaf. In melanoma cells with BRaf mutations (V599E, V599D, L596V and K600E), however, neither of the two Sprys was able to complex with BRaf. This finding suggest that these residues on BRaf are important for the interaction between Raf and Spry2.

Furthermore, transient down regulation of Spry2 by using Spry2-specific siRNA resulted in an increase in the activation of ERK in melanomas with wild type BRaf, whereas no effect was observed in melanomas with mutated BRaf. This suggests that mutant BRaf can bypass the inhibitory effect of Spry2 [372]. The latter may be an important mechanism by which BRaf escapes normal regulatory mechanism and induces malignant transformation.

Another proposed mechanism by which Spry inhibits RTK signaling involves an interaction between *D. m.* Spry and Drk, the *D.m.* homolog of Ras^{GAP1} [327]. Through this interaction Spry facilitates the recruitment of GAP1 at Ras signaling complexes, thereby promoting the inactivation of Ras.

A final line of evidence to support the contention that Spry inhibits RTK signaling upstream of Ras/Raf originates from a study with murine lung epithelial cells, where ectopic Spry2 complexes with Grb2, FRS2, Raf, Shp2, GAP1, FGFR2b and SOS [331]. FGF stimulation enhanced the interactions of Spry2 with Grb2, Raf, and FRS2 while diminishing the interaction between Spry2 and Shp2 or GAP1. Ectopic Spry2 decreased MAPK activation in response to FGF stimulation, suggesting that the inhibitory role of Spry2 is a result of its ability to differentially interact with key components upstream of MAPK [331].

Although Spry inhibits RTK signaling through distinct mechanisms, the biological phenotype resulting from this activity includes a decrease in growth factor-induced proliferation, differentiation and angiogenesis [329, 356, 373]. The inhibitory effect of Spry on cellular proliferation is mediated not only by the ability of Spry to inhibit ERK activation, but also, by its ability to represses SRE- and Elk-mediated transcription [329, 340]. Moreover, ectopic expression of Spry proteins ectopic expression of Spry proteins represses the ability of fibroblasts and leiomyoma cells to form colonies in soft agar, an observation that is consistent

with the ability to repress MAPK activation and cellular proliferation [329, 368]. In addition, Spry attenuates the differentiation of rat phaeochromocytoma (PC12) cells induced by FGF [329], consistent with the fact that Spry proteins inhibit FGF signaling. Spry also inhibits the branching and sprouting of small vessels during angiogenesis [335].

Activation of epidermal growth factor receptor signaling

EGFR is a key mediator of signal transmission in response to extracellular cues. EGFR regulates many cellular functions including proliferation, differentiation and survival [374]. As such, EGFR must be strictly regulated in order to ensure normal cellular function. Uncontrolled function of EGFR can lead to enhanced proliferation, which may lead to cancer formation. Although there are a number of mechanisms to turn off the signaling activity of EGFR, the one that is affected by the function of Spry involves receptor ubiquitination and proteosomal/lysosomal degradation.

The necessity to turn off EGFR signaling following ligand activation is met in part by c-Cbl, an E3 ubiquitin ligase enzyme that catalyzes the poly-ubiquitination of EGFR. The consequence of EGFR ubiquitination is apparent upon the internalization of the ligand-bound receptor. When the receptor is not ubiquitinated it is endocytosed and recycled back to the plasma membrane following the disruption of the ligand-receptor complex (i.e. the inactivation of the receptor). Instead, when the receptor is poly-ubiquitinated it is targeted for

degradation by the proteasomal/lysosomal pathway. The function of c-Cbl is mediated through distinct domains including an atypical SH2 domain that interacts with phosphotyrosine residues located on c-Cbl's substrates. c-Cbl, also contains a RING-finger domain, which is critical for ubiquitination, because it interacts with E2 ubiquitin conjugating enzymes, thus recruiting such factors to the location of the substrate [361]. There, E2 enzymes catalyze the ubiquitination of the substrate. In certain situation more than one ubiquitin moiety is added to the substrate, a modification that targets the substrate for degradation by the proteasome.

Upon EGF stimulation, Spry2 can sustain RTK signaling, leading to sustained activation of ERK. Mechanistically this is attributed to the ability of Spry2 to interact with c-Cbl and inhibit the c-Cbl-induced degradation of EGFR [360, 363]. The interaction between Spry and c-Cbl reduces the level of EGFR ubiquitination by c-Cbl, and thus Spry can sustain EGFR levels and signaling activity. The later has been demonstrated in PC12 cells, which have been paradigmatically used to study the effects of RTK signaling on cell fate, given the fact that they can be induced to proliferate or differentiate, depending on the duration of RTK signaling [375]. In PC12 cells, some growth factors, such as EGF, induce transient activation of MAPK and promote cellular proliferation, whereas other growth factors, such as nerve growth factor (NGF) or FGF, induce sustained ERK activation leading to differentiation and neurite outgrowth. However, when PC12 cells expressing Spry2 are stimulated with EGF, they display sustained ERK

activation and undergo differentiation, consistent with the ability of Spry2 to prevent EGFR degradation induced by c-Cbl [363].

The ability of Spry proteins to interact with c-Cbl is conserved in D. m. Spry and mammalian Spry1 and Spry2. Nevertheless, this function has almost exclusively been studied in the context of Spry2. Spry4 fails to interact with c-Cbl and cannot inhibit EGFR degradation induced by c-Cbl [360]. As described above, the interaction between Spry2 and c-Cbl, involves Spry2 phosphotyrosine residue on position 55 and c-Cbl's SH2 domain [334]. Furthermore, amino acids flanking Y55 on Spry2 are also important for this interaction [355, 362]. Structural differences in this region between Spry2 and Spry4 may account for their different ability to regulate EGFR degradation. In fact, mutation of the Spry2 residues surrounding Y55 to the amino acids present in the same region of Spry4, abrogates the ability of Spry2 to bind c-Cbl [355].

In addition to preventing EGFR degradation, Spry2 can also diminish EGFR endocytosis at an early stage of the internalization/trafficking process [363, 376]. In the absence of Spry2, EGFR localizes in endocytotic vesicles upon EGF-stimulation, whereas upon expression of Spry2, EGFR remains at the plasma membrane.

The role of Spry2 in the regulation of EGFR endocytosis is also supported by recent evidence that Spry2 can interact *in vivo* and *in vitro* with CIN85 [343].

CIN85 is part of an endocytotic complex that assists endocytosis by c-Cbl. It has been proposed that CIN85 leads to clustering of c-Cbl, facilitating c-Cbl-induced EGFR endocytosis and degradation. CIN85 contains three SH3 domains (A, B and C) through which it interacts with proline rich regions (PxxxPR). Spry2 contains two such motif on positions 64 and 309, which interact with the SH3A domain of CIN85. In addition, Spry2 contains another similar motif (xxPxPR) on position 72, which interacts with the SH3C domain of CIN85.

These proline-rich motifs are also present in Spry1, thus enabling this Spry to interact with CIN85. *D. m.* Spry and Spry4, however, lack such motifs and cannot interact with CIN85 [343].

The interaction of Spry2 with CIN85 is important for the inhibition of c-Cbl induced degradation of EGFR, because a Spry2 mutant that cannot interact with CIN85 ($Spry2^{R64,72,309A}$) fails to inhibit EGFR degradation, regardless of the presence of wild type Y55 residue (i.e., this mutant Spry2 retains an intact ability for c-Cbl interaction). Finally, Spry2 associates with both c-Cbl and CIN85 to form a ternary complex that is necessary for inhibition of EGFR degradation and promotion of neurite outgrowth in PC12 cells [343].

The ability of Spry2 to inhibit c-Cbl induced degradation of EGFR implies that Spry may prevent the degradation of additional c-Cbl substrates. However, this function of Spry2 may be restricted only to EGFR, because although c-Cbl targets

FGFR and FRS2 for degradation, Spry2 doesn't seem to have an inhibitory effect under these circumstances [362].

Regulation of integrin-mediated cell spreading by Sprouty-4

The ability of Spry to regulate cell spreading has been studied only in the context of Spry4. Spry4 interacts with TESK1 through its carboxy-terminal region [377]. TESK1 is a serine/threonine kinase that phosphorylates cofilin, an actin binding protein that is involved in actin depolymerization and severance of actin stress fibers [378, 379]. TESK1 phosphorylation inhibits cofilin, and cofilin-induced actin disassembly. Also, TESK1 plays an important role in integrin-mediated actin remodeling and cell spreading. The interaction between Spry4 and TESK results in the inhibition of the kinase activity of TESK1 *in vitro,* as well as in the decrease in the levels of phosphorylated cofilin [380]. Thus, Spry4 negatively regulates cell spreading through the inhibition of TESK1, which results in sustained cofilin activity. Phosphorylation of the conserve tyrosine residue on the N-terminal of Spry4 (Y75) is not necessary for its interaction with TESK1, or for its ability to suppress cell spreading [380].

Regulation of cell migration by Sprouty

Spry proteins inhibit cell migration during *Xenopus* embryogenesis [332], as well as in wound healing assays with mammalian cells [373]. In the latter case, Spry1, Spry2 and Spry4 antagonize growth factor stimulated cellular migration. This

ability of Spry is dependent on its cysteine rich carboxy-terminal [373], and appears to be mediated in part by PTP1B and $p130^{Cas}$ [381, 382]. These studies, in which Spry2 protein was transduced in HeLa cells, demonstrated an increase in the levels and activity of PTP1B phosphatase in the soluble fraction. $p130^{Cas}$, a substrate for PTP1B, exhibits reduced levels of phosphorylation following transduction of Spry2, a finding that is consistent with the increase in the activity of PTP1B by Spry2. What's more expression of $p130^{Cas}$ attenuates Spry2's inhibition of growth factor induced cellular migration [381].

Furthermore, Spry2 represses the activation of Rac1 in wound healing assays, suggesting that the regulation of cellular migration by Spry2 is dependent of its effect of Rac1 activity. Expression of constitutively active Rac1 attenuates the inhibition of migration by Spry2 [382]. Instead, constitutive active Rac1 had no effect on the inhibitory function of Spry2 in cellular proliferation [381], suggesting that Spry can engage specific pathways to carry through its effect on cellular function.

Sprouty deficient mice

Recently, Basson et al [383], Shim et al [384],. and Taketomi et al. [370] successfully generated knockout mice models for *Spry1* and *Spry2*. These models have elucidated important roles of *Spry* genes in mammalian development

***Sprouty-1*$^{-/-}$**

Homozygous *Spry1*$^{-/-}$ mice, which were born at expected Mendelian ratios, display reduced post-natal viability [383]. A small subset (21%) of Spry1-/- mice die within 48 hrs, whereas another population (71%) die within five months. The surviving animals have significant kidney malformations.

Normal kidney development involves the outgrowth of a single ureteric bud from the Wolfian duct during early development [385]. Glial derived growth factor (GDNF), and its tyrosine kinase receptor c-Ret are part of a signaling pathway that is important for ureteric bud formation[386-388]. *Spry1-/-* mice develop supernumerary ureteric buds from the Wolfian duct, which results in multiple ureters and a multiplex kidney [383]. An important characteristic of *Spry1-/-* mice is their sensitivity to GDNF signaling. This is indicated by the levels of active ERK in ectopic sites, as well as by the expression of Wnt11. Normally, Wnt11 is expressed at the tips of ureteric buds [389], where Ret signaling is active, but in *Spry1*$^{-/-}$ mice Wnt11 expression is extended to more anterior sites and in discrete ectopic sites suggesting an overactive c-Ret signaling pathway [383, 390]. The phenotype of *Spry1*$^{-/-}$ mice is mediated by the GDNF sensitization of kidney tissue, because reduction of GDNF gene dosage, as a result of crossing Spry1$^{-/-}$ with Gdnf$^{+/-}$ (*Spry1*$^{-/-}$;*Gdnf*$^{+/-}$) mice, reverts the phenotype of *Spry1*$^{-/-}$ mice. Together, these findings suggest that Spry1 regulates normal ureteric bud and kidney development, through suppression of GDNF/Ret signaling.

Sprouty-2$^{-/-}$

Half of the *Spry2*$^{-/-}$ mice die within six weeks after birth, while the rest can survive for at least six months. These animals are smaller in size compared to littermates [370]. Notably, the surviving *Spry2*$^{-/-}$ mice are characterized by hearing impairment [384], as well as neuronal hyperplasia and esophageal achalasia (constriction of the lower part of the esophagus) [370].

The sense of hearing involves the conversion of the sound waves into vibrational energy at the tympanic membrane and the transmission through the middle ear to the organ of Corti in the inner ear, where vibrational energy is converted into electrical impulses that are transmitted into the brain. The hearing impairment in *Spry2*$^{-/-}$ mice reflects a disruption in the architecture of the organ of Corti [384]. While this organ normally consists of three rows of longitudinal outer hair cells, the organ of Corti in *Spry2*$^{-/-}$ mice consists of four rows. In addition, *Spry2*$^{-/-}$ contained an ectopic pillar cell, leading to the formation of an ectopic Corti-like tunnel.

The normal development of the organ of Corti is dependent in part on FGF8, which is secreted by inner hair cells and acts on the FGFR3 receptors, which are located on the outer hair cells and the pillar cells [384, 391, 392]. It is possible that in *Spry2*$^{-/-}$ mice, FGFR signaling is overactive, resulting in the noted malformations in the organ of Corti. Indeed, reduction of *FGF8* gene dosage in Spry2 null mice, achieved by crossing these mice with *FGF8*$^{+/-}$ mice (*Spry2*$^{-/-}$;*Fgf8*$^{+/-}$) prevents the extra pillar formation and partially reverts the hearing loss

phenotype of Spry2$^{-/-}$ mice. This suggests that Spry2 suppresses FGF8 signaling in the organ of Corti and promotes normal development of this organ [384].

Phenotypically, *Spry2*$^{-/-}$ mice are also characterized by abnormal intestinal motility and esophageal achalasia, due to lower esophageal sphincter (LES) hypercontraction [370]. *Spry2*$^{-/-}$ mice demonstrate hyperplasticity in the enteric nervous plexus (ENS) and have elevated levels of muscarinic (M2)-acetylcholine receptors in the neuro-muscular junctions of esophagus, which may be responsible for the hypercontraction of LES. GDNF/Ret signaling, which is necessary for the survival of ENS [386] is increased in neuronal cells of *Spry2*$^{-/-}$ mice, suggesting that the ability of Spry2 to repress GDNF signaling is important for preventing ENS hyperplasia, and esophageal achalasia. In fact, anti-GDNF antibodies can correct ENS hyperplasia and reduce the esophageal dilation in *Spry2*$^{-/-}$ mice [370].

Sprouty proteins in cancer

The ability of Spry proteins to regulate RTK makes them good candidates as cancer genes. The expression of *Spry2* gene is altered in several types of cancer. Spry1 and Spry2 are down regulated in breast cancer [393], and expression of Spry2 in the breast cancer cells (MCF7) reduces the ability of these cells to form tumors in athymic mice. Spry2^{Y55A}, a mutant that cannot repress growth factor induced ERK activation, fails to abrogate the tumor forming ability of MCF7 cells [393].

Spry1 and Spry2 are also down regulated in prostate cancer. Spry1 was found to be decreased in 40% of prostate cancers, when these were compared with matched normal prostate [394]. Spry2 expression is decreased in invasive prostate cancer and clinical prostate cancer, when these were compared to benign prostatic hyperplasia (BPH) [395]. This reduced expression is attributed to epigenetic inactivation, through hypermethylation of the Spry2 promoter. The *Spry2* gene contains a large CpG island spanning positions -500 and +950 relative to the putative transcription start site. This region is hypermethylated in the high grade clinical prostate cancers, whereas in BPH this region is relatively non-methylated [395].

Interestingly, Spry2 expression is elevated in melanomas with $BRaf^{V600E}$ mutations or in melanomas with $NRas^{Q61R}$ mutations, compared to cells without mutations [396]. An independent study found that Spry2 was expressed at higher levels in melanomas with $BRaf^{V599E}$ compared to melanomas with wild type *BRaf* and normal melanocytes [372].

Although additional studies are needed to determine the precise role of Spry in cancer, initial evidence supports the hypothesis that Spry proteins repress malignant transformation, through their ability to repress RTK signaling. Nevertheless the increased expression of Spry2 in some types of cancer, as well

as its ability to sustain EGFR signaling, suggests that Spry2 promotes cancer progression. Whether this is true remains to be seen.

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Chapter II. Sprouty 2 is necessary for tumor formation by *HRas* oncogene-transformed human fibroblasts

Running title: Spry2 is necessary for HRas-transformation

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Abstract

Sprouty 2 (Spry2) plays a regulatory role in the signaling pathways induced by a number of growth factors. One aspect of the function of Spry2 is to prevent the c-Cbl-induced degradation of epidermal growth factor receptor (EGFR). We report that human fibroblasts malignantly transformed by $HRas^{V12}$ oncogene, exhibited an increase in the expression of Spry2, compared to the parental cells. To determine whether Spry2 plays a role in *HRas*-transformation, we down-regulated the expression of Spry2 using *Spry2*-specific shRNA. HRas-transformed cells with down-regulated levels of Spry2 failed to form tumors when injected into athymic mice, indicating that Spry2 in necessary for tumor formation by HRas-transformed cells. In cells expressing oncogenic *HRas*, Spry2 sustained not only the level of EGFR, but also the activation of ERK. What is more, HRas interacted with Spry2 in HRas-transformed cells, and HRas interacted with c-Cbl and CIN85 in a Spry2-dependent fashion, suggesting that HRas regulates the turnover of EGFR through Spry2. We also found that expression of Spry2 in immortalized human fibroblasts, did not affect EGFR levels, while it inhibited HRas and ERK activation. The effect on ERK was diminished when Spry2 was expressed at a higher level. These data show that Spry2 has distinct functions in pre-malignant and in malignant fibroblasts transformed by HRas. While in the former Spry2 can inhibit EGF signaling, in the latter, the inhibitory function of Spry2 is bypassed and the ability of Spry2 to sustain EGFR takes center stage.

Introduction

Carcinogenesis is a multistep process by which cells acquire neoplastic characteristics through a series of genetic and/or epigenetic changes. To study this process, McCormick and colleagues developed a model system which mimics the pattern by which normal human fibroblasts become malignant [1]. In these experiments, normal foreskin-derived human skin fibroblasts were transfected with a v-Myc oncogene, giving rise to a clonal population of cells, which spontaneously acquired an infinite life span in culture [2]. This cell strain, which has a normal diploid karyotype, was designated MSU-1.0. As MSU-1.0 cells were being propagated in culture, one cell underwent two chromosomal translocations, giving rise to a cell strain that is chromosomally stable, near-diploid, and partially growth factor independent [2]. This cell strain, designated, MSU-1.1, has been malignantly transformed by the overexpression of Ras oncogenes [2-5] or by exposure to a carcinogen, followed by selection of focus-forming cells [6, 7]. When MSU-1.1 cells expressing high levels of $HRas^{V12}$ oncoprotein are injected subcutaneously into athymic mice, they form fibrosarcomas within three weeks. A malignant cell strain derived from such tumors, designated PH3MT, is completely growth factor independent and exhibits anchorage independent growth [4].

Sprouty was first identified in *Drosophila* as an inhibitor of fibroblast growth factor (FGF)-induced tracheal branching [8] and epidermal growth factor (EGF)-induced eye development [9]. Mammalian species express four isoforms of Sprouty (Spry1-4) [8, 10, 11], which act as inhibitors of growth factor-induced cellular differentiation, migration, and proliferation [12-15].

In addition to its inhibitory function, Spry2 also sustains the EGFR signaling [16-21]. This

function is mainly the result of the interaction of Spry2 with c-Cbl, an E3 ubiquitin ligase that catalyzes the ubiquitination of EGFR, targeting this receptor for lysosomal degradation [22]. By binding to c-Cbl, Spry2 prevents the interaction between c-Cbl and EGFR, and this interference blocks the degradation of the receptor. This in turn leads to sustained EGFR-induced ERK activity [23-26].

The expression of *Spry2* gene is altered in several types of cancer. Spry2 is down - regulated in breast cancer [27], and expression of Spry2 in breast cancer cells (MCF7) reduces the ability of these cells to form tumors in athymic mice [27]. Spry2 is also down-regulated in prostate cancer, which is attributed to epigenetic inactivation, through hyper-methylation of the Spry2 promoter [28]. Spry2 expression is also elevated in some cancer subtypes, including melanomas that express activated Ras signaling pathways [29, 30], suggesting that Spry2 contributes to the malignant phenotype in cells expressing oncogenic *Ras*.

The present study was designed to investigate the role of Spry2 in tumor formation in malignant cells with activated Ras signaling. We found that HRas-transformed human fibroblasts (PH3MT) expressed a higher level of Spry2 protein than that found in their parental cells (MSU-1.1). When we stably down-regulated the expression of Spry2 in PH3MT cells, we observed a complete loss of tumor-forming ability by these cells. In PH3MT cells Spry2 sustained the level of EGFR and ERK activation. Interestingly, independent expression of Spry2 in MSU-1.1 cells resulted in the inhibition of EGF signaling and was insufficient to malignantly transform these cells.

Results

Determination of the Expression of Spry2 in HRas-transformed Cells.

To detetermine the effect of HRas-transformation on the expression of Spry2 we examined the cells of the MSU lineage by Northern and Western blotting (Fig. 1A and B). Although compared to MSU-1.0 cells, MSU-1.1 cells exhibit only a modest increase in Spry2 expression, the cells malignantly transformed by $HRas^{V12}$ oncogene (PH3MT) exhibit a significant increase in Spry2 expression. An independent HRas-transformed cell strain (PH2MT) was found to have the same high expression of Spry2 (Fig. 1C). These data suggest that oncogenic *HRas* is responsible for the high level of Spry2 protein found in PH3MT cells. To determine if the same was true for oncogenic *NRas*, we examined the levels of expression of Spry2 protein in MSU-1.1 cell strains malignantly transformed by the $NRas^{V12}$ oncogene, i.e., N-Ras-2T and N-Ras-3T [5]. NRas-transformed cells expressed Spry2 at a level similar to that present in the HRas-transformed cells, i.e., PH2MT and PH3MT (Fig. 1C).

We also examined the level of expression of Spry2 in a four patient-derived fibrosarcoma and seven patient-derived pancreatic carcinoma cell lines. As shown in Fig. 1D, all four fibrosarcoma-derived cell lines expressed high levels of Spry2, compared to normal foreskin-derived fibroblasts. Of seven pancreatic carcinoma-derived cell lines analyzed for expression of Spry2, three cell lines

Figure 1. Expression profile of Spry2 in Ras-transformed cells and in patient derived cancer cells. Northern (A) and Western (B) blots showing the expression of Spry2 in immortalized human fibroblasts strains MSU-1.0 and MSU-1.1, and in MSU-1.1 cells malignantly transformed by the *HRas* oncogene (PH3MT). (C) The expression of Spry2 protein in MSU-1.1 cells malignantly transformed by *HRas* and *NRas* oncogenes. Cell strains that were derived by the malignant transformation of MSU-1.1 cells with *HRas* (PH2MT and PH3MT) or *NRas* (NRas2T and NRas3T) oncogenes, were analyzed by Western blotting with the indicated antibodies. (D) The expression of Spry2 protein in patient-derived fibrosarcoma cell lines and in normal, foreskin-derived, fibroblast cell lines (SL68 and SL89). (E) The expression of Spry2 protein in pancreatic carcinoma cell lines and in an infinite life span, pancreatic cell line (ps-1).

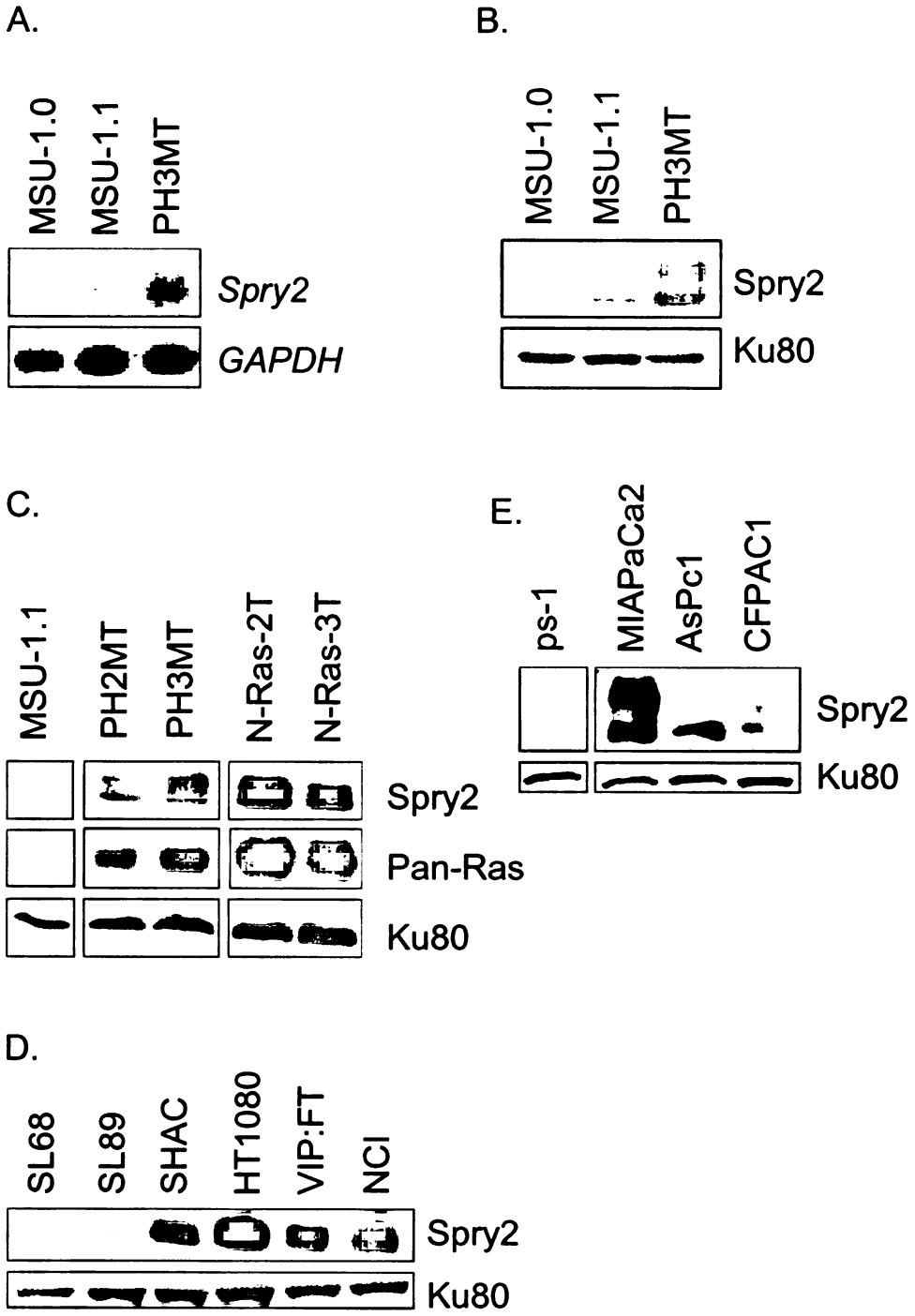

A.
MSU-1.0
MSU-1.1
PH3MT
Spry2
GAPDH
B.
MSU-1.0
MSU-1.1
PH3MT
Spry2
Ku80
C.
MSU-1.1
PH2MT
PH3MT
N-Ras-2T
N-Ras-3T
Spry2
Pan-Ras
Ku80
D.
SL68
SL89
SHAC
HT1080
VIP:FT
NCI
Spry2
Ku80
E.
ps-1
MIAPaCa2
AsPc1
CFPAC1
Spry2
Ku80

Figure 1

expressed high levels of Spry2 protein compared to a normal infinite life span pancreatic cell line (Fig. 1E).

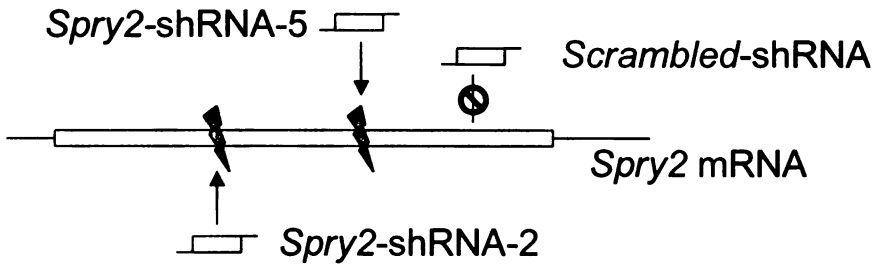
Effect of Spry2 on Tumor Formation by HRas-transformed Cells.

In order to study its role we down-regulated Spry2 in PH3MT cells, by using short hairpin RNA (shRNA). The shRNA molecules were designed to target position 399 or position 492 of the *Spry2* coding region. A scrambled shRNA molecule that does not target Spry2 was included as a control (Fig. 2A). Cell stains expressing a vector without a shRNA molecule (PH3MT-VC), or a vector encoding the scrambled shRNA molecule (PH3MT-SC), were used as controls. We identified two cell strains expressing the Spry2-specific shRNA molecule that targets position 399 (PH3MT-2A3 and PH3MT-2B9), as well as a cell strain expressing the Spry2-specific shRNA molecule that targets position 492 (PH3MT-5A3). The cell strains expressing shRNA specific for either position of the Spry2 coding region were found to express lower levels of the Spry2 protein, compared to the level in the control cell strains (Fig. 2B).

To determine the role of Spry2 in HRas-transformation we first examined the effect of Spry2 depletion on the anchorage independent growth of PH3MT cells. Depletion of Spry2 resulted in fewer and smaller colonies in agarose compared to control cells (Fig.2C).

Figure 2. Effect of Spry2 on the anchorage independent growth of HRas-transformed fibroblasts. (A) A schematic diagram of the positions targeted by the Spry2-specific shRNA constructs. *Spry2*-shRNA-2 targets position 399, whereas *Spry2*-shRNA-5 targets position 492 of the Spry2 coding region. A nonspecific shRNA molecule, designated *scrambled*-shRNA, was also constructed. These constructs were stably expressed in PH3MT cells as described in the Material and Methods section. (B) PH3MT cell strains stably expressing the indicated constructs were analyzed by Western blotting to determine the expression of Spry2. The PH3MT-2A3 and PH3MT-2B9 cell strains were infected with *Spry2*-shRNA-2, whereas the PH3MT-5A3 cell strain was infected with *Spry2*-shRNA-5. (C) The indicated cell lines were grown in agarose, in the presence of 10% serum (+ Serum) or 2.5% serum (- Serum) in the culture medium as described in the Material and Methods section. This graph represents the number of colonies with a diameter greater than 120 μm in each of the analyzed cell strains. A representative of at least two experiments with each of the two control clones and each of the three clones with down-regulated Spry2 is shown. (D) Whole cell lysates from the indicated cell lines were pulled down with RafRBD conjugated beads as described in Materials and Methods. The total amount of Ras in the whole cell lysate (WCL) was determined using a Pan-Ras-specific antibody.

A.


Spry2-shRNA-5
Scrambled-shRNA
Spry2 mRNA
Spry2-shRNA-2

B.

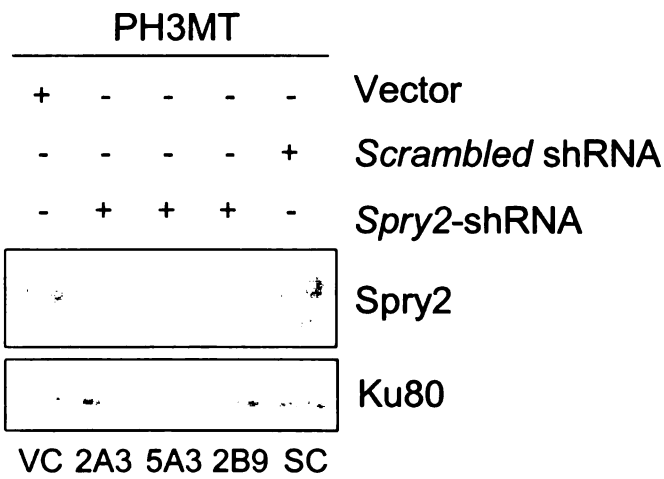
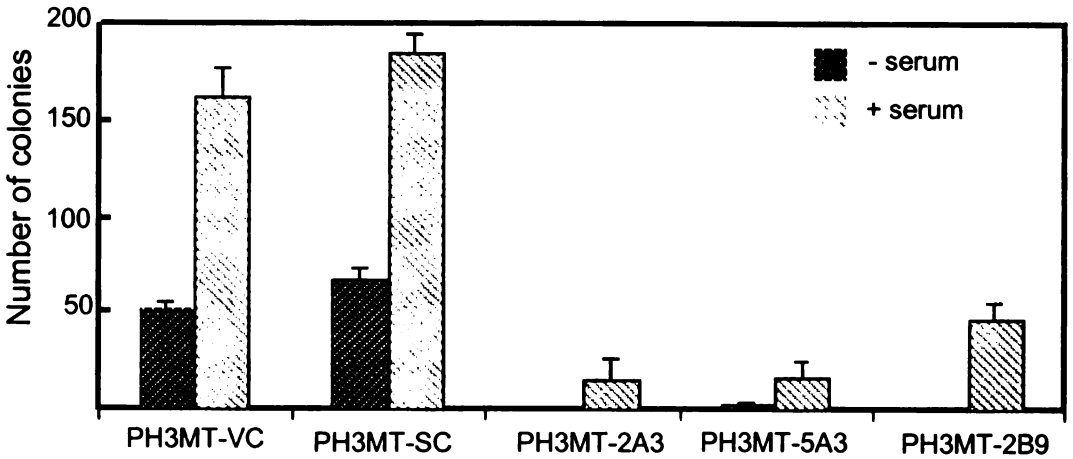

PH3MT
+ - - - - Vector
- - - - + Scrambled shRNA
- + + + - Spry2-shRNA
Spry2
Ku80
VC 2A3 5A3 2B9 SC

Figure 2

C.



D.

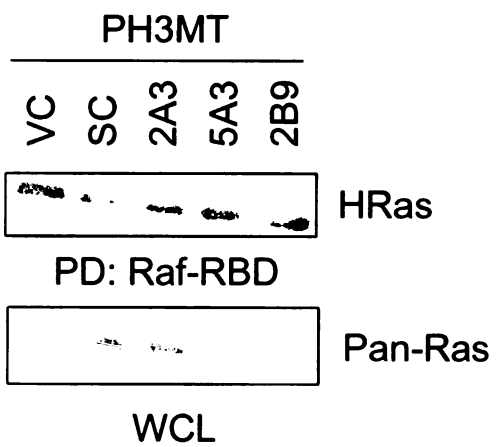


Figure 2 (Cont')

These same cell strains were tested for their ability to form tumors in athymic mice (Table I). The control cell strains formed 0.5 cm^3 tumors in 21 days. In contrast, the three cell strains with down-regulated expression of Spry2 failed to form tumors of any size, indicating that Spry2 is necessary for the malignant phenotype of, HRas-transformed, PH3MT cells. Given the significant effect that the down-regulation of Spry2 had on $HRas^{V12}$ transformed cells, we investigated whether this down-regulation reduced the level of activated HRas by using a Ras-activation assay. Down-regulation of Spry2 had no effect on the levels of active HRas (Fig. 2D), suggesting that Spry2 acts downstream of HRas oncogene to regulate HRas-induced malignant transformation.

Effect of HRas-transformation on the Level of EGFR protein.

Several studies have demonstrated that Ras regulates the signaling activity and endocytosis of EGFR through its effector proteins [31-35]. We found that transformation of immortalized human fibroblasts with $HRas^{V12}$ was associated with an increase in the levels of EGFR. As shown in Fig. 3A, stimulation of immortalized human fibroblasts (MSU-1.1) with EGF resulted in a decrease in the level of EGFR protein. Even though a similar effect was observed in MSU-1.1 cells malignantly transformed by *HRas* oncogene (PH3MT cells), these cells exhibited increased and sustained levels of EGFR compared to their parental cells (Fig. 3A).

Table I The tumorigenicity of the cell strains with down-regulated Spry2

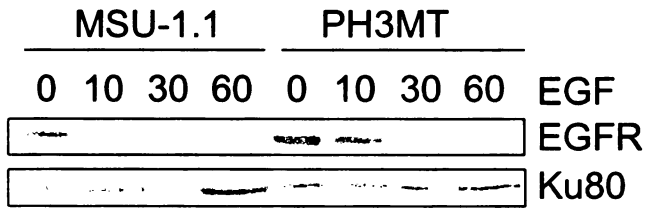
Cell strain	$HRas^{V12}$	Spry2-shRNA	Tumor incidence[a]	Days for tumor to reach 0.5 cm^3 volume
MSU-1.1	-	-	0/6[b]	-
PH3MT	++	-	6/6	21
PH3MT-VC	++	-	12/12	21
PH3MT-SC	++	-	12/12	21
PH3MT-2A3	++	+	0/12	-
PH3MT-5A3	++	+	0/12	-
PH3MT-2B9	++	+	0/12	-

[a]Ratio of tumors formed to the number of sites injected subcutaneously. If no tumors arose 6 months after the injection the mice were sacrificed.

[b]Ten million MSU-1.1 cells were injected in each site. The rest of the cell strains were injected at one million cells per site

Figure 3. Effect of HRas-transformation on the level of EGFR. (A) Immortalized human fibroblasts (MSU-1.1) and their $HRas^{V12}$-transformed derivatives (PH3MT) were serum deprived for 12 h. and then stimulated with EGF (100 ng/mL) for the indicated time points (shown in minutes). (B) MSU-1.1 and PH3MT cells were grown in media with 0.1% serum in the presence (open triangles and squares, respectively) or absence (closed triangles and squares, respectively) of the selective EGFR inhibitor AG1478 (6 μM). The effect of AG1478 in the growth of PH3MT cells was greater than the effect of the same inhibitor in MSU-1.1 cells ($p<0.001$). One of three independent experimental repeats (N=4) is shown.

A.


MSU-1.1
PH3MT
0 10 30 60 0 10 30 60
EGF
EGFR
Ku80

B.

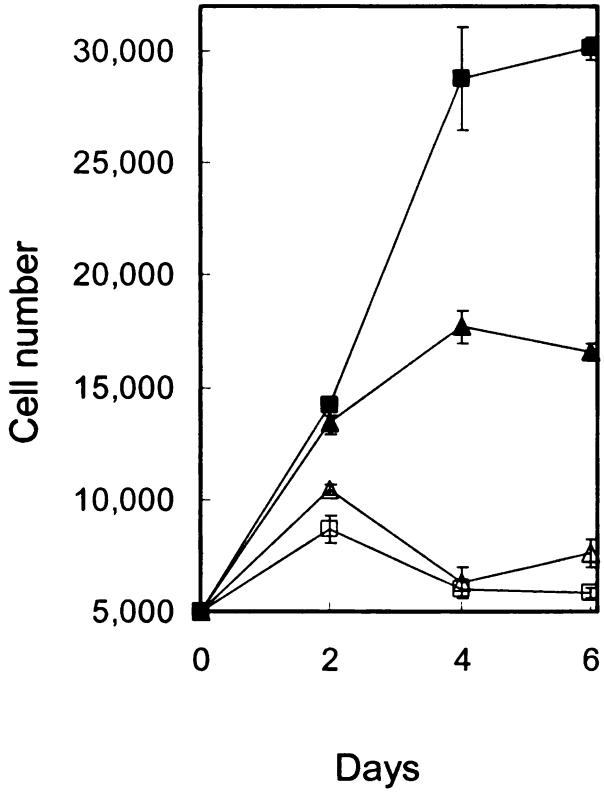

30,000
25,000
20,000
15,000
10,000
5,000
Cell number
0
2
4
6
Days

Figure 3

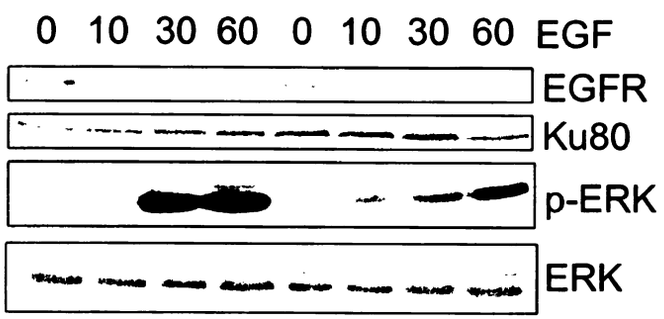
The ability of HRas oncoprotein to induce growth factor independence is an important property in cancer formation. To determine if the activity of EGFR is necessary for the growth factor independence of HRas-transformed fibroblasts, we measured the ability of PH3MT cells to grow in the presence of AG1478, a selective inhibitor of EGFR tyrosine kinase activity [36]. The inhibition of EGFR activity by AG1478 resulted in a decrease in the ability of HRas-transformed cells to grow in medium with reduced serum. This decrease was significantly higher than the corresponding change observed in MSU-1.1 cells (Fig. 3B). These data suggest that EGFR activity is required for the ability of PH3MT cells to grow in the absence of exogenous growth factors.

Effect of Depletion of Spry2 Protein on the Level of EGFR.

To determine if Spry2 sustains EGFR levels in HRas-transformed human fibroblasts, we compared the cell strains with down-regulated Spry2 with their control cell strains for the level of EGFR. Consistent with previous studies [23-26, 37, 38], our results showed that, even after 10 minutes of EGF-stimulation, the level of EGFR was decreased in all three cell strains with down-regulated Spry2 (data not shown). In contrast, the control cells did not show a decrease in EGFR under the same conditions (data not shown). We also examined the time course of EGFR decrease in control cells and in cells with down-regulated Spry2 (Fig. 4A). The cells with down-regulated Spry2 exhibited a more pronounced decrease in the level of EGFR compared to control cells, indicating that Spry2 expression is necessary for the ability of HRas oncoprotein to sustain the level of EGFR.

Figure 4. Effect of Spry2 down-regulation on the level of EGFR in HRas-transformed cells. (A) The indicated cell strains were analyzed as in Fig. 3A. (B) The indicated cell strains were grown in media with 0.1% serum. One of two experimental repeats (N=4) is shown.

A.


PH3MT-SC
PH3MT-2A3
0 10 30 60 0 10 30 60 EGF
EGFR
Ku80
p-ERK
ERK

B.

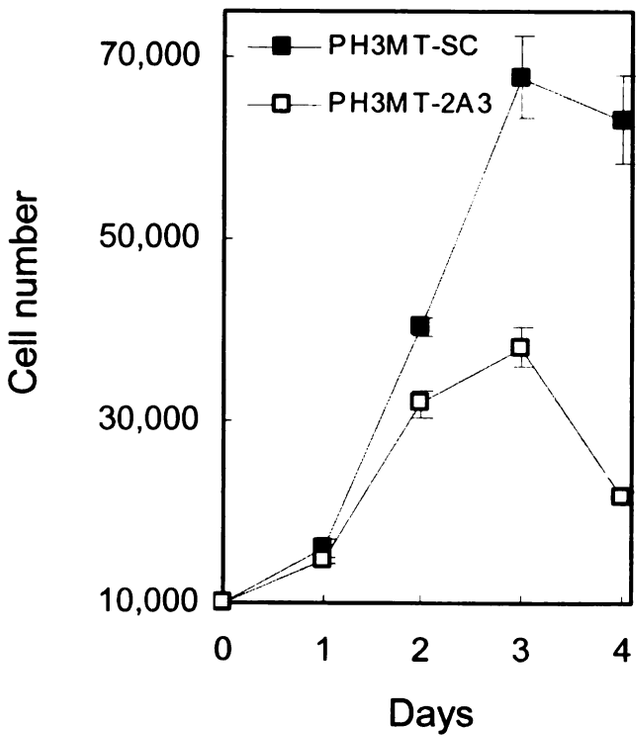

PH3MT-SC
PH3MT-2A3
70,000
50,000
30,000
10,000
Cell number
0
1
2
3
4
Days

Figure 4

To determine whether the expression of Spry2 in HRas-transformed cells was associated with an increase in activation of the MAPK cascade, we examined the EGF-induced activation of ERK. As shown in Fig. 4A, the cell strain with down-regulated Spry2 level had decreased levels of activated ERK compared to the level found in the control cell strain.

We also examined whether depletion of Spry2 had an effect on the growth factor independence of PH3MT cells. We found that HRas-transformed cells with decreased levels of Spry2 protein exhibited a decrease in their ability to grow in media with reduced serum compared to control HRas-transformed cells (Fig. 4B).

Interaction of Spry2 with HRas.

To determine how Spry2 mediated the effect of HRas on EGFR levels we examined whether Spry2 forms a complex with HRas in HRas-transformed cells (PH3MT) by using co-immunoprecipitation experiments. We found that endogenous Spry2 co-immunoprecipitated with HRas, suggesting that Spry2 interacts with HRas *in vivo* (Fig. 4A). The interaction between Spry2 and HRas was enhanced upon stimulation of cells with EGF (data not shown).

Figure 5. Interaction of HRas with Spry2 and Spry2 binding-partners c-Cbl and CIN85. (A) Whole cell lysates from HRas-transformed cells (PH3MT) were immunoprecipitated with an antibody specific to HRas or a nonspecific IgG and immunoblotted with the indicated antibodies. (B, C) Whole cell lysates form control cells (PH3MT-SC), as well as cells with down-regulated Spry2 (PH3MT-2A3) were immunoprecipitated with anti-HRas and immunobloted with the indicated antibodies.

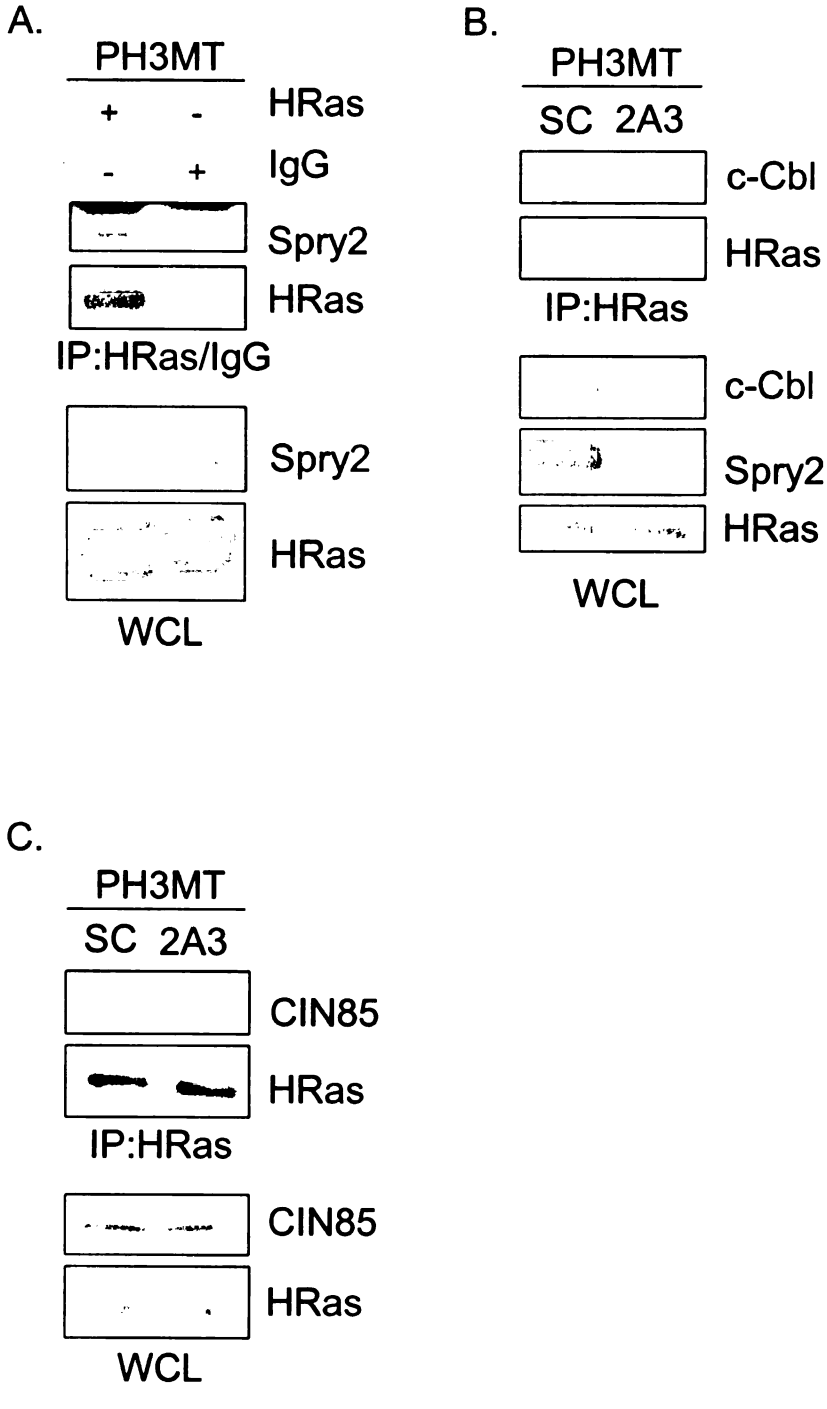

A.
PH3MT
+ -
HRas
- +
IgG
Spry2
HRas
IP:HRas/IgG
Spry2
HRas
WCL
B.
PH3MT
SC 2A3
c-Cbl
HRas
IP:HRas
c-Cbl
Spry2
HRas
WCL
C.
PH3MT
SC 2A3
CIN85
HRas
IP:HRas
CIN85
HRas
WCL

Figure 5

Interaction of HRas with c-Cbl and CIN85 in a Spry2-dependent Fashion.

Because in PH3MT cells Spry2 interacts with both HRas and c-Cbl (data not shown), we hypothesized that Spry2 mediates an association between HRas and c-Cbl. By means of co-immunoprecipitations we found not only that endogenous c-Cbl was bound to HRas, but also, that this interaction was abolished in HRas-transformed cells in which Spry2 has been down-regulated (Fig. 4B).

Recent evidence indicates that in addition to c-Cbl, Spry2 interacts with CIN85 [39]. In particular, Spry2, c-Cbl and CIN85 form a tertiary complex, resulting in the inhibition of EGFR-degradation by the lysosome. With this in mind we sought to determine whether HRas also associates with CIN85 in HRas-transformed cells. We found that endogenous CIN85 co-immunoprecipitated with HRas in cells expressing Spry2, but not in cells with down-regulated Spry2 (Fig. 4C). These findings suggest that HRas interacts with c-Cbl and CIN85 in a Spry2-dependent manner and that HRas regulates the turnover of EGFR at the level of the Spry2/c-Cbl/CIN85 complex.

Effect of Spry2 Expression in Immortalized Human Fibroblasts.

To determine the role of Spry2 in EGF signaling and cancer formation in the absence of activated HRas signaling, we stably expressed Spry2 in MSU-1.1 cells (Fig. 6A). MSU-1.1 cells expressing a low level of Spry2-V5 (MSU-1.1S41) exhibited a decrease in EGF-induced ERK activation, when compared to the control cells (MSU-1.1VC) (Fig. 5B).

Figure 6. Effect of Spry2 expression in immortalized human fibroblasts. (A) MSU-1.1 cells were stably transfected with an empty vector (MSU-1.1-VC), or a vector encoding V5-tagged Spry2 (MSU-1.1S41 and S62). Whole cell lysates from the indicated cell strains were analyzed by Western blotting to determine the expression of Spry2. (B,C) The indicated cells lines were analyzed as in Fig. 3A. (D) Whole cell lysates from the indicated cell lines were analyzed as in Fig. 2D. (E) The indicated cell strains were grown in media with 0.1% serum. One of two experimental repeats (N=4) is shown.

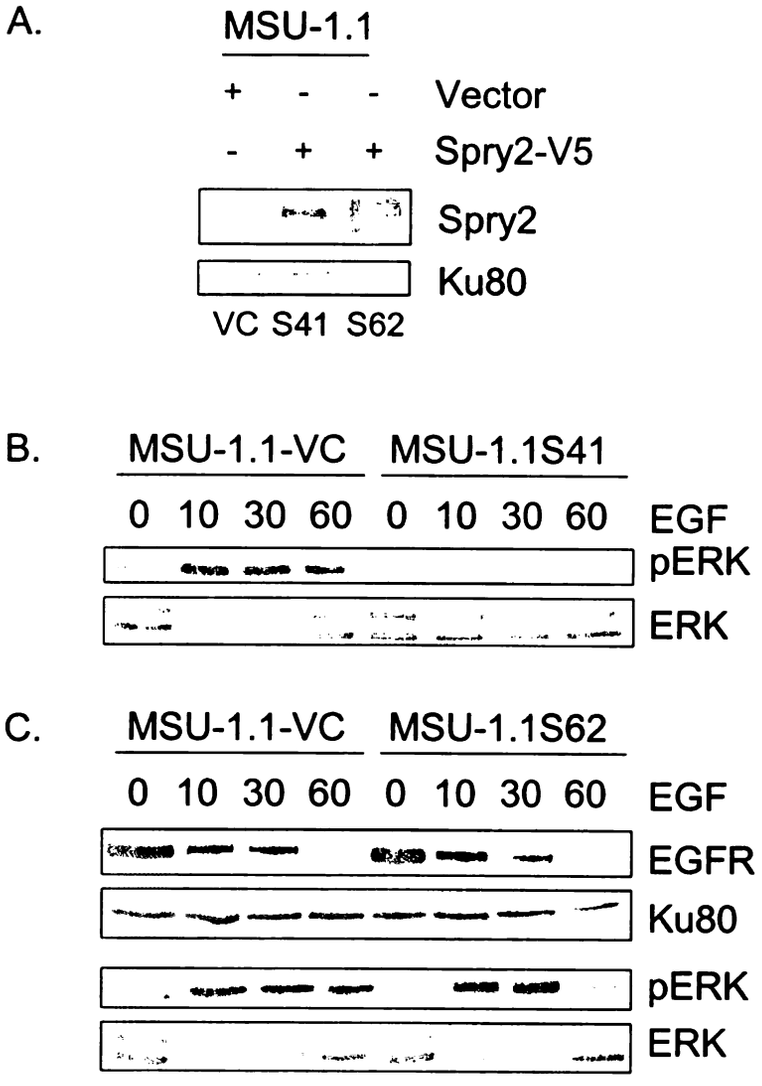
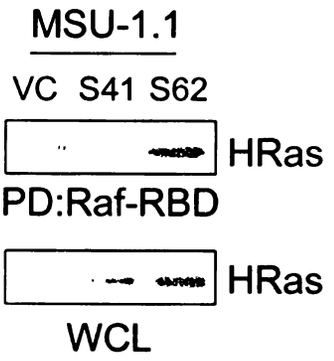

A.
MSU-1.1
+ - - Vector
- + + Spry2-V5
Spry2
Ku80
VC S41 S62
B.
MSU-1.1-VC
MSU-1.1S41
0 10 30 60 0 10 30 60 EGF
pERK
ERK
C.
MSU-1.1-VC
MSU-1.1S62
0 10 30 60 0 10 30 60 EGF
EGFR
Ku80
pERK
ERK

Figure 6

D.



E.

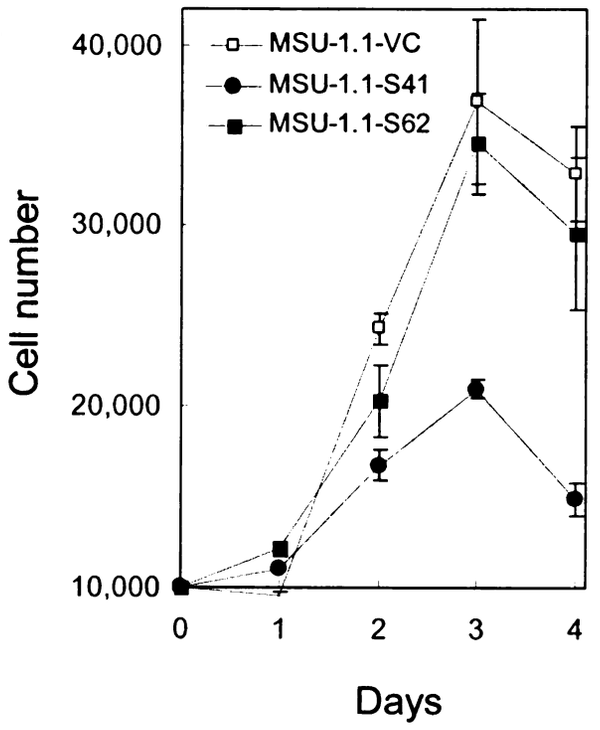


Figure 6 (Cont')

Interestingly, this inhibitory function of Spry2 was less pronounced in MSU-1.1 cells expressing higher levels of Spry2-V5 (MSU-1.1S62), which exhibited an ERK activation profile similar to that of control cells (Fig. 5C). In MSU-1.1S62 cells, Spry2 did not have an effect on the level of EGFR (Fig. 5C). The level of Spry2-V5 expression in MSU-1.1S62 cells was similar to the level of endogenous Spry2 expression in PH3MT cells (data not shown). Exogenous Spry2 inhibited HRas activation in MSU-1.1 cells, an effect that was diminished when at higher levels of Spry2 expression (Fig. 5D). Consistent with these findings, low levels of exogenous Spry2 expression resulted in a decrease in the ability of MSU-1.1 cells to grow in the absence of growth factors (Fig. 5E, MSU-1.1S41). This inhibitory effect was not observed in cells expressing higher levels of Spry2 (Fig. 5E, MSU-1.1S62). Consistent with these results, control cells and cells expressing low or high levels of exogenous Spry2 failed to form tumors upon injection in athymic mice (Table II).

Table II The tumorigenicity of the cell strains expressing Spry2

Cell strain	$HRas^{V12}$	Spry2	Tumor incidence[a]	Days for tumor to reach 0.5 cm^3 volume
MSU-1.1	-	-	0/6[b]	-
MSU-1.1VCA1	-	-	0/6[c]	-
MSU-1.1VCA6	-	-	0/6[c]	-
MSU-1.1S41	-	+	0/6[c]	-
MSU-1.1S62	-	++	0/6[c]	-
MSU-1.1L98	+	-	0/3	-
PH3MT	++	++[d]	6/6	21

[a]Ratio of tumors formed to the number of sites injected subcutaneously. If no tumors arose 6 months after the injection the mice were sacrificed.
[b]MSU-1.1 derivatives were injected at ten million cells per site. PH3MT cells were injected at one million cells per site.
[c]Data reflect a period of 3 months
[d]Represents endogenous level of Spry2

Discussion

Our study also shows that Spry2 makes distinct contributions to the regulation of the EGFR signaling pathway in pre-malignant (MSU-1.1), and in malignant human fibroblasts with active Ras signaling (PH3MT). We found that PH3MT cells have elevated levels of EGFR compared to its parental cell strain and that intact EGFR is necessary for growth factor independence and anchorage independence (data not shown) in these cells. This is consistent with studies showing that EGFR signaling is necessary for HRas-induced cell transformation [31-35].

The elevated level of EGFR in PH3MT cells is dependent on Spry2. Spry2 interacts with HRas, suggesting that Spry2 acts as an effector of HRas, at least in the context of cells overexpressing $HRas^{V12}$. Because Spry2 interacts with several proteins involved in Ras signaling, including Raf [30, 40, 41], it is possible that the interaction between Spry2 and Ras is indirect. Nevertheless, Spry2 facilitates an interaction between HRas, and Spry2-binding partners, c-Cbl and CIN85, which act at the level of EGFR to regulate its endocytosis and degradation [42, 43]. The established function of Spry2 is to inhibit EGFR endocytosis and lysosomal degradation induced by c-Cbl and CIN85 [39]. Although Spry2 was able to sustain EGFR in HRas-transformed cells, Spry2 was unable to do so when expressed at similar levels in their parental cells, suggesting that HRas regulates the ability of Spry2 to prevent EGFR degradation.

In MSU-1.1 cells Spry2 appears to inhibit ERK, whereas in PH3MT cells Spry2 sustains ERK activation. However, the inhibitory function of Spry2 in MSU-1.1 cells was diminished, when Spry2 was expressed at a similar level as the endogenous level in PH3MT cells. This suggests that inhibitory function of Spry2 is dependent on the amount of Spry2 protein within cells. In addition, while in PH3MT cells Spry2 acted downstream of HRas, in MSU-1.1 cells Spry2 acted downstream of EGFR and upstream of HRas. The latter suggests that the inhibitory function of Spry 2 in MSU1.1 cells is mediated by the ability of Spry2 to inhibit RTK signaling at the level of Grb2:SOS.

Based on these findings, we propose that in malignant cells expressing oncogenic *HRas* (PH3MT), activated Ras signaling is responsible for switching the function of Spry2 form inhibitory to activating. First, the inhibitory role of Spry2 at the level of Grb2:SOS complex is bypassed in cells with constitutively active Ras. In addition, HRas induces a high expressional level of Spry2, at which levels the inhibitory activity of Spry2 is diminished when Spry2 is expressed independently of $HRas^{V12}$. Finally, the interaction between HRas and Spry2 may be the additional regulatory element required if Spry2 is to sustain the activation of EGF signaling, and contribute to tumor formation, rather that play an inhibitory role.

Spry2 negatively regulates RTK signaling [44-46], and is down regulated in breast and prostate cancer [27, 28, 47]. Spry2 has a tumor suppressive function in the MCF-7 breast cancer cell line [48] and in a leiomyosarcoma cell line [49]. Our data show that, although Spry2 cannot by itself malignantly transform human fibroblasts, Spry2 expression is necessary for the malignant phenotype of HRas-

transformed fibroblasts. Spry2 appears to be a significant mediator of HRas-transformation, because even though the levels of active Ras were unaltered, down-regulation of Spry2 abrogated tumor formation by HRas-transformed cells.

To our knowledge, the role of Spry2 in cancer has not been examined in the context of cells with activated Ras signaling. It is noteworthy that Spry2 is up-regulated in melanoma cell lines with BRaf- and/or NRas-activating mutations [50], a finding that is consistent with our observation that HRas- and NRas-transformed fibroblasts exhibit a high level of Spry2 protein expression. Because these melanoma cell lines resemble PH3MT cells, in that they contain activated Ras signaling, it would be interesting to determine if Spry2 promotes tumor formation by these cancer cells.

Materials and methods

Cells and Cell Culture.

The derivation of the cells of the MSU lineage has been described [2, 4]. The SL68 and SL89 cell lines are normal neonatal foreskin-derived fibroblasts. SHAC, HT1080, VIP:FT and NCI cells are derived from fibrosarcoma patients [51]. The immortalized pancreatic cell ps-1 was provided by Dr. C. C. Chang (Michigan State University). Pancreatic carcinoma cell lines MIAPaCa-2, AsPc1 and CFPAC1 were obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured in Eagle's MEM, supplemented with L-aspartic acid (0.2 mmol), L-serine (0.2 mmol), pyruvate (1 mmol) and 10% supplemented calf serum (Hyclone, Logan, UT), penicillin (100 units/mL), and streptomycin (100 μg/mL, culture medium) at 37°C in a humidified incubator with 5% CO_2.

Northern Blot Analysis.

Total RNA was extracted from cells with RNA-Zol reagent from Tel-Test (Friendswood, TX) according to the manufacturer's protocol. Northern blotting was performed according to standard procedures. A Spry2-specifc probe was used for the detection of Spry2. Equal loading was determined with a probe specific for GAPDH.

Western Blot Analysis.

Whole cell lysates were prepared as described [52]. The protein content was quantified with Coomasie protein reagent from Pierce (Rockford, IL). Whole cell lysate (50 μg) was

separated by SDS-PAGE. The protein was transferred to polyvinylidene fluoride membrane (Millipore, Billerca, MA), and immunoblotting was carried out using standard techniques. The signal was detected with the Super Signal reagent (Pierce). Antibodies against EGFR, ERK, pERK, c-Cbl and HRas were purchased form Santa Cruz (Santa Cruz, CA); Spry2 from Calbiochem (San Diego, CA); Pan-Ras from Cytoskeleton (Denver, CO) and Ku80 from Serotec (Raleigh, NC). Ku80 protein expression was used as a loading control.

Preparation of Spry2-shRNA Constructs.

The down regulation of Spry2 in PH3MT cells was carried out with the pSuper.retro system by Oligoengine (Seattle, WA). The shRNA constructs were designed according to the manufacturer's protocol. Briefly, the coding region of Spry2 was analyzed with Oligoengine's RNAi design tool and two 19-nucleotide long regions centered on positions 399 and 492 of the Spry2 coding region were selected as target sites. For each of these target sites, a 65-base pair, double-stranded oligonucleotide was synthesized, which encoded the sense and antisense orientations of the Spry2 target site separated by a hairpin sequence of nine base pairs. The double stranded oligonucleotides were synthesized to contain BglII and HindIII overhangs. The sequences for the oligonucleotides targeting positions 399 and 492 on the Spry2 coding region are: 5'GATCCCCGAGACTGCTAGGATCATCCTTCAAGAGAGGAGTGATCCTAGCA GCTCTTTTTGGAAA and 5'GATCCCCGCCA CTGAGCAAGGAAGATTTCAAGAGAATCTTCCTTGCTCAGTGGCTTTTTGGAA A, respectively. In addition to the two Spry2-specific oligonucleotides, we also

synthesized a nonspecific oligonucleotide in which the sense and antisense sequences were scrambled. Following their synthesis, the double stranded oligonucleotides were ligated into the pSuper.retro vector.

Stable Infection.

The pSuper.retro vectors encoding the spry2-specifc or the nonspecific shRNA constructs were transiently transfected into the Phoenix packaging cell line by using Lipofectamine 2000 from Invitrogen (Carlsbad, California) according to the manufacturer's directions. In each case, we used five micrograms of DNA and a 1:4 DNA to Lipofectamine ratio. Forty-eight hours post-transfection the medium was collected, centrifuged at 1,500 rpm for five min and the retrovirus-containing supernatant was collected. One milliliter of retrovirus-containing medium was mixed with 4 μg /mL polybrene and was added to PH3MT cells which had been plated at a density of 200,000 cells per 60 mm dish, 24 h prior to infection. The retrovirus-containing medium was removed after 18 h and the cells were allowed to recover for 30 h. Forty-eight hours post-infection the cells were passaged into 100 mm-diameter dishes at a 1:10 dilution, and 24 h latter the infected cells were selected with puromycin (20 μg /mL). Puromycin-resistant clones were isolated and screened by Western blotting for down-regulation of Spry2. The three independent clones with down-regulated Spry2 that were used in this study have been screened multiple times, after being in culture for different time periods, ranging from days to months. The expression of Spry2 in these cells was down-regulated compared to the control cells in every case.

AG1478 Inhibitor Study.

Cells were plated at a density of 5,000 cells per 60 mm-diameter dish in medium with 0.1% serum, and allowed to grow in the presence or absence of AG1478 at a concentration of 6 μM. Cell growth was monitored by measuring the number of cells every two days. Three independent experiments were performed. Each experimental repeat included at least two replicates for each cell strain analyzed.

Immunoprecipitation Reactions.

Briefly, whole cell lysates (250-500 μg) were precleared with an appropriate IgG anbtibody for 30 min., and then incubated with an antibody specific to HRas for 2 hrs, followed by incubation with protein-G for 1hr. to overnight at 4°. In the case when a HRas agarose conjugate was used (Ras-cCbl/CIN85), the lysates were incubated with the agarose conjugate 3 hrs to overnight. The immunoprecipitated fraction was washed several times with lysis buffer and assayed by Western blotting. This experiment was repeated 3-4 times for the Ras-Spry2 and Ras-c-Cbl interactions, and two times for the Ras-CIN85 interaction.

Anchorage independence assay.

Cells were assayed for their ability to form colonies in agarose as described [52]. Briefly, 5,000 cells were plated in 60-mm-diameter dishes with 0.33% top agarose and 2% bottom agarose. The cells were overlaid with 2.5 mL of culture medium,

which was replaced weekly. After 3 weeks the cells were fixed with 2.5 % gluteraldehyde, and colonies in random fields were analyzed using the NIH Image 1.62 software. The number and the size of the each colony was determined with the Quantity One software by Bio-Rad (Hercules, CA). This experiment was repeated at least twice for each of the two control cell strains and the three cells strains with down-regulated Spry2. Each experimental repeat was performed using two to three replicates for each cell strain.

Tumorigenicity Assay.

Cells were assayed for their ability to form tumors in athymic mice as described [52]. The mice were examined weekly for tumor growth, and the tumors were removed when they reached a volume of 0.5 cm^3. In the absence of tumor formation six months after the injection, the mice were sacrificed. For the study of the MSU-1.1 derivatives, one week prior to injection of cells, the mice were implanted subcutaneously with an absorbable gelatin sponge ($1cm^3$) from Pharmacia (Kalamazoo, MI).

Ras Activation Assay.

Whole cell lysates (2 mg) were pulled down with Raf-Ras binding domain (RBD) conjugated beads from Cytoskeleton (Denver, CO) according to the manufacturer's instructions. The pulled down fractions were immunoblotted with a

HRas specific antibody to determine the level of active HRas. Three experimental repeats were performed.

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Chapter III: Sprouty-2 prevents apoptosis in HRas-transformed human fibroblasts

Running title: Spry2 prevents apoptosis

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Abstract

Sprouty-2 (Spry2) has been reported to prevent epidermal growth factor receptor (EGFR) degradation, which results in sustained signaling activity form this receptor. We have previously shown that Spry2 interacts with HRas and that Spry2 is necessary for the ability of $HRas^{V12}$-transformed fibroblasts to form tumors. Because Ras is reported to reduce the sensitivity of cells to DNA damage-induced apoptosis, we hypothesized that Spry2 plays a role in this process. In a cell strain of *HRas*-transformed human fibroblasts, which express high levels of Spry2, the ability of HRas to prevent UV-induced apoptosis is dependent on intact PI3K- and Rac1-activity. By comparing HRas-transformed cells with endogenous levels of Spry2 to HRas-transformed cells in which Spry2 has been down-regulated, we found that Spry2 enhanced the level of active PI3K and Akt. Also, Spry2 enhanced the level of active Rac1 in HRas-transformed cells, in part by modulating the interaction between HRas and Tiam1, a GDP-releasing factor for Rac1. Consistent with these findings, the down-regulation of Spry2 resulted in increased levels of UV-induced apoptosis. What is more, down-regulation of Spry2 resulted in an increase in the level of p53, paralleled by a decrease in the level of MDM2 phosphorylated at Ser166, a site that is reported to be phosphorylated by Akt. Expression of Spry2 in immortalized human fibroblasts resulted in a decrease in the level of UV-induced apoptosis, and in a decrease in the level of p53 protein. Taken together these findings suggest that Spry2 is an important mediator of survival signals induced by oncogenic *HRas*.

Introduction

The *Ras* proto-oncogenes *HRas*, *NRas* and *KRas* are important regulators of cellular functions, such as proliferation and survival [1, 2]. Ras is a small GTPase that oscillates between an active conformation, which is GTP-bound, and an inactive conformation, which is GDP-bound. Ras serves as a molecular switch to regulate a number of intracellular signaling pathways, including those mediated by Raf, PI3K and Ral [3]. Thirty percent of human tumors contain activating mutations within Ras genes, suggesting that these oncogenes play a causal role in the formation of various types of cancer [4]. These mutations give rise to a constitutively active form of Ras, leading to constitutive activation of Ras-effector pathways [5]. Although initial evidence indicated that Raf is the main effector that mediates Ras-transformation, especially in mouse fibroblasts, more recent studies have indicated the importance of Ras effectors PI3K and Ral in the transformation of human cells [6, 7].

PI3K is a lipid kinase that phosphorylates phosphoinositides on the 3' position of the inositol ring [8, 9]. PI3K consists of two subunits; a regulatory subunit, p85 and a catalytic subunit, p110. The regulatory subunit interacts with phosphorylated tyrosine residues on activated growth factor receptors[10, 11]. This association of p85 with activated growth factor receptors recruits the catalytic subunit to the plasma membrane, were it can phosphorylate phosphoinositides. The p110 catalytic subunit of PI3K interacts with GTP-bound Ras, a process that also results in the activation of PI3K [12]. Activated PI3K converts $PI(4,5)P_2$ into

$PI(3,4,5)P_3$, which activates several effector proteins, including the serine/threonine kinase Akt [13]. Akt phopshorylates a number of substrates, which regulate cellular survival pathways. Akt substrates include BAD, NFκB and Mdm2 [14-17].

Rac1, a member of the Rho family of GTPases, plays an important role in the transformation of fibroblasts by Ras [18]. The activation of Rac1 by Ras occurs either indirectly, through PI3K, or directly, through the activation of Tiam1, a GDP-releasing factor for Rac1 [19, 20]. Rac1 is mainly involved in the regulation of migration, adhesion and division [21]. However, a number of studies, also implicate Rac1 in the regulation of apoptosis [18, 22-25].

p53 functions as a homotetrameric transcription factor, and regulates cell cycle arrest, apoptosis and DNA repair [26-28]. Under physiological conditions, p53 is maintained at a low level by Mdm2, an E3 ubiquitin ligase. MDM2 ubiquitinates p53, targeting it for degradation by the proteasome [29]. The transcriptional activity of p53 is activated in response to cellular stresses that induce DNA damage. Upon DNA damage, the ubiquitination of p53 by Mdm2 is abolished, and p53 translocates to the nucleus, where it induces the transcriptional activation of genes, such as *p21* and *Bax* [30]. Active p21 arrests the cell cycle by inhibiting Cyclin/CDK complexes [31], thus allowing more time for the repair of DNA damage. If the damage is not repaired, p53 induces apoptosis via the activation of BAX, a pro-apoptotic factor that inhibits the anti-apoptotic factor Bcl-2 [32].

Sprouty Sprouty was identified as a repressor of RTK signaling in *Drosophila melanogaster (*D.m.) [33-35]. Mammalian cells express four Spry isoforms (Spry1-4) that are considerably smaller than the *Drosophila* ortholog [36-38]. Mammalian Spry proteins also exert an inhibitory function in RTK signaling. Hiwver, Spry2 also potentates RTK signaling, particularly in response to EGF stimulation [39-41]. This function is the result of the interaction of Spry2 with c-Cbl [42], an E3 ubiquitin ligase that catalyzes the ubiquitination of EGFR, targeting this receptor for proteasomal degradation [43]. In addition, Spry2 binds to CIN85, which is part of an endocytotic complex that assists in the activity of c-Cbl [44]. By binding to c-Cbl and CIN85, Spry2 prevents the interaction between c-Cbl and EGFR, and this interference blocks the degradation of the receptor. This in turn leads to sustained EGFR-induced signaling.

In a previous study (Lito et al., manuscript in preparation) we found that Spry2 interacts with HRas and is necessary for tumor formation by HRas-transformed fibroblasts. In the present study we determined the function of Spry2 in the regulation of apoptotic pathways in response to UV damage. We found that a HRas-transformed cell strain (PH3MT), which expresses high levels of Spry2, exhibited a decrease in the level of UV-induced apoptosis compared to their parental cell strain (MSU-1.1). This process was dependent on intact PI3K and Rac1 activity. Spry2 sustained both the level of active PI3K and the level of active Rac1, in HRas-transformed cells. In the same cellular context, Spry2 contributed

to a low level of p53 protein and was necessary for the ability of HRas to inhibit UV-induced apoptosis. Finally, expression of Spry2 in a cell strain of immortalized human fibroblasts resulted in a decrease in the level of p53 and a decrease in UV-induced apoptosis. These findings suggest that Spry2 has an anti apoptotic function, through the activation of cellular pathways that suppress the level of p53 protein.

Results

Effect of HRas oncogene-transformation on DNA-damage induced apoptosis

Ries et al. have demonstrated that Ras protects NIH3T3 fibroblasts from DNA-damage induced apoptosis [45]. Before we determined the role of Spry2 in this process we verified whether this effect could be reproduced in a HRas-trasnformed cell strain (PH3MT), which has a higher expression of Spry2 compared to its parental cell strain (MSU-1.1) cells (Fig. 1A). To this end, we exposed both MSU-1.1 and PH3MT cells to UV radiation and measured the percentage of cells undergoing apoptosis by staining the cells with AnnexinV-FITC and analyzing the stained cells by flow cytometry. Consistent with the findings of Reis et al., we found that oncogenic *HRas* de-sensitized immortalized human fibroblasts to early apoptotic events induced by DNA-damage (Fig. 1B). What is more, a cell strain derived from the transformation of MSU-1.1 cells with *NRas* oncogene, which expresses high levels of Spry2, was also resistant to UV-

Figure 1. Effect of HRas-transformation on UV-induced apoptosis. (A) Expression levels of Spry2 and HRas in immortalized human fibroblasts (MSU-1.1) and in their *HRas* oncogene-transformed derivatives (PH3MT). (B) The indicated cell lines were plated at a density of 200,000 cells per dish and allowed to grow overnight. The cells were stimulated with UV and allowed to grow under normal conditions for four hours. The cells were stained with Annexin V and analyzed by flow cytometry. Live cells are represented in the lower left quadrant. Early apoptotic cells are represented in the lower right quadrant. Late apoptotic and necotic cells are represented in the upper right quadrant. Dead cells are represented in the upper left quadrant. (C) HRas-transformed cells (PH3MT) were analyzed as in B, in the presence or absence of Wortmannin (50nM). (D) HRas-transformed fibroblasts (PH3MT) were stably transfected with an empty vector or a vector encoding a Myc-tagged, dominant negative form of Rac1 ($Rac1^{N17}$). Whole cell lysates from the stable clones were analyzed by Western blotting to determine the expression of dominant negative Rac1. (E) PH3MT cells expressing an empty vector (PH3MT-VC) and PH3MT cells expressing $Rac1^{N17}$ (PH3MT-RC1) were analyzed as in B.

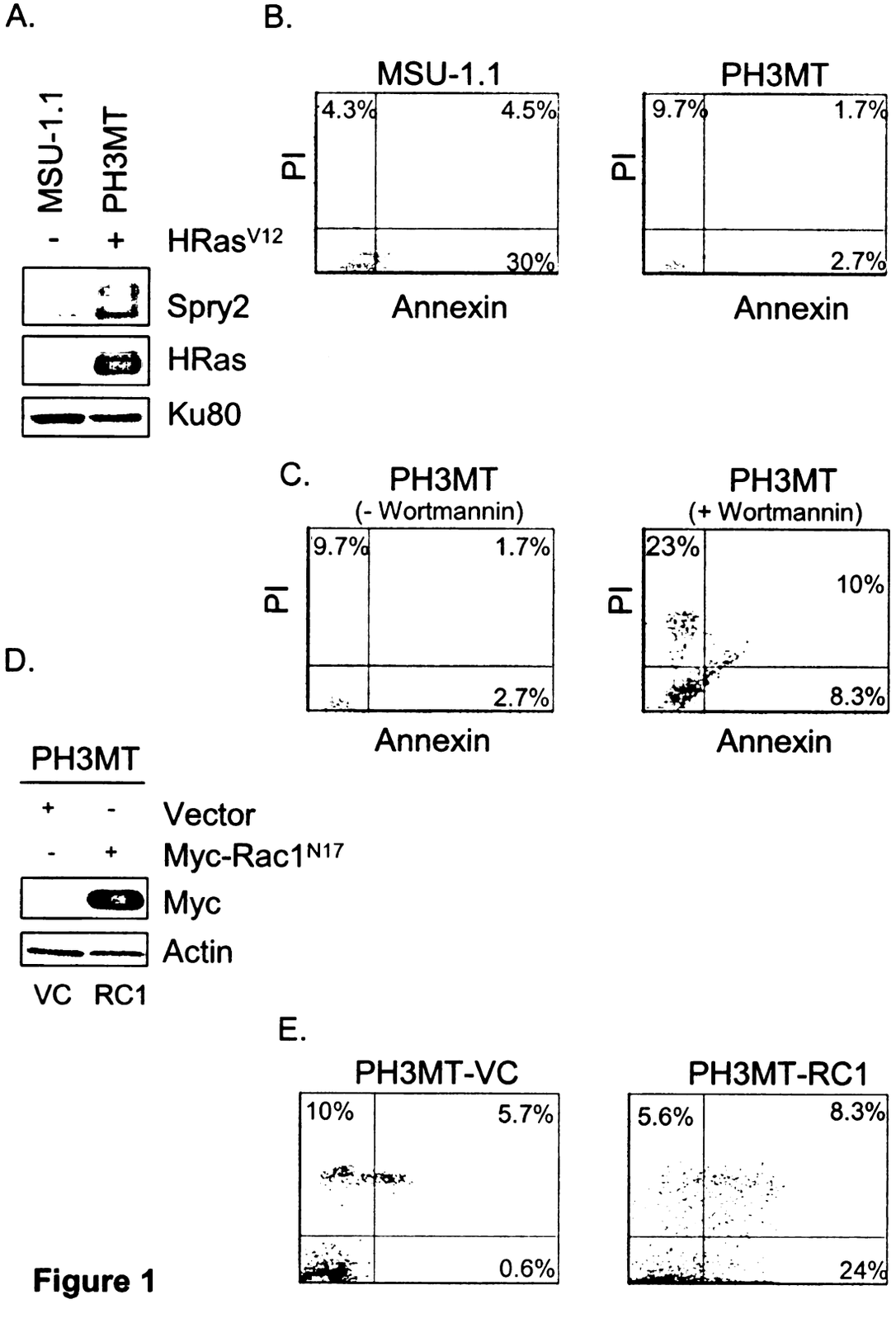

A.
MSU-1.1
PH3MT
-
+
HRas^{V12}
Spry2
HRas
Ku80
B.
MSU-1.1
PH3MT
PI
4.3%
4.5%
30%
9.7%
1.7%
2.7%
Annexin
C.
PH3MT
(- Wortmannin)
PH3MT
(+ Wortmannin)
9.7%
1.7%
2.7%
23%
10%
8.3%
D.
PH3MT
+
-
Vector
-
+
Myc-Rac1^{N17}
Myc
Actin
VC
RC1
E.
PH3MT-VC
PH3MT-RC1
10%
5.7%
0.6%
5.6%
8.3%
24%

Figure 1

induced apoptosis, suggesting that the ability to suppress this type of apoptosis process is shared among these Ras isoforms (data not shown).

Because PI3K activity is necessary for the activation of survival pathways downstream of Ras, as well as for the transformation of human cells by *Ras* oncogene [46], we determined whether PI3K activity is also necessary for the resistance of HRas-transformed cells to DNA damage induced apoptosis. To determine this, we measured UV-induced apoptosis in HRas-transformed cells in the presence or absence of Wortmannin, an inhibitor of PI3K. In the absence of Wortmannin treatment, only a small percentage of HRas-transformed cells underwent DNA-damage induced apoptosis (Fig 1C). In the presence of Wortmannin treatment, however, the percentage of cells undergoing apoptosis was increased (Fig 1C). This suggests that the ability of HRas to protect human fibroblasts from UV-induced apoptosis is at least in part dependent on PI3K activity. Treatment with AG1478, a selective inhibitor of EGFR, also increased the number of Ras-transformed cells undergoing apoptosis in response to UV radiation (data not shown), suggesting that EGFR signaling is also important for the ability of HRas to protect cells from UV-induced apoptosis.

The transformation of MSU-1.1 immortalized fibroblasts by *HRas* oncogene is dependent in part on intact Rac1 activity (Appledorn and McCormick, manuscript in preparation). Because Rac1 can also have a protective effect against UV-induced apoptosis [47], we determined if Rac1 activity was necessary for the

inhibition of UV-induced apoptosis in HRas-transformed cells (PH3MT). Two PH3MT-derrived cell lines, PH3MT-RC1, expressing a dominant negative form of Rac1 ($Rac1^{N17}$), and PH3MT-VC, expressing an empty vector (Fig. 1D), were analyzed for induction of apoptosis under the same conditions as above. The cell line expressing $Rac1^{N17}$ displayed enhanced apoptosis compared to the control cell line (Fig. 1E). This finding suggests that Rac1 is also necessary for the resistance of HRas-transformed cells to UV-induced apoptosis.

Effect of Spry2 on the activation of the PI3K pathway in HRas-transformed cells

PI3K activation involves its recruitment to phospho-tyrosine residues on the cytoplasmic tail of activated RTKs, a process that is mediated by the SH2 domain of the p85 subunit [10]. Because in HRas-transformed cells Spry2 sustains the levels of EGFR, which activates PI3K [48-52], we hypothesized that Spry2 contributes to the activation of PI3K. To test this hypothesis we analyzed a PH3MT-derived cell strain in which Spry2 has been down-regulated by the expression of a *spry2*-specific shRNA (PH3MT-2A3), and a control PH3MT cell strain expressing a scrambled shRNA (PH3MT-SC)(Fig. 2A). We determined the level of phosphorylated p85 in control cells and in cells with down regulated Spry2, because the activation of PI3K correlates with the level of p85 phosphorylation [53-55]. We found that down-regulation of Spry2 in HRas-transformed cells resulted in a decrease in the level of p85 phosphorylation (Fig.

Figure 2. Effect of Spry2 on PI3K signaling in HRas-transformed cells. (A) HRas-transformed cells (PH3MT) were stably infected with a vector encoding a scrambled shRNA (PH3MT-SC), or a vector encoding a *Spry2*-specific shRNA. Whole cell lysates from the stable clones generated were analyzed by Western blotting to determine the expression of Spry2. (B) Control cells (PH3MT-SC) and cells with down-regulated Spry2 (PH3MT-2A3) were analyzed by Western blotting with the indicated antibodies. (C) Non tumorigenic, immortalized human fibroblasts (MSU-1.1) were stably transfected with an empty vector (MSU-1.1-VC) or a vector encoding V5-tagged Spry2 (MSU-1.1-S62). Whole cell lysates were analyzed by Western blotting to determine the expression level of Spry2.

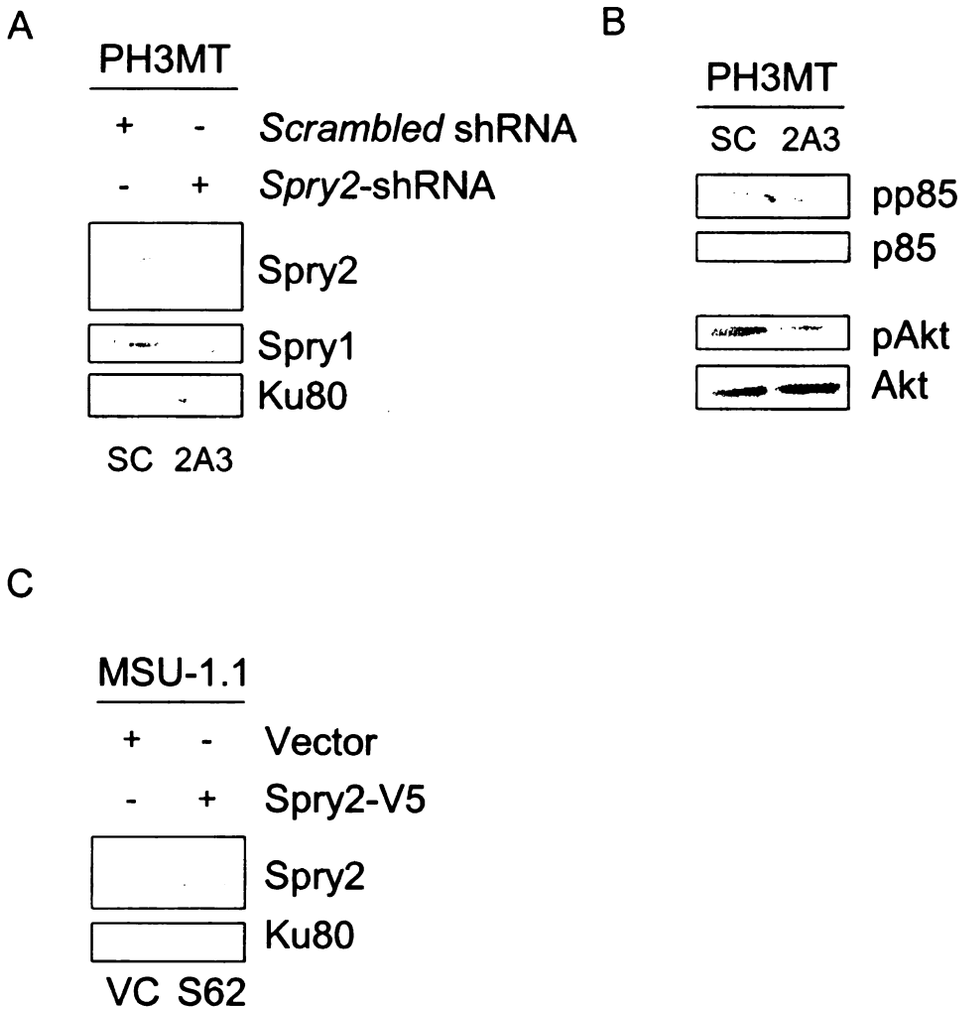

A
PH3MT
+ - Scrambled shRNA
- + Spry2-shRNA
Spry2
Spry1
Ku80
SC 2A3
B
PH3MT
SC 2A3
pp85
p85
pAkt
Akt
C
MSU-1.1
+ - Vector
- + Spry2-V5
Spry2
Ku80
VC S62

Figure 2

2B), a finding that is consistent with the ability of Spry2 to sustain EGFR signaling.

Because Spry2 sustains the activation of PI3K we examined the effect of Spry2 down-regulation in the activation of Akt, a downstream effector of PI3K. We found that cells expressing Spry2 had higher levels of phosphorylated Akt compared to the cells with down-regulated Spry2 (Fig. 2B), suggesting that Spry2 sustains the activation of the PI3K/Akt pathway in HRas-transformed cells.

Effect of Spry2 on the activation of Rac1 in HRas-transformed cells

To determine if Spry2 plays a role in the activation of Rac1, we carried out a Rac1 activation assay to determine the level of activated Rac1 in control HRas-transformed cells (PH3MT-SC) and in HRas-transformed cells with down regulated Spry2 (PH3MT-2A3). The HRas-transformed cell strain with endogenous levels of Spry2, contained a significantly higher level of active Rac1, compared to cell strain with down-regulated Spry2 expression (Fig. 3A).

One function of Rac1 is to inhibit stress fiber formation [21]. To further validate the hypothesis that Spry2 contributes to Rac1 activation in HRas-transformed cells, we determined the level of stress fibers in control cells (PH3MT-SC) and in cells with down-regulated Spry2 (PH3MT-2A3). These cells with decreased Spry2 expression contained more pronounced stress fibers compared to cells with

Figure 3. Effect of Spry2 on the activation Rac1 in HRas-transformed cells. (A) Whole cell lysates from control cells (PH3MT-SC) and cells with down-regulated Spry2 (PH3MT-2A3) were pulled down with PAK-CRIB (Cdc42- and Rac1-interacting domain) conjugated beads. The amount of Rac1 that was bound to the beads, as well as the amount of Rac1 present in whole cell lysates (WCL) is shown. (B) The indicated cell stains were stained with Alexa-fluor 488-conjugated Phalloidin stain, to detect actin-stress fiber formation. (C) A MSU-1.1 cell strain expressing exogenous Spry2 (MSU-1.1-S62), and a MSU-1.1 cell strain expressing an empty vector (MSU-1.1-VC) were analyzed as in A. (D) Whole cell lysates from the indicated cell strains were immunoprecipitated with an antibody specific to HRas, and then immunobloted with the inbdicated antibodies.

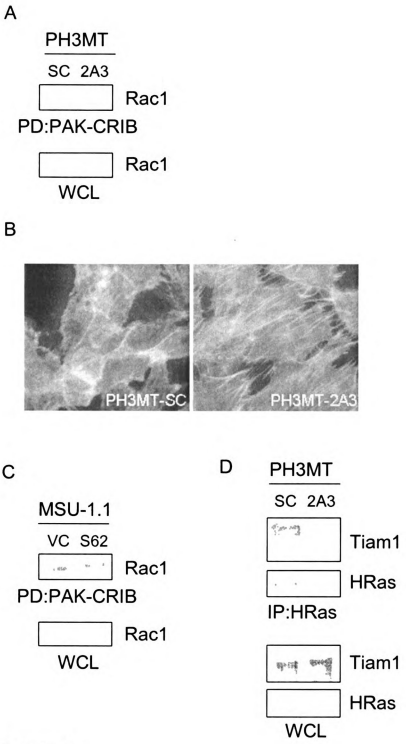

A
PH3MT
SC 2A3
Rac1
PD:PAK-CRIB
Rac1
WCL
B
PH3MT-SC
PH3MT-2A3
C
MSU-1.1
VC S62
Rac1
PD:PAK-CRIB
Rac1
WCL
D
PH3MT
SC 2A3
Tiam1
HRas
IP:HRas
Tiam1
HRas
WCL

Figure 3

endogenous levels of Spry2, consistent with the lower level of Rac1 activation in these cells (Fig. 3B).

To determine if Spry2 has an effect on Rac1 activation independently of Ras, we generated a stable cell strain of immortalized human fibroblasts expressing V5-tagged Spry2 (MSU-1.1-S62) (Fig. 2C). MSU-1.1 cells are the same precursor cells used to generate the HRas-transformed cells in study. Spry2 expression in these cells did not have a significant effect on Rac1 activation (Fig. 3C), suggesting that Spry2 cannot activate Rac1 independently of HRas-transformation.

To determine the role of Spry2 in the activation of Rac1 by HRas, we focused at the level of Ras-Tiam1 interaction. Tiam1 is a guanine nucleotide releasing factor for Rac1, which has been shown to interact with Ras, a process that leads to Rac1 activation [19]. To determine the role of Spry2 in this interaction we carried out co-immunoprecipitation experiments between HRas and Tiam1, in control cells (PH3MT-SC) and in cells with down-regulated Spry2 (PH3MT-2A3). The amount of endogenous Tiam1 that co-immunoprecipitated with HRas was reduced in the in the cells with down-regulated Spry2 expression, compared to control cells (Fig. 3D).

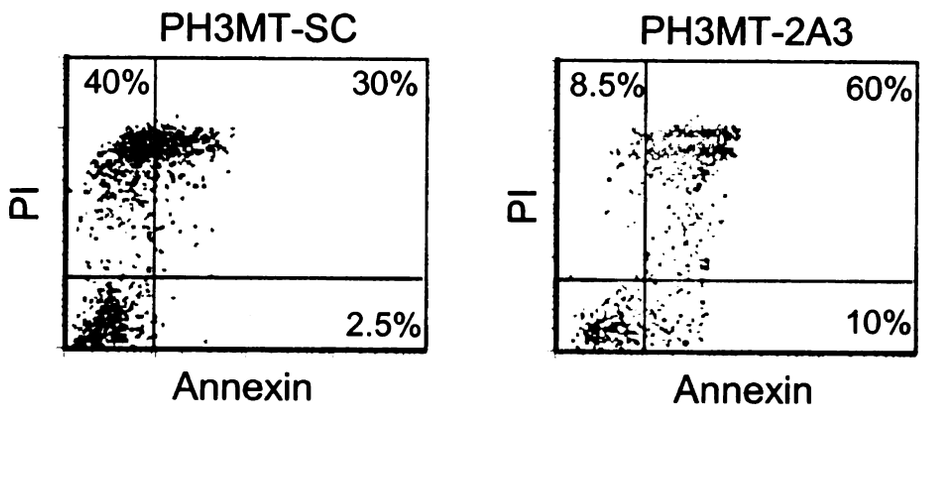
Effect of Spry2 on the induction of apoptosis in response to DNA-damage

Because we found that Spry2 contributes to the activation of PI3K and Rac1, both of which are responsible for the ability of HRas to inhibit apoptosis in response to DNA damage in PH3MT cells, we hypothesized that Spry2 itself is required for such activity. To test this hypothesis, we compared HRas-transformed cells in which Spry2 has been down-regulated (PH3MT-2A3) with control H-Ras-transformed cells (PH3MT-SC). The cells with down regulated Spry2 exhibited an increase in the percentage of cells undergoing apoptosis in response to UV damage, suggesting that Spry2 is necessary for the ability of $HRas^{V12}$ to protect the cells from UV-induced apoptosis (Fig. 4A). It should be noted that the down-regulation of Spry2 had no effect on the induction of apoptosis when the cells were cultured at normal conditions (data not shown).

To determine whether Spry2 plays a role in the regulation of apoptosis independently of HRas-transformation, we also examined the level of apoptosis induced by UV-stimulation in MSU-1.1-derived cell strains expressing either exogenous Spry2-V5 (MSU-1.1-S62) or an empty vector (MSU-1.1-VC). Consistent with the role of Spry2 in HRas-transformed cells, expression of Spry2 in immortalized human fibroblasts (MSU-1.1-S62) diminished the ability of these cells to undergo apoptosis in response to UV-induced DNA damage (Fig. 4B). This is the same type of response induced by expression of *HRas* oncogene in the same cell line (see Fig. 1B).

Figure 4. Effect of Spr2 on UV-induced apoptosis. Control HRas-transformed cells (PH3MT-SC), as well as HRas-transformed cells with down regulated Spry2 (PH3MT-2A3) were analyzed as in Fig. 1B. (B) Control immortalized fibroblasts (MSU-1.1-VC) and immortalized fibroblasts expressing Spry2-V5 (MSU-1.1-S62) were analyzed as in Fig 1B.

A


PH3MT-SC
40%
30%
2.5%
PI
Annexin
PH3MT-2A3
8.5%
60%
10%
PI
Annexin

B

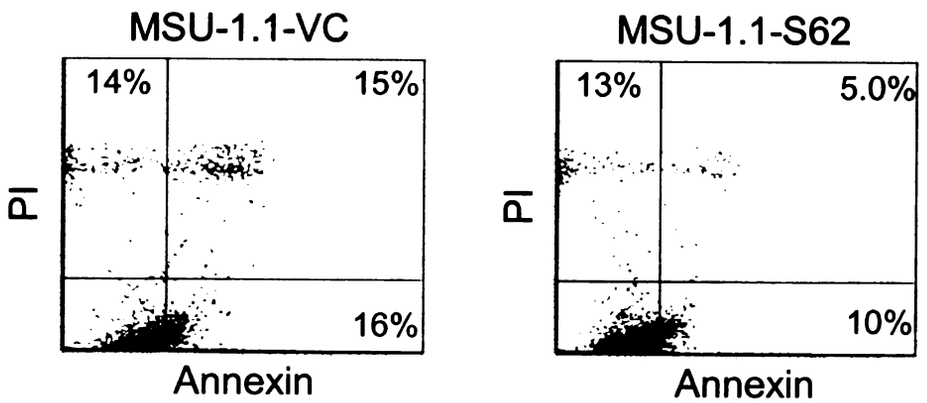

MSU-1.1-VC
14%
15%
16%
PI
Annexin
MSU-1.1-S62
13%
5.0%
10%
PI
Annexin

Figure 4

Effect of Spry2 on the MDM2/p53 pathway

The MDM2/p53 pathway plays a critical role in UV-induced apoptosis [56]. Akt phosphorylates MDM2 on serine residue 166, resulting in the stabilization of MDM2 [57]. This enhances the ubiquitination of p53 by MDM2, which is followed by degradation of the p53. Because Spry2 prevents UV-induced apoptosis, while sustaining the activation of Akt in HRas-transformed fibroblasts, it is likely that Spry2 promotes the Akt-mediated stabilization of Mdm2 and subsequent decrease in the level of p53 protein. To test this hypothesis, we determined the amount of MDM2 phosphorylated at Ser166, in the cell strain with down regulated Spry2 (PH3MT-2A3) and in the contol cell strain (PH3MT-SC). We found that the cell strain with down-regulated Spry2 exhibited a decrease in the amount of MDM2 that is phosphorylated at Ser166 (Fig.5A), which correlates with the decreased in the level of activated Akt in these cells. We also found that the levels of p53 were increased in the cell strain with down-regulated Spry2 (Fig. 5A).

We next determined the stability of MDM2 and p53 in control cells and in cells with decreased expression of Spry2. The cells were radiated with UV and assayed to determine the protein expression levels of MDM2 and p53. We found that HRas-trasnformed cells with down-regualted Spry2 (PH3MT-2A3) exhibited a decrease in the level of MDM2 following UV treatment, when compared to control

Figure 5. Effect of Spry2 on the MDM2/p53 pathway. (A) Control HRas-transformed cells (PH3MT-SC) and HRas-transformed cells with down-regulated Spry2 (PH3MT-2A3) were analyzed by Western blotting with the indicated antibodies. (B) The same cell strains were treated with UV and allowed to grow under normal conditions for the indicated time periods following UV treatment. Whole cell lyses prepared following this treatment were analyzed by Western blotting. (C) Immortalized fibroblasts expressing either an empty vector (MSU-1.1-VC) or Spry2-V5 (MSU-1.1-S62) were analyzed to determine the level of MDM2 and p53. (D) HRas-transformed cells with down-regulated Spry2 (PH3MT-2A3) were stably transfected with a vector encoding GFP-Rac1^{V12} (2A3-R1) or with a vector encoding GFP alone (2A3-VC). Whole cell lysates form these cells were analyzed by Western blotting with the indicated antibodies.

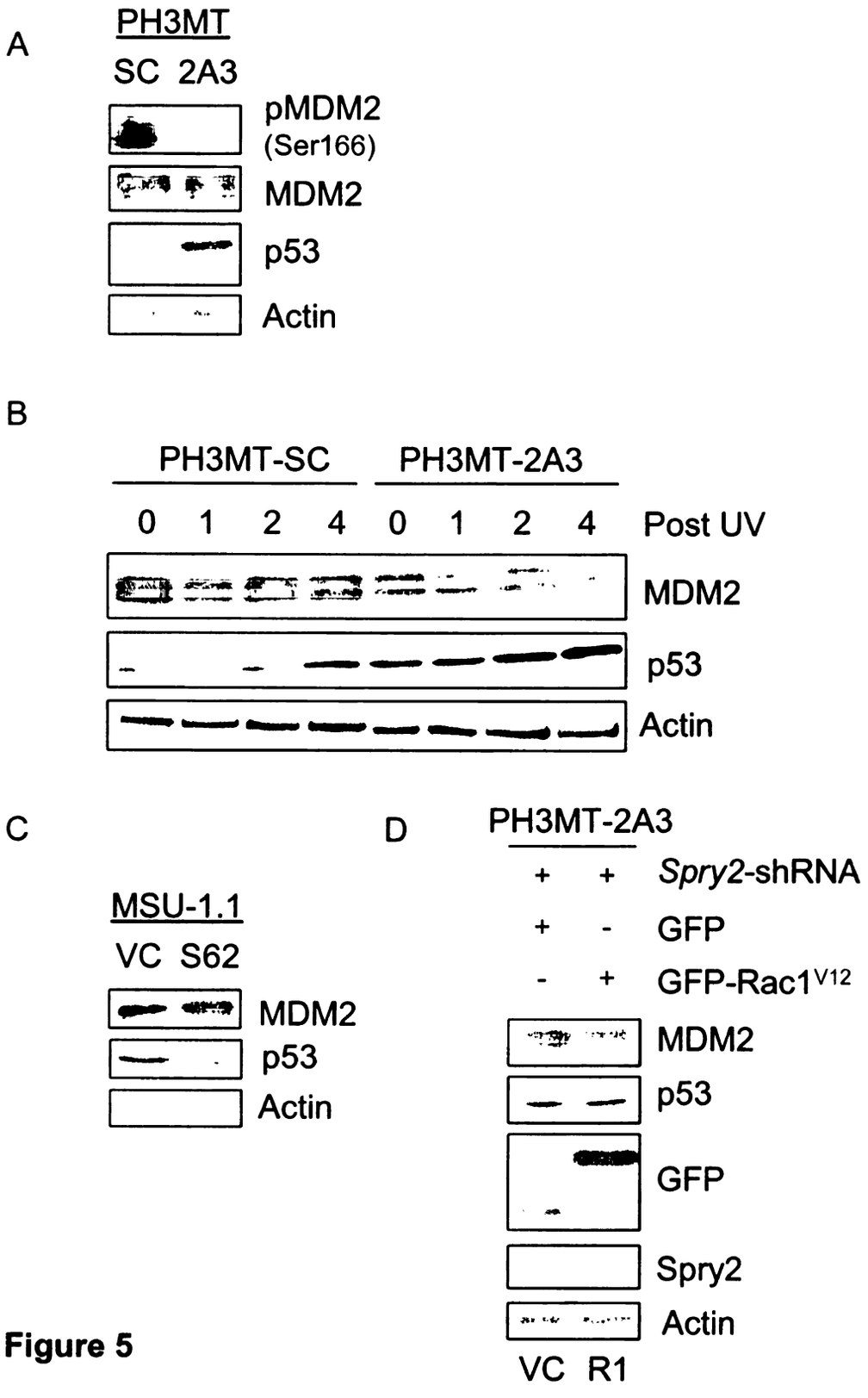

A
PH3MT
SC 2A3
pMDM2
(Ser166)
MDM2
p53
Actin
B
PH3MT-SC
PH3MT-2A3
0 1 2 4 0 1 2 4
Post UV
MDM2
p53
Actin
C
MSU-1.1
VC S62
MDM2
p53
Actin
D
PH3MT-2A3
+ + Spry2-shRNA
+ - GFP
- + GFP-Rac1V12
MDM2
p53
GFP
Spry2
Actin
VC R1

Figure 5

HRas transformed cells (PH3MT-SC) (Fig. 5B). This finding is in agreement with the decrease in the activation of Akt, and the decrease in the phosphorylation that stabilizes MDM2, in the cell strain with down-regualted Spry2. The level of p53 was higher in the cell strain with down-regulated Spry2, compared to the control cell strain, even though both cell strains exhibited an increase in the amount of p53 in response to UV stimulation (Fig. 5B). These findings are consistent with the ability of Spry2 to inhibit UV-induced apoptosis in HRas-transformed cells.

Because we found that Spry2 prevented UV-induced apoptosis upon expression in immortalized human fibroblasts (MSU-1.1) (see Fig. 4B), we determined if expression of Spry2 in these cells had an effect on the level of p53. Consistent with the function of Spry2 in HRas-transformed cells, the MSU-1.1 cell strain expressing Spry2 (MSU-1.1-S62) exhibited a decrease in the level of p53, when compared to the control cell line (MSU-1.1-VC) (Fig. 5C).

We also determined whether Rac1 plays a role in the ability of Spry2 to regulate the MDM2/p53 pathway in HRas-transformed cells. Since the down regulation of Spry2 in HRas-transformed cells resulted in a decrease in the level of active Rac1, we stably expressed GFP-tagged, constitutively active Rac1 ($Rac1^{V12}$) in the cell strain with down-regulated Spry2 (PH3MT-2A3). When compared to a cell strain expressing GFP alone (2A3-VC), the cell stain expressing GFP-$Rac1^{V12}$ (2A3-R1) did not exhibit a difference in the level of MDM2 or p53, both under normal culture conditions (Fig. 5D), as well as following UV-treatment of the cells

(data not shown). This suggests that the effect of Spry2 on the MDM2/p53 pathway is independent of Rac1.

Discussion

In the present study we report that Spry2 contributes to the ability of HRas-transformed cells to evade apoptosis in response to DNA damaging agents, such as UV. The ability of Ras to inhibit apoptosis and promote cellular survival is well documented [58]. This process is mediated not only by Ras-effector pathways including Raf, PI3K and Rac1 signaling [13, 25, 45, 59], but also by the MDM2/p53 pathway. Consistent with these findings, the ability of a HRas-transformed cell strain (PH3MT) to resist DNA-damage induced apoptosis was dependent on intact PI3K and Rac1. In this cell strain Spry2 contributes not only to the activation of PI3K/Akt- and Rac1- pathways, but also, Spry2 sustains the stabilization of MDM2 resulting in a decrease in the level of p53. This suggests that Spry2 functions through these pathways to regulate DNA damage induced apoptosis in HRas-transformed cells.

Some Ras effector pathways converge at the regulation of MDM2/p53. Activation of the Raf/MAPK pathway results in the transcriptional activation of MDM2 [45]. Activation of the PI3K/Akt pathway, results in a decrease in the interaction between Arf and MDM2, thereby stabilizing MDM2 [60] and enhancing the ubiquitination of p53 by MDM2 [17, 57]. Because Spry2 interacts with HRas (Lito et al. manuscript in preparation) it is possible that Spry2 is also a mediator of the

ability of HRas to regulate MDM2 and p53. The ability of Spry2 to regulate MDM2/p53 independently of HRas-transformation, adds more significance to the effect of Spry2 in the regulation of apoptotic pathways. Our data suggest that the effect of Spry2 on MDM2/p53 is mediated by the PI3K/Akt pathway and not by the Rac1 pathway. This is consistent with a report showing that the activation of Rac1 promotes cellular survival through the activation of NFκB [25].

Spry2 sustains the levels and signaling activity of EGFR [36]. This function of Spry2 is observed in HRas-transformed cells as well (Lito et al., manuscript in preparation). RTK activation results not only in the recruitment of the p85 subunit of PI3K, but also in its phosphorylation. Because Spry2 sustained the level of phosphoryaltion of p85 it is possible that Spry2 acts through EGFR to sustain the activation of PI3K. Activation of EGFR reduces UV-induced apoptosis, in a PI3K/Akt dependent pathway [52, 60], a finding that is consistent with the function of Spry2 in HRas-transformed cells.

Spry2 has been found to inhibit Rac1 during wound healing, a property that is necessary for the inhibition of cellular migration by Spry2 [61]. We found that Spry2 sustains the activation of Rac1 in HRas-transformed cells, when these cells are at log-phase growth under normal conditions. A decrease in Spry2 expression enhances stress fiber formation, a process that is consistent with loss of Rac1 activity. In addition Rac1 contributed to VEGF and uPA secretion in PH3MT cells (Appledorn et al Manuscript in preparation) as well as in other HRas-transformed

cells [62-64]. Down-regulation of Spry2 in PH3MT cells resulted in a decrease in the amount of VEGF and uPA secreted by these cells (data not shown), a finding that supports our conclusion that Spry2 contributes to Rac1 activation in HRas-transformed cells. Because we found that Spry2 does not have a significant effect on the activation of Rac1 independently of HRas-transformation, it is likely that Spry2 only mediates the activation of Rac1 by HRas, a possibility that is in agreement with the contribution of Spry2 to Ras-Tiam1 interaction, a step that is critical for the direct activation of Rac1 by Ras. Alternatively, Spry2 may contribute to the activation of Rac1 indirectly, because Spry2 sustains PI3K activation, and Rac1 can be activated by PI3K, a process that is also mediated by Tiam1 [20].

The treatment of cancers by radiation or chemotherapy relies strongly on the induction of apoptosis. Inactivation of pro-apoptotic pathways in cancer cells, as observed during the transformation of cells by HRas, compromises the efficacy of such treatments. Spry2, best known for its negative regulation of RTK signaling, sustains the activation of Ras effector pathways that mediate cellular survival. Through this contribution, Spry2 provides a protective signal during UV-induced apoptosis, and plays a role in the resistance of immortalized and HRas-transformed fibroblasts to this biological process. In light of our findings, targeting the expression and/or function of Spry2 in cancer cells may enhance their responsiveness to chemotherapeutic treatments.

Material and Methods

Cells and Cell Culture.

The derivation of the human fibroblast cell line MSU-1.1 has been described [65, 66]. The PH3MT cell strain was derived from tumors formed in athymic mice by the injection of MSU-1.1 cells malignantly transformed by an overexpressed *HRas* oncogene [65]. The cells were cultured in Eagle's MEM, supplemented with L-aspartic acid (0.2 mmol), L-serine (0.2 mmol), pyruvate (1 mmol) and 10% supplemented calf serum (Hyclone, Logan, UT), penicillin (100 units/mL), and streptomycin (100 μg/mL, culture medium) at 37°C in a humidified incubator with 5% CO_2.

Apoptosis assay

Apoptosis was detected by staining the cells with AnnexinV-FITC (BD Biosciences, San Jose, CA) according to manufacturer's recommendations. Briefly, cells were plated at a density of 200,000 cells per 60 mm-dish. Sixteen hours later, the cells were irradiated with UV at a dose of 30-60 J/m^2 and incubated at 37^o under normal growing conditions for varying time periods. Subsequently, the cells were collected, washed twice with AnnexinV binding buffer and incubated with AnnexinV-FITC at room temperature for 15 min. The cells were also stained with propidium iodide (PI) to distinguish between live and dead cells. AnnexinV-FITC positive cells were determined by flow cytometry under standard conditions. Only the cells that stained positive for AnnexinV, and

not those that were positive for both annexinV and PI, were considered apoptotic. All experiments were repeated at least three times.

Western blotting

Whole cell lysates were prepared as described [67]. The protein content was quantified with Coomasie protein reagent from Pierce (Rockford, IL). Whole cell lysate (50 μg) was separated by SDS-PAGE. The protein was transferred to polyvinylidene fluoride membrane (Millipore, Billerca, MA), and immunoblotting was carried out using standard techniques. The signal was detected with the Super Signal reagent (Pierce, Rockford, IL). Antibodies against pp85, p85, c-Cbl, Mdm2, p53 and H-Ras were purchased form Santa Cruz (Santa Cruz, CA); Spry2 from Calbiochem (San Diego, CA); pAkt and Akt from Cell Signaling (Danver, MA) and Ku80 from Serotec (Raleigh, NC). Ku80 protein expression was used as a loading control.

Rac1 activation

Whole cell lysates (2 mg) were pulled down with PAK-Cdc42/Rac1 interacting region (CRIB)-conjugated beads from Cytoskeleton (Denver, CO) according to the manufacturer's instructions. The pulled down fractions were immunoblotted with a Rac1 specific antibody to determine the level of active H-Ras. this experiemtn was repreated three times in HRas-transforemd cells and twice in immortalized fibroblasts.

Staining for stress fibers

Stress fibers were detected with Alexa-fluor 488 conjugated Phalloidin stain from Invitrogen (Carlsbad, CA) according to the manufacturer's instructions. Briefly, cells at log phase growth were fixed in 10% normal buffered formalin solution, for 10 min., they were washed twice in phosphate buffered saline (PBS), and then they were treated with 0.1 Triton X-100 in PBS for 3-5 min. at room temperature. Subsequently, the cells were incubated with blocking solution (1% BSA in PBS) for 20 min. at room temperature. Finally, the cells were incubated with Phalloidin stain for 30 min. and analyzed by fluorescent microscopy.

Immunoprecipitation Reactions.

Briefly, whole cell lysates (250-500 μg) were precleared with an appropriate IgG anbtibody for 30 min., and then incubated with an antibody specific to HRas for 2 hrs, followed by incubation with protein-G for 1hr. to overnight at 4^{o}. In the case when a HRas agarose conjugate was used, the lysates were incubated with the agarose conjugate 3 hrs to overnight. The immunoprecipitated fraction was washed several times with lysis buffer and assayed by Western blotting. This experiment was repeated three times.

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Appendix A: Analysis of expressional changes between MSU-1.0, MSU-1.1 and PH3MT cells

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Introduction

Carcinogenesis is now recognized as a multistep process during which cells acquire neoplastic characteristics through a series of genetic and/or epigenetic changes. Initially, such changes confer to a normal cell some advantage, leading to its clonal expansion and the emergence of a new population of progeny cells with a more malignant status. By means of the reiteration of this pattern, a single cell can emerge that has acquired all of the changes required to form a cancer.

In order to study the process of carcinogenesis in fibroblastic malignancies, McCormick and colleagues established the human fibroblast MSU-1 cell lineage as a model system [1-3]. This system is comprised of cell strains with either normal, intermediate pre-malignant, or malignant phenotypes. Given that all of these cell strains originate from the same normal parental fibroblastic cell line, this model system provides a fertile ground for the investigation of the genetic changes that convert normal cells into cancer cells.

To develop this system, normal human skin fibroblasts (LG1) were transfected with the *v-Myc* oncogene. A clonal population of cells expressing *v-Myc* was propagated in culture, and after several months the cells became senescent. Nonetheless, a small number of cells developed an endogenous mechanism to evade senescence, and continued to divide, giving rise to a new cell strain named MSU-1.0. MSU-1.0, just like its parental strain, expresses the *v-Myc* oncogene, is diploid, and is karyotypically stable [2]. In addition, the MSU-1.0 cells

endogenously expresses the telomerase gene, expression of which has been reported to immortalize cells [4].

As the MSU-1.0 cells were grown continuously in culture to determine whether they had infinite life spans, a variant clonal derivative arose spontaneously. This cell strain, named MSU-1.1, is chromosomally stable, nearly diploid and also expresses the *v-Myc* oncogene, which was integrated in the same chromosomal site as it was in the parental MSU-1.0 cells. However, MSU-1.1 cells contain two marker chromosomes that distinguish them from their progenitors. These marker chromosomes were formed from a fusion between the q arm of chromosome 1 and chromosome 11 (Marker 1), and a fusion between the p arm of chromosome 15 and the q arm of chromosome 12 (Marker 2) [2].

The MSU-1.1 cell strain has a more rapid growth rate compared to its precursor. Also, it is partially growth factor independent, and can grow better in media containing reduced Ca^{2+} concentrations in comparison to the MSU 1.0 cell strain [5].

The growth characteristics of MSU-1.1 cells suggested that they might be able to be transformed into malignant cells. Indeed, when MSU-1.1 cells were transfected with the *HRas* oncogene, they were able to form tumors in athymic mice with a latency of only few weeks [3]. The cells derived from these tumors (PH3MT) had the same chromosomal structure as MSU-1.1 cells, including the

two marker chromosomes. In addition, MSU-1.1 cells have been malignantly transformed by ionizing radiation [6, 7] or by chemical carcinogen treatment [8], to form tumors in athymic mice. Interestingly, MSU-1.0 cells have never been malignantly transformed into tumor cells, regardless of many attempts, similar to those used to transform MSU-1.1 cells.

In this study, we used the MSU-1 lineage of human fibroblasts as a model system to identify novel genes that are involved in fibrosarcomas. To accomplish this goal, we relied on the Affymetrix Gene Chip technology, and performed a comparative analysis of the expression profiles of MSU-1.0, MSU-1.1 and PH3MT cells.

When we focused our attention on the significant gene changes, we found that 62 genes were up regulated, while 45 genes were down regulated in MSU-1.1 cells, compared to MSU-1.0 cells. These genes retained their type of change in the tumorigenic PH3MT cells. The changes for several of these genes were validated using Northern blotting. From the pool of genes with elevated expression in MSU-1.1 and PH3MT cells (i.e genes that could act as oncogenes), we selected *Sprouty-2* (*Spry2*) for further analysis. *Fibulin-5* (*Fib5*), which belonged to the group of genes with down regulated expression in MSU-1.1 and PH3MT cells (i.e. genes that could acts as tumor suppressors), was also selected for further studies. The products of these two genes will be analyzed to determine if they act as oncogenes or as tumor suppressor genes.

Results

Comparison of the RNA expression profiles of MSU 1.0, MSU 1.1 and PH3MT cells

The expression profiles of MSU-1.1 and PH3MT cells were each compared to that of MSU-1.0 cells. The fold-changes reported for each gene represent those comparisons. Although in this study we placed an emphasis on the difference between MSU-1.0 and MSU-1.1 cells, both the MSU-1.1 vs. MSU-1.0 and the PH3MT vs. MSU-1.0 comparisons were used to determine important genes. The reason for this choice was our assumption that, if the change in expression for a particular gene in MSU-1.1 cells, is retained, at a similar or a more pronounced level, in PH3MT cells, then that gene would be more likely to play a causal role in cancer formation. The comparison between MSU-1.1 and MSU-1.0 cells yielded 108 genes with a 3-fold or more change between these two cell lines. Interestingly, all but one gene retained at least a 2-fold change when PH3MT cells were compared to MSU-1.0 cells. These genes exhibited fold-changes at the 95% confidence interval. Of the differentially expressed genes, 62 were up regulated, and 45 were down regulated in MSU-1.1 and PH3MT cells, with respect to MSU-1.0 cells. To determine which of these genes exhibit similar

Figure 1. Gene chip comparison of MSU1.1 and PH3MT cells to MSU-1.0 cells. Hierarchical clustering of the statistically significant gene changes (107 genes). Genes with an elevated expression levels in MSU-1.1 and PH3MT cells, compared to MSU-1.1 cells are shown in (A), whereas genes with a decreased expression level in MSU-1.1 and PH3MT cells compared to MSU-1.1 are shown in (B). Abbreviations: 1.0: MSU-1.0, 1.1: MSU-1.1, 3MT: PH3MT. To obtain the full name of the gene from the abbreviation or the accession numbers used here refer to the table that follows and/or consult Affymetrix.com.

A.

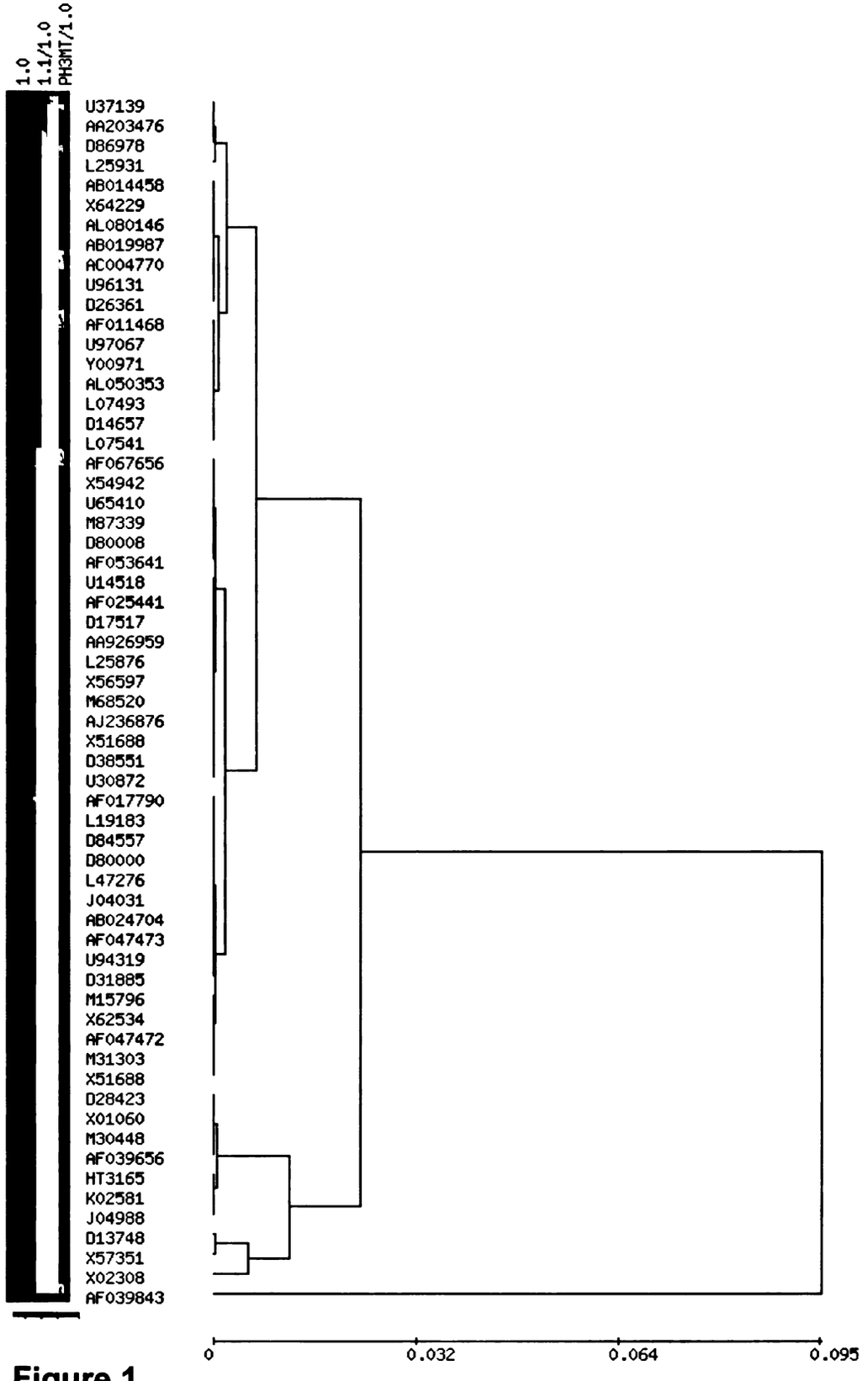
1.0
1.1/1.0
PH3MT/1.0
U37139
AA203476
D86978
L25931
AB014458
X64229
AL080146
AB019987
AC004770
U96131
D26361
AF011468
U97067
Y00971
AL050353
L07493
D14657
L07541
AF067656
X54942
U65410
M87339
D80008
AF053641
U14518
AF025441
D17517
AA926959
L25876
X56597
M68520
AJ236876
X51688
D38551
U30872
AF017790
L19183
D84557
D80000
L47276
J04031
AB024704
AF047473
U94319
D31885
M15796
X62534
AF047472
M31303
X51688
D28423
X01060
M30448
AF039656
HT3165
K02581
J04988
D13748
X57351
X02308
AF039843
0
0.032
0.064
0.095

Figure 1

B.

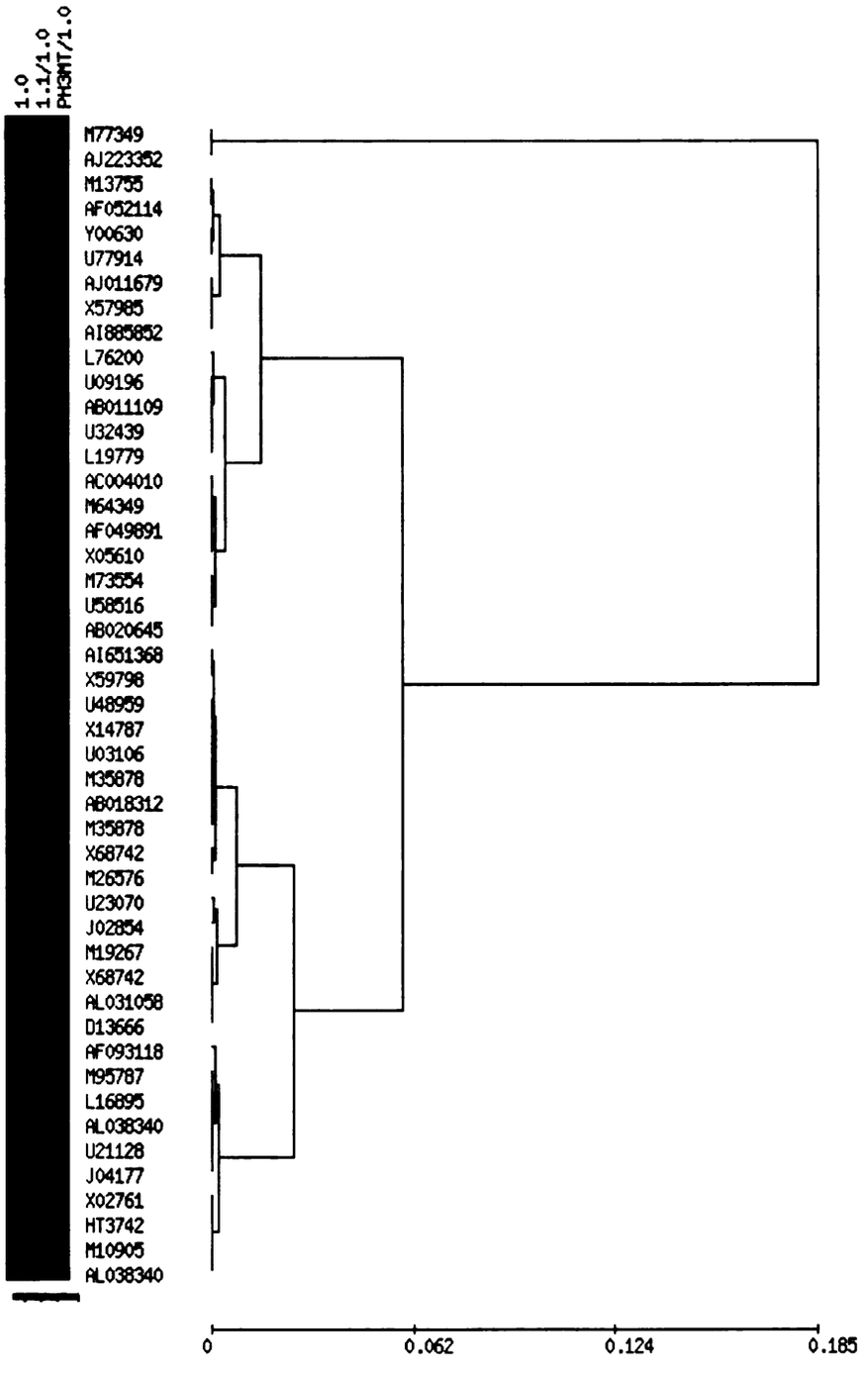

Figure 1 (Cont')

patterns of change, we used the EPCLUSTER software[1] to perform hierarchical, as well as K- means clustering of these genes. (Fig. 1 and 2).

Differentially expressed genes were grouped together according to their functional characteristics (Table I). This was done in order to obtain a global view of the genotypic changes that take place as cell stains in the MSU-1 lineage became malignantly transformed. For a broader picture, some genes with marginal (less than 3-fold) changes between MSU 1.1 and MSU-1.0 cells were also included. The examination of these groups revealed some noteworthy traits. First, all of the genes that encode transcription factors or chromatin remodeling enzymes were up regulated in MSU-1.1 and PH3MT cells. Second, the type of change for the majority of the genes involved in cell cycle control supports a faster G1-S transition, and therefore a faster growth rate in the MSU-1.1 and PH3MT cell strains. This is consistent with prior studies that report the MSU-1.0 cell strain having a slower growth rate compared to the cell strains derived from it. Third, the majority of the genes encoding extracellular matrix (ECM) proteins, or proteins involved in cell adhesion and motility, were found to be down regulated in MSU-1.1 and PH3MT cells. Finally, genes involved in calcium signaling, or genes with calcium-binding domains, were down regulated in MSU-1.1 and PH3MT cells.

[1] This is available for use at the European Bioinformatics Institute's web site: http://ep.ebi.ac.uk/EP/

Figure 2. K-means clustering of the differentially expressed genes. The y-axis represents the logarithmic fold-change in gene expression from the MSU-1.1 vs. MSU-1.0 comparison (first point of discontinuity), and the PH3MT vs. MSU-1.0 comparison (second point of discontinuity). Each line in the graphs represents a gene. Red indicates an increase, while green indicates a decrease in gene expression compared to MSU-1.0 cells. Each graph shows genes with similar patterns of change.

3.00
2.00
1.00
-1.00
-2.00
-3.00
CONTENTS:
ISG15
size=1
CONTENTS:
COL11A1
EST
EST
size=3
CONTENTS:
ZWINT
size=1
CONTENTS:
TSP1
CYCLIN D1
p21
MYLK
RAB6
JAG1
SERBINB2
GUK1
size=21
CONTENTS:
EIF4A1
AXL
CDK2
CYCLIN A2
FBL
BUB3
TFRC
TK
size=19
CONTENTS:
IGFBP3
H2AFO
H2AFQ
COL4A2
size=11
CONTENTS:
CDKN3
CYCLIN B2
USP1
STK6
CATENIN
HMGB2
DEK
INTB3BP
MCM6
CENPA
size=19
CONTENTS:
INTEGRIN A1
FN1
LUMICAN
FIBULIN 5
MYL9
LOX
size=10
CONTENTS:
MAD2L1
PCNA
TYRO3
LBR
CSE1
RFC3
RPA3
TOP2A
size=23

Figure 2

Other investigators have shown that some of the genes reported here are important for cancer formation. Genes like *p21* and *tsp1*[2], whose protein levels were indeed down regulated in MSU-1.1 and PH3MT cells (data not shown), are known tumor suppressor genes. Similarly, *axl* and *tyro* are both members of a novel family of receptor tyrosine kinases (RTK) that can transform fibroblasts when expressed [9, 10]. The genes for *tfr*, and *sema3C* have also been reported to be up regulated in different types of cancer, while *igfbp3* has been reported to be decreased in cancer cells [11]. To some extent, these findings validate the integrity of our gene chip data, and the effectiveness of the MSU-1 lineage of cells as a model system to study the formation of fibroblastic tumors.

Northern blot analysis and confirmation of the gene chip data

We began the validation of the gene chip data by focusing on genes that were functionally related to the phenotypic characteristics of the MSU-1.1 cells, which differentiate them from MSU-1.0 cells. Characteristics such as the faster growth rate of MSU-1.1 cells, as well as their ability to grow in media with reduced calcium, prompted us into assigning a high priority for analysis to genes involved in growth factor signaling pathways (e.g. *spry2, axl, tk1, igfbp3* etc.) and/or exhibit their functions in a calcium dependent manner (e.g. *s.jag1, fib5* etc.). Based on the fact that MSU 1.1 and PH3MT cells contain two marker chromosomes, we also wanted to focus on genes that are located in the chromosomal regions that

[2] Abbreviations: *tsp1: thrombospondin-1, tfr: transferrin receptor, sema3C: semaphorin-3C, igfbp3: insulin like growth factor-3, spry2: sprouty-2, tk1: thymidine kinase, s.jag1: soluble jagged-1, fib5: fibulin-5, lox: lysyl oxidase.*

TABLE I. Grouping of differentially expressed genes according to their function

Gene name	Acc#	Fold change 1.1/1.0	Fold change 3MT/1.0	Description*
Apoptosis				
Bcl1	M73554	-5.3	-6.5	pro apoptotic
CDK inhibitor 1A (p21, Cip1)	U03106	-3.3	-5.1	regulates CDK:cyclin association
Serine proteinase inhibitor B2	Y00630	-3.2	-2.3	
Immediate early response 3	S81914	-3.2	-1.3	involved in TNF induced apoptosis
Caspase 8	X98172	2.3	3.1	protease
ADP-ribosyltransferase-like 2	AJ236876	3.1	3.3	protein ADP-ribosylation; DNA repair
catenin alpha-like 1	U97067	3.6	4.7	
Chromosome segregation 1-like	AF053641	3.7	4.1	involved in TNF induced apoptosis
Gene expression and chromatin control				
high-mobility group box 2	X62534	5.2	4.4	transcriptional activator
β3 endonexin	U37139	4.0	5.9	coactivator for nuclear receptors
DEK oncogene	X64229	3.9	4.6	changes DNA topology
v-myb -like 1	X66087	3.3	1.4	transcriptional activator
pituitary tumor-transforming 1	AA203476	3.0	4.6	inibits p53 transcriptional activity
TRIP13	U96131	2.9	3.2	thyroid hormone receptorsignaling
high mobility group AT-hook 1 (HMGA1)	L17131	2.7	5.0	enhanceosome assembly
Cell Cycle control				
PRAD	M73554	-5.3	-6.5	G1/S transition,
CDK inhibitor 1A (p21, Cip1)	U03106	-3.3	-5.1	G1/S transition,
CDK 2	M68520	2.9	2.7	G2/M transition
BUB3	AF047472	3.0	2.7	mitotic checkpoint
cyclin A2	X51688	3.1	2.8	G1/S and G2/M progression
Mitotic arrest deficient-like 1	U65410	3.3	3.5	mitotic checkpoint
CDC28 protein kinase 2	X54942	3.3	3.6	regulation of CDK activity
cyclin B2	AL080146	3.7	4.3	mitosis
PCNA	M15796	4.3	3.4	DNA binding
CDK inhibitor 3	L25876	4.6	4.5	G1/S transition, cell cycle arrest
MCM6	D84557	4.3	3.7	DNA replication liscencing factor
Transmembrane proteins				
Lamin B receptor	L25931	3.1	4.5	lamin and chromatin binding
AXL receptor tyrosine kinase	M76125	3.2	2.3	RTK
TYRO3 protein tyrosine kinase	D17517	3.4	3.6	RTK
Transferrin receptor (p90, CD71)	X01060	3.6	2.7	binds transferrin; iron transport
Protein Degradation				
Cathepsin K (pycnodysostosis)	X82153	3.0		cysteine-type peptidase
Ubiquitin specific protease 1	AB014458	4.9	5.7	cysteine-type protease

TABLE I. Grouping of differentially expressed genes according to their function (Cont')

Gene name	Acc#	Fold change 1.1/1.0	Fold change 3MT/1.0	Description*
Extracellular Matrix and Cytoskeleton				
Collagen, type IV, alpha 1	M26576	-8.4	-29.4	binds integrins
Insulin-like growth factor binding protein 3	M35878	-7.4	-14.7	binds IGF
Collagen, type XI, alpha 1	J04177	-6.2	-107.6	binds integrins
Collagen, type IV, alpha 2	X05610	-6.1	-9.0	binds integrins
Thrombospondin 1	X14787	-4.2	-7.7	interacts with ECM; integrin ligand
Fibronectin 1	X02761	-3.8	-22.9	binds integrins, collagen
Integrin, alpha 1	X68742	-3.6	-7.1	collagen receptor
TGF β-induced, 68kDa	M77349	-3.6	-1.6	ligand for integrin receptor
Lysyl oxidase	L16895	-3.4	-30.5	collagen crosslinking enzyme
Serine proteinase inhibitor B2	Y00630	-3.2	-2.3	inhibits plasminogen activators
Lumican	U21128	-3.2	-32.0	interacts with collagen
Fibulin 5	AF093118	-3.0	-38.1	interacts with integrins
Semaphorin 3C	AB000220	2.6		ligand for plexin receptor
Integrin β 3 binding protein	U37139	4.0	5.9	
Ca^{2+} Signaling and Ca^{2+} Binding Proteins				
Jagged 1, soluble	U77914	-5.7	-3.4	
Thrombospondin 1	X14787	-4.2	-7.7	
Myosin light polypeptide kinase	U48959	-3.5	-6.1	binds calcium-calmodulin
Myosin, light polypeptide 9, regulatory	J02854	-3.6	-13.9	
Fibulin 5	AF093118	-3.0	-38.1	
Signal Transduction				
Insulin-like growth factor binding protein 3	M35878	-7.4	-14.7	comlexes with IGF1 and IGF2
TGF β-induced, 68kDa	M77349	-3.6	-1.6	
Myosin light polypeptide kinase	U48959	-3.5	-6.1	phosphorylates myosin light chain
CDK inhibitor 1A (p21, Cip1)	U03106	-3.3	-5.1	
Fibulin 5	AF093118	-3.0	-38.1	
Guanylate kinase 1	L76200	-2.8	-2.5	phosphorylates GMP
Sprouty 2	AF039843	2.0	5.9	interacts with Cbl2, Grb2
MAPKAP3	U09578	2.6	5.4	activatesCREB
Thymidine kinase , soluble	K02581	2.8	2.2	phosphorylates thymidine
CDK2	M68520	2.9	2.7	binds cyclins
Casein kinase 2β	M30448	3.1	2.5	phosphorylates p53; binds AP2
AXL receptor tyrosine kinase	M76125	3.2	2.3	RTK; binds GAS6
TYRO3 protein tyrosine kinase	D17517	3.4	3.6	RTK; binds PROS1
Transferrin receptor (p90, CD71)	X01060	3.6	2.7	binds transferrin; iron transport
CSE1 chromosome segregation 1-like	AF053641	3.7	4.1	importin alpha export receptor
Serine/threonine kinase 6	AF011468	4.5	6.4	
CDK inhibitor 3	L25876	4.6	4.5	

Abbreviations: Acc#: accession number, 1.0: MSU-1.0, 1.1: MSU1.1, 3MT: PH3MT
(*) Although some of these genes may have more that one function,
only the function which relates to the group that they are part of is indicated here

were involved in the formation of the marker chromosomes (e.g. *lox, tsp1* etc.). Finally, genes with high fold-changes in expression (e.g. *hmg2, zwint*, etc.) were considered to be important as well.

One will notice that *sprouty-2 (spry2)*, a gene that we assigned with a high priority for analysis, exhibits only a two-fold change between MSU-1.1 and MSU-1.0 cells. The reason for this assignment was the fact that the gene chip data was initially analyzed with older software (MAS4.0, from Affymetrix). According to that analysis, *spry2* exhibited a 3-fold change between MSU-1.1 and MSU-1.0 cells. Recently, a new software (MAS5.0) has been developed that employs a different analytical algorithm. When this software was used to reanalyze the data, we found that the change in expression of *spry2* between MSU-1.1 and MSU-1.0 cells was only 2-fold. The change in PH3MT cells was high in both analyses. Regardless of this discrepancy, we still pursued the study of Spry2.

In subsequent analyses, which were performed to validate the gene chip data for our genes of interest, we have also included several other malignant cell lines. Of these, some have been generated from the transformation of MSU-1.1 cells either by Ras-oncogene expression (PH2MT), by chemical carcinogen treatment (6A/SB1) or by gamma irradiation (MW7.3 and MW16); or they are human patient-derived fibrosarcoma cell lines (SHAC, HT1080, VIP:FT, NCI and 8387).

To date we have been able to confirm the gene chip data for *spry2, lysyl oxidase (lox), fibulin-5 (fib5)* and *soluble jagged-1* (s.*jag1)* (Fig. 3). MSU-1.1 cells show only a modest increase in the level of *spry2*. However, Ras-transformed fibroblasts, as well as patient-derived fibrosarcoma cells, express pronounced levels of this transcript. In an independent study, dominant negative Cdc42 and Rac1 were expressed into PH3MTcausing a significant decrease in the tumorigenicity of those cells. The *spry2* level in those cells was down regulated (Dao et al., unpublished results). This offers an additional clue that Spry2 might have a role in the transformation of cells in the MSU-1 lineage. Since SHAC and HT1080 fibroblasts both carry Ras-activating mutations, we also examined the expression of *spry2* in VIP:FT and 8387 fibroblasts, which do not carry such mutations. The *spry2* level in those cells was as elevated as that in SHAC and HT1080 cells (data not shown).

The *fib5* level in MSU-1.1 and PH3MT cells was down regulated, when compared to MSU-1.0 cells, consistent with the gene chip data. Fibrosarcoma-derived cells, regardless of whether they carried Ras-activating mutations, had an undetectable level of this transcript (Fig. 3 and data not shown). Carcinogen transformed cells, however, showed elevated levels of this gene, which suggests that *fib5* might have a transformation-type specific role.

The analysis of *lox* expression showed a very similar profile to that of *fib5*, consistent with the gene chip data and the fact that these two genes clustered

Figure 3. Northern blot analysis that validates the gene chip data for *spry2, lox, fib5* and *s.jag1*. *GAPDH* was used as a loading control.

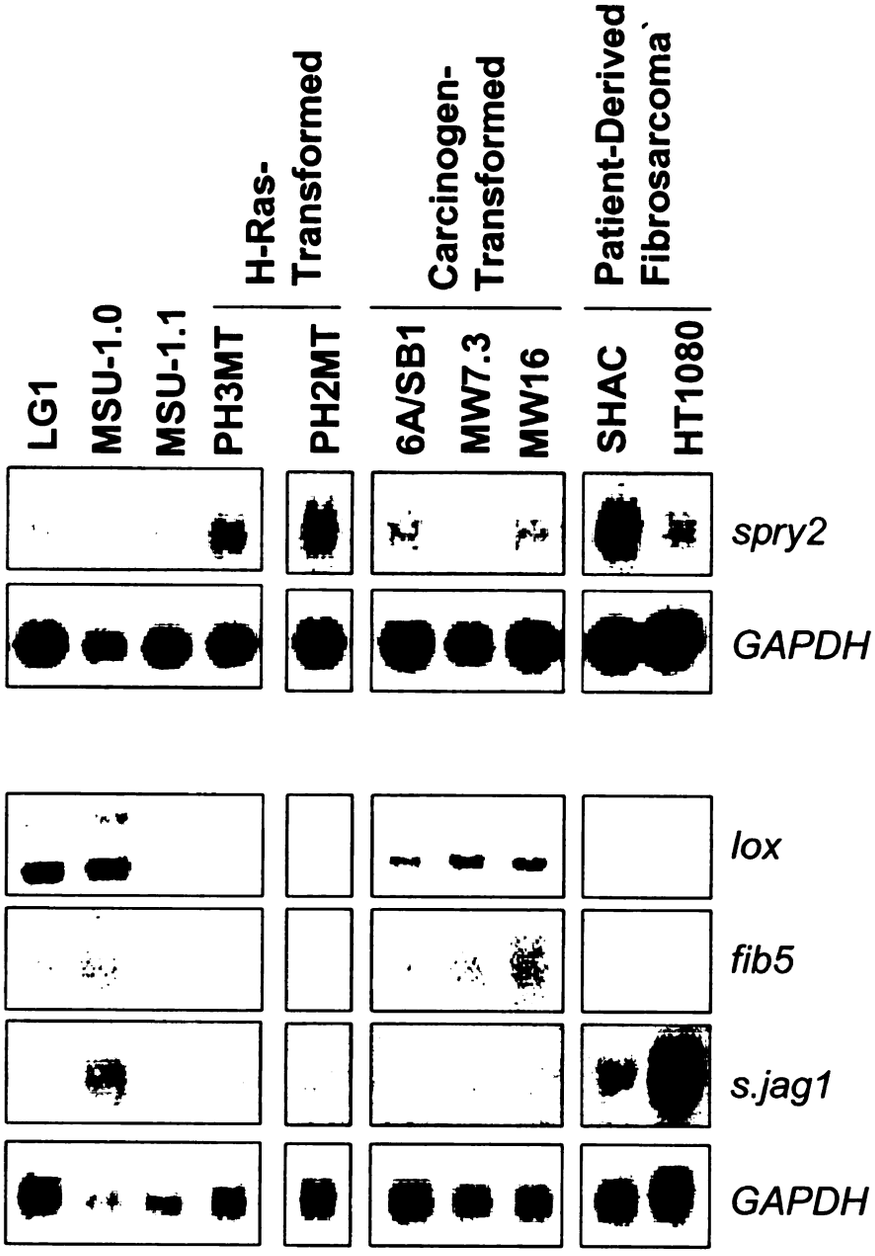
LG1
MSU-1.0
MSU-1.1
PH3MT
H-Ras-Transformed
PH2MT
Carcinogen-Transformed
6A/SB1
MW7.3
MW16
Patient-Derived Fibrosarcoma
SHAC
HT1080
spry2
GAPDH
lox
fib5
s.jag1
GAPDH

Figure 3

together functionally, as well as statistically. Finally, the down regulation of *s.jag1* in MSU-1.1 and PH3MT cells was also confirmed. However, the level of *s.jag1* in SHAC and HT1080 fibroblasts was elevated compared to the malignant cells derived from MSU-1.1 cells (Fig. 3).

Discussion

In this study we used a gene chip analysis to compare the expression profiles of MSU-1.1 and PH3MT cells, to that of MSU-1.0 cells. The analysis of the gene chip data revealed that 62 genes were significantly up regulated and 45 genes were significantly down regulated, in MSU-1.1 and PH3MT cells.

Based on the phenotypic differences between the cell lines examined, we selected several genes for further analysis by Northern blotting. Additional cells lines, derived from different types of tumors, were used to determine if these genes' expression was altered during malignant transformation in general. This showed that *spry2* was up regulated in MSU-1.1 cells, in Ras- and carcinogen-transformed MSU-1.1-derivatives, as well as in human patient-derived fibrosarcoma cells, when these cells were compared to the normal MSU-1.0 cells. *fib5*, *lox* and *s.jag1* were down regulated in MSU-1.1 and PH3MT cells. Although, *fib5* and *lox* transcripts were decreased in patient-derived fibrosarcoma cells, they were present in a substantial amount in carcinogen-transformed MSU-1.1 cells. In contrast, while maintaining a reduced expression in carcinogen-transformed cells, *s.jag1* was up regulated in patient-derived fibrosarcoma cells.

As mentioned earlier, cancer usually arises from genetic changes that affect gene expression or gene function. The gene chip analysis used in this study, cannot assess the mutational status of genes in the cells under study. It can only determine the expression level of a particular transcript. However, while genes with oncogenic or tumor suppressive potential, may not show a change in expression between normal and neoplastic phenotypes, they may have mutations that alter their functions. With this in mind, we are aware of the possibility that some of the genes excluded during the gene chip data analysis, might be important in cancer. Nevertheless, we believe that the genes, which we found to exhibit significant changes in expression, between the cells in our model system, are strong candidates for a role in cancer. Indeed, a number of them have already been reported to play a role in this disease.

In relation to these genes, we found that the majority of genes encoding for extracellular matrix proteins were significantly down regulated in MSU-1.1 and PH3MT cells, when these cells were compared to MSU-1.0 cells. In particular, these genes encoded ligands of integrin receptors. Integrins have been shown to regulate many cellular functions such as adhesion, motility, proliferation and differentiation. Interestingly, the adhesiveness of MSU-1.0 cells to tissue culture dishes is significantly higher that that of MSU-1.1 and PH3MT cells. Additionally, MSU-1.1 and particularly PH3MT cells, display a more rounded morphology compared to their parental strain. These phenotypic changes could be a response to the decreased expression of ECM ligands (e.g. fibronectin, collagen etc.) in

MSU-1.1 and PH3MT cells. These ligands serve as anchors of cells to their stratum, as well as activators of many intracellular signaling molecules that are responsible for the determination of cell shape and attachment (e.g. FAK[3], Cdc42, Rac1 and others) [12, 13].

Another observation regarding the gene chip data was that a number of genes encode proteins involved in growth control, a finding that is consistent with the differences in growth that have been observed between MSU-1.0, MSU-1.1 and PH3MT cells.

To conclude this chapter we ought to say that some of the genetic differences represented here are very likely to contribute to the phonotypical differences of MSU-1.0, MSU-1.1 and PH3MT cells. Such genetic changes may also be responsible for the ability of MSU-1.1 cells to be malignantly transformed by HRas oncogene expression, whereas their precursors, MSU-1.0 cells, are unable to be malignantly transformed by the same oncogene.

[3] Abbreviation: FAK: focal adhesion kinase

Materials and Methods

Total RNA extraction

The total RNA extraction was performed with the RNA Bee reagent according to the manufacturer's instructions. The RNA, at all times, was extracted from cells growing at a logarithmic rate (approximately 60% confluent).

Gene Chip Analysis

The RNA isolated from MSU-1.0, MSU-1.1 and PH3MT cells was subjected to a reverse transcription reaction to produce double stranded cDNA. The first strand synthesis was performed with the Superscript II RTase (Invitrogen), and an oligo-dT primer, which also contained the core promoter region recognized by the T7 RNA polymerase (RNAP). The second strand synthesis was performed, as recommended by Affymetrix, by using DNA polymerase I, RNaseH, and DNA ligase. The double-stranded cDNA (containing the T7 RNAP promoter), was then used as substrate in an *in vitro* transcription reaction, with T7 RNAP and biotin-labeled nucleotides. The product of this reaction (biotin-labeled cRNA) was fragmented into smaller fragments, which were subsequently used to hybridize a U95 Human GeneChip®, purchased from Affymetix. The hybridized chip was then washed, stained and scanned to determine the amount of transcripts present in the cells under study.

The U95 GeneChip® contains 12625 probe sets, each of which recognizes a specific transcript. A probe set is comprised of 32 probe cells, 16 that are called perfect match cells (PM), and 16 that are called mismatch (MM) cells. Each of the probe cells contains 25-mer oligonucleotides that are complementary to different regions of the target. While PM cells contain oligonucleotides that perfectly complement with the transcript, the MM cells contain a mutation on the 13^{th} nucleotide of the 25-mer oligonucleotide.

Data analysis

We first determined which transcripts were present in the cells examined. To this end we analyzed each cell line independently, by using the detection algorithm of Microarray Suite 5.0 (MAS 5.0). The presence of the transcript was determined from the p-value and the detection call, which is a user-defined parameter that was set at 0.04. Transcripts with detection p-values greater than 0.04, were considered to be absent from the cells in study.

The next step in the data analysis was the determination of the signal intensity (i.e. the relative abundance of a transcript). This was done by taking the sum of the logarithm ($\log_2$) of the difference between PM and MM intensities.

Two independent gene chip experiments were performed for MSU-1.0 cells, while MSU-1.1 and PH3MT cells were each examined three times. For each cell line, each experimental repeat was individually analyzed as described.

In order to determine the changes in gene expression between two cell lines, we relied on the change algorithm of MAS 5.0. Each MSU-1.1 experimental repeat was compared to each of the MSU-1.0 experimental repeats. This produced six comparisons between MSU-1.1 and MSU-1.0 cells (1.1 vs. 1.0). In each comparison, the MSU-1.0 array was set to be the baseline. The signal log-ratio (i.e. the change in gene expression) was determined by taking the $\log_2$ of the ratio of the signal intensity of a gene in the experimental array (which in this case represents MSU-1.1 repeats) to the signal intensity of that gene in the baseline array (MSU-1.0 repeats). The statistical significance of the change, was calculated form the change p-value and the change call, a user defined parameter that was set to 0.0025. In this study, we focused only on genes that were increased or decreased within this statistical margin in all comparisons. To determine the fold-change in expression for a particular transcript between MSU-1.1 and MSU-1.0 cells, we averaged the signal $\log_2$ ratios from the six comparisons made between these two cells. The same method was followed to determine the fold-change in the expression of a gene, between PH3MT and MSU-1.0 cells (3MT vs. 1.0). Understandably, the logarithmic fold-change in expression for a gene in MSU-1.0 cells was zero. In this study, only the gene changes that were equal to, or greater than 3-fold in the 1.1 vs. 1.0 comparison, and only the gene changes that were equal to, or greater than 2-fold in the 3MT vs. 1.0 comparison, were considered to be significant

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Appendix B: The role of Sprouty-2 in the malignant phenotype of patient derived fibrosarcoma cell lines

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Keywords: Sprouty; *H-Ras* oncogene; malignant transformation; epidermal growth factor receptor

Introduction

As described in the previous chapters Spry2 is up regulated in HRas-transformed cells and is necessary for the ability of these cells to form tumors in athymic mice. In addition, we found that HRas interacts with Spry2 and c-Cbl suggesting a role for HRas in the regulation of EGFR degradation by c-Cbl.

The expression of Spry proteins is deregulated in a number of human tumors. In particular, Spry1 and Spry2 are down regulated in breast cancer and prostate cancer. In the context of breast cancer, Spry2 functions to suppress tumor formation, consistent with the inhibitory function of this protein in RTK signaling. Interestingly, Spry2 is up regulated in melanomas containing $NRas^{Q59}$ or $BRaf^{V599}$ mutations. The role that Spry2 plays in the malignancy of these tumors remains unanswered.

In addition to being up regulated in cell strains derived by the transformation of immortalized human fibroblasts by the Ras oncogenes, (i.e. $HRas^{V12}$ and $NRas^{V12}$), the expression of Spry2 is also increased in several fibrosarcoma cells including, HT1080 which expresses oncogenic $NRas^{Q59}$, and VIP:FT, which expresses wild type *Ras*.

To determine the role of Spry2 in the malignant phenotype these cells, we generated stable cell strains with down regulated Spry2 expression and analyzed their ability to form tumors in athymic mice.

Results

The role of Spry2 in the malignant phenotype of patient derived fibrosarcoma cell lines

We have previously found that Spry2 is necessary for the ability of HRas ^{V12}-transformed fibroblasts to form tumors in athymic mice suggesting that Spry2 promotes cancer formation. To determine if Spry2 has a broader role in cancer formation we determined the role of Spry2 in HT1080 and VIP:FT cells, two patient-derived fibrosarcoma cell lines expressing oncogenic NRasQ59, or wild type Ras isoforms, respectively. We first determined if the presence of oncogenic NRas in HT1080 cells corresponded to higher levels of active Ras in these cells compared to normal fibroblasts. To this end HT1080 and VIP:FT cells were analyzed with a Ras activation assay. Consistent with the status of *Ras* mutations, the HT1080 cell line contained higher levels of active Ras, compared to both VIP:FT cells and normal fibroblasts (Fig. 1A).

To determine the role of Spry2 in the malignant phenotype of these cells, we down regulated the expression of Spry2 in these cells by stably expressing a vector encoding a Spry2-specific shRNA as described in Chapter II. A vector encoding a scrambled shRNA molecule was used as a control. Stable expression of the Spry2-specific shRNA in HT1080 (Fig. 1B), as well as in VIP:FT cells (Fig. 1C), resulted in the down regulation of Spry2. The levels of Spry2 expression were unaltered in the cells expressing the scrambled shRNA (control cells).

Figure 1. Down regulation of Spry2 in fibrosarcomas with wild type Ras or $NRas^{Q59}$ expression. (A) Whole cell lysates form normal foreskin-derived fibroblasts (SL68 and SL89) and human-patient derived fibrosarcoma cell lines (HT1080 and VIPFT) were pulled down with RafRBD conjugated beads to determine the level of active Ras in each cell line. The total amount of Ras in the whole cell lysate (WCL) was determined using a Pan-Ras-specific isoform. (B, C) Down regulation of Spry2 expression in fibrosarcoma cell lines expressing $NRas^{Q59}$ (HT1080, A) and in fibrosarcoma cells expressing wild type Ras (VIP:FT, B). In both cases, stable cell lines were generated by infection of the parental cells with a retrovirus expressing either a Spry2-specific or a scrambled shRNA. (D, E) The indicated cell lines were serum starved and stimulated with EGF (100ng/mL) for the indicated time points. WCL were analyzed by western blotting to determine the expression of the indicated proteins.

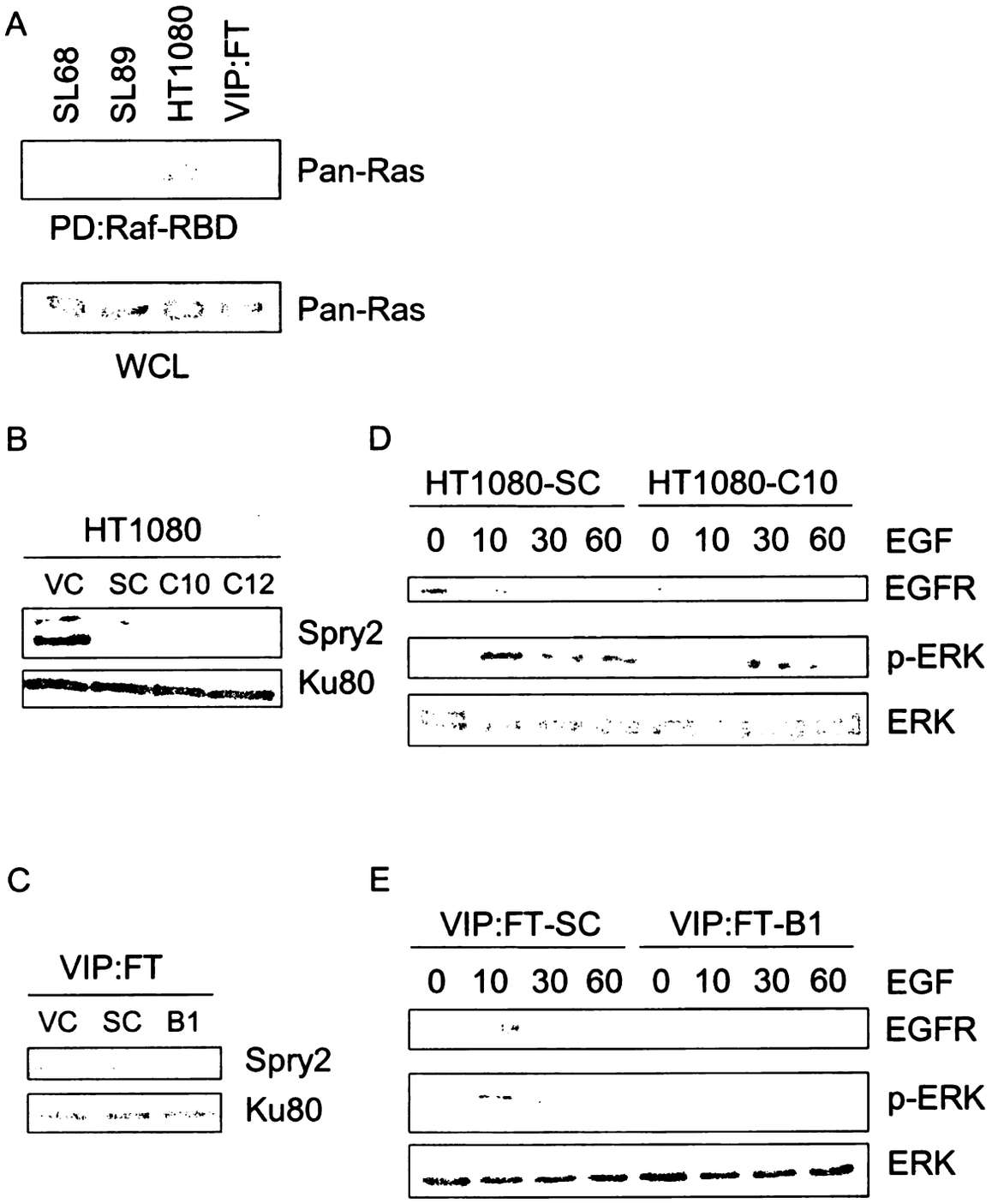
A
SL68
SL89
HT1080
VIP:FT
Pan-Ras
PD:Raf-RBD
Pan-Ras
WCL
B
HT1080
VC SC C10 C12
Spry2
Ku80
C
VIP:FT
VC SC B1
Spry2
Ku80
D
HT1080-SC
HT1080-C10
0 10 30 60 0 10 30 60
EGF
EGFR
p-ERK
ERK
E
VIP:FT-SC
VIP:FT-B1
0 10 30 60 0 10 30 60
EGF
EGFR
p-ERK
ERK

Figure 1

Control cells and cells with down regulated Spry2 were then analyzed for their ability to form tumors in athymic mice. Parental HT1080 cells consistently form sarcomas in athymic mice (100 tumors/100 injected sites) with a latency of three to four weeks. HT1080 cells expressing the scrambled shRNA, formed tumors at a similar ratio as did the parental cells, i.e. 7 tumor/8 sites. HT1080 cells with down regulated Spry2 formed fewer tumors, i.e. 6 tumors/10 sites (Table I).

Parental VIP:FT cells also form tumors in athymic mice with the same consistency as do parental VIP:FT cells (37 tumors/37 injected sites), although they exhibit a longer latency (approximately five weeks). VIP:FT cells expressing the scrambled shRNA formed tumors at a ratio resembling that of the parental cell line, i.e., 4 tumors/6 sites, with a latency of approximately five weeks. VIP:FT cells with down regulated Spry2, however, formed significantly fewer tumors upon injection in athymic mice, i.e. 6 tumors/16 sites. The latency of the tumors formed was the same as in the parental and control cells (Table II).

These preliminary results suggest that both in the presence of wild type as well as in the presence of mutant Ras, Spry2 contributes to the ability of cells to form tumors in athymic mice. According to these findings, Spry2 plays a more prominent role in the tumorgenicity of VIP:FT cells (wild type Ras), than it does in HT1080 cells ($NRas^{Q59}$). In addition to expressing $NRas^{Q59}$, HT1080 cells, also overexpress PDGF. The latter may contribute significantly to the malignancy of these cells. Interestingly, Spry2 inhibits ERK activation and proliferation in

Table I The tumorigenicity of the HT1080 cell lines with down-regulated Spry2

Cell strain	shRNA	Tumor incidence*	Days for tumor to reach 0.5 cm^3 volume
HT1080	-	100/100	21-28
HT1080-SC	*scrambled*	7/8 (87.5%)	35-63
HT1080-C10	*spry2*	6/10 (60%)	35-63

* Ratio of tumors formed to the number of sites injected subcutaneously. The mice were examined for tumor formation for at least 6 months after injection

response to PDGF stimulation, while it sustains ERK activation and growth in soft agar in HRas oncogene-transformed cells. These findings lend the preliminary hypothesis that in HT1080 cells, the ability of Spry2 to sustain RTK signaling in cells with activated Ras may be balanced, to some extent, by its ability to inhibit the same pathway in response to PDGF-stimulation, resulting in a diminished effect in tumor formation.

Effect of Spry2 on EGF-induced cell cycle progression

We have previously shown that Spry2 sustains the levels of EGFR and the signaling activity from this receptor, in fibrosarcomas containing $HRas^{V12}$ mutations. To determine if Spry2 has a similar function in fibrosarcomas expressing mutant or wild type Ras, we examined the expression of EGFR following EGF-stimulation in the presence and absence of Spry2 expression in HT1080 and VIPFT cells. As expected, the levels of EGFR and the levels of active ERK were decreased in both HT1080 and VIP:FT cells with down regulated Spry2, compared to control cells (Fig. 1D and 1E).

Since the down regulation of Spry2 in VIP:FT cells resulted in a more prominent decrease in tumorigenicity, compared to the effect of Spry2 down regulation in HT1080 cells, we focused our attention on function of Spry2 in these cells. Growth factor induced activation of ERK is an important step for cell cycle progression. To determine if the decrease in the activation of ERK, observed in the fibrosarcomas with down regulated Spry2, was associated with changes in

Table II The tumorigenicity of the VIP:FT cell lines with down-regulated Spry2

Cell strain	shRNA	Tumor incidence*	Days for tumor to reach 0.5 cm^3 volume
VIP:FT	-	37/37	28-35
VIP:FT-SC	*scrambled*	4/6 (60%)**	28-84
VIP:FT-B1	*spry2*	6/16 (37.5%)	28-84

* Ratio of tumors formed to the number of sites injected subcutaneously. The mice were examined for tumor formation for at least 6 months after injection

**The remaining 2/6 injections formed tumors that regressed to normal

cell cycle progression we examined the cell cycle distribution of VIP:FT cells expressing the scrambled shRNA to the distribution of VIP:FT cells expressing Spry2 shRNA. The cells were serum starved for a period of 60 hrs, and then stimulated with EGF to induced cell cycle progression. As soon as 2hrs after EGF stimulation, the proportion of control VIP:FT cells in G1 decreased, while the proportion in S increased, indicating progress from G1 to S phase (Fig. 2). Instead, in VIP:FT cells with down regulated Spry2, the proportion of cells in G1 remained unaltered 2 hrs after EGF stimulation, suggesting a delay in S phase entry. Furthermore, the population of control VIP:FT cells at G2 was prominent after ten hours of EGF-stimulation. We were unable to detect a significant population of VIP:FT cells with down regulated Spry2 that were at G2 at least until after 15 hrs of EGF stimulation. The proportion of cells at S phase was similar between the two cell strains. Importantly, the proportion of cells with DNA content below 2n, possibly apoptotic cells, was increased in the cells with down regulated Spry2.

Discussion

The data presented here suggest that Spry2 promotes tumor formation in human patient-derived fibrosarcoma cell lines, consistent with the role of Spry2 in PH3MT cells. Also, the ability of Spry2 to promote tumor formation by these cells is independent of the presence of activating Ras mutations. It should be noted that several months after their generation, the cell lines with down regulated Spry2 had regained expression of Spry2, most likely due to loss of expression of

Figure 2. Down regulation of Spry2 in VIP:FT cells delays progression through the cell cycle. VIP:FT cells expressing either scrambled shRNA or Spry2-specific shRNA were analyzed as described in Materials and Methods.

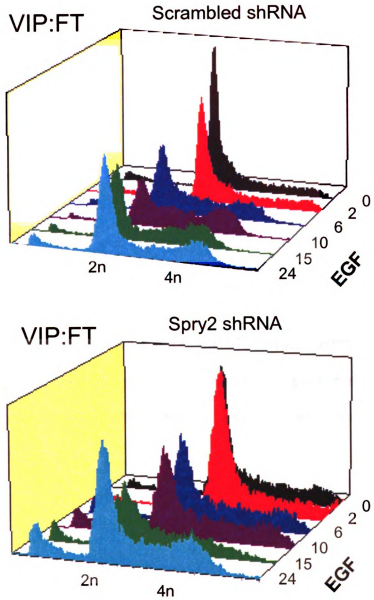
VIP:FT
Scrambled shRNA
2n
4n
0
2
6
10
15
24
EGF
VIP:FT
Spry2 shRNA
2n
4n
0
2
6
10
15
24
EGF

Figure 2

the *spry2*-specific shRNA. This may account for the lesser effect that Spry2 had in the tumor forming ability of HT1080 and VIP:FT cellc compared to the effect of Spry2 on the tumor forming ability of PH3MT cells. Alternatively, Spry2 may have a more pronounced effect in HRas-transformed cells, because Spry2 may play a Ras isoform specific function.

Material and methods

Cell cycle analysis

Cells plated at a density of 200,000 per 10cm-dish, were serum deprived for 48 to 72 hrs., and then stimulated with EGF (300 ng/mL) for varying time periods. After the period of stimulation was over, the cells were collected and fixed in 80% ethanol solution. Fixed cells were stained with propidium iodide (50 ng/mL) for 1 hr. at room temperature. The cell cycle analysis was performed by using FACS, using standard sttings.