Ras modulates Myc activity to repress thrombospondin-1 expression and increase tumor angiogenesis

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Summary

Tumor angiogenesis is postulated to be regulated by the balance between pro- and anti-angiogenic factors. We demonstrate that the critical step in establishing the angiogenic capability of human cells is the repression of the critical anti-angiogenic factor, thrombospondin-1 (Tsp-1). This repression is essential for tumor formation by primary epithelial cells and kidney cells engineered to express SV40 early region proteins, hTERT, and H-RasV12. We have discovered the signaling pathway leading from Ras to Tsp-1 repression. Ras induces the sequential activation of PI3 kinase, Rho, and ROCK, leading to activation of Myc through phosphorylation; phosphorylation of Myc via this mechanism enables it to repress *Tsp-1* expression. We thus describe a novel mechanism by which the cooperative activity of the oncogenes, *ras* and *myc*, leads directly to angiogenesis and tumor formation.

Introduction

The process of tumorigenic transformation involves the sequential acquisition of a number of genetic and epigenetic alterations by the genomes of evolving, premalignant cell populations (Hanahan and Weinberg, 2000). These alterations mimic deregulation of the growth-controlling circuitry of cells. Among other biological changes, these alterations provide the tumor cell with constitutive mitogenic signaling, deregulate the control of the cell cycle, and, as shown in recent years, enable the maintenance of telomeric DNA (Artandi and DePinho, 2000; Blasco, 2002; Bodnar et al., 1998; Kiyono et al., 1998).

In addition, the events that take place during tumor progression enable the tumor to interact with its stromal environment in ways that enhance its ability to proliferate in the primary site and, in highly malignant tumors, to metastasize to distant sites in the body (Brown et al., 1999; MacDougall and Matrisian, 1995). One of the key components of the tumor-associated stroma is the vasculature, which supplies oxygen, nutrients, and growth-promoting signals to the tumor cells and removes metabolic waste generated by the tumor cells (Berse et al., 1992). The newly acquired vasculature may also serve as a conduit through which tumors can metastasize to distant sites (Chiarugi and Christofori, 2000; Folkman, 1985; Hanahan and Folkman, 1996). These observations underscore the importance of elucidating the cancer cell-specific processes that enable tumors to interact with the existing vasculature and recruit neovasculature.

Observations of tumor growth have indicated that small tumor masses of 1–2 mm diameter can persist in a tissue without any tumor-specific vasculature (Coussens et al., 1999; Hanahan et al., 1996; Holmgren et al., 1995). The growth arrest of nonvascularized tumors of this size has been attributed to the effects of hypoxia at the center of the tumor since the diffusion of oxygen through living tissue is effectively limited to distances less than 200 μm (Olive et al., 1992). It has been suggested that tumors emerge from this growth arrest by developing a neovasculature, a change that has been termed the "angiogenic switch" (Hanahan et al., 1996). However, direct proof for the existence of this switch in spontaneously arising human tumors and an elucidation of its mechanism have remained elusive. Indeed, while the cell-to-cell signaling mechanisms that enable tumor cells to evoke angiogenesis have been intensively stud-

SIGNIFICANCE

Tumor dormancy is a critical yet poorly understood phenomenon affecting both the diagnosis and treatment of human cancers. This is due in large part to the lack of model systems available to study dormant tumor cells. We have developed such cells via the ectopic expression of the SV40 early region, hTERT, the catalytic subunit of telomerase, and H-RasV12. Modest overexpression of H-RasV12 results in cells that form tumors unable to progress beyond approximately 2 mm in diameter. However, when VEGF is overexpressed, or when thrombospondin-1 (Tsp-1) is repressed via antisense, the resultant cells form tumors that are unfettered in their growth potential. This observation provides a mechanistic model for one form of tumor dormancy that is governed by the regulation of the angiogenic potential of the tumor cells.

ied, relatively little is known about the cell-autonomous processes that enable tumor cells to induce angiogenesis.

Several growth factors act as positive regulators of angiogenesis. Foremost among these are vascular endothelial growth factor (VEGF) (Leung et al., 1989), basic fibroblast growth factor (bFGF) (Nguyen et al., 1994), and angiogenin (Hu et al., 1994; Soncin, 1992). Proteins such as thrombospondin (Tsp-1) (Good et al., 1990), angiostatin (O'Reilly et al., 1996), and endostatin (O'Reilly et al., 1997) function as negative regulators of angiogenesis.

Tsp-1 was the first naturally occurring inhibitor of angiogenesis to be identified (Good et al., 1990). Tsp-1 inhibits the activity of MMP-9 (Rodriguez-Manzaneque et al., 2001), an extracellular matrix (ECM) metalloproteinase that releases VEGF sequestered in the ECM (Ribatti et al., 1998). In addition, Tsp-1 can act directly to inhibit angiogenesis by binding to the CD36 receptor protein, which is present on endothelial cell surfaces (Dawson et al., 1997).

Both serum- and platelet-derived growth factor (PDGF) treatments upregulate the transcription of Tsp-1 (Framson and Bornstein, 1993; Majack et al., 1987). The Ras oncoprotein, in direct contrast, inhibits the expression of Tsp-1 (Rak et al., 2000). These conflicting responses suggest both positive and negative effects of the mitogenic signaling pathway on Tsp-1 expression. In contrast to its effects on Tsp-1 expression, oncogenic Ras stimulates VEGF expression (Rak et al., 1995), suggesting that Ras is an important regulator of the balance of pro- and anti-angiogenic factors.

The creation of genetically defined human cancer cells has provided the opportunity to define with some precision the regulatory pathways that contribute to the tumorigenic phenotype of human cancer cells (Hahn et al., 1999). In this lab, we have focused much attention on derivatives of three normal human cell types (embryonic kidney cells, foreskin fibroblasts, and mammary epithelial cells) that have been transformed by the retroviral transduction into these cells of the SV40 early region, the catalytic subunit of human telomerase (hTERT), and the oncogenic G12V allele of H-ras (Elenbaas et al., 2001; Hahn et al., 1999). Following introduction of these genes, all three cell types were tumorigenic and angiogenic when injected subcutaneously into nude mice.

In an effort to more closely recapitulate the signaling conditions that operate in spontaneously arising human tumors, we created versions of these human cancer cell lines that expressed lower levels of H-RasV12 (Elenbaas et al., 2001) than those used in the initial transformation experiments. In so doing, we discovered that the human mammary and human embryonic kidney cells expressing the SV40 early region proteins, hTERT, and relatively low levels of H-RasV12 were either unable to form tumors when injected subcutaneously into nude mice or did so only with long latency. As described herein, we have discovered that the prime defect of these cells is their inability to effectively provoke neoangiogenesis. We therefore set out to determine how signaling by the Ras oncoprotein enables cells to emerge from a non-angiogenic, poorly tumorigenic state.

Results

Effect of Ras oncoprotein levels on Tsp-1 expression

Human mammary epithelial- and kidney-derived cells that express the SV40 early region, hTERT, and relatively low levels of oncogenic Ras (~3–7× above endogenous levels of wild-type Ras expression, Figure 1C) form small tumors, approximately 1–2 mm in diameter, that never progress beyond this size. This behavior is in direct contrast to that of cells expressing higher levels of Ras (12–50× above endogenous levels), which succeed in forming tumors of substantial size (1.5 cm diam.) within 3–7 weeks after implantation into host mice (Figure 1A). We speculated that the inability of the low Ras-expressing cells to grow beyond the diameter of 1–2 mm was attributable to a deficiency in angiogenesis.

To test this hypothesis directly, we examined expressed VEGF in both the mammary and kidney cells. Indeed, overexpression of VEGF (400 pg/ml as measured by ELISA) resulted in the conversion of both low Ras expressing cell types from a nontumorigenic to a tumorigenic phenotype that rivaled the high Ras-expressing cells both in frequency of tumor formation and in growth kinetics (Figures 1A and 1B).

These observations suggest that high levels of Ras oncoprotein cause significant levels of VEGF production, resulting in the robust tumorigenic growth of high Ras-transformed cells; conversely, the low Ras-expressing cells, we imagined, were unable to express and release significant levels of VEGF, explaining their weak tumorigenicity. Accordingly, we proceeded to measure the amount of VEGF secreted by the cells expressing no oncogenic Ras, low levels of oncogenic Ras, or high levels of oncogenic Ras. We found that the level of VEGF secreted by cells grown in 0.1% serum and 0.4% O_2 was dramatically greater by cells expressing low levels of Ras cells than by those carrying no ectopically expressed Ras oncoprotein (Figure 2B). However, the further increase in levels of VEGF secretion observed when comparing the low Ras- to the high Ras-expressing cells was a modest one of approximately 1.4-fold. These results suggested that the marginal differences in VEGF expression levels by the low Ras versus high Ras cells could not account for the marked disparity in the tumorigenicity of these two cell populations.

The balance of pro-and anti-angiogenic factors regulates angiogenesis

These various observations, when taken together, suggested that the differences in angiogenicity between the two cell populations might be traced to regulators of neovascularization other than VEGF. Consequently, we turned our attention to the anti-angiogenic factor, Tsp-1, and its expression. An immunoblot analysis of proteins extracted from both the mammary and kidney cells revealed that the level of Tsp-1 expression was essentially unchanged in the low Ras-expressing cells compared to the cells not expressing oncogenic Ras (Figure 2A). However, both cell types expressing high levels of oncogenic Ras exhibited dramatically reduced levels of Tsp-1. In both the kidney cells and mammary epithelial cells, the difference in Tsp-1 expression between the low and high Ras-expressing cells was approximately 8-fold. This suggested that the poor angiogenicity of the low Ras-expressing cells was due to the high levels of Tsp-1 that they produced.

We speculated that an unfavorable ratio of VEGF to Tsp-1 might preclude neoangiogenesis in the tumors formed by the low Ras-expressing cells. This model predicted that we could confer a tumorigenic phenotype on the low Ras-expressing cells by decreasing the amount of Tsp-1 that they expressed. To examine this possibility, we created a retroviral vector specifying antisense Tsp-1 and introduced this construct, via infection,

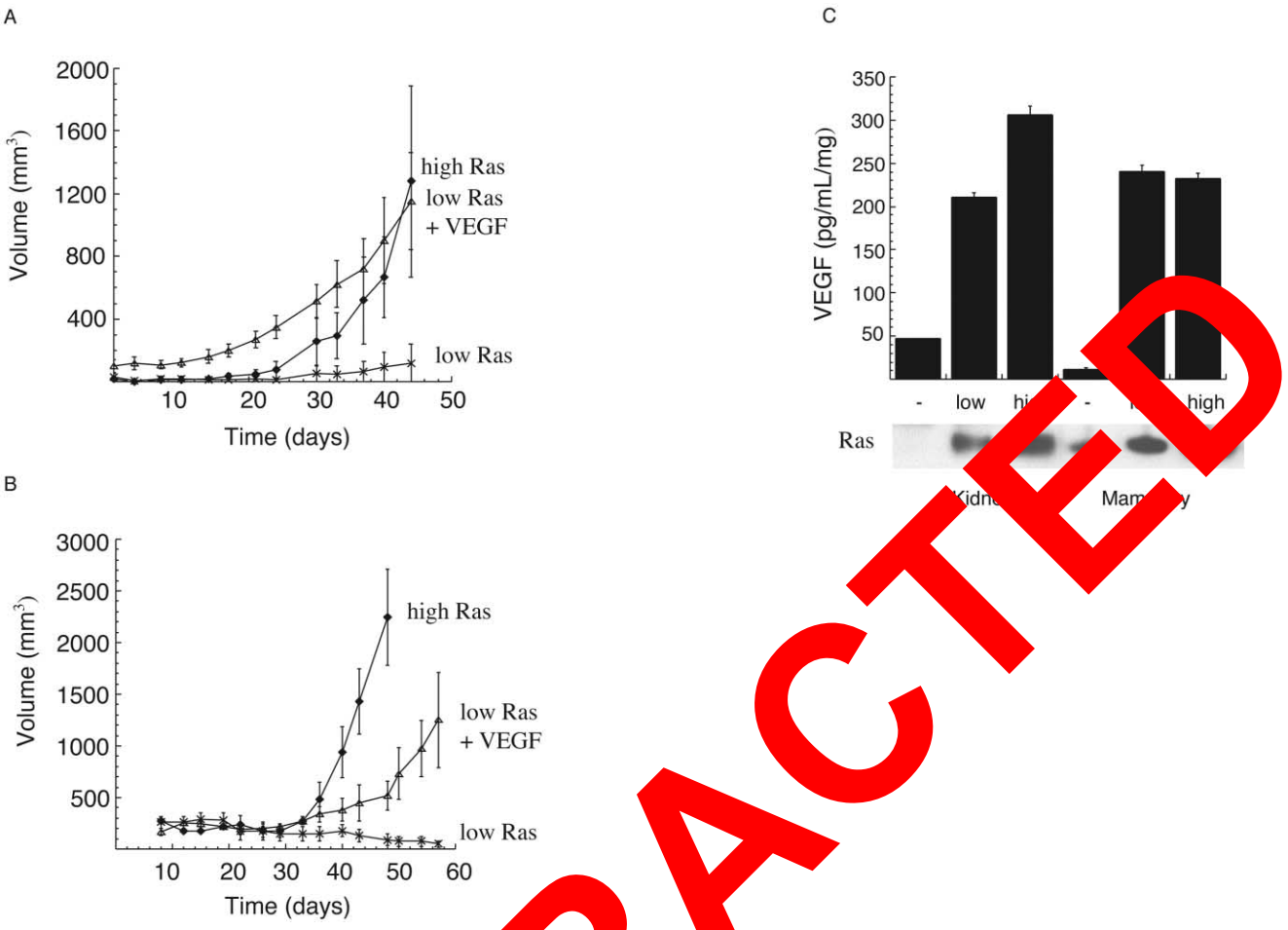

Figure 1. Effects of VEGF on tumor formation

Growth curve of tumors formed by kidney-derived cells (**A**) and least-differentiated cells (**B**) expressing low levels of Ras, low levels of Ras + VEGF, or high levels of Ras.

C: ELISA of secreted VEGF by kidney and mammary derived cells expressing no (—), low, and high levels of oncogenic Ras grown in 0.1% O_2.

into low Ras-expressing kidney cells. As hoped, the antisense construct reduced the level of Tsp-1 protein expression in the low Ras-expressing cells approximately 4-fold (Figure 2B) (Castle et al., 1991). These cells formed subcutaneous tumors in nude mice with a latency and kinetics comparable to those of the high Ras expressing cells, reaching the diameter of ~1.5 cm within 7 days of one another (i.e., 34 versus 41 days) (Figure 2C). As anticipated, the parental low Ras cells expressing only the resistance marker (zeocin) were unable to form tumors during this period (Figure 2C).

To further confirm the role of Tsp-1 in tumor formation and angiogenesis, we transduced the high Ras-expressing kidney cells with a construct specifying Tsp-1. The resultant cells expressed Tsp-1 levels similar to the parental cells expressing no oncogenic Ras (Figure 2D). When the two cell types were injected into nude mice, the cells expressing Tsp-1 formed tumors approximately 60% smaller than those expressing vector alone (Figure 2E). Furthermore, microscopic examination revealed that the center of the tumors formed by the cells ectopically expressing Tsp-1 were completely necrotic (comprising 60%–90% of total tumor volume) (Figure 2F, panel ii) with viable cells comprising only the periphery of the tumor (Figure 2F, panel iv). This end-stage necrosis was characterized by a total absence of intact cells and the presence of fragmented nuclear and cytoplasmic debris. Consistent with an impairment in angiogenesis, this rim of viable cells was no more than 200 μm in thickness at any given point (Figure 2F, panel ii). On the other hand, the tumors formed by the cells expressing high Ras and vector alone formed solid masses that had only sparse patches of necrosis comprising 5%–10% of the tumor (Figure 2F, panel i). When tumor size and viability are taken into account, the viable tumor burden of the mice bearing tumors of high Ras-expressing cells was more than 8-fold greater than that of the mice bearing tumors formed by Tsp-1-expressing cells. These observations confirmed that the ratio between secreted VEGF and Tsp-1 strongly influences the angiogenicity of these tumor cells, and that angiogenicity and tumorigenicity can be achieved either by increasing the expression of VEGF or by reducing the expression of Tsp-1.

The role of Myc in Tsp-1 regulation

Ras signaling has been demonstrated to affect the stability of the Myc protein (Sears, et al., 2000). Furthermore, previous

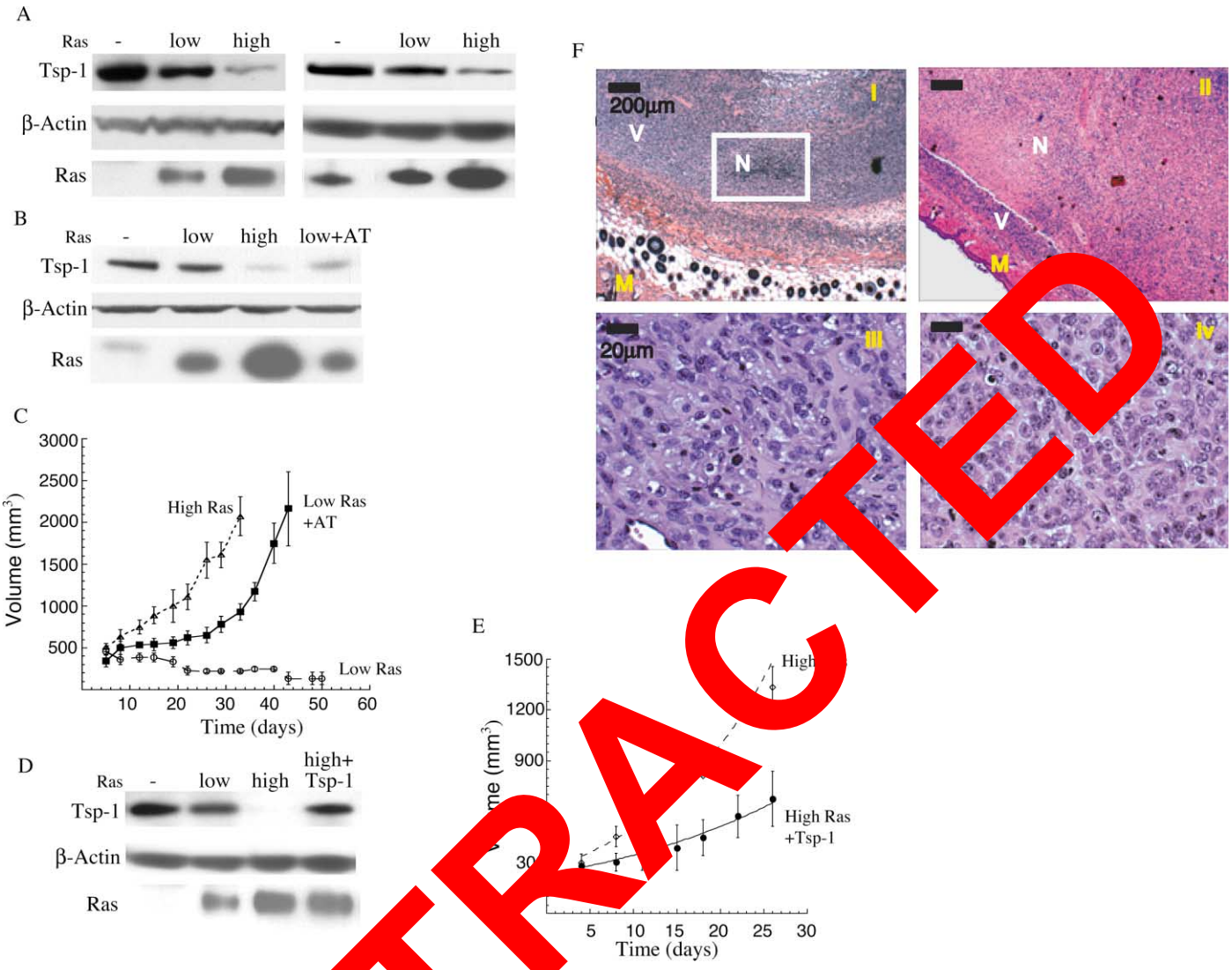

Figure 2. Tsp-1 expression and tumor formation

A: Immunoblot analysis of Tsp-1, β-actin, and Ras proteins expressed in kidney- and breast-derived derived cells.
B: Immunoblot analysis of Tsp-1, β-actin, and Ras expressed by kidney-derived cells expressing no (—), low, or high levels of Ras or of cells expressing low Ras plus antisense Tsp-1 (AT).
C: Growth curves of tumors formed by kidney-derived cells expressing no (—), low, or high levels of Ras and cells expressing low Ras plus antisense Tsp-1 (AT).
D: Immunoblot analysis of Tsp-1, β-actin, and Ras expressed by kidney-derived cells expressing no (—), low, or high levels of Ras or of cells expressing high Ras plus Tsp-1.
E: Growth curves of tumors formed by kidney-derived cells expressing no (—), low, or high levels of Ras and cells expressing high Ras plus Tsp-1.
F: H+E staining of tumors formed by cells expressing high levels of Ras plus control vector (i and iii) and high levels of Ras plus Tsp-1 (ii and iv). Upper panels are 4× magnification and lower panels are 40× magnification. M denotes normal mouse tissue, V denotes areas of viable tumor cells, N denotes areas of necrosis.

work has suggested a role for Myc in the repression of Tsp-1 expression (Ngo et al., 2000; Tikhonenko et al., 1996). Consequently, we examined whether the Ras-induced repression of Tsp-1 also depended on the activation of Myc. To this end, we introduced an inducible dominant-negative version of Myc (DNMycER) into the high Ras-expressing kidney cells. This version of Myc lacks the box 2 region (amino acid residues 106–143) and is able to bind its cofactors, but is unable to form a functional transcription complex (MacGregor et al., 1996). In addition, this version of Myc has been fused to a modified version of the estrogen receptor that is activated only by tamoxifen (4-HT) (Littlewood et al., 1995). In the absences of tamoxifen, this protein is functionally inactive and sequestered in the cytoplasm. Following 4-HT addition, it is rapidly activated and migrates to the nucleus. This hybrid protein therefore makes it possible to induce DN Myc activity through the addition of 4-HT to the growth medium.

In high Ras-expressing kidney cells that expressed DNMycER, treatment with 4-HT induced the level of Tsp-1 to rise within 4 hr, eventually reaching that of the cells expressing no oncogenic Ras by 8 hr after 4-HT addition. In contrast, mock-treated and control cells were unchanged in their expression of

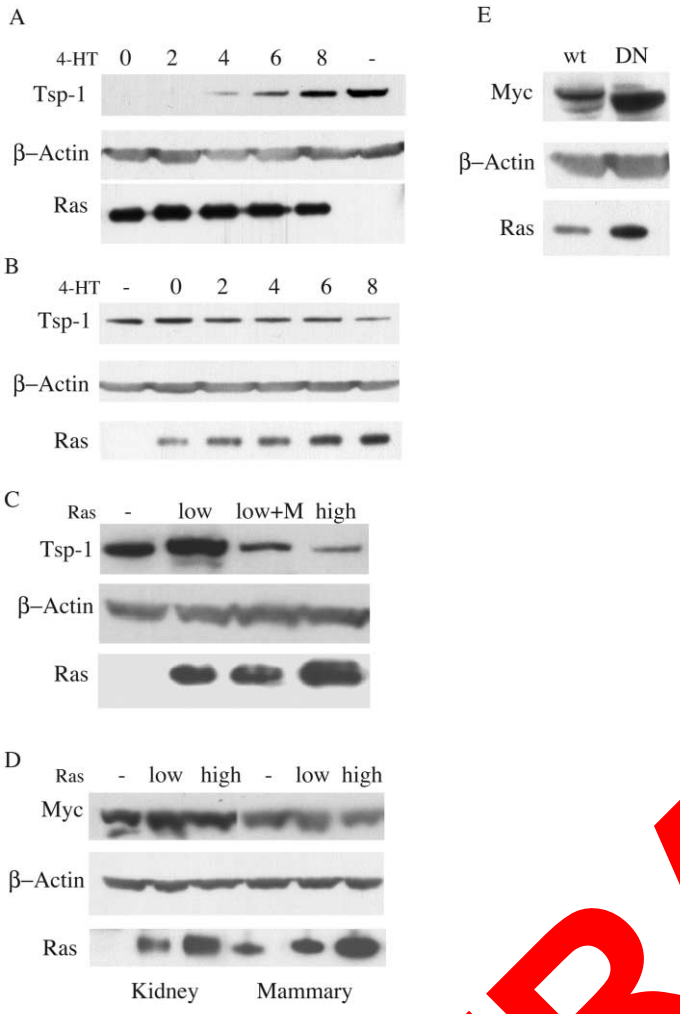

Figure 3. Effects of Myc activity on Tsp-1 expressi

A: Immunoblot analysis of Tsp-1, β-actin, ar proteins ex ed by kidney-derived cells expressing no onco (—) or high s of oncogenic Ras, or high levels of oncoge c Ras p dominant-negative MycER (DER), at 2, 4, 6, and 8 hr afte eatment with

B: Immunoblot analysis of Tsp-1, in, and Ras pro expressed by kidney-derived cells expressing ncoge Ras (—) or lo levels of oncogenic Ras plus MycER (MER) 4, 6, hr after treatment with 4-HT.

C: Immunoblot analysis of Tsp and Ra proteins expressed by kidney-derived cells expressing ri ogenic R —), low levels of oncogenic Ras, high leve ogeni or levels of oncogenic Ras plus a transfected myc ogene

D: Immunoblot alysis of al Myc an β-actin proteins expressed by kidney- and b deriv ressing no oncogenic Ras (—), low levels of oncoge high of oncogenic Ras.

E: Immunoblot analy wtMycER or DNMycER, β-actin, and Ras proteins expressed by kidney-d d cells expressing low levels of oncogenic Ras plus MycER (wt) or high of oncogenic Ras plus dominant-negative MycER (DN).

Tsp-1 protein (Figure 3A). This provided evidence that the Myc protein of the high Ras-expressing cells was participating in the repression of Tsp-1 expression.

We then attempted the opposite experiment by introducing a vector expressing MycER into the low ras-expressing kidney cells. This MycER construct specifies a wild-type Myc protein; as before, this fusion protein is activatable by addition of 4-HT to the culture medium (Littlewood et al., 1995). Upon treatment with 4-HT, these low Ras cells exhibited reduced expression of Tsp-1 protein within 4 hr. By 8 hr after 4-HT addition, the levels of Tsp-1 expression in these low Ras kidney cells decreased to the level of Tsp-1 expression seen in the high Ras kidney cells; mock-treated cells were unchanged in their expression of Tsp-1 (Figure 3B). Expression of both the wtMycER and DNMycER proteins was confirmed by Western blot analysis (Figure 3E). Furthermore, low Ras-expressing kidney cells transfected with a construct specifying wt Myc also exhibited reduced expression of Tsp-1 protein at a lev able to that seen in the high Ras-expressing kidne lls (Fig C).

The fact that overexpression of wt in low Ras xpressing cells is able to repress Tsp-1 press o levels mparable to the high Ras-expressing is indicat hat Ras signaling pathway is functional ese c s. How the inability of these cells to activate th dog us Myc protein suggests that either the level of as g is ins cient to activate the endogenous M protein, at th vel of Myc protein in these cells is i cient. We c ed from these results that Myc activi s re d for the re pression of Tsp-1 by Ras, and that interference wit dogenous Myc activity is sufficient to aboli epression.

Ha g established a role for Myc in the Ras-induced repression o sp-1, we p eeded to examine the effect of Ras signaling o e levels o yc protein expression in the mammary cells an dney c doing so at 0.1% serum in order minimize the effects genic signals from sources other than onco- Ras. We observed that Myc protein levels were unaffected by t ssion levels of the Ras oncoprotein (Figure 3D). Th uled out the possibility that high levels of Ras were inducing e accumulation of increased levels of Myc, thereby mimicking overexpression of Myc observed in several types of human t mors (Little et al., 1983; Seshadri et al., 1989; Trent et al., 1986). Hence, if high levels of Ras were acting through Myc to repress Tsp-1, we reasoned that this action must depend on a mechanism distinct from any effects on the levels of the Myc protein.

Effect of Ras signaling on Myc phosphorylation and activity

We reasoned that if high levels of Ras were not inducing increased levels of Myc, perhaps Ras might be affecting the phosphorylation state of Myc. To pursue this possibility, we examined the phosphorylation state of the Myc protein in these various cell populations. When cells were grown in 0.1% serum, we observed a modest increase in the level of phosphorylated Myc in low Ras cells compared to those not expressing oncogenic Ras (Figure 4A). However, the amount of phosphorylated Myc was dramatically increased in the cells expressing high levels of oncogenic Ras when compared to the level seen in low Ras cells. The level of phosphorylated Myc was determined by immunoblot analysis with an antibody that recognized Myc protein either singly phosphorylated at T58 or doubly phosphorylated at residues T58 and S62 (Figure 4A). These results demonstrate that Myc phosphorylation modulated by Ras correlates with the repression of Tsp-1 expression.

Phosphorylation of Myc at residues T58 and S62 alters its ability to transactivate gene expression (Gupta et al., 1993; Seth et al., 1991) in addition to its effects on metabolic stability (Sears et al., 2000). Furthermore, mutation of S62 to alanine inhibits

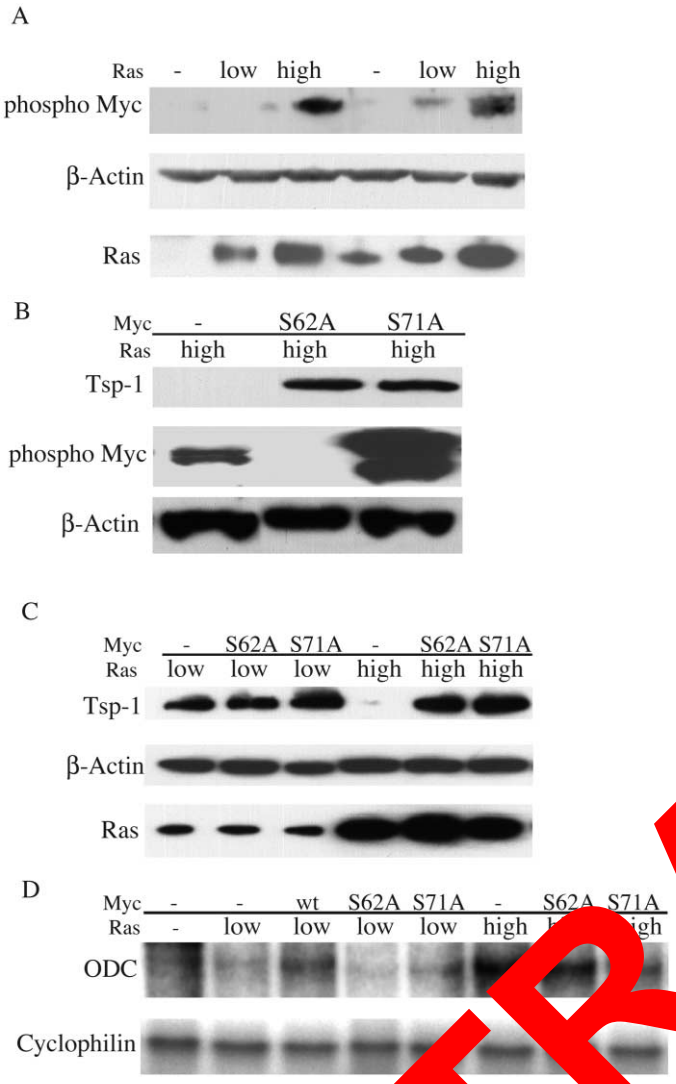

Figure 4. Effects of Myc phosphorylation on Tsp-1 expression

A: Immunoblot analysis of phospho Myc and β-actin proteins expressed by kidney- and breast-derived cells expressing no oncogenic Ras (—), low levels of oncogenic Ras, or high levels of oncogenic Ras.

B: Immunoblot analysis of Tsp-1, phospho Myc, β-actin, and Ras proteins expressed by kidney-derived cells expressing high levels of oncogenic Ras transfected with *S62AMyc* (62) or *S71AMyc* (71) genes.

C: Immunoblot analysis of Tsp-1, β-actin, and Ras proteins expressed by kidney-derived cells expressing low levels of oncogenic Ras, or high levels of oncogenic Ras that were mock transfected or transfected with *S62AMyc* (62) and *S71AMyc* (71) genes.

D: Ribonuclease protection assay for ornithine decarboxylase (ODC) and cyclophilin expressed by kidney-derived cells expressing no oncogenic Ras (—), low levels of oncogenic Ras, or high levels of oncogenic Ras that were mock transfected or transfected with wtMyc (Myc), *S62AMyc* (62), or *S71AMyc* (71) genes.

soft agar colony formation conferred by this protein (Pulverer et al., 1994). In order to assess the role of Myc phosphorylation more directly, we utilized phosphorylation-defective mutants of Myc containing alanine substitutions at two of the sites that have been shown to be phosphorylated, specifically, S62A and S71A (Noguchi et al., 1999). We anticipated that if phosphorylation at residue 62 were required for Myc-mediated Tsp-1 repression, MycS62A should act in a dominant-negative fashion by titrating out Myc partner proteins such as Max (Blackwood and Eisenman, 1991). To test this directly, we utilized the transformed human kidney cells, which, in contrast to the mammary cells, can be readily transfected. Indeed, as expected, transient transfection of high Ras-expressing cells with MycS62A resulted in the loss of Tsp-1 repression (Figure 4B). Furthermore, by immunoblot analysis with anti-phosphoMyc antibody, we determined that phosphorylation at residue 58 was also abolished by the S62A mutant (Figure 4B). This could be attributed to the degradation of Myc protein phosphorylated at T58, or to phosphorylation at residue 62 being a prerequisite for phosphorylation at residue 58 (Lutterbach and Hann, 1994).

While no role for phosphorylation at residue S71 has previously been identified, we found, in fact, that transient expression of a mutant Myc carrying a single amino acid substitution at residue 71 (i.e., S71A) also acted in a dominant-negative fashion to relieve repression of Tsp-1 (Figures 4B and 4C). All the while, transient transfection of low Ras-expressing kidney cells with MycS62A and MycS71A had no effect on the level of Tsp-1 protein (Figure 4C). In addition, expression of MycS71A had no effect on the phosphorylation of Myc at T58 and S62 in the high Ras-expressing cells (Figure 4C). This result suggests that phosphorylation of Myc at S71 is the ultimate activating event responsible for the repression of Tsp-1.

The effects of the phosphorylation-defective mutants were then assayed on established target of Myc—the gene encoding ornithine decarboxylase (ODC)—using a ribonuclease protection assay (RPA). Transient transfection of the low Ras-expressing kidney cells with wild-type Myc resulted in the upregulation of ODC mRNA, while S62A and S71A had no effect (figure 4D). At the same time, transient transfection of the high Ras-expressing kidney cells with either S62AMyc or S71AMyc resulted in the downregulation of ODC mRNA (Figure 4D). These results confirmed that phosphorylation at S62 and S71 is required for Myc function both as a transactivator and as a repressor.

Mechanism of repression of Tsp-1 expression by Ras

The above experiments indicated that signaling downstream from Ras was regulating the repression of Tsp-1, and that this repression was dependent upon the activity of Myc. We therefore decided to further characterize the functional interactions between these two proteins and the *Tsp-1* gene. To begin, we sought to determine which of the effector pathways downstream of Ras was responsible for suppressing Tsp-1 expression. At least three signaling pathways have been shown to be controlled by the Ras protein (White et al., 1995), and several others may also be perturbed in still poorly understood ways. The three major Ras effector pathways enumerated to date involve the Raf-MAPK cascade, the phosphatidyl inositol-3 kinase (PI3K) enzyme, and the Ral guanine nucleotide dissociation stimulator (RalGDS) protein.

In order to dissect the contributions of these three Ras effector pathways to Myc-mediated Tsp-1 repression, we used chemical inhibitors of the Raf and PI3K pathways. Treatment of the high Ras-expressing kidney cells with U0126, a specific inhibitor of MEK1/2 (Favata et al., 1998) that blocks ERK1/2 phosphorylation in the Raf-MAPK pathway, had no effect on

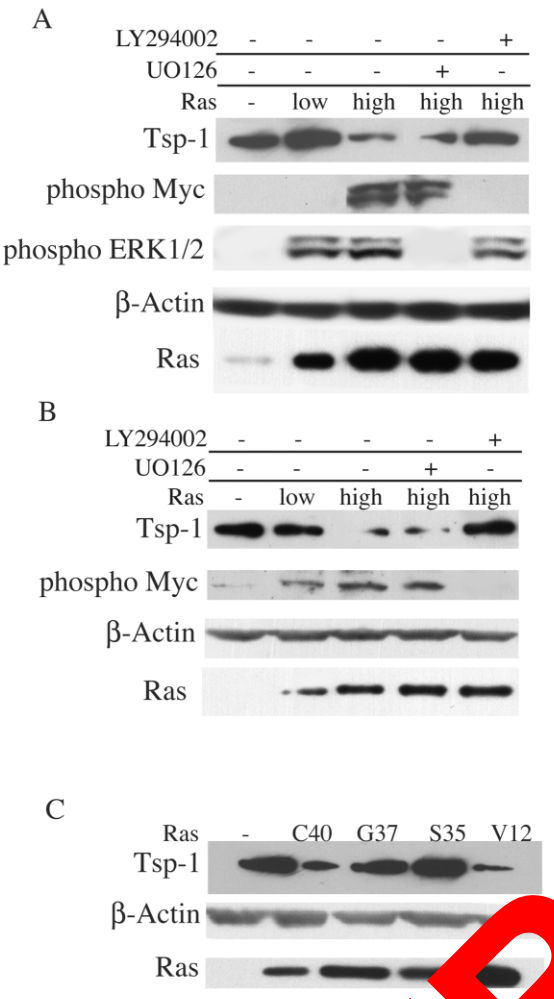

Figure 5. Effects of Ras signaling pathways on [illegible] phosphorylation and Tsp-1 expression

A: Immunoblot analysis of Tsp-1, phospho-Myc, phospho ERK1/2, β-actin, and Ras proteins expressed by kidney-derived cells expressing no oncogenic Ras (—), low levels of oncogenic Ras, or high levels of oncogenic Ras that were otherwise untreated or treated with either U0126 or LY294002.
B: Immunoblot analysis of Tsp-1, phospho-Myc, β-actin, and Ras proteins expressed by breast-derived cells expressing no oncogenic Ras (—), low levels of oncogenic Ras, or high levels of oncogenic Ras that were otherwise untreated or treated with either U0126 or LY294002.
C: Immunoblot analysis of Tsp-1, β-actin, and Ras proteins expressed by kidney-derived cells expressing no oncogenic Ras (—), high levels of oncogenic Ras, RasV12C40 (C40), RasV12G37 (G37), and RasV12S35 (S35).

the level of Tsp-1 protein (Figure 5A). In contrast, treatment of the high Ras-expressing cells with the PI3K inhibitor LY294002 (Vlahos et al., 1994) completely abrogated Tsp-1 repression and restored Tsp-1 protein levels to those seen in cells not expressing oncogenic Ras (Figure 5A). Consistently, Myc phosphorylation was strongly inhibited in both high Ras-expressing cells treated with LY294002, while treatment with U0126 had no effect on Myc phosphorylation (Figure 5B). These results allowed the tentative conclusion that it is the PI3K effector pathway that plays a dominant role in the Ras-mediated phosphorylation of Myc and repression of Tsp-1.

These results were confirmed and extended by analyzing Tsp-1 expression in cells ectopically expressing effector loop mutants of Ras that signal primarily through only one of the three major downstream effector pathways (White et al., 1995). Only the PI3-kinase effector loop mutant (C40), which retains PI3K-activating ability but lacks the other two effector functions of Ras, was able to downregulate Tsp-1 expression. In contrast, the Ras mutant that retains the ability to activate the RalGDS (G37) pathway had no effect on Tsp-1 levels, and selective activation of the Raf pathway by the third mutant (S35) actually increased Tsp-1 expression (Figure 5C). Taken together, these results indicated that the ability of Ras to regulate Tsp-1 expression is attributable largely, if not entirely, to its ability to activate PI3K.

PI3 kinase repression of Tsp

We next sought to determine the downstream effectors of PI3K involved in the repression of Tsp-1. The best-studied effect of PI3K involves its activation of Akt/PKB kinase (Franke et al., 1995). Accordingly, we attempted to mimic the actions of PI3K by expressing a constitutively active mutant of Akt that contains a myristoylation sequence at its carboxyl terminus (Ramaswamy et al., 1999). Cells not expressing oncogenic Ras but expressing the constitutively active version of Akt failed to downregulate Tsp-1 protein levels (Figure 6A). An essential role of Akt/PKB in Tsp-1 repression could be further excluded by retroviral transduction of a dominant-negative mutant of Akt (Hoover et al., 2001) into the high Ras-expressing kidney cells. This mutant had no effect on the expression of Tsp-1, whereas it was able to block the phosphorylation of Bad, a downstream target of Akt (data not shown). Hence, Akt signaling was neither necessary nor sufficient for the repression of Tsp-1.

Having excluded a role for Akt in Tsp-1 repression, we turned our attention to other molecules activated by PIP3, the product of the PI3K enzyme. Several guanine exchange factors (GEFs) for the Rho family of GTPases have been identified that contain PH domains which bind to PIP3 (Holsinger et al., 1995). To determine whether Rho proteins were likely to be involved in mediating the PI3K effects on Myc and Tsp-1, we performed GST-Rhotekin pulldown assays to assess the levels of GTP-bound Rho in the no Ras-, low Ras-, and high Ras-expressing cells (Ren and Schwartz, 2000). Indeed, Rho-GTP levels were approximately 10-fold greater in the high Ras-expressing cells than in the low Ras-expressing cells, while total Rho protein levels were constant (Figure 6B). Furthermore, treatment of the high Ras cells with LY294002 reduced the level of GTP bound Rho to that of the no Ras- and low Ras-expressing cells (Figure 6B).

These observations indicated a correlation between the levels of Myc phosphorylation, Tsp-1 repression, and Rho activation. This suggested the possibility that PI3K was acting through Rho proteins to achieve Myc phosphorylation and Tsp-1 repression. To assess the possible role of Rho as an intermediate in this signaling cascade, we introduced a dominant-negative mutant allele of the *RhoA* gene (RhoAN19) (Olson et al., 1995) into the high Ras-expressing kidney cells. Ectopic expression of RhoAN19 relieved the repression of Tsp-1 in high Ras-expressing cells, restoring the level to that seen in the low Ras-expressing cells (Figure 6C). We further explored the possible involvement of Rho by ectopically expressing mutant, constitutively active versions of RhoA and RhoC in the low Ras cells. Indeed, when these cells were infected with a retroviral vector

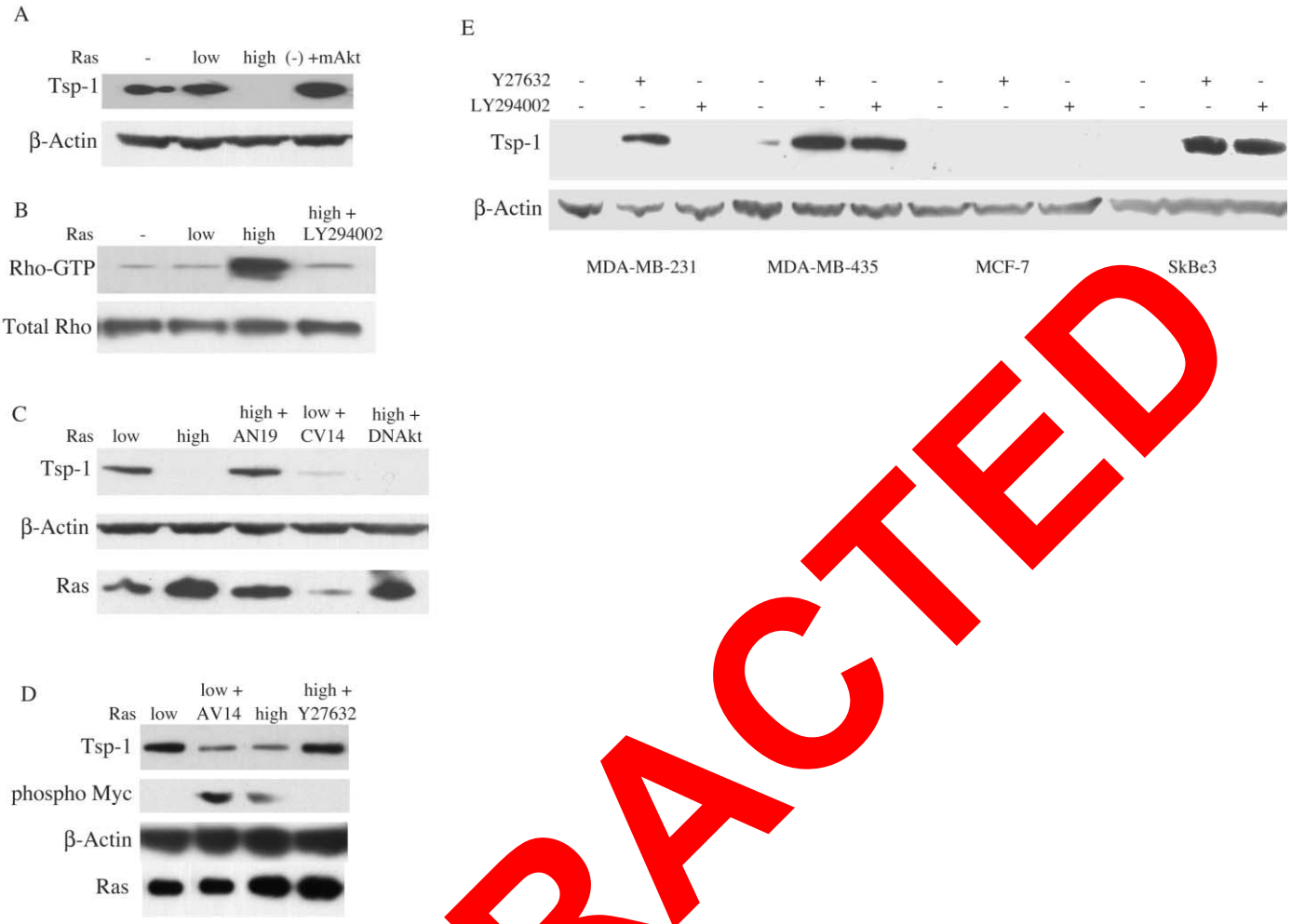

Figure 6. Effects of Rho signaling on Myc phospho[illegible]ion [illegible]-1 expression

A: Immunoblot analysis of Tsp-1 and β-actin p[illegible]ns expresse[illegible]kidney-derived cells expressing no oncogenic Ras (—), low levels of oncogenic Ras, or high levels of oncogenic Ras, or no oncog[illegible]plus myristoy[illegible]Akt (mAkt).

B: Immunoblot analysis of GTP bound Rho[illegible]d to[illegible]o in kidney-d[illegible]rived cells expressing no oncogenic Ras (—), low levels of oncogenic Ras, high levels of oncogenic Ras, or high levels of on[illegible]ogenic Ras [illegible]Y294002.

C: Immunoblot analysis of Tsp-1, β-a[illegible] and Ras protei[illegible]pressed by kidney-derived cells expressing low levels of oncogenic Ras, low levels of oncogenic Ras plus RhoCV14 (CV14), high [illegible]s of oncogenic Ras, [illegible]gh levels of oncogenic Ras plus RhoAN19 (AN19), and high Ras plus dominant-negative Akt (DNAkt).

D: Immunoblot analysis of Tsp[illegible]hos[illegible]Myc, β-actin, and Ras proteins expressed by kidney-derived cells expressing low levels of oncogenic Ras, low levels of oncogenic Ras plus Rh[illegible]AV14), hi[illegible]evels of oncogenic Ras, or high levels of oncogenic Ras plus Y27632.

E: Immunoblot analysi[illegible]-1 an[illegible]ctin e[illegible]ssed in human breast cancer cell lines treated with Y27632, LY294002, or mock treatment (—).

expressing Rho[illegible] or transfected transiently with a vector expressing RhoAV[illegible] Tsp-1 expression was suppressed (Figures 6B and 6C). Tak[illegible]ogether, these observations provided strong indication that Rho serves as a conduit through which the Ras protein signals to induce the repression of Tsp-1 expression.

We next sought to determine the downstream effector of Rho involved in the repression of Tsp-1. Two of the major effectors of Rho are p160ROCKI and ROCKII (two Rho-associated coiled-coil containing protein kinases) (Leung et al., 1996; Matsui et al., 1996). Inhibition of ROCK with the specific inhibitor Y27632 (Uehata et al., 1997) inhibits Ras-induced focus formation and transformation (Sahai et al., 1999). Treatment of high Ras-expressing cells with Y27632 relieved the repression of Tsp-1 expression, restoring the level to that of low Ras-expressing cells, and at the same time abolished the phosphorylation of Myc (Figure 6C). Together, these lines of evidence indicated that Rho signaling is necessary and sufficient for Tsp-1 repression, and that this repression was achieved through the actions of the Rho-associated kinase, ROCK. The involvement of ROCK in the regulation of angiogenesis represents a novel activity, as the only effects of ROCK activation reported to date have been related to cytoskeletal rearrangement and motility (Amano et al., 1996; Kawano et al., 1999).

Repression of Tsp-1 in human breast cancer cell lines

We then sought to determine whether the PI3K/Rho pathway was active in establishing the angiogenic program in human

tumor cell lines. To this end, we made use of the human breast cancer cell lines MDA-MB-231, MDA-MB-435, BT549, MCF7, and SkBr3. We found that Tsp-1 expression was undetectable in all of the above cell lines. Furthermore, when MDA-MB-231, MDA-MB-435, and SkBr3 were treated with the PI3 kinase inhibitor, LY294002, or the ROCK inhibitor, Y27632, Tsp-1 levels dramatically increased after 8 hr of treatment (Figure 6E). This result further confirms that this pathway is active in several human breast cancer cell lines and plays a role in establishing the angiogenicity of human tumors.

Discussion

In previous work, we demonstrated the genetic requirements for the formation of experimentally transformed human cells (Elenbaas et al., 2001; Hahn et al., 1999). This work shed no light, however, on the mechanisms whereby these cells acquired an essential attribute of tumorigenicity, specifically angiogenicity. In the course of the present work, we have uncovered a novel signaling pathway that leads from the Ras oncoprotein via Myc to the repression of expression of the potent anti-angiogenic protein, Tsp-1. The present results suggest that in human cells, repression of Tsp-1 expression is a critical step in the acquisition of angiogenicity and tumorigenicity. One method of achieving Tsp-1 repression is via a PI3K-mediated activation of Myc.

Once sufficient levels of PI3K activity are achieved, they act, as demonstrated here, via a hitherto unidentified signal transduction cascade, which leads through a Rho GEF to Rho and ROCK to the activation of Myc. The identity of the kinase or kinases directly responsible for the phosphorylation and functional activation of Myc at residues 62, and 71 is not addressed by the present work. This may be achieved directly by ROCK, or alternatively, ROCK may act via a cascade of intermediary kinases to modify the Myc protein (Figure 7). In addition, we have discovered that this pathway is also involved in the repression of Tsp-1 in several human breast cancer cell lines.

It has been known for some time that *myc* oncogenes can cooperate with a *ras* oncogene to transform recipient cells (Land et al., 1983). We have demonstrated here that activation of *myc* + *ras* cooperation in human cells is also required for steps subsequent to the initial transformation event, namely the acquisition of the angiogenic phenotype. The results presented here also provide insight into novel regulation of Myc, a protein that has been implicated in numerous forms of human cancer (Escot et al., 1986; Lit[illegible] et al., 1[illegible]; Trent et al., 1986). The ability of Myc to function as an oncoprotein inducing anchorage-independent growth has been found to be connected to its state of phosphorylation (Henriksson et al., 1993; Pulverer et al., 1994). We demonstrate here that activation of Myc via phosphorylation is sufficient to confer an angiogenic phenotype by repressing the expression of Tsp-1, even in the absence of Myc overexpression. Indeed, levels of Myc expression are unaltered by signaling from the Ras oncoprotein. Moreover, this observation suggests that the various contributions of the *myc* gene to tumorigenesis have not been fully enumerated.

In a murine model of melanoma that utilizes a doxycyclin-inducible transgene specifying H-RasV12, Ras signaling is required for the maintenance of the tumor vasculature (Chin et al., 1999). Interestingly, in this model, withdrawal of doxycyclin and subsequent loss of Ras expression led to blood vessel regression. This blood vessel regression could not be rescued by the ectopic expression of VEGF, suggesting that Ras was doing more than merely inducing VEGF expression. These observations are compatible with the presently demonstrated role of Ras in repressing expression of Tsp-1, a protein known to be able to cause apoptosis of endothelial cells (Guo et al., 1997).

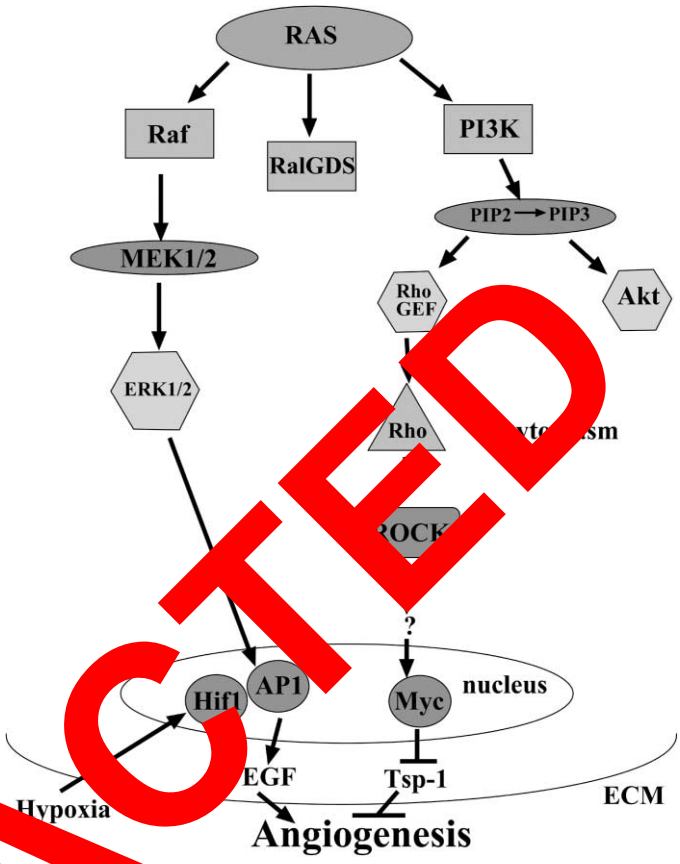

Figure 7. Schematic diagram of signaling pathway from Ras to Tsp-1

More recently, a murine transgenic model of pancreatic cancer revealed that Myc expression was required for both the establishment and maintenance of the tumor vasculature (Pelengaris et al., 2002). This model made use of the tamoxifen-inducible MycER fusion protein and, similar to the observations made with inducible Ras, withdrawal of tamoxifen and subsequent inactivation of Myc resulted in blood vessel regression. The results of these two studies in a rodent experimental system strongly suggest that both Ras and Myc play dominant roles in triggering angiogenesis. Our findings provide a mechanism explaining these observations and extend them to human cells.

While previous work has shown that supra-physiologic levels of oncogenic Ras are capable of downregulating Tsp-1 expression (Rak et al., 2000), and that ectopic expression of PTEN can lead to an increase in Tsp-1 expression (Wen et al., 2001), the signaling pathway(s) that regulate this repression have remained uncharacterized. The present results indicate that oncogenic Ras, expressed at near-physiologic levels, is not sufficient to repress Tsp-1. Our observations indicate that there are at least two processes that are required in order for tumors to downregulate the expression of thrombospondin-1. They are

amplification or overexpression of *myc*, as is seen in many human tumors (Escot et al., 1986; Little et al., 1983; Nau et al., 1984), and hyperactivation of the PI3K/Rho pathway. The latter event may be achieved by point mutation in the *ras* oncogene, mutation or overexpression of a growth factor receptor such as Her2/neu, or loss of the PTEN tumor suppressor protein. This hypothesis is supported by our observation that overexpression of wt Myc in low Ras-expressing cells is necessary and sufficient to repress Tsp-1 expression.

Furthermore, we have identified three breast cancer cell lines that repress Tsp-1 via the PI3K/Rho pathway. These cell lines activate the PI3K/Rho pathway via distinct mechanisms, either through mutation in K-Ras (MDA-MB-231), overexpression of HER2 (SkBr3), or alteration of the PI3 kinase pathway (MDA-MB-435) (Kozma et al., 1987; Singhal et al., 1994). At the same time, MCF7 cells, which have amplified the *myc* locus, and in which only minimal signaling, from Ras or PI3 kinase, may be required to repress Tsp-1, were insensitive to chemical inhibitors that affect this pathway. This opens the possibility that there is at least one other mechanism for repressing Tsp-1 expression. For example, expression of Tsp-1 has been shown to be silenced by methylation in both colorectal cancer cell lines and hematopoietic malignancies (Li et al., 1999). Additionally, it has recently been shown that Id1 is required for the repression of Tsp-1 in both murine embryonic fibroblasts and endothelial cells (Volpert et al., 2002). Therefore, it is possible that Tsp-1 expression is regulated differently in various cell types or during distinct stages of development.

The mechanism by which Myc represses Tsp-1 transcription remains unclear. Previous reports have demonstrated that Myc is capable of inhibiting transcription of a *Tsp-1* promoter construct (Thomas-Tikhonenko et al., 1996). However, M also been hypothesized to affect the stability of the *T* tran ipt (Ngo et al., 2000). Transcriptional repression b yc ha een demonstrated for several genes including p21CIP (Mitchell and El-Deiry, 1999; Staller et al 01). In t cases, this repression has been demonstrate occur via b g to an INR element (Li et al., 1994). Ho er, arch of the enomic TSP-1 DNA sequence has ot revealed onsensus INR site in proximity to the trans ional start site

As previously stated, t Rho p hway is the downstream signaling cascade that is vate PI3 kinase and is required for the repression of Tsp- e 7). Th volvement of Rho could also be signi in ot spec of tumor progression. For instance, it be demon that overexpression of RhoC increa both metasta potential and motility of human B16F ela and A375P amelanotic cells (Clark et al., 20 herefore, the ability of RhoC to increase the metastatic pot l of tumor cells could be accomplished via its ability to incre both the motility and migration of the cells at the primary site and also their angiogenicity at their metastatic destination via downregulation of Tsp-1. It is likely that the functions that have been ascribed to RhoC will be applicable as well to RhoA. The two proteins are 92% identical in their amino acid sequences and both have been shown to be able to stimulate ROCK (Leung et al., 1996; Ridley, 1997; Sahai and Marshall, 2002). Moreover, as demonstrated here, RhoA and RhoC are both able to stimulate Myc phosphorylation and Tsp-1 repression.

The regulation of angiogenesis is an essential, rate-limiting process in tumor formation. Much work has gone into the elucidation of the signaling pathways that regulate the expression of VEGF, one of the most potent angiogenic factors. However, to date, relatively little has been learned of the mechanisms governing the expression of Tsp-1. The results presented here support the hypothesis that neoangiogenesis and thus tumor progression are governed by the relative levels of pro- and anti-angiogenic factors, specifically VEGF and Tsp-1 (Hanahan and Folkman, 1996). Significantly, the ability of Ras to promote angiogenesis and hence tumorigenicity is governed in the presently studied cells far more by its ability to repress Tsp-1 expression than its effects in upregulating VEGF expre se findings raise the hope that chemical inhibitor at disru he pathway(s) leading to Tsp-1 repression ma ove to be e ctive in diminishing the angiogenicity of ertain an tumo and, in turn, slow or even halt their fu r progres

Experimental procedures

Cell lines and construct

The retroviral construc xpressing m E w created by digesting the vector pmVEGF164 rom Bruce S man, Dana Farber Cancer Institute) with Ba and 1, and ligating it to similarly digested pBabe-Zeo or pWZLBlast to create eZeo-VEGF and pWZLBlast-VEGF. The retroviral v WZLBlast-Ts as created by digesting the vector pCDNA (a gift from Michael etmar, Harvard Medical School) with EcoRI Sall and ligating the *tsp-1* DNA to similarly digested pWZLBlast. The re ral vector pW Blast-DNmycER was created by digesting pBabe-Puro-D cER (a gen us gift from Gerard Evan, UCSF) with EcoRI, and ligating th A to pW last and orientation confirmed by restriction digest with BamHI. viral vector pBabeZeo-AT was created by digesting Blast-Tsp1 with EcoRI and SalI, blunting the ends using the Klenow frag NA polymerase (Roche, Indianapolis, Indiana), and ligating it int abe digested with SnaB1; the antisense orientation was confirmed estriction digest. The constructs pMIG-DNRhoA and pMIG-RhoCV14 re gifts from Richard O. Hynes (MIT Center for Cancer Research). The struct pEXV-RhoAV14 was a gift from Alan Hall (MRC, London). The 2H-Ras mutants (a gift of Dr. Julian Downward) (Stratagene) were subcloned from pSG5 into the EcoRI site of the pWZL-Blast retroviral vector and directionality was confirmed by immunoblot analysis using a Ras-specific antibody (SantaCruz Biotechnologies, Santa Cruz, California).

The generation of the human embryonic kidney cells HA1E, HA1EhR, HA1EpR and the human mammary epithelial cells HMLE, HMLEhR, HMLEpR was described previously (Elenbaas et al., 2001; Hahn et al., 1999). HA1EhRV and HA1EpRV were generated by retroviral transduction of the parental cells with pBabeZeo-VEGF, whereas HMLEhRV and HMLEpRV were transduced with pWZLBlast-VEGF. HA1EhRAT was generated by transducing HA1EhR with pBabeZeo-AT. HA1ERasC40, G37, and S35 cell lines were created by transducing the parental HA1E cells with pWZL-RasC40, pWZL-RasG37, and pWZL-RasS35. HA1EpR-RhoAN19 and HA1EhR-RhoCV14 were created by transducing the parental cell lines with pMIG-RhoAN19 and pMIG-RhoCV12. Retroviruses were produced as previously described (Elenbaas et al., 2001).

Tumor formation assays

Tumorigenicity of the cell lines created above was assessed by injecting 2×10^6 cells subcutaneously, either with or without Matrigel (Becton Dickinson, Palo Alto, California), into nude mice that had been irradiated with 4 grays 24 hr prior to injection. The tumor diameter was measured using calipers and the diameter converted to volume using the equation $4/3\pi r^3$.

ELISA assays

The kidney cells were grown in MEMα + 10% IFS in either 0.1% oxygen or 20% oxygen for 48 hr. The mammary cells were grown in a 1:1 ratio of DMEM and F12 media with 5% fetal calf serum and 10 μg/ml insulin, 10 ng/ml hEGF, and 1 μg/ml hydrocortisone (Sigma Chemicals, St. Louis, Missouri). The conditioned media were filtered through 0.45 μm syringe filters, and the levels of VEGF were measured using an ELISA kit from R&D that was specific for either murine or human VEGF (Minneapolis, Minnesota).

VEGF levels were normalized against total protein from the cells used in the assay.

Western blotting

For Western blot analysis, the human embryonic kidney-derived cells were grown in MEMα containing 10% IFS and then switched to MEMα containing 0.1% inactivated fetal calf serum (IFS) for 12 hr. The mammary epithelial-derived cells were grown in a 1:1 mixture of DMEM and F12 + 5% fetal calf serum (FCS) with 10 μg/ml insulin, 10 ng/ml hEGF, and 1 μg/ml hydrocortisone (Sigma Chemicals) and then switched to DMEM containing 2.5% of the standard growth media for 24 hr. For experiments involving kidney cells expressing DNMycER, cells were switched to MEMα containing 0.1% IFS for 4 hr followed by addition of 100 nM 4-OH Tamoxifen for 8 hr (Sigma Chemicals). For experiments utilizing the chemical inhibitors (Calbiochem, San Diego, California), cells were grown in MEMα containing 0.1% IFS for 4 hr followed by addition of 10 μM LY294002, 5 μM UO126, or 10 μM Y27632 for 8 hr. Human breast cancer cell lines MDA-MB-231, MDA-MB-435, MCF-7 were grown in DMEM containing 10% IFS and were switched to 0.1% IFS for 2 hr followed by treatment with 10 μM LY294002, Y27632 or mock treatment for 8 hr. SkBr3 and BT549 were grown in RPMI containing 10% FCS and switched to 0.1% FCS for 2 hr followed by treatment with 10 μM LY294002, Y27632 or mock treatment for 8 hr.

Cells were lysed in 50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1% NP40, 1 mM sodium orthovanadate, 5 mM NaF, 20 mM β-glycerophosphate, and complete protease inhibitor (Roche). Fifty micrograms of protein, as determined by the BioRad protein assay (Bio-Rad, Hercules, California), were loaded per well onto a 4%–12% pre-cast polyacrylamide gradient gel (Invitrogen, Carlsbad, California). The extracts were electrophoresed and transferred to an Immobilon-P membrane (Millipore, Bedford, Massachusetts). The membranes were blocked in 5% nonfat milk and incubated in primary antibody to Ras (c-20, Santa Cruz Biotechnology), Tsp-1 (Ab11, Lab Vision, Fremont, California), β-actin (Abcam, Cambridge, United Kingdom), c-*myc* (hybridoma 9E10), phospho-c-*myc*, phospho-Akt, and phospho-p44/42 ER (Cell Signal Transduction, Beverly, Massachusetts). The membranes were then washed in PBS + 0.1% Tween-20 and incubated with either HRP-conjugated goat anti-mouse or goat anti-rabbit secondary antibody (Jackson Immunoresearch Laboratories, West Grove, Pennsylvania) for another wash. The membranes were then developed with Supersignal Ultura extended (Pierce Chemicals, Rockford, Illinois) and exposed to film.

Rho-GTP assays

The level of GTP bound Rho was assayed by lysing (in 25 mM Tris-Cl [pH 7.5], 150 mM NaCl, 5 mM $MgCl_2$, 1% NP-40, 1 mM DTT, and 5% glycerol plus complete protease inhibitors [Roche], serum starved kidney cells expressing no Ras, low Ras, or high Ras for 12 hr, in the presence or absence of LY294002 for the last 8 hr and then incubation with GST-Rhotekin and GST swell gel for 1 hr (Pierce Chemical). The GST-Rhotekin containing gel was then washed three times with lysis buffer. Bound protein was then eluted by addition of 2× sample loading buffer (125 mM Tris-Cl [pH 6.8], 2% glycerol, 4% SDS, 0.05% bromophenol blue, and 100 mM β-mercaptol ethanol) and boiling at 100°C. Western blotting was then performed as described above.

Transient transfection

Kidney-derived cells expressing either low or high levels of oncogenic H-RasV12 were transiently transfected with 5 μg pCMV2-Flag or pCMV2-FLAG expressing wtMyc, S62AMyc, S71AMyc (gifts from Yoshiyuki Kuchino, National Cancer Center Research Institute, Tokyo, Japan), pBabepuro-MycER (a gift from Gerard Evan, UCSF), or pEXVRhoAV14 using FuGENE 6 transfection reagent. The media was changed 12 hr posttransfection, and 12 hr later, the cells were switched to media containing 0.1% IFS. Cells transfected with pBabepuroMycER were grown in 0.1% serum for 6 hr followed by addition of 4-HT for 18 hr. Cells were harvested and lysed after an additional 24 hr and analyzed by Western blot as described above.

Ribonuclease protection assays

Human embryonic kidney-derived cells, described above, were transfected with 5 μg of pCMV2-Flag or pCMV2-Flag expressing wtMyc, S62AMyc, or S71AMyc. Following serum deprivation, RNA was prepared from transfected cells using the Trizol protocol (Invitrogen). The probe specific for Cyclophilin was prepared via T7 in vitro transcription from linearized pTRIPLEscript-cyclophylin (Ambion, Austin, Texas) incorporating [α-^{32}P] UTP (NEN, Boston, Massachusetts) using MaxiScript T7 kit (Ambion). The probe specific for ODC was prepared via T7 in vitro transcription from linearized pDP18-ODC incorporating [α-^{32}P] UTP using MaxiScript T7 kit (Ambion). RPAs were then performed using the RPA III kit (Ambion). The protected fragments were run on a Criterion 5% TBE-Urea gel (BioRad, Hercules, California), dried on 3 mm filter paper, and visualized by autoradiography.

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