

Activated Ras induces a proangiogenic phenotype in primary endothelial cells

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Angiogenic factors alter endothelial cell phenotype to promote the formation of new blood vessels, a process critical for a number of normal and pathological conditions. Ras is required for the induction of the angiogenic phenotype in response to vascular endothelial growth factor (VEGF). However, VEGF generates many signals, several of which are not dependent upon Ras activation. Our current study investigates the sufficiency of Ras activation for driving angiogenic responses. An activated Ras^{V12} mutant induces prominent membrane ruffling, branching morphogenesis on three-dimensional collagen, DNA synthesis, and cell migration in primary endothelial cells. An upregulation of PI3K/AKT, Erk, and Jnk signaling pathways accompany these phenotypic changes. The inhibition of Erk blocked cell proliferation, but only partially attenuated migration. Blocking PI3K had no effect on DNA synthesis, but caused a modest reduction in cell migration. Lastly, Jnk played a significant role in both the proliferation and migration response. These effects of Ras^{V12} are not the result of increased autocrine secretion of VEGF. These data suggest that the acquisition of activating Ras mutations can lead to a proangiogenic conversion in the phenotype of primary endothelial cells. Furthermore, these data raise the possibility that chronic Ras activation in endothelial cells may be sufficient to promote angiogenesis and the development of vascular anomalies.

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Introduction

Endothelial cells line blood vessels as a quiescent barrier maintaining the homeostasis of tissues and organs in the body. When new vessels are required to vascularize hypoxic tissues, heal wounds, or nourish tumors, endothelial cells undergo changes in cell phenotype,

including increased migration, proliferation, and tubulogenesis, collectively termed angiogenesis (Carmeliet and Jain, 2000). The molecular regulation of the angiogenic phenotype requires coordinated input from a number of signaling molecules. One potential important regulator of the angiogenic response is the small molecular weight GTPase Ras, which is proximally positioned upstream of a number of important signal transduction networks (Ehrhardt *et al.*, 2002).

Recently, we reported that Ras activation is required for the proangiogenic response to vascular endothelial cell growth factor (VEGF), including proliferation, migration, and branching morphogenesis (Meadows *et al.*, 2001). Several lines of evidence implicate Ras in developmental angiogenesis. Mice deficient in either p120-rasGAP or NF-1, proteins that facilitate Ras inactivation, fail to form organized vascular networks (Henkemeyer *et al.*, 1995; Gitler *et al.*, 2003). Similarly, deletion of *Sos1*, a positive regulator of Ras activation, leads to cardiovascular and yolk sack defects, and embryonic lethality (Wang *et al.*, 1997). Lastly, disruption of the Ras effector, B-Raf, results in vascular defects in mice and mid-gestational death (Wojnowski *et al.*, 1997).

Activating Ras mutations have also been linked to the formation of endothelial-derived tumors, including liver angiosarcomas and cardiac hemangiosarcomas induced by chemical carcinogens (Marion *et al.*, 1991; Hong *et al.*, 2000). In addition, immortalized endothelial cells expressing oncogenic Ras converted to an angiogenic phenotype and formed angiosarcomas in mice (Arbiser *et al.*, 1997). However, it is unclear from these studies if activation of Ras in endothelial cells was sufficient to mediate this phenotype switch, or if it required immortalization. In addition, the tumor growth and angiogenic conversion may have been the result of changes in the paracrine expression of VEGF induced by Ras (Arbiser *et al.*, 1997), and ultimately the generation of a number of Ras-independent signals, as is well documented in tumors of several origins (Rak *et al.*, 2000a, b). Therefore, we sought to determine if Ras activation is sufficient to induce the initiation of the angiogenic phenotype in primary endothelial cells.

Using adenovirus delivery of a Ras^{V12} mutant to primary endothelial cells, we found that Ras activation is sufficient to drive branching morphogenesis, cell migration, membrane ruffling, and cell cycle progres-

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sion. An increase in Akt, Erk, and Jnk signaling accompanied these changes. The signaling requirements for the angiogenic change in phenotype bifurcated; proliferation required ERK, while migration was influenced by both ERK and PI3K. Taken together, these results suggest that Ras is capable of driving an angiogenic switch in the phenotype of primary endothelial cells, in the absence of immortalization or angiogenic factors.

Results

To introduce activated Ras into primary endothelial cells, we generated an adenovirus carrying an active HA-tagged Ras^{V12} mutant, coexpressing a GFP marker. Primary endothelial cells were infected with adenoviruses at an MOI of 5–10. Under these conditions, we observed no cellular toxicity. Western blotting confirmed expression of the HA-tagged Ras^{V12} mutant. Ad.GFP and Ad.Ras^{V12} infected cells to similar levels as demonstrated by levels of GFP expression (Figure 1a) and as verified by fluorescence microscopy (data not shown).

Next, we evaluated the activation of Erk, PI3K, and SAPK/Jnk in endothelial cells expressing Ras^{V12}. Growth factors often stimulate these signals in a Ras-dependent manner (Minden *et al.*, 1994; Klinghoffer *et al.*, 1996; Matsumoto *et al.*, 1999; Meadows *et al.*, 2001), and these signals are known to regulate cell responses critical for angiogenesis including motility, growth, and survival (Fox *et al.*, 1996; Thakker *et al.*, 1999; Yu and Sato, 1999; Meadows *et al.*, 2001).

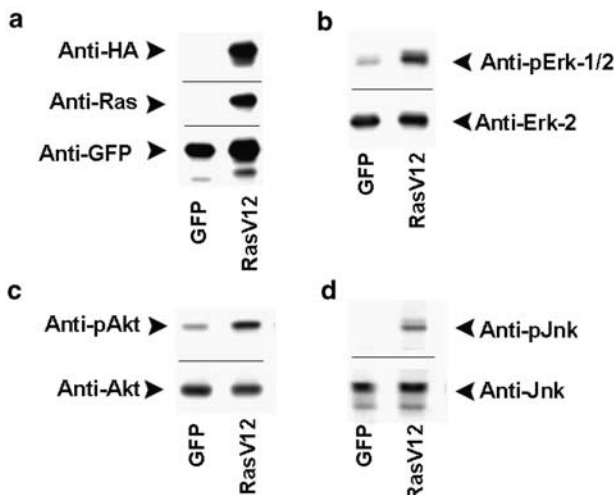


Figure 1 Ras^{V12} alters intracellular signals in primary endothelial cells. (a) Western blot analysis using an HA and Ras antibody confirms the expression of Ad.Ras^{V12} in HUVEC lysates. Anti-GFP shows GFP levels in Ad.GFP- and Ad.Ras^{V12}-infected cells. (b–d) HUVECS infected with Ad.GFP or HA-Ras^{V12} under serum-free conditions were probed with antibodies to phosphorylated Erk, Jnk, and Akt. The major phosphor-Erk band corresponds to Erk-2. Blots were stripped and re probed for the respective proteins in their unphosphorylated state

Western blotting with phosphospecific antibodies to Erk, Jnk, and Akt, a kinase activated downstream of PI3K, demonstrated that all these signals were elevated in response to activated Ras (Figure 1b–d).

In the presence of angiogenic factors, endothelial cells elongate, scatter, and form branching networks in three-dimensional cultures (Montesano *et al.*, 1983). We previously demonstrated that branching morphogenesis, in response to VEGF, is Ras dependent (Meadows *et al.*, 2001). In our current study, we sought to determine whether Ras activation alone would be sufficient to drive this response. We found that cells expressing Ras^{V12} formed branching networks on collagen gels in the absence of added angiogenic factors; in contrast, cells expressing GFP formed monolayer structures with only occasional sprouts (Figure 2). Branching morphogenesis is a complex phenotype mediated by a combination of growth factor signaling, matrix interactions, and physical forces. We sought to define further the effects of Ras activation on individual endothelial cell phenotypes altered in response to angiogenic stimuli, that is, cell migration and proliferation.

The migration of endothelial cells is a major component of the angiogenic response (Carmeliet, 2000), and several reports link Ras signaling to cell

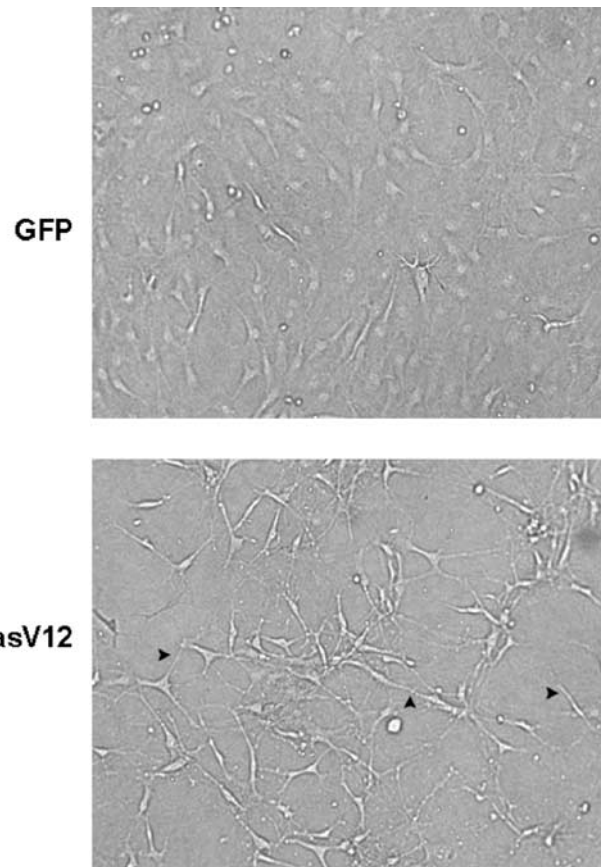


Figure 2 Ras^{V12} induces branching morphogenesis in BLMVECS. BLMVECS infected with Ad.GFP or Ad.Ras^{V12} were seeded into 12 wells containing three dimension-collagen gels in the presence of reduced serum (2.5% FBS) media. After 48 h, cells were fixed and photographed. Arrows indicate branching structures

motility (Sosnowski *et al.*, 1993; Fox *et al.*, 1994; Suzuki *et al.*, 2000; Meadows *et al.*, 2001). We investigated whether Ras activation is sufficient to drive endothelial cell motility. Ad.Ras^{V12} induced prominent membrane ruffles and loss of stress fibers in human umbilical vein endothelial cells (HUVECS) cultured on glass coverslips coated with collagen, while Ad.GFP-infected cells maintained a mix of circumferential and transcellular actin fibrils, with no distinct lamellapodia (Figure 3a). As the induction of membrane ruffles is a morphological phenotype characteristic of motile cells (Ridley *et al.*, 1992), we measured the effect of Ras^{V12} expression on endothelial migration. Ad.Ras^{V12} promotes a 2.5-fold increase in cell migration in the absence of angiogenic stimuli (Figure 3b), a response equivalent to the VEGF-induced cell migration in control GFP cells. Interestingly, VEGF further enhanced the Ras^{V12} migratory

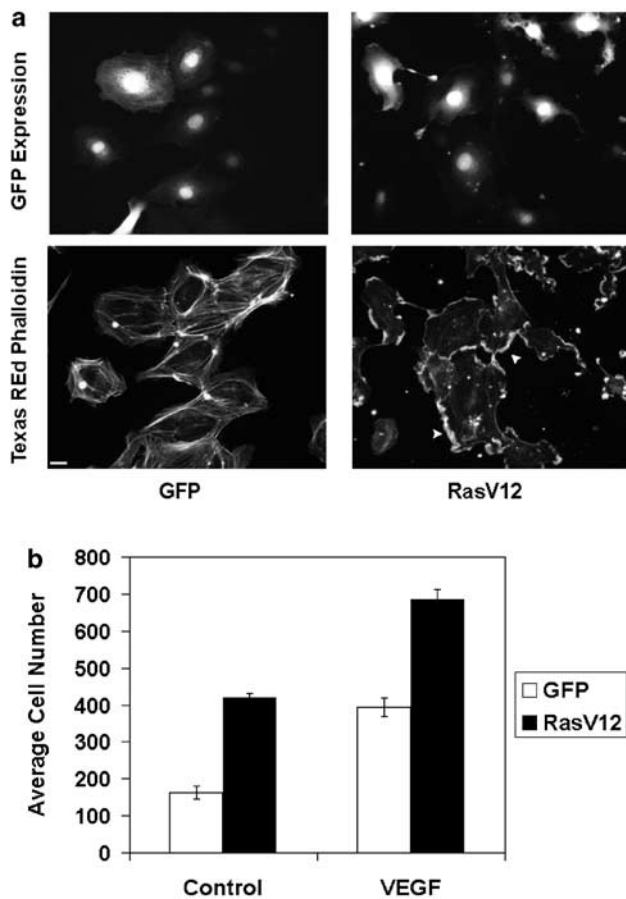


Figure 3 Expression of HA-Ras^{V12} enhances cell motility in HUVECS. **(a)** HUVECS infected with Ad.GFP or HA-Ras^{V12} were seeded onto glass coverslips for 4 h. Cells were labeled with Texas Red-X Phalloidin (red); the GFP marker indicates infected cells (green). Images were captured under $\times 40$ magnification. Scale bar, 20 μ m, arrows indicate membrane ruffling. **(b)** HUVECS infected with Ad.GFP or Ad.Ras^{V12} were seeded onto collagen-coated tissue culture inserts ± 50 ng/ml VEGF in the outer chamber. After 4 h, cells that crossed through the transwell were stained with Hoechst dye, visualized with a digital fluorescent microscope, and counted. The results are from a single experiment with values representing the average cell number \pm s.e.m. Similar results were found in three independent experiments

response, suggesting that VEGF may regulate the migration response through a distinct pathway.

Endothelial cell proliferation is also required for new blood vessel growth (Takeshita *et al.*, 1995; Carmeliet, 2000). Our previous studies demonstrated that Ras activity was essential for VEGF-induced DNA synthesis (Meadows *et al.*, 2001). Therefore, we were interested in the effect of activated Ras^{V12} on cell cycle progression in primary endothelial cells. Oncogenic Ras exerts either positive or negative effects on growth, depending on the cellular context. Active Ras induces proliferation and cellular transformation in immortalized cells (Land *et al.*, 1983; Newbold and Overell, 1983; Yoakum *et al.*, 1985) and some primary cell types (Gire *et al.*, 1999). In contrast, several primary cell types exhibit cycle arrest (Serrano *et al.*, 1997; Woods *et al.*, 1997; Goi *et al.*, 1999). We found that the expression of Ras^{V12} resulted in an increase in DNA synthesis, as measured by [³H]thymidine incorporation (Figure 4a). Similar results were found when we performed this experiment using BrdU incorporation; an assay normalized for cell number, demonstrating that this effect was not due to survival differences (not shown). VEGF did not further augment the Ras^{V12} response. Western blotting showed an increase in Cyclin D1 expression in cells infected with Ad.Ras^{V12} (Figure 4b), consistent with increased proliferation (Weber *et al.*, 1997; Gille and Downward, 1999). In contrast, no substantial increases in p21^{CIP/WAF} or p27^{kIP} were detected (Figure 4b), proteins often associated with Ras/Raf-mediated cell cycle arrest (Lloyd *et al.*, 1997; Pumiglia and Decker, 1997; Sewing *et al.*, 1997; Woods *et al.*, 1997).

Levels of ERK and PI3K activity are enhanced in response to activated Ras (Figure 1). These effector pathways have been linked to cell proliferation and motility in some cases, but not in others (Klemke *et al.*, 1997; Reiske *et al.*, 1999; Meadows *et al.*, 2001; Zeng

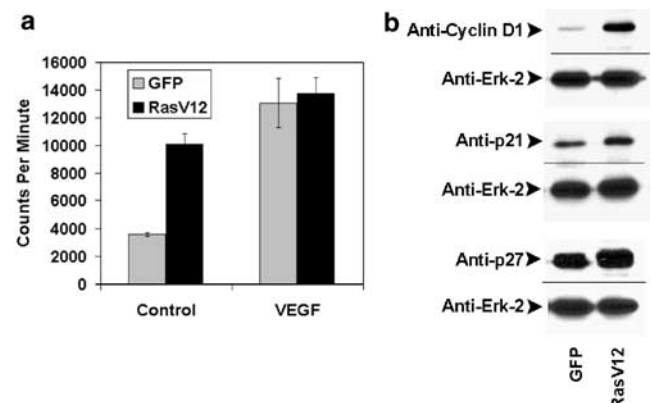


Figure 4 Ras^{V12} induces cell cycle progression in HUVECS. **(a)** Cells infected with Ad.GFP or Ad.Ras^{V12} for 24 h were stimulated \pm VEGF for 16 h and pulsed with [³H]thymidine for 3 h. A scintillation counter was used to detect [³H]thymidine incorporation. The results are from a single experiment and represent an average of the counts per minute \pm s.e.m. Similar results were found in three independent experiments. **(b)** Cells infected with Ad.GFP or Ad.Ras^{V12} were lysed, resolved using SDS-PAGE, and blotted with antibodies to cyclin D1, P21^{WAF/CIP} or p27; blots were reprobbed with Anti-Erk2 to normalize loading

et al., 2001; Goncharova *et al.*, 2002). We sought to determine if Erk and PI3K were signaling intermediates in Ras^{V12}-induced proliferation and migration in endothelial cells, utilizing the well-characterized pharmacological inhibitors LY294002 (inhibits PI3K activity) and U0126 (inhibits Erk activation). The effectiveness of these agents was documented under conditions identical to those described for the migration and proliferation assays. We show that LY294002 (10 μ M) completely inhibited the phosphorylation of Akt (a PI3K-dependent response) induced by Ras^{V12} (Figure 5a). Similarly, Ras^{V12}-mediated Erk phosphorylation was inhibited by incubation with 1 μ M U0126 (Figure 5a).

We used U0126 and LY294002 to investigate the role of the Erk and PI3K signals in mediating the Ras-induced proliferation response. While inhibiting PI3K caused no change in cell proliferation, inhibiting Erk activation reduced DNA synthesis mediated by Ras^{V12} to levels observed in serum-starved cells expressing GFP alone (Figure 5b). As the basal levels of DNA synthesis were suppressed following inhibition of ERK, there appears to be an inductive response to Ras that is Erk independent. However, the induction seen under these conditions only achieves levels seen under quiescent conditions.

Cell migration was stimulated in Ad.Ras^{V12}-infected cells despite the presence of the inhibitors LY294002 and U0126; however, each compound produced a partial inhibition of the migratory response (Figure 5c). These data suggest that the two pathways may be acting in a cooperative or parallel manner to regulate migration. In order to follow up on this possibility, we added both inhibitors simultaneously, resulting in complete inhibition of the Ras^{V12}-induced migration response (Figure 5d).

Ras^{V12} stimulates Jnk activation and this coupled with the demonstrated effects of Jnk-kinase in mediating the effects of VEGF (Pedram *et al.*, 1998) led us to investigate the role of Jnk in Ras^{V12}-induced cell cycle progression and migration. We utilized a kinase dead, Flag-tagged dominant-negative Jnk mutant to inhibit Jnk signal transduction (Li *et al.*, 1996; Dan *et al.*, 2002). Western blot analysis with antibodies to the Flag and HA tags confirmed the expression of our mutant proteins (Figure 6a). We found that Jnk mediates both cell proliferation and migration stimulated by activated Ras. Endothelial cells coinfecting with dominant-negative Jnk mutant and Ras^{V12} showed a reduction in Sphase entry when compared to cells infected with Ras^{V12} alone (Figure 6b). In addition, dominant-negative Jnk inhibited the number of migrating cells stimulated by Ras^{V12} (Figure 6c).

The release of angiogenic factors into the local environment is a mechanism that tumors use to recruit new blood vessels to facilitate its growth (Lazar-Molnar *et al.*, 2000). Mutant Ras proteins increase VEGF production in immortalized epithelial cells and fibroblasts transfected with oncogenic Ras (Rak *et al.*, 2000a). Therefore, we reasoned that the Ras^{V12} phenotype we observed might be the result of an autocrine secretion of VEGF stimulated by Ras. To address this

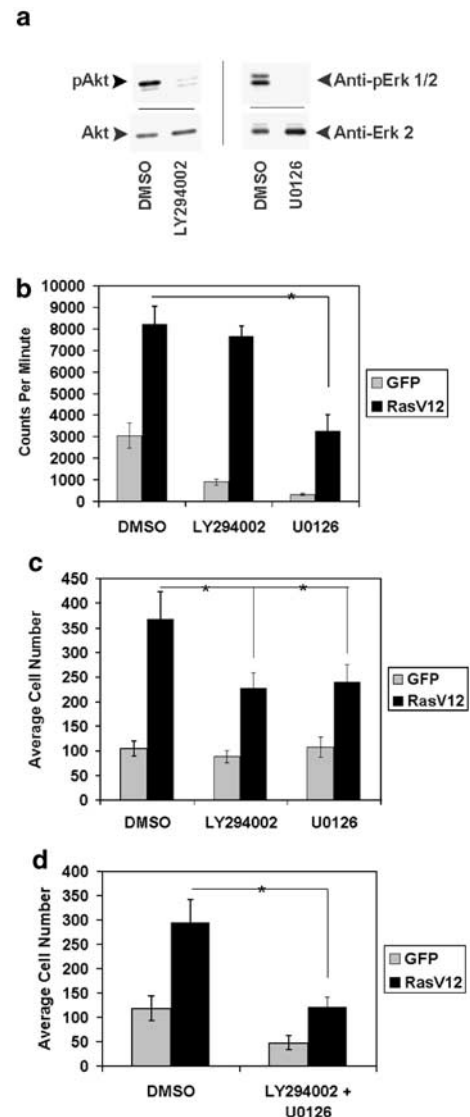


Figure 5 Erk and Akt signaling mediate Ras^{V12}-induced cell migration, while only Erk activity mediates DNA synthesis stimulated by Ras^{V12}. Western Blots for phospho-Akt (a) and phospho-Erk in Ras^{V12}-expressing cells demonstrate that pharmacological inhibitors LY294002 and U0126 inhibit PI3K and MEK activity, respectively. Blots were reprobbed with antibodies to detect total AKT and Erk2. (b) Cells were infected with Ad.GFP or Ad.Ras^{V12} for 24 h, treated with LY294002 (10 μ M) and U0126 (1 μ M) for 16 h, and pulsed with [³H]thymidine for 3 h. [³H]thymidine incorporation was detected with a scintillation counter. The results are from three-pooled experiments. Multivariate ANOVA and *post hoc* Newman-Keuls determined statistical significance between treatment groups. *P*-values <0.01. (c) Ad.GFP- and Ad.Ras^{V12}-infected cells were seeded onto collagen-coated tissue culture inserts in the presence or absence of pharmacological inhibitors LY294002 or U0126. Cells were stimulated \pm VEGF and the number of migratory cells was quantified after 4 h. (d) Migration experiments performed as in (c), except that both the inhibitors were added together

question, we measured VEGF levels in the cell lysates and conditioned media of Ad.Ras^{V12}-infected cells. As positive controls, we also analysed cells infected with Ad.VEGF or Ad.GFP and stimulated with and without VEGF (50 ng/ml). We were able to detect by Western

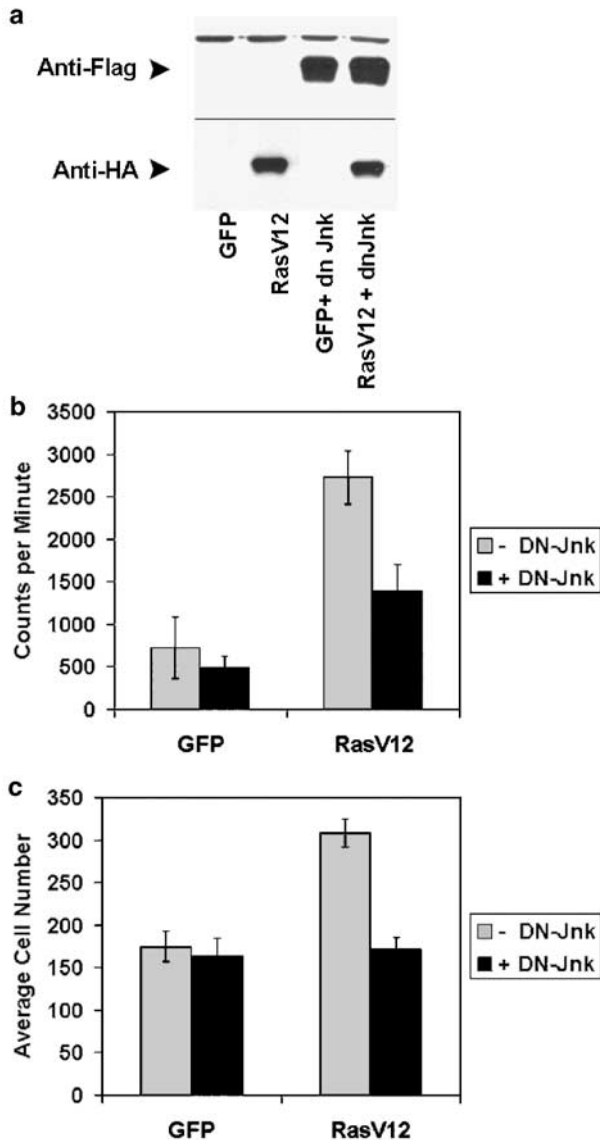


Figure 6 Jnk signaling regulates cell cycle progression and migration stimulated by Ras^{V12}. HUVECS were infected with Ad.GFP, Ad.DN-Jnk, and Ad.RasV12 or a combination of Ad.DN-Jnk and Ad.GFP or Ad.DN-Jnk and Ad.RasV12. (a) Expression of Flag-tagged DN-Jnk and HA-tagged Ras^{V12} was confirmed by Western blot analysis using antibodies to Flag and HA. (b) Cells were infected in serum-free media and incubated for 48 h. Cells were pulsed with [³H]thymidine for 3 h, and [³H]thymidine incorporation was detected on a scintillation counter. The results are from a single experiment and represent an average of the counts per minute \pm s.e.m. (c) Infected cells seeded onto collagen-coated tissue culture transwells and migrated for 4 h in the absence of growth factors. Hoechst dye was used to label cells adhered to the bottom surface of the transwells. The results are from a single experiment and represent the average number of migrating cells \pm s.e.m. Similar results were obtained in three independent experiments

analysis VEGF levels as low as 1 ng/ml (Figure 7a), which is well below the ED₅₀ for VEGF (Conn *et al.*, 1990). Western blotting demonstrated that cells infected with Ad.VEGF and Ad.GFP-infected cells treated with VEGF contained readily detectable levels of VEGF present in their conditioned media; however, no VEGF

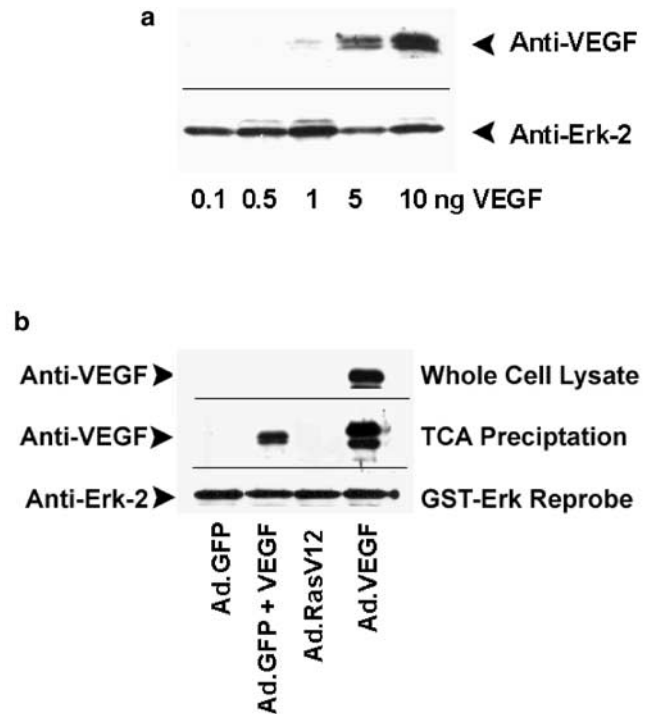


Figure 7 Absence of autocrine VEGF secretion in Ras^{V12}-expressing endothelial cells. (a) 0.1, 1, 5, and 10 ng of VEGF was TCA precipitated in the presence of GST-Erk and BSA. VEGF was detected by Western blot analysis with anti-VEGF; Erk labeling demonstrates loading. (b) HUVECS were infected with Ad.GFP, Ad.Ras^{V12}, or Ad.VEGF for 16 h, and stimulated \pm 50 ng/ml VEGF. Cells were lysed and TCA precipitated in the presence of GST-Erk and BSA. VEGF was detected by Western Blot analysis; samples were reprobated with Erk2

was detected in the conditioned media of Ad.Ras^{V12}-infected cells. We also measured whole-cell lysates, in the event that cell-secreted VEGF was strongly associated with the extracellular matrix. While cells infected with Ad.VEGF ectopically expressing VEGF contained large amounts of VEGF in whole-cell lysates, cells infected with Ad.GFP alone or Ad.Ras^{V12} contained no detectable levels of VEGF. These data suggest that Ras^{V12} does not induce an upregulation in VEGF in primary endothelial cells (Figure 7b), and that the Ras^{V12} is directly inducing the angiogenic change in cell phenotype. To confirm these observations, we measured VEGF levels using an ELISA. VEGF concentrations from the conditioned media of cells infected with GFP and Ras^{V12} were comparable, yielding 11.1 ± 0.32 and 10.7 ± 0.40 pg/ml, respectively. In contrast, VEGF was detected at a concentration of 2015.7 ± 73.7 pg/ml in conditioned media taken from a VEGF-secreting human renal cell carcinoma cell line, 786-O.

Discussion

Our data indicate that Ras activation in primary endothelial cells is sufficient to enhance several cellular phenotypes that contribute to the angiogenic response including, branching morphogenesis, cell migration, and

cell proliferation. Elevated levels of active Akt, Erk, and Jnk accompanied this change. The signaling requirements for the enhanced angiogenic phenotype were divergent. Erk, Jnk, and PI3K signaling regulate the Ras^{V12}-induced migratory response, while only Jnk and PI3K appear to control endothelial proliferation induced by Ras^{V12}. Importantly, we found no evidence of secretion of VEGF production in Ras^{V12}-expressing endothelial cells, as has been described in other cell systems, ruling this out as a mechanism driving the Ras^{V12} phenotype. This may result from the presence of normal p53 in our primary cultures, as previous investigations have demonstrated a strong repressive effect of p53 on oncogene-induced VEGF transcription (Mukhopadhyay *et al.*, 1995; Zhang *et al.*, 2000; Pal *et al.*, 2001).

Ras activation is required for motility in several systems including growth factor-induced chemotaxis in myoblasts (Suzuki *et al.*, 2000), and bFGF-mediated wound closure in endothelial cells (Sosnowski *et al.*, 1993; Fox *et al.*, 1994). In addition, we demonstrated previously a requirement for Ras activation in VEGF-induced endothelial cell migration (Meadows *et al.*, 2001). Our current findings show that Ras^{V12} is sufficient to induce a motile phenotype in primary endothelial cells. The induction of membrane ruffling by Ras^{V12} is consistent with the promigratory phenotype generated by activated Ras in fibroblasts through a Rac-dependent mechanism (Ridley *et al.*, 1992). Several papers have recently demonstrated the importance of Rac in endothelial cell motility (Soga *et al.*, 2001; Ghosh *et al.*, 2002; Zeng *et al.*, 2002). Several lines of evidence suggest that Rac activation is a likely contributor to the motile phenotype induced by Ras: (1) we observe strong activation of the Rac effector Jnk; (2) dominant-negative Jnk inhibits the Ras^{V12}-induced migratory response, and (3) a dominant-negative Rac^{N17} inhibits membrane ruffling and migration in response to Ras^{V12} (Meadows *et al.*, 2003 unpublished data).

Interestingly, VEGF stimulation further enhances the Ras^{V12} response, suggesting that there may be several parallel pathways controlling endothelial cell migration. Consistent with our hypothesis that Ras may be inducing the activation of Rac, a recent report demonstrates that activated Rac and VEGF produce additive effects on migration (Soga *et al.*, 2001). One possibility for the additive effect of VEGF may come from work demonstrating a role for p38 in VEGF-induced cell migration in endothelial cells (Rousseau *et al.*, 1997); we found that p38 is not activated by Ras (data not shown).

The expression of Ras^{V12} in immortalized cells correlates with transformation; however, expression in primary cells results in various outcomes with respect to proliferation. It promotes cell cycle arrest in some primary cell types (Serrano *et al.*, 1997; Zhu *et al.*, 1998), but stimulates cell proliferation in others (Lemoine *et al.*, 1990; Gire *et al.*, 1999). We found increased DNA synthesis following the expression of Ras^{V12}. Consistent with this, cyclin D1 levels were elevated in Ras^{V12}-infected endothelial cells, while p21^{CIP} and p27^{KIP}

levels remained largely unaffected. In contrast to the migration response to Ras^{V12}, the proliferation response seemed to show identical signaling requirements to those previously reported for VEGF (Pedram *et al.*, 1998; Meadows *et al.*, 2001; Zeng *et al.*, 2001). Interestingly, while Ras^{V12} expression does not induce acute cell cycle arrest, studies in our laboratory, investigating the role of Raf activation, demonstrate that activation of Raf in primary endothelial cells results in the induction of p21^{CIP} and premature senescence (McMullen *et al.*, 2003 unpublished data). The long-term effects of expressing oncogenic Ras in primary endothelial cells are unclear. Adaptive changes might take place beyond the window of the experiments conducted in this study, including loss of a proliferation response. This possibility is currently under investigation.

Interestingly, while the current studies were underway, two recent reports reported that oncogenic Ras promotes cell cycle arrest in primary endothelial cells mediated by p21^{CIP}. We cannot be certain as to the differences in these reported results. In the studies by Suzuki *et al.* (2002), Ras^{V12} induced cell cycle arrest in contact-inhibited endothelial cells. In contrast, our studies utilized subconfluent cells. Given the dynamic signal modulation capabilities of the adherens junctions (Conacci-Sorrell *et al.*, 2002), and the necessity of cells to maintain contact inhibition, an exciting possibility is that signals mediated by cadherin engagement may control the balance between pro- and antiproliferative signals mediated by Ras. Indeed, it has previously been demonstrated that engagement of VE-Cadherin results in the inhibition of endothelial cell proliferation (Caveda *et al.*, 1996). In the other report, Ras^{L61} induced cell cycle arrest only at higher viral titers; p21^{CIP} was not induced at lower viral titers, comparable to those used in our study (Spyridopoulos *et al.*, 2002). Previous investigators have demonstrated that differential effects on cell proliferation can be achieved by variations in the 'strength of the signal', that is, lower levels of Raf activation result in cell proliferation, while higher levels of Raf activation induce cell cycle arrest (Woods *et al.*, 1997).

Taken together, these data begin to define the complex inter-relationship between Ras activation, Ras effectors, and the manifestation of the angiogenic phenotype. In our studies, activated Ras promotes endothelial cell migration, proliferation, as well as branching morphogenesis; these are all requisite steps in new vessel formation. These data suggest that Ras activation may be sufficient to induce many of the requisite phenotypes for angiogenesis and coupled with our previous data, define Ras as a potential target for antiangiogenic therapies. Our observations also hint at an intriguing possibility: the acquisition of activating Ras mutations, through carcinogen exposure or inheritance, may create an angiogenically predisposed population of endothelial cells. Increased proliferation rates of these cells could be predisposing for the acquisition of additional mutations and eventually impact the development of vascular tumors and/or anomalies in vascular structure. Congruent with this hypothesis, Ras mutations have been documented in tumors of endothelial

cell origin that arise following carcinogen exposure (Marion *et al.*, 1991; Hong *et al.*, 2000), and introducing immortalized endothelial cells expressing active Ras into mice resulted in the development of lesions resembling angiosarcoma (Arbiser *et al.*, 1997; MacKenzie *et al.*, 2002). Similarly, a line of mice generated in the process of creating a tetracycline-regulated Ras allele developed spontaneous hemangioma (Fisher *et al.*, 2001). In the absence of secondary mutations and progression to vascular tumors, it is possible that Ras mutations will create a hyper-responsive angiogenic environment and thereby function as an important accelerant for the progression of cancer and other diseases dependent upon angiogenesis.

Materials and methods

Generation of recombinant adenovirus

An H-Ras^{V12} cDNA obtained from Channing Der (University of North Carolina, Chapel Hill, NC, USA) was PCR amplified with an N-terminal hemagglutinin tag (HA) (Meadows *et al.*, 2001). The HA-tagged Ras^{V12} was subcloned into the pAd-Track-CMV and the *AdEasy* system was used to generate recombinant adenoviruses (He *et al.*, 1998; Meadows *et al.*, 2001). Viruses were titered as previously described (O'Carroll *et al.*, 2000), and used at a multiplicity of infection (MOI) of 5. This results in moderate expression in nearly 100% of cells (not shown) as monitored by fluorescence microscopy (not shown). The GFP adenovirus was from Q-Biogene (Carlsbad, CA, USA).

Cell culture

HUVECS from pooled donors were purchased from VEC Technologies (Troy, NY, USA). Cells were cultured on tissue culture dishes coated with 0.2% gelatin at 37°C and 5% CO₂ in the presence of M199 media supplemented with 20% FBS, 1% penicillin/streptomycin, 10 µg/ml heparin, and 50 µg/ml of endothelial cell growth supplement (Collaborative Biomedical Products). Bovine lung microvessel endothelial cells (BLMVECS) were purchased from VEC Technologies (Troy, NY, USA) and were cultured in MCDB-131 medium supplemented with 20% FBS, 10 mM L-glutamine, and 1% penicillin/streptomycin on tissue culture dishes coated with 0.2% gelatin. Experiments were conducted in the presence of serum-free MCDB media supplement with or without VEGF or in MCDB containing 2.5% FBS and VEGF as indicated.

Western blotting

Western Blot analysis was conducted using the following antibodies: phospho-ERK, ERK2, p21, p27, and VEGF (Santa Cruz, Santa Cruz, CA, USA), HA (BabCo, Richmond, CA, USA), cyclin D1 (NeoMarkers, Fremont, CA, USA), phospho-Akt, Akt, phospho-Jnk, and Jnk (New England Biolabs, Beverly, MA, USA). Confluent HUVECS were infected with adenoviruses overnight in serum-free MCDB. Cells were stimulated with 50 ng/ml VEGF (R&D, Minneapolis, MN, USA) for 15 min. For phospho-Erk analysis, cells were stimulated with VEGF for 5 min, as it elicits the maximal VEGF-induced Erk activation (Meadows *et al.*, 2001). In some studies, cells were pretreated for 30 min with 10 µM LY294002 or 1 µM U0126, specific pharmacological inhibitors of PI3K

and MEK, respectively. Western blotting conditions were as previously described (Meadows *et al.*, 2001).

Branching morphogenesis assay

Collagen gels were prepared using a neutral solution of 1 mg/ml collagen in 1 × DMEM, supplemented with 50 ng/ml VEGF as indicated. Following polymerization, BLMVECS infected with Ad.GFP or Ad.Ras^{V12} were seeded at 1 × 10⁵ per 12 well and were grown in MCDB supplemented with 2.5% FBS and 1% penicillin/streptomycin for 48 h. Cells were fixed with 3.7% formaldehyde and photographed with a Spot-2 Digital Camera as previously described (Lee *et al.*, 1999; Meadows *et al.*, 2001).

Cellular migration

Transwell migration assays were performed essentially as described previously (Meadows *et al.*, 2001). Infected HUVECS were trypsinized and 5 × 10⁴ cells were seeded onto the upper well of 8 m tissue culture inserts precoated with 10 µg/ml collagen. The lower chamber contained 50 ng/ml VEGF or vehicle and the cells were allowed to migrate for 4 h. Cells were fixed in 3.7% formaldehyde/PBS and quantified (Meadows *et al.*, 2001).

Labeling of actin cytoskeleton

HUVECS infected with Ad.GFP or Ad.HA-Ras^{V12} were trypsinized and 5 × 10⁴ cells were seeded onto 12 mm circle glass coverslips coated with 10 µg/ml collagen. After 4 h, in order to mimic the migration assay, cells were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100/PBS for 30 min and stained for filamentous actin with 0.5 U/ml of Texas Red-X Phalloidin (Molecular Probes) as previously described (Meadows *et al.*, 2001).

Measurement of DNA synthesis

HUVECS were seeded at 1.2 × 10⁴ cells per 24 well in the presence of complete M199 media and allowed to adhere overnight. Cells were infected with Ad.GFP or Ad.HA-Ras^{V12} for 16 h in serum-free MCDB-131, and stimulated with or without 25 ng/ml VEGF for an additional 16 h. In some experiments, cells were preincubated with inhibitors LY294002 or U0126 for 16 h prior to the addition of VEGF. S-phase nuclei were labeled by incubating cells with 0.1 µCi of [³H]thymidine for 3 h. Cells were treated with 10% TCA and recovered in 0.5 ml solvable tissue and gel solubilizer (Packard Bioscience). Samples were transferred to Ultima Gold Scintillation Fluid (Packard Bioscience). Incorporation of [³H]thymidine was measured with a scintillation counter.

VEGF detection

Proteins from cell supernatants of HUVECS infected with Ad.GFP, Ad.Ras^{V12}, Ad.VEGF, and Ad.GFP stimulated with 50 ng/ml VEGF were precipitated with TCA. The supernatants were spiked with 500 ng of GST-Erk to control for equal recovery and 10 µg of BSA to serve as a protein carrier. Proteins were pelleted and washed several times with cold acetone. Pellets resuspended in Laemmli sample buffer were sonicated and analysed by Western blotting.

VEGF concentrations of conditioned media from cells infected with Ad.GFP and Ad.Ras^{V12} for 24-h were determined by ELISA using a Quantikine human VEGF kit (R&D, Minneapolis, MN, USA). A VEGF secreting human renal cell carcinoma cell line 786-0 was used as a positive control. A

VEGF standard curve ranging from 15 to 1000 pg/ml was used to calculate VEGF concentrations.

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