K-Ras Promotes Angiogenesis Mediated by Immortalized Human Pancreatic Epithelial Cells through Mitogen-Activated Protein Kinase Signaling Pathways

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Abstract

Activating point mutations in the K-Ras oncogene are among the most common genetic alterations in pancreatic cancer, occurring early in the progression of the disease. However, the function of mutant K-Ras activity in tumor angiogenesis remains poorly understood. Using human pancreatic duct epithelial (HPDE) and K-Ras4B^{G12V}– transformed HPDE (HPDE-KRas) cells, we show that activated K-Ras significantly enhanced the production of angiogenic factors including CXC chemokines and vascular endothelial growth factor (VEGF). Western blot analysis revealed that K-Ras activation promoted the phosphorylation of Raf/mitogen-activated protein kinase kinase-1/2 (MEK1/2) and expression of c-*Jun*. MEK1/2 inhibitors, U0126 and PD98059, significantly inhibited the secretion of both CXC chemokines and VEGF, whereas the c-*Jun* NH$_2$-terminal kinase inhibitor SP600125 abrogated only CXC chemokine production. To further elucidate the biological functions of oncogenic K-Ras in promoting angiogenesis, we did *in vitro* invasion and tube formation assays using human umbilical vein endothelial cells (HUVEC). HUVEC cocultured with HPDE-KRas showed significantly enhanced invasiveness and tube formation as compared with either control (without coculture) or coculture with HPDE. Moreover, SB225002 (a CXCR2 inhibitor) and 2C3 (an anti-VEGF monoclonal antibody) either alone or in a cooperative manner significantly reduced the degree of both Ras-dependent HUVEC invasiveness and tube formation. Similar results were obtained using another pair of immortalized human pancreatic duct–derived cells, E6/E7/st and its oncogenic K-Ras variant, E6/E7/Ras/ st. Taken together, our results suggest that angiogenesis is initiated by paracrine epithelial secretion of CXC chemokines and VEGF downstream of activated oncogenic K-Ras, and that this vascular maturation is in part dependent on MEK1/2 and c-*Jun* signaling. (Mol Cancer Res 2009;7(6):799–808)

Introduction

Pancreatic cancer is the fourth leading cause of cancer-related deaths in the United States, with approximately 32,000 newly diagnosed cases and an equal number of deaths occurring annually (1). The poor prognosis of pancreatic cancer is attributable to its tendency for late presentation, aggressive local invasion, early metastases, and poor response to chemotherapy (2). As a result, a better understanding of the fundamental nature of this cancer is needed to improve the clinical outcome. The majority of pancreatic cancers arise from cells of ductal origin, and one of the earliest genetic events in the progression of these normal ductal epithelia to premalignant pancreatic intraepithelial neoplasia is mutation of the K-Ras oncogene (3, 4). Moreover, because mutational activation of Ras proteins is seen with such high frequency (90%) in pancreatic ductal adenocarcinoma (5), it is reasonable to consider that clarifying the role of K-Ras in pancreatic cancer carcinogenesis and targeting this signaling pathway is fundamental to improving clinical response.

The growth of malignant solid tumors is dependent on the development of new blood vessels that provide oxygen and nutrients to the tumor cells (6), and it is well established that tumor growth beyond the size of 1 to 2 mm is angiogenesis-dependent (7-9). Furthermore, given that pancreatic cancer usually presents clinically with distal metastasis and this malignant spread is often via the vasculature, neoangiogenesis is a critical element of both primary tumor growth and subsequent spread of the disease. Because oncogenic K-Ras mutation is one of the earliest

genetic events in the progression of these normal ductal epithe-
lia to premalignant pancreatic intraepithelial neoplasia, it is rea-
sonable to hypothesize that angiogenesis is affected by
increased K-Ras signaling. However, little is known about the
role of oncogenic K-Ras mutation in angiogenesis in the early
stages of pancreatic cancer.

Angiogenesis is a complex process involving extracellular
matrix remodeling, endothelial cell migration and proliferation,
and capillary tube formation (10). Angiogenesis is determined
by a balance between angiogenic and angioinhibitory factors
(11, 12). Many reports have shown the expression of various
proangiogenic factors in pancreatic cancer angiogenesis.
Among them, vascular endothelial growth factor (VEGF) and
CXC chemokines, including CXCL1/growth-related onco-
gene-α, CXCL5/epithelial-neutrophil activating protein-78,
and CXCL8/interleukin-8, were described as key players of an-
giogenesis in pancreatic cancer (13-15). Ikeda et al. showed the
relation between K-Ras gene and VEGF expression by quanti-
tative reverse transcriptase-PCR (RT-PCR) analysis and immu-
nohistochemical analysis (16). However, the biological role of
oncogenic K-Ras in VEGF production from pancreatic duct ep-
ithelial cells has not been clearly elucidated. Also, there are few
reports detailing the correlation between K-Ras mutation and
CXC chemokine expression in pancreatic cancer.

In the present study, we show that oncogenic K-Ras pro-
motes the production of angiogenic CXC chemokines and
VEGF from immortalized human pancreatic duct–derived epi-
thelial cells, and that this enhancement is in part dependent on
mitogen-activated protein kinase kinase-1/2 (MEK1/2) and c-
Jun signaling. Our in vitro biological assays also showed that
up-regulated VEGF and CXC chemokine secretion enhance the
invasion and tube formation potencies of human umbilical vein
endothelial cells (HUVEC). To our knowledge, this is the first
report describing the biological effects of the oncogenic K-Ras
on angiogenesis in human pancreatic duct epithelial (HPDE)
cells.

Results

Expression of Oncogenic K-Ras Activates Multiple Downstream Effector Pathways in HPDE-KRas Cells

We initially confirmed up-regulated Ras activation in
HPDE-KRas cells by Ras-GTP-Raf affinity precipitation assay
(Fig. 1A) as first shown by Tsao and colleagues (17). We next
examined the effect of oncogenic K-Ras on the proliferation
and invasion of HPDE cells. Despite the activation of several
growth-promoting (VEGF, pMEK1/2, c-Jun; Fig. 1B) and anti-
apoptotic and survival (cIAP1, Bcl-2, Bcl-xL, cyclin D1;
Fig. 1C) pathways, mutant K-Ras did not affect either prolifer-
ative (in agreement with previous observations; ref. 17) or in-
vasive potency in HPDE cells (Supplementary Fig. S1). Based
on these data, we focused our attention on the paracrine effects
resulting from oncogenic K-Ras expression in epithelial cells.

Because Raf kinases are commonly activated by GTP-
loaded K-Ras, we sought to determine if the Raf/MEK/extra-
cellular signal-regulated kinase (ERK) pathway is persistently
activated in HPDE-KRas cells. Although the levels of activated
MEK1/2 were increased in HPDE-KRas compared with control
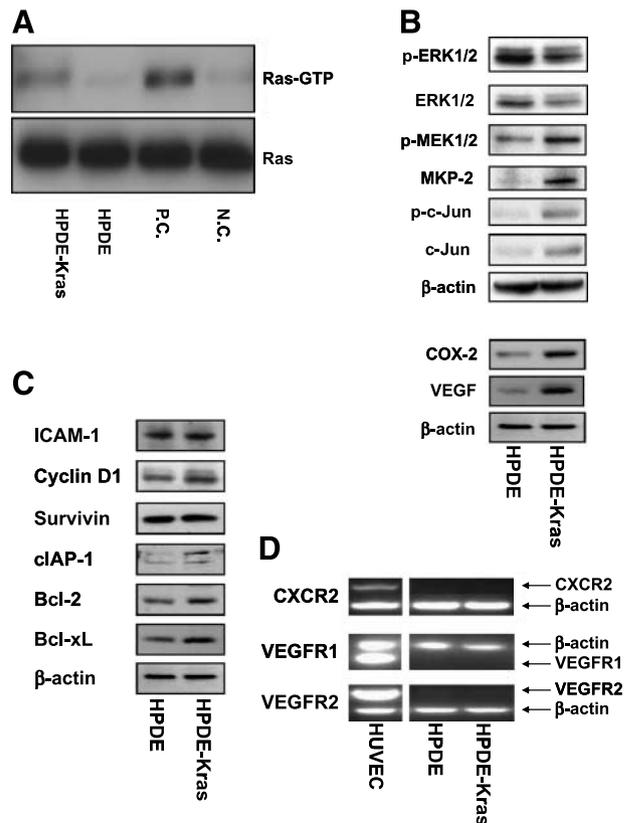HPDE cells, expression of both activated and total ERK1/2 was

FIGURE 1.   Oncogenic K-ras stimulates downstream signaling of
multiple effectors. **A.** Confirmation of K-Ras oncogene expression in
HPDE-KRas: total and GTP-bound Ras proteins were detected by West-
ern blotting using a pan-Ras antibody. Positive controls (P.C.) and nega-
tive controls (N.C.) for Ras activation as described in Materials and
Methods. **B** and **C.** Activation of regulatory, signaling, and survival pro-
teins in HPDE-KRas cells. **B,** whole-cell lysate Western blot analyses
for p-ERK1/2, total ERK1/2, pMEK1/2, MKP-2, p-c-Jun, total c-Jun,
COX-2, VEGF, and β-actin (n = 3 independent experiments); **C,** ICAM-
1, cyclin D1, survivin, cIAP-1, Bcl-2, and Bcl-xL (n = 2 independent experi-
ments) as described in Materials and Methods. **D.** Detection of CXCR2,
VEGFR1, and VEGFR2 mRNA in HPDE and HPDE-KRas cells by RT-
PCR as described in Materials and Methods. HUVEC transcript was used
as a positive control.

decreased. This contrasts with observations made in the major-
ity of cell models of Ras transformation, in which ectopic ex-
pression of mutant Ras causes persistent ERK activation (18,
19). However, these data are concordant with those reported
for pancreatic carcinoma cell lines, in which elevated MEK
phosphorylation was independent of ERK activation (20), and
previous reports have shown that mitogen-activated protein ki-
nase phosphatase-2 (MKP-2) expression increased with the ac-
tivation of MEK (21). We also found that MKP-2 expression in
HPDE-KRas was significantly higher than that of HPDE, pos-
sibly contributing to decreased steady-state phosphorylated
ERK1/2 (p-ERK1/2). Whereas previous reports showed that
up-regulated ERK phosphorylation downstream of oncogenic
K-Ras in these cells, those data resulted from confluent cultures
under starvation conditions (17). We did not starve subconflu-
ent populations of cells prior to harvesting for Western blotting,
and the differences in these culture conditions may also have
contributed to the decreases in p-ERK that we consistently

observed. We next determined the activation of several other known Ras effector pathways in this model system. Compared with HPDE cells, the mutant Ras-expressing counterparts showed elevated levels of both total c-*Jun* and activated c-*Jun* (Fig. 1B). We did an electrophoretic mobility shift assay to assess the effect of oncogenic K-Ras on nuclear factor κB (NF-κB) activity. Mutant K-Ras did not elevate the NF-κB activity in HPDE cells (data not shown). Finally, using Western blot analysis, we showed elevated expression of both the angiogenic enzyme cyclooxygenase-2 (COX-2) and the angiogenic cytokine VEGF as a consequence of oncogenic K-Ras mutation (Fig. 1B).

Expression of CXCR2, VEGFR1, and VEGFR2 in HPDE and HPDE-KRas Cell Lines

As our focus of this study was K-Ras–induced CXC chemokine and VEGF secretion from pancreatic ductal epithelium cells as paracrine stimuli on vascular endothelial cells, we investigated the expression of the target receptors CXCR2, VEGFR1, or VEGFR2 in HPDE and HPDE-KRas cells to reveal any potential autocrine effects of these cytokines. We used HUVEC as a positive control. No mRNA expression of these receptors could be seen in parental HPDE or HPDE-KRas cell lines (Fig. 1D). We further showed a lack of autocrine signaling for these cytokines in both HPDE cell lines by performing an *in vitro* proliferation assay (MTS assay). Agonists CXCL8 and VEGF or inhibitors SB225002 and 2C3 did not affect either HPDE or HPDE-KRas proliferation (Supplementary Fig. S2).

Oncogenic K-Ras Induces Both CXC Chemokines and VEGF Production from HPDE Cells through MEK and/or c-Jun Pathways

To evaluate whether oncogenic K-Ras expression promotes the production of angiogenic factors from HPDE cells, we next measured the concentrations of CXC chemokines or VEGF in HPDE or HPDE-KRas supernatants by ELISA. K-Ras activation significantly enhanced both CXC chemokine and VEGF production from HPDE cells (Fig. 2A and B).

Because K-Ras activated the downstream signaling molecule pMEK1/2, we examined the effect of MEK1/2 inhibitors U0126 and PD98059 on Ras-induced CXC chemokines and VEGF production. Both inhibitors significantly reduced both CXC chemokines and VEGF production driven by oncogenic K-Ras (Fig. 2C and D).

FIGURE 2. Oncogenic K-Ras expression in HPDE-Ras cells triggers the secretion of CXC and VEGF chemokines via mitogen-activated protein kinase pathways. **A** and **B.** Cultured (24 or 48 h) media from HPDE and HPDE-KRas cells were collected. The summed (Σ) concentrations of CXCL1, CXCL5, and CXCL8 chemokines (**A**), or VEGF (**B**) were measured by ELISA and are expressed as mean ± SD ($*, P < 0.01; **, P < 0.05$ as described in Materials and Methods). **C** and **D.** Reduction of CXC chemokine and VEGF production from HPDE and HPDE-KRas cells by blocking MEK or c-*Jun* signaling. **C.** HPDE or HPDE-KRas cells were treated with U0, PD, or SP. Media (48 h) CXC chemokines concentration was measured as described in Materials and Methods ($*, P < 0.01$ compared with control). **D.** Similarly, the effect on VEGF production from HPDE or HPDE-KRas cells by U0, PD, or SP was determined as described in Materials and Methods. Columns, mean values representative of two or more independent experiments done in triplicate; bars, SD ($*, P < 0.01$ compared with control).
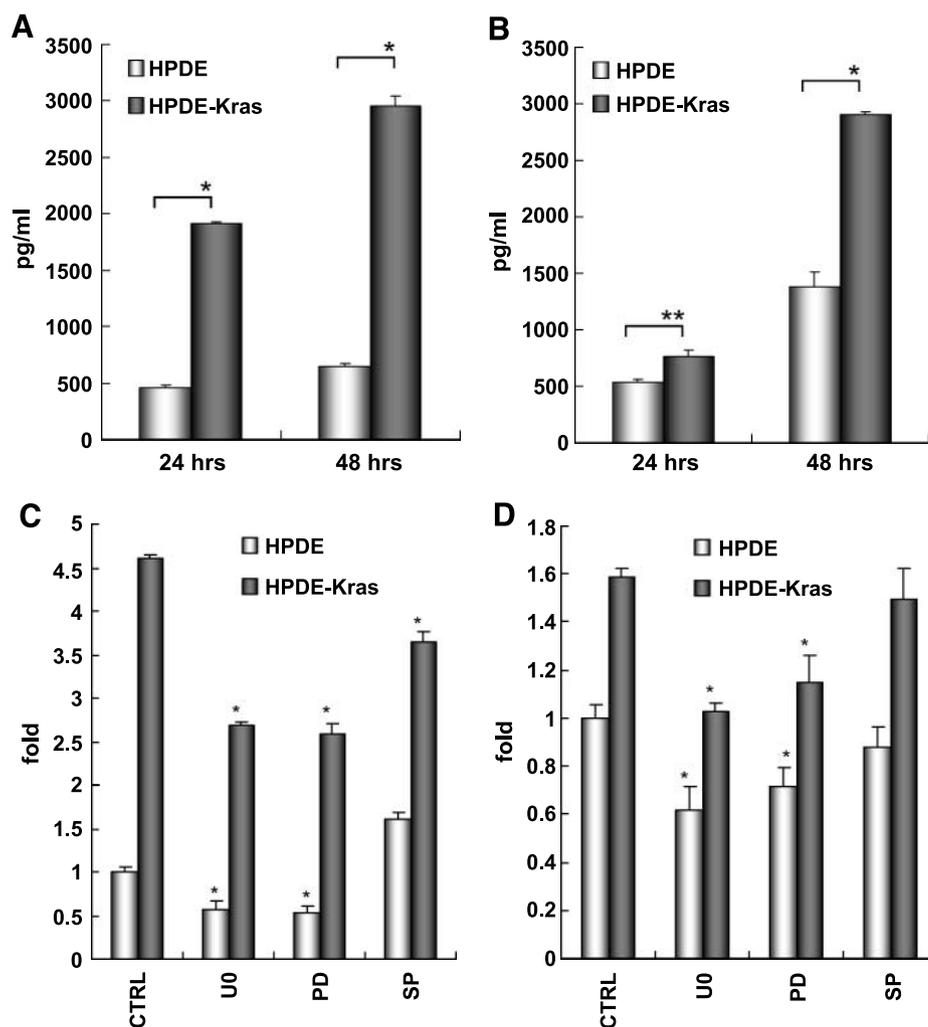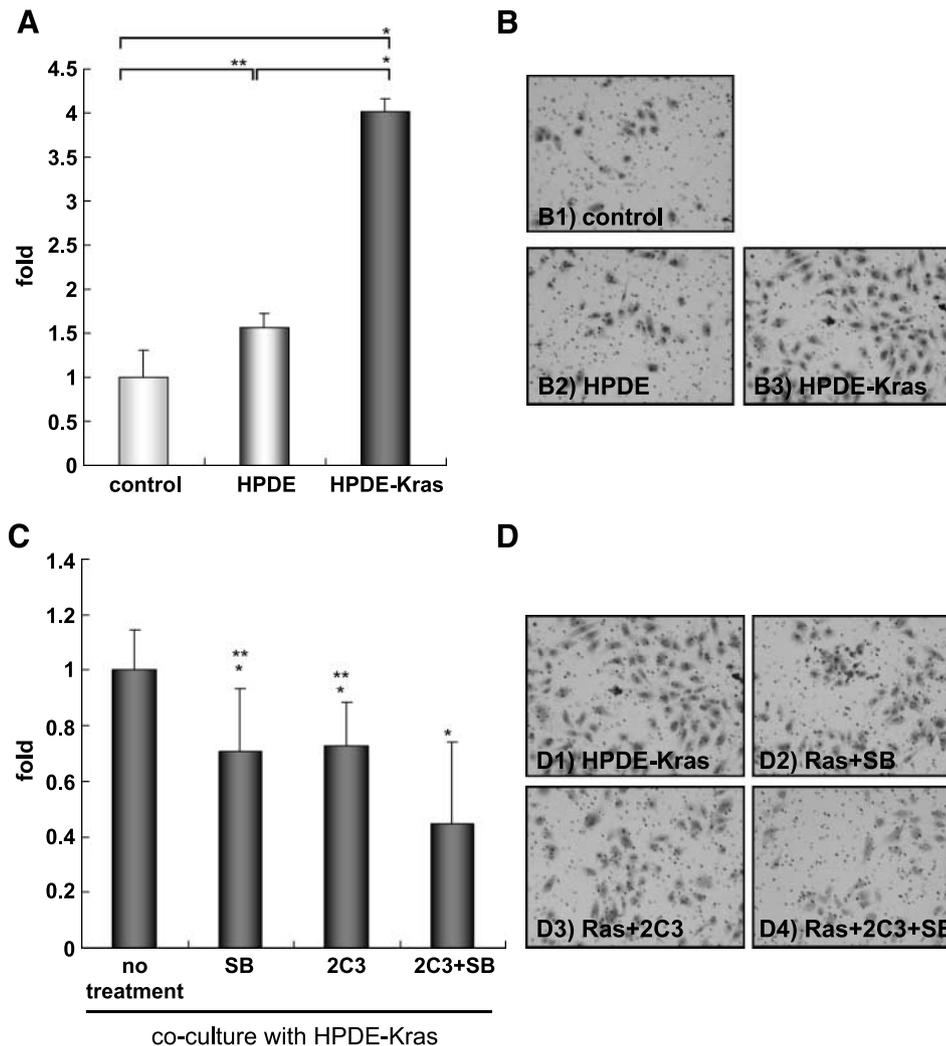
FIGURE 3. HPDE-KRas enhances HUVEC invasion via both CXCR2 and VEGFR2 signaling. **A** and **B.** The alteration of HUVEC invasion through Matrigel by coculture with HPDE or HPDE-KRas cell lines: HUVEC cells were seeded into Matrigel transwell chambers cocultured with HPDE or HPDE-KRas cells or medium only (control). Invading cells were fixed at 16 h, stained and counted as described in Materials and Methods. Columns, mean fold (compared with control); bars, SD (*, $P < 0.01$; **, $P < 0.05$). **C** and **D.** Blockade of CXCR2 and/or VEGFR2 signaling inhibits HUVEC invasion. HUVEC were pretreated with SB225002 and/or 2C3 for 1 h before HUVEC invasiveness was determined, as described in Materials and Methods. Columns, mean fold (compared with control); bars, SD (*, $P < 0.01$ compared with no treatment; **, $P < 0.01$ compared with 2C3 + SB). Columns, mean values representative of two or more independent experiments done in triplicate; bars, SD.

As oncogenic K-Ras activation promoted the expression and phosphorylation of c-*Jun*, we wanted to see if this pathway had a role in the release of CXC chemokines and VEGF. Treatment of cells with the c-*Jun* inhibitor SP600125 significantly reduced CXC chemokine secretion from HPDE-KRas (Fig. 2C). In contrast, SP600125 did not affect oncogenic K-Ras–induced VEGF production (Fig. 2D).

Oncogenic K-Ras Enhances HUVEC Invasion and Endotube Formation by Promoting CXC Chemokine and VEGF Secretion from HPDE Epithelial Cells

Because active K-Ras enhanced the production and secretion of proangiogenic factors from HPDE cell lines, we hypothesized that these agents might result in K-Ras–related angiogenesis. To examine the interaction between HPDE cell lines and HUVEC, we used a double-chamber coculture system to look at HUVEC motility. Coculture with HPDE-KRas cells significantly enhanced HUVEC invasion potency compared with either medium alone (control) or coculture with HPDE cells (Fig. 3A and B). Additionally, to elucidate the mechanism of K-Ras–induced invasion of vascular endothelial cells, we treated HUVEC with the CXCR2 antagonist SB225002 and/or 2C3, an antibody against VEGFR2. Although SB225002 and 2C3 had no effect on the limited activity of HPDE cells to stimulate HUVEC invasion, both drugs significantly inhibited the enhanced HUVEC invasion driven by oncogenic K-Ras. Moreover, the combination of SB225002 and 2C3 completely blocked the enhanced HUVEC invasion by HPDE-KRas (Fig. 3C and D), indicating that both CXC and VEGF epithelial production downstream of activated K-Ras have paracrine effects on vascular endothelial cell motility.

Following these studies on HUVEC invasion, we next examined the paracrine effect of oncogenic K-Ras on HUVEC tube formation. To show an interaction between HPDE cell lines and HUVEC, we again used the two-chamber coculture system. Coculture of HUVEC with HPDE-KRas cells significantly enhanced HUVEC endotube formation as compared with growth medium alone (control) or coculture with HPDE cells (Fig. 4A and B). To further investigate the role of mutant K-Ras–induced CXC chemokines and VEGF in HUVEC tube formation potency, we treated HUVEC with SB225002 and 2C3. In contrast to observations with invasion assays, HPDE-augmented endotube formation was partially inhibited by the CXCR2 inhibitor SB225002 or a neutralizing antibody against

the receptor (Supplementary Fig. S4). Both SB225002 and 2C3 inhibited HUVEC tube formation enhanced by oncogenic K-Ras; the combination of the two inhibitors completely blocked both enhanced HUVEC tube formation and native tube growth potential (Fig. 4C and D). These inhibitors indicate that the normal ability of HUVEC to organize into structures (control in Fig. 4B1) is at least in part dependent on endogenous VEGF and CXCR signaling.

Oncogenic K-Ras Enhances the Production of Both CXC Chemokines and VEGF from E6/E7/st Cell Lines

To solidify our findings on the effect of oncogenic K-Ras on angiogenesis in more detail, we used another pair of immortalized human pancreatic duct–derived cells: E6/E7/st and its oncogenic K-Ras variant, E6/E7/Ras/st. Initially, we did an ELISA of CXC chemokines and VEGF, and showed that production of both cytokines from E6/E7/st cell lines was significantly enhanced by mutant K-Ras expression (Fig. 5A and B). Although K-Ras–driven CXC chemokine production was significantly inhibited by treatment with MEK1/2 inhibitors,

U0126 and PD98059, the c-*Jun* NH$_2$-terminal kinase inhibitor SP600125 did not affect the secretion of CXC chemokines (Fig. 5C). All three inhibitors, U0126, PD98059, and SP600125, diminished VEGF release from E6/E7/Ras/st cells (Fig. 5D).

Mutant K-Ras Stimulates Both HUVEC Invasion and Endotube Formation in E6/E7/Ras/st Cells via Multiple Signaling Pathways

Western blot analysis confirmed that activated K-Ras enhanced the expression of the proangiogenic factors COX-2 and VEGF in E6/E7/Ras/st cells compared with parental controls (Fig. 6A). E6/E7/Ras/st cells showed significantly enhanced HUVEC invasion compared with E6/E7/st cells, and the enhancement was inhibited by the combination of SB225002 and 2C3 (Fig. 6B; Supplementary Fig. S3A). E6/E7/Ras/st cells significantly augmented the length and complexity of HUVEC endotube structures compared with controls (without coculture) and E6/E7/st cells. This enhancement was significantly inhibited by SB225002 and/or 2C3 (Fig. 6C; Supplementary Fig. S3B).
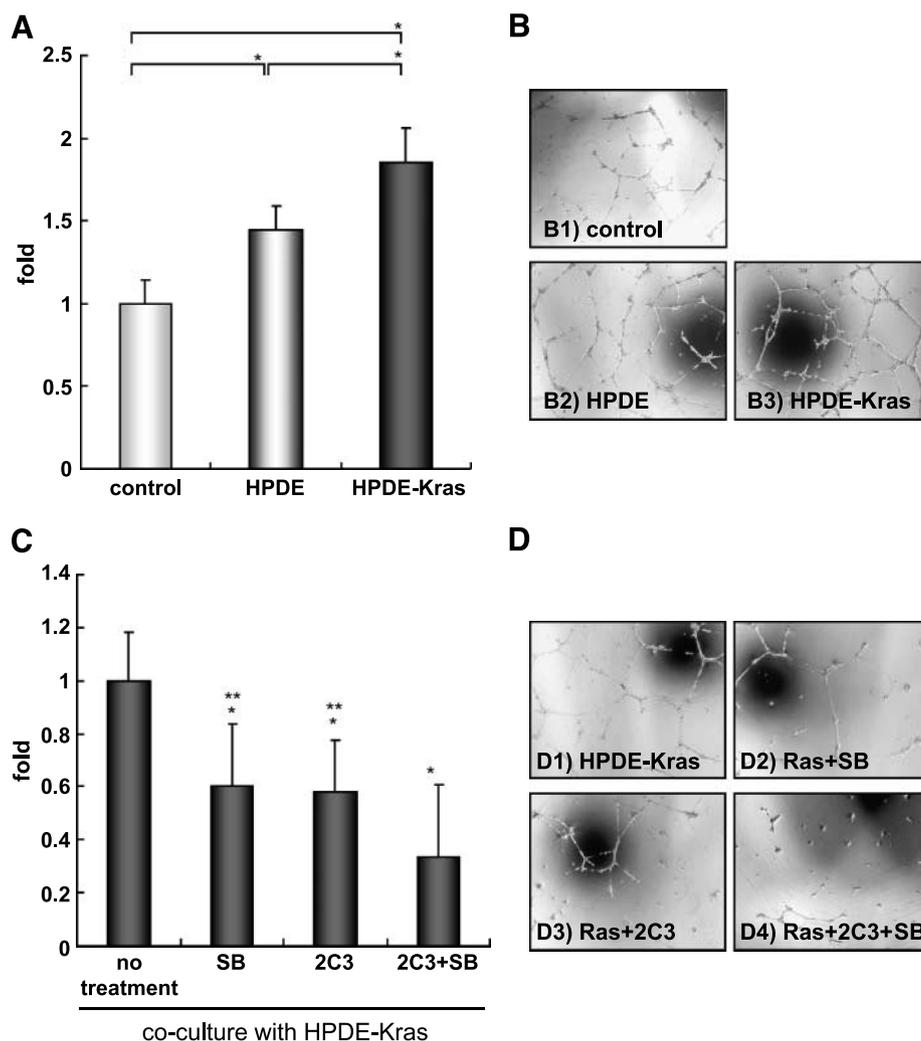
FIGURE 4. HPDE-KRas enhances angiogenesis via both CXCR2 and VEGFR2 signaling. **A** and **B.** HUVEC were cocultured with HPDE or HPDE-KRas cells as described in Materials and Methods. Columns, mean fold of control; bars, SD (*, *P* < 0.01). **C** and **D.** Blockade of CXCR2 and/or VEGFR2 signaling inhibits the HUVEC tube formation enhanced by HPDE-KRas. HUVEC were pretreated with SB225002 and/or 2C3 for 1 h. HUVEC tube formation was determined as described in Materials and Methods. Columns, mean; bars, SD (*, *P* < 0.01 compared with no treatment; **, *P* < 0.01 compared with 2C3 + SB). Columns, mean values representative of three or more independent experiments done in triplicate; bars, SD.

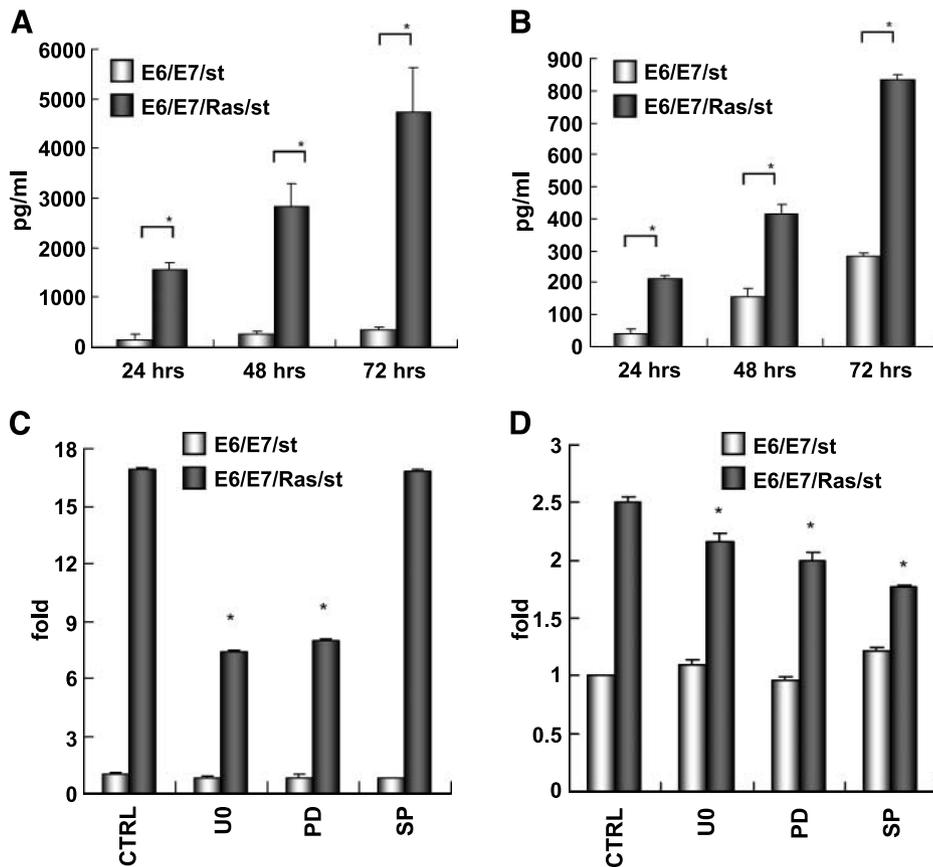**FIGURE 5.** Oncogenic K-Ras expression in E6/E7/Ras/st cells stimulates the secretion of CXC and VEGF chemokines via mitogen-activated protein kinase pathways. **A** and **B.** CXC chemokine production from E6/E7/st or E6/E7/Ras/st cells. Culture media from growing E6/E7/st or E6/E7/Ras/st cells were collected (24, 48, and 72 h) and summed concentrations of CXCL1, CXCL5, and CXCL8 chemokines (**A**) or VEGF (**B**) were measured by ELISA as described in Materials and Methods. Columns, mean; bars, SD (*, $P < 0.01$). **C** and **D.** CXC chemokines and VEGF production is reduced by blocking MEK and c-*Jun* signaling. Media from E6/E7/st or E6/E7/Ras/st cells treated with U0, PD, or SP were collected at 48 h as described in Materials and Methods. CXC chemokines (**C**) or VEGF (**D**) concentrations are expressed as mean fold ± SD (*, $P < 0.01$ compared with control). Columns, mean values representative of two or more independent experiments done in triplicate; bars, SD.

Discussion

It has been well established that the initiation and progression of pancreatic cancer is driven in part by a series of genetic mutations, including mutations of K-Ras, CDKN2A, TP53, BRCA2, and SMAD4/DPC4, which occur early in the development of ductal cell dysplasia (3, 22). Less clear is which of the many mutations are critical for pancreatic cancer development, and what the roles of the different proteins may be. Because
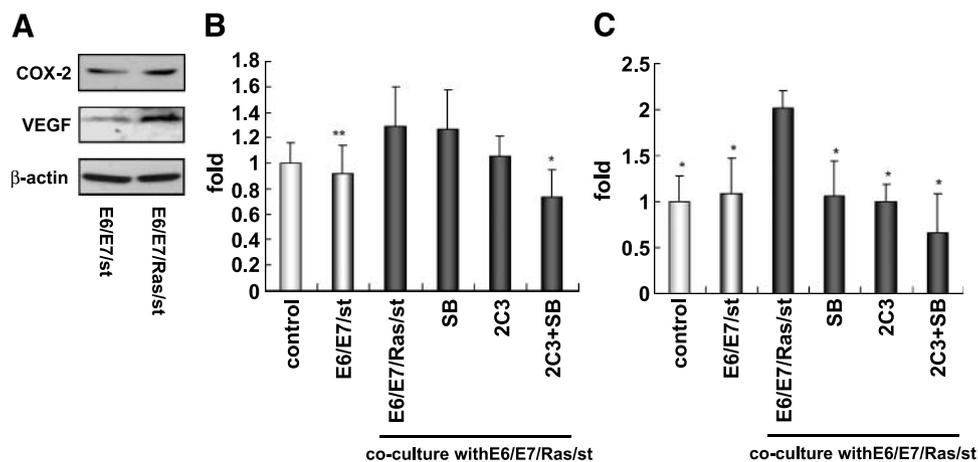

FIGURE 6. Oncogenic K-ras expression in E6/E7/st/Ras cells stimulates proangiogenic factor expression and paracrine invasion and tubule formation of HUVEC. **A.** Western blot of COX-2 and VEGF expression as described in Materials and Methods, representative of $n = 3$ experiments. **B.** E6/E7/Ras/st enhances the invasion of HUVEC via both CXCR2 and VEGFR2 signaling. Naïve HUVEC cells or HUVEC pretreated with SB and/or 2C3 for 1 h were cocultured with E6/E7/st or E6/E7/Ras/st cells or medium only (control) for 16 h as described in Materials and Methods (*, $P < 0.01$; and **, $P < 0.05$ compared with E6/E7/Ras/st). **C.** E6/E7/Ras/st enhanced the tube formation by HUVEC via both CXCR2 and VEGFR2. HUVEC and E6/E7/st or E6/E7/Ras/st were cocultured for 16 h as described in Materials and Methods, and endotubes were counted from nine random fields per sample. As above, HUVEC were pretreated with SB and/or 2C3 for 1 h. Columns, mean fold; bars, SD (*, $P < 0.01$ compared with E6/E7/Ras/st). Columns, mean values representative of two or more independent experiments done in triplicate; bars, SD.

activating mutations of K-Ras are in evidence in early pancreatic intraepithelial neoplasias, and K-Ras mutations are found in >90% of patients with pancreatic cancer (23), and because downstream signaling due to K-Ras can contribute to all of the characteristics of malignant progression (24), it is tempting to believe that mutant K-Ras is critical for many aspects of pancreatic cancer. In addition to the direct changes in epithelial cells as a result of genetic alterations, a growing area of interest includes the signaling cross-talk that happens among tumor epithelial cells, the surrounding stroma, and vascular tissue. Our goal for this experimental study was to investigate such paracrine effects of hyperactive K-Ras protein in pancreatic duct–derived epithelial cells on vascular endothelial cells. Our data clearly indicated that oncogenic K-Ras induced the production of angiogenic factors from HPDE cells, and that as a consequence, invasion and tube formation of HUVEC were significantly enhanced.

We initially showed that in spite of Ras-dependent increases in both growth-promoting and antiapoptotic/survival proteins, there was no significant difference in proliferation between HPDE and HPDE-KRas. However, Tsao and colleagues have previously shown that the tumorigenic potential of HPDE-KRas cells in severe combined immunodeficiency mice was significantly increased compared with HPDE cell lines (17). This result suggests that tumor growth was enhanced by the paracrine effects of oncogenic K-Ras as opposed to simple augmented growth rate. Because one of the most important interactions between the tumor and its microenvironment is angiogenesis, and because neoangiogenesis is a rate-limiting step in tumor expansion, in the present study, we focused our attention on vascular network initiation. The production of the known angiogenic factors CXC chemokines and VEGF were significantly enhanced (5-fold and 2-fold at 48 hours, respectively) by the expression of mutant K-Ras in the epithelial cells. These enhanced cytokines did not affect HPDE-KRas cells in an autocrine manner, as evidenced by the absence of change in proliferation, but significantly enhanced both invasion and tube formation of HUVEC in coculture assays. These results seem to explain the previous report that oncogenic K-Ras had little effect on HPDE contact–independent growth but enhanced tumorigenic potential *in vivo* (17), and suggests that paracrine cross-talk between tumor cells and the microenvironment is important in the malignancy of pancreatic cancer.

The best-characterized effector pathway in oncogene K-Ras function is the Raf/MEK/ERK cascade (25). In our HPDE and HPDE-KRas system, mutant K-Ras expression enhanced MEK activation but did not up-regulate ERK activation, similar to results reported for pancreatic carcinoma cell lines, in which elevated MEK was independent of ERK activation (20). Instead, similar to previous results in other pancreatic cell systems (21, 26), our data showed that oncogenic K-Ras increased the expression of the phosphatase MKP-2 in these HPDE cell systems. Although activation of the Ras/MEK/ERK signaling axis has been shown to increase proliferation, it is possible that hyperactivation of these pathways may be detrimental to cell growth. Others have similarly shown a disconnect between activated MEK and activated ERK, suggesting that p-ERK may not be the most relevant marker of pathway stimulation in all situations (27, 28). Whether augmented MEK-dependent MKP-2 expression plays a pro-oncogenic role or is simply a compensatory mechanism to reduce overactive ERK1/2 is unclear and warrants further study in the future. It is tempting to believe, however, that because this increase in MKP-2 is seen in a wide range of both pancreatic cancer cell lines and Ras-transformed pancreatic cells, this phosphatase plays an important role in the progression of this tumor type. We next examined whether enhanced MEK activation by oncogenic K-Ras promoted the production of angiogenic factors from HPDE cell systems. Blocking of MEK signaling by U0126 or PD98059 significantly inhibited the production of both CXC chemokines and VEGF from HPDE-KRas, indicating that proangiogenic factor secretion is modulated at least in part by Ras-dependent MEK1/2 activation. It is possible that substrates of MKP-2 that remain phosphorylated in the face of decreased MKP-2 activity due to MEK inhibition could play a role both in the changes seen in VEGF and CXC chemokine release and in angiogenesis phenotype. This area remains an intriguing opportunity for our ongoing research.

Previous reports showed that K-Ras oncogene expression up-regulates the activity of NF-κB (29). NF-κB is an important transcription factor involved in the production of VEGF and interleukin-8 in pancreatic cancer (30, 31), and has been associated with pancreatic carcinogenesis (32-35). Indeed, previously published data showed that NF-κB is constitutively activated in most (>70%) human pancreatic cancer cell lines and primary tumor specimens (35). We did electrophoretic mobility shift assays to assess NF-κB activity in both HPDE and HPDE-KRas cells, using TNF-α stimulated KBM-5 cells as a positive control, but found no significant difference in NF-κB activity between the cell lines (data not shown). These results indicate that NF-κB is not activated by oncogenic K-Ras in this model system, and NF-κB may not participate in the production of angiogenic factors in the early stages of pancreatic cancer. It does not, however, preclude a role for NF-κB–driven VEGF at other points of pancreatic cancer development, particularly the vascular expansion necessary for distal metastasis. Because the phosphoinositide 3-kinase/AKT pathway is another major downstream effector cascade stimulated by K-Ras signaling (36, 37), we examined the effect of phosphoinositide 3-kinase/AKT pathway on the Ras-induced production of angiogenic factors in the HPDE cell system. Using the phosphoinositide 3-kinase inhibitor LY294002, we saw no significant effect on factor secretion (data not shown), suggesting that activation of phosphoinositide 3-kinase is not critical for CXC and VEGF release.

Previous reports have indicated that the K-Ras downstream c-*Jun* pathway plays an important role in cell survival regulation in pancreatic cancer (38). Indeed, our Western blot analysis showed increased c-*Jun* activation by hyperactivated K-Ras in HPDE cell systems. Our present study showed that the c-*Jun* NH$_2$-terminal kinase inhibitor SP600125 significantly inhibited CXC chemokine production, but not VEGF production, in the HPDE cell system. In contrast, VEGF production from the E6/E7/st cell system was significantly inhibited by SP600125, but SP600125 did not inhibit the CXC chemokine production from the E6/E7/st cell model. It may be that more complex mechanisms might be involved in the mutant K-Ras/c-*Jun* pathways and one of the future directions of our study will be to clarify this signaling.

In summary, we found that oncogenic K-Ras enhanced the production and secretion of CXC chemokines and VEGF from both HPDE and E6/E7/st immortalized pancreatic epithelial cell systems that were in part dependent on MEK1/2 and c-*Jun* signaling. Consequently, these enhanced proangiogenic factors promoted the invasion and tube formation of HUVEC in coculture systems *in vitro*. The results of our experiments clearly showed that oncogenic K-Ras mutation in ductal epithelial cells contributes in a paracrine manner to the initiation and maturation of vascular endotubes, a critical hurdle in the early development of pancreatic cancer. These data further illustrate the importance of the K-Ras signaling cascade in pancreatic cancer malignancy, the necessity to understand the multifaceted roles of Ras in order to advance therapeutic options for pancreatic cancer (39), and the increasing field of research focused on cross-talk among epithelial, stromal, and vascular cell populations in tumor development.

Materials and Methods

Cell Culture

HUVECs were obtained from Lonza Walkersville, Inc. HUVEC were maintained in endothelial growth media-2 (EGM-2; Lonza) with EGM-2 singlequots (Lonza), which contained 2% FCS. The HPDE cells and K-Ras4B^{G12V}–transfected HPDE (HPDE-KRas) cells were generous gifts from Dr. Ming-Sound Tsao (17, 40). These cells were cultured in keratinocyte serum-free medium supplied with 5 ng/mL of epidermal growth factor and 50 μg/mL of bovine pituitary extract (Invitrogen). A second pair of immortalized normal human pancreatic duct epithelial cell lines with or without mutant oncogenic K-Ras (G12D; E6/E7/Ras/st and E6/E7/st, respectively) were also used. Briefly, these cells were pancreatic duct–derived cells from the healthy pancreas of a 52-year-old accident victim. The cells were immortalized by ectopic expression of the catalytic subunit of human telomerase and HPV16 E6 and E7, and were subsequently transformed by expression of SV40 small T antigen and mutant K-Ras^{G12D} (26, 41, 42). These were maintained in M3/5 growth medium [four parts high-glucose DMEM to one part M3F (INCELL Corp.) supplemented with 5% FCS]. All cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Reagents and Antibodies

The following antibodies against VEGF, COX-2, and MKP-2 were obtained from Santa Cruz Biotechnology. The antibodies against p-ERK1/2, ERK1/2, p-Raf/MEK, p-c-*Jun*, and c-*Jun* were obtained from Cell Signaling Technology, Inc. Antibodies against β-actin was obtained from Sigma-Aldrich, Co. 2C3, anti-VEGF monoclonal antibody, which is shown to block the interaction of VEGF with VEGFR2, was kindly given by Dr. Rolf A. Brekken (University of Texas Southwestern Medical Center, Dallas, TX). MEK inhibitors U0126 and PD98059, c-*Jun* NH$_2$-terminal kinase inhibitor SP600125, and the antagonist of G protein–coupled chemokine receptor CXCR2, SB225002 were obtained from Calbiochem.

Ras Activation Assay

Initially, we reconfirmed the expression of activated Ras in HPDE and HPDE-KRas cells by Ras Activation Assay kit (Up-state Biotechnology, Inc.) following the instructions of the manufacturer. Briefly, cells were washed twice with ice-cold PBS and lysed in 1× Mg^{2+} lysis/washing buffer containing protease inhibitors (Roche Molecular Biochemicals) for 15 min at 4°C. Cell lysates were centrifuged at $1,000 × g$ for 20 min and the protein concentrations of the supernatants were then determined by BCA protein assay kit (Pierce). Equal amounts of samples (400 μg) were immediately affinity-precipitated using 20 μg of Ras assay reagent (agarose-conjugated Raf-1 RBD) for 45 min at 4°C. The precipitates were washed thrice with 1× Mg^{2+} lysis/washing buffer and eluted by boiling in 2× SDS-PAGE reducing sample buffer. The proteins were separated on a 12% SDS-polyacrylamide gel and then immunoblotted with a pan-Ras antibody supplied in the Upstate kit (RAS10). The same antibody was used to determine total K-Ras amounts. Positive and negative assay controls for Ras activation were cell lysate ectopically loaded with GTPγS and GDP, respectively.

Western Blot

Total cell lysates from confluent cultures were prepared using ice-cold lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0), 1% Nonidet P40, 0.1% SDS, 0.5% deoxycholate-Na, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride, plus protease inhibitors]. Fifty micrograms of protein from cell lysates were separated on 10% or 12% SDS-PAGE gels and transferred to Immobilon transfer membranes. The membrane was incubated in blocking buffer (5% nonfat dry milk in TBS containing 0.1% Tween 20) for 1 h at room temperature. The membrane was incubated with primary antibody overnight at 4°C. The membrane was washed with TBS containing 0.1% Tween 20, and incubated for 1 h with secondary anti-mouse or anti-rabbit peroxidase-linked antibodies in blocking solution at room temperature. After washing, protein-antibody complexes were visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). The membrane was washed and stripped using Restore Western blot stripping buffer (Pierce) and β-actin was detected by the same method.

ELISA

HPDE, HPDE-KRas, E6/E7/st, and E6/E7/Ras/st cells were seeded at a density of $2 × 10^5$ cells/mL into a 24-well plate and cultured overnight. Medium was then exchanged and cells were cultured for a further 24, 48, or 72 h. The culture media were then collected and microfuged at 1,500 rpm for 5 min to remove insoluble matter, and the supernatants frozen at −80°C until use in the ELISA. The concentrations of CXCL1, CXCL5, CXCL8, and VEGF were measured using an ELISA kit (R&D Systems) according to the instructions of the manufacturer. The concentrations of individual CXC chemokines were added because they are all agonists of CXCR2. To examine the roles of MEK and c-*Jun* signaling in enhanced production of both CXC chemokines and VEGF by oncogenic K-Ras, all cell lines were treated with MEK inhibitors U0126 (5 μmol/L) or PD98059 (10 μmol/L), or c-*Jun* NH$_2$-terminal kinase inhibitor SP600125 (20 μmol/L). After 48 h of incubation, the supernatants were collected and the concentrations of CXC chemokines or VEGF were measured as described above.

RT-PCR Analysis

Total RNA was prepared from all cell lines using an RNeasy Mini Kit by Qiagen. RT-PCR was done according to the instructions of the manufacturer using a One-step RT-PCR kit by Qiagen. For RT-PCR, we used the following pairs of forward and reverse primer sets: 5′-aacatggagagtgacagctttg-3′ and 5′-ttagagagtagtggaagtgtg-3′ (CXCR2; PCR product size is 1,071 bp), 5′-ctatcactgcaaagccacca-3′ and 5′-aagccctcttccaagtgat-3′ (VEGFR1; PCR product size is 329 bp), and 5′-gcatggtcttctgtgaagca-3′ and 5′-ttcctccaactgccaatacc-3′ (VEGFR2; PCR product size is 806 bp). PCR was done for 35 cycles with denaturation at 94°C for 60 s, annealing at 54°C for 60 s, and extension at 72°C for 60 s. Amplified DNA fragments were resolved by electrophoresis on 1.2% agarose gels containing ethidium bromide. β-Actin was used as a positive control.

HUVEC Proliferation Assay

To confirm the effects of oncogenic K-Ras on the proliferation of HPDE cells, we did a CellTiter 96 AQ$_{ueous}$ One Solution Cell Proliferation Assay (MTS assay; Promega) according to the instructions of the manufacturer. Briefly, HPDE or HPDE-KRas cells were seeded at a density of 2×10^3 cells/100 μL in 96-well plates. After 12, 36, 60, and 84 h of incubation, 20 μL of CellTiter 96 AQ$_{ueous}$ One Solution Reagent was added to each well and the trays were incubated for 3 h at 37°C, after which absorbance was measured using a microplate reader with a test wavelength of 490 nm.

HUVEC Invasion Assay

The invasive potency of HUVEC was determined by invasion assays using the BD Bio-Coat Matrigel invasion assay system (BD Biosciences) which was done according to the instructions of the manufacturer. We used a double-chamber method to determine the effect of coculturing with HPDE, HPDE-KRas, E6/E7/st, or E6/E7/Ras/st cells on HUVEC invasiveness. HUVEC cells (5×10^4) were seeded into the upper transwell chambers with 8 μm pores, which were then placed into 24-well plates in which HPDE, HPDE-KRas, E6/E7/st, or E6/E7/Ras/st cells (1×10^5 cells) or medium only (control) were preseeded. After 16 h of incubation, the upper surface of the transwell chambers was wiped with a cotton swab and the invading cells were fixed and stained with Diff-Quik stain (Dade-Behring). Invading cells were counted in five random microscopic fields (×200). Similarly, to examine the effect of oncogenic K-Ras–induced CXC chemokines or VEGF on HUVEC invasiveness, HUVEC were pretreated with CXCR2 antagonist SB225002 (100 nmol/L) and/or anti-VEGFR2 antibody 2C3 (100 nmol/L) for 1 h. Then the effect on HUVEC invasiveness was determined as above.

Tube Formation Assay for Angiogenesis on Matrigel

HUVEC tube formation potency was measured by angiogenesis assay on Matrigel (BD Biosciences). For reconstitution of a basement membrane matrix, Matrigel was diluted 2-fold with cold DMEM (without FCS) and added to the 24-well tissue culture plate (250 μL/well) at 4°C. The 24-well plate was incubated for 2 h at 37°C to allow the Matrigel to solidify. HUVECs were trypsinized, counted, resuspended in basal medium, and added on top of the reconstituted basement membrane (5×10^4 cells/well). To evaluate the effect of the oncogenic K-Ras on HUVEC tube formation, we used a double-chamber method. HPDE, HPDE-KRas, E6/E7/st, or E6/E7/Ras/st cells (1×10^4 cells) were seeded into transwell chambers consisting of polycarbonate membranes with 0.4 μm pores (BD Biosciences) and allowed to adhere overnight. The chambers were then placed into the HUVEC tube formation assay system. Cells were incubated for 16 h to allow the formation of capillary-like structures. These endotubes were quantified by counting nine random microscopic fields (×40) per sample, with each condition being assessed in triplicate. Similarly, to examine the effect of cell line–induced CXC chemokine or VEGF secretion on HUVEC tube formation, HUVEC were pretreated with CXCR2 antagonist SB225002 (100 nmol/L) and/or anti-VEGFR2 antibody 2C3 (100 nmol/L) for 1 h. HUVEC tube formation potency was determined in the same way.

Statistical Analysis

Multiple group comparisons were done by using one-way ANOVA with a post hoc test, Student-Newman-Keuls test, or the Dunnett test for subsequent individual group comparisons. Differences between the two groups were evaluated using Student's t test. $P < 0.05$ was considered statistically significant. Mean values and SD were calculated for experiments done in triplicate (or more).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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