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Activated Kras<sup>G12D</sup> is associated with invasion and metastasis of pancreatic cancer cells through inhibition of E-cadherin

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BACKGROUND: Pancreatic cancer (PC) harbours an activated point mutation (Kras<sup>G12D</sup>) in the Kras proto-oncogene that has been demonstrated to promote the development of PC.

METHODS: This study was designed to investigate the effect of the oncogenic Kras<sup>G12D</sup> allele on aggressiveness and metastatic potential of PC cells. We silenced the oncogenic Kras<sup>G12D</sup> allele expression in CD18/HPAF and ASPC1 cell lines by stable expression of shRNA specific to the Kras<sup>G12D</sup> allele.

RESULTS: The Kras<sup>G12D</sup> knockdown cells exhibited a significant decrease in motility (P < 0.0001), invasion (P < 0.0001), anchorage-dependent (P < 0.0001) and anchorage-independent growth (P < 0.0001), proliferation (P < 0.005) and an increase in cell doubling time (P < 0.005) in vitro and a decrease in the incidence of metastases upon orthotopic implantation into nude mice. The knockdown of the Kras<sup>G12D</sup> allele led to a significant increase in the expression of E-cadherin (mRNA and protein) both in vitro and in vivo. This was associated with a decrease in the expression of phospho-ERK-1/2, NF-κB and MMP-9, and transcription factors such as δEF1, Snail and ETV4. Furthermore, the expression of several proteins involved in cell survival, invasion and metastasis was decreased in the Kras<sup>G12D</sup> knockdown cells.

CONCLUSIONS: The results of this study suggest that the Kras<sup>G12D</sup> allele promotes metastasis in PC cells partly through the downregulation of E-cadherin.

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Pancreatic cancer (PC) has one of the worst prognoses among all known cancers, with a mortality to incidence ratio of ~0.83 (Jemal et al, 2009). In the United States, it remains the fourth leading cause of cancer-related deaths with an incidence of ~12.3 per 100 000 people (Jemal et al, 2009). The median survival of patients with PC is a mere 4.1 months with the overall 5-year survival rate being <5% (Heinemann et al, 2008; Sultana et al, 2008; Jemal et al, 2009). At the time of diagnosis, >85% of patients have metastatic disease, which makes surgical and medical interventions largely ineffective (Matsuno et al, 2004). One of the reasons for the poor outcome of PC is the lack of early detection markers and limited efficacy of existing treatment regimes. Therefore, there is an urgent need to understand the pathogenesis of PC in order to discover early detection marker(s), novel molecular targets and new therapeutic strategies.

Recent advances in molecular genetics have revealed a compendium of genetic lesions associated with the progression and metastasis of PC (Hezel et al, 2006). Of these mutations, Kras is found to be mutated in almost all cases (75–90%) of PC and represents an early event in the development and progression of this malignancy (Almoguera et al, 1988; Shibata et al, 1990; Caldas and Kern, 1995; Dergham et al, 1997; Moskaluk et al, 1997; Wang et al, 2002). Kras is a member of the highly homologous Ras family of proteins and has potent transforming ability (Barbacid, 1987). It is a 21 kDa size monomeric membrane-localised guanine nucleotide (GTP/GDP)-binding protein. A wide variety of extracellular stimuli can activate Kras, and the activated form, in turn, activates a cascade of signals that ultimately regulate cell growth, differentiation (McCormick, 1989) and apoptosis. Mutations in Kras occur most frequently at codon 12 (Gonzalez-Cadavid et al, 1988; Grunewald et al, 1989; Mariyama et al, 1989; Nagata et al, 1990; Shibata et al, 1990; Van Laethem et al, 1995), and less frequently at codons 13 and 61 (Motojima et al, 1991, 1993; Caldas and Kern, 1995). All of these mutations can abolish the intrinsic ability of the Kras protein to hydrolyse GTP, resulting in continuous stimulation of cell proliferation (Barbacid, 1987; Ellis and Clark, 2000).

Recently, several approaches, including short interfering RNAs, antibodies, mutant Kras-specific peptide inhibitors, adenoviruses expressing antisense Kras, dominant-negative Kras, antisense oligonucleotides and different drugs have been investigated for their ability to target the mutant form of Kras (Aoki et al, 1995, 1997; Adjei, 2001). The major drawback of dominant-negative Kras and pharmacological Kras inhibitors is the lack of specificity (Kohl et al, 1994; Feig, 1999; Bolick et al, 2003; de Bono et al, 2003), whereas the antisense oligonucleotides downregulate the wild-type
Kras, which is essential for the normal function of all cells in the body. RNA interference (RNAi) has become a novel approach to target the mutant form of this oncogene specifically (Devì, 2006; Gaither and Iourgenko, 2007). Kras is a potent tumour initiator as evidenced by observations that activating mutations in Kras (G12D) are required for the development of pancreatic intraepithelial neoplasms (PanINs), which precede invasive adenocarcinoma (Aguirre et al, 2003). There is also evidence to suggest that several point mutations in codon 12 can result in constitutive activation of Kras (Karapetis et al, 2008). A point mutation (GGT GAT) resulting in a single amino-acid change from glycine to aspartic acid in codon 12 (KrasG12D) is observed in many cases of PC as well as in many PC cell lines (Hohne et al, 1992). Various studies have shown the role of mutant KrasG12D in enhanced cell proliferation and transformation of normal pancreatic epithelial cells (Hingoranî et al, 2003; Tuveson et al, 2004); however, its function in the late stage of PC progression remains unknown.

The objective of this study was to investigate the role of mutant KrasG12D allele in PC by knockdown of this allele in the highly metastatic PC cell lines CD18/HPAF and ASPC1, followed by examination of the effects on cellular functions (through in vitro and in vivo functional studies) and intracellular signalling cascades. Altogether, our data indicate that silencing of KrasG12D causes a significant reduction in the motility, invasion and metastatic potential of PC cells. This is done through an upregulation of E-cadherin and downregulation of Snail, ßE1 and ßV4 transcription factors, and signalling pathways such as Akt, FAK and ERK1/2.

MATERIALS AND METHODS

Cell culture, plasmid construction and transfection

CD18/HPAF, Capan-1, ASPC-1 cells were cultured in DMEM, whereas BXPC-3 cells were grown in RPMI and HPDE cells in Keratinocyte media, respectively, supplemented with 10% fetal calf serum and antibiotics (penicillin and streptomycin 0.1 µg ml–1). The pSUPER.retro.puro vector was digested with Bgl II and HindIII restriction enzymes and dephosphorylated with calf intestinal alkaline phosphatase (CIAP). Two complementary oligonucleotides, 5′-GATCCCGCTTTGAGCTGGATCTCATGAGACCTAGCCATAGCTGGC AATTTCC-3′ and 5′-AGCTTTTCCAAAAAGTGGAGCTGGATCTCATGAGACCTAGCCATAGCTGGCGAATTTCC-3′, corresponding to the mutant KrasG12D gene with BglII and HindIII sites were synthesised, annealed, dephosphorylated and ligated into the digested pSUPER vector (Restriction sites at 3′ and 5′ ends are underlined while the sequence of the hairpin loop sequence is indicated by underline italics). The primary antibodies for the activated form and total FAK, Kras, cyclins D1, E and A and NF-B were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), CMyc and p21WAF1/CIP from Epitomics (Burlington, CA, USA), activated and total Akt and ERK, Caspase-3 and Cleaved-Caspase-9 from Cell Signaling (Danvers, MA, USA), matrix metalloproteinase-9 (MMP-9) and E-cadherin were gifts from Dr Rakesh Singh and ß-actin was from Sigma Aldrich (St Louis, MO, USA).

Immunoblot analysis

Immunoblot analysis was done as described previously (Moniaux et al, 2008). The expression level in the scramble RNA-transfected cells.

Growth kinetics, clonogenicity and apoptosis assays

Cells (1.0 × 10⁶ cells per ml of medium containing 1.0% FBS) were seeded in six-well plates and allowed to grow for different time intervals. The growth of the cells was monitored by counting the number of viable cells on a Vi-CELL (Boulevard, CA, USA) counter every day for 8 days. The cell population doubling time (Td) was calculated during the exponential growth phase (96–144 h) using the following formula: Td = 0.693 t/ln (Nf/N0), where t is the time difference (in h), Nf is the cell number at time t (144 h) and N0 is the cell number at the initial time (96 h) (Zhang et al, 2002). To assess clonogenic potential, the cells were trypsinised and either plated in 0.3% agarose with a 0.5% agarose overlay (1 × 10³ cells per well in 24-well plate) for assessment of anchorage-independent growth or on plastic coated petri dishes for anchorage-dependent respectively. The number of foci >100 µm was counted after 14 days. Apoptosis was measured by Annexin V FITC staining as described previously (Chaturvedi et al, 2007). This assay is based on the principle that during the process of apoptosis, phosphatidyl serine (normally localised on the inner leaflet of the plasma membrane) is flipped out. Annexin V has a high affinity for phosphatidyl serine and binds to it. However, at this stage (called early apoptosis), the cell membrane is still intact and hence propidium iodide (PI) is excluded from these cells. Therefore, early apoptotic cells are defined by a positive staining for Annexin V and a negative staining for PI.
As apoptosis progresses, the cell membrane permeability increases, leading to increased entry of PI that binds to the DNA. Hence, cells during the later stage of apoptosis are positive for both Annexin V and PI. A similar process also takes place during necrosis, making it impossible to distinguish late apoptosis from necrosis with this assay.

**Tumourigenicity assay**

Subconfluent cultures of CD18/HPAF-derived clones were trypsinised and washed with phosphate-buffered saline. Cell viability was determined by Trypan blue staining and single-cell suspensions of >90% viability was used for the orthotopic injections. The cells were resuspended in a normal saline (NS) solution at a concentration of 5 × 10⁴ cells per 50 μL. Immune-deficient mice were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD, USA). The mice were treated in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines. The orthotopic implantation was performed as previously described (Choudhury et al., 2004). All mice were killed after 21 days of implantation. The presence of metastatic lesions in the different organs was determined thorough gross inspection and histological analysis. Pancreatic tumours were excised, weighed and measured.

**Motility and invasion assay**

For motility assays, 1 × 10⁶ cells suspended in serum-free medium were plated in the top chamber of polyethylene teraphthalate membranes (six-well insert, pore size 8 μm) (Becton Dickinson, Franklin Lakes, NJ, USA). Then, 2 ml of 10% serum-containing medium was added to the lower chamber of the well and the cells were allowed to migrate for 22 h under chemotactic drive. After incubation, the cells that did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. The migrated cells on the lower side of the membrane were stained with Diff-Quick cell stain kit (Dade-Behring Inc., Newark, DE, USA) and photographed in 10 random fields of view at ×100 magnification. Cell numbers were counted and expressed as the average number of cells per field of view. For invasion assay, cells (1 × 10⁵) were seeded on Matrigel-coated membrane inserts (BD Biosciences, Bedford, MA, USA) and photographed in 10 random fields of view under a microscope. Three independent experiments were done in each case. The data were represented as the average of the three independent experiments with the standard error of mean (s.e.m.).

**Oligonucleotide array gene expression analysis**

Human oligonucleotide array containing probes for 39 200 genes was constructed at the Microarray Core Facility of University of Nebraska Medical Center. Total RNA was isolated from shK-ras and K-ras scramble transfected CD18/HPAF cells by Qiagen RNEasy kit (Qiagen Sciences, Valencia, CA, USA) according to the manufacturer’s directions. The procedure for the microarray hybridisation and the subsequent analysis has been previously described by us (Chaturvedi et al., 2007).

**Statistical analysis**

For analysis of microarray data, a gene chip containing 39 200 genes was used. The data were normalised using BRB Array Tools. Random-variance paired t-tests were used to determine which genes are differentially expressed between tumour samples and the normal samples. The random-variance paired t-test allows for sharing information among genes about variation without assuming that all genes have the same variance, which gives a more accurate estimate of the variability when sample sizes are small (2). A significance level of 0.001 was selected to help limit the false discovery rate (FDR) due to multiple comparisons. The FDR was limited to <10%. Parametric data were compared using the two-tailed Student’s t-test, whereas nonparametric data were analysed using a two-way ANOVA or χ² test. Data were analysed using the MedCalc for Windows version 9.6.4.0 software (MedCalc Software, Broekstraat, Mariakerke, Belgium). A P-value of <0.05 was considered significant.

**RESULTS**

**Targeting of mutant KrasG12D allele by stable expression of KrasG12D-shRNA leads to decreased oncogenic Kras expression**

To target the KrasG12D mutant allele, siRNA oligos were designed that were 64 nucleotides long and covered with a point mutation in codon 12 (G→D) of the Kras gene. They were cloned into the pSUPER RETRO mammalian expression vector. Similarly, a scramble expression vector construct (pSUPER Kras-Scr) was made using scramble shRNA oligonucleotides. The resultant constructs (pSUPER-shKrasG12D and pSUPER Kras-Scr) were transfected into CD18/HPAF pancreatic adenocarcinoma cells. Pooled populations of CD18/HPAF-shKrasG12D and CD18/HPAF-Kras-Scr were selected for puromycin resistance. The effective inhibition of the mutant KrasG12D allele was determined by real-time PCR using a primer set that selectively amplifies the mutated Kras allele but not the wild-type (WT) allele. RNAs isolated from BXPC3, HPDE and Capan-1 cells were used as a control as it is known that BXPC3 and HPDE cells express only the WT alleles and Capan-1 has a G12V mutation instead of the G12D mutation. The CD18/HPAF-shKras cells had a significantly decreased expression of KrasG12D mRNA compared with the CD18/HPAF-Kras-Scr cells (Figure 1A). Furthermore, the expression of total Kras protein was reduced in the CD18/HPAF-shKrasG12D cells compared with Kras-Scr-transfected cells (Figure 1B). Similar results were observed with transient knockdown of the oncogenic Kras allele in ASPC1 cells (Figure 1C).

In order to confirm the specificity of the oligos, we transiently transfected BXPC3 cells (KrasG12D negative) with pSUPER-shKrasG12D. The pSUPER-Kras-Scr vectors revealed no significant decrease in Kras total protein between the BXPC3 Scr and BXPC3 shKras (Figure 1D).

**Silencing of mutant KrasG12D allele leads to altered morphology, decreased growth rate and reduced clonogenicity of pancreatic cancer cells**

The morphology and growth rates of the pooled populations of CD18/HPAF-shKras and CD18/HPAF-Kras-Scr cells were monitored after inhibiting oncogenic Kras (mutant allele) in tumour cells. The CD18/HPAF-shKras cells showed a tendency to grow as clumps when compared with the CD18/HPAF-Kras-Scr cells (Figure 1E). Similar growth pattern was observed with silencing of mutant KrasG12D allele in ASPC1 cells (Figure 1F). A growth curve was plotted to determine the effect of Kras knockdown on the cell doubling time. Calculation of population doubling time during the exponential phase (96–144 h) demonstrated a significant (P<0.0045) increase in cell doubling time in the CD18/HPAF-shKras cells (61.0 h) compared with scramble siRNA-transfected cells (27.0 h; Figure 2A). Furthermore, on the last day (day 8), there was nearly a 90% reduction in the number of cells in...
the CD18/HPAF-shKras group when compared with the CD18/HPAF-Kras-Scr group (Figure 2A).

The effect of silencing oncogenic Kras<sup>G12D</sup> expression on the clonogenic properties of CD18/HPAF cells was studied in anchorage-independent and anchorage-dependent conditions. We observed that the CD18/HPAF-shKras<sup>G12D</sup> cells had a significantly reduced ability to divide as evidenced by the reduction in the number of colonies formed in both anchorage-independent (\(P < 0.0001\)) and anchorage-dependent conditions (Figure 2B and C). Analysis of PI- and Annexin V-positive cells by flow cytometry indicated a significant increase in the number of apoptotic cells (\(P < 0.05\); with Annexin V-positive but PI-negative staining) and late apoptotic/necrotic cells (\(P < 0.002\); with Annexin V-positive and PI-positive staining) in CD18/HPAF-shKras cells when compared with CD18/HPAF-Kras-Scr cells (Figure 2D).

Oncogenic Kras knockdown results in an inhibition of cell motility and invasion

Several studies have reported that invasive and metastatic properties of tumour cells are partly influenced by their phenotypic characteristics such as motility and invasion. Silencing of oncogenic Kras<sup>G12D</sup> leads to a significant (\(P < 0.0001\)) reduction in cellular motility and invasive ability (~6- and 10-fold, respectively) in CD18/HPAF cells (Figures 3A and B).

Selective inhibition of oncogenic Kras<sup>G12D</sup> in pancreatic cancer cells results in the suppression of tumourigenicity and metastasis

To examine the effect of oncogenic Kras knockdown in vivo, a pooled population of CD18/HPAF-shKras<sup>G12D</sup> and CD18/HPAF-Kras-Scr cells was orthotopically implanted into the pancreas of nude mice. The animals were killed at 21 days post-implantation and the pancreatic tumours were removed and weighed. We carried out haematoxylin and eosin staining (Figures 4A and B). Liver, lung, diaphragm, intestine, kidney and mesenteric lymph nodes were examined for the presence of metastatic lesions. A primary pancreatic tumour and metastatic lesions in the spleen and on the intestinal wall were found in all the mice implanted with CD18/HPAF-Kras-Scr cells. In this group, some animals also had metastasis in the liver (\(n = 2\)) and/or kidney (\(n = 1\)). In contrast, animals injected with CD18/HPAF-shKras cells had
Effect of Kras<sup>G12D</sup> silencing on downstream signalling

Kras mutation has previously been reported to be associated with the upregulation of cyclins D and E and downregulation of p27<sup>kip1</sup> (Fan and Bertino, 1997). As shown in Figures 5A and B, the sequence-specific knockdown of the activated Kras allele led to a decreased expression of cyclins D1 and E in the CD18/HPAF-shKras and ASPC1-shKras cells in comparison with the Kras-Scr-transfected cells, whereas no change was observed in cyclin A levels. Similarly, the expression of p27<sup>kip1</sup>, caspase-3 and cleaved caspase-9 was also increased in shKras<sup>G12D</sup> transfected cells compared with the Kras-Scr-transfected CD18/HPAF and ASPC1 cells. Inhibition of the Kras<sup>G12D</sup> allele expression resulted in a significant decrease in the activation of downstream signalling molecules, including phospho-ERK1/2, phospho-Akt and phospho-FAK, MMP-9, c-Myc, NF-kB in the shKras-transfected cells compared with Kras-Scr-transfected cells (Figures 5C–F). The level of total ERK-1/2, Akt and total FAK, however, remained unchanged. Furthermore, immunoblot analysis also revealed an increased expression of E-cadherin in the CD18/HPAF-shKras and ASPC1-shKras cells compared with the scrambled population (Figures 5C and D). In agreement with these results, immuno-fluorescence analysis also revealed an increase in E-cadherin expression and membrane localisation in CD18/HPAF-shKras-transfected cells when compared with CD18/HPAF-Kras-Scr knockdown cells (Figure 5G). Furthermore, immunohistochemical analysis of the primary orthotopic tumour sections (from the orthotopically implanted mice) revealed an increased E-cadherin expression in the CD18/HPAF-shKras tumours compared with the scramble vector-transfected cells (Figure 5H).

Alteration in signalling pathways because of knockdown of mutant Kras allele in CD18/HPAF cells

In order to identify the pathways dysregulated in PC cells because of the knockdown of the mutant Kras allele, we compared the gene expression profiles of CD18/HPAF-shKras and scramble cells by global microarray analysis. The microarray analysis revealed that many genes were significantly up- or down-regulated more than two-fold in CD18/HPAF-shKras cells compared with the scrambled cells (Supplementary Tables 2 and 3). Notably, the functional classes of genes affected by Kras silencing included tumour suppressors (HMMR, CAV1 and BHLHE41), cell adhesion molecules (CDH1, LGALS4 and PVRL3), genes regulating cellular motility and invasion (ETV4, NT5E and ALDH1A1), cell growth

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(GCNT3), cell cycle (HPGD, CDKN1A and CAV1), metastasis (CD82) and signal transduction (TM4SF4 and NR2F1). Out of these pathways, the pathway modulated by the transcription factor ETV4, SNAIL and δEF1 appeared to be highly perturbed. Using the Ingenuity Pathway Analysis (Ingenuity Systems, Mountain View, CA, USA) software, the differentially expressed genes were grouped into several gene networks (Supplementary Figure 1). Some of the differentially expressed genes were validated by real-time PCR (Figure 6A). The real-time PCR also showed a reduced expression of SNAIL and δEF1 transcription factors in Kras knockdown cells compared with control cells (Figure 6B). The gene ontology-based clustering analysis revealed that many of the genes differentially regulated upon silencing of Kras were involved in cell adhesion and metastasis. In summary, the microarray analysis suggests that Kras signalling is important in the process of PC metastasis and may crosstalk with other signalling pathways.

**DISCUSSION**

Cancer development involves a multistep process in which tumour cells acquire various genetic and epigenetic changes to grow and metastasise to distant organs. Investigation of the molecular genetics of pancreatic adenocarcinoma has revealed a specific pattern of genetic lesions that occur during the initiation and progression of PC (Aguirre et al, 2003; Hezel et al, 2006). Out of these, mutations in the Kras gene are reported to be an early event, being observed in virtually all cases of PC (75–95%; Almoguera et al, 1988; Wang et al, 2002). These mutations in Kras have an important role in the initiation and progression of PC. During the later part of the disease, other genetic and epigenetic alterations occur in EGFR, HER2, p16(Ink4a), p53, Smad/DPC4, and other genes (Aguirre et al, 2003; Hezel et al, 2006) that facilitate the development of pancreatic adenocarcinoma and, subsequently,
its metastasis (Aguirre et al., 2003; Hingorani et al., 2005; Bardeesy et al., 2006; Hezel et al., 2006). The dominant nature of the mutant Kras allele results in the cells exhibiting a transformed ability even when a single allele of mutant Kras is expressed. Consequently, inhibition of the oncogenic Kras allele expression in human cancers is a promising approach for tumour-specific gene therapy (Friday and Adjei, 2005). Previous studies have shown that Kras<sup>G12D</sup> has lower intrinsic GTPase activity than WT Kras. Furthermore, this mutant is also insensitive to p-120-GAP (Bollag et al., 1996), leading to constitutive activation of Ras-mediated downstream signalling pathways in cells expressing the mutant Kras allele. Therefore, studies on the molecular and cellular functions associated with this mutant hold paramount importance for therapeutic purposes.

In this study, the expression of the mutated Kras<sup>G12D</sup> allele was selectively inhibited by shRNA, specifically targeting the mutant allele in CD18/HPAF and ASPC-1 PC cells. Subsequently, we studied the effect of Kras<sup>G12D</sup> silencing on the function of PC cells in vitro (motility, invasion and clonogenicity) and in vivo (tumourigenesis and metastasis) and its impact on downstream signalling pathways by using pooled populations. In the case of CD18/HPAF cells, suppression of the oncogenic Kras<sup>G12D</sup> allele led to a significant reduction in their tumourigenic and metastatic potential in vivo. Microarray analysis identified several genes associated with cell growth, proliferation and metastasis that were significantly altered in the CD18/HPAF-Kras<sup>G12D</sup> knockdown cells.

The stable expression of a shRNA targeting the mutant Kras<sup>G12D</sup> allele led to a decreased expression of the mutant allele at the

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Table 1 Incidence of metastases developed by orthotopic implantation of pooled populations of CD18/HPAF Scr cells and shKras clones in immunodeficient mice

| Cell type                  | Spleen | Liver | Peritoneum | Mesenteric lymph nodes | Kidney | Intestinal wall |
|----------------------------|--------|-------|------------|------------------------|--------|-----------------|
| CD18/HPAF scramble         | 4/6 (67%) | 2/6 (33%) | 4/6 (67%) | 4/6 (67%) | 1/6 (17%) | 5/6 (83%) |
| CD18/HPAF shK-ras           | 0/6 (0%) | 0/6 (0%) | 0/6 (0%) | 1/6 (17%) | 0/6 (0%) | 0/6 (0%) |

P-value (χ² test) 0.06 0.45 0.06 0.24 1.0 0.01

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Figure 5 Western blot analysis comparing the expression of key molecules involved in cell proliferation (c-myc, cyclins A, D1, E and p27), apoptosis (caspase-3 and 9), metastasis and invasion (E-cadherin, MMP-9, NF-κB, phospho and total FAK, phospho and total Akt and phosphor and total ERK-1/2) in CD18/HPAF (A, C, E) and ASPC-1 (B, D, F) Scr vs shKras<sup>G12D</sup> cells. (H) Immunohistochemical analysis of primary tumours developed following orthotopic implantation of CD18/HPAF-shKras<sup>G12D</sup> and scramble cells in immunodeficient mice. Tumour sections were stained for E-cadherin using specific anti-mouse monoclonal antibody (original magnification × 100). Tissue derived from CD18/HPAF-shKras<sup>G12D</sup> tumours showed a stronger membrane staining for E-cadherin (arrowheads). (G) Confocal analysis showed increased E-cadherin expression on the cell membrane in CD18/HPAF-shKras<sup>G12D</sup> cells compared with scramble cells (*Normal mouse pancreatic tissue, primary tumour with E-cadherin expression).
mRNA level in CD18/HPAF-shKras cells (Figure 1A). The total Kras protein expression was also significantly decreased in the CD18/HPAF-shKras ASPC1-shKras cells (Figures 1B and C). These results suggest that siRNAs can serve as powerful tools for sequence-specific inhibition of an oncogenic mutant allele. Our study also corroborates previous studies in human (Capan-1 KrasG12D) and murine (C26 colorectal cells, KrasG12D) cells, wherein the stable knockdown of mutant Kras allele resulted in a reduced expression of both the oncogenic and total Kras protein and mRNA levels (Brummelkamp et al., 2002; Smakman et al., 2005).

It is now clearly established that activating mutations in Kras are found in the majority of PCs and that these mutations, along with other genetic lesions, contribute to the increased aggressiveness of the tumour (Almoguera et al., 2011). The Ras pathway has also been implicated in cytoskeletal rearrangements, altering the expression of integrins and cell migration through activation of the PI3-K/Akt pathway (Potempa and Ridley, 1998; Okudela et al., 2004; Fleming et al., 2005). Inhibition of KrasG12D by shRNA has been previously shown to decrease tumourigenicity in Capan-1 cells upon subcutaneous implantation (Brummelkamp et al., 2002) and in murine C26 colorectal cancer cells in vitro. The Kras knockdown cells formed fewer tumours and did not cause morbidity (Smakman et al., 2005). We also noted that inhibition of KrasG12D resulted in a significant decrease in the tumourigenic and metastatic potential of CD18/HPAF (Figure 4A and Table 1). However, there was no decrease in the incidence of tumours with 100% of the animals injected with the CD18/HPAF-shKrasG12D cells forming tumours. The possible explanations for this observation include the presence of heterogeneous cells in the pooled clonal population and the partial compensation of the anti-tumourigenic effect of KrasG12D knockdown by other unknown signalling pathways. Although these earlier studies indicated that oncogenic Kras has a key role in the proliferation of PC cells, its role in regulating metastasis of PC has remained largely unexplored. In order to determine the role of activated Kras in metastasis, we utilised an orthotopic model that revealed a significant inhibition of metastasis upon selective silencing of KrasG12D (Table 1). Our results suggest that the constitutively active form of Kras not only regulates tumour cell growth, but it also has an important role in modulating the invasive nature of the malignant cells.

Tumour invasion through the ECM and tissue barriers requires the combined effects of increased cell motility and proteolytic degradation. We observed decreased levels of activated FAK (pY925) in the CD18/HPAF-shKras and ASPC1-shKras cells when compared with that in the scramble cells (Figures 5E and F). The decreased activation of FAK may be responsible for the reduced motility observed in the CD18/HPAF-shKras cells compared with scramble cells similar to that reported in an earlier study (Sieg et al., 2000).

Additionally, the phenotypic changes associated with epithelial mesenchymal transition (EMT) include both an increased cellular motility (Thiery, 2002) and an increased production of ECM-degrading enzymes, accompanied by disruption of E-cadherin-mediated cell–cell adhesion (Takeichi, 1995; Christofori and Semb, 1999). The function of epithelial E-cadherin is altered in most epithelial tumours and can be disrupted by various genetic and epigenetic mechanisms, including modulation by signalling molecules. Loss of E-cadherin activates signals that promote tumour cell migration, invasion and dissemination (Thiery, 2002).
The increased expression of E-cadherin in the Kras knockdown cells (Figures 5C, 6A, and Supplementary Figure 1) seems to suggest that oncogenic KrasG12D can inhibit E-cadherin function partly by suppressing its expression. Furthermore, the expression of MMP-9, a key mediator of the invasive property of malignant cells (Fridman et al, 2003), was also decreased upon silencing of the oncogenic Kras allele. Kras-mediated ERK activation is known to induce MMP-9 that, in turn, causes cleavage of E-cadherin leading to the disruption of cell-cell contacts (Wang et al, 2009). Our results suggest that a MMP-9-mediated decrease in E-cadherin expression may contribute to the highly metastatic property of CD18/HPAF cells. This suggests that activated Kras contributes to the metastatic nature of CD18/HPAF cells.

To elucidate the global cellular pathways that are altered in PC cells upon downregulation of the mutant Kras allele, we conducted microarray analysis and observed that most of the genes differentially expressed in the Kras-Scr compared with the shKras cells were associated with cell proliferation, motility and metastatic behaviour of tumour cells (Supplementary Tables 2 and 3). Of those genes that were altered because of silencing of oncogenic Kras, alteration in the expression of the transcription factor Snail supports Kras-mediated E-cadherin regulation. Snail has been shown to promote tumour cell invasion by either inducing the transcription of MMP-9 (Jorda et al, 2005) or suppression of transcription of E-cadherin (Huber et al, 2005). Recently, a report showed that δEF1-ZEB1 binds to the promoter of the E-cadherin and represses its expression (Eger et al, 2005). In the present study, real-time PCR analysis showed that the expression of both SNAIL and δEF1/ZEB1 was decreased in the CD18/HPAF-shKras cells (Figure 6B). Altogether, these results, including the increase in E-cadherin, point to SNAIL and δEF1/ZEB1-mediated regulation of E-cadherin as a target of Kras in PC cells. In addition, the microarray analysis identified several differential genes associated with cell proliferation, motility and metastatic behaviour of tumour cells (Supplementary Tables 2 and 3 and Supplementary Figure 1). Taken together, the results of our study provide an insight into the diverse pathways altered in PC cells upon the sequence specific inhibition of the mutant Kras allele. This study was aimed at understanding the pathological role of the highly oncogenic KrasG12D allele in PC. The specific silencing of the oncogenic Ras allele downregulated multiple signalling pathways that are involved in promoting cell proliferation, inhibiting apoptosis, breaking cell-cell contacts and regulating expression of protease like MMP-9. The observed inhibition of cell proliferation in the Kras knockdown cells may be mediated through the inhibition of the MAPK pathway, whereas the increase in the metastatic property of KrasG12D expressing cells might be at least partly because of the activation of FAK and a reduction in the expression of E-cadherin (Figure 7). Finally, our studies demonstrated that PC cells harbouring the KrasG12D mutation are dependent on Ras signalling and suggest that shRNA-mediated gene silencing could be an effective approach for selective inhibition of activated Kras. The results of our study could be useful to target novel proteins downstream of activated Kras in order to disrupt Ras-mediated oncogenic signalling pathways.

Conflict of interest
The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)

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Figure 7 Proposed model for Kras-mediated signalling events that promote progression and metastasis of pancreatic cancer. A glycine (G) to aspartate (D) mutation in the Kras protein makes it constitutively active by making it insensitive to inactivation by GTPase-activating proteins (GAPs). The constitutively active Kras (Kras(G12D)) can then activate several pathways involved in cell proliferation, migration, invasion and metastasis of PC cells. The activated Kras upregulates cyclin D1 and downregulates the expression of p27, leading to cell proliferation. Mutant Kras-mediated activation of extracellular signal-regulated kinase (ERK) can activate downstream signalling pathways that promote cell proliferation, survival, migration and metastasis. Likewise, Kras-mediated activation of focal adhesion kinase (FAK) leads to the activation of Akt, and thus promotes cell migration and survival. The activated FAK can also directly regulate cell motility, Kras-mediated upregulation of NF-kB leads to an upregulation of the transcription factors SNAIL and δEF1 that, in turn, repress the expression of E-cadherin, a key molecule regulating cell migration/invasiveness. At the same time, it also upregulates ETV4 (a transcription factor) that, in turn, promotes the expression of matrix metalloproteinase-9 (MMP-9), a gelatinase that promotes cancer cell invasion.

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