# Stable suppression of tumorigenicity by virus-mediated RNA interference

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## Summary

**Most human tumors harbor multiple genetic alterations, including dominant mutant oncogenes. It is often not clear which of these oncogenes are continuously required and which, when inactivated, may inhibit tumorigenesis. Recently, we developed a vector that mediates suppression of gene expression through RNA interference. Here, we use a retroviral version of this vector to specifically and stably inhibit expression of only the oncogenic *K-RAS*$^{V12}$ allele in human tumor cells. Loss of expression of *K-RAS*$^{V12}$ leads to loss of anchorage-independent growth and tumorigenicity. These results indicate that viral delivery of small interfering RNAs can be used for tumor-specific gene therapy to reverse the oncogenic phenotype of cancer cells.**

## Introduction

Human carcinogenesis is a multistep process, often requiring between 7 and 10 discrete (epi)genetic events that endow the cells with ever-increasing proliferative advantage (Hanahan and Weinberg, 2000). To develop effective anticancer therapies, it is essential to know which of these events are still required late in the process of tumor progression to maintain an oncogenic phenotype. Simplified mouse models for cancer, in which fewer genetic alterations are present, have provided evidence that continued activity of certain oncogenes is required for tumor growth (Hahn and Weinberg, 2002). It is far from clear, however, which of the many alterations in human cancers are still required late in tumor progression. This highlights the need for new technology to assess the contribution of individual cancer-causing genes to the oncogenic phenotype of late-stage human cancers.

*RAS* genes are frequently mutated in human cancers, particularly in pancreatic (85%) and colon carcinoma (40%) (Barbacid, 1987; Bos, 1989). The proteins encoded by the *RAS* genes (*K-RAS*, *H-RAS*, and *N-RAS*) are guanine nucleotide binding proteins that associate with the inner plasma membrane and transduce external signals to the interior of the cell. They regulate a broad spectrum of cellular activities, including proliferation, differentiation, and cell survival (Campbell et al., 1998). Mutant *RAS* oncogenes often contain point mutations that alter only a single amino acid, which locks the oncogenic RAS proteins in a persistently activated GTP-bound state (McCormick, 1989, 1990). In mouse models of lung cancer, somatic activation of oncogenic *K-ras* was necessary for early onset of tumors, and its continued production for maintenance of tumor viability (Chin et al., 1999; Fisher et al., 2001; Jackson et al., 2001; Johnson et al., 2001). A complication in using *RAS* oncogenes as targets in anticancer therapy is that at present it is not possible to specifically inhibit only the oncogenic *RAS* alleles. This may be essential, since the wild-type *K-RAS* gene appears to be required for viability, as evidenced by the embryonic lethal phenotype of mice nullizygous for *K-ras* (Johnson et al., 1997). Therefore, tools are required to effectively inhibit the activity of oncogenic K-RAS, but not that of the wild-type K-RAS protein in normal tissues. We report here that oncogenic alleles of *K-RAS* can be specifically and stably inactivated in human cancer through use of a viral RNA interference vector, leading to loss of tumorigenicity.

### SIGNIFICANCE

**Oncogenes found in human cancer often differ from their normal counterparts by single base mutations only. No technology is available to date to specifically inactivate only the mutant versions of such oncogenes. This may be essential for safe and effective anticancer therapy, as the corresponding wild-type alleles may be required for viability of normal cells. We show here that retroviral delivery of short interfering RNAs can specifically inhibit the mutant *K-RAS*$^{V12}$ allele in human pancreatic carcinoma, while leaving the wild-type *K-RAS* allele untouched. In spite of the fact that pancreatic carcinoma cells have many genetic alterations, loss of *K-RAS*$^{V12}$ expression leads to loss of tumorigenicity, indicating that this oncogene is still required late in tumorigenesis to maintain an oncogenic phenotype.**

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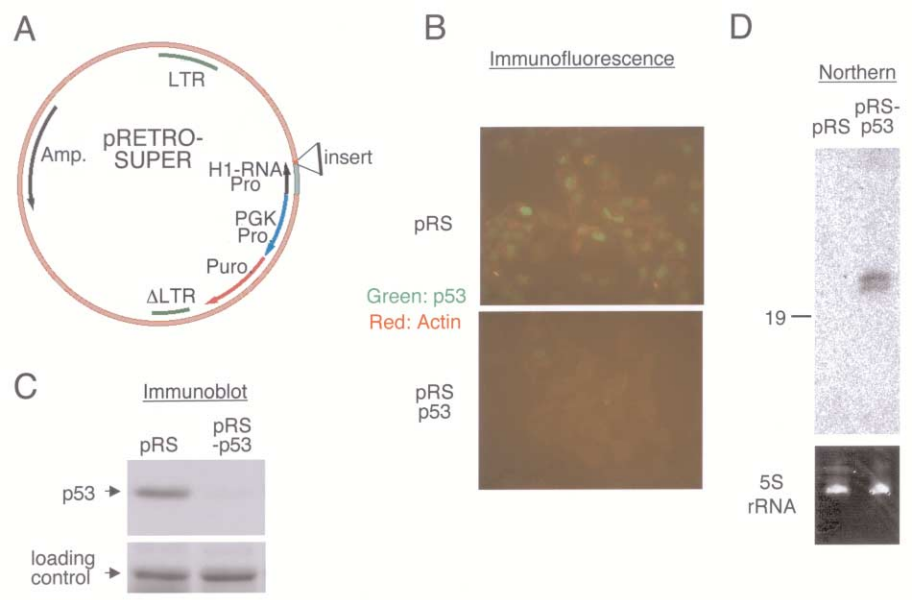


**Figure 1.** A retroviral vector that mediates RNA interference

**A:** A schematic drawing of retroviral pRETRO-SUPER RNA interference vector (pRS). DNA fragments containing the H1-RNA promoter with no insert or with an insert to target human p53 (as described in Brummelkamp et al., 2002) were digested (EcoRI-XhoI) from corresponding pSUPER constructs and cloned into a self-inactivating pMSCV to generate pRS and pRS-p53, respectively.

**B:** Human U2-OS cells that stably express the murine ecotropic receptor (to allow retroviral entry into cells) were infected with pRS and pRS-p53 retrovirus and selected for one week with puromycin. Polyclonal populations of puromycin-resistant cells were immunostained for p53 (in green) and for actin (in red).

**C:** Whole cell extracts were made from the same infected polyclonal populations of U2-OS cells as in **B**, separated by SDS-polyacrylamide gel electrophoresis (PAGE), and immunoblotted to detect p53 protein.

**D:** Northern blot analysis with total RNA from the same infected cell population described in **B** was preformed and probed with the sense 19 nucleotide targeting p53 sequence.

## Results and Discussion

We have recently developed a vector (pSUPER) that directs the synthesis of short interfering RNAs (siRNAs) in mammalian cells (Brummelkamp et al., 2002). Through RNA interference, these short transcripts can inhibit gene expression to the extent that their function is abrogated (Brummelkamp et al., 2002; Tuschl, 2002). To accomplish more efficient delivery of short interfering RNAs, we tested whether retroviruses that carry the pSUPER cassette can mediate gene silencing. We cloned the entire pSUPER expression cassette from a p53 knockdown vector (Brummelkamp et al., 2002) into a self-inactivating pMSCV-puro retroviral vector (pRETRO-SUPER-p53 (pRS-p53), Figure 1A) (*19*). Viral stocks were generated from this vector and control pRETRO-SUPER vector and used to infect U2-OS cells that express the murine ecotropic receptor to allow infection by ecotropic virus. After infection, cells were drug-selected and immunostained for p53 protein. Figure 1B shows that the vast majority of the cells which were infected with the pRS-p53 virus stained only weakly for p53, whereas all the pRS-control infected cells showed a clear nuclear p53 staining. As expected, the red staining of the control actin protein was similar in both polyclonal populations. Western blot analysis of these cells confirmed clear suppression of *p53* expression mediated by pRS-p53 virus infection (Figure 1C). Consistent with this, Northern blot analysis with the sense-19 nt p53 target sequence as a probe detected 21–22 nt siRNAs generated only by the pRS-p53 construct (Figure 1D).

It was shown recently that RNA viruses are sensitive to RNA interference (Gitlin et al., 2002; Jacque et al., 2002; Novina et al., 2002). Nevertheless, we were able to obtain high-titer retroviral supernatants of pRS-p53 ($10^6$/ml) in spite of the fact that the full-length retroviral transcript produced by pRS-p53 also contains the p53 sequence that is targeted by the virally encoded siRNAs. Apparently, the full-length retroviral transcript does not fall victim to self-inflicted RNA interference. One possible explanation could be that the intramolecular base pairing of the p53 target sequence with its complementary sequence within the retroviral transcript precludes siRNA recognition. Alternatively, rapid packaging of retroviral transcript in a viral coat may render the full-length transcript relatively resistant to RNA interference. Whatever the explanation, these results indicate that retroviral vectors can be used to mediate efficient integration of pSUPER cassettes in human cells and direct the synthesis of siRNAs to suppress gene expression.

To study the effects of inhibition of oncogenic *RAS* expression on the tumorigenic phenotype of human cancer cells, we targeted the expression of the endogenous mutant *K-RAS$^{V12}$* allele with our siRNA vector in the human pancreatic cell line CAPAN-1 (Figure 2A). We have shown recently that a single base mismatch in the 19 nt targeting sequence of siRNAs generated by pSUPER completely abrogates suppression of gene expression (Brummelkamp et al., 2002). To target specifically the mutant *K-RAS$^{V12}$* allele, we cloned a 19 nt targeting sequence spanning the region encoding valine 12 of mutant K-RAS into the pSUPER vector, yielding pSUPER-K-RAS$^{V12}$ (*20*). Figure 2B shows that CAPAN-1 human pancreatic carcinoma cells transiently transfected with pSUPER-K-RAS$^{V12}$ had signif-

**Table 1.** Oncogenic properties of cells with knockdown of K-RAS$^{V12}$

**A:** Growth in soft agar. The average number of soft agar colonies from three independent experiments is represented.

| Cell line | pRS | pRS-K-RAS$^{V12}$ | pRS-p53 |
|---|---|---|---|
| CAPAN-1 | 150–200 | 0–2 | 150–200 |
| EJ | 300–400 | 300–400 | 300–400 |

**B:** Tumorigenicity in athymic nude mice of cells infected with K-RAS$^{V12}$ or control knockdown vector.

| Cell line | pRS | pRS-K-RAS$^{V12}$ |
|---|---|---|
| CAPAN-1 | 6/6 | 0/6 |



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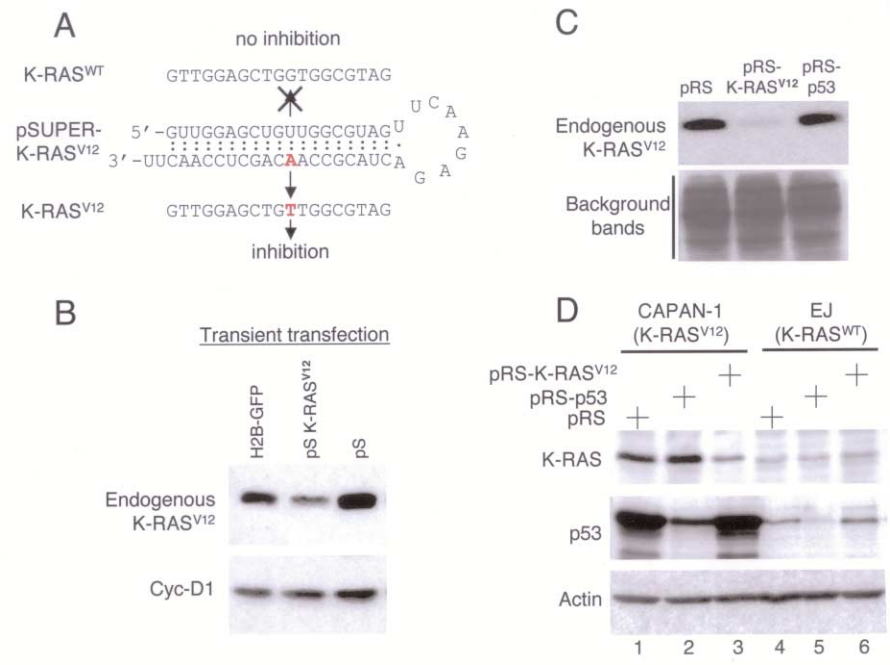


**Figure 2.** Selective suppression of oncogenic *K-RAS*$^{V12}$

**A:** Sequences of the wild-type and V12 mutant alleles of human K-RAS and the predicted mutant-specific short hairpin transcript encoded by pSUPER-K-RAS$^{V12}$.

**B:** The 19 nt sequence spanning the V12 mutation of K-RAS$^{V12}$ was used to generate a pSUPER-K-RAS$^{V12}$ (pS-K-RAS$^{V12}$) construct, as described (Brummelkamp et al., 2002). This construct, an empty pSUPER (pS), and H2B-GFP plasmids were electroporated into CAPAN-1 cells and whole cell extracts were prepared as described (Agami and Bernards, 2000). Immunoblot analysis was preformed using a specific anti-K-RAS antibody and anti-cyclin-D1 as control.

**C:** The pSUPER cassette, containing the K-RAS$^{V12}$ targeting sequence, was cloned into pRS as described in Figure 1, and virus stock was produced. A stable polyclonal pool of CAPAN-1 cells that expresses the murine ecotropic receptor was infected with the indicated viral stocks. Cells were selected with puromycin for three days and whole cell extracts were used for immunoblot analysis to detect K-RAS protein and the controls p53 and actin.

**D:** Stable polyclonal pools of CAPAN-1 and EJ cells that express the ecotropic receptor were infected with the indicated virus stocks, drug selected, and immunoblotted to detect K-RAS, p53, and actin proteins.

icant suppression of endogenous *K-RAS*$^{V12}$ expression, whereas control cyclin-D1 protein levels were unaffected (Figure 2B). We then cloned the pSUPER-K-RAS$^{V12}$ cassette into the pRETRO-SUPER retroviral vector. pRS-K-RAS$^{V12}$ virus was then used to infect CAPAN-1 cells stably expressing the murine-ecotropic receptor. Parental pRS and pRS-p53 viral stocks were used for control infections. Following drug selection, a Western blot analysis with anti-K-RAS specific antibodies revealed that the K-RAS$^{V12}$ expression in the pRS-K-RAS$^{V12}$-infected CAPAN-1 cells was markedly suppressed compared to control

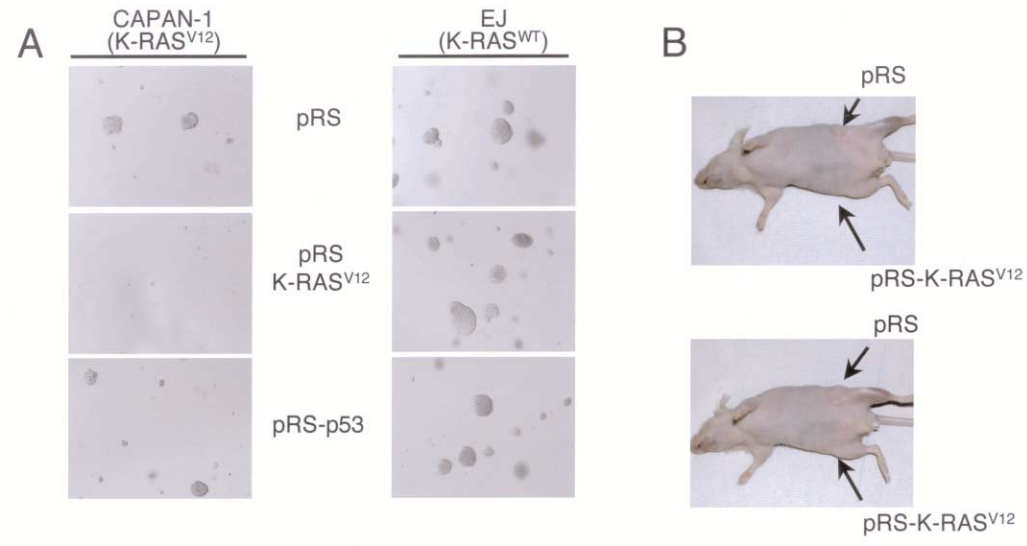


**Figure 3.** Stable and selective loss of tumorigenicity by a retroviral vector that targets the *K-RAS*$^{V12}$ oncogene

The same CAPAN-1 (harbor mutant K-RAS$^{V12}$) and EJ (harbor wild-type K-RAS) cell populations as in Figure 2 were infected with the indicated RETRO-SUPER viruses and selected for three days using 3 μg/ml puromycin.

**A:** $2 \times 10^4$ selected cells from the indicated infections were plated in duplicates in 2.5 cm diameter plates containing soft agar. Shown is one representative of three independent experiments.

**B:** $1 \times 10^6$ selected cells from pRS and pRS-K-RAS$^{V12}$ infections were injected subcutaneously into athymic nude mice as indicated. Five weeks later, mice were inspected for the presence of tumors at the site of injection.



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infections (Figure 2C). Growth curves and flow cytometry analyses showed that knocking down *K-RAS$^{V12}$* had no significant effect on the ability of CAPAN-1 cells to proliferate adherently using standard tissue culture conditions (data not shown). Next, we tested the specificity of our targeting construct by examining the expression of wt *K-RAS*. We used EJ cells, which endogenously express two wild-type *K-RAS* alleles, but harbor oncogenic *H-RAS$^{V12}$*. Western blot analysis revealed that comparable levels of wt K-RAS protein were expressed in EJ cells, irrespective of whether they were infected with the same pRS-K-RAS$^{V12}$, pRS-p53, or pRS retroviral stocks used for the CAPAN-1 cells (Figure 2D, lanes 1, 3, 4, and 6). In contrast, *p53* expression was suppressed equally by pRS-p53 in both EJ and CAPAN-1 cell types, ruling out the possibilities that the EJ cells were not infected or lacked components necessary for RNA interference (lanes 2 and 5). Thus, the RNA interference response provoked by the pRS-K-RAS$^{V12}$ retrovirus is powerful and sufficiently selective to distinguish between the wild-type and *K-RAS$^{V12}$* alleles, which differ by one base pair only.

The presence of oncogenic *K-RAS* alleles is frequent in human tumors, but almost invariably associated with multiple other genetic events. To address the question of whether the oncogenic phenotype of late stage human tumors still depends on the expression of oncogenic mutant *K-RAS*, we again used CAPAN-1 cells. One phenotype that is associated with tumorigenicity is the ability to grow independent of anchorage when plated in a semisolid media (soft agar assay). We infected CAPAN-1 and EJ cells with either pRS-K-RAS$^{V12}$ or with control pRS-p53 and pRS virus. After drug selection, $2 \times 10^4$ cells were plated in soft agar and allowed to grow for three weeks. As expected from transformed human tumor cell lines, both CAPAN-1 and EJ cell lines were able to grow and form colonies when infected with pRS and pRS-p53 control viruses (Figure 3A and Table 1A). In contrast, infection of pRS-K-RAS$^{V12}$ abolished almost completely the colony growth of CAPAN-1 cells in this assay. Importantly, the effect of pRS-K-RAS$^{V12}$ was specific, as soft agar growth of EJ cells (which contain the *H-RAS$^{V12}$* oncogene) was unaffected (Figure 3A and Table 1A).

Finally, we tested if downregulation of *K-RAS$^{V12}$* expression in CAPAN-1 cells affected their ability to form tumors in nude mice. We infected CAPAN-1 cells with either a pRS-K-RAS$^{V12}$ virus or pRS control virus and drug selected for three days to eliminate uninfected cells. After this, we injected $1 \times 10^6$ infected cells subcutaneously into athymic nude mice. As shown in Figure 3B and Table 1B, control pRS-infected CAPAN-1 cells gave rise to tumors within 5 weeks in all mice, whereas none of the six animals infected with the pRS-K-RAS$^{V12}$ virus developed tumors.

These results demonstrate that viral vectors can be used to integrate expression cassettes in the genomes of human cells, which mediate RNA interference to induce persistent loss-of-function phenotypes. Vectors like these have at least two potential applications. In gene therapy, the selective downregulation of only the mutant version of a gene allows for highly specific effects on tumor cells, while leaving the normal cells untouched. This feature greatly reduces the need to design viral vectors with tumor-specific infection and/or expression. By designing target sequences that span chromosomal translocation breakpoints found in cancer, these vectors may also be used to specifically inhibit the chimeric transcripts of these translocated chromosomes. The recent demonstration that siRNAs can inhibit gene expression in vivo provides further support for the notion that oncogene-specific RNA interference may be a viable approach to treat cancer (Lewis et al., 2002; McCaffrey et al., 2002). However, the success of such approaches still awaits the development of an efficient delivery system that can affect most of the tumor cells. In addition, these vectors can be used to efficiently identify the genetic events that are required for cancer cells to manifest a tumorigenic phenotype. Through use of this technology, out of the many genetic alterations present in most human cancer cells, the most effective targets for drug development can be rapidly identified.

## Experimental procedures

### Antibodies and plasmid construction

The antibodies used in this study were: Human p53 (Do-1) and FL393, cyclin D1 (M20), K-RAS (F234), and Actin (C-11) from Santa Cruz.

pSUPER and pSUPER-p53 constructs were described previously (Brummelkamp et al., 2002). To generate the pSUPER-K-RAS$^{V12}$, the pSUPER vector was digested with BglII and HindIII and the annealed oligos (5′gatccccGTTGGAGCTGTTGGCGTAGttcaagagaCTACGCCAACAGCTCCAACtttttggaaa3′ and 5′agcttttccaaaaaGTTGGAGCTGTTGGCGTAGtctcttgaaCTACGCCAACAGCTCCAACggg3′ were ligated into the vector. The 19 nt *K-RAS$^{V12}$* target sequences are indicated in capitals in the oligonucleotide sequence; the G-T mutation that generates the Gly-Val substitution in the twelfth amino acid of K-RAS is underlined).

To generate pRETRO-SUPER (pRS) constructs, a self-inactivating murine stem cell virus (pMSCV) plasmid was used. The 3′ LTR of the pMSCV was inactivated by an internal (NheI-XbaI) deletion to generate a self-inactivating virus (ΔLTR). Upon integration to the genome of the virus produced from this vector, the 3′ ΔLTR is duplicated to the 5′ LTR to generate a provirus that lacks all of LTR's enhancer-promoter activities. EcoRI- and XhoI-digested inserts from pSUPER, pSUPER-p53, and pSUPER-K-RAS$^{V12}$ (containing the Polymerase III promoter and the targeting inserts) were cloned into the same sites in the self-inactivating pMSCV viral construct to generate the corresponding pRS constructs.

### Cell culture, transfection, and retroviral infection

All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Ecotropic retroviral supernatants were produced by transfection of phoenix packaging cells by calcium-phosphate precipitation. 48 hours posttransfection, the tissue culture medium was filtered through a 0.45 μm filter, and the viral supernatant was used for infection of cells after addition of 4 μg/ml polybrene. Cells were infected for at least 6 hr and allowed to recover for 24 hr with fresh medium. Infected cells were selected with puromycin 1–3 μg/ml for 48 hr. High efficiency electroporation of cells was performed as described (Agami and Bernards, 2000).

Polyclonal pools of U2OS, EJ and CAPAN-1 cells stably carrying the murine ecotropic receptor were generated to allow infections with ecotropic retroviral supernatants.

### Western blot analysis and immunofluorescence

Western blots were carried out using whole cell extracts, sepa-



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rated on 10%–15% SDS-PAGE gels, and transferred to polyvinylidene difluoride membranes (Millipore). Western blots were developed using enhanced chemiluminescence (Amersham Biosciences, Inc.) following the instructions of the manufacturer. For immunofluorescence, the cells were fixed in 4% paraformaldehyde and stained using antibodies FL-393 against p53 (1:100) and Phalloidin (1:100) against actin. The second antibody used was fluorescein isothiocyanate-conjugated goat anti-mouse (1:75, DAKO).

#### Northern blot analysis

Cells were infected and selected as described above, and total RNA (30 μg) was extracted one week later. RNA was loaded on an 11% denaturing polyacrylamide gel, and separated and blotted as described (Lee et al., 1993). Membranes were probed with a $^{32}$P-labeled sense p53 19 nt probe corresponding to the target sequence. The control 5S-rRNA band was detected with ethidium bromide staining.

#### Soft agar assay

In DMEM containing 10% serum, $2 \times 10^4$ cells were resuspended in 2 ml 0.4% low melting point agarose (Sigma type VII, catalog no. A-4018) and seeded, in duplicate, into six-well plates coated with 1% low melting point agarose in DMEM containing 10% serum. The number of foci was scored after 3 weeks.

#### Tumor growth in mice

For analysis of the tumorigenic capacity of CAPAN-1 cells in vivo, $1 \times 10^6$ cells, infected and selected with either pRS or pRS-K-RAS$^{V12}$ as described above, were injected subcutaneously in each flank of athymic nude mice. Tumor growth was scored after 5 weeks.

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