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Activation of *Ras/Raf* protects cells from melanoma differentiation-associated gene-5-induced apoptosis

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Abstract

Melanoma differentiation-associated gene-5 (*mda*-5) was the first molecule identified in nature whose encoded protein embodied the unique structural combination of an N-terminal caspase recruitment domain and a C-terminal DExD/H RNA helicase domain. As suggested by its structure, cumulative evidences documented that ectopic expression of *mda*-5 leads to growth inhibition and/or apoptosis in various cell lines. However, the signaling pathways involved in *mda-5*-mediated killing have not been elucidated. In this study, we utilized either genetically modified cloned rat embryo fibroblast cells overexpressing different functionally and structurally distinct oncogenes or human pancreatic and colorectal carcinoma cells containing mutant active *ras* to resolve the role of the Ras/Raf signaling pathway in *mda*-5-mediated growth inhibition/apoptosis induction. Rodent and human tumor cells containing constitutively activated Raf/Raf/MEK/ERK pathways were resistant to *mda*-5-induced killing and this protection was antagonized by intervening in this signal transduction cascade either by directly inhibiting *ras* activity using an antisense strategy or by targeting *ras*-downstream factors, such as MEK1/2, with the pharmacological inhibitor PD98059. The present findings provide a further example of potential cross-talk between growth-inhibitory and growth-promoting pathways in which the ultimate balance of these factors defines cellular homeostasis, leading to survival or induction of programmed cell death.

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Abbreviations: Ad, adenovirus; APC, allophycocyanine; AS, antisense; CARD, caspase recruitment domain; CdkIs, cyclin-dependent kinase inhibitors; CREF, cloned rat embryo fibroblasts; ERK, extracellular signal-regulated kinase; IFN-β, fibroblast (beta) interferon; MAPK, mitogen-activated protein kinase; *mda*-5, melanoma differentiation-associated gene-5; MEK, MAPK/ERK kinase; MEZ, mezerein; m.o.i., multiplicity of infection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; pfu, plaque-forming units; PI3K, phosphatidylinositol-3-kinase; PKC, protein kinase C; SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase.

Introduction

In human melanoma cells, combinatorial treatment with interferon-β and mezerein, a protein kinase C (PKC)-activating compound, leads to terminal differentiation characterized by a rapid and irreversible loss in proliferation, changes in antigenic phenotypes and gene expression, and a decrease in tumorigenic potential in nude mice.[1–3] Melanoma differentiation-associated gene-5 (*mda*-5) was initially identified by subtraction hybridization as an upregulated transcript in terminally differentiated HO-1 human melanoma cells.[3,4] The encoded MDA-5 protein was recognized as the first molecule with a unique structural combination: an N-terminal caspase recruitment domain (CARD) and a C-terminal putative RNA helicase domain.[3,4] Our previous studies demonstrate that *mda*-5 is an early interferon inducible gene, and its expression requires an intact interferon signaling pathway, as there is no detectable expression of *mda*-5 in cells containing defects in the Jak/Stat signaling pathway upon interferon treatment.[4]

Based on the presence in the MDA-5 protein of a CARD motif, a proapoptotic element present in many caspases that facilitates cell death through homophilic protein–protein interactions, we proposed that *mda*-5 might play a role in mediating programmed cell death.[3] This hypothesis was later proven correct.[4] When ectopically expressed, *mda*-5 promotes growth inhibition in many tumor cells, as demonstrated by both cell viability and colony formation assays. Induction of membrane blebbing, formation of an internucleosomal DNA ladder on agarose gels and Annexin V binding on the exposed phosphatidylserine of dying cells, all characteristics of apoptosis, provide direct confirmation that *mda*-5-induced cell death occurs by apoptosis.[4] The ability of *mda*-5 to mediate apoptosis in a variety of tumor cells embodying various genetic mutations, including Rb, p53 and bax, indicates that *mda*-5-induced killing is independent of many

key cell death-regulating factors (unpublished data). The mouse homolog of *mda*-5 (Helicard) has also been shown to cause apoptosis by facilitating DNA fragmentation in *in vitro* experiments.[5] However, the underlying mechanism of *mda*-5-induced and Helicard-induced apoptosis is presently unknown.

The Ras/Raf/MEK/ERK pathway plays a pivotal role in cell proliferation and prevention of apoptosis.[6] Ras, a GTP-binding protein, is a common upstream activator of several signaling pathways, including Raf/MEK/ERK.[6–8] Raf is a serine/threonine kinase and can be activated through recruitment to the plasma membrane by interacting with Ras, followed by dimerization between Raf proteins, and phosphorylation of different Raf domains. Additionally, impairment of *ras* activity hinders *raf* activation.[6,9–11] Aberrant activation of this pathway is frequently linked with tumor progression and malignancy, which can contribute to the low efficacy of chemotherapy in patients.[6] Mutations leading to constitutive activation of *ras* have been found in approximately 30% of all human cancers.[12] For example, *ras* mutations contribute as a molecular defect to 85–90% of pancreatic cancers and about 50% of colorectal cancers.[13–16] Although extensive evidence has confirmed an effect of *ras* on tumorigenesis in immortal cell lines,[17] other studies have also demonstrated antiproliferative influences of oncogenic *ras* in nontransformed cells via induction of premature G_1 arrest and senescence by $p15^{Ink4b}$ and $p16^{Ink4a}$ through the Raf/MEK/ERK pathway.[18]

Our current study expands on our previous findings[3,4] corroborating the ability of *mda*-5 to induce apoptosis when overexpressed using a recombinant replication-incompetent adenovirus, Ad.*mda*-5, in a spectrum of tumor cell lines. Additionally, we have examined the effects of specific oncogenic mutations, including *HPV-18*, a specific temperature-sensitive mutant of type 5 adenovirus (*H5hr1*), *src*, *ras* and *raf*, on *mda*-5-mediated killing in an immortal normal rat embryo fibroblast cell line (CREF), which was developed in our laboratory to analyze the role of discrete genetic changes in the process of cancer progression.[19,20] CREF cells exhibit properties of untransformed rat embryo cells, including contact inhibition, failure to grow in an anchorage-independent manner in agar and lack of tumorigenic potential when injected into athymic nude mice or syngeneic Fischer rats.[19–21] CREF cells are susceptible to oncogenic transformation by different cellular and viral oncogenes, including *Ha-ras*, *v-src*, *v-raf*, *HPV-18* and *H5hr1*.[15,20–23] Unlike human tumor cells, which frequently harbor numerous mutations in several genes, CREF cells and their single oncogene-transformed counterparts reflect a homogeneous genetic background, thereby facilitating a precise determination of the role of specific oncogenic changes in susceptibility to *mda*-5-induced apoptosis. Based on these considerations, we used this well-defined system to examine the effects of *ras* and *raf* as well as other oncogenes, including *H5hrl*, *HPV* and *src*, on *mda*-5-induced killing. Among the different transformed variants of CREF cells, only *ras*- and *raf*-transformed CREF cells (CREF-*ras* and CREF-*raf*) were protected from cell death induced by *mda*-5. Based on these observations, studies were extended to human tumor cells, including colorectal and pancreatic carcinoma cells with a well-defined *ras* status, again highlighting a role for this genetic change in mediating resistance to *mda*-5. A direct functional relationship between *ras* activation and protection of tumor cells from *mda*-5-induced death was confirmed by inhibiting *ras* function using antisense or genetic deletion approaches, thereby eliciting sensitivity to *mda*-5-induced apoptosis. Furthermore, PD98059, a specific inhibitor of MEK1/2 that has been shown to inhibit MEK/ERK pathway activation, also diminished the protection of mutated active *ras* in preventing *mda*-5-induced cell death. These studies support an antiapoptotic role of the Ras/Raf/MEK/ERK signaling pathway in regulating *mda-5*-mediated death of tumor cells.

Results

Ad.*mda*-5 induces growth suppression and apoptosis in parental CREF cells, but not in mutant *ras*- or *raf*-transformed CREF cells

Parental nontransformed and different oncogene-transformed CREF cells were infected with either Ad.*null* (a recombinant adenovirus lacking the *mda*-5 gene) or Ad.*mda*-5 at a multiplicity of infection (m.o.i.) of 50 plaque-forming units (pfu) per cell, as described in Materials and Methods. After 48 h, adenovirus-mediated expression of *mda*-5 was detected at the mRNA and protein levels by Northern and Western blot analyses, respectively (Figure 1a). Parental and transformed CREF cells produced both *mda*-5 mRNA and protein after Ad.*mda*-5 infection, but not following Ad.*null* infection (Figure 1a). A quantitative analysis of the level of *mda*-5 expression using densitometer scanning confirmed comparable levels of *mda*-5 in the different CREF cells (data not shown).

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays were used to determine cell proliferation and viability. At 5 days after Ad.*mda*-5 infection (100 pfu/cell), significant growth inhibition and/or viability decreases were apparent in CREF, CREF-*H5hrl*, CREF-*HPV* and CREF-*src* cells, but not in CREF-*raf* and CREF-*ras* cells (Figure 1b). Ad.*mda*-5-infected CREF, CREF-*H5hrl*, CREF-*HPV* and CREF-*src* cells grew at a rate of 49, 46, 66 and 58% of uninfected cells, respectively (Figure 1b). Ad.*null* infection did not affect cell growth in any of the cell lines in comparison with their uninfected counterparts, ruling out the possibility that adenoviral infection at 100 pfu/cell might have contributed to this growth inhibition and/or viability decrease. Colony formation assays further demonstrated that Ad.*mda*-5 infection at 100 pfu/cell led to significant decreases in colony numbers in CREF, CREF-*H5hrl*, CREF-*HPV* and CREF-*src* cells, but not in CREF-*raf* or CREF-*ras* cells (Figure 1c). The colonies formed by CREF, CREF-*H5hrl*, CREF-*HPV* and CREF-*src* after Ad.*mda*-5 infection accounted for 60, 46, 66 and 58% of colonies formed by uninfected cells, respectively. Ad.*null* infection alone did not change the number of colonies formed in any cell type as compared to uninfected control cells (Figure 1c). Growth inhibition shown by MTT or colony formation assays may result from cell growth arrest or cell death. As *mda*-5 is known to induce apoptosis, the mechanism of growth inhibition in CREF, CREF-*H5hrl*, CREF-*HPV* and CREF-*src* cells after Ad.*mda*-5 infection was examined using apoptosis assays. At 2 days after infection with either

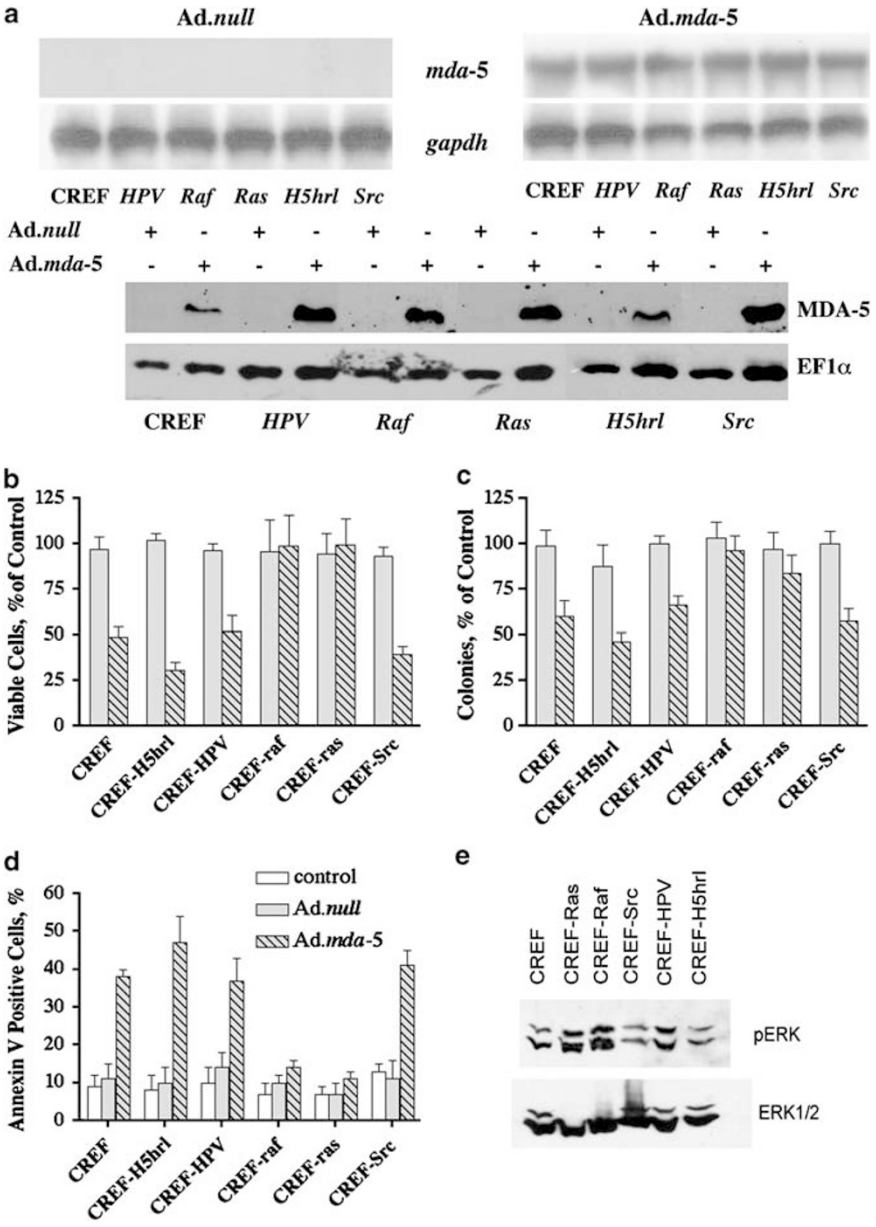

Figure 1 *Ras*- and *raf*-transformed CREF cells are resistant to *mda-5*-induced cell death. Cells were infected with Ad.*null* or Ad.*mda*-5 at an m.o.i. of 100 pfu/cell. (**a**) (Top) mRNA levels of *mda-5* determined by Northern blot analysis. (Bottom) Protein levels of MDA-5 determined by Western blotting analysis. (**b**) Cells plated at 1000 cells per well in 96-well plates on the previous day were infected with either Ad.*null* or Ad.*mda*-5, and cell viability was measured by MTT assay on the fifth day after infection. (**c**) Effect of Ad.*mda*-5 infection on the cloning (colony-forming) ability of CREF clones. Cells infected on the previous day were replated at 300 cells/60-mm plate. (**d**) CREF clones plated on six-well plates (20 000 cells/well) on the previous day were infected with Ad.*null* or Ad.*mda*-5 and Annexin V binding was determined 48 h later. (**e**) The level of phospho- and total ERK in the indicated cell lines was determined by Western blot analysis

Ad.*null* or Ad.*mda*-5 at 100 pfu/cell, parental and transformed CREF cells were incubated with Annexin V-allophycocyanine (APC) and positively stained cells, indicative of apoptotic cells, were measured by flow cytometry. Although there was some level of spontaneous apoptosis (up to 13% in CREF-*src* cells) in uninfected control cells, Ad.*mda*-5 infection resulted in 38, 47, 37 and 41% Annexin V-positive cells in CREF, CREF-*H5hrl*, CREF-*HPV* and CREF-*src* cells, respectively (Figure 1d). Ad.*null* infection did not cause any statistically relevant level of apoptosis above the basal level in any of the CREF cells. In contrast, CREF-*raf* and CREF-*ras* cells displayed no apoptosis induction following Ad.*mda-5* infection (100 pfu/cell) when compared with uninfected or Ad.*null*-infected cells (Figure 1d). The level of activation of pathways downstream of *ras* or *raf* was analyzed by Western blot analysis for phosphorylated ERK in different oncogene-transformed CREF cell lines. Higher phospho-ERK level was observed in CREF-*ras* and CREF-*raf* cells than the other cell lines (Figure 1e). The level of total ERK was similar in all the cell lines. These results argue that activation of *ras* or *raf*

protects rodent cells from apoptosis induction resulting from overexpression of *mda*-5.

To further confirm an antiapoptotic role of *ras*/*raf* in *mda*-5-induced cell death in CREF cells, we used a replication-incompetent adenovirus expressing antisense (AS) K-*ras* (Ad.K*ras*(AS)) to block *ras* function. As shown by MTT assay in Figure 2a, none of the three Ads, Ad.*null*, Ad.*mda*-5 or Ad.K*ras*(AS), when used alone at 100 pfu/cell, caused growth suppression or viability loss in CREF-*raf* or CREF-*ras* cells. However, the combination of Ad.*mda*-5 plus Ad.K*ras*(AS) applied at 50 pfu/cell each led to induction of growth inhibition in CREF-*ras* cells to a comparable level (56% of untreated) as that found in CREF cells infected with Ad.*mda*-5 alone (59% of untreated) or combined with Ad.K*ras*(AS) (52% of untreated control). In contrast, the combinatorial treatment of CREF-*raf* with Ad.*mda*-5 and Ad.K*ras*(AS) failed to suppress cell proliferation, further confirming that *raf* activation is downstream of *ras* activation (Figure 2a). Annexin V staining demonstrated that the induction of growth inhibition in CREF cells by Ad.*mda*-5 and in CREF-*ras* cells by Ad.*mda*-5 plus Ad.K*ras*(AS) resulted from apoptosis, and neither Ad.*mda*-5 alone nor in combination with Ad.K*ras*(AS) promoted apoptosis in CREF-*raf* cells (Figure 2b).

In agreement with the findings that *v-ras*- or *v-raf*-transformed cells display elevated activation of the mitogen-activated protein kinase (MAPK) pathway,[24] the expression of oncogenic forms of *ras* and *raf* in CREF cells also activated this pathway.[15] To resolve whether *ras/raf* activation exerts its antiapoptotic effect through the MEK/ERK pathway, we used PD98059, a pharmacological inhibitor of MEK1/2 capable of blocking the *ras/raf*/MEK/ERK signaling pathway. The *mda*-5-induced killing effect in the presence or absence of PD98059 was determined by colony formation assay. As expected, when PD98059 was applied along with Ad.*mda*-5, colony formation was reduced significantly, not only in CREF-*ras* (49% of uninfected or Ad.*null*-infected cells) but also in CREF-*raf* cells (52% of uninfected or Ad.*null*-infected cells), whereas Ad.*mda*-5 alone did not cause any significant decrease in colony formation in either cell type (Figure 2c). In CREF parental cells, Ad.*mda*-5 resulted in a similar decrease in colony numbers regardless of whether PD98059 was present or absent (Figure 2c). The combinatorial treatment with Ad.*mda*-5 and Ad.K*ras*(AS) promoted colony reduction in CREF-*ras*, but not in CREF-*raf* (Figure 2c), which was consistent with MTT and Annexin V data (Figure 2a and b).

Experiments were performed to determine whether the antagonistic effects of *ras* and *raf* were mediated by their ability to modulate the level of overexpressed MDA-5 protein. Western blot analysis of CREF, CREF-*raf* and CREF-*ras* cells infected with Ad.*mda*-5 alone or in combination with Ad.K*ras*(AS), or in the presence of PD98059, displayed similar levels of MDA-5 protein (Figure 3), indicating that inhibition of Raf/Raf/MEK/ERK by either AS K-*ras* or PD98059 did not modulate MDA-5 protein levels. Employment of specific K-Ras and c-Raf antibodies revealed that CREF-*ras* contains the highest level of K-ras protein, and CREF-*raf* has the most abundant expression of c-Raf protein among the three cell lines examined, thus confirming the genetic authenticity of these cell lines (Figure 3). Ad.K*ras*(AS) infection reduced *ras* protein levels in CREF-*ras* cells

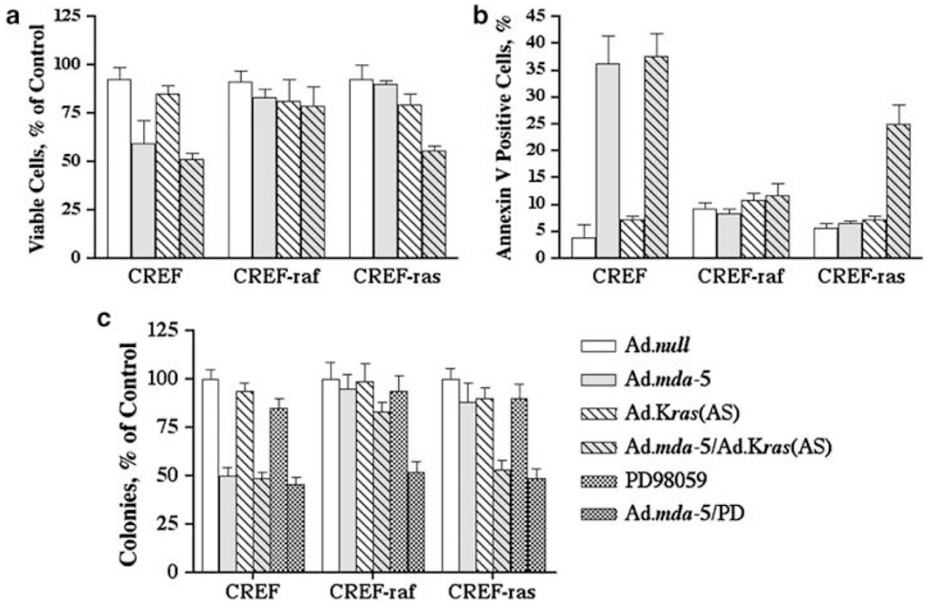

Figure 2 Inhibition of Ras/Raf/MEK/ERK activation by AS K-*ras* sensitizes CREF-*ras*, but not CREF-*raf*, to *mda*-5-induced growth inhibition and apoptosis. (**a**) The indicated cells plated at 1000 cells per well in 96-well plates on the previous day were infected with Ad.*null*, Ad.*mda*-5 or Ad.K*ras*(AS) alone at 100 pfu/cell or infected with Ad.*mda*-5 plus Ad.K*ras*(AS) at 50 pfu/cell each. Cell viabilities were measured by MTT assays on the fifth day after infection. (**b**) The indicated cells were plated in six-well plates (20 000 cells/well) on the previous day and infected with Ad.*null*, Ad.*mda*-5 or Ad.K*ras*(AS) alone at 100 pfu/cell, or infected with Ad.*mda*-5 plus Ad.K*ras*(AS) at 50 pfu/cell of each virus. After 2 days, cells were stained with Annexin V-APC and analyzed by flow cytometry. (**c**) Colony (clone)-forming ability was determined after infection with Ad.*mda*-5 in the presence or absence of Ad.K*ras*(AS) or PD98059. CREF, CREF-*raf* and CREF-*ras* cells were infected with Ad.*null*, Ad.*mda*-5, or Ad.K*ras*(AS) alone at 100 pfu/cell, or with Ad.*mda*-5 plus Ad.K*ras*(AS) at 50 pfu/cell of each virus, or with Ad.*mda*-5 (100 pfu/cell) in the presence of PD98059 (10 μM) for 24 h and replated at 300 cells/60-mm plate for colony formation assay

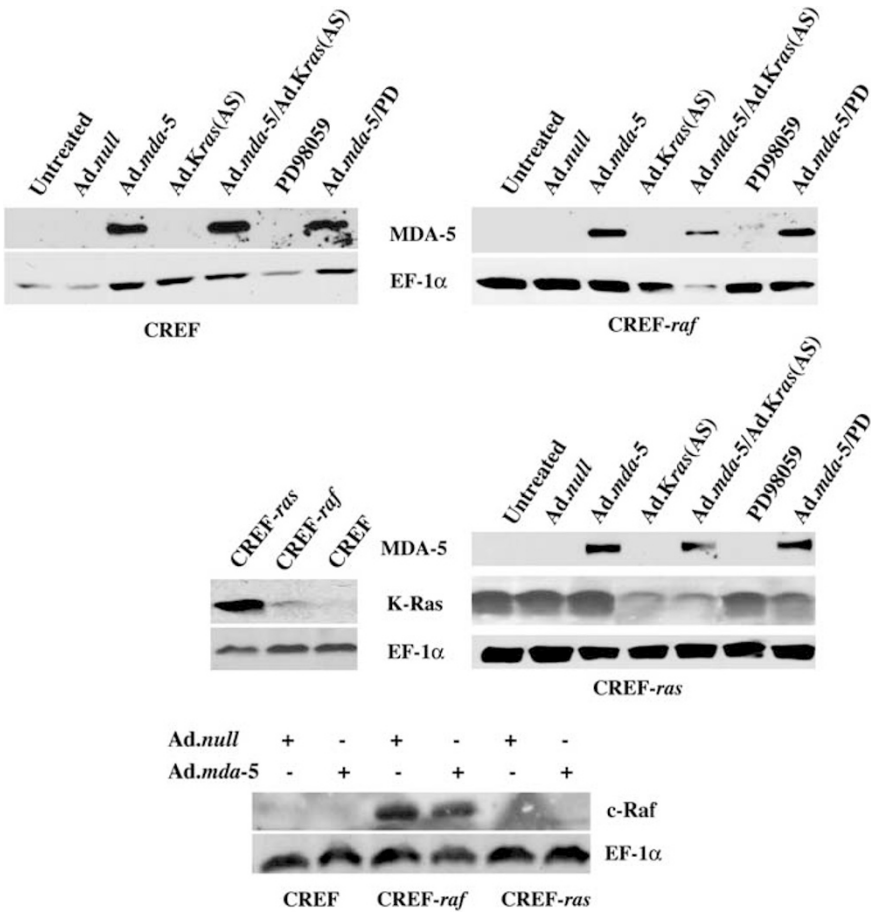

Figure 3 Expression of MDA-5, K-Ras and c-Raf proteins in CREF, CREF-*raf* and CREF-*ras* cells following Ad.*mda*-5 infection in the presence or absence of Ad.K*ras*(AS) or PD98059. Cells were infected with Ad.*null*, Ad.*mda*-5 or Ad.K*ras*(AS) alone at 100 pfu/cell, or with Ad.*mda*-5 plus Ad.K*ras*(AS) at 50 pfu/cell of each virus, or with Ad.*mda*-5 (100 pfu/cell) in the presence of PD98059 (10 μM) for 48 h. Proteins were extracted and resolved on SDS-PAGE, followed by immunoblotting with MDA-5, K-Ras or Raf-1 antibodies. Expression of EF-1α was used to normalize sample loading

(Figure 3), which correlated with the reversal of *mda*-5-induced killing in these cells when infected with both Ad.*mda*-5 and Ad.K*ras*(AS) (Figure 2). It should be noted that Ad.*null* and Ad.*mda*-5 infection did not alter the level of Raf protein in CREF-*raf*, CREF or CREF-*ras* cells (Figure 3). In total, these findings suggest that the *ras*/*raf*/MEK/ERK signaling pathway is pivotal in protecting rodent cells from *mda*-5-mediated lethality.

Mutant *ras* activation protects human pancreatic carcinoma cells from Ad.*mda*-5-induced growth inhibition and apoptosis

Studies were performed to determine if *ras* protection against *mda*-5-induced killing in rodent CREF cells represents a general phenomenon that also extends to human cancer cells. To initially test this possibility, we used four pancreatic tumor cell lines, BxPC-3 cells which contain wild-type K-*ras* and PANC-1, MIA PaCa-2 and AsPC-1 cells that contain mutant active K-*ras*. These cell lines have been intensively studied and well-characterized in our laboratory and by others.[16,25]

We initially examined the effect of Ad.*mda*-5 (100 pfu/cell) infection on cell growth by MTT assays. The three pancreatic tumor cells, PANC-1, MIA PaCa-2 and AsPC-1, expressing mutant active K-*ras* did not display significant levels of growth inhibition and/or cell viability loss following a single infection with either Ad.*mda*-5 or Ad.K*ras*(AS) in comparison with Ad.*null*-infected cells or following treatment with PD98059 (Figure 4a). However, a combination treatment of these cells with both Ad.*mda*-5 and Ad.K*ras*(AS) or Ad.*mda*-5 and PD98059 resulted in significant decreases in cell viability. Among these cells, PANC-1 were most susceptible to Ad.*mda*-5 plus Ad.K*ras*(AS) infection or Ad.*mda*-5 and PD98059 treatment, demonstrating an $\sim$90% decrease in cell viability in comparison with Ad.null-infected cells (Figure 4a). Infection of MIA Paca-2 and AsPC-1 cells with Ad.*mda*-5 and Ad.K*ras*(AS) resulted in an $\sim$60 and $\sim$30% decrease in cell viability, respectively, when compared with their Ad.*null*-infected counterparts, whereas Ad.*mda*-5 and PD98059 treatment resulted in an $\sim$70 decrease in cell viability in both these cells (Figure 4a). In contrast, in BxPC-3 cells manifesting wild-type K-*ras*, neither Ad.*mda*-5 alone nor Ad.*mda*-5 plus Ad.K*ras*(AS) or Ad.*mda*-5 and PD98059 treatment affected growth, indicating that the transformation of this cancer cell line may be *ras*-independent and that

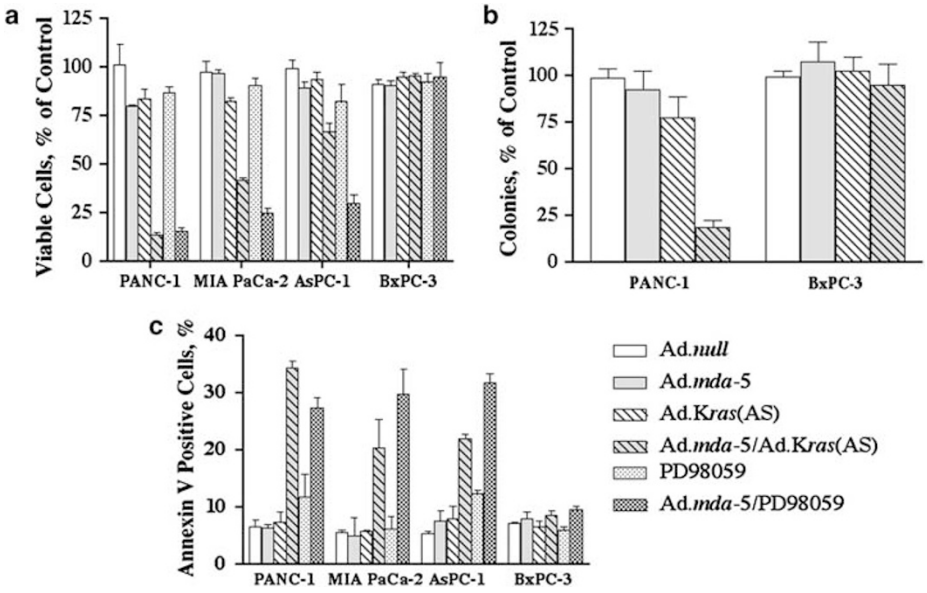

Figure 4 Inhibition of K-*ras* activity by adenovirus expressing AS K-*ras* or by treatment with PD98059 renders mutated K-*ras* human pancreatic carcinoma cells susceptible to *mda*-5-induced growth suppression and apoptosis. BxPC-3, PANC-1, MIA PaCa-2 and AsPC-1 were infected with Ad.*null*, Ad.*mda*-5 or Ad.K*ras*(AS) alone at 100 pfu/cell, or with Ad.*mda*-5 plus Ad.K*ras*(AS) at 50 pfu/cell of each virus or infected with Ad.*mda*-5 plus treated with PD98059 (10 μM). (**a**) Cells plated at 1000 cells per well in 96-well plate on the previous day were infected with the indicated virus or treated with PD98059, and cell viabilities were measured on the fifth day after infection. (**b**) BxPC-3 and PANC-1 were infected as described for panel a for 24 h and replated at 500 cells/60-mm plate. (**c**) Cells were plated in six-well plate (20 000 cells/well) on the previous day, infected with the indicated viruses or treated with PD98059, and analyzed after 2 days by FACS for Annexin V staining

this pathway also protects from *mda*-5-induced lethality (Figure 4a).

Clonal assays further documented that combined infection with Ad.*mda*-5 and Ad.K*ras*(AS) at 50 pfu/cell promoted a dramatic decrease in colony numbers in PANC-1 cells expressing mutant K-*ras*, but not in BxPC-3 cells containing wt K-*ras* (Figure 4b). Although Ad.*mda*-5 infection alone at 100 pfu/cell did not change the number of colonies formed in PANC-1 cells, the combinatorial infection with Ad.*mda*-5 and Ad.K*ras*(AS) decreased the colony-forming ability by 83% in comparison with uninfected control cells (Figure 4b). Neither Ad.*mda*-5 alone nor Ad.*mda*-5 plus Ad.K*ras*(AS) elicited a significant effect on clonigenicity in BxPC-3 cells, which corroborates the data obtained using MTT assays (Figure 4a).

Annexin V staining followed by fluorescence-activated cell sorting (FACS) analysis revealed that the growth inhibition induced by combined infection with Ad.*mda*-5 and Ad.K*ras*(AS) was due to apoptosis (Figure 4c). In parallel with the viability assays, PANC-1 cells displayed more apoptotic cells (36%) than MIA PaCa-2 (16%) and AsPC-1 (21%) cells following Ad.*mda*-5 plus Ad.K*ras*(AS) infection (Figure 4c). Ad.*mda*-5 and PD98059 treatment resulted in $\sim$25–30% apoptosis in these three cell lines. These findings were recapitulated by using siRNA specific for MEK1 and MEK2 (data not shown). There was no evidence of apoptosis in wt K-*ras* expressing BxPC-3 cells, even after combined treatment with Ad.*mda*-5 and Ad.K*ras*(AS) (Figure 4b).

Experiments were performed to explore the mechanism underlying the resistance of pancreatic carcinoma cells to *mda*-5. Infection of all four pancreatic carcinoma cells with Ad.*mda*-5 (100 pfu/cell), alone or in combination with Ad.K*ras*(AS), resulted in *mda*-5 mRNA (Figure 5a). The relative levels of *mda*-5 mRNA were higher in PANC-1 and MIA PaCa-2 cells with lower levels in BxPC-3 cells and lowest levels in AsPC-1 cells. In contrast, infection with only Ad.*mda*-5 did not result in detectable MDA-5 protein in any of these pancreatic carcinoma cells (Figure 5b). However, when the three mutant K-*ras* pancreatic cancer cell lines (PANC-1, MIA PaCa-2 and AsPC-1), but not wild-type K-*ras* BxPC-3 cells, were infected with a combination of Ad.*mda*-5 and Ad.K*ras*(AS) MDA-5 protein was detected. This effect was associated with a marginal increase in *mda*-5 mRNA in PANC-1, MIA PaCa-2 and AsPC-1 cells, most evident in AsPC-1 cells. In contrast, no change in *mda*-5 mRNA was apparent in BxPC-3 following co-infection with Ad.*mda*-5 and Ad.K*ras*(AS). The levels of *ras* proteins in the pancreatic tumor cells were measured by Western blotting using anti-*ras* antibody, confirming that the Ad.K*ras*(AS) virus was able to decrease levels of K-*ras* in all four cell types (Figure 5b). The ability of this virus to decrease K-*ras* protein levels in mutant and wild-type K-*ras* cells was predicted and relates to the fact that the antisense construct was designed to target the initiation codon of this gene, thereby displaying no preferential activity toward mutant *versus* wild-type K-*ras.* This interesting finding suggests that a 'translational block' for *mda*-5 exists in mutant K-*ras* pancreatic cancer cells, which can be ablated by blocking K-*ras* expression in a manner similar to that observed with the novel apoptosis-inducing cytokine gene *mda*-7/IL-24 in these cells.[16,25] An additional mechanism such as increased degradation of protein might also be involved in regulating MDA-5 protein level in pancreatic cancer cells. Initial studies using proteasome inhibitors show an increase in the level of MDA-5 protein (data not shown), indicating that

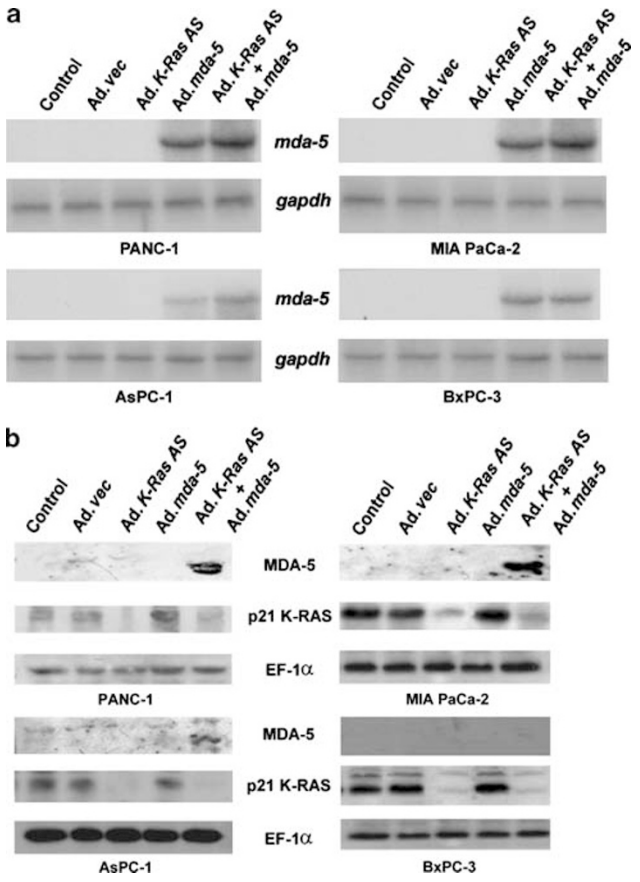

Figure 5 Expression of *mda*-5 mRNA and MDA-5 and K-Ras proteins in pancreatic carcinoma cells following Ad.*mda*-5 infection in the presence or absence of Ad.K*ras*(AS). (**a**) The indicated cell type was uninfected or infected with 100 pfu/cell with the indicated virus and *mda*-5 and *gapdh* mRNA levels were determined 48 h later by Northern blotting. (**b**) Cells were infected with the indicated virus (total m.o.i. was 100 pfu/cell) for 48 h and protein levels of MDA-5, p21 K-Ras and EF-1α were determined by Western blotting using appropriate antibodies

multiple mechanisms control MDA-5 protein expression level in these cells.

Mutant *ras* activation protects human colorectal carcinoma cells from Ad.*mda*-5-induced growth inhibition and apoptosis

After confirming a protective effect of K-*ras* from *mda*-5-induced killing in human pancreatic tumor cells, we further tested this protection in human colorectal cancer cells, the second leading human cancer containing *ras* mutations.[14,26] Two sets of human colorectal tumor cell lines were utilized, HCT 116 and Hkh2, and DLD-1 and DKO-4.[27] Among these cell types, HCT 116 and DLD-1 contain mutated active K-*ras*, whereas Hkh2 and DKO-4 are respective derivatives with their K-*ras* alleles knocked out by homologous recombination.[27]

As many colorectal carcinoma cells are sensitive to adenoviral infection (unpublished data), a total viral input of 25 pfu/cell was used. We first examined cell viability by MTT assays. Compared with uninfected cells, Ad.*null* at 25 pfu/cell did not significantly affect cell growth in HCT 116, Hkh2, DLD-1 or DKO-4 cells (Figure 6a). Although Ad.*mda*-5 (25 pfu/cell) resulted in significant growth inhibition in both K-*ras*-deleted cells, Hkh2 and DKO-4, demonstrating the cell viability of only 57 and 58% of their uninfected (data not shown) or Ad.*null*-infected counterparts, respectively, it did not lead to any significant decrease in cell viability in either of the K-*ras* constitutively active cells, HCT 116 or DLD-1 cells (Figure 6a). In HCT 116 and Hkh2, Ad.K*ras*(AS) infection at 25 pfu/cell alone caused a significant decrease in cell growth that was 70 and 42% of the uninfected cells, respectively (Figure 6a). Ad.K*ras*(AS) only marginally inhibited DLD-1 cell growth, whereas it produced an $\sim$41% decrease in cell viability in DKO-4 cells (Figure 6a). The inhibitory effect elicited by Ad.K*ras*(AS) may result from the inhibition of K-*ras* in HCT 116, and inhibition of H-*ras* by AS K-*ras* owing to the homology between different *ras* isoforms in Hkh2 and DKO-4 cells. However, the combination of Ad.*mda*-5 (12.5 pfu/cell) and Ad.K*ras*(AS) (12.5 pfu/cell) resulted in a significant decrease in growth potential in all four cell lines, further highlighting the protective role of active *ras* in *mda*-5-mediated growth suppression (Figure 6a). The relative resistance of DLD-1 to the combined growth inhibition was consistent with its decreased response to Ad.K*ras*(AS) infection (Figure 6a).

Annexin V staining and FACS analysis confirmed that cell growth suppression induced by Ad.*mda*-5, Ad.K*ras*(AS) alone or the combination of the two viruses in human colorectal cancer cells resulted to a great extent from apoptosis (Figure 6b). Consistent with the decreased cell viability in HCT 116, Hkh2 and DKO-4 cells confirmed by MTT assays, these cells exhibited high levels of apoptotic cells (40, 47 and 44%, respectively) after Ad.*mda*-5 plus Ad.K*ras*(AS) infection (Figure 6b). Although DLD-1 was relatively resistant to the combinatorial treatment, displaying only 20% apoptotic cells, this level was still statistically higher than apoptosis induced by Ad.*null* (7%), Ad.*mda*-5 alone (12%) or by Ad.K*ras*(AS) alone (10%) (Figure 6b).

Clonal assays of DLD-1 and DKO-4 cells generated results consistent with those of MTT and Annexin V staining assays. At 25 pfu/cell, Ad.*null*-infected cells gave rise to numbers of colonies comparable to that of uninfected cells (114 and 95% of that of uninfected DLD-1 and DKO-4 cells, respectively) (Figure 6c). Although Ad.*mda*-5 alone did not affect the colony-forming ability of mutant active K-*ras* expressing DLD-1 cells, it induced a decline in colony numbers when applied in the presence of either Ad.K*ras*(AS) or PD98059 (Figure 6c). However, Ad.*mda*-5 alone caused a significant decrease in colony formation in DKO-4 cells lacking K-*ras* activity. Considering the antiapoptotic role *ras* plays under many conditions, the reduced colony-forming ability of Ad.K*ras*(AS) in both DLD-1 and DKO-4 cells is likely due to a knockdown of K-*ras* activity in DLD-1 cells, and a further inhibition of H-*ras* activity in DKO-4 cells (Figure 6c).

Adenovirus-mediated expression of MDA-5 protein in these four colorectal cancer cells was determined by Western blot analysis using anti-MDA-5 antibody (Figure 7). In all four colorectal carcinoma cells, there were comparable levels of MDA-5 protein in Ad.*mda*-5-infected cells, and neither

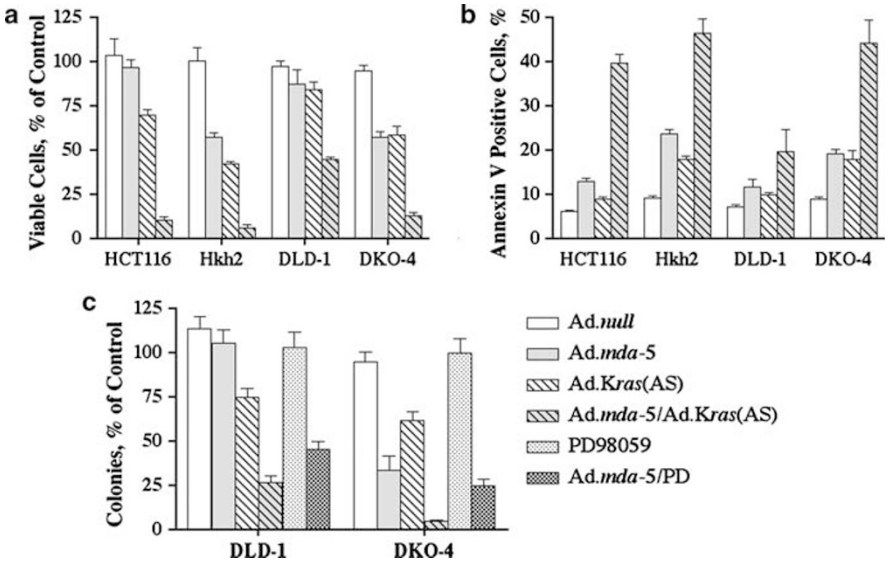

Figure 6 Blocking Ras/Raf/MEK/ERK activation using AS K-*ras* or PD98059 or by genetic knockout of K-Ras sensitizes mutant K-*ras* expressing human colorectal carcinoma cells to *mda*-5-induced growth inhibition and apoptosis. (**a**) The indicated cell type was plated at 1000 cells per well in a 96-well plate on the previous day and were either infected with Ad.*null*, Ad.*mda*-5 or Ad.K*ras*(AS) at 25 pfu/cell or infected with Ad.*mda*-5 plus Ad.K*ras*(AS) at 12.5 pfu/cell of each virus. Cell viabilities were measured on the fifth day after infection. (**b**) The indicated cell type was plated in six-well plates (20 000 cells/well) on the previous day and infected as in (**a**), stained with APC-labeled Annexin V and analyzed by FACS. (**c**) The indicated cell type was infected with Ad.*null*, Ad.*mda*-5 or Ad.K*ras*(AS) at 25 pfu/cell, or with Ad.*mda*-5 plus Ad.K*ras*(AS) at 12.5 pfu/cell of each virus, or with Ad.*mda*-5 (25 pfu/cell) in the presence of PD98059 (10 μM) for 24 h and replated with 500 cells/60-mm plate for colony formation assay

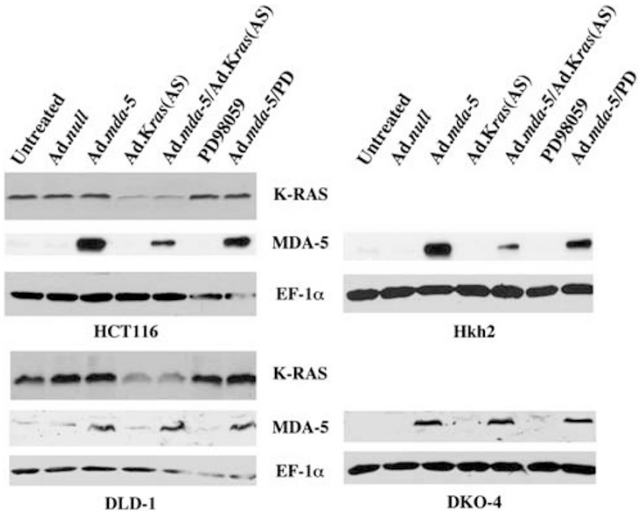

Figure 7 Expression of MDA-5 and K-Ras proteins in colorectal carcinoma cells with mutated or knocked out K-Ras following Ad.*mda*-5 infection in the presence or absence of Ad.K*ras*(AS) or PD98059. HCT 116, Hkh2, DLD-1 and DKO-4 cells were infected and/or treated as described in Figure 6, lysates were collected 48 h post-infection and Western blot analysis was performed using MDA-5, K-Ras and EF-1α antibodies

infection with Ad.K*ras*(AS) nor treatment with PD98059 lead to a change in Ad.*mda*-5-mediated MDA-5 protein levels (Figure 7). Western blotting also confirmed the levels of K-*Ras* in the four colorectal cancer cells, with HCT 116 and DLD-1 expressing abundant levels of K-*Ras* (Figure 7), Hkh2 and DKO-4 did not contain detectable levels of K-*Ras* proteins (data not shown). Ad.K*ras*(AS) reduced the endogenous level of K-*ras* by $\sim$80% in HCT 116 cells and by $\sim$40% in DLD-1 cells (Figure 7), accounting for their decreased response to *mda*-5-mediated killing in the presence of Ad.K*ras*(AS) (Figure 6a and b).

Studies have shown that different isoforms of *ras*, K-, H- and N-*ras* can play differential and overlapping roles in cell proliferation, differentiation and apoptosis.[26] We therefore employed a clone of Hkh2, HrasCL10, in which H-*ras* was introduced by transfection and stably expressed,[28,29] to test whether H-*ras* activity could replace/restore the antiapoptotic function of K-*ras* in *mda*-5-mediated cell death. Both MTT and colony formation assays showed a statistically significant restoration of growth potential and colony-forming ability in HrasCL10 cells after Ad.*mda*-5 infection compared to the significantly impaired conditions in Hkh2 (Figure 8a and b). Interestingly, cell viability and cloning ability in HrasCL10 cells did not recover completely ($\sim$70%) to that in the parental mutant active K-*ras* expressing HCT 116 cells (Figure 8a and b), which could be owing to the differential protective action of K-*ras versus* H-*ras* or alternatively the fact that the level of H-*ras* in HrasCL10 is significantly lower than that of K-*ras* in HCT 116 cells (Figure 8c).[28,29]

Unlike pancreatic carcinoma cells (Figure 5b), Ad.*mda*-5 infection of the different colorectal carcinoma cells produced equivalent amounts of MDA-5 protein, data shown for HCT 116, Hkh2 and HrasCL10 cells (Figure 8c). This finding argues against the possibility that the differential killing induced by Ad.*mda*-5 in these cells might result from differences in transduced MDA-5 protein levels (Figure 8c). As shown previously,[28,29] HCT 116 cells expressed high levels of K-*ras*, whereas there were only marginal levels of total *ras* protein in Hkh2 and HrasCL10 cells (Figure 8c).

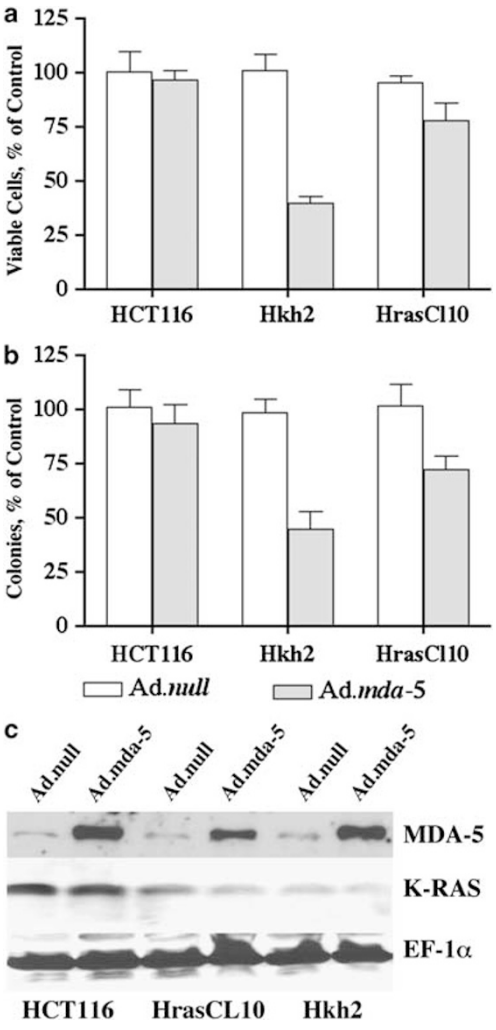

Figure 8 Stable expression of a H-*ras* oncogene in Hkh2 colorectal carcinoma cells lacking K-*ras* activity restores protection against *mda*-5-induced growth suppression in Hkh2. HCT 116, Hkh2 and HrasCL10 cells were infected with Ad.*null* or Ad.*mda*-5 at 25 pfu/cell for 2 or 5 days, and cell growth and viability were analyzed by MTT (**a**) or Annexin V staining assays (**b**), respectively. (**c**) Expression of MDA-5 and K-RAS proteins in HCT 116, Hkh2 and HrasCL10 cells 2 days after Ad.*mda*-5 infection. Cells were treated as indicated, lysates collected 48 h post-infection and Western blot analysis was performed using MDA-5, K-Ras and EF-1α antibodies

Discussion

We presently document a protective role of activation of the Ras/Raf/MEK/ERK pathway in mediating growth suppression and apoptosis by the novel *mda*-5 gene in both oncogenically transformed rodent and human pancreatic and colorectal carcinoma cells. Inhibiting Ras/Raf/MEK/ERK activation by abolishing *ras* activity, by genetic deletion, antisense strategies or by inhibiting the *ras*-downstream product MEK1/2 function by its specific pharmacological inhibitor PD98059 reverses resistance to *mda*-5-induced apoptosis. Conversely, partial restoration of *ras* function by stably transfecting H-*ras* into colorectal carcinoma cells with genetically deleted (knockout) K-*ras* partially restores the antiapoptotic and prosurvival capabilities against *mda*-5-induced proliferation inhibition and apoptosis induction.

Immortal CREF cells, which display a normal cellular phenotype, were sensitive to *mda*-5-induced growth suppression and induction of apoptosis. Among different oncogene-transformed CREF cells, only CREF-*ras* and CREF-*raf* demonstrated resistance to *mda*-5-mediated apoptosis, whereas type 5 adenovirus (*H5hrl*), *HPV-18* and *src* transformation of CREF cells did not afford protection (Figure 1). In many cell types, transformation by v-*ras* or v-*raf* can activate MAPK pathways.[24] In CREF cells, in addition to activating the MAPK pathway, transformation with the Ha-*ras* or v-*raf* oncogene also results in morphological changes and acquisition of anchorage-independent growth and tumorigenic competence in nude mice, whereas inhibition of MAPK signaling reduces anchorage-independent growth.[15,22,23] The colony-forming ability of each cell line has been found to be approximately proportional to the degree of MAPK activation,[15] suggesting that MAPK signaling may play a paramount role in this process. MAPK pathways can transduce a multitude of extracellular signals, leading to various cellular responses, including cell growth, differentiation, apoptosis or inflammation.[30] In most cases, MAPK pathways are activated by small G proteins, such as Ras, Rac and Rap1, although they can be activated by other enzymes. In mammalian cells, there are three major MAPK pathways, MAPK/ERK, SAPK/JNK and p38 MAPK.[30] Among them, the Ras/Raf/MEK/ERK pathway is positioned at the center of the signaling cascade, thereby influencing cell proliferation, differentiation and survival.

The present experiments support the hypothesis that K-*ras* activation is a primary contributor to the inability of *mda*-5 to induce apoptosis in human pancreatic and colorectal carcinoma cells, tumors in which *ras* mutations occur at high frequencies. Not only blocking K-*ras* by AS or genetic deletion but inhibiting its downstream pathway MEK1/2 allows *mda*-5 to kill K-*ras* mutant cancer cells. This combinatorial treatment-induced apoptosis appears to be a synergistic effect between *mda*-5 and K-*ras* suppression. Previously, we documented that *mda*-7/IL-24 (melanoma-differentiation-associated gene-7/interleukin-24), a tumor-specific cell death-inducing cytokine gene,[31] induces apoptosis specifically in K-*ras* mutant human pancreatic tumor cells upon inhibition of K-*ras* expression.[16,25] In mutant, but not wild-type, K-*ras* pancreatic tumor cells, *mda*-7/IL-24 mRNA is expressed following infection with 100 pfu/cell of Ad.*mda*-7, whereas MDA-7/IL-24 protein and apoptosis are only induced in these cells when K-*ras* expression is nullified. These studies uncovered an interesting phenomenon involving a message-specific 'translational block' in pancreatic tumor cells that correlated with mutant K-*ras* expression. A parallel process may also exist relative to *mda*-5 in mutant K-*ras* pancreatic carcinoma cells, as infection with Ad.*mda*-5 (at doses resulting in MDA-5 protein expression in other cell types) results in *mda*-5 mRNA with no detectable MDA-5 protein (Figure 5). When K-*ras* is inhibited using Ad.K*ras*(AS) in mutant, but not wild-type, K-*ras* pancreatic tumor cells, MDA-5 protein is detected and apoptosis is induced (Figures 4 and 5). The lack of an effect of Ad.*mda*-5 plus Ad.K*ras*(AS) in wild-type K-*ras* BxPC-3 cells suggests that additional pathways may be involved in modifying expression and/or retention of MDA-5 protein in different pancreatic tumor cells. Indeed, initial studies with proteasome inhibitors (data not shown)

indicate that accelerated degradation of MDA-5 protein might also control the expression level of MDA-5 protein. The mechanism underlying suppression of specific message translation and its reversion in pancreatic tumor cells is currently under investigation.

Numerous studies have suggested that aberrant activation of the Ras/Raf/MEK/ERK pathway is a primary reason why many tumors are refractory to current therapies. Mutant active *ras* has been a major contributor to the inappropriate activation of this pathway, and direct inhibition of *ras* activity or targeting its downstream pathway, such as MEK1/2, has proven to be effective in tumor suppression.[16] Ras/Raf/MEK/ERK activation exerts multiple roles in maintaining a cell's homeostasis, including cell growth, differentiation and apoptosis.[30] The final outcome of this activation may be cell type context dependent and the contribution of different isoforms of RAS proteins, as well as the particular *raf* gene activated. There are three isoforms of Ras proteins identified in mammals, K-, H- and N-*ras*. Some studies have shown that K-*ras* and H-*ras* have distinct and overlapping specificities to downstream signaling pathways: K-*ras* preferentially activates Raf/MEK/ERK cascade, whereas H-*ras* activates the PI3K/Akt cascade.[7,28] Additionally, there is a higher mutation frequency of K-*ras* than that of H-*ras* in human tumors,[17,26] implicating the Raf/MEK/ERK pathway as a common target for tumorigenesis. However, depending on the cell culture system used and in diverse cellular contexts, different conclusions can be drawn. For instance, some studies have documented that K- and H-*ras*-mediated signaling pathways exert different effects on apoptosis and the K-ras downstream Raf/MEK/MAPK pathway is required for the induction of apoptosis in endometrial cells. The constitutively activated K-ras promoted apoptotic cell death, whereas the H-ras mutant rescued rat endometrial cells from apoptosis.[32] Our findings suggest that in different cell systems the *ras/raf* signaling pathway plays a general role in mediating resistance to Ad.*mda-5*-induced cell death.

We have employed both K-*ras*-mutated human carcinoma cells and H-*ras*-activated rodent-transformed CREF cells. Both Ras isoforms provide protection against *mda*-5-mediated killing, suggesting that both K-*ras* and H-*ras* can elicit an antiapoptotic function through their downstream targets, in our system most likely Raf/MEK/ERK. This is further illustrated by using an isogenic human carcinoma cell system. The parental HCT 116 cells have mutant active K-*ras*, and recombinant deletion of its K-RAS D13 leads to the generation of Hkh2 cells lacking K-*ras* activity.[27] As shown earlier, Hkh2 cells also have $\sim$70% reduced basal ERK1/2, AKT and JNK1/2 activity.[29] A third cell line HrasCL10 was made by genetically introducing H-RAS V12 into Hkh2 that lacks the activated K-*ras* molecule. The expression of H-*ras* restores the basal ERK1/2 and AKT activity.[29] In our study, HCT 116 presents with high resistance to *mda*-5-mediated killing, which is extinguished in Hkh2 and partially regained in HrasCL10 cells (Figure 8). This is consistent with previous data that HrasCL10 cells show increased cell survival after radiation compared with Hkh2.[29] The fact that the protection regained in HrasCL10 cells against *mda*-5-induced apoptotic effect is not 100%, as is the case with HCT 116 cells, may indicate that H-*ras* and K-*ras* initiate different downstream pathways with different strengths. However, it is equally possible that this differential effect reflects the quantitative levels of these proteins, as the endogenous K-*ras* activity greatly exceeds the transfected H-*ras* activity (Figure 8). These possibilities are highlighted by the fact that in HrasCL10 cells there is no restored JNK1/2 activity as in parental HCT 116 cells.[29] Further experiments are required to examine the involvement of JNK1/2 in *mda*-5-mediated killing.

The Ras/Raf/MEK/ERK pathway has been shown to exert its cell cycle progression effects by inducing cell cycle regulatory proteins, such as cyclin-dependent kinase inhibitors (CdkIs), including p21$^{waf\text{-}1,Cip1,mda\text{-}6}$ and cyclins, and transcription factors, including NF-κB, c-Myc, Ets, CREB and AP-1.[17] After membrane translocation initiated by Ras, activated Raf proteins associate with cell proliferation in murine and human cytokine-dependent hematopoietic cells, murine NIH-3T3 fibroblasts and A10 smooth muscle cells.[10,30] Src can also activate raf-1 by phosphorylating Raf-1 at S338 and S339.[10] However, in our study, we found that src-transformed CREF cells, CREF-*src*, remain responsive to *mda*-5-induced apoptosis. This suggests that other signaling events may also be required to activate the Raf/MEK/ERK pathway. Additionally, overexpression of Src tyrosine kinase can be proapoptotic. In human gallbladder epithelial cells, transfection of v-src sensitizes cells to taxotere-induced apoptosis in a H-ras-, Raf-1-, PI3K- and PKC-independent manner.[33] In NIH3T3 cells, a tyrosine phosphorylation event was involved in the negative feedback regulation of Raf-1. Inhibition of a Src family tyrosine kinase appears to alleviate this tyrosine kinase-mediated inhibition of Raf-1 and allows activating modification(s) of Raf-1 to proceed.[34] Scaffolding proteins aid the transmission of signals from Raf-1 to its downstream kinases MEK and ERK. Two proteins form complexes with members of the Raf/MEK/ERK pathway. The kinase suppressor of Ras (KSR) forms complexes with Raf, MEK and ERK, whereas the MEK partner 1 (MP1) protein forms a complex with MEK and ERK.[17,35] These complexes assure that the different members of this cascade are in the appropriate stoichiometry for efficient signal transduction. Analyzing the effect of Ad.*mda*-5 infection combined with inhibition of these scaffolding proteins will facilitate the dissection of the protective role of different members of the *ras* signaling cascade. In the present study, the inability of *mda*-5 to induce apoptosis in CREF-*raf* cells even after blocking K-*ras* activity by Ad.K*ras*(AS) approaches indicates a Ras-independent Raf activation, which was also documented in previous studies.[34,36] However, the susceptibility of CREF-*raf* to *mda*-5-induced killing after MEK1/2 inhibition by PD98059 further confirms the involvement of Raf/MEK/ERK in *mda*-5-mediated apoptosis. Studies are in progress to analyze the association of Ras/Raf/MEK/ERK pathway with different aspects of the apoptotic machinery involved in Ad.*mda*-5-induced apoptosis such as bcl-2 family members, as they have been related to Ras/Raf/MEK/ERK-modulated cell survival.[17]

The present studies are beginning to shed light on the role of defined signaling pathways in mediating sensitivity or resistance to *mda*-5-induced apoptosis. A role for *ras* and *raf* in mediating resistance and an involvement of its downstream signaling component, MEK1/2, is presently illuminated. This effect has been confirmed in the context of a genetically

homogenous CREF background as well as in human pancreatic and colorectal cancer cells. In these model systems, overexpression of *ras* and in the CREF context *raf* protects cells from *mda*-5-induced killing and ablating these pathways or their downstream target MEK1/2 renders these cells sensitive to apoptosis induction by *mda*-5. Further studies are in progress to precisely define the relevance of this important signaling pathway, Ras/Raf/MEK/ERK and the putative role of other signaling pathways in mediating response to the novel apoptosis-inducing gene *mda*-5. Elucidating the mechanism of action of the proapoptotic gene *mda*-5 will shed light on several significant processes, including interferon signaling and response, cellular differentiation, apoptosis induction and viral pathogenicity.[3,4,37–40]

Materials and Methods

Reagents and antibodies

All chemicals were purchased from Sigma (St Louis, MO, USA) unless indicated. PD98059 was purchased from Calbiochem (Cat# 513000, La Jolla, CA, USA). Anti-RAF-1 (Cat# 07-396) and EF1α (Cat# 05-235) antibody were obtained from Upstate Biotechnology (Waltham, MA, USA). c-K-*RAS* antibody was purchased from Oncogene Research Products (Cat# OP24, OP24L, San Diego, CA, USA). MDA-5 antibody was produced in rabbits by repeated injections of GST-MDA-5 recombinant protein, MDA-5_{1-335}.

Cell lines and culture conditions

Parental CREF cells are a specific clone of Fischer F2408 cells.[19] CREF cells transformed by H-*ras*, v-*raf*, v-*src*, *HPV*-18 and a specific temperature-sensitive mutant of type 5 adenovirus *H5hrl* were obtained by transfecting CREF cells and isolating morphologically transformed foci.[15,41] BxPC-3, PANC-1, MIA PaCa-2 and AsPC-1 pancreatic carcinoma cells were obtained from the American Type Culture Collection. Human colorectal carcinoma cells HCT 116, Hkh2 (HCT 116 without mutant K-*ras*), DLD-1 and DKO-4 (DLK without mutant K-*ras*) were provided by Dr. Bert Vogelstein, Johns Hopkins University, MD, USA. HrasCL10 (HCT 116 without mutant K-*ras* and expressing mutant H-*ras*) were generated as described previously.[29] All cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% (for parental and transformed CREF cells) or 10% (human pancreatic and colorectal carcinoma cells) fetal bovine serum and penicillin/streptomycin (100 U/100 μg/ml) in a 5% CO_2, 95% air-humidified incubator. All cells were routinely screened for mycoplasma contamination.

Adenovirus construction and infection

The replication-incompetent adenoviruses (Ad.*null*, Ad.*mda*-5 and Ad.K*ras*(AS)) were constructed and amplified as described previously.[3,4] Amplified virus was purified with BD Adeno-X virus purification kit (BD Clontech) and viral titers were determined by plaque assay in HEK 293 cells. Adenovirus was inoculated onto cells plated the previous day at the indicated m.o.i. with a minimal volume of media providing cell coverage. After 2 h of incubation with rotation, the infected cells were refed with fresh media. Infected cells were treated and analyzed as indicated in the figure legends.

MTT cell growth and viability assays

MTT assays were performed as described previously.[16] Cells were seeded in 96-well plates and were treated next day as indicated in Results. On the day of the assay, cells were exposed to 100 μl of complete media containing 0.5 mg/ml MTT. After 4 h, an equal volume of 10% SDS–0.01 N HCl solution was added to each well and the plate was incubated at 37°C overnight to facilitate lysis of the MTT metabolite. Metabolic conversion of MTT by living cell was measured by A595 with Model 555 Microplate Reader (BioRad, Hercules, CA, USA).

RNA isolation and Northern blot analysis

Total RNA was extracted with Qiagen RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. An *Eco*RI fragment (2.5 kb) of the *mda*-5 cDNA and a 0.7-kb PCR fragment of *gapdh* were used as probes.

Western blot analysis

Protein extracts were prepared by lysis in 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 0.1% SDS, 0.1% Triton X-100, 0.1% Nonidet P-40, 0.5% Na-deoxycholate, 1 mM DTT and protease inhibitors (Roche) and centrifuged at 13 000 r.p.m. for 15 min at 4°C Equal aliquots (50 μg) of protein samples were resolved in 8% (for MDA-5 and EF-1α) or 12% (p21 K-RAS) SDS-PAGE and transferred to a nitrocellulose membrane. Immunodetection of the Western blot was performed as described previously.[4]

Apoptosis assays

Annexin V binding assays were used to determine apoptosis induction under various treatment conditions. Briefly, 48 h after treatment, cells (5×10^5 cells per sample) were trypsinized, washed twice with ice-cold PBS, resuspended in 100 μl of binding buffer containing 10 mM HEPES-NaOH (pH 7.4), 140 mM NaCl and 5 mM $CaCl_2$, and stained with APC-labeled Annexin-V (BD Biosciences, Palo-Alto, CA, USA) for 15 min at room temperature. Within 30 min after addition of 400 μl of propidium iodide in the binding buffer to a final concentration of 0.5 μg/ml, the cells were analyzed with FACS Calibur (Becton Dickinson, San Jose, CA, USA).

Colony-forming (clonal) assays

Cells were plated at 1×10^6 in a 10-cm dish 1 day before infection with adenoviruses at the indicated m.o.i. At 1 day after infection, cells were trypsinized and replated at 300–800 cells/6-cm dish with 6 ml of complete growth media. After 2 weeks, cells were fixed with 3.7% formaldehyde and stained with 5% Giemsa solution. Colonies with $\geq$50 cells were enumerated.

Acknowledgements

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