Oncogenic K-ras activates p38 to maintain colorectal cancer cell proliferation during MEK inhibition

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Abstract. Background: Colon carcinomas frequently contain activating mutations in the K-ras proto-oncogene. K-ras itself is a poor drug target and drug development efforts have mostly focused on components of the classical Ras-activated MEK/ERK pathway. Here we have studied whether endogenous oncogenic K-ras affects the dependency of colorectal tumor cells on MEK/ERK signaling.

Methods: K-ras mutant colorectal tumor cell lines C26, HCT116 and L169 were used. K-ras or components of the MEK/ERK and p38 pathway were suppressed by RNA interference (RNAi). MEK was inhibited by U0126. p38 was inhibited by SB203850.

Results: MEK inhibition, or suppression of MEK1/2 or ERK1/2 by RNA interference, reduced the proliferation rate of all colorectal cancer cell lines. However, cell proliferation returned to normal after two weeks of chronic inhibition, despite the continued suppression of MEK or ERK. In contrast, K-ras-suppressed tumor cells entered an irreversible senescent-like state following ERK pathway inhibition. MEK inhibition or ERK1/2 suppression caused activation of p38α in a K-ras-dependent manner. Inhibition or suppression of p38α prevented the recovery of K-ras mutant tumor cells during prolonged MEK inhibition.

Conclusion: Oncogenic K-ras activates p38α to maintain cell proliferation during MEK inhibition. MEK-targeting therapeutics can create an acquired tumor cell dependency on p38α.

Abbreviations

MAPK Mitogen-activated protein kinase;
ERK Extracellular signal-regulated kinase;
MEK MAPK/ERK kinase;
EGFR Epidermal growth factor receptor;
PI3K Phosphatidylinositol-3-kinase.

1. Introduction

Activating mutations in the KRAS/K-ras proto-oncogene are found in approximately 40% of colorectal tumors. Deletion or suppression of endogenous oncogenic Ras alleles from human and mouse colon tumor cells strongly reduces their tumorigenic potential [2,30,32,35]. Efforts to generate effective Ras onco- protein inhibitors have so far remained unsuccessful [7,9]. Ras-activated signaling intermediates may serve as alternative targets for therapy. Indeed, components of the classical Ras-activated MEK/ERK pathway, in particular RAF and MEK, have served as targets for the development of novel anti-cancer drugs [10,28,29]. MEK inhibitors are especially effective in tumor cells with activating mutations in the BRAF oncogene [33]. However, tumor cell lines with activating mutations in KRAS/K-ras display a highly variable response to MEK inhibitors [6,23,33,39,41]. Furthermore, the levels of steady state ERK phosphorylation in these cell lines also vary extensively and do not predict response to MEK inhibition [41]. In mice, intestine-specific expression of K-rasD12 induces hyperplasia which depends on activation of the ERK pathway [11, 36]. However, the ERK pathway is no longer activated in intestinal tumors generated by oncogenic K-ras in cooperation with mutant APC and these tumors fail to respond to MEK inhibitors [11,36]. In colon cancer patients, the majority of tumors display elevated levels of phosphorylated ERK and MEK when com-
pared to normal mucosa [14,19]. Although MEK inhibitors can effectively suppress ERK phosphorylation in human tumors, this does not correlate with robust anti-tumor responses [1, 27]. This demonstrates the existence of resistance mechanisms also in human tumors [1, 27]. The mechanisms underlying resistance to MEK-targeted therapy are incompletely understood. Recent work has shown that activation of the PI(3)-kinase pathway, by activating mutations in PIK3CA or inactivating mutations in PTEN, is a major cause of tumor cell resistance to MEK inhibitors [40].

The aim of this study was to assess whether tumor cells that are dependent on endogenous KRAS/K-ras, also depend on the MEK/ERK pathway. We show that oncogenic Ras does not cause addiction to this pathway, but allows tumor cells to recover from its inhibition by activating p38α. The finding that ERK pathway inhibition can create an acquired dependency on p38α may have implications for the use of MEK and p38-targeted therapeutics.

2. Materials and methods

2.1. Cell lines

The colorectal cancer cell lines C26 (K-rasG12D) and HCT116 (KRASG13D) were obtained from ATCC. L145 and L169 were freshly isolated from colorectal liver metastases, and were established as spheroid cultures. Both spheroid populations contain a mutant KRASG12D allele. We previously established C26 cell lines in which the endogenous K-rasD12 allele is stably suppressed by mutant specific RNA interference, using a lentiviral vector (C26-K-rasKD) [32]. Control C26 cells were transduced with a lentiviral shRNA construct targeting luciferase (see below). C26, all its derivatives, and HCT116 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Dulbecco, ICN Pharmaceuticals, Costa Mesa, CA, USA) supplemented with 5% (v/v) fetal calf serum, 2 mM glutamine, 0.1 mg/ml streptomycin, and 100 U/ml penicillin. Human intestinal epithelial cells (HIEC) were kindly provided by Prof. J.F. Beaulieu.

Human colorectal tumor specimens were obtained in accordance with the ethical standards of the institutional committee on human experimentation from patients undergoing a colon or liver resection for metastatic adenocarcinoma. Informed consent was obtained from both patients. The spheroid cells were cultured in advanced DMEM/F12 (Gibco) supplemented with 0.6% glucose (BDH Lab. Supplies), 2 mM L-glutamine (Biowhittaker), 9.6 µg/ml putrescine (Sigma), 6.3 ng/ml progesterone (Sigma), 5.2 ng/ml sodium selenite (Sigma), 25 µg/ml insulin (Sigma), 100 µg/ml apotransferrin (Sigma), 5 mM hepes (Gibco), 0.005 µg/ml trace element A (Cellgro), 0.01 µg/ml trace element B (Cellgro), 0.01 µg/ml trace element C (Cellgro), 100 µM β-mercapto ethanol (Merck), 10 ml antibiotic-antimycotic (Gibco), 4 µg/ml gentamicine (Invitrogen), 0.002% lipid mixture (Sigma), 5 µg/ml glutathione (Roche) and 4 µg/ml Heparin (Sigma). Growth factors (20 ng/ml EGF (Invitrogen) and 10 ng/ml b-FGF (Abcam)) were added to the cell culture medium freshly each week.

All cell culture was carried out in non-tissue culture treated flasks (BD Falcon) at 37°C in a 5% CO2 humidified incubator. Spheroid cultures were maintained in low-adhesion flasks in stem cell medium without serum.

Two-monthly mycoplasm tests confirmed that all experiments were performed in mycoplasm-free cell cultures.

2.2. Antibodies and inhibitors

The following antibodies were obtained from Cell Signaling Technology Inc., Danvers, MA, USA: rabbit anti-pERK p44/42 (thr 202/tyr 204), rabbit anti-pAKT (ser 473), rabbit anti-pMEK1/2 (#9121) and the secondary antibody peroxidase conjugated anti-rabbit IgG. The following antibodies were all obtained from Santa Cruz biotechnology, Heidelberg, Germany: rabbit anti-MEK1 (sc219), rabbit anti-MEK2 (sc524), rabbit anti-p21 (sc397), mouse anti-cyclin D1 (sc450), goat anti-phospho-pRb (sc12901), mouse anti-p53 (sc126), rabbit anti-p16 (sc468) and goat anti-AKT1 (sc1618). Anti-p38α (#9218) and phospho-p38 (T180/ T182) (#9211) were from Cell Signalling. Anti-phospho-MPM2 was from Millipore (#05-3669). Anti-p27 (#554069) was from BD Biosciences (Alphen aan den Rijn, The Netherlands) and anti-actin (NB 600501) was from Novus Biological (Littleton, CO, USA). The MEK inhibitor U0126 was from Promega, Madison, WI, USA and the PI3K inhibitor LY 294002 and the p38α/β inhibitor SB203850 were from Sigma, Saint Louis, MO, USA.

2.3. Lentiviral constructs

To stably knock down ERK1, ERK2, MEK1 and MEK2 we used short hairpin RNAs (shRNAs) express-
ed by lentiviral vectors. The ERK1 and ERK2 constructs were kindly provided by Dr. Brambilla and were previously described [37]. For knockdown of MEK1 and MEK2, we obtained lentiviral constructs from the TRC-library (Open Biosystems, Huntsville, AL, USA). For MEK1 and MEK2 we used the target sets NM_008927 and NM_023138. TRCN0000025214 and TRCN0000055063 produced the most effective MEK1 and MEK2 knock down respectively and were used for all subsequent experiments. For p38 we used the target set NM_011951 of which TRCN0000055223 produced the most effective knock down. The lentiviral construct targeting luciferase (control) harbored the targeting sequence TGACCAGGCATTCACAGA AAT. Lentivirus production was performed as described before [31]. Lentiviral infection was performed according to the Open Biosystems protocol.

2.4. Western blotting

Western blotting was performed as described in [17], using 50 µg of cell lysate.

2.5. Proliferation and population doubling assays

Cells were plated at a density of 5000 cells/well in 96-well plates. The relative number of viable cells in each well was then analyzed for 3–6 consecutive days by standard 3-(4,5 dimethylthiazolyl-2)-2,5-diphenyltetrazoleumbromide (MTT) assays (Roche Diagnostics) according to the manufacturer’s instructions. All proliferation assays were performed at least two times in triplicate. For population doubling assays the cells were seeded at a density of 30,000 cells/well on 6 wells plates and each well was passed at confluence in a 1:4 dilution (2 population doublings). The inhibitors U0126, LY 294002 and SB were used at a concentration of 10 µM, unless stated otherwise. All population doubling experiments were performed at least twice. For measuring growth rates the [# population doublings/# days] was determined for all control and inhibitor-treated cell populations. The mean growth rate in control-treated cell populations was set to 100% and was used to calculate the relative growth rate in inhibitor-treated cell populations.

2.6. Statistical analysis

Differences between the distinct treatment groups were evaluated using the Student’s t-test. Asterisks indicate statistical significance, based on two-tailed analyses of the data sets. Differences with p-values < 0.05 were considered statistically significant.

3. Results

3.1. Suppression of oncogenic K-ras sensitizes C26 colon tumor cells to MEK/ERK inhibition

First, we tested whether K-rasD12-dependent C26 colon tumor cells [32] are also dependent on MEK/ERK pathway activity. Pharmacologic inhibition of MEK by U0126 reduced the rate of cell proliferation and caused extensive cell flattening (Fig. 1A and B left upper and middle panel). U0126-treated C26 cells displayed strongly reduced levels of phosphorylated ERK1 and ERK2 as expected (Fig. 1C), and accumulated in G1 with a concomitant reduction in S and M-phase cells (Fig. 1D and E). Cell viability was not affected by U0126 treatment (Fig. 1B and D). After approximately 2 weeks of chronic MEK inhibition C26 cell proliferation returned to normal and this was accompanied by a partial restoration of the original cell morphology, despite continued suppression of ERK1/2 phosphorylation (Fig. 1A–C). Strikingly, U0126 treatment of C26 cells in which oncogenic K-ras is suppressed (C26-KrasKD [32]) caused extensive cell flattening and a complete loss of cell proliferation from which the cells did not recover (Fig. 1A–C). Similarly, U0126 treatment of primary human intestinal epithelial cells caused a lasting growth inhibition from which cells were unable to recover (Suppl. Fig. 1: www.qub.ac.uk/isco/JCO/).

Next we tested whether K-ras suppression would similarly sensitize cells to inhibition of other Ras-effector pathways. To this end, C26 and C26-KrasKD cells were treated with the PI(3)K inhibitor LY294002 and cell proliferation was followed over time. LY294002 reduced the rate of cell proliferation in both cell types to a similar extent (by approximately 30%), without inducing gross alterations in cell morphology and without affecting cell viability (Suppl. Fig. 2A and B: www.qub.ac.uk/isco/JCO). The levels of pAKT were stably suppressed in both cell types during the course of the experiment (Suppl. Fig. 2C: www.qub.ac.uk/isco/JCO). These results show that endogenous oncogenic K-ras provides resistance to MEK inhibition, but not to PI3K inhibition.

3.2. Oncogenic K-ras reduces tumor cell dependency on MEK and ERK

We next assessed the relative importance of MEK1 and MEK2 in maintaining proliferation of tumor cells in the presence or absence of K-rasD12. To this end, expression of either MEK1 or MEK2 was suppressed by
Fig. 1. Oncogenic K-ras reduces C26 tumor cell dependency on MEK activity. (A) C26 and C26-K-rasKD cells were treated with 10 µM U0126, which was refreshed every 3 days. Cells were passed 1:4 at confluence so that each passage correlates with 2 population doublings. The graphs show population doublings of control and U0126 treated cells over time. The selective recovery of C26 cells from U0126-mediated growth arrest was observed in four independent experiments. A representative experiment is shown. (B) Photomicrographs showing C26 and C26-K-rasKD cells (left and right panels) treated with 10 µM U0126 for three days (middle panels) and 25 days (lower panels). (C) C26 and C26-K-rasKD cells were treated as in A and the phosphorylation of ERK1 and ERK2 was assessed by anti-phospho-ERK1/2 Western blot analysis.

using lentiviral RNA interference (RNAi) vectors. Stable knockdown of either MEK1 or MEK2 had no effect on the proliferation of C26 cells (Fig. 2A and B). In contrast, knockdown of either MEK1 or MEK2 had a profound and lasting inhibitory effect on the proliferation of K-rasD12-suppressed cells (Fig. 2A and B), although the effect was less dramatic than that observed following U0126 treatment (Fig. 1A). In both cell types MEK1 and MEK2 expression were successfully and stably suppressed (Fig. 2B).

The classical MEK targets are ERK1 and ERK2. Suppression of either kinase by RNA interference had a transient but reproducible inhibitory effect on the proliferation of C26 cells, but cell proliferation returned to normal after approximately two weeks of cell culture (Fig. 2C and D). However, ERK1 or ERK2 knockdown had a far more dramatic effect on K-rasD12-suppressed cells, causing profound and long-term inhibition of cell proliferation (Fig. 2C and D). Taken together, the results show that endogenous K-rasD12 reduces the dependency of C26 cells on MEK and ERK.

3.3. Oncogenic K-ras prevents senescence induction following ERK silencing

The inhibition of MEK in cells lacking K-rasD12 resulted in complete cessation of cell proliferation, but did not induce cell death (Fig. 1A). This prompted us to investigate whether the suppression of ERK1 and ERK2 in the absence of K-rasD12 would be sufficient to induce senescence. To this end, C26 cells and C26-K-rasKD cells were transduced with a combination of the ERK1 and ERK2-targeting RNAi vectors. Combined
knockdown of ERK1 and ERK2 induced cell flattening and reduced cell proliferation in C26 cells (Fig. 3A–C), similar to U0126 treatment (Fig. 1A). Cell proliferation returned to normal after approximately two weeks of cell culture similar to what was observed following treatment with U0126, or after single ERK1 or ERK2 knockdown. However, the combined knockdown of ERK1 and ERK2 in C26-K-rasKD cells caused a complete cessation of cell proliferation after a single passage three days after initiation of selection (Fig. 3A–C). The cells did not die but obtained a morphology that was reminiscent of the morphology of senescent cells (i.e., large flattened pancake-shaped cells; Fig. 3B). An important hallmark of senescent cells is their inability to respond to growth factors. Indeed, EGF-stimulated MEK and AKT phosphorylation in control C26-K-rasKD cells, but failed to do so in the senescence-like ERK1/2 knockdown cells (Fig. 3D). In addition, the senescence-like cells also displayed loss of cyclin D1 expression and reduced phosphorylation of the retinoblastoma tumor suppressor protein pRb (Fig. 3E). Although the cyclinD1/cdk4 inhibitor p16 is usually strongly expressed in senescent cells its expression was lost in ERK1/2-suppressed cells, which further implicates ERK1/2 signaling in the control of p16 expression [20]. The senescence-like cells also displayed activation of the p53 tumor suppressor and its target p21, and of the related cell cycle inhibitor p27 (Fig. 3E). Taken together the data show that oncogenic K-ras prevents senescence induction as a result of ERK silencing.

3.4. Ras-dependent activation of p38α allows cell proliferation during ERK suppression

We next tested whether recovery of C26 cells from the U0126-imposed cell cycle arrest was correlated with activation of other MAP kinase pathways. Indeed, we found that MEK inhibition by U0126 or ERK1/2 suppression increased the phosphorylation of p38α in a K-rasD12-dependent manner, both in C26 cells and in HCT116 cells (Fig. 4). To assess whether p38α activation was involved in mediating recovery from MEK/ERK inhibition we generated stable p38α knockdown cells and made use of the p38 inhibitor SB203580. Knockdown of p38α or treatment with SB203580 had no discernable effect on cell proliferation or on the cell cycle profile of C26 cells (Fig. 5A–C). In addition, SB203580 had no effect on long-term proliferation of two additional human colorectal cancer cell lines expressing endogenous oncogenic K-ras (HCT116 and L169) (Fig. 5A). U0126 treatment caused a temporary decrease in the growth rate of all three colorectal cancer cell lines (C26, HCT116, L145) (Fig. 5A). However, all three cell types recov-
Fig. 2. Oncogenic K-ras reduces C26 tumor cell dependency on MEK1–2 and ERK1–2. (A) C26 and C26-K-rasKD cells were transduced with lentiviral shRNA constructs targeting MEK1, MEK2 or firefly luciferase (control). After puromycin selection population doubling assays were performed. The selective proliferation-suppressing effect of MEK1 and MEK2 knockdown in the C26-K-rasKD cells was observed in four independent experiments. A representative experiment is shown. Short-term mitochondrial activity assays (MTT) were carried out 35 days post-selection in triplicate (indicated by the arrow). *Indicates statistically significant differences (p < 0.01). (B) At the indicated times following puromycin selection cell lysates were prepared and expression of MEK1 and MEK2 were determined by Western blotting.

4. Discussion

Our results show that oncogenic K-ras reduces the dependency of colon tumor cells on ERK pathway activity and that it prevents senescence induction as a result of MEK inhibition. The results from clinical studies so far indicate that treatment of cancer patients with MEK inhibitors can effectively lower the
levels of pERK1/2 in different tumor types, including those of colorectal origin, but that this is not clearly correlated with changes in tumor cell proliferation or tumor progression [1,27]. Tumor cells with oncogenic Ras depend on the continued presence of this oncogene for maintaining tumorigenic potential [2,30, 32,35]. However, its presence does not make tumor cells dependent on MEK/ERK pathway activity, but can even cause resistance to inhibition of this pathway [39,40]. Identification of the K-ras-activated pathway(s) that desensitize(s) colon tumor cells to ERK pathway inhibition may therefore be the key to effective MEK-targeted therapy. A recent study showed that suppression of the PI(3)K pathway is an important determinant of colorectal tumor cell sensitivity to MEK inhibitors and that its inhibition greatly sensitizes tumor cells with oncogenic K-ras to MEK inhibition [40]. Our study identifies the p38α pathway as a second K-ras-activated resistance pathway to MEK inhibitors, p38α was activated as a result of MEK inhibition in a Ras-dependent manner. Furthermore, this was required for recovery from cell cycle arrest and restoration of proliferative capacity in three independent colorectal cancer cell lines. The effect of p38α on cell cycle inhibition or progression is highly context-dependent [38]. Most studies indicate that p38α primarily acts as a tumor suppressor (reviewed in [38]). The mechanisms of tumor suppression by p38α may vary under different conditions and in different cell types [4,16,24] and include suppression of EGFR signaling [24,42], JNK/c-Jun signaling [15,16] and activation of p53 [4,13]. Indeed, p38α-suppression caused
increased JNK phosphorylation and EGFR signaling in our cells (MdB, unpublished results) but this had no discernable stimulatory effect on tumor cell proliferation. p38α also plays a role in the G2/M checkpoint that halts cell cycle progression after DNA damage [3,21]. The latter function of p38 may be less relevant in the context of MEK/ERK suppression which causes accumulation of cells in G1. Furthermore, we did not observe an increased number of cells entering mitosis following inhibition or suppression of p38α.

In addition to its tumor-suppressive activity, p38α can also stimulate cell proliferation. This is mostly observed in established tumor cell lines, for instance by contributing to cyclin D1 and cyclin E expression [5,8,12,18,22,25,26]. In colorectal HT29 cells p38α contributes to cell proliferation and cyclin E expression [5]. These authors also demonstrated that p38α inhibition ultimately caused autophagic cell death in colorectal cancer cell lines and suggested that p38α could serve as a target for therapy in colorectal cancer [5]. In addition to p38α, p38γ may also promote colorectal tumor cell proliferation, as its inhibition reduced cell proliferation in HCT116 cells [34]. However, p38γ is insensitive to SB203580 and its expression requires ERK pathway activity [34], suggesting that it does not play a role during recovery form MEK inhibition.

Future research should elucidate under which (stressed) circumstances colorectal tumor cells come to
Fig. 3. (Continued.) (C) The cells in B were lysed and expression of ERK and ERK2 was assessed by Western blotting using a polyclonal anti-ERK1/ERK2 antibody. (D) Fourteen days after transduction of the control (luciferase) and ERK1/2 targeting shRNA vectors, the cells were stimulated with EGF (20 ng/ml, 3 min) and analyzed for AKT and MEK phosphorylation by Western blotting using phospho-specific antibodies. (E) C26-K-rasKD control and senescent-like cells were lysed and analyzed for expression of the indicated markers of cell cycle arrest and senescence by Western blotting.

Fig. 4. MEK/ERK inhibition causes K-ras-dependent p38α phosphorylation. C26, C26-K-rasKD and HCT116 cells were treated overnight with U0126 as indicated. Alternatively, C26 and C26-K-rasKD cells were transduced with shRNA vectors targeting luciferase (control) or ERK1 and ERK2 (ERK1/2) as indicated. After puromycin selection, cells were lysed and analyzed for the levels of total and phosphorylated p38α, ERK1 and ERK2. Both experiments were performed three times with similar results.

rely on p38α. The results presented here suggest that ERK inhibition can induce an acquired dependency on p38α, which offers a possibility for therapeutic exploitation.

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Fig. 5. p38α mediates Ras-dependent tumor cell recovery from MEK inhibition. (A) C26 cells were transduced with lentiviral shRNA vectors targeting luciferase (control) or p38α. A western blot showing successful p38α knockdown is shown on the right. Alternatively, cells were treated with the p38 inhibitor SB203580 (10 µM). Successful p38 inhibition was demonstrated by Western blot analysis of the phosphorylation state of the p38 substrate MK2. C26 cells in which p38 was either suppressed or inhibited were then treated with U0126 (10 µM) for 30 days. Medium (+/- inhibitors) was refreshed every three days. The growth rate of all treated cell populations relative to control cell populations was measured over time by population doubling assays before and after recovery, as indicated. Means and SEM of three independent experiments are shown.

*BDenotes statistically significant differences (p < 0.05). ns – not significant. Similarly, two human colorectal cancer cell lines (HCT116 and L169) were treated with U0126 in the presence or absence of SB203580. The rate of cell proliferation before and after recovery from U0126 was measured as above. (B) C26 cells were treated with U0126 (10 µM) for 21 days in the presence or absence of SB203580. In addition, C26-p38KD cells were treated with U0126 (10 µM) alone. The figure shows photomicrographs of C26 control and U0126-recovered cells (top panels), and of non-recovered cells in which p38 was either inhibited (middle panels) or suppressed (lower panels). Bars 50 µm.
Fig. 5. (Continued.) (C) C26 cells were treated with U0126 and/or SB203580 as indicated for 2 or for 19 days. Cells were then fixed and stained with propidium iodide and the cell cycle profile was determined by FACS analysis. Means and SEM of three independent experiments are shown. *Denotes statistically significant differences ($p < 0.05$). ns – not significant. (D) C26 cells were treated as in C. Inhibitor- and control-treated cell populations were fixed and the percentage of mitotic cells (phospho-MPM2-positive) was determined by FACS analysis. *Denotes statistically significant differences ($p < 0.05$). ns – not significant.

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