K-Ras and B-Raf oncogenes inhibit colon epithelial polarity establishment through up-regulation of c-myc

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KRAS, BRAF, and PI3KCA are the most frequently mutated oncogenes in human colon cancer. To explore their effects on morphogenesis, we used the colon cancer–derived cell line Caco-2. When seeded in extracellular matrix, individual cells proliferate and generate hollow, polarized cysts. The expression of oncogenic phosphatidylinositol 3-kinase (PI3KCA H1047R) in Caco-2 has no effect, but K-Ras V12 or B-Raf V600E disrupts polarity and tight junctions and promotes hyperproliferation, resulting in large, filled structures. Inhibition of mitogen-activated protein/extracellular signal–regulated kinase (ERK) kinase blocks the disruption of morphology, as well as the increased levels of c-myc protein induced by K-Ras V12 and B-Raf V600E. Apical polarity is already established after the first cell division (two-cell stage) in Caco-2 three-dimensional cultures. This is disrupted by expression of K-Ras V12 or B-Raf V600E but can be rescued by ribonucleic acid interference–mediated depletion of c-myc. We conclude that ERK-mediated up-regulation of c-myc by K-Ras or B-Raf oncogenes disrupts the establishment of apical/basolateral polarity in colon epithelial cells independently of its effect on proliferation.

Introduction

Human colorectal carcinomas have the third highest incidence and are the second most common cause of cancer-related deaths in both men and women (U.S. Cancer Statistics Working Group, 2010). The genetics of colorectal tumors have been extensively studied and defined by a series of stepwise alterations, including disruption of the tumor suppressor functions of APC, p53, and SMAD2/4 (Vogelstein et al., 1988; Fearon and Vogelstein, 1990). The Catalogue of Somatic Mutations in Cancer has revealed that the three most commonly mutated oncogenes in colorectal carcinoma are KRAS (35%) and its effectors BRAF (12%) and PI3KCA (12%; Forbes et al., 2011). Interestingly, colorectal tumors rarely contain both KRAS and BRAF mutations, although ~8% have PI3KCA mutations coexistent with KRAS or BRAF mutations (Rajagopalan et al., 2002; Yuan and Cantley, 2008; Janku et al., 2011).

We have developed a 3D model of morphogenesis with the colon cancer–derived cell line, Caco-2 (Jaffe et al., 2008). Caco-2 cells harbor mutations in APC, p53, and SMAD4 but not in KRAS, BRAF, or PI3KCA. (De Bosscher et al., 2004; Forbes et al., 2011). These cells have been used extensively to study normal and differentiated intestinal epithelium in 2D culture (Grasset et al., 1985; Sääf et al., 2007). When seeded in extracellular matrix containing laminin and collagen, Caco-2 cells form fully polarized cysts that have tight junctions and membrane polarity. Cyst development involves oriented, symmetric cell divisions, such that each new daughter maintains contact with a central apical surface to produce a polarized spherical monolayer (Jaffe et al., 2008). Interestingly, apical/basolateral polarity and tight junction formation are established at the two-cell stage in 3D, likely during or soon after cytokinesis in the first cell division.

3D epithelial cell culture systems provide an opportunity to explore the effects of oncogene expression on morphogenesis, alongside proliferation and growth (Debnath and Brugge, 2005). To date, most work in this area has been conducted with mammary epithelial cells. Activated ErbB2, active AKT, or ectopic c-myc expression in the human breast cancer cell line MCF10A, for example, promotes hyperproliferation and disrupts cyst...
morphogenesis in 3D cultures (Muthuswamy et al., 2001; Debnath et al., 2003; Zhan et al., 2008; Partanen et al., 2009). Further analysis suggested that disruption of cyst morphology and the promotion of proliferation are distinct activities of ErbB2—the former mediated by an interaction of this receptor with the apical polarity complex Par6–atypical PKC (aPKC; Aranda et al., 2006). However, MCF10A cells lack tight junctions, caused by low levels of the apical polarity protein Crumbs, and so are not fully polarized (Fogg et al., 2005; Underwood et al., 2006; Plachot et al., 2009). Primary mouse mammary cells do form fully polarized cysts with tight junctions when grown in a 3D matrix (Jechlinger et al., 2009). In this case, expression of the K-Ras V12 oncogene, together with c-myc, led to large, filled 3D structures, but the relationship between dysregulated proliferation and disruption of morphogenesis was not addressed in this study. Other epithelial cell types, such as lung, colon, and pancreas, have been used in 3D culture conditions, but whether disruption of morphogenesis is a direct effect of oncogene signaling or an indirect consequence of hyperproliferation is unclear (Wang et al., 2009; Makrodouli et al., 2011; Botta et al., 2012).

Here, we report that expression of the K-Ras or B-Raf oncogenes induces hyperproliferation and disrupts morphogenesis in Caco-2 3D cultures. Both oncogenes inhibit the establishment of apical polarity and tight junctions in two-cell structures, and this is mediated through increased levels of c-myc protein. We conclude that the K-Ras and B-Raf oncogenes disrupt apical/basolateral polarity in colon epithelial cells independently of their effects on proliferation.

**Results and discussion**

**Oncogenic K-Ras and B-Raf disrupt Caco-2 3D morphogenesis**

Previous work has shown that Caco-2 cells seeded in extracellular matrix generate hollow, fully polarized structures after 10 d (Jaffe et al., 2008; Durgan et al., 2011). These structures show clear apical localization of aPKC, a member of the Par3–Par6–aPKC apical polarity complex, at the inner luminal surface and are categorized as having “normal” morphology (Fig. 1 A and Video 1). Caco-2 cells expressing an oncogenic version of phosphatidylinositol 3-kinase (PI3K; PI3KCA H1047R) form structures identical to control (Fig. 1, A and B, left). In contrast, Caco-2 cells expressing K-Ras V12 or B-Raf V600E produce filled structures that lack a central lumen and are categorized as “solid” (Fig. 1, A and B, left; and Video 2). These structures show diffuse cytoplasmic staining of aPKC with occasional small patches of aPKC membrane localization. An intermediate category of “multiple layer” describes structures that have a lumen but with multiple cell layers between the apical surface and the outer edge of the structure. K-Ras V12 and B-Raf V600E structures are also much larger than controls (Fig. 1, A and B, right). The functional activity of these expressed oncogenes was confirmed by Western blot analysis, and the expression level of B-Raf V600E is similar (1.5x) to endogenous B-Raf (Fig. S1 A). In a recent publication, B-Raf V600E was also reported to produce solid structures when expressed in Caco-2 in 3D cell culture, but K-Ras V12 had no effect (Makrodouli et al., 2011). The reason for this discrepancy is not clear—perhaps the two Caco-2 isolates differ, or perhaps the level of oncogene expression is a factor.

**K-Ras V12 promotes hyperproliferation and disrupts apical polarity and tight junction formation**

To further characterize the effect of K-Ras V12 expression, cells were reextracted from 3D matrix 10 d after plating, and lysates were analyzed by Western blotting. As expected, increased levels of phospho-extracellular signal–regulated kinase (ERK) and phospho-AKT were apparent (Fig. 2 A). Control cells proliferate for several days when grown in 3D matrix (Fig. 2 B) but then exit the cell cycle (Fig. 2 C). This phenomenon has been attributed to contact inhibition followed by differentiation in 2D cultures (Siäf et al., 2007), but it is not clear why Caco-2 cells stop proliferating in 3D cultures. K-Ras V12–expressing cells, on the other hand, continue to proliferate over a period of ≥10 d, and Ki67 expression suggests that proliferation occurs in cells irrespective of whether they are positioned internally or peripherally (Fig. 2, B and C).

To investigate the effects of K-Ras V12 expression on cell polarity, immunofluorescence was performed on day 10 structures. The apical markers aPKC and ezrin strongly localize to the luminal surface of control structures, but this is disrupted after K-Ras V12 expression, and the proteins become largely cytoplasmic (Fig. 2, D and E). Although apical localization of actin is also disrupted after K-Ras V12 expression (Fig. 2 D), K-Ras V12 appears to have little effect on the deposition of laminin on the outside of peripheral cells (Fig. 2 E). E-cadherin, which localizes to lateral membranes in control cells, remains membrane bound in K-Ras V12 structures (protein levels are unaffected by Western blot analysis; not depicted) but becomes evenly distributed around the periphery of all cells (Fig. 2 F). ZO-1 localizes to the apical tip of the lateral membrane in control cells and is essential for luminal expansion. This is largely absent in K-Ras structures (Fig. 2 F). We conclude that K-Ras and B-Raf (unpublished data) oncogenes promote hyperproliferation and disrupt apical polarity and tight junction formation when expressed in Caco-2 cells.

**Mitogen-activated protein/ERK kinase (MEK) inhibition prevents K-Ras V12-mediated disruption of normal morphology**

To identify signals downstream of K-Ras V12 responsible for these effects, inhibitors of MEK (component of the ERK MAPK pathway) and PI3K were used, and their effectiveness was shown on Western blots (Fig. S1 B). Increasing concentrations of the MEK inhibitor U0126 from 5 to 20 µM prevented K-Ras V12 from promoting hyperproliferation and from disrupting Caco-2 3D morphology (Fig. 3, A and B). At the highest concentration of U0126, and after 10 d, the size and morphology of K-Ras V12 structures were almost indistinguishable from those of control cells. Interestingly, if the MEK inhibitor is washed out of K-Ras V12–expressing cells after a 4-d incubation, the normal morphology of the 3D structures is maintained over the subsequent 3 d (unpublished data). Thus, reactivation of K-Ras...
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K-Ras V12– and B-Raf V600E–expressing cells (Fig. 4 A and Fig. S2 B). Phosphorylation of c-myc by ERK on Ser62 has been shown to promote c-myc stabilization (Sears et al., 2000). This likely accounts for the increased levels of protein observed, although c-myc levels can also be regulated at the transcriptional and translational levels, and we have observed a 2.5-fold increase in c-myc mRNA levels in K-Ras V12 and B-Raf V600E 3D cultures as determined by quantitative PCR (Fig. S2 C).

To determine whether c-myc mediates any of the biological effects of K-Ras V12 or B-Raf V600E expression, two c-myc small hairpin RNAs (shRNAs) were used. Hairpin 2 substantially depleted c-myc levels, whereas Hairpin 1 depletion was partial (Fig. S2 D). Hairpin 2 fully prevented both the disruption of morphology and the hyperproliferation induced by K-Ras V12 and B-Raf V600E expression, whereas Hairpin 1 was partially effective (Fig. 4, B and C).

K-Ras V12 and B-Raf V600E promote hyperproliferation and disrupt morphogenesis through up-regulation of c-myc

ERK activation has a large number of downstream consequences, but numerous studies suggest that a key effector, particularly in the context of cancer, is c-myc (Sears et al., 2000; Lee et al., 2010). To determine whether c-myc protein levels are affected by K-Ras V12 or B-Raf V600E, cells were seeded in 3D culture and, 24 h later, reextracted, lysed, and analyzed on Western blots. K-Ras V12 and B-Raf V600E expression resulted in a 10-fold increase in c-myc protein levels and a corresponding increase in phospho-Ser62 c-myc (Fig. 4 A and Fig. S2 A). Furthermore, the increase in c-myc levels is completely inhibited upon MEK inhibition of K-Ras V12– and B-Raf V600E–expressing cells (Fig. 4 A and Fig. S2 B). Phosphorylation of c-myc by ERK on Ser62 has been shown to promote c-myc stabilization (Sears et al., 2000). This likely accounts for the increased levels of protein observed, although c-myc levels can also be regulated at the transcriptional and translational levels, and we have observed a 2.5-fold increase in c-myc mRNA levels in K-Ras V12 and B-Raf V600E 3D cultures as determined by quantitative PCR (Fig. S2 C).

To determine whether c-myc alone is sufficient to disrupt Caco-2 3D morphogenesis and promote hyperproliferation, c-myc was expressed in cells from a retroviral vector. After selection, the level of c-myc expression in Caco-2 cells was similar to that found in K-Ras V12–expressing cells (Fig. 4 F). c-myc expression disrupted Caco-2 morphology to produce solid structures and had a modest effect on the overall size of the structure (Fig. 4, D and E). Because c-myc expression is known to increase both proliferation and apoptosis, it is likely that other signals prevent c-myc–dependent apoptosis in K-Ras V12 and B-Raf V600E 3D cultures (Evan et al., 1992; Zhan et al., 2008). We conclude that both the disruption of cell polarity and the promotion of hyperproliferation by K-Ras and B-Raf oncogenes are mediated by up-regulation of c-myc.
K-Ras V12 and B-Raf V600E inhibit apical/basolateral polarity establishment at the two-cell stage

To investigate whether the loss of apical polarity and tight junction assembly seen in mature K-Ras V12 and B-Raf V600E structures is a direct effect of increased c-myc expression or an indirect consequence of dysregulated proliferation, 3D cultures were examined after the first cell division in 3D, i.e., at the two-cell stage. The majority of two-cell structures formed from control cells show strong localization of aPKC in an apical domain between the two daughter cells (Fig. 5A). However, in two-cell structures formed from K-Ras V12– or B-Raf
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significant increase in normal polarized morphology of K-Ras V12 3D structures (Fig. S3, D and F). Together, these observations are consistent with the conclusion that disruption of polarity establishment is a direct effect of oncogene expression and not an indirect consequence of dysregulated proliferation.

These data show that the K-Ras V12 and B-Raf V600E oncogenes, which are frequently found in human colon cancer, disrupt apical polarity and tight junctions and promote hyperproliferation in Caco-2 cells. All the effects observed are mediated through ERK-dependent up-regulation of c-myc protein. The analysis of two-cell 3D structures reveals that c-myc disrupts the establishment of polarity during the first cell division. The establishment of apical polarity is critical in epithelial cells—it is required to orient the mitotic spindle to promote symmetric cell division within the plane of a tissue, and it is required to establish tight junctions and maintain a distinct luminal surface (Jaffe et al., 2008). In the absence of an apical surface, the orientation of cell divisions becomes randomized, creating a solid or multilayered structure. Although c-myc is up-regulated in a large number of human cancer types, including colon, and is well known to promote hyperproliferation, it was surprising to find that it directly influences polarity establishment in epithelial cells (Bourhis et al., 1990; Kozma et al., 1994; Deming et al., 2000). c-myc is a master regulator known to both activate and repress gene expression, and so it seems likely that changes in one or more of its transcriptional targets are responsible for the effects of c-myc described here on polarity (Brenner et al., 2005).

To further confirm that disruption of polarity establishment is not a result of cell cycle dysregulation, we first analyzed the levels of cell cycle proteins in 3D structures at the two-cell stage. The levels of cyclin A, B, and E and cell cycle inhibitors p16, p21, and p27 were not significantly different in K-Ras V12 or B-Raf V600E cells from control cells, but cyclin D1 levels and retinoblastoma (RB) phosphorylation were significantly increased (Fig. S3 A). However, in c-myc-overexpressing cells, cyclin D1 levels were not significantly different from control cells. In addition, overexpression of cyclin D1 in Caco-2 cells did not disrupt polarity establishment at the two-cell stage (Fig. S3, B, C, and E). The kinase associated with cyclin D1, cdk4, promotes G1 progression and is responsible for RB phosphorylation. Inhibition of cdk4 with the small molecule PD0332991 blocked cell cycle progression, as expected, but at intermediate concentrations, there was no significant increase in normal polarized morphology of K-Ras V12 3D structures (Fig. S3, D and F). Together, these observations are consistent with the conclusion that disruption of polarity establishment is a direct effect of oncogene expression and not an indirect consequence of dysregulated proliferation.

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Materials and methods

Reagents and antibodies
Primary antibodies used for immunofluorescence are as follows: aPKC (C-20; Santa Cruz Biotechnology, Inc.) at 1:200, Ki67 (M7240; Dako; gift from F. Giancotti, Memorial Sloan-Kettering Cancer Center, New York, NY) at 1:200, E-cadherin [13–1900; Invitrogen] at 1:500, ezrin (E13420; BD) at 1:250, laminin B2 (clone A5; EMD Millipore) at 1:100, and ZO-1 (33–9100; Invitrogen) at 1:500. Secondary antibodies used for immunofluorescence are as follows: Alexa Fluor 488 and 568 (Invitrogen) at 1:200, Cy3 (Jackson Materials and methods

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Figure 5. K-Ras V12 and B-Raf V600E inhibit apical polarity establishment through ERK-mediated regulation of c-myc. (A and B) Single cells expressing control (pQCXIP GFP) or K-Ras V12 (pQCXIP GFP K-Ras V12) were embedded in matrix. After 2 d, cells were fixed and stained for aPKC, actin, and DNA (blue; A) and ZO-1, E-cadherin, and DNA (blue; B). Representative images are shown. (C) Two-cell structures expressing control (pQCXIP GFP), K-Ras V12 (pQCXIP GFP K-Ras V12), B-Raf V600E (pQCFLAP B-Raf V600E), or c-myc (pBabe blast c-myc) were fixed and stained for aPKC and DNA. (Bottom) K-Ras V12–expressing Caco-2 cells were plated as single cells in 3D and treated with 20 µM U0126. Cells stably expressing shmyc 2 were superinfected with K-Ras V12 (pQCXIP GFP K-Ras V12) retrovirus. 4 d later, cells were seeded into 3D culture. Cells were fixed 2 d later at the two-cell stage and stained for aPKC and DNA. Representative images are shown. (D–G) At least 50 two-cell structures were imaged for each condition in three independent experiments and analyzed for proper apical localization of aPKC. The means ± SEM are shown. **, P < 0.005; ***, P < 0.0005. Bars, 50 µm.
Immunofluorescence and imaging
Before fixation, embedded 3D cultures were incubated with 50 U/ml collagenase I (Sigma-Aldrich) diluted in PBS for 15 min at RT. Both embedded and mature structures were fixed in 10% formalin (Sigma-Aldrich) for 30 min at RT. After triple washes with PBS, cells were incubated in immunofluorescence buffer supplemented with 0.5% Triton X-100 and 1% BSA along with phallolidin and DNA stain of either Hoechst or DRAQ5. Cells were again rinsed with PBS 3x for 10 min. Embedded structures were mounted on coverslips using fluorescent mounting medium (Dako). Mature structures were stored in PBS containing 0.2% azide. This protocol was modified from a previously published study (Jaiffe et al., 2008).

Fixation and stained 3D cultures were imaged on a spinning-disk confocal microscope (UltraVIEW ERS; PerkinElmer) with an EM charge-coupled device camera (iXon 897; Andor Technology). Mature structures were imaged using a 40x objective (Plan Apochromat, NA 1.4, oil; Carl Zeiss), whereas embedded structures were imaged using a 63x objective (Plan Apochromat, NA 1.4, oil; Carl Zeiss). All imaging was performed with oil immersion at RT. Images were acquired and analyzed with MetaMorph (Molecular Devices). MetaMorph was also used to adjust the brightness and contrast of images as well as to make videos from confocal z-stacks.
Quantitative PCR

Cells were cultured as described for 3D lysates. Total cellular RNA was isolated from pellets of day 1 3D structures with a purification kit (RNeasy Mini; QIAGEN). RT-PCR was performed on 200 ng of total RNA using a RT-PCR kit (SuperScript One-Step; Invitrogen) for each sample. Quantitative PCR was performed using TaqMan reagents on the real-time PCR system (7500; Applied Biosystems). The Taqman probe Hs00905030_m1 was used to amplify c-myc, and Hs99999905_m1 was used to amplify glyceraldehyde-3-phosphate dehydrogenase.

Statistics

Statistical significance was evaluated using Prism software (GraphPad Software). The unpaired t test was performed with two-tailed p values and 95% confidence intervals.

Online supplemental material

Fig. S1 shows Western blot analysis of K-Ras V12, B-Raf V600E, and PIK3CA H1047R cell lysates and effects of U0126 or PIK-90 inhibitors. Fig. S2 shows c-myc up-regulation and RNAi depletion in K-Ras V12 and B-Raf V600E cells. Fig. S3 shows that promoting proliferation in control cells or inhibiting proliferation in K-Ras V12 cells does not affect 3D morphology. Videos 1 and 2 are stack videos depicting control or K-Ras V12 day 10 3D structures, respectively, stained with actin and DNA. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201201028/DG1.

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