Research Article

APC inhibits ERK pathway activation and cellular proliferation induced by RAS

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Summary
Inactivating mutations in the adenomatous polyposis coli gene (APC), and activating mutations in RAS, occur in a majority of colorectal carcinomas. However, the relationship between these changes and tumorigenesis is poorly understood. RAS-induced activation of the ERK pathway was reduced by overexpressing APC in DLD-1 colorectal cancer cells. ERK activity was increased by Cre-virus-induced Apc knockout in primary Apclox/lox mouse embryonic fibroblasts, indicating that APC inhibits ERK activity. ERK activity was increased by overexpression and decreased by knock down of β-catenin. The activation of Raf1, MEK and ERK kinases by β-catenin was reduced by co-expression of APC. These results indicate that APC inhibits the ERK pathway by an action on β-catenin. RAS-induced activation of the ERK pathway was reduced by the dominant negative form of TCF4, indicating that the ERK pathway regulation by APC/β-catenin signaling is, at least, partly caused by effects on β-catenin/TCF4-mediated gene expression. The GTP loading and the protein level of mutated RAS were decreased in cells with reduced ERK activity as a result of APC overexpression, indicating that APC regulates RAS-induced ERK activation at least partly by reduction of the RAS protein level. APC regulates cellular proliferation and transformation induced by activation of both RAS and β-catenin signaling.

Key words: APC, β-catenin, ERK, RAS, Transformation, Wnt

Introduction
The adenomatous polyposis coli gene (APC) encodes a key tumor suppressor involved in negative regulation of the canonical Wnt/β-catenin signaling cascade (Clevers, 2004; Nathke, 2004). Mutations of APC have been detected in sporadic cancers (Nishisho et al., 1991; Nagase and Nakamura, 1993; Nathke et al., 1996) and in the germline of familial adenomatous polyposis (FAP) patients (Miyoshi, 1992; Howe and Guillem, 1997). APC interacts with many components of the Wnt/β-catenin signaling pathway, including AXIN, GSK-3β and β-catenin (Mimori-Kiyosue and Tsukita, 2001), but the key tumor suppressor function of APC involves destabilization of free β-catenin (Bienz and Clevers, 2000; Munemitsu et al., 1995). Loss of functional APC leads to nuclear accumulation of β-catenin (Korinek et al., 1997), which binds to members of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors such as TCF4 (Karim et al., 2004; Polakis, 2000). Formation of a TCF4/β-catenin complex results in transcriptional activation of target genes, such as MYC and cyclin D1, which are involved in early transformation of the colonic epithelium (He et al., 1998; Korinek et al., 1997; Peifer and Polakis, 2000; Shtutman et al., 1999).

Multiple mutations are necessary for the development of malignancy, and a succession of mutations has been detected during colorectal tumorigenesis (Kinzler and Vogelstein, 1996). APC mutations occur in the early adenoma stages of colorectal tumorigenesis and are likely to be involved in the initiation of colorectal tumors (Behrens and Lustig, 2004; Kinzler and Vogelstein, 1996; Nathke, 2004). RAS mutations, also a major factor of colorectal tumors, usually occur in the mid-stages of tumorigenesis and are implicated in tumor progression (Kinzler and Vogelstein, 1996). A defective APC allele and an activated Ras gene are sufficient to synergistically cause normal colonic epithelial cells to produce carcinomas (D’Abaco et al., 1996), indicating that APC and RAS signals interact in forming tumors.

Extracellular signal regulated kinase (ERK) is a major component of the ERK pathway (often called the MAP kinase pathway); it plays an important role in transmitting RAS-mediated signals for proliferation and transformation (Giehl, 2005; Hancock, 2003; Suzuki et al., 2002). Although APC and RAS mutations are major causes of colorectal tumorigenesis, the relationship between the Wnt/β-catenin and ERK signaling pathways is poorly understood. In NIH 3T3 cells, the ERK pathway was found to be directly activated by WNT3A independently of β-catenin (Yun et al., 2005). However, overexpression of β-catenin by transient transfection caused simultaneous activation of RAF1, MEK, and ERK kinases, indicating that interaction between the Wnt/β-catenin and ERK pathways occurs at several levels (Yun et al., 2005).
In this study, we examined the possible role of the negative regulator of the Wnt/β-catenin pathway, APC, in regulating the ERK pathway activated by oncogenic RAS. A role of APC in ERK pathway regulation was confirmed by overexpression as well as knockout analyses. Regulation occurred at least in part via effects on β-catenin. The involvement of TCF4/β-catenin-mediated transcription in ERK pathway regulation by APC/β-catenin signaling was further investigated by examining the effects of a dominant-negative form of TCF4. In our analysis of the involvement of RAS in ERK pathway inhibition by APC, we observed a reduction in the levels of GTP binding of mutated RAS proteins in colorectal cancer cells. The reduction of RAS protein by APC overexpression indicated that the mutated RAS proteins in colorectal cancer cells. The reduction of the involvement of RAS in ERK pathway inhibition by APC, effects of a dominant-negative form of TCF4. In our analysis of the involvement of RAS in ERK pathway inhibition by APC, we observed a reduction in the levels of GTP binding of mutated RAS proteins in colorectal cancer cells. The reduction of RAS protein by APC overexpression indicated that the mutated RAS proteins in colorectal cancer cells retained mutated RAS. We also investigated the role of APC in inhibiting proliferation and transformation in response to mutated RAS and β-catenin. Our results point to a role of APC in regulating tumor progression caused by activation of RAS and β-catenin.

**Results**

APC inhibits RAS-induced AP-1 and ELK1 reporter activities and ERK activity

We examined the effect of APC on RAS-L61-induced AP1 (Bernstein and Colburn, 1989; Eisenman and Cooper, 1995) activation to assess the role of APC in RAS-induced cell proliferation and malignant transformation. AP1 reporter gene expression was increased 22-fold by Ras-L61 transfection in APC-mutated DLD-1 cells, and this activity was reduced by more than 50% by co-transfection with APC (Fig. 1A; left panel). ELK1-dependent trans-reporter (Hipskind et al., 1991) activity was also increased 20- to 30-fold by Ras-L61 transfection, and this increase was also inhibited by co-transfection with APC although to a lesser extent (Fig. 1A; compare left and right panels). The difference is probably due to the fact that the AP1 reporter is regulated by both Wnt/β-catenin and ERK pathways (Tufan et al., 2002; Tice et al., 2002) whereas the ELK1 reporter is only subject to regulation by ERK signaling with no substantial influence of the Wnt/β-catenin pathway (Hipskind et al., 1991; Tori et al., 2004).

RAS generally stimulates cell proliferation by activating the RAF1→MEK→ERK signaling cascade (Giehl, 2005; Hancock, 2003), with subsequent activation of cell cycle promoting factors, such as AP1 (Hancock, 2003; Wasylyk et al., 1998) and ELK1 (Aplin et al., 2001; Hipskind et al., 1991). To identify the role of APC in RAS-induced ERK activation, we monitored the effect of APC overexpression. ERK activities were increased approximately 4.5-fold by Ras-L61 transfection, and more than 50% of the RAS-induced activity was lost by co-transfection with APC (Fig. 1B; representative results are shown in left panel). Therefore, APC inhibits the stimulation of ERK and its target promoters in response to Ras-L61.

ERK activities are negatively and positively regulated by overexpression and knockout of APC, respectively

In addition to inhibiting RAS-induced ERK activation, APC inhibited the basal ERK activity in DLD-1 cells (Fig. 2A). To confirm inhibition of basal ERK activities by APC, we generated APC-null MEF primary cells by retroviral Cre-induction of Apclox/lox MEF (Shibata et al., 1997). β-catenin levels were two to threefold higher in these cells than in the parental cells (Fig. 2B), indicating that deletion of Apc inhibits β-catenin degradation. ERK and RAF1 activities also increased in the Apc null cells (Fig. 2B). The level of activated AKT (p-Akt) did not change as a result of Apc knockout (Fig. 2B), indicating a specificity of the Apc null effect in activation of the ERK pathway. Inactivation of loxp-flanked Apc by Cre was verified by RT-PCR and western blotting (Fig. 2B).

Regulation of ERK by APC occurs at least in part by degradation of β-catenin

APC regulates the degradation of β-catenin, and loss

![Fig. 1. Effects of overexpressing APC on RAS-induced ERK pathway activation. (A) DLD-1 cells were transfected with 0.5 μg of the AP-1 (left panel) or ELK1 reporter (right panel) plasmids together with 0.5 μg of pCMV or pCMV-APC. Transfection with ELK1 trans-reporter was coupled with transfection of 0.5 μg of pFR-Luc or 25 ng of pFA2-ELK1 (Park et al., 2002). All cases except Mock (transfection with pCMV alone), were coupled with transfection with 0.1 μg of pMT3RAS-L61 (H-Ras-L61). Cells were harvested after 48 hours and the relative levels of expression of the reporters were measured by assaying luciferase. (B) DLD-1 cells were transfected with 0.5 μg of pCMV or pCMV-APC with or without 0.1 μg of pMT3RAS-L61. Cells were harvested after 72 hours. Western blot analyses were performed on whole-cell lysates to detect p-ERK, APC, ERK and α-tubulin. The right bar graph shows quantitative analyses of the intensities of p-ERKs presented as the average of three independent identical experimental results from the left panel.](image-url)
of APC results in the accumulation of β-catenin (Korinek et al., 1997; Polakis, 1997). In contrast to the effect of APC, ERK activities increased when β-catenin was overexpressed by transient transfection of DLD-1 cells (Fig. 3A), and endogenous ERK activity declined when β-catenin levels were reduced by exposure to β-catenin siRNA (Fig. 3B).

To determine whether APC regulates ERK via an effect on β-catenin, we tested whether the ERK activation due to β-catenin overexpression could be reduced by APC co-expression. This proved to be the case (Fig. 4A). In cells co-transfected with both APC and β-catenin, the β-catenin level was significantly reduced (Fig. 4A), probably because of degradation by overexpressed APC. We detected a high molecular weight APC band in cells co-expressing APC and β-catenin (Fig. 4A) presumably reflecting post-translational modification of APC, similar to hyper-phosphorylation of APC by GSK-β (Ikeda et al., 2000).

Fig. 3. Effects of overexpression and reduction of β-catenin on ERK activity. (A) DLD-1 cells were transfected with 1 μg of pcDNA3.0 or with different amounts (0.5 or 1.0 μg) of Flag-β-catenin-pcDNA3.0. They were harvested 48 hours after transfection, and p-ERK, Flag, β-catenin, and ERK were detected by western blotting. Anti-Flag antibody was used to detect Flag-β-catenin. (B) DLD-1 cells were transfected with β-catenin siRNA (or not transfected). They were harvested 72 hours later and p-ERK, β-catenin, ERK, and α-tubulin were detected by western blotting.

Fig. 4. Effects of APC overexpression on β-catenin-induced ERK and ELK1 reporter activation. (A) DLD-1 cells were transfected with 0.5 μg of pcDNA3.0 or Flag-β-catenin-pcDNA3.0 with or without 0.5 μg of pcCMV-APC. They were harvested 72 hours later and western blot analyses were performed to detect p-ERK, Flag-β-catenin, APC and ERK. Anti-Flag antibody was used to detect Flag-β-catenin. (B) DLD-1 cells were transfected with 0.5 μg of pcCMV APC with or without Flag-β-catenin-pcDNA3.0. (Left panel) Co-transfection of 0.5 μg of ELK1 with 0.5 μg of pFR-Luc and 25 ng of pFA2-ELK1; ELK1 trans-reporter activity was measured. (Right panel) pTOPFLASH, pFOPFLASH were co-transfected, and luciferase activities derived from the reporter genes were measured 48 hours later. Wnt/β-catenin reporter activity was calculated by dividing the luciferase activity of pTOPFLASH by that of pFOPFLASH (Korinek et al., 1997).
construct (pTOPFLASH) (Korinek et al., 1997) to that from the TCF4-non-responsive control luciferase reporter gene construct (pTOPFLASH) (Korinek et al., 1997) was increased by β-catenin transfection and decreased by APC co-transfection (Fig. 4B; right panel), confirming the normal role of both APC and β-catenin in TCF4/β-catenin-mediated gene expression.

**ERK pathway regulation by APC-β-catenin signaling is due to β-catenin/TCF4 mediated gene expression**

To identify the route of ERK activation by β-catenin, we measured the activities of the MAP kinase module kinases (RAF1, MEK and ERK), which represent the activation status of the proteins (Park et al., 2002), increased when β-catenin was overexpressed in DLD-1 cells (Fig. 5A). To further characterize ERK regulation by β-catenin, we measured the effect of the MEK inhibitor PD98059 on β-catenin-induced ERK activation. ERK activation by β-catenin was significantly lowered by pre-treatment with PD98059 (Fig. 5B, upper panel). β-catenin-induced ERK activation was further stimulated by co-expression of constitutively active MEK (MEK-CA) and β-catenin (Fig. 5B; lower panel), as well as by transfection with non-degradable S33Y-β-catenin (Fig. 5B; upper and lower panels), indicating that the ERK activity was regulated by β-catenin.

We investigated the effect of dn-TCF4 overexpression on RAS-induced ERK activation by transfection with ΔN-TCF4E (Tetsu and McCormick, 1999) to determine whether inactivation by APC of RAS-induced ERK is the result of β-catenin/TCF4-dependent transcription (Korinek et al., 1997; Biezen and Clevers, 2000). ERK activation by β-catenin was reduced by co-expression of dn-TCF4 (Fig. 6A), indicating that ERK pathway regulation by APC depends at least in part on β-catenin/TCF4-mediated transcription of target genes. The involvement of β-catenin/TCF4 in regulating activation of the ERK pathway by RAS was further investigated by measuring the effect of dn-TCF4 overexpression on RAS-induced ELK1 trans-reporter activity. This was also increased by transfection with Ras-L61, and reduced by co-transfection with dn-TCF4 (Fig. 6B).

**APC inhibit RAS-induced transformation**

The effect of APC transfection on the formation of foci by RAS was measured to assess the role of APC in transformation of cells harboring mutated RAS. DLD-1 cells displayed a transforming phenotype, and formed substantial numbers of foci (Fig. 7). The number of foci was further increased by transfection with Ras-L61 as well as with β-catenin (Fig. 7), and the Ras-L61-induced increase in foci formation was reduced by transfection with APC. The increase of foci caused by β-catenin was also antagonized by APC (Fig. 7).

**APC inhibits morphological transformation and proliferation of NIH 3T3 cells induced by RAS**

We investigated the effect of inhibition of the RAS-induced ERK pathway activation by APC on cell proliferation by measuring BrdU incorporation by NIH 3T3 cells expressing a doxycycline (Dox)-inducible form of Ras-G12R (Lim et al., 2004). Using this inducible system, we could measure the effect of APC on proliferation caused specifically by RAS.
APC regulates ERK pathway

ERK activities increased four- to fivefold in response to Dox-induced Ras-G12R expression, and the cells took on a typical transformed appearance 24 hours after RAS induction (Fig. 8A). The role of APC in anti-transformation was further investigated by monitoring the effect of overexpression of enhanced green fluorescent protein (EGFP)-tagged APC (EGFP-APC) on the Ras-G12R-induced morphological transformation. The typical Ras-G12R-induced morphological transformation resulted in a small round shape of individual cells transfected with EGFP-APC compared to non-transfected cells nearby. The cells expressing EGFP also maintained the transformed morphologies due to RAS (Fig. 8B). To investigate the effect of APC on RAS-induced cellular proliferation, APC-transfected cells were also analyzed by FACS. The intensity of TRITC (FL2-H), which represents the amount of incorporated BrdU was increased from 35.8% to 89.9% by Ras-L61 induction (Fig. 9A).

The basal and Ras-L61-induced fluorescence intensities were reduced 36.2% and 41.8%, respectively, by APC transfection. We did not observe a significant increase in BrdU incorporation in normal NIH 3T3 cells treated with Dox (data not shown), indicating that the proliferation was not caused by Dox itself, but was caused by RAS. BrdU incorporation also increased from 32.0% to 82.3% after transfection with β-catenin, and co-transfection with Apc reduced the basal and β-catenin-induced fluorescence intensities by 41.2% and 39.6%, respectively (Fig. 9B).

APC inhibits RAS-induced ERK activation at least partly by reduction of the RAS level

To further define where APC acts in regulation of ERK activation induced by RAS, we measured the effect of APC on RAS activity by monitoring the level of GTP bound Pan-RAS (GTP-RAS). GTP-RAS that was increased by transfection with mutated Ras-L61 was weakly decreased in DLD-1 cells by co-transfection with APC (Fig. 10A). The increase in Pan-RAS caused by transfection with mutated Ras-L61 was similarly reduced by APC overexpression. We further measured the effect of APC on GTP loading of endogenous RAS in DLD-1 and SW480 colorectal cancer cells retaining mutated RAS (Davies et al., 2002) to further characterize the role of APC in regulation of ERK and RAS. Endogenous basal ERK activities were reduced by APC overexpression in DLD-1 and SW480 cells (Fig. 10B). The levels of GTP-RAS and RAS were reduced in these colorectal cancer cells when APC was
overexpressed. GTP-loading and the RAS protein levels were also reduced by Apc overexpression in NIH 3T3. However, the amount of reduction of GTP-loading of RAS due to APC overexpression was much more significant in NIH 3T3 cells retaining wild-type RAS than in DLD-1 and SW480 colorectal cancer cells retaining mutated RAS.

Discussion
Colorectal tumorigenesis is a multi-step process involving a series of mutations (Kinzler and Vogelstein, 1996; Sancho et al., 2004). APC is a negative regulator of the Wnt/β-catenin signaling pathway (Nathke, 2004; Clevers, 2004), and inactivation of APC by both genetic and somatic mutations initiates colorectal tumorigenesis (Clevers, 2004; Kinzler and Vogelstein, 1996; Fearnhead et al., 2001). The tumor-suppressing activity of APC largely involves facilitating the proteasome-mediated degradation of β-catenin (Korinek et al., 1997; Peifer and Polakis, 2000).

RAS is an upstream component of the ERK pathway (Giehl, 2005), and its activation by genetic alteration is also associated with colorectal tumor progression (Kinzel and Vogelstein, 1996; Sancho et al., 2004). Although both APC and RAS mutations are major causes of transformation, the relationship between the Wnt/β-catenin and the ERK pathways is poorly understood. Several studies have pointed to interaction between the β-catenin/Wnt and the RAS-ERK pathways (Conacci-Sorrell et al., 2003; Weng et al., 2002) without clarifying the mechanism involved. β-catenin cooperates with RAS in the transformation process (Damalas et al., 2001). Patients with RAS mutations are resistant to tumor development in the presence of normal APC, indicating that APC can suppress tumorigenesis induced by RAS (Kinzler and Vogelstein, 1996).

We investigated the role of APC in RAS-induced proliferation and transformation of DLD-1 colorectal cancer cells. APC overexpression reduced the activation of ERK and its target promoters that are induced by transfection with oncogenic Ras-L61, indicating that APC antagonizes the RAS-induced ERK pathway activation that is responsible for proliferation and malignant transformation. APC also inhibited endogenous ERK activities in DLD-1 and SW480 colorectal cancer cells retaining the mutated RAS gene (Fig. 10B), providing a further indication of the role of APC in regulation of RAS-induced ERK activation. APC also reduced basal ERK activities without RAS activation, based on observations of ERK activation in ApcΔ/Δ MEF cells and ERK inactivation within NIH 3T3 cells that overexpressed APC. ERK activity was increased by β-catenin overexpression and decreased by inhibiting β-catenin expression with siRNA. ERK activation by β-catenin has also been observed in NIH 3T3 cells (Yun et al., 2005). These results indicate that APC
recently, Tan et al. (Tan et al., 2005) reported that the RAS were equivalent in colorectal cancer and NIH 3T3 cells. The reduction of the RAS protein by APC, and the reduction of overexpression was much more significant than the reduction of the APC regulates ERK activity regardless of the genetic status of the Ras gene. The reduction in the GTP-RAS level by APC in RAS-activated cells points to a novel mechanism of action on β-catenin. The anti-tumor activity of APC is based on its ability to destabilize free β-catenin (Bienz and Clevers, 2000) and β-catenin-induced ERK activation was abolished by APC overexpression. Since Ras-L61-induced ERK activation was abolished by co-transfection with dn-TCF4 it is likely that APC inhibits RAS-induced activation of ERK signaling at least partly by reducing TCF4/β-catenin-mediated gene transcription.

Inhibition of RAS-induced ERK activation by APC indicates that APC probably functions at Ras or at its downstream component(s). RAS gene mutations render the RAS proteins insensitive to GTPase activating protein (GAP) induced hydrolysis of GTP to GDP, thereby locking them in the GTP-bound active state (Lowy and Willumsen, 1993; Bos, 1989). The reduction of GTP-bound overexpressed mutant RAS by APC co-expression indicates that APC regulates mutated RAS by regulation of the protein level rather than by GAP hydrolysis. This proposal is based on the observation of a reduction in the RAS protein level in cells with reduced GTP-RAS levels due to APC overexpression. The endogenous RAS protein levels were more significantly decreased with a reduction of the GTP-RAS level than in cells that possessed mutated RAS in DLD-1 and SW480 colorectal cancer cells (Davies et al., 2002), further supporting regulation of the RAS protein level by APC. The levels of both GTP-RAS and RAS were also reduced by APC overexpression in NIH 3T3 cells retaining wild-type Ras, in agreement with our observation that APC regulates ERK activity regardless of the genetic status of the Ras gene. The reduction in the GTP-RAS level by APC overexpression was much more significant than the reduction of the Ras gene in NIH 3T3 cells, although the reduction in the amount of the Ras protein by APC, and the reduction of Ras were equivalent in colorectal cancer and NIH 3T3 cells. Recently, Tan et al. (Tan et al., 2005) reported that the epidermal growth factor receptor (EGFR) was a target for the Wnt/β-catenin pathway in the liver, and that the EGFR level was transcriptionally regulated by Wnt/β-catenin signaling (Tan et al., 2005). Therefore, significant regulation of the GTP-RAS level by APC in NIH 3T3 cells is probably due to dual regulation of wild-type RAS in NIH 3T3 cells by both GTP hydrolysis due to EGFR reduction, and reduction of the protein level. However, mutated RAS may be regulated only by reduction of RAS protein level in DLD-1 and SW480 colorectal cancer cells. The mechanism of regulation of the RAS protein level by APC/β-catenin signaling is unknown.

APC inhibits proliferation of colorectal cancer cells by blocking G1 to S phase progression (Heinen et al., 2002). This may be due to its effect on the β-catenin/TCF4 complex and a consequent reduction in the expression of its target genes such as MYC and cyclin D1 (Heinen et al., 2002). APC also inhibits serum-stimulated proliferation of NIH 3T3 cells by blocking the G1 to S phase transition (Baeg et al., 1995). We also observed that APC reduced BrdU incorporation in response to RAS activation, indicating APC involvement in regulation of RAS-induced proliferation. In addition, APC antagonized RAS-induced transformation, as shown by measurement of the effects on foci formation and morphological changes caused by RAS. Inhibition of basal ERK activity is correlated with inhibition of basal proliferation, indicating that APC regulates basal and RAS-induced proliferation by regulation of the ERK pathway.

Mutations in oncogenic RAS frequently occur during the mid-stages of colorectal tumorigenesis and are important for tumor progression (Kinzler and Vogelstein, 1996). However, inactivating APC mutations occur in the initiation stages (Kinzler and Vogelstein, 1996). To the best of our knowledge, there has been no previous report of APC involvement in tumor progression. The present evidence for an anti-proliferative role of APC in RAS-activated cells points to a novel mechanism of colorectal tumor suppression by APC. We also observed that both basal, and β-catenin- and RAS-induced, oncogenic transformation was inhibited by APC. APC is thus capable of regulating tumorigenesis caused by multiple defects involving activation of the RAS and Wnt/β-catenin pathways.

Materials and Methods

Cells and plasmids
NIH 3T3 cells, and DLD-1 and SW480 human colorectal cancer cell lines retaining a RAS mutation (Davies et al., 2002) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The NIH 3T3 cell line containing doxycycline-inducible H-Ras (G12R) has been described by Lim et al. (Lim et al., 2004). DLD-1 and SW480 cells were maintained in RPMI1640 supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/ml of penicillin and 100 μg/ml of streptomycin in 5% CO2 at 37°C. NIH 3T3 and H-Ras-inducible NIH 3T3 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM).
containing 10% fetal bovine serum (FBS), also under the same conditions and with the same analyses.Preparations were performed on cells at 70% confluence unless otherwise stated. pCMV-APC (Morin et al., 1997), pTOPFLASH (Korinek et al., 1997) and pPOFLASH (Korinek et al., 1997) were provided by B. Vogelstein and K. Kinzler of the Johns Hopkins Oncology Center. The AP-1-reporting vector (Lee et al., 1999) and the adenovirus 3/13 reporter (J. Lee, University of Michigan, Seoul, Korea) were kindly provided by J. Jeong of KAIST, Taejon, Korea. The ELK1 trans-reporting pFR-Luc and pFA2-ELK1 trans-activator plasmids were obtained from Stratagene. Flag-β-catenin-pcDNA3.0, Flag-S33Y-β-catenin-pcDNA3.0 (Kolligs et al., 1999), and ΔN-TCF4E (Tetsu and McCormick, 1999) vectors were obtained from Eric R. Fearon of the University of Michigan, and pCDNA3.0-MEK1-CA (Bonnin et al., 1999) was obtained from Michael E. Greenberg of Harvard Medical School. The pEGFP-C1-hApc vector (Rosin-Arbesfeld et al., 2003) was provided by M. Benez of the MRC Laboratory of Molecular Biology.

**Transfection and transient reporter assays**

DLD-1 cells were plated in 6-well plates at 1×10^5 cells per well. After 24 hours of growth, they were transfected with plasmids using Lipofectamine Plus reagent according to the manufacturer’s instructions (Life Technologies, Grand Island, NY, USA). For transient reporter assays, DLD-1 cells were similarly transfected with either the pCMV-APC or the Flag-β-catenin-pcDNA3.0 vector, together with an AP-1-reporting vector, pTOPFLASH, pFLASH or the pFA2-ELK1/pFR-Luc vector construct (Park et al., 2002). Where required, cells were co-transfected with the pCMV vector, pMT3RasL61, or a dominant-negative ΔN-TCF4E. SW480 or NIH 3T3 cells were similarly transfected with pCMV or pCMV-APC. Transfections were performed using Lipofectamine (Invitrogen) and the pCMV-β-gal reporter (Clontech, Palo Alto, CA, USA). Forty-eight hours after transfection, cells were fixed with 3.7% paraformaldehyde for 20 minutes at room temperature to permit complete dissociation of nucleoprotein complexes. One hundred microliters of chloroform was added and the mixture vortexed for 15 seconds followed by centrifugation at 12,000 g for 15 minutes at 4°C. The supernatant was transferred to a new tube and mixed with 250 μl of isopropanol, then incubated at room temperature for 10 minutes. After centrifugation at 12,000 g for 10 minutes at 4°C, the RNA pellet was resuspended in 20 μl of distilled water. First-strand cDNA was synthesized from 1 μg of total cellular RNA by RT using oligo-dT priming for the Apc and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) mRNA. RT was performed in a final volume of 20 μl containing either 5 μl of APC or 2 μl Gapdh cDNA, dNTPs, 1.5 mM MgCl₂, 2.5 units of Taq polymerase (Perkin Elmer-Cetus, Norwalk, CT, USA) and 1 μM of each primer using the Bioneer PCR Thermal Block system (Bioneer, Deajeon, Korea). The following primers were used: Apc forward, 5'-AGT TGC CTC TGC AGG TGC A-3'; Apc reverse, 5'-CCA CTG AGA ACG GAA CCC T-3'; Gapdh forward, 5’-CCC CTT CAT TGA CCT CAA CTA C-3'; Gapdh reverse: 5’-GAG TCC TTC CAC GAT ACC AAA G-3’. Primer amplification was followed by touchdown PCR. PCR conditions were: denaturation at 94°C for 12 seconds, annealing at 52°C for 20 seconds, and extension at 72°C for 55 seconds, for a total of 22 cycles.

**Western blot analysis**

Cells were transfected with a combination of plasmids using Lipofectamine Plus reagent according to the manufacturer’s instructions (Invitrogen). The MEFs were seeded on a coverslip and transfected with Flag-β-catenin-pcDNA3.0, Flag anti-β-catenin-pcDNA3.0, pMT3RasL61 and pCMV-APC was performed, and cells were selected with G418 at 8 μg/ml. After 12 days, the cells were stained with 0.5% Crystal Violet in 20% ethanol. The final siRNAs were purified with a glass fiber filter and transfected into DLD-1 cells with Lipofectamine Plus reagent employing 1.68 μg per 5.3-cm culture dish. The transfected cells were grown for 48 hours at 37°C in a 5% CO₂ incubator, and harvested for western blot analysis.

**siRNA treatment**

β-catenin (GenBank accession numbers NM_001904) mRNA target sequences were designed using an siRNA template design tool (Ambion, Austin, TX, USA), and siRNA was prepared with a Silencer™ siRNA construction kit (Ambion). Twenty-nine-mer oligonucleotides containing the 17-probe sequence (5’-CCTGTCTCTC-3’) were designed [human β-catenin, 5’-AAGCTCTGTATGTTGGAAA-3’ (392-412) and 5’-AACTACTGTTGGACCAAGAC-3’ (1213-1233)]. The dsDNA templates for siRNA were constructed by filling-in using dNTPs and Klenow DNA polymerase, and the 17-mer probe sequences (5’-CCTGTCTCTC-3)’. Single stranded RNAs were synthesized using the T7 RNA polymerase followed by complementary RNA hybridization. Template dsDNAs were removed by DNase and the hybridized RNAs were digested by RNase to produce 21-23 nt overhangs. The final siRNAs were purified with a glass fiber filter and transfected into DLD-1 cells with Lipofectamine Plus reagent employing 1.68 μg per 5.3-cm culture dish.

**Fluorescence microscopy and morphological observation**

RAS-inducible NIH 3T3 cells were grown on 22×22 mm coverslips (Marienfeld, Germany) in 10% FBS medium with 200 μg/ml G418 to a density of 2×10^5 cells/well in 6-well plates. Cells were transfected with pEGFP-C1 (Clontech, Palo Alto, CA, USA) or pEGFP-C1-hApc (Rosin-Arbesfeld et al., 2003) for 24 hours, followed by subsequent induction with 2 μg/ml Dox for 24 hours. The cells were washed with a Nikon Eclipse TE2000-E fluorescence microscope (Model; LHS-H 100P-1), and photographed.

**Immunofluorescence staining and flow cytometric analysis**

For quantitative analysis of proliferation, RAS-inducible NIH 3T3 cells were grown in DMEM containing 10% FBS and 200 μg/ml G418, then seeded in a 6-well plate for 24 hours. The cells were transiently transfected for 48 hours with pCMV or pCMV-APC, and then either treated with 2 μg/ml of doxycyclin or left untreated. For measurement of the effect of APC on β-catenin-induced proliferation, the cells were transfected for 48 hours with a combination of pCMV, pCMV-APC, pCMV-APC, and Flag-β-catenin-pcDNA3.0, BrdU was added to achieve a final concentration of 20 μg/ml of doxycyclin for 48 hours before harvesting cells. For quantification of the BrdU, cells were fixed with 3.7% paraformaldehyde for 20 minutes at room temperature, permeabilized with permeabilization buffer (phosphate-buffered saline without Mg²⁺ and Ca²⁺, 1% FBS, 0.1% saponin), then incubated with anti-BrdU monoclonal antibody, and subsequently with tetramethyl-rhodamine (TRITC)-conjugated goat anti-rabbit IgG (Jackson Immuno Research Laboratories, West Grove, PA, USA). The cell cycle profile was determined using a Becton Dickinson FACS Caliber with the Cell Quest Version 3.3 program (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA).
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APC regulates ERK pathway