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 $Apc^{flox/flox}$ 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 reduction of the level of GTP binding in mutated RAS by APC was at least partly caused by reduction of the RAS protein level 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 Colburm, 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 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; Torii 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 $Apc^{flox/flox}$ 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

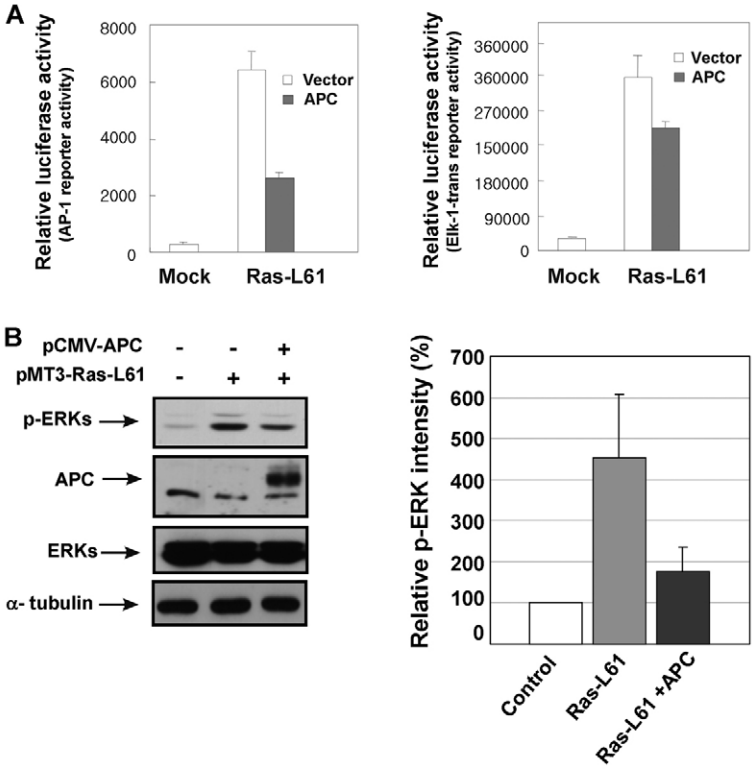

Fig. 1. Effects of overexpressing APC on RAS-induced ERK pathway activation. (A) DLD-1 cells were transfected with 0.5 μg of the AP-1 (left panel) or ELK1 reporter (right panel) plasmids together with 0.5 μg of pCMV or pCMV-APC. Transfection with ELK1 trans-reporter was coupled with transfection with 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.

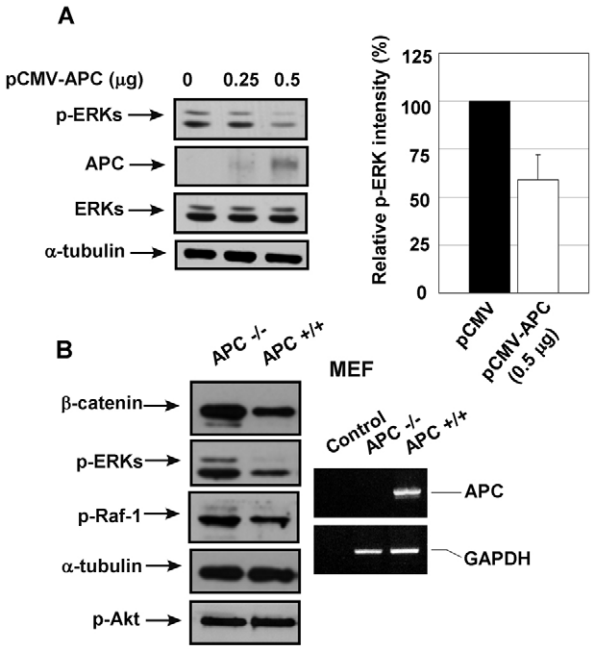

Fig. 2. Effects of overexpression and deletion of APC on ERK activity. (A) Left, DLD-1 cells were transfected with 0.5 μg of pCMV or with 0.25 or 0.5 μg of pCMV-APC. Cells were harvested 72 hours after transfection, and p-ERK, APC, ERKs and α-tubulin were detected in whole-cell lysates by western blotting. The right bar graph shows quantitative analyses of the intensities of p-ERKs shown as the average of three independent identical experimental results for transfection with 0.5 μg of pCMV-APC from the left panel. (B) $Apc^{flox/flox}$ primary mouse embryonic fibroblast (MEF) cells were infected with RCAS-Cre retrovirus (see Materials and Methods). β-catenin, p-ERK, p-Raf1, p-Akt and α-tubulin were detected in whole-cell lysates by western blotting. The right panels shows the *Apc* deletion within the Cre virus-infected MEF cells identified by RT-PCR analysis. Control is the result of PCR in the absence of template.

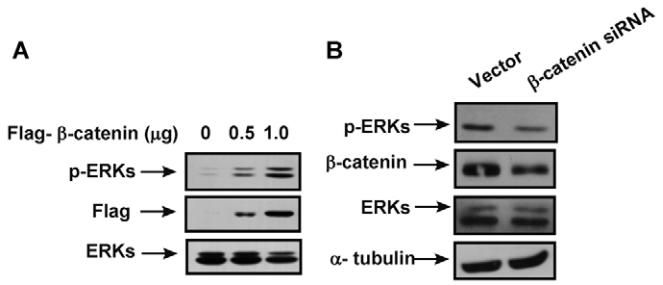

Fig. 3. Effects of overexpression and reduction of β-catenin on ERK activity. (A) DLD-1 cells were transfected with 1 μg of pcDNA3.0 or with different amounts (0.5 or 1.0 μg) of Flag-β-catenin-pcDNA3.0. They were harvested 48 hours after transfection, and p-ERK, Flag-β-catenin, and ERK were detected 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.

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). We also transfected DLD-1 cells with *APC* together with β-catenin, and found that ERK-dependent ELK1 trans-reporter activity was also increased by β-catenin overexpression (Fig. 4B; left panel). β-catenin-induced ELK1-dependent reporter activation was also lowered by co-transfection of *APC* (Fig. 4B; left panel). The ratio of luciferase activity derived from the TCF4-responsive reporter

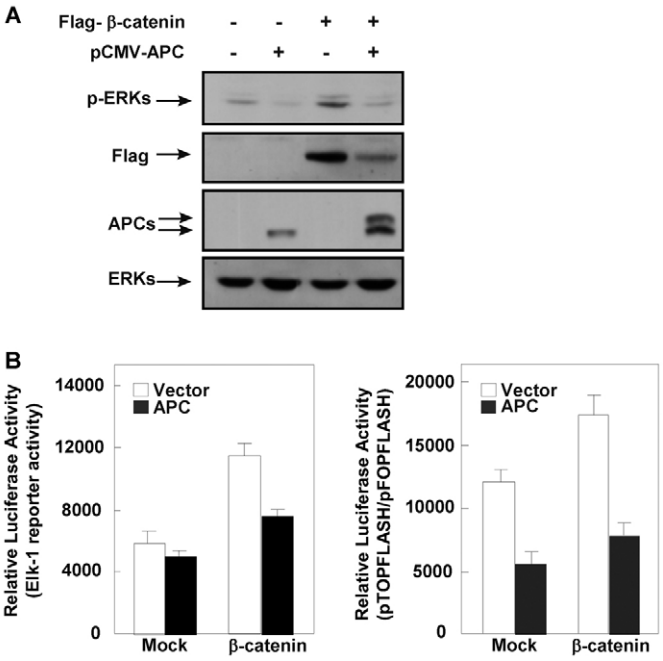

Fig. 4. Effects of APC overexpression on β-catenin-induced ERK and ELK1 reporter activation. (A) DLD-1 cells were transfected with 0.5 μg of pcDNA3.0 or Flag-β-catenin-pcDNA3.0 with or without 0.5 μg of pCMV-APC. They were harvested 72 hours later and western blot analyses were performed to detect p-ERK, 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 pCMV or pCMV-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 (pFOPFLASH) (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) after β-catenin transfection. The levels of phospho-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; Bienz 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

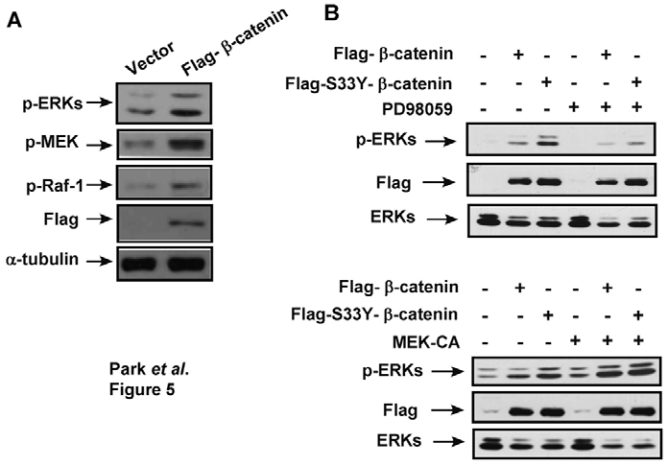

Fig. 5. Effects of β-catenin overexpression on activation of the RAF1→MEK→ERK cascade, and effects of PD98059 and constitutively active MEK on β-catenin-induced ERK activation. (A) DLD-1 cells were transfected with 1.0 μg of pcDNA3.0 or Flag-β-catenin-pcDNA3.0. After 48 hours, they were harvested and p-ERK, p-MEK, p-Raf (Ser-338), Flag-β-catenin and α-tubulin were detected by western blotting. (B) DLD-1 cells were transfected with 1.0 μg of pcDNA3.0, Flag-β-catenin-pcDNA3.0 or Flag-S33Y-β-catenin-pcDNA3.0. Where required, cells were exposed to 20 μM PD98059 for 24 hours before harvesting (upper panel), or 0.2 μg of MEK-CA-pcDNA3.0 was co-transfected with the Flag-β-catenin-pcDNA3.0 (lower panel). After 48 hours p-ERK, Flag-β-catenin and ERK were detected by western blotting.

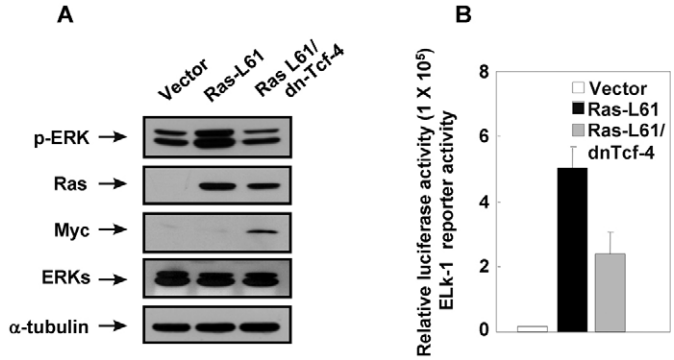

Fig. 6. Effects of a dominant negative TCF4 on RAS-induced activation of ERK and ELK1 reporters. (A) DLD-1 cells were grown and transfected with 0.1 μg of empty pMT3 vector or 0.1 μg of pMT3 RAS-L61. Where required, they were co-transfected with 0.5 μg of the MYC-tagged dnTCF4 expression vector, ΔN-TCF4E (Tetsu and McCormick, 1999). 48 hours after transfection, cell extracts were prepared, and p-ERK, RAS-L61, ΔN-TCF4E (dn-TCF4-MYC), ERK and α-tubulin proteins were detected by western blotting. Anti-MYC antibody was used to detect dn-TCF4-MYC. (B) DLD-1 cells were grown and transfected with 0.1 μg of empty pMT3 vector or 0.1 μg of pMT3 RAS-L61. Where required, they were co-transfected with 0.5 μg of MYC-tagged dn-TCF4 expression vector, ΔN-TCF4E. 0.5 μg of ELK1 trans-reporter was co-transfected, as described in Fig. 1B. Extracts were prepared 48 hours after transfection, and luciferase activities measured. Each data point represents the average of three independent analyses.

activation. 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).

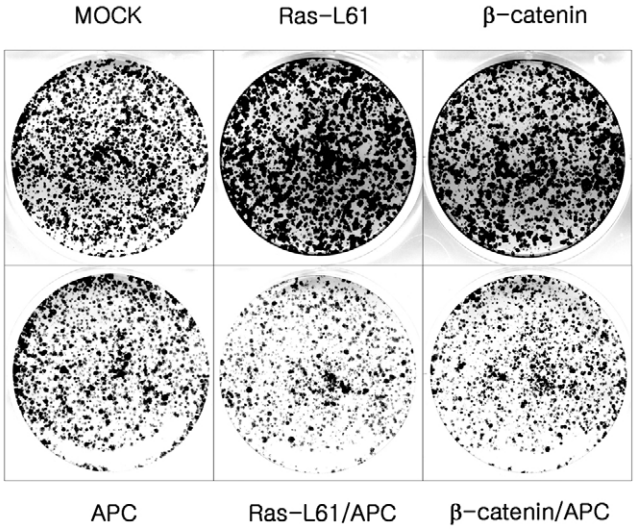

Fig. 7. Effects of *APC* transfection on RAS- and β-catenin-induced foci of transformation in DLD-1 cells. 1×10^4 DLD-1 cells were transfected with 0.5 μg each of different combinations of pCMV, pCMV-APC, pcDNA3.0-Flag-β-catenin, or pMT3 RAS-L61, and selected with G418. After 12 days, the cells were stained with 0.5% crystal Violet in 20% ethanol and the foci photographed.

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

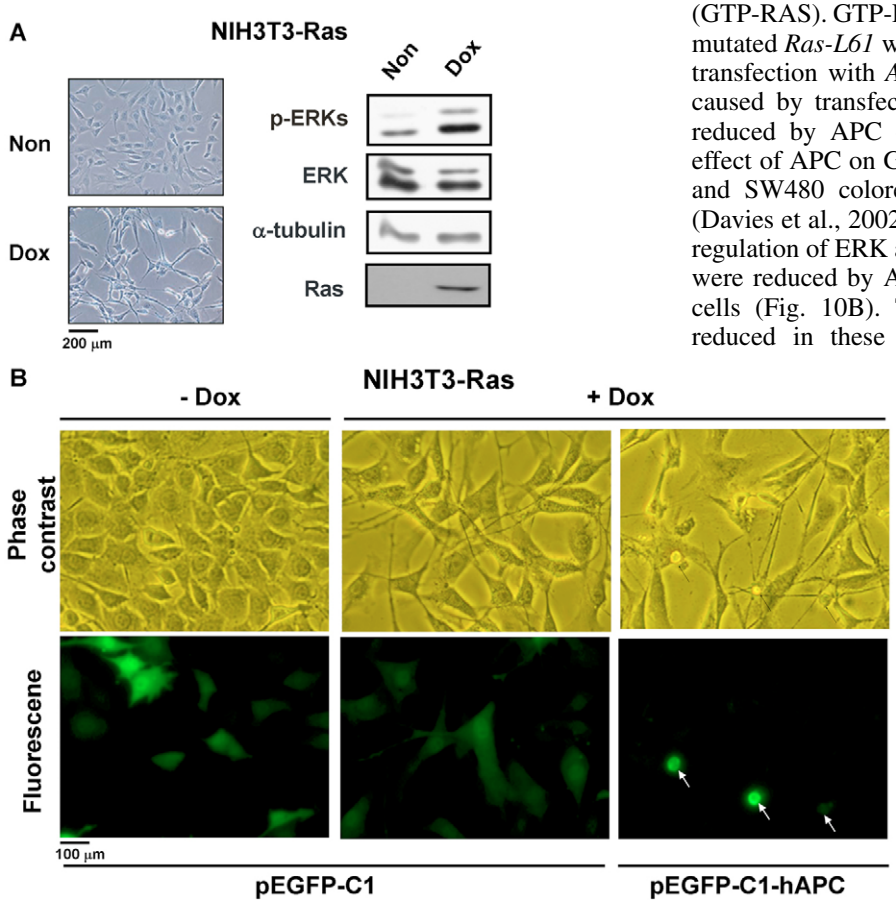

Fig. 8. Effects of APC on RAS-induced morphological transformation in NIH 3T3 cells. (A) NIH 3T3 cells containing doxycyclin (Dox)-inducible H-Ras (G12R) (Lim et al., 2004) were grown in DMEM containing 10% FBS, and were treated (or not) with 2 μg/ml Dox of for 24 hours. They were examined by phase-contrast microscopy, photographed (×200), and p-ERK, ERK, α-tubulin and RAS were detected by western blotting. (B) RAS-inducible NIH 3T3 cells were grown as in A, and transfected with 0.5 μg of either pEGFP-C1 or pEGFP-C1-hAPC. Cells were visualized by fluorescence microscopy. Arrows indicate cells expressing APC-EGFP.

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 (Kinzler 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 $Apc^{-/-}$ 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

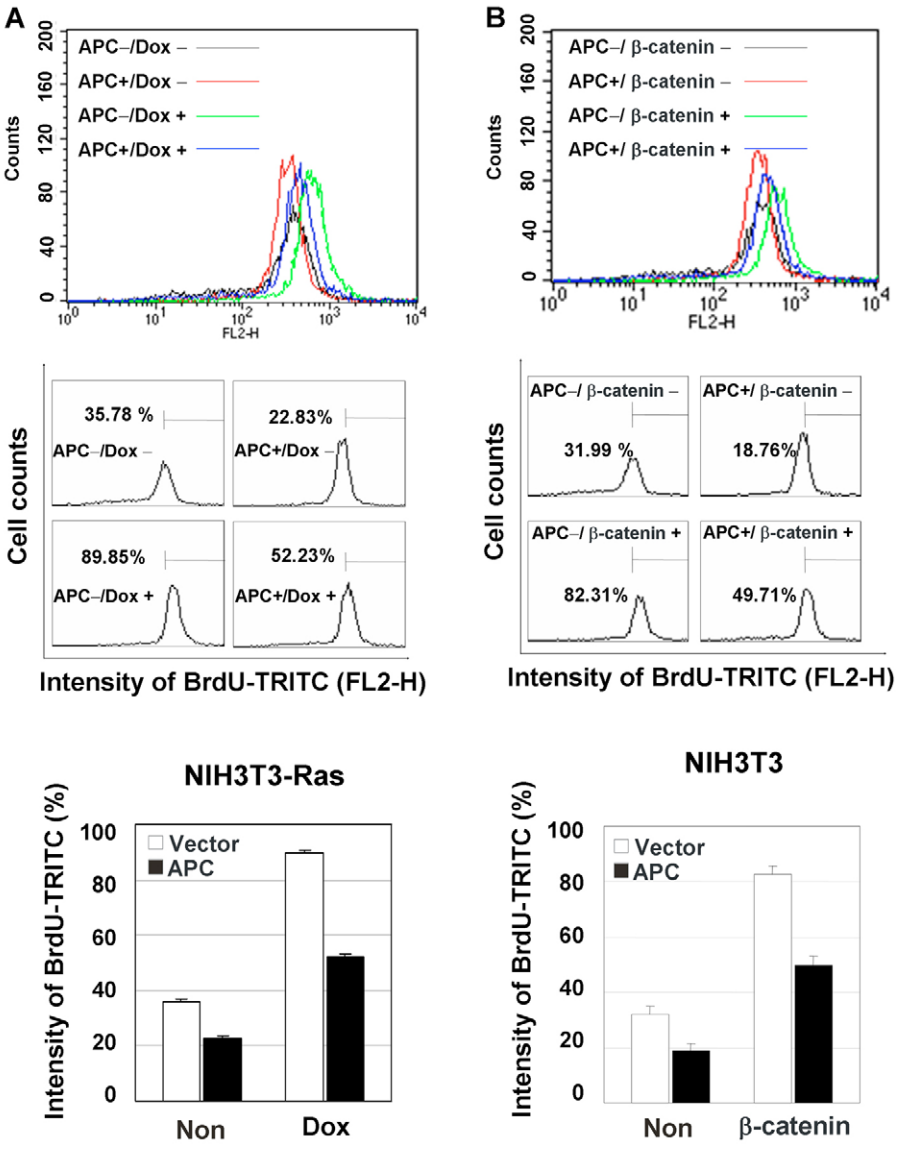

Fig. 9. Effects of APC on RAS- or β-catenin-induced proliferation in NIH 3T3 cells. (A) RAS-inducible NIH 3T3 cells were grown and transfected with 0.5 μg of either pCMV or pCMV-APC, and RAS was induced by doxcyclin treatment as described in Fig. 8A. The cells were fixed, permeabilized and incubated with BrdU antibody and subsequently with TRITC-conjugated goat anti-rabbit IgG. The relative intensities of TRITC-conjugated BrdU were measured using a FACS Vantage system (Becton-Dickinson Immunocytometry systems). The lower panel shows a quantitative analysis of the data in the upper panels. Each data point represents the average of three independent analyses. (B) RAS-inducible NIH 3T3 cells were grown and transfected with 0.5 μg of either pCMV or pCMV-APC together with or without 0.5 μg of pcDNA3.0 or pcDNA3.0-Flag-β-catenin. Immunofluorescence staining and FACS analyses were performed and analyzed as described in A. The lower panel shows a quantitative analysis of the data in the upper panels. Each data point represents the average of three independent analyses.

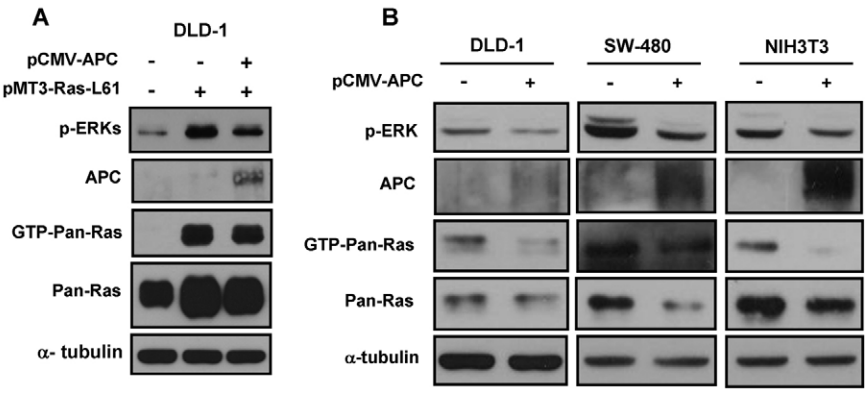

Fig. 10. The effects of APC on GTP-loading and the protein level of Ras. (A) DLD-1 cells were transfected with 0.5 μg of either pCMV or pCMV-APC with or without 0.1 μg of pMT3-RAS-L61. Cells were harvested 72 hours after transfection. RAS pull-down analysis was performed to detect GTP-loaded active RAS (De-Rooij and Bos, 1997) as described in Materials and Methods. Western blot analyses were performed on whole-cell lysates to detect p-ERK, APC, Pan-RAS and α-tubulin. The GTP-loaded Pan-RAS proteins (RAS-GTP) were also detected by western blot analysis using anti-Pan-RAS antibody. (B) DLD-1, SW480 or NIH 3T3 cells were transfected with 0.5 μg of pCMV or pCMV-APC. Cells were harvested 72 hours after transfection, and p-ERK, APC, Pan-RAS and α-tubulin were detected in whole-cell lysates by western blotting. RAS pull-down analysis and detection of RAS-GTP were performed as described in A.

regulates the ERK pathway by its 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 abrogated 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 GTP 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 level 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% CO_2 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 supplements. Analyses were performed on cells at 70% confluence unless otherwise stated. pCMV-APC (Morin et al., 1997), pTOPFLASH (Korinek et al., 1997) and pFOPFLASH (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., 1996) was obtained from S. J. Lee of the Korea Cancer Center Hospital, Seoul, Korea. pMT3RasL61 was provided by J. Jeong of KAIST, Taejon, Korea. The ELK1 trans-reporting pFR-Luc and pFA2-ELK1 *trans*-activator plasmids were purchased 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 (Bonni 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. Bienz 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, pFOPFLASH or the pFA2-ELK1/pFR-Luc vector system (Park et al., 2002). Where required, cells were co-transfected with the pCMV vector, pMT3RasL61, or dominant negative ΔTCF4E. SW480 or NIH 3T3 cells were similarly transfected with pCMV or pCMV-APC. Transfection efficiencies were normalized by co-transfection with 50 ng of the pCMV-β-gal reporter (Clontech, Palo Alto, CA, USA). Forty-eight hours after transfection, cells were rinsed twice with ice-cold phosphate-buffered saline (PBS), harvested and resuspended in reporter lysis buffer (Promega, Madison, WI, USA) for luciferase assay. Luciferase activities were normalized to β-galactosidase levels as an internal control. All analyses were performed in triplicate on independent cell cultures.

Western blot analysis

Cells were transfected with different combinations of plasmids using Lipofectamine Plus reagent (Life technologies). The MEK inhibitor PD98059 (Calbiochem, La Jolla, CA, USA) was added at a concentration of 20 μM where required. Cells were harvested 48 hours after transfection. Attached cells were rinsed three times with ice-cold PBS, harvested and lysed directly in Laemmli SDS sample buffer for western blot analysis (Oh et al., 2002). In the case of mouse embryonic fibroblasts, cell lysates were prepared in a lysing buffer (50 mM Tris, pH 7.4, 1% NP-40, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF, 130 μM bestatin/1 μM leupeptin/0.3 μM aprotinin mix, 1 mM Na_3Vo_4, 1 mM NaF). They were incubated for 20 minutes on ice; cell debris was then removed by centrifugation at 12,000 ***g*** for 20 minutes at 4°C, and the supernatant was used as total protein lysate. Whole cell lysate or 30 μg of total protein lysate from each sample was subjected to 5-10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blot analysis using anti-p-ERK (New England Biolabs, Beverly, MA, USA), anti-ERK (Santa Cruz Biotechnology, Santa Cruz, CA), anti-p-MEK (New England Biolabs), anti-p-RAF1 (Ser-338; Upstate Biotechnology, Lake Placid, NY), APC (C-20, Santa Cruz), anti-Flag (Sigma, St. Louis, MO), anti-β-catenin (Santa Cruz), anti-MYC (Santa Cruz), anti-Pan-RAS (Upstate Biotechnology), anti-p-Akt (Santa Cruz) or anti-α-tubulin (Oncogene) primary antibody followed by matching horseradish peroxidase-conjugated secondary antibody. Protein bands were visualized by enhanced chemiluminescence (Amersham Pharmacia, Uppsala, Sweden).

Preparation of APC-deleted MEF cells

Apc$^{flox/+}$ embryonic stem cells were obtained from Tetsuo Noda of the Cancer Institute of the Japanese Foundation for Cancer Research (Shibata et al., 1997). Mice generated form these cells were crossed to βAKE-TVA transgenic mice to generate *Apc*$^{flox/flox}$; βAKE-TVA progeny. The βAKE-TVA mice, which expressed the retroviral receptor TVA under the control of the β-actin promoter, were obtained from Stephen Hughes of the National Cancer Institute, Frederick, MD, USA (Federspiel et al., 1996). *Apc*$^{flox/flox}$; βAKE-TVA mouse embryonic fibroblast (MEF) primary cells were grown in DMEM supplemented with 10% FBS. After the cells had attached, the medium was removed and the cells were infected with 1 ml of RCAS-Cre retrovirus, obtained from Eric Holland of the Memorial Sloan-Kettering Cancer Center. The cells were swirled and incubated at 37°C for 2 hours. The medium containing the virus was then removed and replaced with fresh medium, followed by incubation for 1 hour at 37°C. After a second infection cycle, the cells were incubated for 2 days at 37°C before harvesting.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from *Apc*$^{-/-}$ and *Apc*$^{+/+}$ MEFs with TRIzol® reagent (Invitrogen). Cells were incubated with 0.5 ml of Trizol reagent for 5 minutes at room temperature to permit complete dissociation of nucleoprotein complexes. One hundred μl 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 isopropyl alcohol, 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 primer. PCR for the *Apc* and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was performed in a final volume of 20 μl containing either 5 μl of *APC* or 2 μl *Gapdh* cDNA, dNTPs, 1.5 mM $MgCl_2$, 2.5 units of Taq polymerase (Perkin Elmer-Cetus, Norwalk, CT, USA) and 1 nM of each primer using the BIONEER PCR Thermal Block system (Bioneer, Deajeon, Korea). The following primers were used; *Apc* forward, 5′-ATG TCC CTC TCC AGG TGC A-3′; *Apc* reverse, 5′-CCA CTG AGA AGC GAA CGC 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′. Primary amplification was performed 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.

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 oligonuclotides containing the T7 promoter sequence (5′-CCTGTCTC-3′) were designed [human β-catenin, 5′-AAGTCCTGTATGAGTGGGAAC-3′ (392-412) and 5′-AAACTACTGTGGACCACAAGC-3′ (1213-1233)], and the dsDNA templates for siRNA were constructed by fill-in using dNTPs and Klenow DNA polymerase, and the T7 promoter sequence (5′-CCTGTCTC-3′). Single stranded RNAs were synthesized with T7 RNA polymerase using the dsDNAs followed by complementary RNA hybridization. Template dsDNAs were removed by DNase and the hybridized RNAs were digested by RNase to produce 5′-UU-3′ 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 3.5-cm culture dish. The transfected cells were grown for 48 hours at 37°C in a 5% CO_2 incubator, and harvested for western blot analysis.

Colony formation assay

DLD-1 cells were plated in 6-well plates at 1×10^4 cells per well. A colony-formation assay on cells transfected with a combination of the control vector, Flag-β-catenin-pcDNA3.0, pMT3RasL61 and pCMV-APC was performed, and cells were selected with G418 at 8 mg/10 ml. After 12 days, the cells were stained with 0.5% Crystal Violet in 20% ethanol.

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 (Clonetech, 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 viewed under 200× magnification using a Nikon Eclipse TE2006-U 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, pcDNA3.0 and Flag-β-catenin-pcDNA3.0, BrdU was added to achieve a final concentration of 20 μM 8 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 Mg^{2+} and Ca^{2+}, 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).

Measurement of RAS activation

The capacity of RAS-GTP to bind to the RAS-binding domain of RAF1 (RBD) was used to analyze the activation status of RAS (De-Rooij and Bos, 1997). Cells were lysed in a culture dish with RAS extraction buffer [20 mM Tris-HCl (pH 7.5). 2 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$, 1% (vol/vol) Triton X-100, 5 mM NaF, 10% (vol/vol) glycerol, 0.5% (vol/vol) 2-mercaptoethanol] plus protease and phosphatase inhibitors. Cleared (10,000 ***g***) lysate was subjected to a RAS-GTP assay as per the manufacturer's instructions (Upstate, Lake Placid, NY, USA). The amount of RAS in the bound fraction was analyzed by western blotting with the anti-Pan-RAS antibody.

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References

Aplin, A. E., Stewart, S. A., Assoian, R. K. and Juliano, R. L. (2001). Integrin-mediated adhesion regulates ERK nuclear translocation and phosphorylation of Elk-1. *J. Cell Biol.* **153**, 273-282.

Baeg, G. H., Matsumine, A., Kuroda, T., Bhattacharjee, R. N., Miyashiro, I., Toyoshima, K. and Akiyama, T. (1995). The tumour suppressor gene product APC blocks cell cycle progression from G0/G1 to S phase. *EMBO J.* **14**, 5618-5625.

Behrens, J. and Lustig, B. (2004). The Wnt connection to tumorigenesis. *Int. J. Dev. Biol.* **48**, 477-487.

Bernstein, L. R. and Colburn, N. H. (1989). AP1/jun function is differentially induced in promotion-sensitive and resistant JB6 cells. *Science* **244**, 566-569.

Bienz, M. and Clevers, H. (2000). Linking colorectal cancer to Wnt signaling. *Cell* **103**, 311-320.

Bonni, A., Brunet, A., West, A. E., Datta, S. R., Takasu, M. A. and Greenberg, M. E. (1999). Cell survival promoted by Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science* **286**, 1358-1362.

Bos, J. L. (1989). Ras oncogenes in human cancer. *Cancer Res.* **49**, 4682-4689.

Clevers, H. (2004). Wnt breakers in colon cancer. *Cancer Cell* **5,** 5-6.

Conacci-Sorrell, M., Simcha, I., Ben-Yedidia, T., Blechman, J., Savagner, P. and Ben-Ze'ev, A. (2003). Autoregulation of E-cadherin expression by cadherin-cadherin interactions: the roles of beta-catenin signaling, Slug, and MAPK. *J. Cell Biol.* **163**, 847-857.

D'Abaco, G. M., Whitehead, R. H. and Burgess, A. W. (1996). Synergy between Apc min and an activated ras mutation is sufficient to induce colon carcinomas. *Mol. Cell. Biol.* **16**, 884-891.

Damalas, A., Kahan, S., Shtutman, M., Ben-Ze'ev, A. and Oren, M. (2001). Deregulated beta-catenin induces a p53- and ARF-dependent growth arrest and cooperates with Ras in transformation. *EMBO J.* **20**, 4912-4922.

Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M. J., Bottomley, A. et al. (2002). Mutations of the BRAF gene in human cancer. *Nature* **417**, 949-954.

De-Rooij, J. and Bos, J. L. (1997). Minimal Ras-binding domain of Raf1 can be used as an activation-specific probe for Ras. *Oncogene* **14**, 623-625.

Eisenman, R. N. and Cooper, J. A. (1995). Signal transduction. Beating a path to Myc. *Nature* **378**, 438-439.

Fearnhead, N. S., Britton, M. P. and Bodmer, W. F. (2001). The ABC of APC. *Hum. Mol. Genet.* **10**, 721-733.

Federspiel, M. J., Swing, D. A., Eagleson, B., Reid, S. W. and Hughes, S. H. (1996). Expression of transduced genes in mice generated by infecting blastocysts with avian leukosis virus-based retroviral vectors. *Proc. Natl. Acad. Sci. USA* **93**, 4931-4936.

Giehl, K. (2005). Oncogenic Ras in tumour progression and metastasis. *Biol. Chem.* **386**, 193-205.

Hancock, J. F. (2003). Ras proteins: different signals from different locations. *Nat. Rev. Mol. Cell. Biol.* **4**, 373-384.

He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B. and Kinzler, K. W. (1998). Identification of c-MYC as a target of the APC pathway. *Science* **281**, 1509-1512.

Heinen, C. D., Goss, K. H., Cornelius, J. R., Babcock, G. F., Knudsen, E. S., Kowalik, T. and Groden, J. (2002). The APC tumor suppressor controls entry into S-phase through its ability to regulate the cyclin D/RB pathway. *Gastroenterology* **123**, 751-763.

Hipskind, R. A., Rao, V. N., Mueller, C. G., Reddy, E. S. and Nordheim, A. (1991). Ets-related protein Elk-1 is homologous to the c-fos regulatory factor p62TCF. *Nature* **354**, 531-534.

Howe, J. R. and Guillem, J. G. (1997). The genetics of colorectal cancer. *Surg. Clin. North Am.* **77**, 175-195.

Ikeda, S., Kishida, M., Matsuura, Y., Usui, H. and Kikuchi, A. (2000). GSK-3beta-dependent phosphorylation of adenomatous polyposis coli gene product can be modulated by beta-catenin and protein phosphatase 2A complexed with Axin. *Oncogene* **19**, 537-545.

Karim, R., Tse, G., Putti, T., Scolyer, R. and Lee, S. (2004). The significance of the Wnt pathway in the pathology of human cancers. *Pathology* **36**, 120-128.

Kinzler, K. W. and Vogelstein, B. (1996). Lessons from hereditary colorectal cancer. *Cell* **87**, 159-170.

Kolligs, F. T., Hu, G., Dang, C. V. and Fearon, E. R. (1999). Neoplastic transformation of RK3E by mutant beta-catenin requires deregulation of Tcf/Lef transcription but not activation of c-myc expression. *Mol. Cell. Biol.* **19**, 5696-5706.

Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B. and Clevers, H. (1997). Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC–/– colon carcinoma. *Science* **275**, 1784-1787.

Lee, H. Y., Dawson, M. I., Walsh, G. L., Nesbitt, J. C., Eckert, R. L., Fuchs, E., Hong, W. K., Lotan, R. and Kurie, J. M. (1996). Retinoic acid receptor- and retinoid X receptor-selective retinoids activate signaling pathways that converge on AP-1 and inhibit squamous differentiation in human bronchial epithelial cells. *Cell Growth Differ.* **7**, 997-1004.

Lim, Y., Han, I., Jeon, J., Park, H., Bahk, Y. Y. and Oh, E. S. (2004). Phosphorylation of focal adhesion kinase at tyrosine 861 is crucial for Ras transformation of fibroblasts. *J. Biol. Chem.* **279**, 29060-29065.

Lowy, D. R. and Willumsen, B. M. (1993). Function and regulation of Ras. *Annu. Rev. Biochem.* **62**, 851-891.

Mimori-Kiyosue, Y. and Tsukita, S. (2001). Where is APC going? *J. Cell Biol.* **154**, 1105-1109.

Miyoshi, Y. (1992). Germ-line mutations of the APC gene in 53 familial adenomatous polyposis patients. *Proc. Natl. Acad. Sci. USA* **89**, 4452-4456.

Morin, P. J., Sparks, A. B., Korinek, V., Baker, N., Clevers, H., Vogelstein, B. and Kinzler, K. W. (1997). Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta catenin or APC. *Science* **275**, 1787-1790.

Munemitsu, S., Albert, I., Souza, B., Rubinfeld, B. and Polakis, P. (1995). Regulation of intracellular beta-catenin levels by the adenomatous polyposis coli (APC) tumor-suppressor protein. *Proc. Natl. Acad. Sci. USA* **92**, 3046-3050.

Nagase, H. and Nakamura, Y. (1993). Mutations of the *APC* (adenomatous polyposis coli) gene. *Hum. Mutat.* **2**, 425-434.

Nathke, I. S. (2004). The adenomatous polyposis coli protein: the Achilles heel of the gut epithelium. *Annu. Rev. Cell. Dev. Biol.* **20**, 337-366.

Nathke, I. S., Adams, C. L., Polakis, P., Sellin, J. H. and Nelson, W. J. (1996). The adenomatous polyposis coli tumor suppressor protein localizes to plasma membrane sites involved in active cell migration. *J. Cell Biol.* **134**, 165-179.

Nishisho, I., Nakamura, Y., Miyoshi, Y., Miki, Y., Ando, H., Horii, A., Koyama, K., Utsunomiya, J., Baba, S. and Hedge, P. (1991). Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients. *Science* **253**, 665-669.

Oh, S. Y., Park, K. S., Kim, J. A. and Choi, K. Y. (2002). Differential modulation of zinc-stimulated p21(Cip/WAF1) and cyclin D1 induction by inhibition of PI3 kinase in HT-29 colorectal cancer cells. *Exp. Mol. Med.* **34**, 27-31.

Park, K. S., Ahn, Y., Kim, J. A., Yun, M. S., Seong, B. L. and Choi, K. Y. (2002). Extracellular zinc stimulates ERK-dependent activation of p21(Cip/WAF1)and inhibits proliferation of colorectal cancer cells. *Br. J. Pharmacol.* **137**, 597-607.

Peifer, M. and Polakis, P. (2000). Wnt signaling in oncogenesis and embryogenesis – a look outside the nucleus. *Science* **287**, 1606-1609.

Polakis, P. (1997). The adenomatous polyposis coli (APC) tumor suppressor. *Biochem. Biophys. Acta* **332**, F127-F147.

Polakis, P. (2000). Wnt signaling and cancer. *Genes Dev*. **14**, 1837-1851.

Rosin-Arbesfeld, R., Cliffe, A., Brabletz, T. and Bienz, M. (2003). Nuclear export of the APC tumor suppressor controls beta-catenin function in transcription. *EMBO J.* **22**, 1101-1113.

Sancho, E., Batlle, E. and Clevers, H. (2004). Signaling pathways in intestinal development and cancer. *Annu. Rev. Cell Dev. Biol.* **20**, 695-723.

Shibata, H., Toyama, K., Shioya, H., Ito, M., Hirota, M., Hasegawa, S., Matsumoto, H., Takano, H., Akiyama, T., Toyoshima, K. et al. (1997). Rapid colorectal adenoma formation initiated by conditional targeting of the Apc gene. *Science* **278**, 120-123.

Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R. and Ben-Ze'ev, A. (1999). The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. *Proc. Natl. Acad. Sci. USA* **96**, 5522-5527.

Suzuki, T., K-Tsuzuku, J., Ajima, R., Nakamura, T., Yoshida, Y. and Yamamoto, T. (2002). Phosphorylation of three regulatory serines of Tob by Erk1 and Erk2 is required for Ras-mediated cell proliferation and transformation. *Genes Dev.* **16**, 1356-1370.

Tan, X., Apte, U., Micsenyi, A., Kotsagrelos, E., Luo, J. H., Ranganathan, S., Monga, D. K., Bell, A., Michalopoulos, G. K. and Monga, S. P. (2005). Epidermal growth factor receptor: a novel target of the Wnt/beta-catenin pathway in liver. *Gastroenterology* **129**, 285-302.

Tetsu, O. and McCormick, F. (1999). Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* **398**, 422-426.

Tice, D. A., Soloviev, I. and Polakis, P. (2002). Activation of the Wnt pathway interferes with serum response element-driven transcription of immediate early genes. *J. Biol. Chem.* **277**, 6118-6123.

Torii, S., Kusakabe, M., Yamamoto, T., Maekawa, M. and Nishida, E. (2004). Sef is a spatial regulator for Ras/MAP Kinase signaling. *Dev. Cell* **7**, 33-44.

Tufan, A. C., Daumer, K. M., DeLise, A. M. and Tuan, R. S. (2002). AP-1 transcription factor complex is a target of signals from both Wnt-7a and N-cadherin-dependent cell-cell adhesion complex during the regulation of limb mesenchymal chondrogenesis. *Exp. Cell Res.* **273**, 197-203.

Wasylyk, B., Hagman, J. and Gutierrez-Hartmann, A. (1998). Ets transcription factors: nuclear effectors of the Ras-MAP-kinase signaling pathway. *Trends Biochem. Sci.* **23**, 213-216.

Weng, Z., Xin, M., Pablo, L., Grueneberg, D., Hagel, M., Bain, G., Muller, T. and Papkoff, J. (2002). Protection against anoikis and down-regulation of cadherin expression by a regulatable beta-catenin protein. *J. Biol. Chem.* **277**, 18677-18686.

Yun, M. S., Kim, S. E., Jeon, S. H., Lee, J. S. and Choi, K. Y. (2005). Both ERK and Wnt/beta-catenin pathways are involved in Wnt3a-induced proliferation. *J. Cell Sci.* **118**, 313-322.