Activated Ras Protein Accelerates Cell Cycle Progression to Perturb Madin-Darby Canine Kidney Cystogenesis

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Background: How the activated Ras oncoprotein induces morphological anomalies in three-dimensional conditions remains unknown.

Results: Activated Ras induces luminal cell filling in the organoids by means of Raf and PI3K.

Conclusion: At least two effectors are required for the morphological phenotypes of Ras.

Significance: This might lead to improved diagnosis and treatment of carcinomas.

In a number of human cancer cells, K-RAS is frequently mutated and activated constitutively, culminating in the induction of continuous cell growth, a hallmark of cancer cells. It is still unclear, however, how the mutated K-RAS induces morphological abnormalities in cancerous tissues. To investigate the mechanism underlying the K-RAS-induced morphological changes, we utilized an auxin-dependent protein expression system, which enabled us to rapidly induce and evaluate constitutively active K-Ras in MDCK (Madin-Darby canine kidney) cysts, a model for polarized epithelial structure. Cells carrying the constitutively active KRasV12 gene were morphologically indistinguishable from normal cells in two-dimensional culture. However, in a gel of extracellular matrix, KRasV12-expressing cells failed to form a spherical cyst. When KRasV12 induction was delayed until after cyst formation, some cells in the cyst wall lost polarity and were extruded into and accumulated in the luminal space. With effector-specific mutants of KRasV12 and inhibitors for MEK and PI3-kinase, we found that both the Raf-MEK-ERK and PI3-kinase axes are necessary and sufficient for this phenotype. Live cell imaging with cell cycle indicators showed that KRasV12 expression promoted cell cycle progression, which was prevented by either MEK or PI3-kinase inhibitors. From these results, we provide a model wherein active-Ras induces cell cycle progression leading to apical cell extrusion through Raf and PI3-kinase in a cooperative manner. The system developed here can be applied to drug screening for various cancers originating from epithelial cells.

RAS proteins operate as molecular switches in signal transduction pathways downstream of tyrosine kinases. Among RAS oncogenes, K-RAS is most frequently mutated in human tumors. A common single-nucleotide K-RAS mutation at codon 12, from glycine (G) to aspartate (D) or valine (V), causes the membrane-associated K-RAS to remain locked in the active form (1).

K-RAS mutation incidence varies widely in organs. For example, K-RAS oncogenes are found in almost 90% of pancreatic cancers, they are present in 50% of colon and 25% of lung adenocarcinomas, and basically absent from prostate and breast cancers (2). Based on experiments using a knock-in strategy in mice, it was suggested that neoplastic growth induced by an endogenous K-Ras oncogene depends upon the cellular context (3). Pancreatic ductal adenocarcinoma (PDAC) accounts for the vast majority of exocrine pancreatic tumors (4). It is now well established that PDAC has precursor lesions, termed PanIN for pancreatic intraepithelial neoplasia. In the course of progression to PDAC, each PanIN stage is associated with increasingly frequent mutations in canonical oncogenes and tumor suppressor genes, such as K-RAS (90–100%), p16INK4a (90–95%), p53 (50–85%), DPC4/SMAD4 (50%), and BRCA2 (10%). Of these mutations, K-RAS mutations are the earliest known genetic abnormalities recorded. Similar stepwise mutation accumulation for carcinogenesis is known in colon cancers (5). These collectively indicate the importance of K-RAS mutations in the carcinogenesis of epithelial organs. Considering that the final diagnosis of cancer is based on the histology of the cancer tissue, unveiling the role of K-Ras mutations on the morphology of the cells will provide a molecular basis for histopathological diagnosis of cancer.

Defining the precise role of the K-RAS oncogene in human cancers is hindered by the sporadic nature of its activation. To reconstitute gene alterations in mice or cultured cells, various methods for rapid activation of the mutated protein have been developed. One system involves conditionally activating Ras by...
Experimental Procedures

Plasmids and Antibodies—The original plasmid for the TIR1 and AID system was purchased from BioROIS Co., Ltd. (Mishima, Japan) (7). TIR1–9myc was amplified by PCR with primers and cloned into retroviral expression vectors pCX4puo or pCX4neoDX. Similarly, AID was amplified by PCR and cloned into a related retroviral expression vector to create pCX4bsr–3HA-AID-AID, which carries a blasticidin resistance-selectable marker. A variety of mutants of K-Ras, RalA, and MEK1 were amplified and cloned into this vector. A FRET biosensor for ERK activity, EKAREV, has been described previously (17). CFP and Venus of a FRET biosensor for caspase 3 activity, SCAT-3 (18), were replaced with Venus and mCherry, respectively, and cloned into pCX4neoDX vector. Apical and basolateral markers GPI-mCherry and GFP-Synaptin4, respectively, were constructed in the pCX4bsr backbone (19). Plasmids for Fucci, CSII-EF-MCS-mCherry-hCdt, and CSII-EF-MCS-Venus-hGem were provided by Dr. Atsushi Miyawaki, RIKEN (20).

Primary antibodies used in this study were: anti-myc (Santa Cruz Biotechnology), anti-HA (Roche Applied Science), and anti-pan-Ras (Calbiochem). Secondary antibodies IRDye 800CW and IRDye 680 (LI-COR) were used for Western blotting, and anti-rat IgG antibody conjugated with Alexa Fluor 488 (Invitrogen) was used for immunostaining.

Cell Culture—MDCK cells were purchased from RIKEN BioResource Center (No. RCBO0995) and maintained in minimal essential medium (MEM) containing Earle’s balanced salt solution (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Equitech-Bio, Woburn, MA), 3% l-Gln, 0.1% nonessential amino acids, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 µg/ml streptomycin, in a 5% CO₂ humidified incubator at 37 °C.

Establishment of Cell Lines—Viruses were produced in BOSC23 cells that were transfected with the expression vectors, the packaging plasmid pG (21), and the envelope plasmid pMD.G (22) or pCMV-VSV-G-Rsv-Rev (provided by Hiroyuki Miyoshi and Atsushi Miyawaki, RIKEN). MDCK cells were first infected with the retrovirus for TIR1–9myc expression, cloned, and designated as MDCK-TIR1. The MDCK-TIR1 cells were next infected with the retrovirus for various 3HA-AID-tagged proteins and selected by antibiotics. To establish the MDCK cells stably expressing the fluorescence markers or Fucci, MDCK-TIR1 cells were infected with retro- or lentiviruses, respectively. For lentivirus, pCAG-HIVgp (provided by Hiroyuki Miyoshi) and pCMV-VSV-G-Rsv-Rev were utilized for virus production. For some experiments, virus-containing medium was concentrated by RetroX (Clontech). After infection, cells were subjected to selection for 2 days with 2 mg/ml G418 for pCX4neo vectors, 2 µg/ml puromycin for pCX4puo vectors, and 20 µg/ml blasticidin for pCX4bsr vectors. For conditional degradation of AID-tagged proteins, the cells were treated with 50 µM synthetic auxin, 1-naphthaleneacetic acid (NAA), in culture medium.

For the MDCK cells stably expressing a FRET biosensor, the expression vectors were introduced into MDCK cells by the Tol2 system (17, 23). After several days, fluorescent cells were sorted by FACS.

Western Blotting—Cells were lysed in 1 × SDS sample buffer (62.5 mM Tris-HCl (pH 6.8), 12% glycerol, 2% SDS, 0.004% Bromo Phenol Blue, and 10% 2-mercaptoethanol). After sonication, proteins were separated by SDS-PAGE on 5–20% Nagaiki precast gels (Oriental Instruments LTD.) and transferred to PVDF membranes (Immobilon-FL, Millipore). After blocking with Odyssey blocking buffer (LI-COR) for 1 h, membranes were blotted with various antibodies diluted 1:2000 in a solution of Odyssey blocking buffer and Tris-buffered saline, followed by incubation with secondary antibodies. Membranes...
were then scanned with an Odyssey IR scanner and analyzed with Odyssey imaging software.

**Immunostaining**—Cells were cultured on 35-mm glass-bottom dishes in medium with or without NAA for 48 h. The cells were fixed by 4% paraformaldehyde for 20 min at room temperature and permeabilized with 0.1% Triton X-100 for 10 min at room temperature. After washing with phosphate-buffered saline (PBS), samples were treated with primary antibody, anti-HA antibody diluted to 1:500, and Alexa Fluor 568 phalloidin (Invitrogen) diluted to 1:1000 for 30 min at 4 °C, followed by incubation with anti-rat IgG antibody conjugated with Alexa Fluor 488. Images were obtained by FV1000 confocal microscopy (Olympus). For cleaved caspase 3 staining, cysts were fixed with 4% paraformaldehyde overnight and permeabilized with 0.25% Triton X-100 for 30 min at room temperature. After washing with PBS, cells were treated with 0.7% fish gelatin/PBS containing 0.025% saponin for 20 min and incubated overnight with anti-cleaved caspase 3 (Cell Signaling Technology) diluted to 1:100, followed by incubation with anti-rabbit IgG antibody conjugated with Alexa Fluor 488 for 4 h. At the same time, Hoechst 33258 (Invitrogen) was added at a dilution of 1:500 for nuclei staining.

**FACS**—MDCK cells expressing fluorescent proteins were trypsinized and resuspended in PBS containing 3% FBS and were sorted by FACS Aria II (BD Biosciences). The blue (488-nm) laser and the FITC channel were used for detection of fluorescence from the FRET probe and GFP. The green (561-nm) laser and the PE channel were used for detection of fluorescence from mCherry.

**Cystogenesis**—Cysts were generated as previously described (19, 24, 25). Briefly, 7.5 × 10^3 MDCK cells were placed on a glass coverslip (13 mm in diameter) coated with 80 μl of polymerized Matrigel (BD Biosciences), and then supplied with culture medium containing 2% Matrigel (BD Biosciences) and then supplied with culture medium containing 10% FBS. Culture dishes were sealed with Parafilm and were maintained at 37 °C during imaging.

**RESULTS**

**Rapid Induction of the AID-KRasV12 Protein in MDCK Cells**—To induce a constitutively active form of K-Ras protein, we utilized the AID system (7). In the presence of NAA, a synthetic auxin, TIR1 plant F-box protein binds to the AID protein, leading to polyubiquitination of AID proteins and thereby degradation of the AID protein by the proteasome (Fig. 1A). Thus, an AID-tagged protein, such as AID-KRasV12, should also be degraded in an auxin-dependent manner. MDCK cells were first infected by a retrovirus carrying the TIR1 gene. After single-cell cloning, a clone with the highest TIR1 expression was designated as MDCK-TIR1 and used thereafter. MDCK-TIR1 cells were infected with a retrovirus carrying the AID-KRasV12 to establish MDCK-KRasV12 cell lines, which were passaged in the presence of NAA (7).

The expression of the AID-KRasV12 protein was induced by removal of NAA. To validate the system, MDCK-KRasV12 cells were cultured for 24 h without or with 50 μM NAA, followed by further incubation without NAA (Fig. 1B). The expression level of AID-KRasV12 was normalized to that in MDCK-KRasV12 cells cultured without NAA (left lane). The average of three independent experiments showed that NAA suppressed AID-KRasV12 expression to 7.5%. Removal of NAA rapidly restored the KRasV12 expression, to 38% in 1 h and 60% in 3 h. Thus, the AID-inducible system allowed us to examine the effect of rapid
induction of KRasV12 in MDCK cells. During the course of experiments, we found that AID-KRasV12 expression could not be restored efficiently when cells were treated with 500 μM NAA (supplemental Fig. S1); therefore, NAA was used at a concentration of 50 μM in the following experiments.

To compare the expression level of AID-KRasV12 with that of the endogenous Ras proteins, MDCK-KRasV12 cells were cultured in the presence or absence of NAA and subjected to a quantitative Western blotting analysis with an anti-pan-Ras antibody (Fig. 1C). We found that the expression level of AID-KRasV12 was 2.0 ± 0.53 (the average ± S.D.) times higher than that of the endogenous Ras proteins. Thus, MDCK-KRasV12 cells allowed us to analyze the effect of the KRasV12 mutant at a physiological expression level. We further examined the subcellular localization of the AID-KRasV12 protein and the effect of KRasV12 expression on the morphology of MDCK cells in two-dimensional growth conditions (Fig. 1D). AID-KRasV12 localized to the plasma membrane as anticipated but did not induce any detectable morphological changes as examined by phalloidin staining (Fig. 1D). This result is consistent with the previous report that expression of KRasV12 does not induce loss of cell-cell contact when the cells are cultured at high density (26).

**Perturbation of Cystogenesis by KRasV12**—A normal MDCK cell embedded in Matrigel grows to form a cyst, whose lumen faces the apical surface of polarized monolayer MDCK cells. Using the AID-inducible expression system, we next examined the effect of KRasV12 expression on cystogenesis. MDCK-KRasV12 cells were cultured in Matrigel with or without NAA for 7 days and stained for the nuclei of live cells with DyeCycle. In the presence of NAA, the MDCK-KRasV12 cells formed a spherical cyst with a central lumen as did the parent MDCK cells (Fig. 2A) (19). In differential interference contrast (DIC) images of MDCK-KRasV12 with NAA, cells were sometimes observed in the lumen as well; however, such cells were dead, judging from the lack of DyeCycle staining. In the absence of NAA, i.e. in the presence of the KRasV12 protein, the MDCK-KRasV12 cells formed a cyst-like cell mass, in which the lumen was mostly filled with cells (right panels).

The frequency of such cyst-like cell masses, hereinafter called “abnormal cysts,” was estimated from DIC images (Fig. 2B). In the absence of NAA, 95% of cysts were abnormal, although 30% of cysts were abnormal even in the presence of NAA, probably due to the leaky expression of KRasV12. To recapitulate the ***in vivo*** cancer condition, MDCK-KRasV12 cells cultured in the presence of NAA for 7 days were deprived of NAA to induce KRasV12 expression. Upon NAA removal, 51.4% cysts became abnormal. These results indicate that expression of active Ras perturbs cystogenesis both in early and late stages. Similar results were obtained from MDCK cysts expressing the constitutively active paralog AID-HRasV12 (supplemental Fig. S2).

**Luminal Cell Filling upon KRasV12 Induction**—We reported previously that forced activation of Rac1 at the apical plasma...
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ERK Activation upon KRasV12 Expression—There are three canonical Ras effectors for cellular transformation; i.e. Raf, RalGEF, and PI 3-Kinase (PI3K) proteins. Extracellular signal-regulated kinase (ERK) is the major downstream molecule located in the Ras-Raf axis, and ERK regulates proliferation and differentiation of many cell types (27). To monitor the activation of KRas in three-dimensional culture conditions, we visualized ERK activity with a FRET biosensor for ERK, EKAREV (supplemental Fig. S3A) (17). Using this biosensor, ERK activity can be visualized in three-dimensional culture by the FRET/CFP emission ratio. MDCK-KRasV12 cysts expressing the EKAREV biosensor were subjected to time lapse imaging for 24 h by a confocal microscope (Fig. 4A). Before NAA removal, ERK activity was heterogeneous at first, varying from cell to cell in the cyst (left). Upon NAA removal, ERK was gradually activated in all cells. Quantitative analysis of individual cysts indicated that ERK activity gradually increased until 15 h (supplemental Fig.

membrane disrupts cell polarity and induces misorientation of the cell division axis, leading to the luminal cell filling (19). To examine whether or not the KRasV12 protein utilizes the same mode of luminal cell filling as does Rac1 activation at the apical membrane, Keima red fluorescent protein-fused histone H1 protein, named H1-Keima, was expressed to follow the migration of the MDCK-KRasV12 cells by time lapse confocal microscopy (Fig. 3A). Upon KRasV12 induction, cells moved into the luminal space without cell division. We next examined epithelial polarity with apical and basolateral markers, GPI-anchored mCherry and Syntaxin4-fused GFP, respectively (Fig. 3B). Several hours after the induction of KRasV12, the apical marker was gradually lost from the plasma membrane facing the lumen. Notably, inward cell movement followed the disappearance of the apical marker at the inner surface of cells. In the cells moving to the lumen, the basolateral marker was distributed around the entire plasma membrane, indicating the loss of epithelial polarity. These results indicate that, unlike the case in Rac1-activated cells, disorientation of the cell division axis was not the cause of the luminal cell filling in cells induced to express KRasV12; rather, cells moving into the luminal space lost their apico-basal polarity.

FIGURE 3. Luminal cell filling and loss of the apical markers upon KRasV12 expression. A, MDCK-KRasV12 cells expressing dKeima-tagged histone H1 were cultured to form cysts in Matrigel containing 50 μM NAA for 5 days. Cysts were further cultured in the absence of NAA for time lapse imaging for 24 h. Arrowheads indicate a cell migrating into the luminal space. Scale bar is 20 μm. B, MDCK-KRasV12 cells were stably transfected with expression vectors for apical and basolateral markers, GPI-mCherry (magenta) and GFP-Syntaxin4 (green), respectively. The day 7 cysts were subjected to time lapse imaging for 24 h. Arrowheads indicate tight junctions between neighboring cells. Note that the disappearance of the apical marker precedes the migration of a cell into the luminal space. Scale bar is 20 μm.

FIGURE 4. Cooperation of Raf and PI3K for the induction of luminal cell filling upon KRasV12 induction. A, MDCK-KRasV12 cells stably transfected with an expression vector for an ERK biosensor, EKAREV, and cultured to form cysts in Matrigel containing 50 μM NAA for 7 days. Cysts in Matrigel without NAA were imaged by time lapse confocal microscopy for 24 h. The FRET/CFP ratio image was generated by MetaMorph software and is shown in the intensity-modulated display modes. Scale bar is 20 μm. B, schematic view of Ras-mediated signaling and the experimental design. The KRasV12T35S mutant activates Raf, but not RalGEF or PI3K, among the three canonical Ras effectors. The KRasV12E37G and KRasV12Y40C mutants activate Raf and RalGEF, and PI3K, respectively. PD184352 and LY294002 (denoted as PD and LY) inhibit MEK and PI3K, respectively. C, cysts of MDCK-KRasV12 cells cultured to form cysts in Matrigel containing 50 μM NAA for 5 days and treated with 2.5 μM PD184352 or 50 μM LY294002 for 2 days in culture medium with or without NAA. Abnormal cysts were counted on DIC images. The averages of three independent experiments are shown ± S.D. (error bars). The numbers of cysts examined are: experiment 1 (n = 243), 171, 305, 170, and 346; experiment 2 (n = 310, 394, 350, 316, 287, and 503); and experiment 3 (n = 191, 170, 230, 258, 269, and 226). *p < 0.05; **p < 0.01 comparison between cysts with and without NAA. D, AID-tagged KRasV12 effector-specific mutants and AID-tagged constitutive active mutants of MEK (MEK1D55E) and RalA (RalAQ72L) introduced into MDCK-TIR cells and cultured on Matrigel with 50 μM NAA for 7 days. Abnormal cysts were scored as in B. The numbers of cysts examined were: experiment 1 (n = 296, 257, 123, 169, 176, 278, 116, 93, 211, 174, 330, 215, 138, 149, 255, 85, and 198 for each of the constructs); experiment 2 (n = 94, 103, 296, 270, 154, 113, 105, 159, 135, 185, 177, 285, 193, 196, 251, 278, 166, and 97); and experiment 3 (n = 171, 178, 122, 86, 174, 137, 127, 118, 112, 251, 254, 188, 85, 97, 150, 126, 142, and 204). Data are the average ± S.D. *p < 0.05; **p < 0.01 compared with cysts with NAA by Student's t test.
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S3B). These results indicate that ERK activity varied between cells in the mature cyst structure and that Ras activation evened out such ERK heterogeneity by universal activation. Of note, ERK activity within the cells was comparable at the lumen and at the cyst wall, ruling out the possibility that asymmetric ERK activity in the cell is the major determinant for infiltration to the luminal space.

Requirement of Raf and PI3K for the Luminal Cell Filling upon K-RasV12 Induction—To examine which of the three effectors, Raf, RalGEF, or PI3K, was required for the luminal cell filling, we used inhibitors and effector-specific mutants. First, MDCK-KRasV12 cells were cultured to form cysts in the presence of NAA and further cultured in the medium containing inhibitors with or without NAA. PD184352 and LY294002 were used to inhibit signaling pathways from ERK and PI3K, respectively (Fig. 4B). In either case, luminal cell filling was significantly inhibited, indicating that both ERK and PI3K pathways are required for the luminal cell filling (Fig. 4C). Similar apical extrusion of a KRasV12-transformed cell has been demonstrated in two-dimensional culture when it was surrounded by normal cells (26). In that report, however, ERK, but not PI3K, was required for the apical extrusion, showing a discrepancy between the two-dimensional and three-dimensional conditions.

We next used effector-specific mutants of KRasV12 to untangle the downstream contributions of KRas effectors. Those mutants, K-RasV12-T35S, -E37G, and -Y40C, preferentially bind to and activate Raf, RalGEF, and PI3K, respectively (28) (Fig. 4B). MDCK-TIR1 was infected with retroviruses encoding the AID-tagged Ras mutants and examined as described (Fig. 4C). The Raf-specific mutant T35S, but not E37G and Y40C, significantly induced luminal cell filling, similar to KRasV12, albeit to a much lesser extent. The lack of luminal cell filling by E37G and Y40C mutants was not due to low expression of the mutant proteins (supplemental Fig. S4A).

We also confirmed that even 16-fold diluted KRasV12 could cause luminal cell filling more efficiently than did the T35S mutant (Fig. 4D, 5th column). We therefore reasoned that both Raf and PI3K might be required for the luminal cell filling and expressed both T35S and Y40C by dual infection. As expected, luminal cell filling was significantly increased by the dual infection, indicating that both ERK and PI3K pathways must be activated for efficient luminal cell filling (Fig. 4D, 6th column). We further examined the role of the Raf and RalGDG pathways with AID-tagged constitutive active mutants of the Raf-MEK and RalGDG pathways, namely MEK1-SDSE and RalA-Q72L, respectively. In accordance with the results above, active MEK, but not active RalA, induced the luminal cell filling to an extent similar to that induced by T35S. From these results, we concluded that the PI3K pathway was required for the efficient luminal cell filling induced by the Raf-MEK-ERK cascade.

Requirement of PI3K for Preventing Apoptosis—The PI3K/Akt pathway is best recognized as a regulator of mammalian cell survival (29). We asked whether this activity of PI3K is required for activated Ras-initiated luminal cell filling. For this we examined the amount of activated, cleaved caspase 3, which proteolytically cleaves many intracellular target proteins during the execution of apoptotic cell death. MDCK-TIR-KRasV12 cells were grown to form cysts in the presence of NAA and treated with DMSO or LY294002 for 2 days in the absence of NAA, followed by fixation and staining with anti-cleaved caspase 3 antibody (green) and Hoechst 33258 (blue). The graph is a quantification of cyst numbers with apoptotic cells in the lumen. The numbers of cysts examined were: experiment 1 (DMSO and LY249002; n = 35, 35); experiment 2 (n = 33, 33); and experiment 3 (n = 31, 35). Data are the average ± S.D. (error bars). *, p < 0.05 comparing cysts between DMSO and LY249002 treatment by Student’s t test. In B, MDCK-KRasV12 cells expressing modified SCAT-3 FRET biosensor (above) were prepared as in A. After 2 days of treatment with DMSO or LY249002, 30 cysts in each condition were imaged with a confocal microscope. mCherry/Venus ratio of cells of cyst wall and luminal space was calculated by measuring fluorescent intensity of mCherry and Venus. ***, p < 0.001.
Accelerated Cell Cycle Progression upon Ras Activation—Raf-MEK-ERK and PI3K-AKT signaling pathways cooperate to promote G0→G1→S cell cycle progression in fibroblasts (30). Similarly, we observed here that ERK and PI3K cooperatively induced the luminal cell filling (Fig. 4D). Naturally, we hypothesized that Ras activation would accelerate cell cycle progression in an ERK- and PI3K-dependent manner in MDCK cysts. To probe this, we utilized a cell cycle indicator, Fucci (20). Fucci consists of mCherry-hCdt1 and Venus-hGeminin, G1 and S/G2/M phase indicators, respectively. MDCK-KRasV12 cells expressing the Fucci indicator were examined as described previously (Fig. 6A). In the presence of NAA, MDCK-KRasV12 cells in S/G2/M phase comprised <20% of total cells. Upon NAA removal, the resulting induction of KrasV12 markedly increased the fraction of cells in S/G2/M phase from approximately 20% to approximately 30% after 12 h and to 45% after 24 h. Notably, both PD184352 and LY294002 suppressed the KRasV12-induced cell cycle progression (Fig. 6B). Thus, both the loss of epithelial polarity and acceleration of cell cycle progression seem to contribute to the luminal cell filling.

DISCUSSION

In this report, we have shown that expression of oncogenic KRasV12 in MDCK cells within a mature cyst structure causes luminal cell filling by perturbation of apico-basal polarity and acceleration of cell cycle progression. For this, Raf and PI3K signaling pathways cooperatively work downstream of Ras.

How does cell proliferation induce luminal cell filling? Is unregulated cell cycle progression sufficient to cause luminal cell filling? There are three clues to answer these questions. First, mature cysts treated with epidermal growth factor increase in cell number and in diameter without luminal cell filling. Thus, at least in the case of EGF-induced cell cycle progression, accelerated cell growth cannot induce luminal cell filling. Second, even though ERK is activated in all KRasV12-induced cells constituting the cysts, only a small fraction of cells extrude into the lumen, and there should therefore be identifiable characteristics of the extrusion-prone cell. Based on this, we found that the extruded cell showed disrupted polarity (Fig. 3B), but ERK activation was comparable with cells remaining in the cyst wall (Fig. 4A). Third, not only extrusion, but also prevention of anoikis after cell extrusion, is important for luminal cell filling (Fig. 5). Considering carcinoma in our body, such apical cell extrusion might be a novel mechanism for cancer cell metastasis. If cells were extruded from the epithelial layer to the lumen, the extruded cells should be transported out of the body. However, if the cells are extruded into a vessel cavity, apical cell extrusion can be the cause of metastasis. During daily pathological diagnosis of various carcinomas, we frequently observe single or multilayered epithelial cells lining endothelial cells. Although it is not clear that cells form cysts or tubes from a single section, morphologically it looks like MDCK cysts with a single lumen formed in a gel. It is therefore tempting to speculate that cell extrusion observed in this study also occurs in cancer cells in vessels undergoing metastasis. Further experiments to connect Ras and polarity are required for a full account of activated Ras-induced cell extrusion.

Consistent with our results (Fig. 4, C and D), several lines of evidence indicate a similar signaling requirement of two major effectors of Ras, Raf, and PI3K. To date, it has been reported that PI3K participates in a Raf-ERK mediated proliferation axis or parallel pathways such as anoikis and polarity formation. In mouse embryonic fibroblasts, the Raf/MEK/ERK pathway was sufficient to sustain normal proliferation, whereas the PI3K/AKT pathway failed to induce it (31). However, the PI3K-mediated pathway cooperated with the Raf-mediated one to reproduce the full response induced by Ras. Similar to this, under conditions where activation of neither Raf nor AKT alone promoted S phase progression, coactivation of both kinases elicited a robust proliferative response in NIH3T3 cells (30). In MCF10A cysts, PI3K activity is necessary for ERK1/2-stimulated proliferation (32). All of these reports indicate the importance of a cooperative effect of Raf and PI3K pathways for cell proliferation. It has already been established that AKT plays a pivotal role to prevent anoikis and apoptosis by protein phosphorylation (33). According to our results, PI3K inhibitor treatment resulted in an increase of apoptosis in the lumen (Fig. 5). Because we assume that cells lost adherence to the substrate during extrusion, it is possible that PI3K is involved in the initial phase of cell extrusion by preventing anoikis. Addition of exog-
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The effect of induced Raf activation in MDCK cysts was previously studied with a Raf-ER fusion protein consisting of a constitutively active mutant of the Raf-1 kinase domain coupled with the ligand-binding domain of the estrogen receptor (35). Upon Raf-1 activation, MDCK cells exhibited a cord-like structure formation (37, 38). In our study, inhibition of PI3K almost completely canceled the effect of Ras activation, indicating that Raf activation alone could not cause the morphological change of the cyst structure of MDCK cells. The discrepancy between that study and ours might be due to the difference in protein expression levels. Ras expression in our system was 2-fold above the endogenous one (Fig. 1C). The expression level of Raf-ER was not compared with that of endogenous Raf-1 in the previous study; however, it is likely that Raf-ER was overexpressed, considering that the endogenous number of molecules per cell of Raf-1 or B-Raf is significantly less than that of Ras proteins (39). Alternatively, it is possible that activated Raf-ER behaves differently from Raf proteins activated by the KRasV12 protein.

Meanwhile, the Ras-induced phenotype observed in our study (Figs. 2–4) is similar to that of activated Raf in MCF10A; that is, larger cysts with luminal filling (40, 32). We expect that either the PI3K pathway may not be required for the luminal filling or that it may be constitutively activated in MCF10A cells.

In summary, we established an in vitro system to investigate the molecular mechanisms by which Ras activation causes aberrant epithelial structures in vivo. We previously found that forced activation of Rac1 at the apical membrane (19) induced polarity loss and luminal cell filling similar to that induced by the active Ras expression reported here. However, live cell imaging revealed that they utilize distinct mechanisms: Rac1 activation induces aberrant cell division, whereas Ras activation accelerates cell cycle progression. Although Ras mutation is not common in renal cell carcinoma, this system can be used to investigate carcinogenesis in other organs such as pancreas, colon, and lung. It is true that epithelial cells express tissuespecific molecules for their distinct function; however, at the same time it is also true that they share common mechanisms to maintain their structure (10, 41). Breaking such organized structure is also a common feature of benign and malignant tumors. Adding various AID-tagged oncoproteins to this system will be useful for categorization of pathological diagnosis. Considering that the majority of sporadic cancers arise from polarized tissues, the strategy established here can be applied for drug screening for various carcinomas. We found that inhibitor treatment was effective to prevent the aberrant cell cycle progression (Fig. 6B) and morphology (Fig. 4C). It has been reported recently that cotreatment with multiple drugs is effective for Ras-induced carcinoma (42) and that development of various inhibitors targeting Raf and PI3K pathways is ongoing (43). Combining various FRET biosensors for activity of signaling molecules to this system will strengthen the screening system and provide an effective and logical treatment for cancers.

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