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SHORT REPORT

Oncogenic K-Ras promotes proliferation in quiescent intestinal stem cells☆,☆☆

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Abstract
K-Ras is a monomeric GTPase that controls cellular and tissue homeostasis. Prior studies demonstrated that mutationally activated K-Ras (K-Ras^{G12D}) signals through MEK to promote expansion and hyperproliferation of the highly mitotically active transit-amplifying cells (TACs) in the intestinal crypt. Its effect on normally quiescent stem cells was unknown, however. Here, we have used an H2B-Egfp transgenic system to demonstrate that K-Ras^{G12D} accelerates the proliferative kinetics of quiescent intestinal stem cells. As in the TAC compartment, the effect of mutant K-Ras on the quiescent stem cell is dependent upon activation of MEK. Mutant K-Ras is also able to increase self-renewal potential of intestinal stem cells following damage. These results demonstrate that mutant K-Ras can influence intestinal homeostasis on multiple levels.

Abbreviations: DOX, doxycycline; Egfp, enhanced green fluorescent protein; TACs, transit amplifying cells

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Introduction

Homeostasis in the intestinal epithelium requires the concerted action of multiple stem and progenitor populations. Some of these populations are highly proliferative, while others are typically quiescent (Barker, 2014). Highly-proliferative, or fast-cycling, stem cells are present throughout the intestine and are responsible for the daily maintenance of the epithelium, and therefore represent the "work-horse" for intestinal homeostasis (Barker et al., 2007). Slowly-cycling, or quiescent, stem cells are less prevalent and have been shown to be able to replace fast-cycling stem cells after injury, and therefore are considered clonogenic reserve cells that can maintain intestinal homeostasis (Buczacki et al., 2013; Tian et al., 2011). While the precise relationships between the

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different types of stem and progenitor cells remain to be determined, it is clear that altering their growth properties can lead to pathology, for example cancer (Barker et al., 2009). We demonstrated previously that activation of the K-Ras oncoprotein promotes expansion and hyper-proliferation within the intestinal transit amplifying cells (TACs), a population that is normally highly proliferative (Haigis et al., 2008). Similarly, oncogenic K-Ras has been shown to accelerate the rate of cell division in $Lgr5^{+}$ stem cells in the small intestine (Snippert et al., 2014). Here, we have studied if/how mutant K-Ras can affect the proliferative and self-renewal properties of intestinal stem cells that are normally quiescent.

Material and methods

Animals and tissues

All mice were cared for according to the guidelines of the Hospital Subcommittee on Animal Research (SRAC). Fabpl-Cre; $K\text{-}Ras^{LSL\text{-}G12D/+}$ animals have been described previously (Haigis et al., 2008). In these animals, Cre is expressed only in the distal small intestinal and colonic epithelia, so mutant K-Ras is expressed only in those regions. Rosa26-M2-rtTA; ColA1-H2B-Egfp transgenic mice have been described previously (Foudi et al., 2009). In this system, the M2 reverse tetracycline transactivator (M2-rtTA) is knocked into the Rosa26 locus, which is expressed in all cells in the adult mouse. Expression of the H2B-Egfp chimeric cDNA from the collagen 1a1 locus is regulated by a doxycycline (DOX) response element. In the absence of DOX, the H2B-Egfp fusion protein is not expressed. When animals are administered with DOX in the drinking water, M2-rtTA binds to the response element and induces expression of H2B-Egfp. Both control (Fabpl-Cre; Rosa26-M2-rtTA; ColA1-H2B-Egfp) and K-Ras mutant (Fabpl-Cre; $K\text{-}Ras^{LSL\text{-}G12D}$; Rosa26-M2-rtTA; ColA1-H2B-Egfp) animals (4–6 week old mice at the beginning of treatment) were given a pulse of DOX (Sigma-Aldrich, 2 mg/ml in water) in the drinking water for 2 weeks. DOX was then removed from the drinking water and animals were sacrificed at defined time points: 1, 3, 5, 7, 9,10, 11, 13, 15, 20, and 25 days. At least 3 animals were analyzed at each time point following DOX removal.

Control and K-Ras mutant animals that were treated with PD0325901 were also given a 2 week pulse of DOX in the drinking water. Following removal of DOX, animals were given a daily intraperitoneal injection of PD0325901 (ChemieTek, 12.5 mg/kg in 10% DMSO) or 10% Dimethyl Sulfoxide (DMSO) and then sacrificed at defined time points: 5, 7, and 13 days. Five animals were analyzed at each time point.

For experiments in which dextran sodium sulfate (DSS) was used to induce epithelial damage, percent damage was determined by dividing the summed length of all damaged regions by the total length of the distal colon. Damaged tissue was defined as any region that was not healthy. Healthy tissue was taken to be any contiguous region that had at least 5 undamaged crypts. For all animals, distal colon was taken to be 5 cm of colon from the distal end.

For tissue collection, animals were sacrificed and the entire intestinal tract was removed and washed with cold PBS. For immunohistochemical analysis, tissues were fixed in formalin overnight. For western blot analysis tissue was lysed in RIPA buffer and stored at −80°C. The proximal small intestine (duodenum) was obtained from tissue immediately adjacent to the stomach. The distal small intestine (ileum) was obtained from tissue immediately adjacent to the cecum. The colon was obtained from tissue immediately adjacent to the rectum.

Flow cytometry, RNA preparation, and qRT-PCR

Rosa26-M2-rtTA; ColA1-H2B-Egfp transgenic mice were given a pulse of doxycycline in the drinking water for 2 weeks. DOX was then removed from the drinking water and animals were sacrificed at 25 days post induction. Lgr5-Egfp-IRES-$CreER^{T2}$ mice were 8–10 weeks of age. Following sacrifice, colons were removed, opened longitudinally and cut into 3-5 mm segments. Tissue was placed in cold PBS (Ca and Mg-free) and vortexed 6 times for 5 s to remove debris and fecal matter before incubation in PBS with 20 mM EDTA at 37 °C for 30 min. The tissue was then transferred to cold PBS and vortexed vigorously to release the crypts. Crypt-containing supernatants were supplemented with 10% FBS, pelleted and resuspended in DMEM for one additional wash. Following spin, crypt pellet was resuspended in 10 ml TrypLE Express (Invitrogen #12605-010) with DNase (1000 u/10 ml, Sigma #D5025), and incubated at 37 °C for 45 min with intermittent mixing to disassociate the crypts into single cells. Cells were filtered through a 40 μm cell strainer (BD Bioscience #352340) into DMEM (Corning #10-013-CV) media containing 5 mM EDTA. Cells were washed twice with DMEM and resuspended in DMEM with 4 mM $MgCl_2$, 200 u/ml DNase, and Propidium Iodide. Because H2B-Egfp animals also show expression of Egfp in immune cells, cells from those animals were stained with CD45-APC-Cy7 (Biolegend #103116) for 10 min at room temperature before resuspension in sorting media. Using the BD Aria flow cytometer, cells were gated on PI negative, CD45 negative populations and then the high Egfp expressing populations (~2% total epithelial cells) and Egfp negative populations were sorted directly into TRIzol. RNA was isolated using TRIzol reagent according to manufacturer's instructions. Subsequently, cDNA was made using High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems) and quantitative real time PCR was performed using TaqMan PCR assays (Applied Biosystems) to detect the expression of Egfp (Assay ID: Mr04097229-mr), Leucine-richrepeat-containing G-protein coupled receptor 5 (Lgr5, Mm00438890_m1), Olfactomedin 4 (Olfm4, Mm01320260_m1), Achaete-scute complex homolog 2 (Ascl2, Mm01268891_g1), Axin 2 (Mm00443610_m1), Mucin 2 (Muc2, Mm01276696_m1), Alkaline phosphatase (Alph, Mm01285814_g1), and Chromogranin A (Chga, Mm00514341_m1). All samples were run in duplicate and normalization was carried out using the $2^{\Delta\Delta CT}$ method relative to 18S rRNA (Mm03928990).

Immunohistochemistry

Tissues were processed for histology via standard protocols and tissue sections were cut to 5 μm. Tissue sections were stained with mouse anti-GFP antibody (Clontech, 632380), rabbit anti-phospho-Histone H3 antibody (Ser10, #9701), and the Vectastain ABC Kit (Vector Laboratories) according to manufacturer's instructions. Reactions were visualized with 3,3′-diaminobenzidine (DAB) and counterstained with hematoxylin.