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Modulation of RAB5A early endosome trafficking in response to KRas mediated macropinocytic fluxes in pancreatic cancer cells

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ABSTRACT

KRAS is the key mutated gene in pancreatic ductal adenocarcinoma (PDAC). Emerging evidence indicates that KRas modulates endocytic uptake. The present study aimed to explore the fate of early endosomal trafficking under the control of KRas expression in PDAC.

Surprisingly, PANC-1 cells lacking KRas exhibited significantly enlarged early and late endosomes containing internalized dextran and epidermal growth factor. Endosome enlargement was accompanied by reduced endosomal degradation. Both KRas silencing and lysosomal blockade caused an upregulation of the master regulator of early endosome biogenesis, RAB5A, which is likely responsible for the expansion of the early endosomal compartment, because simultaneous KRAS/RAB5A knockdown abolished endosome enlargement. In contrast, early endosome shrinkage was seen in MIA PaCa-2 cells despite RAB5A upregulation, indicating that distinct KRas-modulated responses operate in different metabolic subtypes of PDAC.

In conclusion, mutant KRAS promotes endosomal degradation in PDAC cell lines, which is impaired by KRAS silencing. Moreover, KRAS silencing activates RAB5A upregulation and drives PDAC subtype-dependent modulation of endosome trafficking.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest cancers. Less than 5% of affected patients can expect long-term survival, even if the primary, localized tumor is curatively resected and patients undergo adjuvant chemotherapy [1]. Early lymph node metastasis and high resistance toward chemotherapy are essential reasons for the aggressive growth of this malignancy. More than 90% of the PDAC cell lines exhibit activating mutations of the KRAS gene, which is considered the signature mutation within a diverse mutational landscape of the tumors, including genetic and metabolic subtypes [2,3]. KRas primarily localizes to the plasma membrane, but can also translocate to early endosomes following the endocytic pathway. Oncogenic KRAS thereby stimulates macropinocytosis and autophagy to ensure an extra nutrient supply, and to exploit metabolic needs and energy stores (such as during anticancer treatments) [4–7]. Macropinocytosis, autophagy, and signaling events rely on endocytosis and recycling of endosome vesicles. Briefly, the machinery of vesicle degradation consists of the maturation of early endosomes, trafficking, their fusion with the lysosomal compartment, and the hydrolytic cleavage of their content.

One of the master regulators of this process is RAB5, which is a critical determinant of endosomal cargo sorting to degradation or recycling routes, and is essential for macropinosome formation [8–10]. In several types of cancer, RAB5 has been associated with increased tumor invasion and dissemination [11,12]. To date, no such data exist that support the involvement of RAB5 in PDAC progression. The present study aimed to explore the fate of early endosomal trafficking under the control of KRas expression and activity in PDAC.

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2. Material and methods

2.1. Cell lines

The experimental protocol was approved by the local ethics committee of the Technische Universität Dresden (decision number EK 496122016). The human pancreatic cancer cell lines PANC-1, MIA PaCa-2, and BxPC-3 were purchased from ATCC (LGC Standards, Wesel, Germany) and regularly tested for contamination. PANC-1 and BxPC-3 were grown in an RPMI-1640 medium, supplemented with 10% fetal calf serum (FCS). For MIA PaCa-2 cells, Dulbecco's modified Eagle medium, including 10% FCS and 2.5% horse serum, was used. All cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.2. Endocytic assays

For early endosome analysis, the cells were stimulated with 33% FCS for 30 min. Fluid phase endocytosis was analyzed using Alexa Fluor 488–tagged dextrans (10,000 MW, Molecular Probes, Thermo Fisher Scientific, Waltham, Massachusetts, USA) or TMR-dextran (70,000 MW, Molecular Probes) at a final concentration of 1 mg/mL with 30-min incubating time. Epidermal growth factor uptake was performed with Alexa Fluor 555–tagged epidermal growth factor (EGF) (Thermo Fisher Scientific) with a dilution of 1:500 (40 ng/mL) at indicated time points. Degradation was examined by self-dequenching dye-quenched bovine serum albumin (DQ-BSA) (Molecular Probes) at 10 μg/mL with an incubation time of 4 h. For all experiments, cells were prestarved in a serum-free medium for 4–12 h. Degradation blockage was achieved using chloroquine (CQ, Sigma-Aldrich, St. Louis, Missouri, USA) diluted in H_2O and bafilomycin A1 (Sigma-Aldrich) diluted in dimethyl sulfoxide. Experiments were performed with final concentrations of 100 μM (CQ) and 300 nM (bafilomycin A1) overnight.

2.3. Transfection and siRNA

Ribonucleic acid (RNA) interference experiments were performed with two to three different sequences using HiPerfect Transfection Reagent (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The following sequences were targeted by siRNAs: *KRas2.5* 5′-GGCTATATTTACATGCTACTA-3' (Eurofins MWG Operon, Ebersberg, Germany) [24], *KRas3.9* 5′-CUAUGGUCCUAGUAGGAAATT-3′, *KRas4.0* 5′-GCCUUGACGAUACAGCUAATT-3' (#7939 and #7940, Ambion, Thermo Fisher Scientific), RAB5A_2 5′-AGGAATCAGTGTTGTAGTA-3' (Eurofins MWG Operon), and nonsense siRNA (Allstars, Qiagen), which served as the negative control. All siRNA transfections were executed with a final concentration of 60 nM per target gene and an incubation time of 72 h. For the exogenous overexpression of RAB5, a wild-type (WT) RAB5 was used for transient transfection of PANC-1 cells. The experiment was performed using the Lipofectamine2000 Transfection Reagent (Thermo Fisher Scientific).

2.4. Quantitative RT-PCR

The RNA was isolated with the Nucleo Spin II Kit (Macherey-Nagel, Düren, Germany) and converted into cDNA with the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Thermo Fisher Scientific) according to the manufacturers' protocols. The quantitative RT-PCR was performed using Power SYBR Green Master Mix (Life Technologies). The ACTB gene served as a housekeeping gene for normalization of C_t-values. The Delta-Delta-C_t method was used for quantification of gene-expression levels.

2.5. Western blotting

Cells were washed and lysed on ice by a radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors. A Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) was used for concentration measurement. A total mass of 10- to 15-μg protein per sample and lane was separated electrophoretically in a 4–12% Bis-Tris NuPAGE Gel (Thermo Fisher Scientific) and blotted onto a nitrocellulose membrane. Slim Fast powder (5%, Allpharm Vertriebs GmbH, Messel, Germany) dissolved in Tris-buffered saline/0.1% Tween buffer (Sigma-Aldrich) was used for blocking. The blots were then incubated with the primary antibodies mouse anti-KRas (1:100, sc-30, Santa Cruz Biotechnology, Dallas, Texas, USA), rabbit anti-GAPDH (1:5.000, Cell Signaling, Cambridge, United Kingdom), and rabbit anti-RAB5 (1:1000, Cell Signaling) overnight at 4 °C. Horseradish peroxidase–tagged, host-specific secondary antibodies (Cell Signaling) were used. For detection, a chemiluminescence solution (Immobilon Western Chemi, Merck Millipore, Billerica, Massachusetts, USA) was added using G:Box Chemi XT4 (Syngene, Cambridge, United Kingdom). Glyceraldehyde 3-phosphate dehydrogenase served as the loading control. Quantification of protein intensity was performed using ImageJ software (Version 2.0.0; http://imagej.net/).

2.6. Immunofluorescence staining and confocal imaging

The following antibodies were used for immunofluorescence staining: mouse anti-EEA1 (#610456, BD Bioscience, Franklin Lakes, New Jersey, USA), rabbit anti-LAMP1 (L1418, Sigma-Aldrich), and rabbit anti-RAB5A (S-19, Santa Cruz Biotechnology). Cells were grown on collagen-A (0.1 mg/mL) coated glass cover slips and treated with serum starvation and/or siRNA transfection. Immunostaining was performed as described previously [25]. Samples were then incubated with the primary antibody for 1 h. After several washing steps with PBS, cover slips were treated with Alexa Fluor 488- or 633-tagged secondary antibodies (Molecular Probes). Cell nuclei were stained with DAPI (Sigma-Aldrich). Confocal imaging was performed on a Leica SP5 laser-scanning microscope using the 40× oil-immersion objectives (Leica Microsystems, Heidelberg, Germany). The endosomes were measured by ImageJ using an equal vesicle diameter threshold for background subtraction.

2.7. Statistical analysis

The R software package (R version 3.1.3, The R Foundation for Statistical Computing; http://www.R-project.org) was used for statistical calculation. The significance level for all calculations was set at $P = 0.05^{*}/0.01^{**}/0.001^{***}$. Bar plots indicate mean values and standard error of the mean, if not indicated otherwise.

3. Results

3.1. KRas knockdown leads to enlarged early endosomes and a blockade of fluid phase endocytic flux

The PDAC cell lines, PANC-1, MIA PaCa-2 and BxPC-3, were all derived from primary tumors, and exhibited different expression levels of KRAS transcripts. The WT KRAS-expressing BxPC-3 cells had lower KRas mRNA expression than the two cell lines with activating mutations of KRAS. The latter two are classified into different metabolic subtypes: the glycolytic (MIA PaCa-2) and the lipogenic (PANC-1) subtypes [3]. The PANC-1 cell line with heterozygously mutated KRAS had the highest KRas expression (Fig. 1A). To assess whether KRas affects early endosomes in these cells, we silenced the gene by transient RNA interference (siRNA) using at

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