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Down-regulation of Rab5 decreases characteristics associated with maintenance of cell transformation

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ABSTRACT

The early endosomal protein Rab5 is highly expressed in tumor samples, although a causal relationship between Rab5 expression and cell transformation has not been established. Here, we report the functional effects of targeting endogenous Rab5 with specific shRNA sequences in different tumor cell lines. Rab5 down-regulation in B16-F10 cells decreased tumor formation by subcutaneous injection into C57/BL6 mice. Accordingly, Rab5 targeting in B16-F10 and A549, but not MDA-MB-231 cells was followed by decreased cell proliferation, increased apoptosis and decreased anchorage-independent growth. These findings suggest that Rab5 expression is required to maintain characteristics associated with cell transformation.

1. Introduction

Cancer progression is characterized by the acquisition of several traits, including sustained proliferation, resistance to cell death, evading anti-growth factors, replicative immortality, angiogenesis and metastasis [1]. Several of these traits have been shown to be influenced by deregulated organelle and endosome trafficking [2], although the molecular players implicated in these phenomena remain to be fully characterized. Rab GTPases are critical regulators of endosome trafficking with functions ranging from endosome and vesicle formation through tethering, targeting, fusion and transport [3]. In recent years, several Rab proteins have been shown to be altered in cancer, supporting the view that deregulation of components required for intracellular trafficking may be important in cancer progression (reviewed in Refs. [4,5]). In this respect, the early endocytic protein Rab5 is particularly relevant, because it binds a wide range of molecules and effectors, and additional functions have been documented for this GTPase. For instance, Rab5 is required for mitosis progression, by controlling chromosome alignment, kinetochore assembly and nuclear envelop disassembly [6,7]; moreover, Rab5 is known to promote cell migration and invasion in cancer cells [8–10]. Intriguingly, Rab5 levels are upregulated in lung adenocarcinoma [11], breast cancer [12], hepatocellular carcinoma [13], ovarian cancer [14], thyroid adenoma [15] and cervical carcinoma [9]. Importantly, increased expression of Rab5 is associated with elevated incidence of metastasis [12,16]. Despite this evidence, the precise role of Rab5 in determining characteristics associated with malignant transformation and cancer progression remain poorly understood. In this report, we show that Rab5 is important for the maintenance of characteristics associated with malignant transformation in different cancer cell lines, as shown using both *in vitro* and *in vivo* assays.

2. Materials and methods

2.1. Materials

Monoclonal anti-Rab5 (sc46692) was from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit and goat anti-mouse antibodies coupled to horseradish peroxidase (HRP) and anti-actin antibody (number A5316) were from Bio-Rad Laboratories (Hercules, CA). Tissue culture medium, antibiotics and fetal bovine serum (FBS) were from GIBCO Life Technologies (Grand Island, NY)

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and HyClone Laboratories (Logan, UT). The EZ-ECL chemiluminescent substrate was from Pierce Chemical (Rockford, IL). Rab5 lentiviral short hairpin RNAs (shRNA) were from Open Biosystems (Huntsville, AL).

2.2. Cell culture

MDA-MB-231 human breast cancer cells, A549 lung carcinoma cells and B16-F10 murine melanoma cells were cultured in DMEM-F12, DMEM-high glucose and RPMI medium, respectively, supplemented with 10% FBS and antibiotics. Rab5 targeting was performed as previously described, by using shRNA constructs targeting Rab5A [8,10]. To this end, shRNA sequence #B5, #F8 and #F10 (Open Biosystems) were used to target endogenous Rab5A in A549, B16-F10 and MDA-MB-231 cells, respectively. Control cells were infected with a lentivirus encoding a non-specific shRNA sequence (plasmid 1864; Adgene, Cambridge, MA). Stable cell lines were selected and maintained in puromycin-containing culture medium, as previously reported [10].

2.3. Western blotting

Cells were washed twice with ice-cold PBS and lysed in 0.2 mM HEPES (pH 7.4) buffer containing 0.1% SDS, phosphatase inhibitors (1 mM Na_3VO_4), as well as protease inhibitors. Total protein extracts (50 μg/lane, unless indicated) were separated by SDS-PAGE and transferred onto nitrocellulose membrane. Blots were blocked with 5% milk in 0.1% Tween-TBS and then probed with primary antibodies. Bound primary antibodies were detected with HRP-conjugated secondary antibodies and the EZ-ECL system.

2.4. Apoptosis measurement

Cell death by apoptosis was measured by flow cytometry, following propidium iodide (PI) staining, as previously described [26]. Cells were cultured for 24 h in medium supplemented with 0%, 2% and 10% serum, then harvested and resuspended in PBS. Samples were acquired by FACS (BD FACSCanto) and the extent of apoptosis was determined by plotting PI fluorescence versus the forward scatter parameter, using the *WinMDI 2.8* software. Methanol and TNFα-treated cells were used as controls for normodiploid and hypodiploid (apoptotic) cells, as previously described [26].

2.5. Proliferation assay

Cells were seeded on 96-well plates at a density of 1×10^4 cells per well. Cells were incubated for 24, 48 and 72 h, and optical density (O.D.) was assessed with the MTS® kit, by measuring the absorbance at 490 nm, according to the manufacturer's instructions (Promega, Madison, WI, US).

2.6. Anchorage independent growth

Cells (1.5×10^3) were re-suspended in 500 μl culture medium (DMEM-F12, DMEM-HG, RPMI, depending on the cell type) containing 10% FBS and 0.3% low-melting point agarose (Invitrogen, Carlsbad, CA, US). This layer was poured on top of a 500 μl solidified-bottom layer containing 0.8% low-melting point agar in a 24-well plate, allowed to solidify at room temperature and then returned to 37 °C. Samples were photographed at different time points and the number of colonies was determined in 5 random fields per well. Three wells per experiment were analyzed. Data were averaged from three independent experiments. Colony formation was defined as the number of colonies per total of cells in the visual field analyzed.

2.7. Tumor growth assay

Tumorigenicity assays were performed as previously reported by us [27]. Briefly, B16-F10 cells (3×10^5) in 100 μl physiological saline (0.9% NaCl) were injected sub-cutaneously into the flanks of C57/BL6 mice. Appearance of tumors was monitored by palpitation. The largest perpendicular diameters of the resulting tumors were periodically measured, and tumor volumes were calculated according to the following formula: $\text{width}^2 \times \text{length} \times \pi/6$ (Current Protocols in Immunology, 2000). Animals were sacrificed when tumors reached the bioethically permitted limit of 2500 mm^3. Animal survival is defined as the period post-injection of tumor cells until animals were sacrificed. The experimental protocols employed were approved by the institutional bioethics committee (CBA 0271 CMUCH).

2.8. Statistical analysis

Data were analyzed with unpaired Student's t-tests, by using the *GraphPad Prism 5* software (San Diego, CA, US). Three independent experiments were analyzed, and $p < 0.05$ was considered significant.

3. Results

3.1. Down-regulation of endogenous Rab5 in tumor cell lines decreases cell proliferation and promotes cell death

We previously showed that Rab5 is required to sustain migration and invasion of A549 lung cancer and MDA-MB-231 breast cancer cells [10], as well as B16-F10 mouse melanoma cells [8]. Intriguingly, Rab5 has been shown to be increased in several tumor samples, when compared to non-tumor tissue [9,11–15], although the functional relevance of this increase is unknown. Therefore, we sought to target the endogenous protein in different tumor cell lines using a shRNA-based approach. Rab5 was efficiently down-regulated in A549 and MDA-MB-231 cells, as well as B16-F10 cells using species specific shRNA sequences (Fig. 1A). Intriguingly, down-regulation of Rab5 substantially decreased cell proliferation of A549 and to a lesser extent B16-F10 cells, whereas a negligible decrease in proliferation was observed for MDA-MB-231 cells (Fig. 1B). Additionally, the extent of cell death was evaluated in tumor cells treated with shRNA-targeting endogenous Rab5. As anticipated, Rab5 down-regulation was followed by increased apoptosis levels in A549 and B16-F10 cells, but not in MDA-MB-231 cells (Fig. 1C). No differences were observed for other forms of cell death, such as necrosis (data not shown). Since both cell proliferation and apoptosis are key events de-regulated in cancer cells, these observations prompted us to investigate the requirement of Rab5 in maintenance of further characteristics of cell transformation, including anchorage-independent growth and tumor formation.

3.2. Expression of Rab5 is associated with anchorage independent growth and tumorigenicity

The ability of tumor cells with low endogenous Rab5 levels to grow in an achorage independent manner was evaluated in soft agar assays. Indeed, A549, B16-F10 and MDA-MB-231 cells readily formed colonies in soft agar and treatment with a control shRNA did not affect this pattern (data not shown). However, shRNA-mediated targeting of Rab5 decreased the ability of A549 and B16-F10, but not MDA-MB-231 cells, to form colonies in soft agar (Fig. 2). Importantly, decreased anchorage-independent growth in

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