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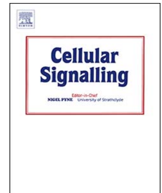

RACK1 cooperates with *NRAS*^{Q61K} to promote melanoma *in vivo*

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ABSTRACT

Melanoma is the deadliest skin cancer. RACK1 (Receptor for activated protein kinase C) protein was proposed as a biological marker of melanoma in human and domestic animal species harboring spontaneous melanomas. As a scaffold protein, RACK1 is able to coordinate the interaction of key signaling molecules implicated in both physiological cellular functions and tumorigenesis. A role for RACK1 in rewiring ERK and JNK signaling pathways in melanoma cell lines had been proposed. Here, we used a genetic approach to test this hypothesis *in vivo* in the mouse. We show that *Rack1* knock-down in the mouse melanoma cell line B16 reduces invasiveness and induces cell differentiation. We have developed the first mouse model for RACK1 gain of function, *Tyr::Rack1-HA* transgenic mice, targeting RACK1 to melanocytes *in vivo*. RACK1 overexpression was not sufficient to initiate melanomas despite activated ERK and AKT. However, in a context of melanoma predisposition, RACK1 overexpression reduced latency and increased incidence and metastatic rate. In primary melanoma cells from *Tyr::Rack1-HA*, *Tyr::NRas*^{Q61K} mice, activated JNK (c-Jun N-terminal kinase) and activated STAT3 (signal transducer and activator of transcription 3) acted as RACK1 oncogenic partners in tumoral progression. A sequential and coordinated activation of ERK, JNK and STAT3 with RACK1 is shown to accelerate aggressive melanoma development *in vivo*.

1. Introduction

Cutaneous melanoma is the deadliest skin cancer. Melanoma has a high metastatic capacity. Despite recent clinical breakthroughs, the majority of metastatic melanoma patients do not survive [1]. The study of a minipig melanoma model revealed an overexpression of *RACK1* (Receptor for activated protein kinase C) mRNA in melanoma cells [2]. RACK1 protein is strongly expressed in melanoma cells of primary tumors and metastases in different mammalian species: patients [2], horses [3] and dogs [4]. In sharp contrast, RACK1 is not detected in normal skin melanocytes or in nævi by immunofluorescence [2–4]. Interestingly, RACK1 increased the survival of human melanoma MeWo cells following UV induced-apoptosis. Moreover, inhibition of RACK1 expression using RNA interference was shown to reduce the tumorigenicity of MeWo cells in a xenograft model [5].

RACK1 is a ubiquitous and abundant protein [6]. It is a scaffold containing seven WD40 repeats considered protein-protein interaction platforms. Through its ability to coordinate the interaction of key

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signaling molecules, RACK1 is thought to integrate various pathways involved in both physiological and tumorigenic cellular functions making it a signaling hub [7]. Yet, the extent to which the multiple binding partners of RACK1 are coordinated has not been much tested *in vivo*. In an attempt to alter RACK1 levels in mammals, the group of S. Biffo obtained one mouse line with a hypomorphic *Rack1* allele. While homozygosity for that hypomorphic *Rack1* allele resulted in a lethal phenotype, heterozygous adult mice showed no major phenotype except for a belly spot and hypopigmented tail and paws [8], typical features of a developmental defect in melanoblast migration.

A role for RACK1 in the crosstalk between ERK (Extracellular signal-regulated kinase) and JNK (c-Jun N-terminal kinase) signaling in melanoma was proposed to set up a feed forward mechanism triggering tumoral progression [9]. In the light of these *in vitro* data, we hypothesized that gain of function of RACK1 targeted to melanocytes in the context of *NRas* constitutive activation would accelerate melanomagenesis by strengthening converging tumoral signaling.

As for other solid cancers, cutaneous melanoma development is considered as a multistep process. Melanomagenesis requires a combination of gain of function mutations in oncogenes and loss of function mutations in tumor suppressor genes [10]. The first spontaneous metastasizing melanoma model harbored the $NRas^{Q61K}$ mutation in a deleted *Cdkn2a* background [11]. To test whether an overexpression of RACK1 was sufficient to trigger melanoma, we created *Tyr::Rack1-HA* transgenic mice in which a hemaglutinin (HA) epitope-tagged-RACK1 is expressed off the *Tyrosinase* promoter. We show here that RACK1 overexpression is not sufficient to trigger nevi or melanomas despite ERK and AKT activation. Yet, in a context of melanoma predisposition, RACK1 melanocytic overexpression reduced latency and increased incidence and metastatic rate. We found activated JNK and STAT3 as partners of RACK1 in melanomagenesis.

2. Materials and methods

2.1. Mice and genotyping

Mouse *Rack1* cDNA was tagged with HA by PCR before insertion in a pBSK-UPT-Tyr-SV40 plasmid [12]. Micro-injection of the linearized vector was made in B6CBAF1/J fertilized oocytes. *Tyr::Rack1-HA* transgenic founders were characterized by Southern blot analysis and PCR genotyping. Data come from the 7th backcross onwards on C57BL/6J background. The $Pax3^{GFP}$ and *-Cdkn2a* alleles and *Tyr::*$NRas^{Q61K}$ transgene have been backcrossed onto the C57BL/6J background for > 15 generations [13]. Animal care and use for this study were approved by the ethical board of Alfort Veterinary School in accordance with European Union Standards (agreement number 16, notice 14/02/12-4). To identify the *Tyr::Rack1-HA* transgene, the following primers were used: forward: 5′-gtcgacatgaccgagcagatgacc-3′ and reverse 5′-tacatggttgcgccatctgcgccgcgggtaccaatag-3′. PCR conditions were 30 s at 94 °C, 30 s at 65 °C, 30 s at 72 °C for 30 cycles and a final extension step at 72 °C for 10 min. The other genotyping conditions were as described [13].

2.2. Histologic analysis and immunofluorescence in mouse samples

Complete necropsy and systematic pathological analysis were performed on all mice as described [14]. Immunofluorescence was performed with mouse monoclonal anti-RACK1 (Transduction Laboratories, dilution 1:150, BD Biosciences, Le Pont de Claix, France), chicken polyclonal anti-GFP (Abcam, 1:600, Paris, France), mouse monoclonal anti-HA (Covance, 1:600, Rueil-Malmaison, France), rabbit anti-cytokeratin5 (Thermo Scientific, 1:100, Fisher Scientific, Illkirch, France) and rabbit polyclonal anti-pERK (Thr202/Tyr204, 1:200) and anti-pAKT (Ser473, 1:50) (Cell Signaling, Ozyme, St Quentin, France) and rabbit anti-ERK 1:100, goat anti-Ki67 1:100, anti-STAT3, anti-JNK (D-2) (Santa Cruz, Heidelberg Germany) antibodies. Nuclear counter-staining was achieved with 4′,6′-diamidino-2-phenylindole (DAPI) (1:1000, Invitrogen). Sections were examined with a Zeiss Axio Observer Z1M ApoTome microscope (Carl Zeiss S.A.S., Le Pecq, France). Controls without the first antibodies showed no unspecific labeling. Images were processed with the *AxioVision* computer program version 4.6 (Carl Zeiss). Figures are representative of the skin samples evaluated (n > 8 for each mouse line). All images shown are individual sections of z series stack. Final figures were assembled with Adobe Photoshop CS3 (Adobe Systems; USA). Quantification of Ki67/GFP positive nuclei was performed on images obtained at 40 × in regions positive for Ki67, counting at least 30 GFP^+ cells per field, 2 fields per mouse, 6 mice per genotype.

2.3. Fluorescent activated cell sorting (FACS), cell culture, soft agar assays and immunofluorescence

Skin melanocytes and melanoma cells from primary tumors (n = 6) or metastases (lymph nodes n = 6; lung n = 4; liver n = 1; brain n = 1) were isolated, FACS-sorted and cultured as previously described [13]. B16 melanoma cell line was grown in DMEM medium with 10% fetal calf serum and penicillin/streptomycin. All cells were grown at 37 °C under 5% CO_2 at pH 7.0–7.1. ERK inhibitor U0126, 5 μM and 10 μM, and JNK inhibitor SP600126 20 μM (Sigma-Aldrich, Saint-Quentin Fallavier, France) were incubated for 24 or 48 h. Human cells UACC903 were cultured as previously described [9]. Soft agar tests were made in 96-well plates as described [13].

For immunofluorescence on cells plated onto coverslips, fixation lasted 15 min in 2% PFA and permeabilization with ice-cold methanol, 10 min. Immunolabeling on cells or agar slices was performed like in tissue section with the omission of the antigen retrieval step. Antibodies used were mouse monoclonal anti-Ki67 (1:100, Novocastra, Newcastle upon Tyne, UK), rabbit polyclonal anti-pPKCα/β_{II} (Thr638/641, 1:100), anti-pJNK (pSAPK Thr183/Tyr185, 1:25), anti-pSTAT3 (Tyr705, 1:100) (Cell Signaling) and as above.

2.4. RNA interference and transduction

Mouse *Rack1* shRNA sequence (ID# 61854) corresponding to a sequence inside exon 2, was obtained from Ambion (Invitrogen): GGTCACTCCCACTTCGTTATT and the scramble sequence used was GTCACTCACCCTTCGGTTATT [15]. Lentiviral vectors with GFP reporter of infections were produced as previously described [16]. Three *Stat3* shRNA (ID# 424803, 424802, 641819) were obtained from Open Biosystem (Thermo Fisher Scientific) as lentiviral vectors. Transduction was performed with at 0.45 ng/μl of lentiviral titer in presence of polybrene. RNA was collected on the third day.

2.5. RNA extraction and quantitative RT-PCR

RNA extractions were performed on 20,000 FACS-sorted cells following RNA XS kit manufacturer instructions (Macherey Nagel, Germany) as described [13]. RNA sequencing (RNA-seq) on *shScramble* and *shRack1*-treated melanocytes, primary melanoma from *Tyr::NRas**; $Pax3^{GFP/+}$ cells with or without *Tyr::Rack1-HA* was performed on technical triplicates of viral infection. Libraries were prepared by selecting polyadenylated mRNA using the TruSeq RNA Sample Prep Kit (Illumina, San Diego, CA). When performed on plated infected cells, RNA was prepared from 10^6 cells in 6 well plates. qPCR assays on cDNA from primary cells infected with *shRack1* were performed using TATAA Granscript cDNA Supermix for reverse transcription and TATAA SYBR GranMaster mix on a Light Cycler480 qPCR instrument (Roche) (TATAA Biocenter, Czech Republic). *Actb*, *Gapdh*, *Tubb5* and *Rnp2* were used as reference genes. Experiments were carried out at least twice in triplicates.

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