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Review Article

The involvement of mutant Rac1 in the formation of invadopodia in cultured melanoma cells

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ABSTRACT

In this article, we discuss the complex involvement of a Rho-family GTPase, Rac1, in cell migration and in invadopodia-mediated matrix degradation. We discuss the involvement of invadopodia in invasive cell migration, and their capacity to promote cancer metastasis. Considering the regulation of invadopodia formation, we describe studies that demonstrate the role of Rac1 in the metastatic process, and the suggestion that this effect is attributable to the capacity of Rac1 to promote invadopodia formation. This notion is demonstrated here by showing that knockdown of Rac1 in melanoma cells expressing a wild-type form of this GTPase, reduces invadopodia-dependent matrix degradation. Interestingly, we also show that excessive activity of Rac1, displayed by the P29S, hyperactive, "fast cycling" mutant of Rac1, which is present in 5–10% of melanoma tumors, inhibits invadopodia function. Moreover, knockdown of this hyperactive mutant enhanced matrix degradation, indicating that excessive Rac1 activity by this mutant can negatively regulate invadopodia formation and function.

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1. Invadopodia-mediated cancer invasion

Invadopodia are actin-based protrusions of the plasma membrane that penetrate into the extracellular matrix (ECM), and enzymatically degrade it [1–3]. They belong to a family of structures, collectively known as invadosomes [4], that facilitate cell invasion through tissues. This process occurs under specific physiological conditions such as wound repair, embryogenesis, and cell differentiation, as well as under pathological conditions such as pathogen infection or cancer metastasis [5].

Invadopodia are unique adhesion structures whose activities – invasion, adhesion and matrix degradation – are each regulated and implemented by a set of multiple proteins. Coordination of their functions occurs on both spatial and temporal levels, controlled by a mechanism that can be referred to as a highly coordinated "grip-soften-push" mechanism [6].

Invadopodia attach to the ECM via their adhesion domain, a ring-shaped structure surrounding the actin core [7–9]. This ring contains transmembrane integrin receptors and various adhesion proteins such as vinculin, paxillin, zyxin, ILK and Hic-5, as well as GTPases and proteases [9]. The adhesion process is critical for invadopodia function, yet it is commonly a transient state [7,9], which is followed by matrix degradation by a variety of proteases belonging to the metalloproteinase (MMPs), ADAM, and serine protease families [2,5,10,11]. The membrane-bound protease, MT1-MMP (or MMP14), functions as a master regulator of invadopodia development [10,12], whose recruitment promotes

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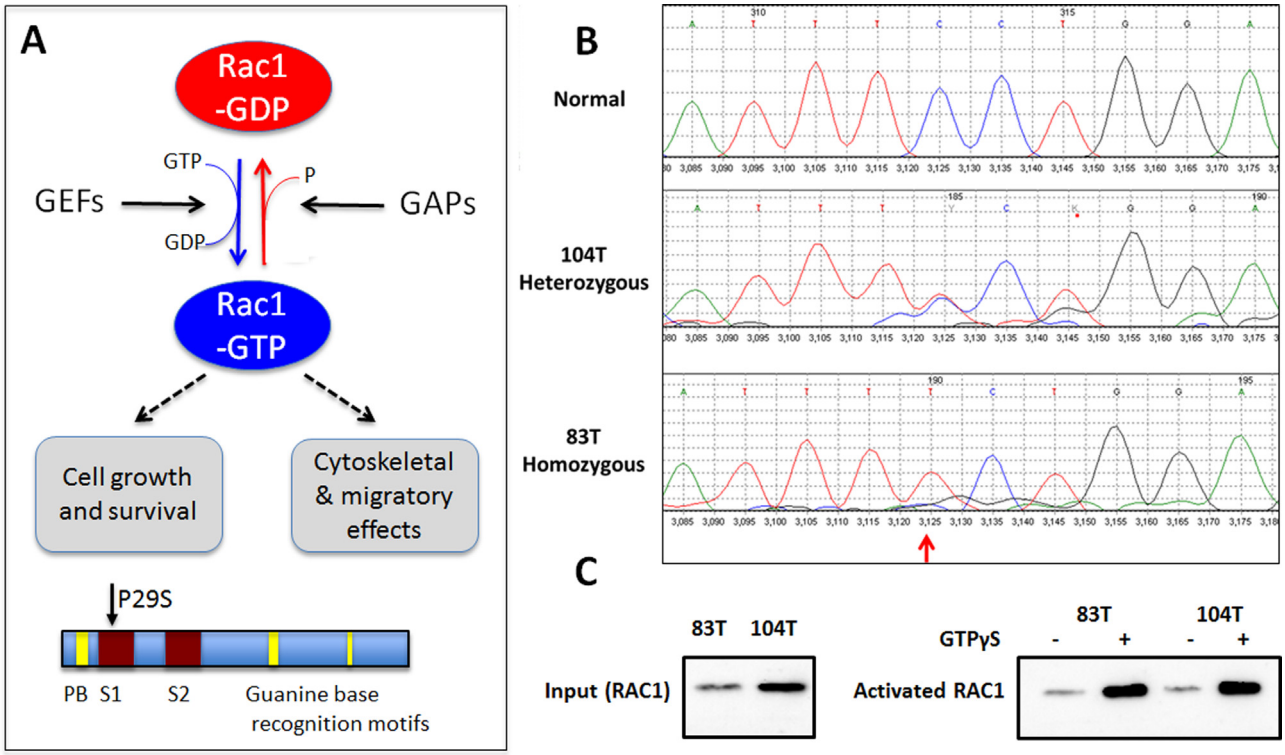

Fig. 1. RAC1-P29S is an active form of RAC1 in cells that harbor the mutation. (A) A schematic drawing depicting the mode of Rac1-mediated signaling, including the activation of the molecule by exchange factors (GEFs), replacing bound GDP with GTP, and its deactivation by Rac-GTPase-activating proteins (GAPs). The active form of Rac1, in turn, is responsible for multiple cellular processes, including regulation of cell growth and survival (e.g., via stimulation of MAP kinases and NFkB) and modulation of cytoskeletal organization (e.g., via activation of the WAVE1-Arp2/3 pathway), which enhances cell migration, invasion and metastasis. At the bottom, a scheme of the Rac1 molecule is presented, highlighting the main domains of the molecule, and pointing to the position of the P29S mutation within the Switch I domain. (B) Sanger sequencing chromatogram showing the zygosity of the 104T and 83T cells. A normal Rac1 chromatogram is shown for comparison. Red arrow indicates the C > T mutation leading to the P29 > S amino acid change in the Rac1 protein (C) RAC1 activation assay. 104T and 83T cells were cultured for 24 hours prior to lysis. Lysates were precipitated using PDB-PAK beads; a small amount was set aside for use as a loading control. Each sample was split into two, and 0.1 mM GTPγS was added to one of the samples.

invadopodia maturation [3] and activation of the soluble MMPs, MMP9 and MMP2 [11,13].

The actin core arises out of the adhesion process, starting when receptor tyrosine kinases such as the EGF receptor are activated [14,15]. This signaling event, in turn, triggers the activation of c-src [16–19,3–6], PKC kinases [20–23] and, eventually, the adaptor protein TKS5 [24], which plays a key role in the nucleation and formation of the actin core bundle. TKS5 can then bind to Ptdlns(3,4)P_2 at the plasma membrane, enhancing the recruitment of actin regulators, such as NCK1, to the membrane [3]. This signaling cascade, which leads to actin polymerization in invadopodia cores, is regulated by small Rho-family GTPases, and drives actin polymerization by the Arp2/3 complex [25–28]. Arp 2/3-driven actin nucleation is promoted by the severing activity of cofillin [29–31]. The actin bundle thus formed is further stabilized and is mostly CDC42-dependent [2,14], which can directly activate the N-WASP-WIP complex; this, in turn, is mechanically reinforced by cortactin [5] and fascin [25,32,33], producing a stable "invasive protrusion" that pushes against the ventral cell membrane, promoting its penetration into the ECM [5,12,14,20,34–36].

The main cause of cancer mortality is the process of metastasis. In order to metastasize, cancer cells must acquire the ability to breach the surrounding extracellular matrix (by various modes of cell invasion [37]), then disperse and penetrate into the surrounding normal tissue, as well as into neighboring blood vessels and lymphatics (by intra- and extravasation) [38]. The invasive potential of cancer cells is mostly correlated with their ability to form invadopodia [39]. This correlation has been demonstrated in many types of cancer cells, among them primary glioma [40], breast cancer [41], and bladder cancer [42].

Modulation of invadopodia components was shown to inhibit cancer cell invasion in *in vitro* models, and cancer metastasis in mice. For example, while overexpression of cortactin in breast cancer cells led to an increase in bone metastatic potential, overexpression of a phosphorylation-deficient mutant cortactin reduced the cell's metastatic potential [43]. Furthermore, expression of various TKS5 adaptor isoforms was shown to regulate the metastatic potential of lung adenocarcinoma in a mouse model: While the short TKS5 isoform inhibits metastasis, the long one promotes it [44].

2. Rho family GTPases and their role in invadopodia-mediated cancer invasion

Studies addressing the regulation of invadopodia formation and function focused much attention around the involvement of Rho-family GTPases in the number and activity of invadopodia in cancer cells. Activation of Rho GTPases was shown to drive invadopodia development and invasion in epithelial ovarian cancer cells [45]. Over-activation of small GTPases through upregulation of intracellular GTP levels was also shown to enhance the ability of melanoma cells to invade and metastasize [46].

Though Rho family GTPases were shown to be involved in invadopodia formation and function [2], until now, most such studies have focused primarily on the small Rho-family GTPase CDC42 [2,28]. Expression of constitutively active CDC42 in RPMI17951 melanoma cells increased formation of invadopodia, while expression of dominant negative RAC1 resulted in a diffuse type of matrix degradation [47]. In glioma cells, inhibition of RAC1

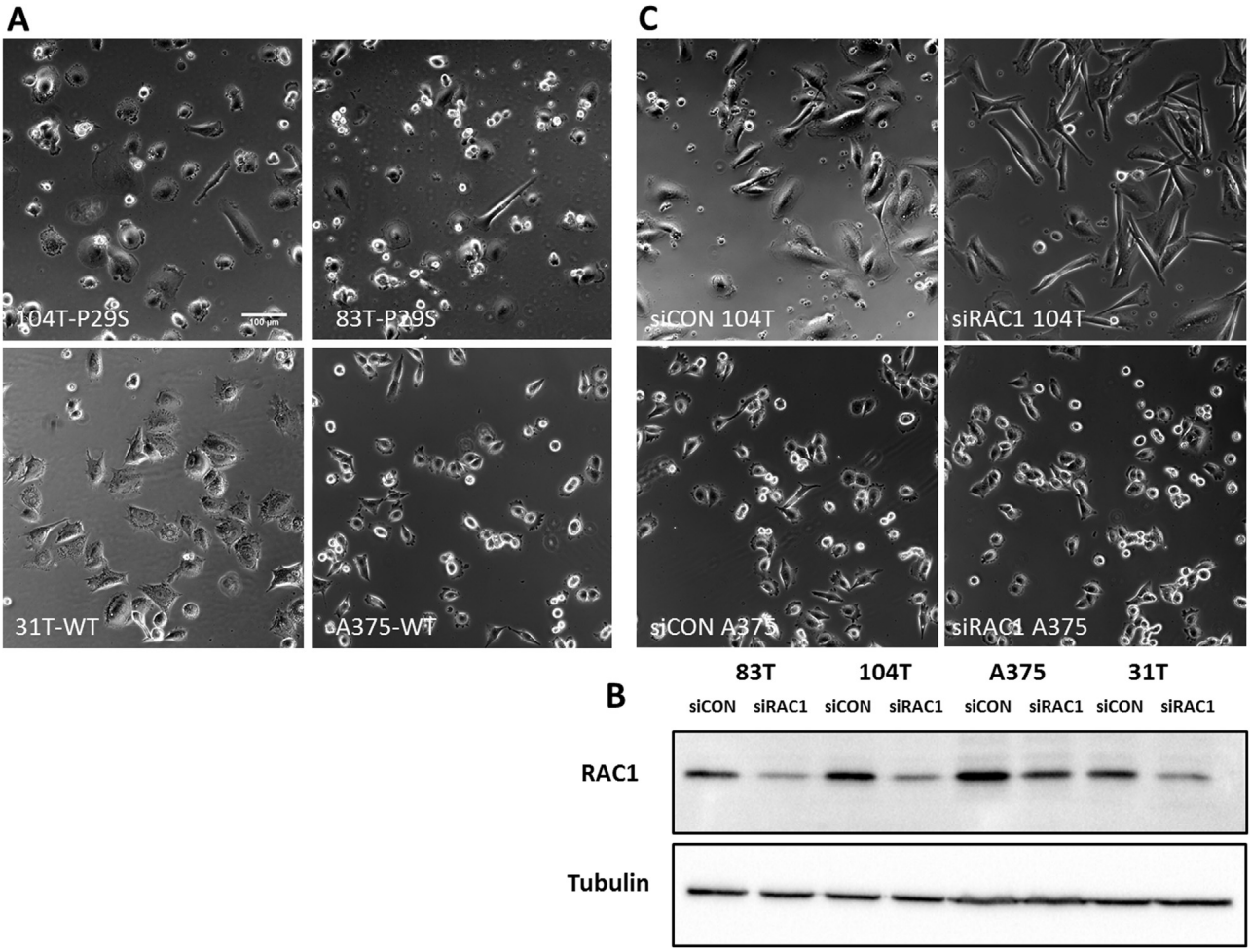

Fig. 2. RAC1-P29S drives lamellipodia formation in cells that harbor the mutation. (A) Snapshots of time-lapse, phase contrast microscopy of 104T, 83T (RAC1-P29S), and 31T, A375 (RAC1-WT) cells. The cells were plated on fibronectin-coated, glass-bottom dishes and cultured for 6 h, to enable cell spreading. Scale bar: 100 um (Supplementary Movie 1). (B) Western blot analysis of RAC1 levels in control (siCON), or RAC1 knockdown (siRAC1) in 83T, 104T, 31T and A375 cells. (C) First and last time point of time-lapse, phase contrast microscopy of control (siCON) versus RAC1 knockdown (siRAC1) of 104T and A375 cells. The cells were plated on fibronectin-coated, glass-bottom dishes and cultured for 6 h, to enable cell spreading. Cells were then imaged every 5 min for 24 h (Supplementary Movie 2). Magnification as in A.

reduced invadopodia formation [48], and in MCF10A cells, such inhibition reduced matrix degradation. Based on such results, it was proposed that RAC1 has a role in invadopodia formation and invasive function, although the mechanisms underlying this process are still poorly understood.

Another instance of actin reorganization occurs during cell migration [37]. This process is mainly regulated by the small GTPases RAC1 and Cdc42 [37], which play an important role in driving the development of lamellipodial and filopodial extensions at the cell's leading edge during cell migration, and are known to promote cancer invasion [49,50].

During the past decade, multiple melanoma oncogenes were identified, several of which were successfully targeted pharmacologically, using small molecular-weight drugs [51,52]. Among them, the small GTPase RAC1, mutated at position 29, was shown to be associated with 5–10% of all melanomas. This mutant was shown to be an active form ("fast-cycling") of RAC1 [53,54], suggesting that excessive Rac1 activity might promote melanoma malignancy. The P29S mutation is located in the switch I region of RAC1, known to be a conserved regulatory element of the GTPase superfamily, important for nucleotide binding and, therefore, interactions with downstream effectors [54–56]. RAC1-P29S was shown to bind more GTP, as well as downstream effectors such as PAK1 and MLK3 [53,54]. It also induces ERK phosphorylation, cell proliferation, membrane ruffling, and transwell migration in normal cells [54,57]. The general mode of action of Rac1, and its P29S mutant, are shown in Fig. 1A.

Due to the importance of RAC1 in cytoskeletal regulation during migration, primarily through its ability to stimulate actin nucleation at the leading edge [53,58] and its newly discovered association with metastatic melanoma, we aimed to characterize the effects of RAC1-P29S on cell adhesion and cytoskeletal organization in melanoma. We specifically focused on the involvement of the RAC1-P29S mutation in the regulation of invadopodia formation and organization in metastatic melanoma cells.

3. RAC1-P29S is an active form of RAC1 in cells that harbor the mutation

To study the effects of RAC1-P29S on adhesion, cytoskeletal organization, and invadopodia formation and function in melanoma cells, we used four melanoma cell lines, two of which express wild-type RAC1 (A375 and 31T) and two that harbor the RAC1-P29S mutation (104T and 83T). From the available Sanger sequencing information, shown in Fig. 1B, we know that one of the mutant cell lines is a homozygote for the mutation (83T), and the other (104T) is a heterozygote (Fig. 1B). To test if the zygosity affects RAC1 activity in the cells, both mutants were analyzed using the RAC1 activation assay (Fig. 1C). As shown, the homozygous

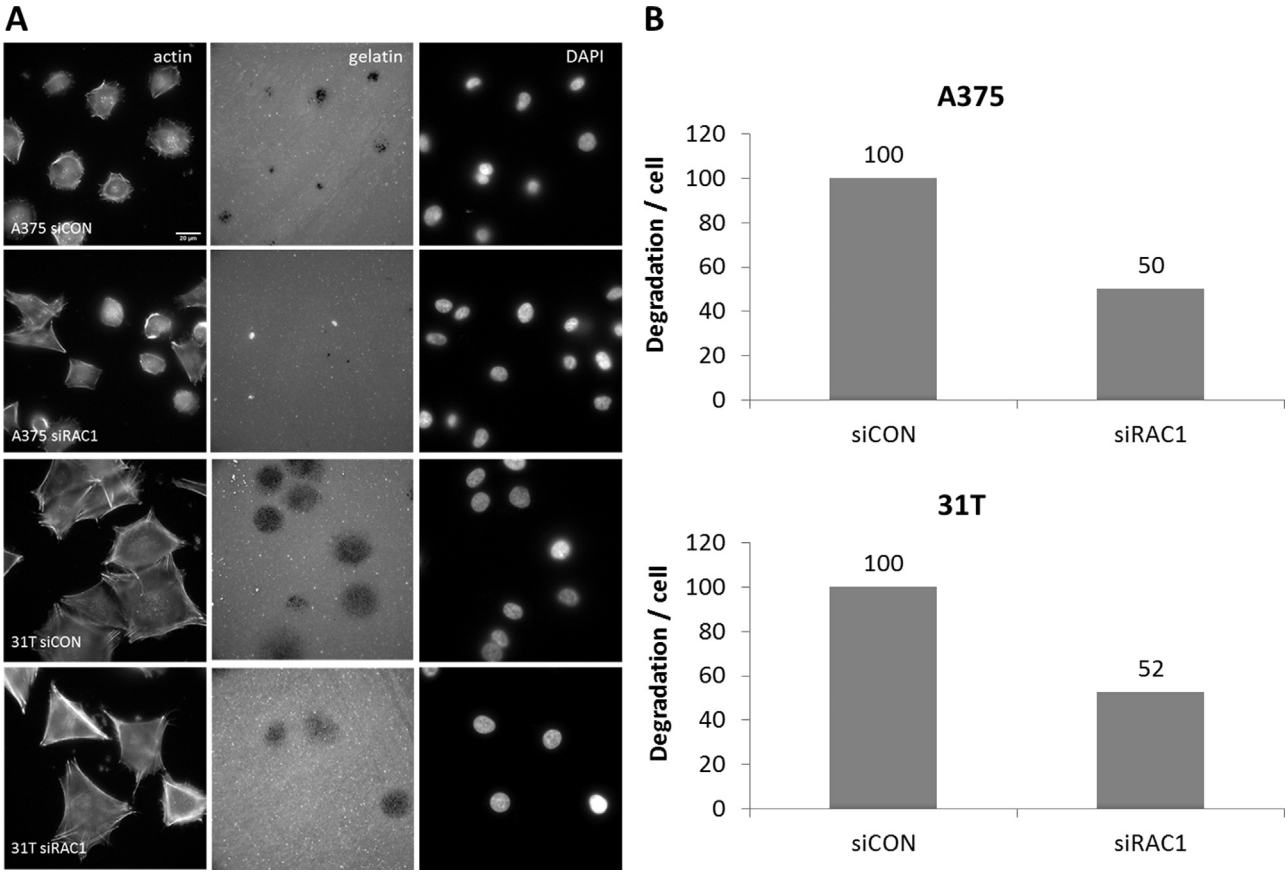

Fig. 3. RAC1 enhances invadopodia function in melanoma cells WT for Rac1. (A) Representative gelatin degradation assay of control (siCON) and RAC1 knockdown (siRAC1) in A375 and 31T (WT) cells. Cells were cultured on fluorescently labeled gelatin for 6 h. Cells were then fixed and stained for actin (as invadopodia marker), and DAPI and gelatin degradation were analyzed. Scale bar: 20um. (B) Invadopodia function analysis of control (siCON) versus RAC1 knockdown (siRAC1) in A375 and 31T (WT) cells. Invadopodia function was quantified using ImageJ software as gelatin degradation area/cell (μm^2) using ImageJ software, and was normalized to the control (percentages)

mutant 83T demonstrated considerably stronger Rac1 activity than the heterozygous mutant, when normalized to the total Rac1 protein present in the cell. This was true both at basal levels of Rac1 activity, and after stabilization of the active Rac1 form by the addition of GTPγS, a non-hydrolysable form of GTP (Fig. 1C). Notably, Rac1 activity levels were very low in the wild-type Rac1 melanoma lines, A375 and 31T (data not shown). These results suggest that the zygosity of RAC1-P29S correlates with RAC1 activation in the cells, strengthening the claim that the mutant encodes for an active RAC1, as previously shown [57].

4. RAC1-P29S drives lamellipodia formation in cells that harbor the mutation

To test the activation of RAC1-P29S at the level of cytoskeletal function, the two RAC1-WT cell lines (A375 and 31T) and the two mutant cell lines (104T and 83T) were monitored for over 24 h by phase contrast, live-cell microscopy (Fig. 2A and Supplementary Movie 1). Both mutant cells formed multiple, rounded lamellipodia per cell, and kept their migratory phenotype throughout the assessment. The migratory behavior of the mutant cells was distinctly different from that of the WT cells, which were more polarized and displayed fewer lamellipodia (Fig. 2A and Supplementary Movie 1).

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.yexcr.2016.02.003.

To verify that lamellipodia formation in the mutant cells is due to RAC1 activity, RAC1 was knocked down in both the 104T mutant cells and the A375 WT cells (Fig. 2B; 104T and A375), and the cells were followed for 24 h using time-lapse microscopy (Fig. 2C, and Supplementary Movie 2). Both WT and mutant RAC1 mutant cells displayed reduced motility upon RAC1 knockdown (siRAC1), compared to control siRNA (siCON). In the mutant 104T cells, treatment with RAC1 siRNA led to a gradual loss of the lamellipodia phenotype, and over time, the cells became polarized and less motile (Fig. 2C, and Supplementary Movie 2). In comparison, control cells (siCON) kept their migratory phenotype throughout the period of monitoring.

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.yexcr.2016.02.003.

5. RAC1-P29S negatively regulates invadopodia function in melanoma cells

As RAC1 is known to be involved in actin remodeling processes, we tested the capacity of the RAC1-P29S mutation to affect invadopodia formation and function. To this end, we knocked down RAC1 by siRNA in the two WT (A375, 31T) and two mutant RAC1 cell lines (104T, 83T) (Fig. 2B), and analyzed invadopodia formation and function using a gelatin degradation assay.

To our surprise, Rac1 knockdown led to a differential effect on invadopodia-mediated matrix degradation in the WT and mutant cells. Knockdown of Rac1 in WT cells caused a twofold reduction in matrix degradation in both cell types (A375 and 31T) (Fig. 3A and B), while knockdown of Rac1 in the mutant cell lines (104T and 83T), caused a dramatic elevation in invadopodia function (Fig. 4A and B). Notably, the effect of increased invadopodia function in 83T cells that harbor a homozygous P29S mutation was

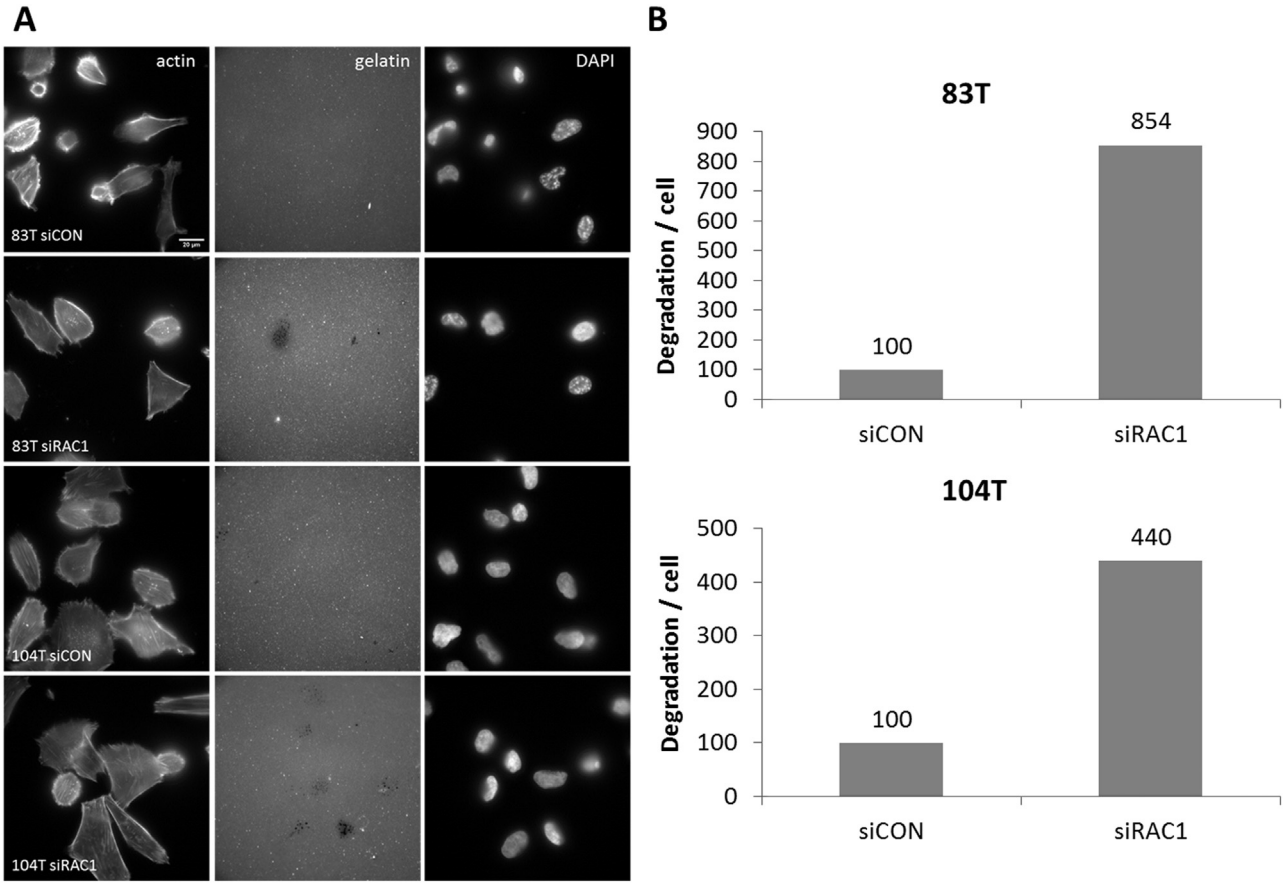

Fig. 4. RAC1-P29S negatively regulates invadopodia function in melanoma cells. (A) Representative experiment of gelatin degradation assay of control (siCON) and RAC1 knock down (siRAC1) in 104T and 83T cells (RAC1-P29S). Scale bar 20 um. (B) Invadopodia function analysis, of control versus RAC1 knock down 104T and 83T cells. Invadopodia function was quantified as gelatin degradation area/cell (μm2) using ImageJ software, and was normalized to the control (percentages).

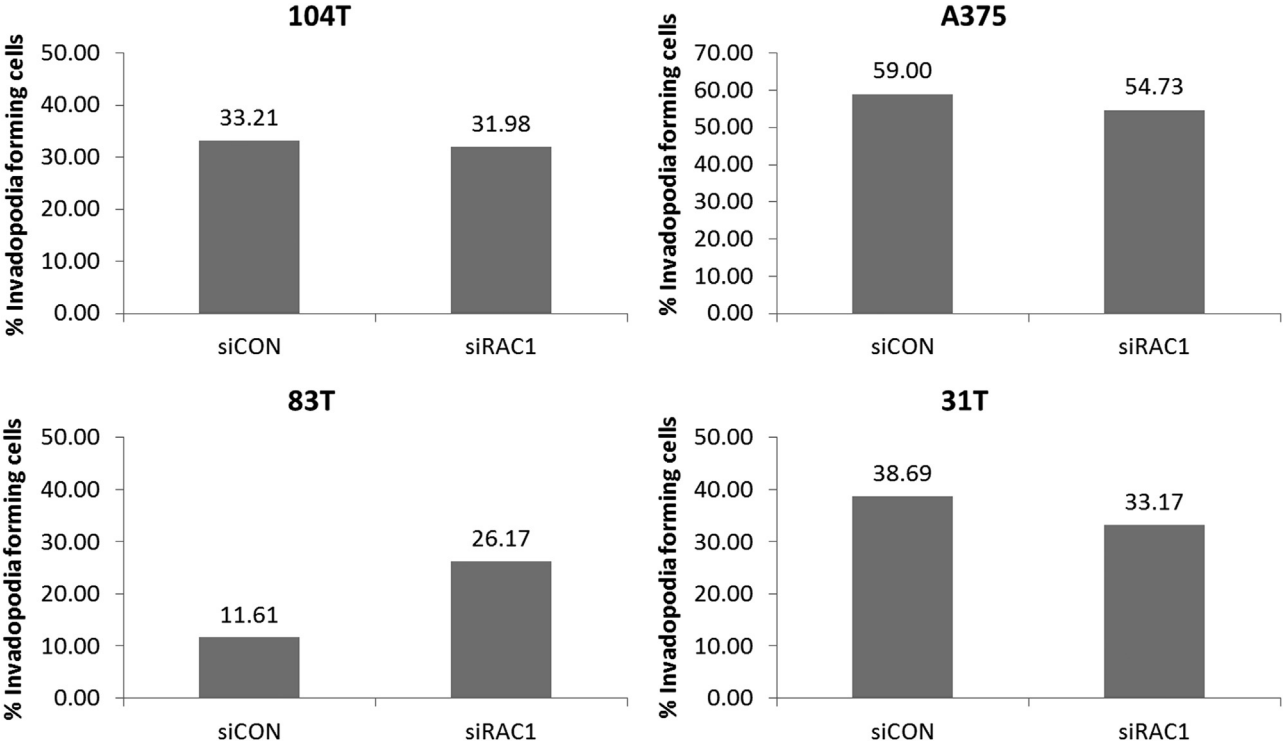

Fig. 5. Effect of Rac1 knockdown on the percentage of invadopodia-forming cells, in cells harboring the Rac1-P29S mutation (104T and 83T) or in cells wild-type for Rac1 (A375 and 31T). The number of invadopodia-forming cells was counted manually in the experiments described in Figs. 3 and 4. As shown, Rac1 knockdown had no effect on the percentage of invadopodia-forming cells in the 31T and A375 lines (both expressing WT Rac1), as well as in the mutant 104T line, suggesting that the effects of the knockdown on the total matrix degradation activity are mostly attributable to the effect on the degradation by individual cells. In the case of the mutant 83T cells, part of the enhancement is attributable to the increase in the number of invadopodia-forming cells (~2 fold).

consistently stronger than that seen in the 104T cells that harbor a heterozygous P29S mutation (Fig. 4A and B), suggesting that WT-RAC1 and P29S-RAC1 regulate invadopodia function in opposite ways.

In addition, it would appear that the overall effect of RAC1 on invadopodia occurs downstream of core formation, since the number of invadopodia-forming cells was similar in control (siCON) and knockdown (siRAC1) in both WT and the heterozygous RAC1-P29S mutant cells. Interestingly, in the homozygous mutant cell line 83T, RAC1-P29S showed a clear capacity to negatively regulate not only invadopodia matrix degradation ability, but also the prominence of invadopodia-forming cells (Fig. 5).

In conclusion, we show here that Rac1 can serve as a potent regulator of invasive cell migration and invadopodia-dependent matrix degradation. Interestingly the effects of the WT Rac1 are quite different from those of the mutant Rac1. As shown here, Rac1 activity in A375 and 31T cells is rather low, compared to that of the mutated 104T and 83T cells, and this difference is manifested in the higher capacity of the mutant to develop lamellipodia and migrate. Surprisingly, and potentially most interesting, is our observation that the hyper-active mutated form, Rac1-P29S, unlike WT Rac1, suppresses matrix degradation while enhancing lamellipodial extension, raising the possibility that the WT and mutant forms of Rac1 operate along different downstream cytoskeletal elements, and possibly signaling pathways. The significance of these differences for understanding the tumorigenic properties of the hyperactive mutant remains to be unraveled.

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