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ORIGINAL ARTICLE

PDE4D promotes FAK-mediated cell invasion in BRAF-mutated melanoma

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The cyclic AMP (cAMP) signaling pathway is critical in melanocyte biology for regulating differentiation. It is downregulated by phosphodiesterase (PDE) enzymes, which degrade cAMP itself. In melanoma evidence suggests that inhibition of the cAMP pathway by PDE type 4 (PDE4) favors tumor progression. For example, in melanomas harboring RAS mutations, the overexpression of PDE4 is crucial for MAPK pathway activation and proliferation induced by oncogenic RAS. Here we showed that PDE4D is overexpressed in BRAF-mutated melanoma cell lines, constitutively disrupting the cAMP pathway activation. PDE4D promoted melanoma invasion by interacting with focal adhesion kinase (FAK) through the scaffolding protein RACK1. Inhibition of PDE4 activity or inhibition of PDE4D interaction with FAK reduced invasion. PDE4D expression is increased in patients with advanced melanoma and PDE4D–FAK interaction is detectable *in situ* in metastatic melanoma. Our study establishes the role of PDE4D in BRAF-mutated melanoma as regulator of cell invasion, and suggests its potential as a target for preventing metastatic dissemination.

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INTRODUCTION

Melanocytes are pigment-producing cells derived from the neural crest, which colonize mainly the basal layer of the epidermis but also the eyes and the ears. The transformation of these cells gives rise to benign lesions such as nevi or malignant tumors, melanoma. The incidence of melanoma has been steadily increasing for the past 30 years and although melanoma accounts for $< 5\%$ of skin tumors, the vast majority of skin cancer deaths are from melanoma.[1] This is due to their highly metastatic behavior and poor response to chemo- and radiotherapies. The limited success of classic therapeutics for melanoma treatment has led to a focus on developing rationally targeted therapies aimed at the molecular mechanisms underlying tumorigenesis. The recent discoveries in signal transduction in melanoma have allowed a better understanding of the molecular biology behind their development. Cutaneous melanoma harbors frequent oncogenic mutations, which activate the MAPK pathway. In particular around 50% of melanoma harbor an activating mutation in BRAF, the V600E substitution being the most frequent, whereas around 20% of melanoma carry a mutation of RAS. BRAF and NRAS mutations are mutually exclusive.[2] The discovery of frequent mutations activating the MAPK pathway has led to the rapid development of a number of pharmacological agents that inhibit key components of this pathway. In particular, inhibitors of the mutated BRAF oncogene, or its target MEK, have been developed with significant results. BRAF and MEK inhibitors have been shown to delay the progression of advanced melanoma and improve overall survival.[3–5] However resistance rapidly develops,[6] suggesting that we still need to improve our knowledge of signal transduction pathways in melanoma in order to find new targeted therapies. Signaling pathways that cooperate with BRAF to transform melanocytes may harbor valuable therapeutic targets to complement the current inhibitors of the MAPK pathway. One such pathway, which is both important for normal melanocyte biology and melanoma development, is the cyclic AMP (cAMP) pathway.[7] The cAMP pathway is activated in melanocytes downstream of melanocytic agonists such as the α-melanocyte-stimulating hormone (α-Msh) acting through the G protein-coupled receptor melanocortin 1 receptor (MC1R). This pathway is closely associated with melanocyte pigmentation because it transcriptionally activates expression of the microphtalmia-associated transcription factor MITF that regulates melanin synthesis.[8] Loss of function mutations in MC1R have been shown to confer a significant risk for developing melanoma, which may in part be independent of pigmentation.[9] MC1R variants are strongly associated with BRAF mutations in melanomas, which are not associated with chronic sun damage, thus highlighting a connection between MC1R and BRAF.[10] Moreover, evidence suggests that the cAMP pathway is inhibited in melanoma, favoring tumor progression.[11,12] For example, melanocyte transformation by oncogenic RAS or BRAF induces their dedifferentiation, and in general melanoma cell lines in culture lose their differentiated characteristics such as the expression of melanin.[13] It has also been shown that the presence of differentiated cells *in situ* was associated with good prognosis,[14] whereas melanoma tumor-initiating cells express very little differentiation markers.[15,16]

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The cAMP pathway is modulated by cAMP-phosphodiesterases (PDE) whose primarily function is to downregulate cAMP levels by specifically catalyzing cAMP hydrolysis, thereby modulating downstream signaling cascades. Previous studies have reported the expression of numerous PDEs in melanoma, although their contribution to tumor pathology has only recently being investigated.[17–19] PDE enzymes are classified into 11 families (PDE1– PDE11) based on their sequence similarity, substrate preference and sensitivity to various inhibitors. Among them, phosphodiesterase 4 (PDE4), which specifically catalyzes hydrolysis of cAMP, are classified into four subtypes (PDE4A, PDE4B, PDE4C and PDE4D) with at least 35 splice variants. PDE4s have a critical role in controlling intracellular cAMP concentrations in physiological conditions and in many cancer cells.[20] Inhibition of PDE4 was shown to suppress tumor growth in prostate, lung, colon and brain cancer.[21–24] Their role in melanocyte biology and melanoma development has recently been highlighted, where PDE4D3 regulated MITF in an homeostatic pathway that controlled melanocyte differentiation.[18]

The prominent role of PDE4 in RAS-mutated melanoma has recently been described. We have shown that cAMP signaling was suppressed by PDE4B and PDE4D in melanoma cell lines expressing mutant RAS.[13] Interestingly, inhibition of PDE4 was sufficient to abrogate transformation of normal melanocytes by oncogenic RAS, and inhibition of PDE4 isoforms could induce cell death in melanoma cells, but not in melanocytes.[25] These data demonstrated that overexpression of PDE4 enzymes is critical for the MAPK activation by oncogenic RAS in melanoma.

The role of PDE4 enzymes in melanoma driven by oncogenic-mutated BRAF is not known and is the subject of this study. We show that the cAMP pathway is constitutively inhibited by PDE4 in melanoma lines mutated on BRAF, which is similar to what we described in NRAS-mutated melanoma. However, PDE4 inhibition in these cells does not inhibit proliferation but instead reduces invasion. We demonstrate that PDE4D regulates melanoma cell invasion by interacting with the focal adhesion kinase (FAK). Furthermore we show that PDE4D expression *in situ* is increased in patients with advanced melanoma.

RESULTS

PDE4 inhibits the cAMP pathway in melanoma cells

To address the regulation of the cAMP pathway in BRAF-mutated melanoma, we analyzed the phosphorylation of a well-characterized PKA substrate, the transcription factor CREB in two BRAF-mutated melanoma cell lines, SkMel28 and WM266.4, compared with normal melanocytes NHEM. α-Msh alone stimulated phosphorylation of CREB in NHEM but not in melanoma cell lines where it had to be combined with the PDE inhibitor IBMX (a non-specific PDE inhibitor) to induce a robust phosphorylation of CREB (Figure 1a, Supplementary Figure 1a). This demonstrates that melanoma cells have a functional MC1R but that cAMP signaling is inefficient, presumably because its degradation by PDE is increased. In these cells, forskolin (activator of adenylyl cyclase) induced CREB phosphorylation, which was further stimulated by PDE inhibition. These results showed that cAMP signaling was uncoupled because of PDE activity in BRAF-mutated melanoma cells unlike normal melanocytes.

The PDE family being large and complex, we used a range of PDE inhibitors with different specificities to evaluate the relative contribution of the different families of PDE to the inhibition of CREB phosphorylation: BRL50481 (specific for PDE7), dipyridamole (specific for PDE5, PDE6 and PDE9-11), rolipram (specific for PDE4) and zaprinast (specific for PDE1/5/6).[26] We found that, in BRAF-mutated melanoma cell lines, CREB phosphorylation was only rescued by combining α-Msh with IBMX or rolipram (Figure 1b, Supplementary Figure 1b). These data showed that PDE4 constantly induced a loss of activation of cAMP in response to α-Msh in BRAF-mutated melanoma cell lines.

PDE4D5 is expressed in melanoma cell lines

In order to characterize the expression profile of PDE4 isoforms in BRAF-mutated melanoma cell lines, we first analyzed the expression of the four *PDE4* genes (*PDE4A, PDE4B, PDE4C* and *PDE4D*) by reverse transcription followed by real-time PCR (RT–PCR) in BRAF-mutated melanoma cell lines (Figure 1c). We showed that NHEM and all studied melanoma cell lines expressed the messenger RNA (mRNA) of *PDE4A, PDE4B* and *PDE4D* but only very weakly *PDE4C*. At the protein level PDE4A was detected in all cell lines but one whereas PDE4C was not expressed. Different isoforms of PDE4B and PDE4D were expressed in NHEM and melanoma cells (Figure 1d). Their molecular weight on western blot suggested that melanocytes expressed PDE4B2 and PDE4D3, whereas melanoma cell lines expressed PDE4B2, PDE4B3 and PDE4D5.[25] As several PDE4B and PDE4D isoforms can co-migrate on a western blot, we used reverse transcription followed by RT–PCR to identify the PDE4B and PDE4D isoforms expressed in NHEM and human melanoma cells. We confirmed that human melanoma cell lines expressed significantly higher level of PDE4D5 than PDE4D3 ($P = 0.004$) (Figure 1e), and both PDE4B2 and PDE4B3 isoforms (Supplementary Figure 2).

PDE4 inhibition reduces melanoma invasion

To investigate whether altered PDE4 expression had a functional effect on cell proliferation in BRAF-mutated melanoma cell lines, as reported in RAS-mutated melanoma,[25] we inhibited all PDE4 isoforms with a saturating concentration of rolipram (10 μmol/l) and combined this treatment with a suboptimal dose of forskolin (1 μmol/l) to reactivate the cAMP pathway.[25,27] We found that treatment by rolipram and forskolin had little effect on the proliferation of BRAF-mutated melanoma cell lines (A375, SkMel5, SkMel28 and WM266.4) in comparison with the BRAF inhibitor PLX 4720 (−18% vs − 70%, $P = 0.001$, Figure 2a). Next, we hypothesized that PDE4 could be involved in other malignant properties, and analyzed the effect of reactivating the cAMP pathway on invasion in the same cell lines. A375, SkMel5, SkMel28 and WM266.4 melanoma cell lines were treated with rolipram and forskolin and deposited on a substitute of basement membrane (Matrigel), and invasive cells were counted after 24 h. Treatment with rolipram and forskolin decreased invasion of all four melanoma cell lines by 50–80% (Figure 2b). Reduction of invasion was significantly higher than reduction of proliferation (−62% vs − 18%, $P = 0.0003$). Thus, under conditions of suboptimal adenylyl cyclase activity, inhibition of PDE4 can suppress the invasion of BRAF-mutated melanoma. To decipher the molecular mechanism of PDE4 involvement in melanoma invasion we analyzed signaling pathway activation in response to PDE4 inhibitors. We showed that treatment with a suboptimal dose of forskolin associated with rolipram induced only a transient phosphorylation of CREB in 4 melanoma cell lines. No downstream targets such as MITF were induced in three out of four cell lines (Figure 2c, Supplementary Figure 3), suggesting that the CREB/MITF axis was not the main pathway involved in the regulation of invasion under PDE4 inhibition. Similarly, reactivation of the cAMP pathway had no effect on ERK activation, which is constitutive due to the presence of the V600E BRAF mutant (Supplementary Figure 4). Furthermore, using a phospho-proteomics assay (R&D Systems, Minneapolis, MN, USA) we evaluated activation of pathways simultaneously in response to a time course treatment of forskolin and rolipram but could not detect any significant changes in phosphorylation of protein kinases except for the transient CREB phosphorylation (data not shown).

As these results suggested that invasion was independent of the classical cAMP/PKA/MITF signaling pathway and the MAPK

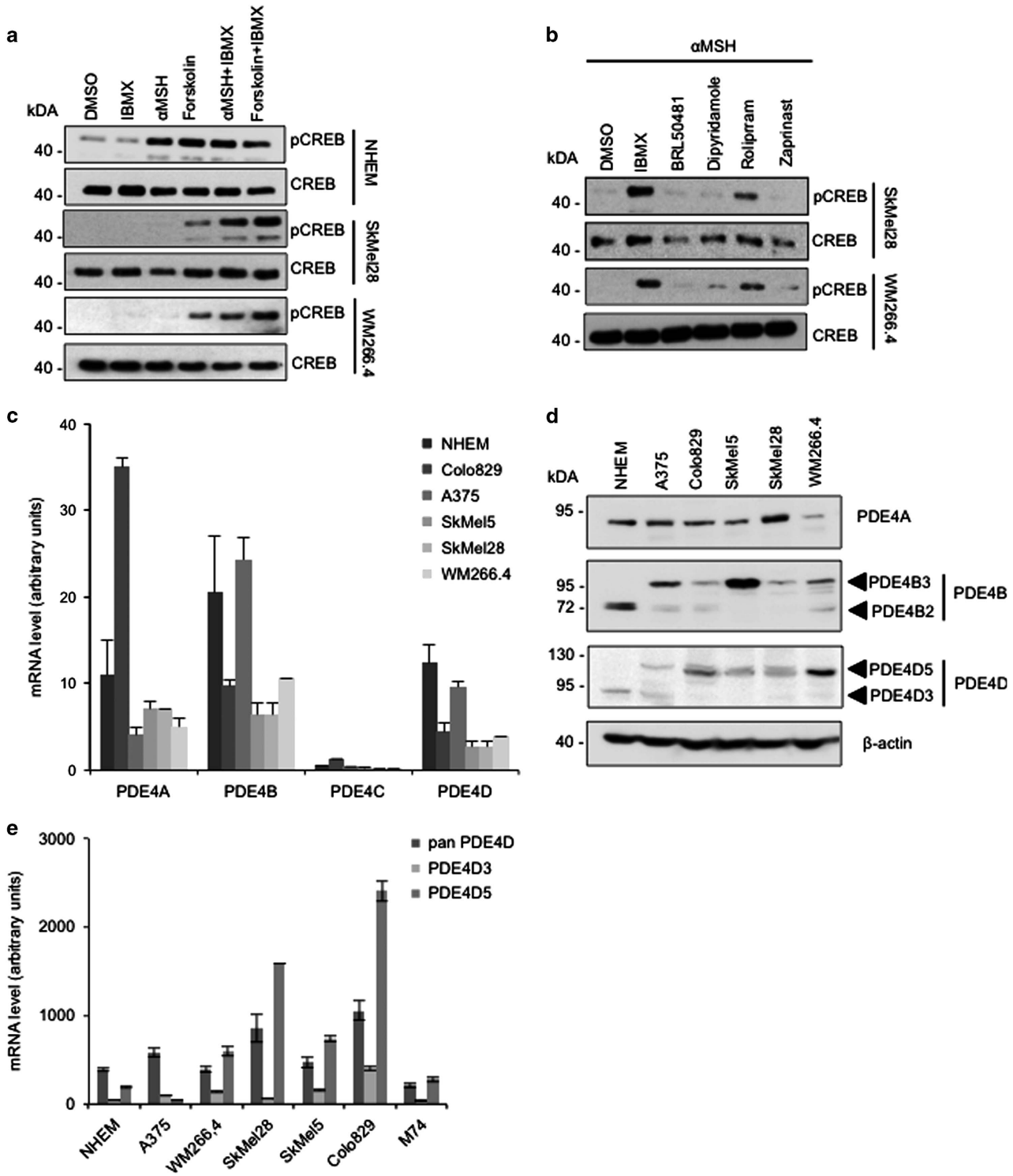

Figure 1. PDE4 constitutively inhibits the cAMP pathway in BRAF-mutated melanoma. (**a**) Immunoblot of phospho-CREB (pCREB) and total CREB in normal human epidermal melanocytes melanocytes (NHEM) and in two BRAF-mutated melanoma cell lines (SkMel28 and WM266.4) treated with DMSO, IBMX (100 μmol/l), αMSH (1 μmol/l), forskolin (1 μmol/l) or a combination of those for 60 min. (**b**) Immunoblot of phospho-CREB (pCREB) and total CREB in SkMel28 and WM266.4 treated for 60 min in the presence of αMSH (1 μmol/l) with the pan-inhibitor of phosphodiesterase IBMX or with selective PDE inhibitors: BRL50481 (PDE7), dipyridamole (PDE5/6/9/10/11), rolipram (PDE4) or zaprinast (PDE1/5/6). Quantification of expression level of phosphorylated CREB and total CREB assessed by western blotting is available in Supplementary Figures 1a and 1b. (**c**) *PDE4A*, *PDE4B*, *PDE4C* and *PDE4D* mRNA expression in NHEM and BRAF-mutated melanoma cell lines assessed by quantitative RT–PCR, related to *GAPDH* mRNA. Bars represent mean ± s.d. (**d**) Immunoblot analysis of PDE4A, PDE4B and PDE4D in NHEM and in BRAF-mutated cell lines (A375, Colo829, SkMel5, SkMel28 and WM266.4). β-actin served as a loading control. (**e**) Quantification of PDE4D isoforms (PDE4D3 and PDE4D5) mRNA assessed by quantitative RT–PCR, normalized to *GAPDH* mRNA level. Pan PDE4D probes detect all isoforms within the PDE4D subfamiliy. Human melanoma cell lines expressed significantly higher level of PDE4D5 than PDE4D3 ($P = 0.004$) (unpaired *t*-test). Bars represent mean ± s.d.

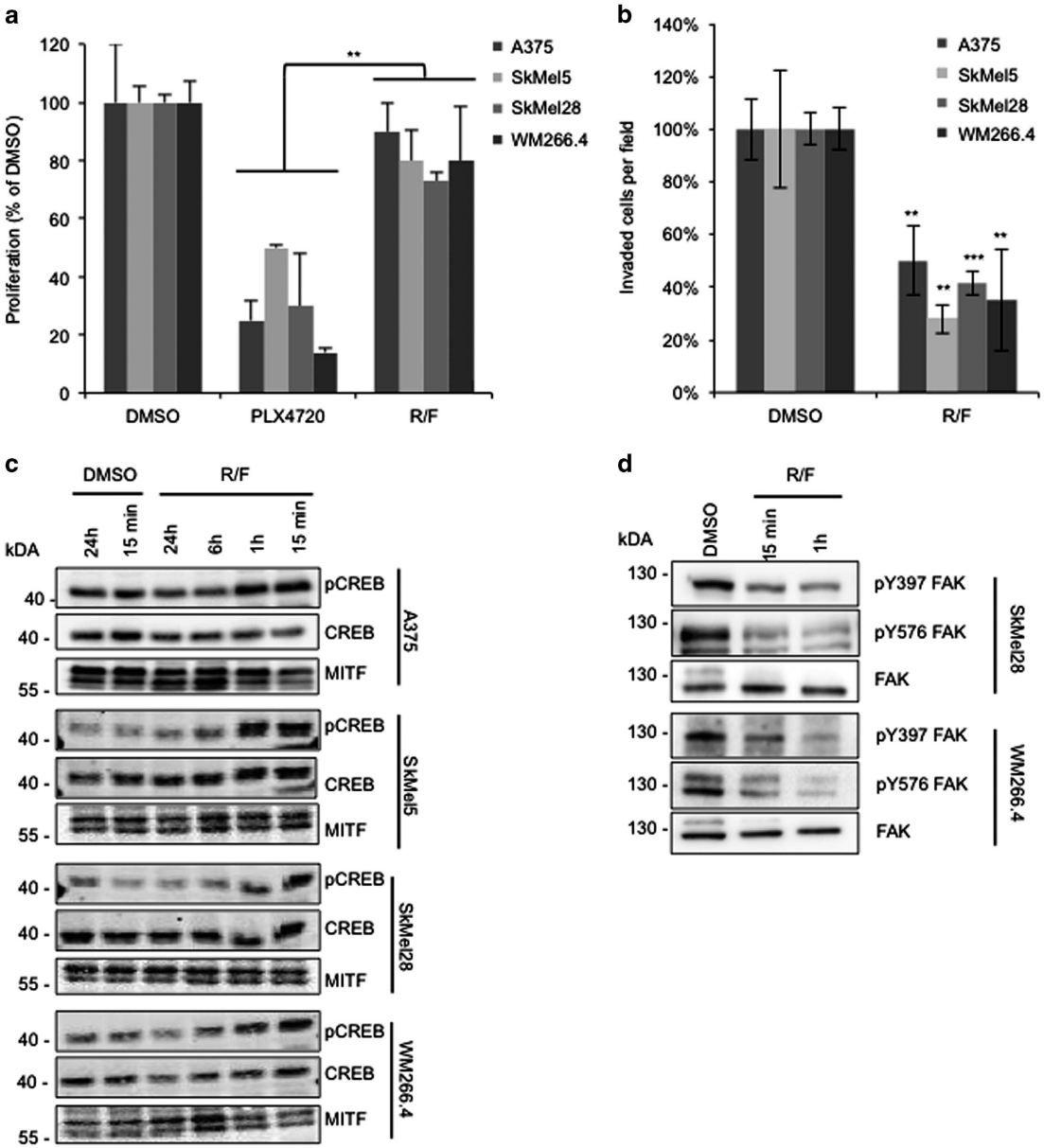

Figure 2. Inhibition of PDE4 reduces melanoma cell invasion in a CREB-/MITF-independent manner. (**a**) Proliferation of four BRAF-mutated melanoma cell lines treated with DMSO, a BRAF inhibitor (PLX 4720 10 μmol/l) or rolipram (10 μmol/l) plus forskolin (1 μmol/l) (R/F) for 96 h. (**b**) Matrigel invasion assay of four BRAF-mutated melanoma cell lines treated with DMSO or rolipram (10 μmol/l) plus forskolin (1 μmol/l) (R/F) for 24 h. Bars represent mean ± s.d. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (unpaired *t*-test). (**c**) Immunoblot analyses of phospho-CREB (pCREB), total CREB and MITF in four BRAF-mutated melanoma cell lines treated with DMSO or rolipram (10 μmol/l) plus forskolin (1 μmol/l) (R/F) for the indicated times. Quantification of expression level of phosphorylated CREB and MITF related to total CREB is available in Supplementary Figure 3. (**d**) Immunoblot analyses of phospho-FAK (pY397 and pY576) and total FAK in SkMel28 and WM266.4 treated with DMSO or rolipram (10 μmol/l) plus forskolin (1 μmol/l) (R/F) for the indicated times. Quantification of expression level of phosphorylated FAK related to total FAK is available in Supplementary Figure 5.

pathway, we looked for other PDE4 partners. FAK is a major component of the integrin signaling pathway that promotes tumor cell invasion.[28] To determine if FAK is involved in the PDE4-mediated invasion, we assessed the level of FAK activation in two BRAF-mutated melanoma cell lines treated with rolipram and forskolin. We found that phosphorylation at both tyrosine 397 and 576, which are early events associated to FAK activation,[29] was reduced in response to PDE4 inhibition (Figure 2d, Supplementary Figure 5). Altogether these data demonstrated that inhibition of PDE4 reduces FAK activation and reduces the invasion of BRAF-mutated melanoma cells.

The PDE4D5 isoform promotes cell invasion in BRAF-mutated melanoma

To evaluate the specific involvement of PDE4D on invasion we performed experiments with the PDE4 inhibitor rolipram alone. We found that the number of invaded A375 and SkMel28 cells through Matrigel was significantly lower in cells treated with rolipram for 24 h (Figure 3a). To confirm the specific role of PDE4D, we silenced its expression using short interfering RNA (siRNA) in WM266.4 melanoma cells. PDE4D silencing, which was confirmed by western blotting, significantly inhibited cell invasion compared with control siRNA-transfected cells ($P < 0.0001$) (Figure 3b).

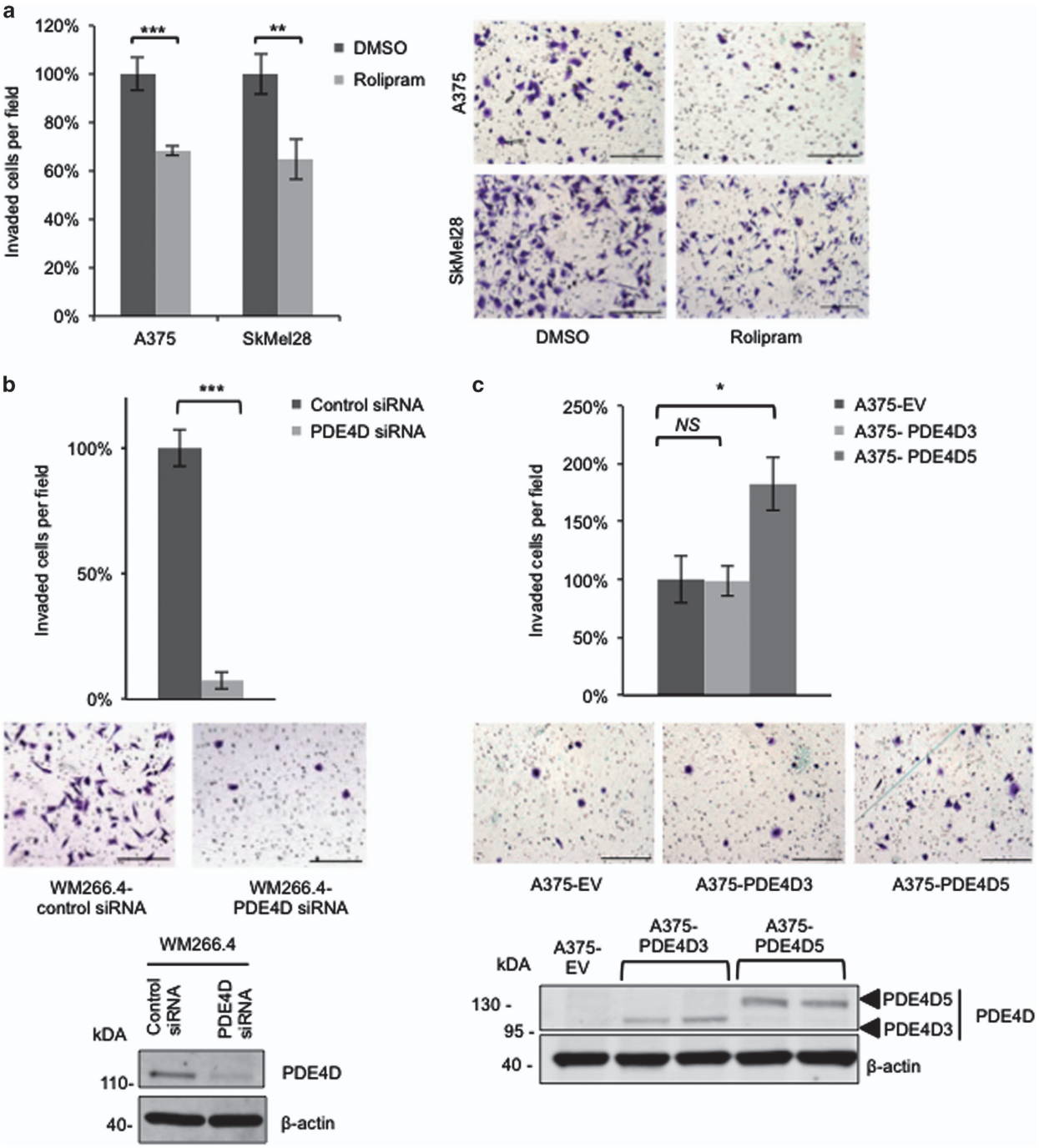

Figure 3. PDE4D5 promotes tumor cell invasion in melanoma. (**a**) Matrigel invasion assay of A375 and SkMel28 treated with DMSO or rolipram (10 μmol/l) for 24 h. Representative images of invaded cells after staining with crystal violet and quantification from three independent experiments performed in duplicate. (**b**) Matrigel invasion assay of WM266.4 transfected with control siRNA or PDE4D siRNA. Representative images of invaded cells after staining with crystal violet and quantification from two independent experiments performed in duplicate are shown. PDE4D expression assessed by western blotting after transfection with control siRNA or PDE4D siRNA for 72 h was performed to confirm PDE4 silencing. β-actin served as a loading control. (**c**) Matrigel invasion assay of A375 transfected with empty vector control (EV), PDE4D3 or PDE4D5. Representative images of invaded cells after staining with crystal violet and quantification from three independent experiments performed in duplicate. Overexpression of PDE4D3 or PDE4D5 was assessed by western blotting after transfection of A375 cells with EV, PDE4D3 or PDE4D5. β-actin served as a loading control. Bars represent mean ± s.d. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (unpaired *t*-test). Scale bar, 250 μm. NS, not significant.

This result demonstrated that PDE4D, and not the general cAMP/PKA/MITF signaling pathway, promoted invasion.

A previous study reported that the PDE4D5 isoform forms a complex with FAK, controlling integrins and cell polarization in cancer cells.[30] Because PDE4D5 is overexpressed in melanoma cells we hypothesized that it controls melanoma invasion through its interaction with FAK.

To specifically study the effect of PDE4D5 isoform on invasion, we overexpressed PDE4D5 in A375, which express low level of PDE4D5 (Figure 1e) and are weakly invasive (Figure 3a). As a

control we transfected A375 cells with the PDE4D3 isoform, which cannot interact with FAK.[30] Overexpression of PDE4D5 or PDE4D3 was confirmed by western blotting. A375 cells overexpressing PDE4D5 showed significantly increased invasiveness in comparison with PDE4D3- or empty vector-overexpressing cells ($P = 0.03$, Figure 3c).

Together these results demonstrated that the PDE4D5 isoform specifically promotes melanoma cell invasion, and that inhibiting PDE4 with rolipram impairs invasive properties of melanoma cells.

PDE4D5 regulates melanoma cell invasion through a PDE4D5/RACK1/FAK complex

RACK1 is a scaffold protein interacting with several proteins such as FAK, Src, integrins and, among PDE, with the PDE4D5 isoform only.[31] Recently RACK1 was reported to connect PDE4D5 to FAK in a model of squamous cancer cells.[30] As our results showed that PDE4D5 expression was elevated in BRAF-mutated melanoma, regulating melanoma cell invasion and FAK phosphorylation, we hypothesized that RACK1 could interact with the signaling complex involving PDE4D5 and FAK in melanoma cells. To detect protein–protein interaction *in situ* we used a proximity ligation assay (PLA), in which a pair of oligonucleotide-labeled secondary antibodies (PLA probes) generates a signal only when they have bound in close proximity.

In highly invasive SkMel28 cells, we found that PDE4D co-localized with FAK and the scaffold protein RACK1, whereas, as a negative control, we showed that PDE4B did not (Figure 4a). PDE4D also interacted with the active phosphorylated FAK (pY576 and pY397; Figure 4b). In SkMel28 cells cultured on Matrigel the number of interactions between PDE4D and total or activated FAK was increased (Figure 4c). These results showed that PDE4D5 interacted with FAK in melanoma cells suggesting that the FAK–PDE4D interaction could be involved in the invasion process in melanoma.

To more precisely investigate the interactions in the PDE4D5/RACK1/FAK trimolecular complex and its role in promoting cell invasion, we generated an inhibitory peptide specifically designed to disrupt the interaction between PDE4D5 and RACK1 (PDE4D5-RACK1). We compared its action with a control peptide that disrupted the interaction of PDE4D5 with β-arrestin (PDE4D5-bAR), a scaffold protein that links to all PDE4 isoforms[30,32] (Figure 4d). Because peptides were FITC labeled, we could confirm peptide internalization by fluorescent imaging and studied PDE4D–FAK interaction by PLA in cells treated with each peptide.

In SkMel28 cells treated with the PDE4D5-RACK1 disrupting peptide, PDE4D–FAK interaction was abolished. The interactions of PDE4D with FAK were only detected in cells treated with the PDE4D5-β arrestin-disrupting peptide or in cells that failed to integrate the PDE4D5-RACK1 disrupting peptide (Figure 4d). These results confirmed that PDE4D5 co-localizes with FAK by interacting with RACK1. We tested the invasion ability of cells treated with the PDE4D5-bAR and PDE4D5-RACK1 peptides and showed that PDE4D5-RACK1 disrupting peptide significantly reduced invasion in SkMel28 cells as compared with control peptide ($P = 0.03$) (Figure 4e).

To conclude, these data demonstrate that PDE4D5 regulates tumor cell invasion through the activation of FAK in BRAF-mutated melanoma. FAK-mediated invasion requires its co-localization with PDE4D5, which is recruited in close proximity to FAK by the scaffold protein RACK1.

PDE4D expression increases in advanced human melanoma tumors

To determine if the role of PDE4D in invasion was significant for disease progression in patients, we set out to extrapolate the results we observed *ex vivo* in human melanoma samples. First we determined whether PDE4D co-localized with FAK in human melanomas as in cultured cell lines. We tested PDE4D–FAK interaction using PLA *in situ* in paraffin-embedded sections obtained from 9 metastatic human melanoma samples (Figure 5a). As observed in cultured cells, we found that PDE4D and FAK co-localized in melanoma tumors. Interestingly, this interaction could also be detected in melanoma non mutated on BRAF, suggesting that PDE4D might be involved in melanoma progression *in vivo* independently of BRAF.

Because invasion is a critical process involved in metastatic dissemination, we investigated whether PDE4D expression level increased in advanced stage melanomas (AJCC 2009 classification). We analyzed the expression of PDE4D in 43 human melanoma tumors (33 primary melanomas and 10 lymph node metastases) by quantitative RT–PCR. PDE4D mRNA was expressed at a very low level in primary melanomas at early stage (Breslow index < 1 mm), but at a higher level in advanced stage (Breslow index > 4 mm) and in lymph node metastases. PDE4D mRNA expression level was significantly increased in advanced tumor stage (Figure 5b). We showed that the expression of PDE4D5 mRNA isoform also increased in advanced stage melanomas confirming the importance of this isoform (Supplementary Figure 6).

To illustrate the results obtained from qPCR, six primary melanomas (Breslow index < 1 mm, $n = 3$ and Breslow index > 4 mm, $n = 3$) samples were stained for PDE4D using immunohistochemistry. PDE4D was detected in melanoma cells at the protein level, and was expressed more abundantly in advanced stages of melanoma (Figure 5c).

DISCUSSION

The purpose of this study was to decipher the role of PDE and the cAMP signaling pathway in BRAF-mutated melanoma, and led to the identification of key signal transduction events that contribute to increased melanoma invasion.

Although the cAMP pathway has a major role in melanocytes and melanoma, the role of PDE, the key regulators of the cAMP pathway, is just starting to be described. In RAS-mutated melanomas the oncogenic signal induced by RAS is transducted through CRAF instead of BRAF in the MAPK cascade, and PDE4 expression is essential for making the switch from BRAF to CRAF.[13,25] Moreover PDE4 inhibition with rolipram associated with a low dose of forskolin is sufficient to induce RAS-mutated melanoma cell line apoptosis, which could be a new potent therapeutic approach.[25] These promising results in melanoma with mutated RAS led us to investigate the role of PDE in BRAF-mutated melanoma, which represents around 50% of melanomas. First we showed that PDE4D is overexpressed in BRAF-mutated melanoma cell lines, constitutively disrupting the cAMP pathway activation by α-Msh. But contrary to what we described in RAS-mutated melanoma, cAMP reactivation through PDE4 inhibition was not associated with inhibition of the MAPK pathway, decreased proliferation or apoptosis induction. However, PDE4 inhibition induced a significant decrease in invasion. This biological effect was independent of MITF induction by the cAMP pathway but instead relied on the interaction between PDE4 and FAK. We demonstrated that cAMP reactivation, induced by PDE4 inhibition and low adenylyl cyclase activation, reduced FAK phosphorylation and suppressed tumor cell invasion. However, it had no durable effect on the level of CREB phosphorylation or the expression of MITF, the classical targets of the cAMP pathway in melanocytic cells. In addition, PDE4 inhibition with rolipram only, which did not stimulate CREB phosphorylation, was sufficient to reduce the level of FAK activation and cell invasion suggesting that the role of PDE4 in promoting melanoma cell invasion may be independent of a global downregulation of the cAMP signaling pathway. PDE4D isoform expression and activity are spatially and temporally

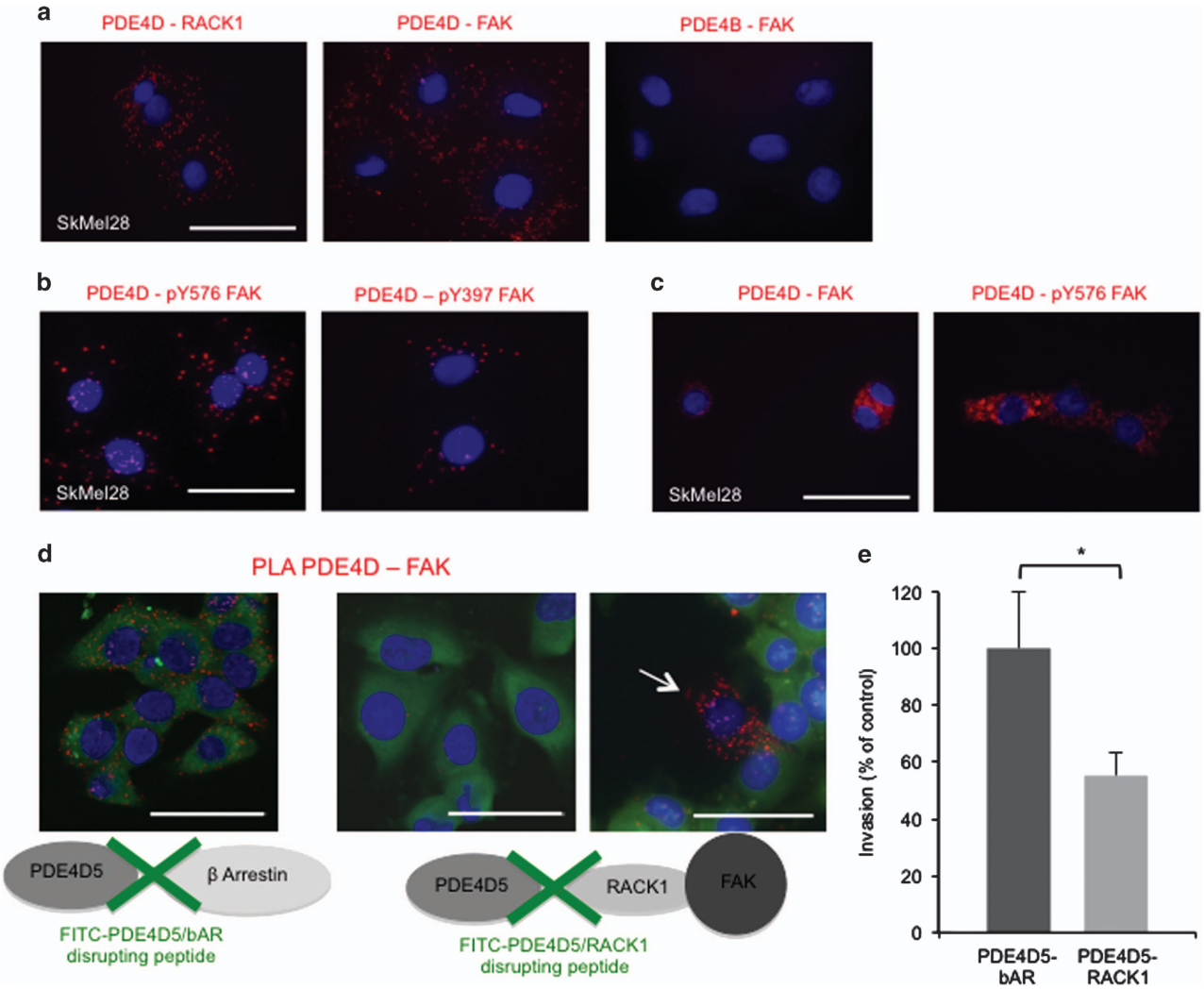

Figure 4. PDE4D5 interacts with RACK1 to promote FAK-mediated invasion in melanoma. (**a**) PDE4D interactions with RACK1 and FAK detected by *in situ* PLA in SkMel28 cultured on coverslips. PDE4B–FAK interaction was tested as a negative control. (**b**) PDE4D interactions with two phosphorylated forms of FAK (pY576 and pY397) in SkMel28 cultured on coverslips, detected by *in situ* PLA. (**c**) PDE4D interactions with phospho-FAK (pY576-FAK) or total FAK detected by *in situ* PLA in SkMel28 cultured on coverslips coated with Matrigel. (**d**) Schematic representation of disrupting peptides designed to study PDE4D–FAK interaction, and representative immunofluorescent staining of SkMel28 cells treated with each peptide for 6 h (10 μmol/l). Left, disrupting PDE4D5-β arrestin peptide (control peptide), and right, disrupting PDE4D5-RACK1 peptide. FITC-labeled peptides internalization was assessed (green). PDE4D–FAK interactions were analyzed using *in situ* PLA and confocal microscopy (red). DAPI-stained cell nuclei (blue). PDE4D–FAK interactions were only detectable in SkMel28 transfected with control peptide PDE4D-bAR (left) or in SkMel28, which failed to be transfected with peptide PDE4D5-RACK1 (right, white arrow). The interactions were visualized as fluorescent red dots. DAPI stained nuclei (blue). Scale bar, 50 μm. (**e**) Invasion assay of SkMel28 transfected with PDE4D5-β arrestin or PDE4D5-RACK1-disrupting peptide. Bars represent mean ± s.d. $*P < 0.05$ (unpaired *t*-test).

regulated, thus allowing subcellular compartmentalization of cAMP signaling.[27] PDE4D5, which is the main PDE4D isoform expressed in melanoma, is the only PDE4 isoform that can interact with FAK through the scaffolding protein RACK1.[31] Therefore, we hypothesized that this PDE4D5/RACK1/FAK complex could induce invasion in melanoma. Indeed, we showed that peptide disrupting PDE4D5/RACK1 interaction, and therefore PDE4D5/FAK interaction, inhibited invasion, demonstrating that PDE4D5 regulated invasion through FAK. We showed here that FAK interaction with RACK1 is not sufficient to promote invasion in melanoma but that it requires interaction with PDE4D5. cAMP signaling responses are spatially and temporally regulated by a balance between adenylyl cyclases and PDE. By controlling cAMP gradients, the different isoforms of PDE regulate distinct sets of intracellular processes depending on cellular type and subcellular compartmentalization.[27] Although the PDE4D5–RACK1 interaction does not affect PDE4D5 activity,[31] the control of cAMP level in the vicinity of the PE4D5/RACK1/FAK complex seems essential to regulate FAK activity.

Although in RAS-mutated melanoma the disruption of the cAMP pathway is a necessary event to allow proliferation of tumor cells, its inactivation does not seem critical for the proliferation of BRAF-mutated melanoma cells. However, previous studies suggest that the cAMP pathway has an opposite role in the resistance to BRAF inhibitors in BRAF-mutated melanoma. cAMP signaling-dependent components such as PKA, adenylyl cyclase and G protein-coupled receptor have been associated with resistance to BRAF inhibitors,[33] whereas high cAMP level has been shown to be a marker of sensitivity to BRAF inhibitor[34] and to decrease cell proliferation.[12] Although we showed that PDE4D5 signals through FAK to promote invasion in BRAF-mutated melanoma cells, we could not detect any signaling mechanism coupling mutated

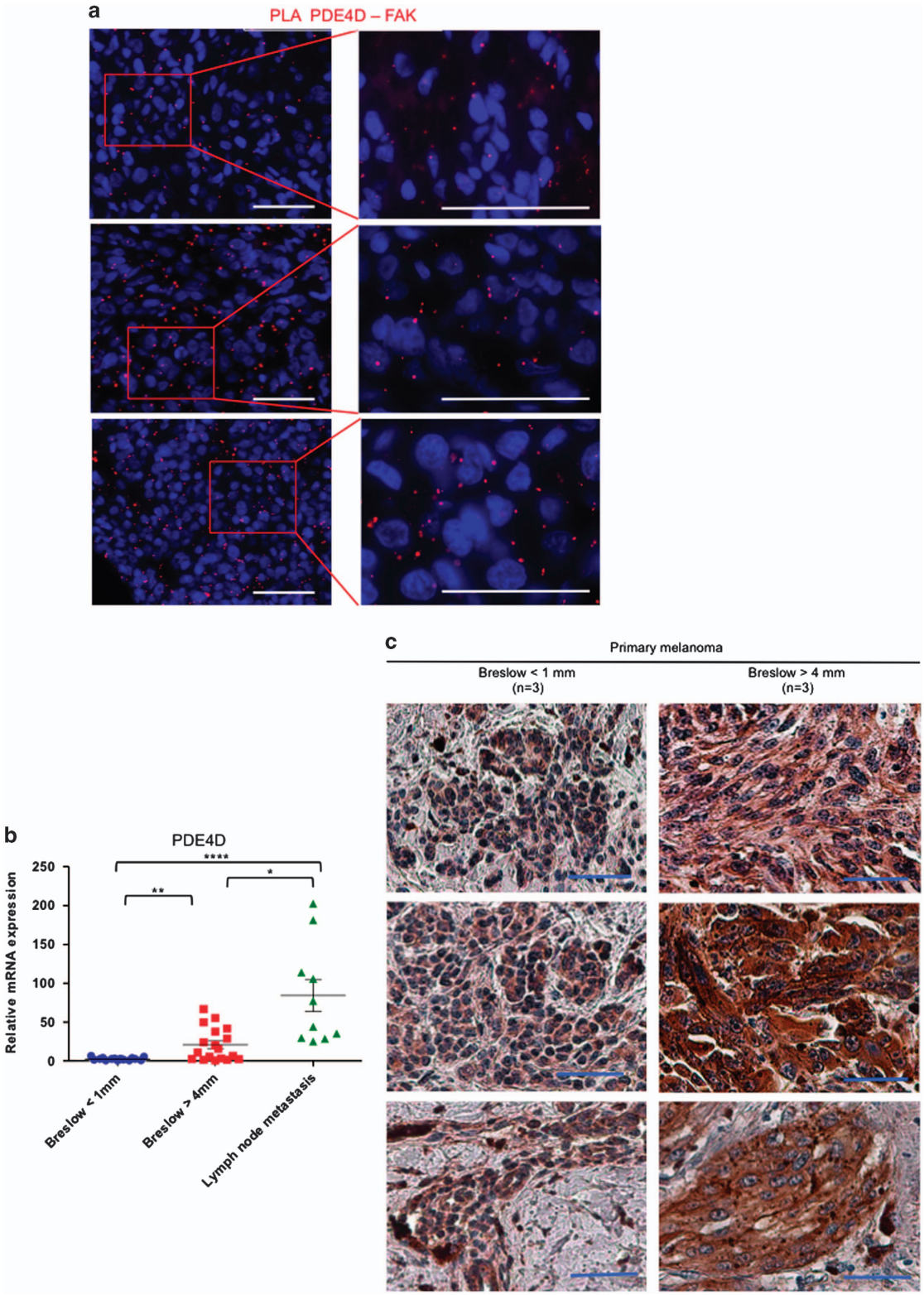

Figure 5. PDE4D is associated with melanoma progression in human melanoma tumors. (**a**) Representative pictures of PDE4D–FAK interactions assessed by *in situ* PLA (red dots) in paraffin sections of 3 out of 9 metastatic human melanoma samples. DAPI-stained cell nuclei (blue). Scale bar, 50 μm. (**b**) PDE4D mRNA expression assessed by qPCR in 43 human melanoma tumors. PDE4D transcript level was normalized to *PPIA* gene expression. Melanoma samples represent three groups of invasion: Breslow index <1 mm ($n=15$), Breslow index >4 mm ($n=18$), lymph node metastases ($n=10$). $^{*}P<0.05$; $^{**}P<0.01$; $^{****}P<0.0001$ (Mann–Whitney test performed among the groups in pairs). Bars represent mean ± s.d. (**c**) Representative images of PDE4D expression assessed by immunohistochemical staining in paraffin sections of six primary human melanoma tumors (Breslow index <1 mm, $n=3$ and Breslow index >4 mm, $n=3$). Scale bar, 50 μm.

BRAF to PDE4D5 expression. Previous studies have implicated oncogenic BRAF in melanoma metastasis through transcription regulation of partners involved in invasion.[35,36] But PDE4D does not seem to be one of these partners, as BRAF inhibition did not alter PDE4D expression (data not shown). This is in agreement with the fact that BRAF mutation is a very early event, whereas PDE4D is expressed in later stage melanoma.

The major health threat arising from malignant melanoma is death from metastatic disease. Therefore targeting key signal transduction events supporting metastasis could result in a more favorable clinical outcome. Invasion, migration and vasculogenic mimicry are all characteristics of an aggressive melanoma phenotype.[29] Focal adhesion kinase is at the intersection of numerous signaling pathways promoting tumor cell invasion and metastases (reviewed in Sulzmaier *et al*.[28]). In melanoma, FAK is phosphorylated on its key tyrosine residues, Tyr397 and Tyr576, in only the most aggressive melanoma cells, which correlates with an increase in invasive behavior.[29] In our study we report that PDE4D5 forms a trimolecular complex with FAK involving the scaffold protein RACK1 that promotes phosphorylation of FAK and melanoma cell invasion. We also observed that the PDE4D–FAK interaction is detectable in human melanoma samples, suggesting that regulating invasion may also be a relevant role for PDE4D *in vivo*. Interestingly, PDE4D expression and PDE4D–FAK interaction were detected *in situ* regardless of BRAF mutation status suggesting that PDE4D-FAK-mediated cell invasion may be a general mechanism in all melanomas. Because inhibition of PDE4D is sufficient to reduce the ability of cells to invade, inhibiting PDE4D could be an alternative strategy to FAK inhibition in treating aggressive melanomas or preventing emergence of melanoma clones with enhanced metastatic capabilities.

The targeted inhibition of PDE4 is a current topic of exploration in cancer[21,22] and PDE4D is overexpressed in numerous types of cancers including melanoma, and ovarian, endometrial, prostatic and gastric cancers.[17,37] Lin *et al*.[17] reported that PDE4D protein level is elevated in tumors compared with adjacent normal tissues, and high PDE4D mRNA expression is correlated to low survival rate in endometrium cancer and head and neck squamous cell carcinomas tumors. In our study we observed that high PDE4D mRNA expression was associated with advanced disease stage, and we confirmed this observation at the protein level in primary human melanomas. One of the challenges in melanoma is the identification of primary melanomas that may give rise to metastases. On the basis of our preliminary results, PDE4D appears a reliable marker for advanced melanoma. But whether PDE4D expression could be used as a predictive marker of metastatic dissemination remains to be confirmed in further studies.

In conclusion, we have found evidence that PDE4D5 is overexpressed in advanced melanoma and that it promotes melanoma cell invasion in BRAF-mutated melanoma. This work helps our understanding of the role of PDE4 in melanoma, its potential as a target for the prevention of metastatic dissemination and its potential as a predictive marker for invasive tumor.

MATERIALS AND METHODS

Cell lines and reagents

Human melanoma cell lines A375, Colo829 SkMel5, SkMel28, WM266.4 and melanocytes NHEM were previously described.[38]

A375, SkMel5 and WM266.4 were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS), Colo829 and SkMel28 in RPMI (Invitrogen) containing 10% FCS and NHEM in medium 154 supplemented with Human melanocyte Growth Supplement (Invitrogen).

Inhibitors were from Selleck Chemicals.

For PDE4D silencing, melanoma cells were transfected with PDE4D siRNA (5′-UGUACAUCAAGGCAAGUUCTT-3′) or scrambled siRNA (5′-ACCGU CGAUUUCACCCGGGTT-3′) as control using lipofectamine-RNAi MAX (Invitrogen), and incubated for 48 h before analysis.

For stable PDE4D isoform expression, PDE4D3 and PDE4D5 complimentary DNA were cloned in the pEF6 vector (Invitrogen); A375 cells were transfected with JetPEI according to the manufacturer's instructions and selected with blasticidin (10 μg/ml; Gibco, Carsbad, CA, USA).

Disrupting peptides were obtained from Proteogenix (Schiltigheim, France). The peptide sequences were HPLWETWADLVHPDAQDILDTLEDN+K (PDE4D5-RACK1) and APDDPEEGRQGQTEKFQFELTLEED+K (PDE4D5-bAR).

Proliferation assay

The cells were plated in a 96-well plate and allowed to adhere for 24 h, and were then cultured in the presence of inhibitors or DMSO for 72 h. MTS assays were performed using a CellTiter 96 Aqueous One Solution cell proliferation assay (Promega, Madison, WI, USA).

Western blotting analyses

Melanoma cells were lysed in RIPA buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na3VO4) supplemented with proteinase inhibitor cocktail. Whole-cell lysates were resolved by SDS–polyacrylamide gel electrophoresis and transferred on nitrocellulose membranes. Membranes were probed with following primary antibodies (obtained from Cell Signaling Technology (Danvers, MA, USA) and used at 1:1000 dilution unless otherwise mentioned): phospho-CREB (pY733), CREB, phospho-ERK1/2 (1/5000, Sigma-Aldrich, St Louis, MO, USA), ERK1/2 (1/5000, Merck Millipore, Billerica, MA, USA), MITF (Acris Antibodies, Herford, Germany), PDE4A (Abcam, Cambridge, UK), PDE4B (Abcam), PDE4D (Abcam), phospho-FAK (pY576 or pY397), FAK and β-actin (1:2000, Acris Antibodies). Labeling was visualized using enhanced chemoluminescence kit (Thermo Fisher Scientific, Lafayette, CO, USA) on an ImageQuant Imaging system and quantified using Image J software (NIH, Bethesda, MD, USA).

RT–PCR analysis

From cultured cells, total RNA was extracted using Nucleospin RNA kit (MACHEREY-NAGEL GmbH & Co., KG Düren, Germany). Reverse transcription was performed with ThermoScript RT–PCR System (Thermo Fisher Scientific). Primers were specifically designed for each transcript using Primer Express 3.0 software (DeNovo Software, Glendale, CA, USA). Transcript levels were measured by qRT–PCR using SYBR green master on a 7300 RT–PCR system (Applied Biosystems, Foster City, CA, USA).

RNA from paraffin-embedded (FFPE) tissue sections was extracted using RNeasy FFPE extraction kit (Qiagen, Redwood City, CA, USA) after xylene treatment according to the manufacturer's protocol. RNA quantity and quality was assessed using the Nanodrop-ND-1000 (Nanodrop Technologies, Wilmington, DE, USA). First-strand complimentary DNA was synthesized using a High-Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer's protocol. Transcript levels were measured by qRT–PCR using Perfect Master Mix-Probe (AnyGenes, Paris, France) on LightCycler-480 (Roche, Indianapolis, IN, USA).

Transcript levels were normalized to the housekeeping β-actin, GAPDH or PPIA (peptidylprolyl isomerase A) transcripts.

Matrigel invasion assays

Invasion assays were performed using a modified Boyden chamber on 8 mm pore filters coated with Matrigel. Cells were seeded on Matrigel in FBS-free medium (and inhibitors or DMSO if required) and allowed to invade for 24 h. Invaded cells were fixed with paraformaldehyde 4% and stained with crystal violet 0.5%. Pictures were taken under a light microscope and cells were counted using Image J software (NIH).

Proximity ligation assay

Cells grown on 12-well culture slides and immediately fixed were subjected to *in situ* PLA using the Duolink Detection kit (Olink Bioscience, Uppsala, Sweden) according to the manufacturer's instructions. Slides were blocked, incubated with antibodies directed against PDE4D (Abcam) and FAK, pY397- or pY576-FAK (Cell Signaling Technology) and thereafter incubated with PLA probes, which are secondary antibodies (anti-rabbit and anti-goat) conjugated to oligonucleotides. Circularization and ligation of the oligonucleotides was followed by an amplification step.

The products were detected by a complementary fluorescently labeled probe. Protein complexes were visualized with an Axiovert fluorescent microscope and a confocal microscope.

Human melanoma samples and immunohistochemistry

From 2007 to 2010, paraffin-embedded (FFPE) tissue specimens were collected from the Dermatology Department of Saint Louis Hospital, Paris, France for 43 patients with primary melanoma ($n = 33$) and lymph node metastases ($n = 10$). All patients gave informed written consent. The research was approved by the Ethics Committee of Saint Louis Hospital.

Five 10 µm sections were extracted for RNA analysis (see above, RT–PCR analysis section).

Six primary melanoma samples were stained for PDE4D using immunohistochemistry. Five micrometer-thick sections were dewaxed in xylene and rehydrated through decreasing concentrations of alcohol. Antigen retrieval was carried out in 10 mM citrate sodium buffer (pH 6) for 15 min at 95 °C in a bain marie. Slides were blocked with 2.5% normal horse serum, incubated with anti-PDE4D antibody (1:200, Abcam) overnight at 4 °C, then incubated with biotinylated secondary antibody followed by incubation with peroxidase–streptavidin complex (Universal Quick Kit RTU, Vector Labs., Burlingame, CA, USA). Color development was performed with DAB (Vector Labs).

Statistics

Data are presented as the mean values ± s.d. Unpaired *t*-test or Mann–Whitney test were used to evaluate differences between the two groups. All *P*-values reported were two-sided. Analyses were performed using Prism 6 (GraphPad Software Inc., La Jolla, CA, USA).

CONFLICT OF INTEREST

CL: scientific advisory board for Roche and Novartis; AB: co-founder of OREGABiotech, Ecully, France; The remaining authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

JD, AS, FL, JA and ND conceived, designed, performed experiments and analysed the data. NO, MB, SM and CL contributed reagents and materials, and performed data analysis. AB and CL provided technical advice and expertise. JD and ND wrote the manuscript. CL and ND supervised the project.

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