FAK competes for Src to promote migration against invasion in melanoma cells

K Kolli-Bouhafs1,2, E Sick1,2, F Noulet1,2, J-P Gies1,2, J De Mey1,2 and P Ronde1,2*

Melanoma is one of the most deadly cancers because of its high propensity to metastasis, a process that requires migration and invasion of tumor cells driven by the regulated formation of adhesives structures like focal adhesions (FAs) and invasive structures like invadopodia. FAK, the major kinase of FAs, has been implicated in many cellular processes, including migration and invasion. In this study, we investigated the role of FAK in the regulation of invasion. We report that suppression of FAK in B16F10 melanoma cells led to increased invadopodia formation and invasion through Matrigel, but impaired migration. These effects are rescued by FAK WT but not by FAKY397F reexpression. Invadopodia formation requires local Src activation downstream of FAK and in a FAK phosphorylation-dependant manner. FAK deletion correlates with increased phosphorylation of Tks-5 (tyrosine kinase substrate with five SH3 domain) and reactive oxygen species production. In conclusion, our data show that FAK is able to mediate opposite effects on cell migration and invasion. Accordingly, beneficial effects of FAK inhibition are context dependent and may depend on the cell response to environmental cues and/or on the primary or secondary changes that melanoma experienced through the invasion cycle.

Cell Death and Disease (2014) 5, e1379; doi:10.1038/cddis.2014.329; published online 14 August 2014

Patients with spreading melanoma diseases have a very poor prognosis with a 5-year survival rate <5%. The metastatic spread of melanoma is a complex process involving several genetic alterations. In melanoma, as in many highly invasive cancer cell types like head and neck squamous cell carcinoma5 or breast carcinoma,6 specialized matrix-degrading organelles termed invadopodia have been identified. Invadopodia consist of dynamic actin-based protrusions of 0, 1 to 2 µm in diameter emanating from the ventral edge of tumor cells.4 Besides their actin scaffold, these structures are enriched in proteolytic enzymes such as matrix metalloproteinases (MMPs), which mediate extracellular matrix (ECM) degradation. Indeed, MMP are upregulated in invasive melanoma and there is extensive evidence that they have a role in promoting the dissemination of melanoma.6–7 Several proteins like integrins, Src and paxillin, found at sites of cell adhesion to the matrix, are also present in invadopodia.3,9 On the other hand, other proteins like the Src substrate proteins cortactin10 and the tyrosine kinase substrate with five SH3 domain (Tks-5)11 are specifically localized at invadopodia and not found at focal adhesion (FA). In addition, reactive oxygen species (ROS)12 have been localized at invadopodia and are supposed to have a prominent role in inducing invadopodia function.13,14 Although significant efforts have been made to characterize components of invadopodia, the precise mechanisms of their regulation, especially in a melanoma context, remain poorly understood.

Tumor invasion is a multistep process that requires cell adhesion to the environing substratum, migration and invasion. In many cell types, migration requires fine control of FA turn-over. FAs are formed by the cluster of up to 200 proteins15 ensuring cell anchorage to the ECM. The cyclic process of FA formation and disruption is crucial for cell migration. Because both anchorage and migration involve cellular interactions with ECM components, FAs are endowed with transmembrane ECM receptor proteins such as integrins that interact with ECM molecules and intra-cellular proteins composed of scaffold proteins, as well as signal-transducing molecules. Among those, focal adhesion kinase (FAK) is a crucial signaling protein that integrates signals from integrins to the actin filaments during cell migration.16 Structurally, FAK is a 125-kDa protein that contains an N-terminal 4.1-ezrin–radixin–moesin domain, a central kinase domain and a C-terminal domain that contains the focal adhesion targeting site.17 The phosphorylation of FAK at Y397 creates a binding site for Src, which can phosphorylate other tyrosines on the FAK sequence, thus creating new binding sites for SH2 domain-containing proteins.

FAK is involved in many aspects of the metastatic process and thus, overexpression, hyperphosphorylation and/or elevated activity of FAK have been reported in a variety of human cancers, including sarcomas and carcinomas of the breast, colon, thyroid, prostate, oral cavity, liver, stomach and ovary.18 In human melanoma cell lines, early studies reported high FAK expression and requirement of FAK for cell substrate adhesion.19 Later, it was reported that FAK promotes the aggressive melanoma phenotype.20 Indeed, immunohistochemical analyses revealed high levels of FAK

1CNRS, UMR 7213, Laboratoire de Biophotonique et Pharmacologie, Illkirch, France and 2Université de Strasbourg, Faculté de Pharmacie, Illkirch, France
*Corresponding author: P Ronde, CNRS, UMR 7213, Faculté de Pharmacie, Université de Strasbourg, 74 rde du Rhin, 67401 Illkirch, France. Tel: + 33 368 85 41 84; Fax: + 33 368 85 43 13; E-mail: philippe.ronde@unistra.fr

Abbreviations: MMP, matrix metalloproteinases; FA, focal adhesion; FAK, focal adhesion kinase; ECM, extracellular matrix; Tks-5, tyrosine kinase substrate with five SH3 domain; mda-9, melanoma differentiation-associated gene-9; PKCγ2, protein kinase Cγ2; ROS, reactive oxygen species; DAPI, 4',6-diamidino-2-phenylindole; FRAP, fluorescence recovery after photobleaching; TIRF, total internal reflection fluorescence

Received 08.2.14; revised 16.6.14; accepted 01.7.14; Edited by Z-X Xiao
phosphorylation at Tyr397 and Tyr576, a marker of FAK kinase activity, in late-stage cutaneous and uveal melanoma, which correlated with their increased invasion and migration properties.\textsuperscript{21} Furthermore, melanoma differentiation-associated gene-9 (mda-9)/syntenin was also reported to mediate adhesion-dependant activation of protein kinase Cz (PKCz) and FAK in melanoma cells. Thus, inhibiting either mda-9/syntenin or PKCz suppressed fibronectin-induced formation of integrin-\beta1/FAK/c-Src signaling complexes and reduced migration and invasion toward fibronectin.\textsuperscript{22} Therefore, FAK appears to be a major player of melanoma invasion, but how this kinase controls the formation and proteolytic activity of invadopodia in melanoma cells was never investigated.

In this study, we uncovered a surprising negative regulation of invadopodia activity in B16F10 cells by FAK. The depletion of FAK was associated with increased ROS production and Tks-5 phosphorylation. Using mutation of FAK at Tyr397, a binding site for Src, we found that these sites are implicated in FAK-mediated inhibition of invadopodia activity. In addition, we report that this mutation induced decreased migration speed but increased invasive properties. Taken together, our data suggest a competition between FA and invadopodia substrates for Src phosphorylation that might depend on environmental cues, thus leading to the engagement of either migration or degradation pathways.

Results

FAK deletion is associated with increased proteolytic activity of invadopodia. FAK has long been known to promote migration and invasion of many cellular cancer types, among which is the murine melanoma B16F10 cell line.\textsuperscript{23} However, whether and how FAK affects invadopodia activity especially in the melanoma context remains poorly documented. To begin investigating the role of FAK in melanoma B16F10 invasion, we compared the ability of invadopodia to degrade the ECM in control and FAK-deficient cells. For this purpose, we used siRNA directed against the 5′-UTR region of FAK to deplete cells from endogenous FAK. Western blot analysis of FAK expression revealed that knockdown efficiency reached 83.33 and 84.24% after 48 and 72 h of siRNA transfection, respectively (Figure 1a). Invadopodia activity was quantified by their ability to degrade the ECM using Alexa-405 gelatin as a substrate. As shown in Figure 1b, B16F10 cells can spontaneously form invadopodia after 16 h of culture on fluorescent gelatin. Surprisingly, quantification of fluorescent gelatin degradation revealed that matrix degradation was 14 times higher in FAK-deficient cells as compared with control cells (Figure 1b).

Ssrc is necessary for the degradation activity of invadopodia downstream of FAK. To investigate whether Src, a binding partner of FAK, is implicated in the process of matrix degradation, control and FAK-deficient B16F10 cells were treated with PP2, an inhibitor of Src kinase. We find that PP2 treatment completely abolished active invadopodia in control (data not shown) and FAK-deficient B16F10 cells (Figure 2a), thus establishing the essential role of Src in the control of matrix degradation. It should also be noted that, although Src inhibition blocked invadopodia activity, further FAK depletion was unable to restore matrix degradation, thus suggesting that Src acts downstream of FAK in the control of invadopodia activity. The question then arises as to whether the increased matrix degradation observed in FAK-deficient B16F10 cells could be due to an increase in Src activity. For this purpose, western blot analysis using a phosphospecific Ab directed against Src-Tyr416, which constitutes a marker of Src activation,\textsuperscript{12,24} was performed. In an unexpected way, no variation in Src phosphorylation at Tyr416 was observed compared with control (Figure 2b). This result suggests that FAK does not alter Src activity in melanoma cells. Therefore, as Src is involved in invadopodia activity, FAK may affect downstream substrates of the Src kinase implicated in invadopodia formation and/or the turnover of Src at specific cellular locations.

Next we evaluated whether the phosphorylation state of relevant Src substrates has been altered in FAK-deficient cells. At invadopodia, several Src substrates are phosphorylated in a way critical for their functional activity, including cortactin\textsuperscript{25} and Tks-5.\textsuperscript{26} Tks-5 appears localized at invadopodia in cancer cells and has a prominent role in their formation.\textsuperscript{27} By western blotting, we show that Tks-5 phosphorylation was increased in FAK-deficient cells as compared with control (Figure 3a). Co-immunoprecipitation experiments confirm the global increase of tyrosine phosphorylation of Tks-5 but not cortactin in FAK-deficient B16F10 cells. Moreover, overexpression of active Src or active Src-mCherry greatly enhances Tks-5 phosphorylation level as
revealed by an immunoprecipitation experiment using a Tks-5 antibody (Figure 3b, left; Supplementary Figure 1). This suggests that the formation of the FAK–Src complex hinders the phosphorylation of Tks-5, although once the FAK–Src complex is disrupted due to FAK depletion, Src acquires the ability to phosphorylate Tks-5. To investigate whether Src is indeed a limiting factor in the phosphorylation of Tks-5, B16F10 cells were transfected with the activated form of Src, SrcY530F. As shown in Figure 3b, expression of the activated form of Src coupled to mCherry did not change the basal level of Tks-5 in both control and FAK-deficient cells. The Graph shows no increase in Src activity after FAK depletion which being invadopodia as revealed by actin/cortactin staining (Figure 4b, top). On the contrary, in FAKsi-treated cells, active Src is no longer localized at FAs (Figure 4a, bottom) but displays increased localization at invadopodia (Figure 4b, bottom). Together with the established role of Src in Tks-5 phosphorylation previously demonstrated, this is consistent with a role of FAK in controlling the localization of active Src, thus leading to the differential regulation of FA and invadopodia activities.

**ROS are necessary for the degradative activity of invadopodia.** Recently, a correlation has been established between ROS production, Tks-5 expression and the formation of invadopodia. As we observed an increase in the phosphorylation state of Tks-5 in our cells, we measured the level of ROS in control and FAK-deficient B16F10 cells. As expected, we found a 38% increase in ROS production on FAK depletion (Figure 5a). Moreover, cells' treatment with the ROS inhibitor MnTMpyP blocked the degradative activity.
of invadopodia in both control and FAK-deficient B16F10 cells, thus confirming the essential role of ROS production in the control of invadopodia activity (Figure 5b). These results suggest that FAK, via its interaction with Src, downregulates Tks-5 phosphorylation, thus altering ROS production.

**FAK phosphorylation at Tyr397 negatively regulates invadopodia activity.** Next we investigated which molecular determinants are responsible for FAK-mediated decreased invadopodia activity in B16F10 cells. For this purpose, we first explored the role of the FAK-Tyr397 phosphorylation site, as this site has been previously shown to be a binding site for the SH2 domain of Src. Thus, B16F10 cells expressing FAK WT or FAK-Y397F coupled to GFP derivatives were generated and transfected with siRNA directed against the 5'-UTR region of FAK. As this region is exclusively found in endogenous FAK but not in either FAK WT or FAK-Y397F constructs, targeting the 5'-UTR region allows an almost complete extinction of endogenous FAK without affecting the expression of exogenous FAK forms as demonstrated in Figure 6a. The subcellular localization of the FAK forms revealed that, although both FAK-GFP and FAK-Y397F-GFP were correctly addressed at FAs, none of the constructs were found at invadopodia as demonstrated by the lack of colocalization of FAK with actin and cortactin (Figure 6b) known to be co-enriched at invadopodia.
FAK regulates invadopodia activity in melanoma cells
K Kolli-Bouhafs et al

To examine the effect of FAK and FAK mutant reexpression in a FAK-null context, we quantified the number of invadopodia in each context. Our results show that cells reexpressing FAK WT but not FAK-Y397F displayed significantly less invadopodia as compared with FAK-deficient cells, thus suggesting that FAK phosphorylation at Tyr397 in FAs is necessary for FAK-mediated inhibition of invadopodia formation. As FAK promotes a reduction in the number of invadopodia displayed per cells, we analyzed the degradative activity of FAK-null cells after reexpression of FAK WT or FAK-Y397F. As shown in Figure 7a, the expression of FAK-Y397F but not FAK WT increased the degradative activity of invadopodia reaching 24.10 μm²/cell, close to the value obtained with FAK depletion (24.90 μm²/cell). This shows that the loss of phosphorylation at Tyr397 has a similar effect on the degradative activity of invadopodia as FAK depletion, pinpointing that the phosphorylation at Tyr397 is necessary for FAK to negatively regulate invadopodia function. Moreover, this FAK mutant also triggered an increase in Tks-5 phosphorylation (Figure 7b) and ROS production (Figure 7c), suggesting that the disruption of FAK–Src interaction had enabled Src to promote invadopodia activity by phosphorylating Tks-5 and subsequent ROS production.

FAK deletion reduces cell migration but increases cell invasion. We next investigated whether FAK deletion impaired cell migration. The migration properties of control and FAK siRNA-treated cells reexpressing wild-type FAK or FAK-Y397F cells were evaluated in the Boyden chamber assay with 10% FBS used as a chemoattractant. This showed that the migration speed of FAK siRNA-treated cells was decreased by 58.4% as compared with non-treated cells. This effect was abolished by reexpression of wild-type FAK but not that of FAK-Y397F (Figure 8a). Thus, as already demonstrated in various other cancer and non-cancer cell lines, FAK expression is necessary for melanoma cell migration. As FAK deletion increases invadopodia activity but decreases cell migration, the invasive properties of B16F10 melanoma cells were evaluated in invasion assays in which the cells degrade and migrate through Matrigel. To evaluate the invasive properties, without accounting for the intrinsic migration properties, we calculated the invasion index by dividing the percentage of cells invading through Matrigel-coated inserts with the percentage of cells migrating through non-coated insert. This showed that FAK-deficient cells were significantly more invasive than control cells. This effect was abolished by reexpression of wild-type FAK but not that of FAK-Y397F (Figure 8b).

FAK is phosphorylated and highly dynamic at FAs. As FAK is not localized at invadopodia but phosphorylated FAK alters invadopodia activity, we analyzed the localization of FAK phosphorylated at Tyr397 in B16F10 cells. Using a phosphospecific Ab directed against FAK-Tyr397, we found that FAK is mainly phosphorylated at FAs with no specific staining at invadopodia. Therefore, FAK residency time at FAs will determine its phosphorylation state and thus the degree of Src accumulation at FAs. To analyze the dynamics of FAK at FA, the mobility of FAK was assessed by fluorescence recovery after photobleaching (FRAP) experiments. To this end, the GFP moiety contained in the FAK fusion protein present in FAs randomly selected at the periphery of the cells was photobleached with short high-power 491-nm excitation using a Cobolt Calypso Laser, and the recovery of fluorescence in the bleached regions was followed by time-lapse imaging over the ensuing 100 s (Figure 9). Because adhesions are localized at thin cell edges near the interface with the glass, high signal/noise ratio could be achieved using total internal reflection fluorescence (TIRF) microscopy for time-lapse imaging. Using this method, we found unexpectedly fast dynamics of FAK at FAs, yielding a t½ of 7.53 ± 1.7 s together with a high mobile fraction of 92.03%.
Discussion

Despite recent progress in our knowledge of the molecular mechanisms implicated in melanoma dissemination, metastatic melanoma still accounts for more than 80% of deaths by skin cancer. This is mainly due to its high plasticity and high resistance to existing therapies. Therefore, identification of new therapeutic targets is urgently needed. Today, invadopodia are thought to promote the invasive properties of cancer cells by clustering proteases involved in ECM degradation. Recently, these structures have been identified in many metastatic melanoma from both human and mouse origin. Although significant advances have been made in understanding the role of FAK in migration processes, its role in the regulation of invadopodia dynamics and activity remains poorly documented. Nevertheless, as FAK is at the crossroad of FA and actin signaling, it is generally thought to have an important role in the regulation of invadopodia. Here we found that FAK deletion led to an unexpected upregulation of invadopodia activity together with a clear increase in the invasion index in melanoma cells. Moreover, although we show that Src is absolutely required for invadopodia activity, as already reported, FAK has no effect on the activation state of Src in B16F10 cells. Further investigation of potential molecules involved in the control of invadopodia activity revealed that both Tks-5 phosphorylation and ROS production were increased on FAK deletion, suggesting that they may be part of a common signaling pathway in the control of melanoma invasion. This is in agreement with previous result showing that Tks-5 is expressed in human cancer tissues and particularly in breast cancers and melanomas. Moreover, although we show that Src is absolutely required for invadopodia activity, as already reported, FAK has no effect on the activation state of Src in B16F10 cells. Further investigation of potential molecules involved in the control of invadopodia activity revealed that both Tks-5 phosphorylation and ROS production were increased on FAK deletion, suggesting that they may be part of a common signaling pathway in the control of melanoma invasion. This is in agreement with previous result showing that Tks-5 is expressed in human cancer tissues and particularly in breast cancers and melanomas. Moreover, although we show that Src is absolutely required for invadopodia activity, as already reported, FAK has no effect on the activation state of Src in B16F10 cells. Further investigation of potential molecules involved in the control of invadopodia activity revealed that both Tks-5 phosphorylation and ROS production were increased on FAK deletion, suggesting that they may be part of a common signaling pathway in the control of melanoma invasion. This is in agreement with previous result showing that Tks-5 is expressed in human cancer tissues and particularly in breast cancers and melanomas.

Figure 6 FAK mutation at Tyr397 increases invadopodia formation. (a) B16F10 cells were transiently transfected with either control siRNA or FAK siRNA and FAK-GFP or FAK Y397F-GFP. Representative western blots showing FAK expression in the different conditions. Note the reexpression of FAK in siRNA-treated cells transfected with FAK WT or FAK Y397F. (b) Confocal images of fixed FAK siRNA-treated cells expressing wild-type or mutant FAK-GFP (middle right panel) and immunostained for actin (left panel) or cortactin (middle left panel). Note the localization of FAK at FAs (arrowheads) and the absence of FAK at invadopodia (arrows) identified by actin/cortactin staining in cortical dot-like structures. Scale bars: 5 μm. (c) Analysis of the number of invadopodia per cell in the different conditions above. Invadopodia segmented using both actin and cortactin staining appear in yellow on the FAK image (right). Quantification reveals a significant reduction in the number of invadopodia in FAK siRNA-treated cells transfected with FAK WT but not FAK Y397F (*P < 0.02, n = 5 with 65–170 invadopodia analyzed per conditions).

Figure 7 FAK mutation on Tyr397 increases invadopodia activity, ROS production and Tks-5 phosphorylation. (a) B16F10 cells transiently transfected with FAK siRNA and FAK WT or FAK Y397F were cultured on Alexa fluor 405 gelatin coverslips (gray) and labeled for actin (red) and cortactin (cyan). Scale bars: 20 μm. Quantification of degradation areas shows increased invadopodia activity in cells depleted for FAK, which is overcome by FAK WT but not FAK Y397F reexpression (n = 3, ***P < 0.0001). (b) Extracts from B16F10 cells transiently transfected with FAK siRNA and FAK Y397F were analyzed by immunoblotting and probed for Tks-5 and phosphoryrosines. Note the increase in Tks-5 phosphorylation on FAK deletion (n = 3, ***P < 0.0001). (c) ROS production was quantified in B16F10 cells transiently transfected with FAK siRNA and FAK Y397F using the CM-H2DCF-DA method. Results show an increase in ROS production in cells deficient for FAK, which is not compensated by reexpression of mutant FAK (n = 3, ***P < 0.0001).
increased Tks-5 phosphorylation in WT cells but had no additional effect in a FAK-null context, suggesting that in the latter condition, Tks-5 already reached a maximum level of phosphorylation. Taken together, our study suggests that Src promotes Tks-5/ROS-dependent invadopodia activity downstream of FAK in melanoma cells. This raised the question of how FAK may regulate Src activity. To answer it, we first analyzed the cellular localization of active Src in B16F10 cells. We found that active Src is located at FAs and invadopodia in control cells, whereas in FAK-deficient cells, active Src was almost completely absent at FAs while displaying increased localization at invadopodia. We next analyzed the cellular localization of FAK and found that FAK is located at FAs but not at invadopodia in B16F10 cells. We also show that, in the absence of FAK, melanoma cells display more numerous invadopodia. Conflicting results have been described regarding the cellular localization of FAK at invadopodia. In v-Src-transformed cells, it has been shown that Src enhanced the interaction between β1 integrin and FAK at invadopodia.35 Similarly, FAK was also detected in invadopodia from MCF10A-CA1 breast cancer cells where it alters the dynamics of these structures.60 On the other hand, in the human breast cancer cells, MDA-MB-231 and MTLn3, which display both FA and invadopodia, FAK is not present at invadopodia as well as in rat mammary adenocarcinoma cells where it alters the dynamics of these structures.36,41 It is interesting to note that one recent study has mentioned FAK to be dispensable for dot-shaped podosomes formation but essential for the assembly of rosette-shaped structures in Src-transformed fibroblasts and human lung adenocarcinoma.42 Finally, in some cancer cell lines, like the HT-1080 fibrosarcoma or the PAN-1 pancreatic cancer cells, FAK has been shown to target MT-MMP1 at FA to mediate matrix degradation.43 Therefore, the role of FAK in invasion may depend on the invasive structures being formed by cells originating from different cancer types. Nevertheless, in agreement with previous study,41 we found that mutation of FAK at Tyr397, a tyrosine residue that on phosphorylation is a binding site for Src, increases invadopodia formation and activity in a manner similar to FAK knockdown. This mutated form of FAK also activates Src downstream effectors, Tks-5 and NoxA, as demonstrated by the increase in Tks-5 phosphorylation and ROS production. Moreover, we show for the first time that FAK deletion in melanoma cells, although decreasing migration, increases the invasion index through Matrigel. These effects were also mimicked by reexpression of FAKY397F but not FAK wild type. Thus, our results suggest that, depending on the phosphorylation state of FAK at Tyr397, Src may activate either a migration pathway by triggering FA disassembly via formation of a FAK/Src complex at these sites44–46 or an invasion pathway, via specific targeting of Src to invadopodia, leading to matrix degradation. Alternatively, FAK expression may also be environmentally controlled to specify which pathway should be activated (Figure 10).

It has been reported that the lifetime of invadopodia is relatively high, ranging from minutes to hours,33 thus allowing significant matrix degradation 3–4 h after cell plating. Therefore, for efficient ECM proteolysis leading to dissemination of melanoma cells, high migration speed should be either avoided or redirected toward the degradation area. In this respect, our results show that downregulation of FAK is sufficient to both promote increased matrix degradation and reduce migration speed. This leads to the question whether FAK expression could be regulated between different stages of melanoma progression. In their early phase, melanoma cells have been shown to express mainly proliferative genes...
which upon unknown signaling change to an invasive signature gene set.41 This change may be triggered by subtle alterations of the microenvironment. For example, hypoxia drives metastatic progression by promoting such a switch in melanoma gene signature.48 The possible transformation of FAs into invadopodia could represent another example of melanoma plasticity directed toward increased invasion. Indeed, as FA and invadopodia share many structural components, several studies have suggested that invadopodia might derive from FA. Thus, in Src-transformed fibroblasts, invadopodia formation is initiated at adhesions in response to the focal generation of phosphatidylinositol-3,4,5-trisphosphate. Therefore, approaches to identify key molecules that drive the transition between FAs and invadopodia have been used and led to the recent discovery that the crosstalks that drive the transition between FAs and invadopodia to promote invadopodia activity and cell invasion while inhibiting cell migration

Figure 10 Theoretical model for FAK-mediated regulation of matrix degradation. (left) Active Src is in equilibrium between two compartments: invadopodia and FAs. FAK is located at FAs but not at invadopodia. When phosphorylated at Tyr397, FAK recruits Src to mediate phosphorylation of downstream substrates like paxillin, thus promoting FAs turn-over and cell migration. (right) Depletion of FAK or inhibition of Src binding to FAK releases Src from FAs, thus mediating phosphorylation of FAK-S5 at invadopodia to promote invadopodia activity and cell invasion while inhibiting cell migration.

FAK regulates invadopodia activity in melanoma cells K Kolli-Bouhafs et al

In conclusion, our results identify FAK as a key molecule at the crossroad between invasion and migration pathways in melanoma cells. How FAK is environmentally regulated during melanoma progression and whether FAK expression, phosphorylation and/or dynamics represent the key determinants of this transition will need further investigation.

Materials and Methods

Antibodies and other reagents. The following mouse monoclonal antibodies were used: anti-FAK (BD Transduction Laboratories, Le Pont de Clai, France), anti-cortactin (Millipore, Molsheim, France), anti-Tks-S5 (Tebu-bio, Le Perray-en-Yvelines, France), anti-Tks-S5/5 PLX domain, anti-Tks-S5 SH3 domain, anti-phosphoTyr59 (Millipore), anti-talin, anti-paxillin, anti-phospho-Y397 FAK (Invitrogen, Camarillo, CA, USA) and anti-jil-1 (Sigma-Aldrich, St Louis, MO, USA). Anti-Src and anti-phospho-Src anti-bodies were from Cell Signalling (Danvers, MA, USA) or Invitrogen (Grand Island, NY, USA). Horseradish peroxidase-conjugated goat anti-mouse was from Promega (Madison, WI, USA). Actin antibody conjugated to Alexa fluor 647 was from Santa Cruz (Heidelberg, Germany). PP2 was from Calbiochem (Billenica, MA, USA) and MnTMPyP from MERCK Biosaences (Darmstadt, Germany). G-2-Sepharose beads and ECL plus kit were from GE healthcare (Freiburg, Germany). MnP, CM-H2DCF-DA, Alexa Fluor 488-conjugated goat anti-mouse and Alexa-405-conjugated kit and Lipofectamine 2000 were from Invitrogen. Gelatin from porcine skin and mouse monoclonal Ab was from Sigma-Aldrich.

Cell culture, DNA constructs and stable transfection. The B16F10 melanoma cell line was from ATCC (Manassas, VA, USA). Cells were maintained in DMEM (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum, 1 mM L-glutamine and 1% penicillin/streptomycin antibiotics. Plasmid pAcSF1- hyg-C1-FAK was obtained as previously described.41 Punctual mutations were performed on the FAK coding sequence through directed mutagenesis (Quick-changel XL site-directed mutagenesis, Agilent Technologies, Massy, France). Y397 was replaced by phenylalanine and the FAK mutation was verified by sequencing. B16F10 cells were transfected with 4 μg DNA using lipofectamine 2000 (Invitrogen), according to the manufacturer instructions.

siRNA transfections. B16F10 cells were plated at 1.5 × 10^5 cells in six-well plates. Cells, 18 h later, were transfected with 50 pmol of siRNA for 5 μl Lipofectamine 2000 in 500 μl Opti-MEM (Gibco, Gibco Island, NY, USA). Stealth siRNA oligonucleotides (Invitrogen) used were control siRNA 5'-GAAGUACAUUCUUUGCUAGAC-3' and FAK siRNA 5'-UGCAAGAUUGCUACGAUGCAUGCUAACAA-3'. These sequences have already been described to target the 5^'-UTR region of FAK in rat MTLn3 cells.41 However, a sequence alignment was performed using the link http://www.ba.itb.cnr.it/BIG/Blast/BlastUTR.html to check out sequence homology in Mus musculus as B16F10 cell line stems from murine lineage. Transfected cells were incubated for 48 h (unless otherwise indicated) at 37 °C before use.

Cell treatment with PP2, MnTMPyP. One hour after seeding on fluorescent gelatin, B16F10 cells were treated with PP2 5-(4-chlorophenyl)-7-(dimethylamino)pyrazolo[3,4-d]pyrimidine) for 3 h at 10 μM to inhibit Src activity, and with MnTMPyP (manganese (III) tetrakis(1-methyl-4-pyridyl)porphyrin) for 1 h at 37 °C at the concentration of 300 μM to inhibit ROS formation.

In vitro fluorescent matrix digestion assay and immunofluorescence. Cells transfected or not with siRNA were plated at 5000 cells/cm² in a two-well lab-tek and incubated overnight at 37 °C on fluorescent-labeled Alexa-405 gelatin as described elsewhere.32 Cells, 18 h later, were fixed using 4% paraformaldehyde solution for 15 min, and permeabilized using triton X-100 at 0.05%. Unspecific sites were blocked by incubating cells in 1% of bovine serum albumin (BSA) at room temperature. Cells were then labeled for 1 h with anti-cortactin Ab (1/1000), anti-talin Ab (1/500), anti-FAK Ab (1/1000), anti-paxillin Ab (1/μg/ml), anti-P-Tyr416-Src Ab (1/250) or Alexa-647-conjugated phalloidin. After three washes with PBS, cells were incubated with Alexa-488-conjugated goat anti-rabbit Ab (1/500), rhodamine-conjugated donkey anti-mouse Ab (1/400), Alexa-
555-conjugated goat anti-mouse Ab (1/250) or CY 5-conjugated donkey anti-rabbit Ab (1/250) for 1 h, washed three times with PBS and then observed using either a Leica confocal microscope TSC SPE (× 63 HCX PL Apo 1.32 NA objective, Wetzlar, Germany) or a Leica DMIRE2 microscope (× 40 HCX PL Apo 1.25 NA and × 63 HCX PL Apo 1.32 NA objectives). Areas of degradation were identified as black holes on fluorescent gelatin and quantified using ImageJ software downloaded from the NIH website.

Western blot and immunoprecipitation. Total cell proteins were obtained by lysing cells for 30 min at 4 °C in RIPA buffer for western blot (1 M Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM sodium orthovanadate, 10 mM NaF, 5 mM Na pyrophosphate) or IP buffer for immunoprecipitation (150 mM NaCl, 1% Triton X-100, 1% Nonidet P-40, 50 mM Tris-HCl, pH 8.0, 10 mM NaF, 2 mM Na3VO4) both supplemented with Protease Inhibitor Cocktail (complete Mini, Roche, Mannheim, Germany). Lysates were clarified by centrifuging at 14 000 r.p.m. Cells were subsequently suspended in 300 μl DMEM supplemented with 10% FCS and 100 units/ml penicillin and streptomycin.

ROS measurement. Cells were plated at subconfluent in complete medium. Cells, 18 h later, were incubated with 25 μM of the fluorescent ROS probe CM-H2DCF-DA (the 5-(and-6)-carboxy-2', 7'-dichlorodihydrofluorescein diacetate) in HBSS supplemented with 10% FBS for 20 min at 37 °C in the dark. Cells were then washed carefully three times with HBSS, collected with EDTA for 1 min and resuspended in HBSS 10% FBS, then centrifuged for 5 min at 1000 r.p.m. Cells were subsequently suspended in 300 μl HBSS and analyzed by flow cytometry at 545 nm. Quantification of fluorescence was performed using the FACS Aria software (San Jose, CA, USA).

Cell invasion and migration assay. Invasion was assessed using 24-well plates having Matrigel-coated inserts (BD Biosciences, San Jose, CA, USA) and migration was assessed using uncoated inserts. Transfected B16F10 cells were plated for 1 h at room temperature using 0.5% BSA in TBS-T (pH 7.4) containing 100 μg/ml Tris, 150 mM NaCl and 0.1% Tween 20. Then, membranes were incubated with primary antibody diluted in TBS-T containing 5% BSA or 5% non-fat milk overnight at 4 °C. After washing membranes three times in TBS-T for 10 min, specific horseradish peroxidase-labeled secondary antibodies (Promega) were incubated with membranes for 1 h at room temperature. Signals were detected using an ECL™ kit.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. This work was supported in part by grants from the Ligue Contre le Cancer (Comités du Bas-Rhin and du Haut-Rhin) to P.R. K.K.-B. was supported by a doctoral fellowship from the Ministère de la Recherche. R. Vauchelles from the PIQ (Quantitative Imaging Platform, Faculté de Pharmacie, Université de Strasbourg) is thanked for technical assistance in data analyses. We also thank T. Steffan for excellent technical assistance, C. Auger for his help with the ROS measurements and V. Schini-Kerth for providing reagents.

9

FAK regulates invadopodia activity in melanoma cells

K Kolli-Bouhafs et al

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. This work was supported in part by grants from the Ligue Contre le Cancer (Comités du Bas-Rhin and du Haut-Rhin) to P.R. K.K.-B. was supported by a doctoral fellowship from the Ministère de la Recherche. R. Vauchelles from the PIQ (Quantitative Imaging Platform, Faculté de Pharmacie, Université de Strasbourg) is thanked for technical assistance in data analyses. We also thank T. Steffan for excellent technical assistance, C. Auger for his help with the ROS measurements and V. Schini-Kerth for providing reagents.

9
FAK regulates invadopodia activity in melanoma cells
K Koli-Bouhafs et al

23. Liu JD, Chen SH, Lin CL, Tsai SH, Liang YC. Inhibition of melanoma growth and metastasis by combination with (-)-epigallocatechin-3-galllate and dacarbazine in mice. J Cell Biochem 2001; 83: 631–642.

24. Hamada A, Deramautd TB, Takeda K, Ronde P. Src activation and translocation from focal adhesions to membrane ruffles contribute to formation of new adhesion sites. Cell Mol Life Sci 2002; 59: 304–309.

25. Ahmed S. Nanoscopy of cell architecture: the actin-membrane interface. Bioarchitecture 2007; 445: 851–857.

26. Miller AJ, Mihm MC Jr. Melanoma. N Engl J Med 2006; 355: 51–65.

27. Destaing O, Block MR, Planus E, Albiges-Rizo C, DerMardirossian C, Bokoch GM. Direct interaction between Tks proteins and the paxillin-binding protein Tks5/Fish is required for podosome formation and function, and for the protease-driven invasion of cancer cells. Cancer Cell 2003; 7: 155–165.

28. Gray-Schopfer V, Wellbrock C, Marais R. Melanoma biology and new targeted therapy. Nature 2007; 445: 851–857.

29. Miller AJ, Mihm MC Jr. Melanoma. N Engl J Med 2006; 355: 51–65.

30. Destaing O, Block MR, Planus E, Albiges-Rizo C, DerMardirossian C, Bokoch GM. Direct interaction between Tks proteins and the paxillin-binding protein Tks5/Fish is required for podosome formation and function, and for the protease-driven invasion of cancer cells. Cancer Cell 2003; 7: 155–165.

31. Murphy DA, Courthe Edre SA. The ‘ins’ and ‘outs’ of podosomes and invadopodia: characteristics, formation and function. Nat Rev Mol Cell Biol 2011; 12: 413–426.

32. Liu JD, Chen SH, Lin CL, Tsai SH, Liang YC. Inhibition of melanoma growth and metastasis by combination with (-)-epigallocatechin-3-galllate and dacarbazine in mice. J Cell Biochem 2001; 83: 631–642.

33. Okawa T, Oyama M, Kohama H, Uehara S, Udawaka N, Saya H et al. Tks5-dependent formation of circumferential podosomes/invadopodia mediates cell-cell fusion. J Cell Biol 2012; 197: 553–568.

34. Hauck CR, Hauck CR, Hauck CR. The ‘ins’ and ‘outs’ of podosomes and invadopodia: characteristics, formation and function. Nat Rev Mol Cell Biol 2011; 12: 413–426.

35. Destaing O, Block MR, Planus E, Albiges-Rizo C, DerMardirossian C, Bokoch GM. Direct interaction between Tks proteins and the paxillin-binding protein Tks5/Fish is required for podosome formation and function, and for the protease-driven invasion of cancer cells. Cancer Cell 2003; 7: 155–165.

36. Murphy DA, Courthe Edre SA. The ‘ins’ and ‘outs’ of podosomes and invadopodia: characteristics, formation and function. Nat Rev Mol Cell Biol 2011; 12: 413–426.

37. Gray-Schopfer V, Wellbrock C, Marais R. Melanoma biology and new targeted therapy. Nature 2007; 445: 851–857.

38. Gray-Schopfer V, Wellbrock C, Marais R. Melanoma biology and new targeted therapy. Nature 2007; 445: 851–857.

39. Hamada A, Deramautd TB, Takeda K, Ronde P.Src activation and translocation from focal adhesions to membrane ruffles contribute to formation of new adhesion sites. Cell Mol Life Sci 2002; 59: 304–309.

40. Alexander NR, Branch KM, Parekh A, Clark ES, Inoue H, Bharti K, Hirsch DS, Nie Z, Yoon HY et al. Src-dependent phosphorylation of ASAP1 regulates podosomes. J Cell Sci 2007; 27: 8271–8283.

41. Pan YR, Chen CL, Chen HC. FAK is required for the assembly of podosome rosettes. J Cell Biol 2011; 195: 113–129.

42. Wang Y, McViven MA. Invasive matrix degradation at focal adhesions occurs via protease recruitment by a FAK-p30Cas complex. J Cell Biol 2012; 196: 375–385.

43. Carragher NB, Westhoff MF, Finchem VF, Schaller MD, Frame MC. A novel role for FAK as a protease-targeting adaptor protein: regulation by p42 ERK and Src. Curr Biol 2003; 13: 1442–1450.

44. Carragher NB, Westhoff MF, Finchem VF, Schaller MD, Frame MC. A novel role for FAK as a protease-targeting adaptor protein: regulation by p42 ERK and Src. Curr Biol 2003; 13: 1442–1450.

45. Webb DJ, Donais K, Whitmore LA, Thomas SM, Turner CE, Parsons JT et al. FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. Nat Cell Biol 2004; 6: 154–161.

46. Deramautd TB, Dujardin D, Hamadi A, Noulet F, Koli K, De Mey J et al. FAK phosphorylation at Tyr-925 regulates cross-talk between focal adhesion turnover and cell protrusion. Mol Biol Cell 2011; 22: 964–975.

47. Hock KS, Eichhoff OM, Schlegel NC, Dobbeling U, Kobern T, Schraer L et al. In vivo switching of human melanoma cells between proliferative and invasive states. Cancer Res 2008; 68: 650–656.

48. Widmer DS, Hoek KS, Cheng PF, Eichhoff OM, Biedermann T, Raajmakers MI et al. Hypoxia contributes to melanoma heterogeneity by triggering HIF1alpha-dependent phenotype switching. J Invest Dermatol 2013; 133: 2436–2443.

49. Yoshino D, Jouquin J, Emmons SW, Miller T, Golgof M, Costello K et al. Network analysis of the focal adhesion to invadopodia transition identifies a PI3K-PKalpha-invasive signaling axis. Sci Signal 2012; 5: ra66.

50. Timpson P, Jones GE, Frame MC, Bruton VG. Coordination of cell polarization and migration by the Rho family GTPases requires Src tyrosine kinase activity. Curr Biol 2001; 11: 1936–1946.

51. Le Devedec SE, Geverts B, e Bont H, Yan K, Verbeek FJ, Houtsmuller AB et al. The residence time of focal adhesion kinase (FAK) and paxillin at focal adhesions in renal epithelial cells is determined by adhesion size, strength and life cycle status. J Cell Sci 2012; 125(Pt 19): 4498–4500.

52. Deramautd TB, Dujardin D, Noulet F, Martin S, Vauchelles R, Takeda K et al. Altering FAK-paxillin interactions reduces adhesion, migration and invasion processes. PLoS One 2014; 9: e92039.

53. Arty DM, Yamada KM, Mueller SC. ECM degradation assays for analyzing local cell invasion. Methods Mol Biol 2009; 522: 211–219.

Supplementary Information accompanies this paper on Cell Death and Disease website (http://www.nature.com/cddis)