Focal adhesion kinase and paxillin : mediators of breast cancer cell migration

Verkoeijen, S.

CHAPTER 6

Paxillin serine 178 phosphorylation determines mammary tumor cell migration and metastasis formation through regulating EGFR expression

Saertje Verkoeijen [*], Sylvia Le Dévédec [*], Yafeng Ma [*], Wies van Roosmalen, Reshma Lalai, Martine van Miltenburg, Marjo de Graauw and Bob van de Water

Division of Toxicology, Leiden/Amsterdam Center for Drug Research (LACDR), Leiden University, Leiden, the Netherlands

[*]These authors contributed equally to the manuscript.

Running title:
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ABSTRACT

Paxillin is a multidomain scaffold protein that localizes at focal adhesions and is involved in regulation of the dynamics of matrix adhesion which is required for cell migration. Phosphorylation of paxillin at serine 178 is mediated by EGF-induced JNK activation, while JNK signaling is required for EGF-driven cell migration. So far the significance of paxillin Ser178 phosphorylation in tumor cell migration and breast cancer metastasis remains unknown. Here we show that expression of the phospho-defective mutant paxillinS178A in the metastatic mammary adenocarcinoma MTLn3 cell-line significantly decreased EGF-induced cell migration which was correlated with impaired focal adhesion turnover. This was associated with attenuated lung metastasis formation by paxillinS178A in an orthotopic *in vivo* mammary gland tumor/metastasis model. EGFR signaling is regulated and trans-activated at focal adhesions by both integrins and FAK. Interestingly, paxillinS178A expression caused a decreased expression of EGFR compared to wt-paxillin. Re-expression of wt-EGFR in MTLn3-paxillinS178A fully restored the EGF-driven cell motility and focal adhesion dynamics. Furthermore, re-expression of EGFR in MTLn3-paxillinS178A rescued spontaneous metastasis from breast to lung. Our data show a role for paxillin Ser178 in the regulation of EGFR and, thereby, in the regulation of EGF-driven cell migration and metastasis formation.

INTRODUCTION

Breast cancer represents the most common type of cancer among women. The formation of metastases, which is a determinant of the prognosis of cancer patients, involves distinct cellular processes including cell migration, invasion, intra- and extravasation and proliferation. These processes are regulated by growth factors, cytokines and cellular matrix molecules (1). An important regulator of cell proliferation and migration is the receptor tyrosine kinase epidermal growth factor receptor (EGFR) whose stimulation by epidermal growth factor (EGF) results in the activation of downstream signaling pathways including different mitogen-activated protein kinase (MAPK) cascades: extracellular signal-regulated kinase (ERK), p38 and c-Jun NH2-terminal kinase (JNK) (2).

The JNK group of kinases has essential roles in cancer development including regulation of the survival/proliferation balance as well as cell migration (3-6). Dual Thr and Tyr phosphorylation of JNK by upstream MAP kinase kinases results in JNK activation and nuclear translocation. In the nucleus, JNKs phosphorylate and activate transcription factors including members of the AP-1 family such as c-Jun. The JNK-AP-1 pathway regulates the expression of genes involved in the cell cycle, survival and apoptosis and extracellular matrix (ECM) homeostasis (7-13). Besides phosphorylation of transcription factors, several cytoskeleton-associated adaptor and signaling proteins have recently been identified as direct JNK substrates including β-catenin (14) and paxillin (3). Since both proteins are well known to be involved in cell-matrix and cell-cell contacts, the direct JNK-mediated phosphorylation of these targets may also influence cell migration.

Paxillin is a 68 kD multidomain adaptor protein associated with focal adhesions, contacts between cells and ECM (15-17). It functions as a scaffold to integrate multiple signaling pathways involved in matrix adhesion dynamics and cell migration (18-22). Recent studies have identified paxillin as a novel JNK substrate (2,3). EGF-driven JNK activation results in JNK-mediated phosphorylation of paxillin at Ser178 and affects the migration of different cell-types (3). Interestingly, expression of a Ser178 to Ala mutant of paxillin, inhibits the migration in some cells (3). How JNK-mediated phosphorylation of paxillin regulates cell migration is still largely unknown. Moreover it is unclear whether this phosphorylation event is relevant for cancer metastasis formation.

Here we explored the role and mechanism of paxillin Ser178 phosphorylation in breast tumor progression using the highly metastastic breast tumor cell line MTLn3 as a model. We show that ectopic expression of paxillinS178A mutants significantly decreased EGF-dependent signaling and cell migration. Using an orthotopic mammary gland tumor/metastasis model, we demonstrate that the JNK-mediated phosphorylation of paxillin at Ser178 is essential for efficient metastasis of MTLn3 cells to the lung. Expression of paxillinS178A was found to reduce expression of EGFR, whereas re-expression of EGFR rescued the defected tumor cell migration and metastasis formation. Our data indicate that the phosphorylation of Ser178 of paxillin by JNK can regulate cell migration and metastasis formation via modulation of the EGFR-signaling pathway.

MATERIALS AND METHODS

Chemicals and antibodies

Alpha modified minimal essential medium without ribonucleosides and deoxyribonucleosides (α-MEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), trypsin and geneticin (G418 sulphate) were from Life Technologies. Rat tail collagen type I was from Upstate Biotechnology. LipofectAMINE Plus transfection reagents were from Invitrogen. Primary antibodies were anti-paxillin (BD), anti-tubulin, anti-GFP (Sigma, St. Louis, MO), anti-JNK-PT183/PY185 (Promega), anti-ERK-PT202/PY204, anti-AKT-PS473 (Cell Signaling), anti-paxillin-PS178 (Abcam, Cambridge, UK), anti-EGFR for Western blot (rabbit polyclonal, Cell Signaling Technology) and FACS/immunostaining (mouse monoclonal, Calbiochem). All secondary antibodies were from Jackson. The Western-Star immunodetection system (Tropix kit) was from Applied Biosystems and ECL Plus reagent was from Amersham. Hoechst 33258 and rhodamine-phalloidin were from Molecular Probes and Aqua Poly/Mount was from Polysciences.

Cell Culture

MTLn3 cells were cultured as before (23). To generate stable cell lines, MTLn3 cells were transfected with GFP-paxillin or GFP-paxillinS178A along with empty vector pcDNA3 using LipofectAMINE plus reagents according to manufacturer's

procedures. Stable transfectants were selected using G418 and individual clones were picked and kept at 100 μg/ml G418. For EGF experiments, 70-80% confluent cells were starved for 4 hours followed by exposure to EGF (10 nM).

Luciferase reporter assay

MTLn3 cells were transiently transfected with the reporter constructs pGL3-Tata-5xE3AP1, pGL3-Tata-5xcollTRE, pGL3-Tata-5xJun2 using Lipofectamine Plus reagent (23). After 16 hours cells were serum-starved for 4 hours followed by analysis of luciferase activity using a luminescence plate reader.

Proliferation, attachment and wound healing assay

For proliferation assay, cells were plated in complete medium in 6-wells plates and cultured for 24, 48, 72 or 96 hours at which time point the amount of cells was determined by counting. For cell attachment assay, 1 hour serum-starved cells were replated in complete medium on collagen-coated 6-wells plates for 30, 60, 90 and 120 minutes, and following a PBS wash, attached cells were trypsinized and counted. For wound healing assays, monolayer cells were scratched using a pipette tip to generate a wound followed by a wash with medium and incubation in α-MEM supplemented with 1% (v/v) FBS for 20 hours. Wounds were photographed using phase contrast microscopy with a Nikon Coolpix digital camera directly after scratching and after 20 hours. Wound closure was determined using Image J software.

Live cell imaging

Random cell migration-Cells were cultured in glass-bottom plates overnight and starved for 4 hours followed by imaging for 1-3 hours on a Nikon TE 2000-E microscope in a humid climate of 37°C and 5% CO2 with either DIC (Differential Interference Contrast) or fluorescence microscopy. Subsequently, cells were treated with EGF and movies were captured with 20x objective. When used, the JNK inhibitor SP600125 (20 μM) was added 30 minutes prior to stimulation. Cell speed was determined with a homemade macro written in Image-Pro Plus (Media Cybernetics Inc., Silver Spring, MD).

TIRF and FRAP-Total internal reflection fluorescence (TIRF) microscopy was performed with a Nikon TE 2000-E microscope in a climate control chamber. To determine the turnover of GFP-tagged paxillin proteins in individual focal

adhesions, fluorescence recovery after photobleaching (FRAP) was performed as follows: photobleaching was applied to a small area covering a single focal adhesion for 1 s with laser intensity of 50 µW. Redistribution of fluorescence was monitored with 100 ms time intervals at 7.5 µW starting directly after the bleach pulse. Approximately 20 focal adhesions (each in distinct cells) were averaged to generate one FRAP curve for a single experiment. All measurements were performed at 37°C and the experiment was performed on three different days. The relative fluorescence intensity of individual focal adhesion was calculated at each time interval as follows: Irel(t) = (FAt / FA0), where FAt is the intensity of the focal adhesion at time point t after bleaching and FA0 is the average intensity of the focal adhesion before bleaching.

Gel electrophoresis and immunoblotting

Equal protein amounts (25 µg, Bradford protein assay) were separated on 7.5% polyacrylamide gels and transferred to PVDF membranes (Millipore). Membranes were blocked in 5% (w/v) BSA in TBS-T and probed with primary antibody overnight followed by sufficient washes and incubation with secondary antibodies. Alkaline phosphatase-conjugated secondary antibodies for phospho-proteins were detected with the Western-Star immunodetection system. For detection of horseradish peroxidase-conjugated antibodies, ECL Plus reagent was used, followed by visualization on a Typhoon Imager 9400.

Immunofluorescence

Cells were plated on collagen-coated glass coverslips. Cells were briefly washed in PBS, followed by fixation in 3.7% formaldehyde for 10 minutes at room temperature. After washing, coverslips were blocked in TBP (0.1% (w/v) Triton X-100, 0.5% (w/v) BSA in PBS, pH 7.4). Incubation with primary antibodies diluted in TBP containing 0.05% (w/v) NaN3 was carried out overnight at 4°C. Coverslips were mounted on glass slides using Aqua Poly/Mount.

***In vivo* tumor growth and metastasis formation**

6 week-old Rag2 -/- γc -/- mice were obtained from in-house breeding. Animals were housed in individually ventilated cages under sterile conditions containing 3 mice per cage. Sterilized food and water were provided *ad libitum*. To measure spontaneous metastasis, tumor cells ($5x10^5$) were injected into the right thoracic

mammary fat pads as described previously (24). After 4 weeks, the lungs were excised as well as the primary tumor which was also weighed. For quantification of all GFP-paxillin-positive macro- and micro-metastases, the flat side of the right lung was analyzed with the immunofluorescence microscope using a 10x objective lens (NA 0.25). Next the right lung and primary tumor were fixed in 4% paraformaldehyde. Paraffin sections of the lungs (5 μm) were stained with H&E followed by histological analysis.

Statistical Analysis

Student's t test was used to determine significant differences between two means ($p<0.05$).

RESULTS

EGF-induced cell migration of MTLn3 cells is dependent on JNK activity and associated with paxillin Ser178 phosphorylation.

First we determined the role of JNK-paxillin signaling in EGF-induced migration of the highly metastatic MTLn3 rat mammary adenocarcinoma cell line. Exposure to EGF caused a rapid onset of lamellipodia formation, random cell migration and scattering of MTLn3 cells (Fig. 1A (and supplemental movie M1, not shown)). An inhibitor of JNK, SP600125, blocked the migration almost completely which was associated with increased cell clustering of the MTLn3 cells. This clustered phenotype was associated with increased localization of β-catenin at cell-cell contacts (Fig. 1B). Quantitative analysis of the movies indicates that inhibition of JNK drastically reduces persistent movement (Fig. 1C) and velocity of both individual and clustered cells (Fig. 1D). By immunofluorescence, we observed that active phosphorylated JNK co-localizes with paxillin at focal adhesions in MTLn3 cells (data not shown) as was previously observed in renal epithelial cells (25). Treatment with EGF induced the transient phosphorylation of paxillin at Ser178 in association with JNK activation. Importantly, this phosphorylation was dependent on JNK, since SP600125 prevented the efficient phosphorylation of paxillin at Ser178 (Fig. 1E). These data indicate that in MTLn3 cells EGF-induced JNK activation mediates cell migration possibly via the phosphorylation of paxillin at Ser178.

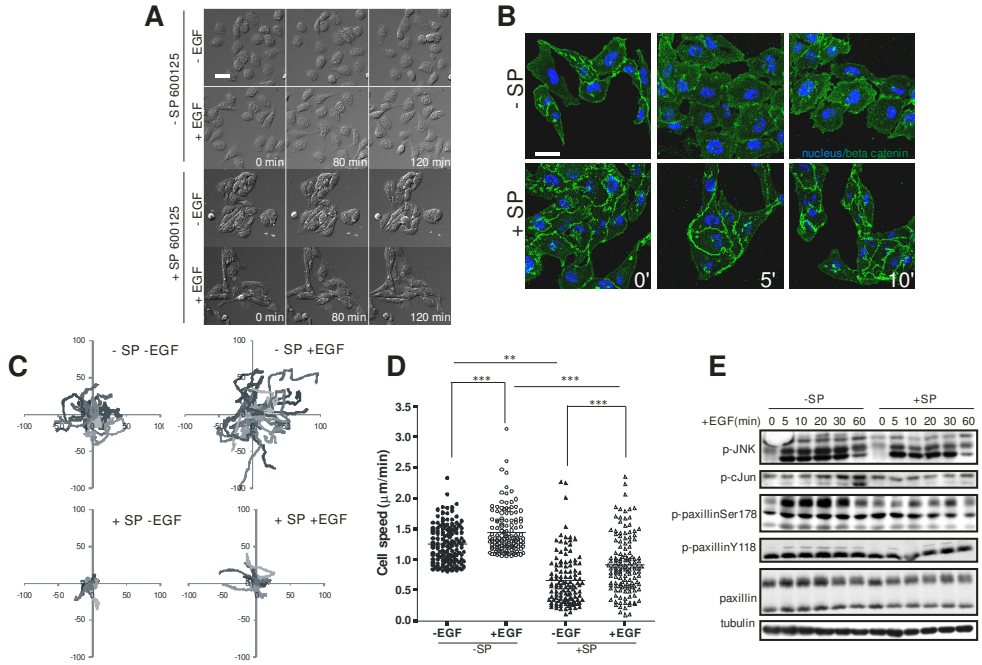

Figure 1. JNK-mediated phosphorylation of paxillin Ser178 plays a role in tumor cell migration. (A) MTLn3 cells are observed by DIC microscopy after EGF stimulation (10 nM) or not for 3 hours with or without JNK inhibitor SP600125 (20 µM). Snapshots of the time-lapse made for 2 hours are shown, scale bar is 50 µm. See movie M1. (B) Inhibition of JNK results in localization of β-catenin at cell-cell contacts, MTLn3 cells were fixed 0, 5 and 10 minutes after EGF stimulation with or without SP600125 and stained for the nucleus (blue) and β-catenin (green). Scale bar is 20 µm. (C) Plots represent the overall trajectories of individual cells of representative time-lapse movies of the 4 conditions (+/-EGF and +/-SP600125). X and Y axis are in µm. (D) JNK inhibition reduces cell speed of MTLn3 cells in both control and EGF treatments ($p<0,001$). (E) Inhibition of JNK results in the absence of c-Jun phosphorylation after EGF stimulation and significantly reduces phosphorylation of paxillin Ser178.

PaxillinS178A mutant inhibits MTLn3 cell motility *in vitro*.

To further investigate the role of paxillin Ser178 in cell migration, we generated MTLn3 cell-lines stably expressing either GFP-tagged wt-paxillin or phospho-defective mutant GFP-paxillin in which the serine residue 178 was replaced by alanine (further referred to as paxillinS178A). Three independently obtained wt-

paxillin clones and three paxillinS178A clones were selected for further experiments. MTLn3 clones stably expressing GFP-wt-paxillin or GFP-paxillinS178A were evaluated by flow cytometry, Western blotting (supplemental data 1A) and immunofluorescence (Fig. 2A). Expression levels were approximately equal in all clones. Although GFP-paxillinS178A still localized at focal adhesions, cells were smaller and clustered with enhanced cell-cell contacts while the wt cells were stretched with large lamellipodia and almost no cell-cell contacts (Fig. 2A and supplemental data 1B). In contrast to wt-paxillin cells, paxillinS178A cells formed clusters with clear β-catenin and E-cadherin-positive cell-cell junctions (Fig. 2B and supplemental data 1C), reminiscent to the effect of SP600125 in control cells (compare Fig. 1B). Given the prominent role of paxillin in focal adhesion formation and dynamics that determines cell spreading; we next examined the effect of paxillinS178A expression on cell attachment and spreading. Significantly less paxillinS178A cells attached compared to wt-paxillin cells (Fig. 2C-a). Furthermore, while most of wt-paxillin cells had already spread, most of the paxillinS178A cells remained rounded and presented a smaller surface area even after three hours of spreading (Fig. 2C-b). We also determined the effect of paxillinS178A on directed cell migration in an artificial wound healing assay (Fig. 2D and supplemental data 1D). While wt-paxillin cells had closed the wound by 83%, paxillinS178A cells had only closed 25% of the wound after 20 hours.

PaxillinS178A expression affects EGF-induced cell migration and focal adhesion dynamics.

In a random cell migration assay wt-paxillin cells rapidly formed lamellipodia and became highly motile while paxillinS178A cells showed decreased cell motility and responded less to EGF stimulation (Fig. 3A (and supplemental movie M2, not shown)). Since paxillinS178A most likely acts as a dominant negative construct in these cells, it may compete for the localization of endogenous paxillin at focal adhesions and prevent the phosphorylation of endogenous paxillin at Ser178 by JNK. Indeed, EGF stimulation of paxillinS178A cells induced negligible Ser178 phosphorylation of endogenous paxillin whereas in wt-paxillin cells, both endogenous and GFP-wt-paxillin were phosphorylated at Ser178 after EGF treatment (supplemental data 2). Importantly, an EGF-induced mobility shift of endogenous paxillin was observed in both wt and S178A cell-lines, indicating that most of the other paxillin modifications were unaffected (supplemental data 2).

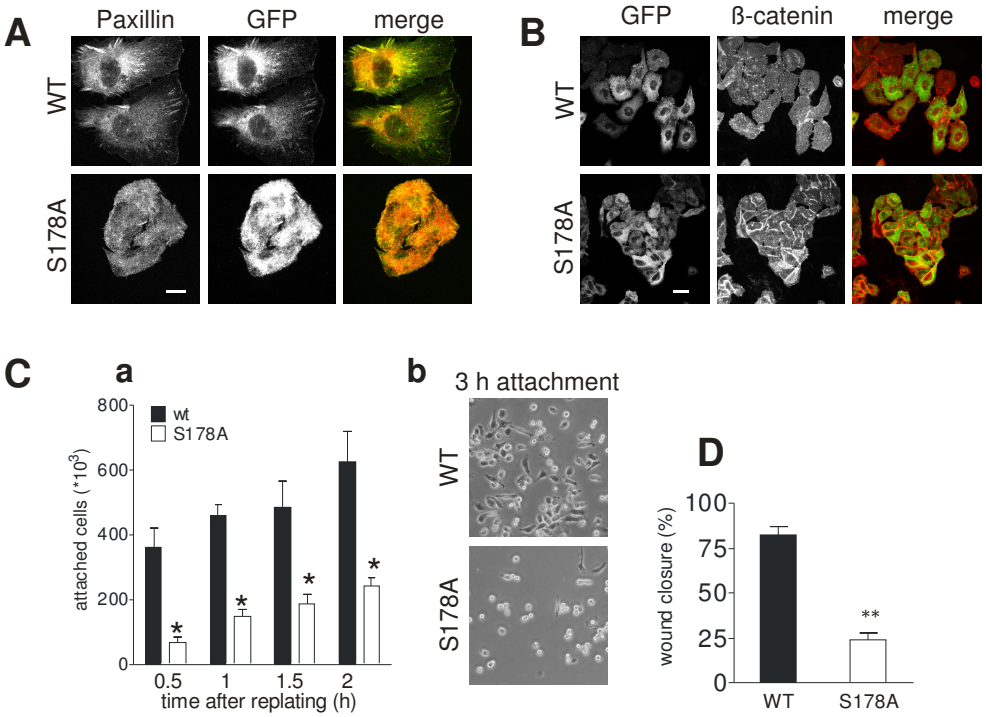

Figure 2. Expression of paxillinS178A decreases cell spreading and directed cell migration. Three different clones for GFP-wt-paxillin and GFP-paxillinS178A MTLn3 cells were selected for further research. (A) Immunostaining of paxillin (red) colocalizes with ectopic expression of GFP-wt-paxillin and GFP-paxillinS178A (green). Scale bar is 10 µm. (B) Immunostaining of β-catenin (red) and GFP-paxillin (green). Scale bar is 20 µm. (C) Cell adhesion (a) and spreading assay (3 hours) (b). (D) In a scratch assay, the wound was almost closed after 20 hours in the wt cells, while the mutant cells closed only 25% of the wound.

To understand the mechanism of the inhibitory effect of paxillinS178A on cell migration, we determined the dynamics of focal adhesions in wt and mutant cells using TIRF microscopy. MTLn3 cells expressing wt-paxillin show a high focal adhesion turnover which was enhanced upon EGF stimulation. In contrast, mutant cells show a much slower rate of FA disassembly either in the presence or absence of EGF (Fig. 3B (and supplemental movie M3, not shown)).

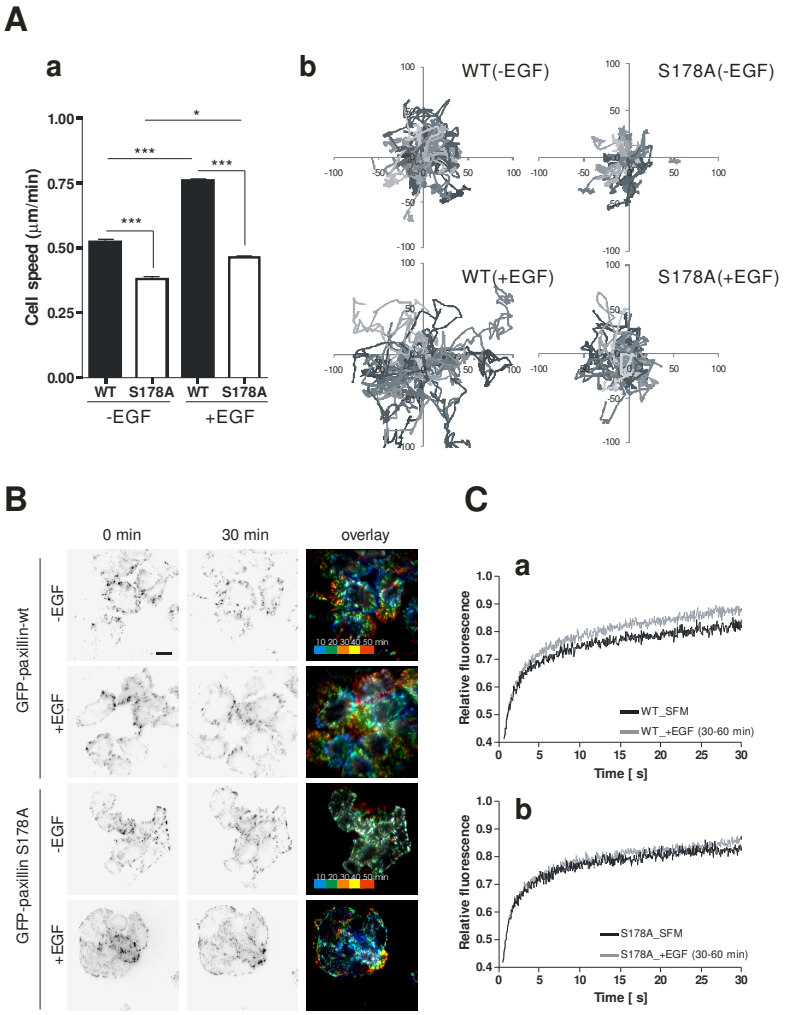

Figure 3. EGF-driven cell migration is inhibited in paxillinS178A cells because of impaired focal adhesion turnover. (A) PaxillinS178A reduces cell speed (a) and directional cell movement (b) of MTLn3 cells. See also movie M2. Cells were followed for approximately 10 hours with epi-fluorescence ($p<0,001$). (B) Matrix adhesions dynamics in MTLn3 cells was visualized with TIRF microscopy. See also movie M3. Overlay of images at different times in red, green and blue produces a white/grey image if no focal adhesion positions remain unchanged. Scale bar is 20 µm. (C) Protein dynamics was measured with the spot bleaching technique in serum-free medium (SFM) and upon EGF exposure (GFP-wt-paxillin (a) and GFP-paxillinS178A (b)).

The decreased focal adhesion dynamics was associated with reduced mobility of GFP-paxillinS178A as determined by FRAP experiments. Although under serum-free conditions the rates and percentages of fluorescence recovery of GFP-wt-paxillin and GFP-paxillinS178A were similar, an increase in the mobile fraction of GFP-wt-paxillin was measured in cells exposed to EGF after 30 minutes (Fig. 3C-a) while this was not observed for GFP-paxillinS178A (Fig. 3C-b). This suggests that pSer178-paxillin modifies its affinity for binding partners at FAs resulting in decreased stability of paxillin when MTLn3 cells are stimulated with EGF.

GFP-paxillinS178A expression impairs metastasis formation of MTLn3 cells in an orthotopic breast tumor model.

We next determined whether paxillin Ser178 was important for spontaneous metastasis formation. The MTLn3 cell line has been established as a suitable cell model to study metastasis formation from mammary gland tumors to the lung (24). We injected GFP-wt-paxillin (clone #2) and GFP-paxillinS178A (clone #2) cells into the mammary fat pads of immunodeficient Rag2-/- γ-/- mice. After three weeks mice were sacrificed for the analysis of the primary mammary gland tumors as well as lung metastases. All primary tumors remained GFP-positive, indicating expression of wt or mutant GFP-paxillin continuously during the experiment. The edges of the GFP-wt-paxillin tumors were more invasive-like compared to those of GFP-paxillinS178A tumors (Fig. 4A). Yet, the weight of the primary tumor was not significantly altered by GFP-paxillinS178A (Fig. 4B). GFP-paxillinS178A MTLn3 cells formed significantly less spontaneous lung metastases than GFP-wt-paxillin MTLn3 cells (Fig. 4C and D). Interestingly, while wt-paxillin cells formed relatively more small micro-metastatic lesions compared to mutant cells, these metastases also had a more scattered phenotype (Fig. 4C). For the first time, our data indicate an important role of the residue Ser178 of paxillin in breast cancer progression.

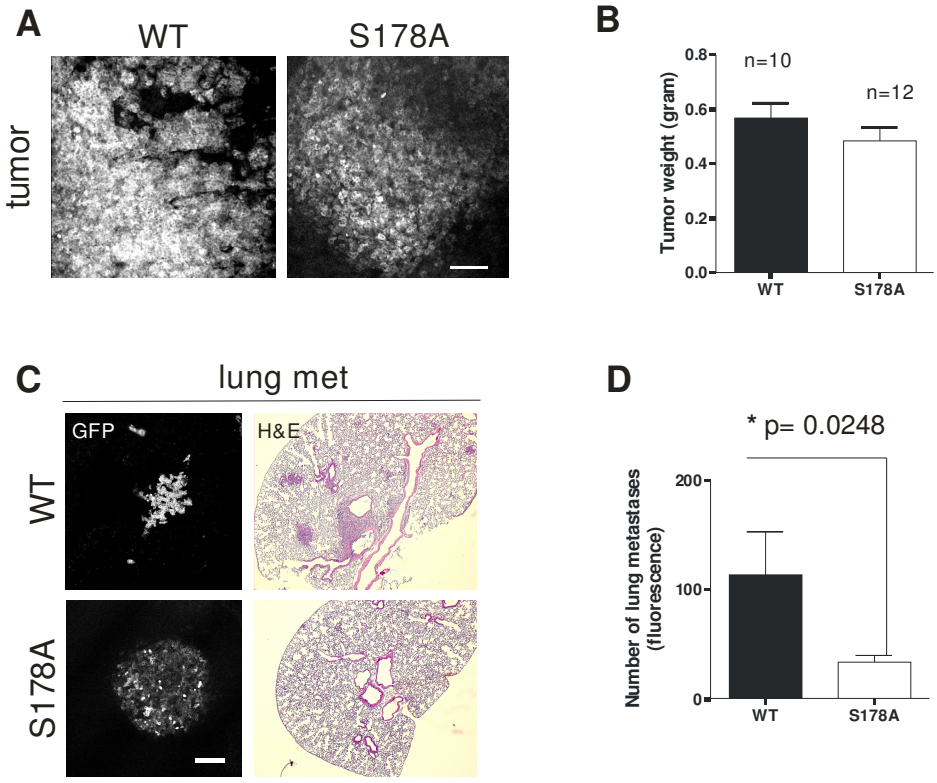

Figure 4. Expression of paxillinS178A impairs lung metastasis formation *in vivo*. (A). Clones #2 of GFP-wt-paxillin and GFP-paxillinS178A MTLn3 cell-lines were used for *in vivo* experiments. Two-photon intravital imaging of primary tumors. Scale bar is 100 µm. (B) No significant difference in tumor weight was measured between the wt mice (n=10) and S178A mice (n=12). (C) Two-photon intravital microscopy of lung metastases. Scale bar is 100 µm. Representative H&E staining of lung slices of both groups are shown. (D) Number of GFP-positive lung metastases in the wt and mutant groups.

Ectopic paxillinS178A expression results in EGFR downregulation at mRNA and protein levels.

Next we sought to determine the possible mechanism by which paxillinS178A affects tumor cell migration and metastasis formation. EGFR signaling is regulated and transactivated at focal adhesions by both integrins (26,27) and FAK (28,29). Therefore we reasoned that paxillinS178A may disturb the EGFR signaling pathway. Interestingly, all three paxillinS178A clones had much lower levels of

EGFR protein than wt clones (Fig. 5A). This was associated with reduced mRNA expression of EGFR in paxillinS178A cells (Fig. 5B). As a consequence, downstream EGFR signaling towards Akt and ERK were also reduced in S178A clones (Fig. 5C). Since EGF also caused JNK activation in control MTLn3 cells (Fig. 1), we also determined the activation of JNK signaling pathway in these cells. Indeed paxillinS178A expression also inhibited EGF-induced JNK activation (Fig. 5C), which was associated with a reduction in c-Jun transcriptional activity as determined by luciferase reporter assays (Fig. 5D). These data indicate that paxillinS178A affects the expression of EGFR possibly through the regulation of c-Jun-mediated EGFR transcription (30,31), thereby disturbing downstream signaling pathways that are essential in the cell migration process.

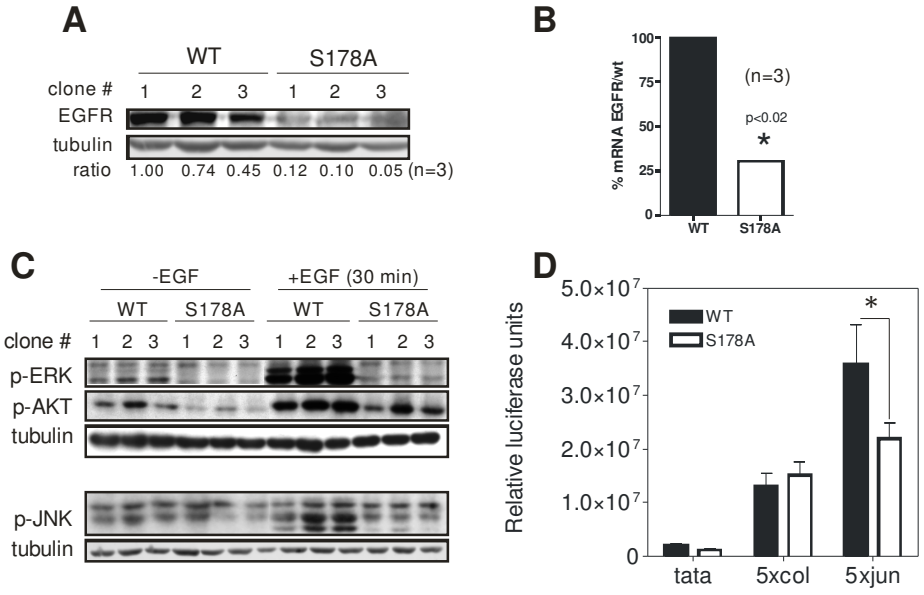

Figure 5. EGFR and EGFR-signaling is downregulated in paxillinS178A mutant cells. (A) Protein expression of EGFR examined by Western blot in all wt and S178A clones. Ratios of EGFR levels relative to wt1 clone are indicated below the blot. (B) mRNA levels of EGFR in all wt and S178A clones. (C) EGFR downstream signaling towards Akt, ERK and JNK is affected in S178A clones. (D) Activity of AP-1 member c-Jun determined by TATA-luciferase reporter assay in all the different clones in serum-free conditions.

Re-expression of wt-EGFR in paxillinS178A cells restores EGF-driven cell motility and lung metastasis formation.

Finally, we determined the role of EGFR downregulation in the paxillinS178A cells. We re-expressed EGFR in paxillinS178A cells using retroviral transfection followed by FACS sorting (Fig. 6A). EGFR re-expression induced a more spread phenotype in paxillinS178A cells (see Fig. 6A). Both EGF-driven cell migration was rescued as well as the protein turnover of paxillinS178A at focal adhesions upon EGF stimulation (Fig. 6B; compare with Fig. 3C). Next we determined whether EGFR re-expression also restored the capacity of MTLn3 paxillinS178A cells to metastasize to the lungs. For this purpose we injected GFP-paxillinS178A cells and EGFR-GFP-paxillinS178A cells into the mammary fat pads of immunodeficient Rag2-/- γ-/- mice. The tumor growth of paxillinS178A and EGFR- paxillinS178A cells was similar in the tested animals (Fig. 6C-b) and all tumor cells in the primary tumors were GFP-positive (Fig. 6C-a). Importantly, there was an approximately threefold increase in the lung tumor burden in mice injected with GFP-paxillinS178A cells that expressed EGFR (Fig. 6D). These data indicate that the reduced metastasis formation of GFP-paxillinS178A cells is directly related to the expression levels of EGFR.

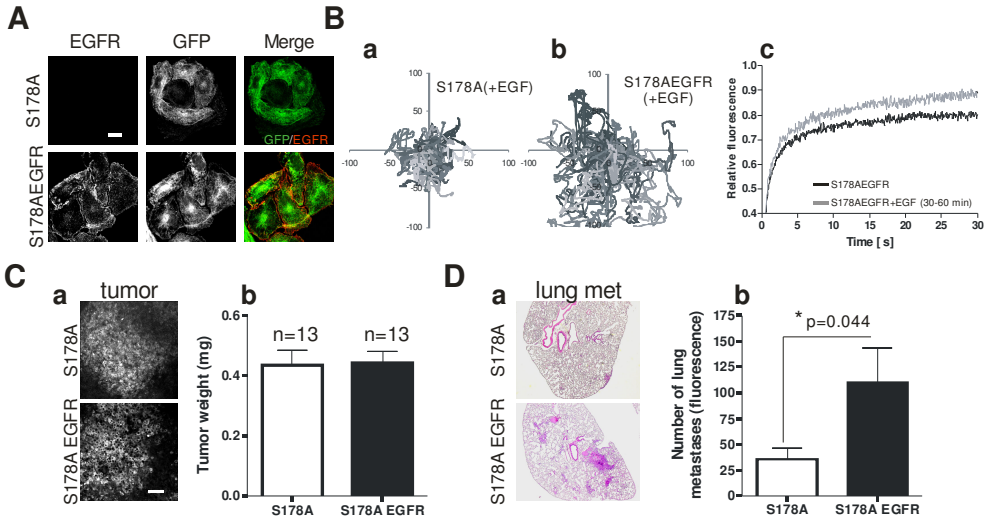

Figure 6. Re-expression of wt-EGFR fully restores EGF-driven cell motility and lung metastasis formation. (A) Immunostaining with a specific mouse monoclonal antibody against human EGFR confirmed the ectopic expression of EGFR in S178A cells; paxillinS178A (green) and human EGFR (red). Scale bar is 10 μm. (B) Trajectories of the movements of S178A and EGFR-S178A cells are plotted in (a) and (b) respectively (X and Y are in μm). Protein dynamics was quantified with spot bleaching technique (c). (C) Two-photon imaging of GFP-positive lung metastases, scale bar is 100 μm. (a). There was no significant difference in tumor weight of S178A (n=13) and EGFR-S178A (n=13) groups (b). (D) H&E staining of lung slices of S178A and EGFR-S178A groups (a). Number of GFP-positive metastases counted in the fresh lungs at time of sacrifice is shown (b).

DISCUSSION

Our data indicate that EGF-induced JNK activation is essential for MTLn3 cell migration. Phosphorylation of paxillin on Ser178 in MTLn3 cells was induced upon EGF stimulation in a manner sensitive to the JNK inhibitor, and expression of paxillin-S178A inhibited cell motility. Such a role for paxillin Ser178 in cell migration fits with observations in several different tumor cell lines (3,32-37). Here we further demonstrate that the JNK-paxillin axis regulates both the dynamics of focal adhesions through modulation of paxillin protein dynamics at focal adhesions as well as the stability of E-cadherin-based adherence junction formation. We do

not yet know how exactly the phosphorylation at Ser178 affects paxillin dynamics, but it is conceivable that Ser178 phosphorylation induces a conformational change affecting paxillin interactions with other focal adhesion components. Alternatively, the decreased EGFR expression in paxillinS178A cells may affect downstream signaling pathways that indirectly affect focal adhesion dynamics as well as adherence junction stability. Indeed, re-introduction of EGFR in paxillinS178A cells reversed the epithelial-like phenotype and rendered paxillinS178A again less immobile at focal adhesions, suggesting that this is not an intrinsic characteristic of paxillinS178A, but is rather due to altered signaling in cells mediated by downstream EGFR signaling pathways. This needs further investigation.

So far no *in vivo* data on the specific role of paxillin Ser178 in metastasis formation have been presented (38). Here, for the first time, we demonstrate that the Ser178 of paxillin is essential for spontaneous metastasis formation in an orthotopic breast tumor/metastasis model. Our findings are indicative for a role for the JNK-paxillin pathway in the regulation of the expression of EGFR, thereby severely affecting the capacity of tumor cells to migrate and metastasize. Thus, reduced levels of EGFR were observed in three independent paxillinS178A MTLn3 clones, resulting in decreased EGF-induced activation of signaling pathways downstream of EGFR. Given the fact that EGFR is often highly expressed in advanced breast cancer, and that EGFR antagonists inhibit spontaneous metastasis formation of MTLn3 cells, we anticipated that the reduced EGFR expression was an essential component of the inhibited cell migration and metastasis formation. Indeed, re-expression of the wt-EGFR in the paxillinS178A cells did rescue their migratory defects the migratory phenotype of MTLn3-paxillin-S178A cells as well as their metastatic capacity under *in vivo* conditions. This regulation of the EGFR by paxillin phosphorylation has not been described before. Interestingly, paxillinS178A MTLn3 cells showed reduced c-Jun transcriptional activity. In MCF7 cells stable overexpression of c-Jun induces an increase in EGFR expression suggesting that AP-1 transcription factors can regulate EGFR transcription levels (31); such a regulation is also observed in keratinocytes (30). We propose a role of the JNK-c-Jun signaling pathway in the regulation of EGFR expression at transcriptional level. Our preliminary data in MCF7 cells that have increased ectopic expression of EGFR demonstrate that paxillin is involved in EGFR recycling and downstream signaling (de Graauw, unpublished observations). Thus, an alternative explanation may be that JNK-

mediated phosphorylation of paxillin Ser178 is required for efficient EGFR endocytosis and recycling, an essential component in its activation (39). Hence this will affect proper JNK activation and AP-1 transcription factor activation thereby providing a positive feedback for EGFR expression. Alternatively, paxillin may affect the expression of EGFR by its known role in the regulation of gene expression through its interaction with ERK (22), poly-A-binding protein (40), Abl (41) and steroid receptors, or through its own ability to undergo nucleocytoplasmic shuttling (22,42).

Our findings indicate that the JNK-paxillin axis modulates the scattered phenotype of MTLn3 cells. Both inhibition of JNK as well as ectopic expression of paxillinS178A mutant reversed the scattered phenotype towards a more epithelial-like morphology with the formation of E-cadherin/β-catenin cell-cell junctions. This morphological switch was observed under serum-starved and EGF-treated conditions. The (in)direct tyrosine phosphorylation of β-catenin by EGFR and other receptor tyrosine kinases such as c-Met is known to destabilize its binding to E-cadherin. Because of the low EGFR expression in the paxillin mutant cells, we anticipate that this results in de-phosphorylation of β-catenin leading to its localization to the cell-cell contacts. These data suggest that paxillinS178A does not affect the intrinsic molecular components and machinery required for the scattered phenotype and support the notion that the effect of paxillinS178A is rather related to the defects in the EGFR signaling pathways. Consequently, the defects in *in vivo* metastasis formation of MTLn3-paxillinS178A cells are most likely largely due to defects EGF signaling. This fits with our observations that ectopic EGFR expression itself is sufficient to again allow metastasis formation of paxillinS178A cells.

In summary, our data provide insight into the crucial role of JNK-mediated phosphorylation of paxillin Ser178 in tumor cell migration and metastatic spread, which is directly related to maintenance of EGFR expression. Given the essential role of Ser178 phosphorylation in the migration and metastasis formation, monitoring this paxillin phosphorylation in tumor samples from patients may be indicative of the activation of this pro-metastatic pathway and possibly predictive for the disease prognosis. Moreover, with the further development of specific JNK inhibitors it is anticipated that novel targeted therapies that antagonize the migratory/invasive behavior of tumor cells may be used in the clinic in the future. Further work in this area is required.

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SUPPLEMENTAL DATA

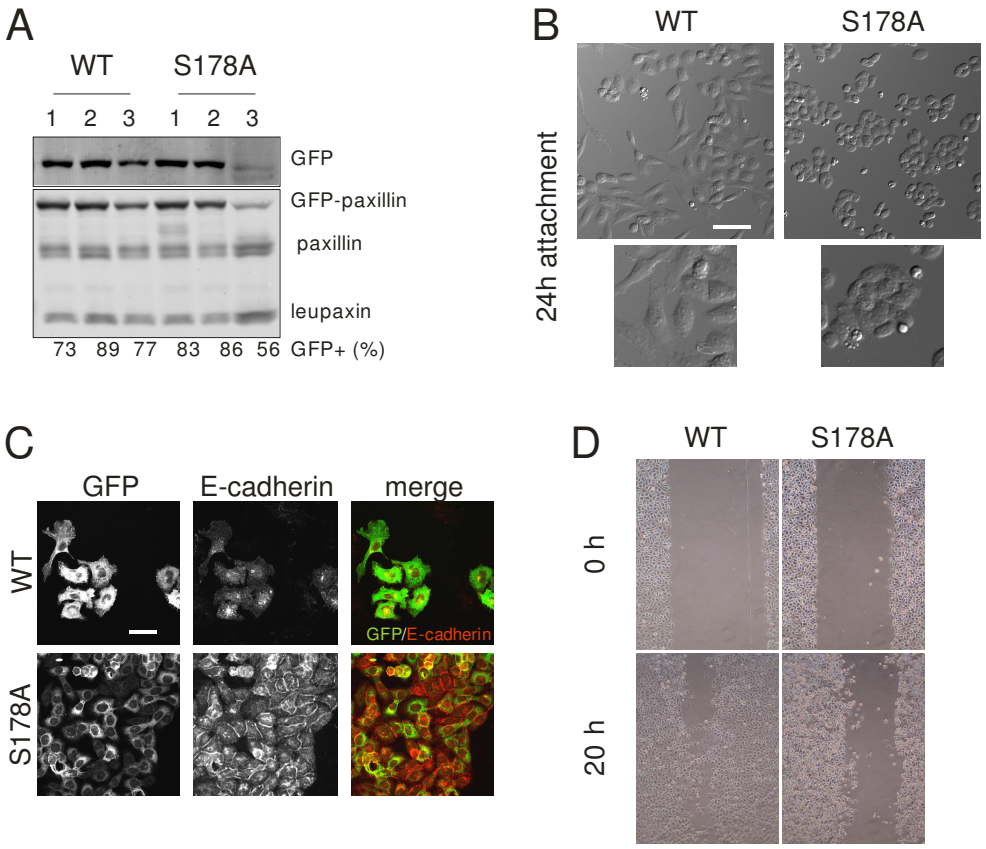

Figure S1. Expression of paxillinS178A mutant decreases cell spreading and directed cell migration. (A) Expression of GFP-paxillin examined by Western blotting. The GFP signal was also detected by FACS analysis; percentage of positive cells is indicated. (B) wt and S178A cells after 24 hours attachment. Scale bar is 50 μm. (C) Immunostaining of E-cadherin (red) and GFP-paxillin (green). Scale bar is 20 μm. (D) Snapshots of the wound made in wt and S178A cells at 0 hours and 20 hours.

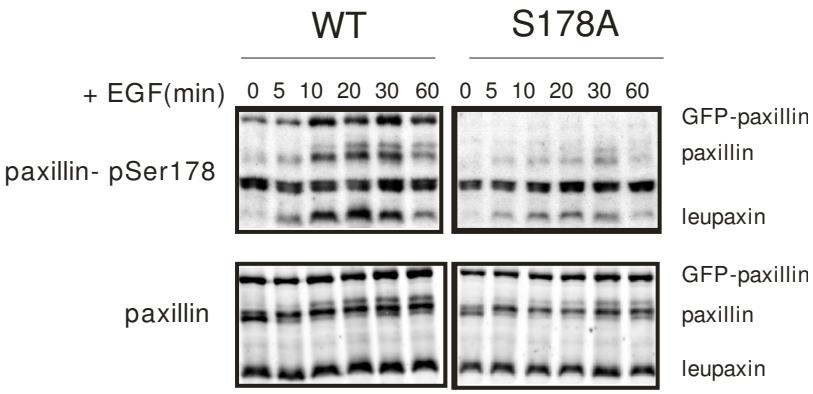

Figure S2. Phosphorylation of endogenous paxillin Ser178 after treatment with EGF for 60 minutes. PaxillinS178A mutant overexpression impairs phosphorylation of endogenous paxillin at Ser178 after EGF treatment for 0, 5, 10, 20, 30 and 60 minutes.