ARTICLE
Journal of Cellular Biochemistry 114:1744–1759 (2013)

Journal of Cellular Biochemistry

Dynamics of Focal Adhesions and Reorganization of F-Actin in VEGF-Stimulated NSCs Under Varying Differentiation States

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ABSTRACT

Precise migration of neural stem/progenitor cells (NSCs) is crucially important for neurogenesis and repair in the nervous system. However, the detailed mechanisms are not clear. Our previous results showed that NSCs in varying differentiation states possess different migratory ability to vascular endothelial growth factor (VEGF). In this study, we demonstrate the different dynamics of focal adhesions (FAs) and reorganization of F-actin in NSCs during spreading and migration stimulated by VEGF. We found that the migrating NSCs of 0.5 and 1 day differentiation possess more FAs at leading edge than cells of other states. Moreover, the phosphorylation of focal adhesion kinase (FAK) and paxillin in NSCs correlates closely with their differentiation states. VEGF promotes FA formation with broad lamellipodium generation at the leading edge in chemotaxing cells of 0, 0.5, and 1 day differentiation, but not in cells of 3 days differentiation. Furthermore, cells of 1 day differentiation show a maximal asymmetry of FAs between lamella and cell rear, orchestrating cell polarization and directional migration. Time-lapse video analysis shows that the disassembly of FAs and the cell tail detachment in NSCs of 1 day differentiation are more rapid, along with the concurrent enlarged size of FAs at the leading edge, leading to the most effective chemotactic response to VEGF. Collectively, these results indicate that the dynamics of FAs and reorganization of F-actin in NSCs that undergo directional migration correlate closely with their differentiation states, contributing to the different chemotactic responses of these cells to VEGF. J. Cell. Biochem. 114: 1744–1759, 2013.

KEY WORDS: NEURAL STEM CELLS (NSCs); VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF); DIRECTIONAL MIGRATION; DIFFERENTIATION; FOCAL ADHESIONS (FAs)

Neural stem/progenitor cell (NSC) migration is an essential process for the development of the central nervous system (CNS) and the ongoing neurogenesis that occurs in the mature CNS of most vertebrate species, as well as the transplantation of NSCs for neural regeneration and nerve repair after injury [Sun et al., 2004; Okano and Sawamoto, 2008]. It has been reported that only a small subpopulation of the transplanted NSCs successfully reach the damaged brain areas [Lindvall et al., 2004; Shah et al., 2008], and that NSCs migrating out of the neurospheres derived from the subventricular zone of newborn rats in response to VEGF stimulation are immunoreactive for GFAP and nestin [Mani et al., 2010], suggesting that tropism for pathology in the brain is likely to be exhibited by a subpopulation of NSCs that is at a specific differentiation stage. In line with this, we have proved that the directional migration of NSCs closely correlates with their differentiation states [Liu et al., 2011]. However, the underlying mechanisms are not yet clear.

During cell migration, new focal adhesions (FAs), which are highly dynamic and undergoing continuous assembly and disassembly (named as turnover) [Burnette et al., 2011; Kuo et al., 2011], are established at the leading edge [Nobes and Hall, 1995] after a membrane protrusion being generated and driven by

The authors declare no conflict of interest.

Additional supporting information may be found in the online version of this article.

Grant sponsor: National Natural Science Foundation of China; Grant numbers: 31071220, 30870642; Grant sponsor: Suzhou City Science and Technology Project; Grant number: SYS201203.

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Manuscript Received: 10 December 2012; Manuscript Accepted: 4 February 2013

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 26 February 2013

DOI 10.1002/jcb.24517 • © 2013 Wiley Periodicals, Inc.

actin polymerization in the direction of movement [Powner et al., 2011], then a contractile force drives the cell body forward, and the rear part of the cell is detached from the substrate with FAs disappeared [Zaidel-Bar et al., 2003]. Initial assembly of matrix contacts (called as focal complexes) are smaller and display more rapid turnover than FAs [Broussard et al., 2008]. In order for cells to undergo persistent motility, FA dynamics must also be coordinated with the assembly of new F-actin-containing protrusions, which are under the control of several actin-nucleating and elongating molecules [Le Clainche and Carlier, 2008]. Within the cell body, F-actin is bundled into stress fiber, generating contractile forces by pulling against FAs to induce retraction of the rear cell membrane during migration [Wozniak et al., 2004].

FA assembly and turnover are associated with tyrosine phosphorylation of FA proteins such as 68-kDa protein paxillin and 125-kDa protein focal adhesion kinase (FAK) [Panetti, 2002]. In addition to binding directly to integrin cytodomains, paxillin contains numerous protein-binding modules that interact with a variety of structural and signaling proteins and is therefore classified as a molecular adaptor or scaffold protein [Turner, 2000]. Tyrosine phosphorylation of paxillin has been shown to be important for the formation of FAs [Richardson et al., 1997; Jackson et al., 2011], and phosphorylation at Y31 (tyrosine 31) and Y118 is activated by integrin gather or several tyrosine kinases (such as Src, FAK) during cell migration [Richardson et al., 1997; Nakamura et al., 2000; Jasinski et al., 2011]. Overexpression of Y31/118-paxillin enhances lamellipodial protrusion and formation of focal complexes and FAs, whereas cells overexpressing a non-phosphorylatable paxillin mutant display prominent stable fibrillar adhesions, which are developed from FAs by actomyosin contractility [Zaidel-Bar et al., 2007]. FAK, an integrin-binding nonreceptor tyrosine kinase that is activated to autophosphorylate at Y397 [Schaller et al., 1994], is recruited to Y31/118-paxillin and its overexpression triggers FA disassembly [Zaidel-Bar et al., 2007]. Knockdown of FAK or mutation of FAK at Y397 fails to induce FA disassembly and induces large and stable FAs leading to migratory deficits [Sieg et al., 1999; Wagner et al., 2008].

Previous results in our lab demonstrated that vascular endothelial growth factor (VEGF), a well-known factor that regulates the directed migration of NSCs [Zhang et al., 2003], induces chemotaxis migration of NSCs by activation of PI3-kinase and MAPKs pathways depending on their differentiation status, and that NSCs of 1 day differentiation exhibit the most effective directional migration [Liu et al., 2011]. However, the dynamics of FAs and reorganization of F-actin in NSCs during directional migration remain unclear.

In this study, we analyzed the dynamics of FAs and reorganization of F-actin in VEGF-stimulated NSCs with respect to their differentiation states. We found that the formation of FAs and organization of F-actin in NSCs in varying differentiation states during cell spreading after seeding are different. By using a wound model in which a denuded area (wound area) was produced by scratching a monolayer of NSCs in varying differentiation states and cells migrated into the wound area, we found that more FAs at leading edge after wounding were formed in NSCs of 0.5 and 1 day differentiation, than cells of undifferentiation and 3 days differentiation. Furthermore, in the lamellipodium generated in the front of undifferentiated NSCs, Y31-paxillin distally gathers in both small focal complexes and FAs while Y118-paxillin accumulates at stable FAs close to cell center. VEGF induces the remodeling of FAs in a temporal and spatial manner with stress fiber formation, the degree of which varies, depending on cell differentiation states: first, 5 ng/ml VEGF increases the number of FAs in cells of 0, 0.5, and 1 day differentiation, but not in cells of 3 days differentiation, whereas the distribution of FAs in cells of 0.5 and 3 days differentiation is changed by stimulation, which is not observed in cell in 0 or 1 day differentiation state; second, when NSCs are exposed to VEGF at concentration ranging from 5 to 100 ng/ml for 30 min, the number of FAs at the periphery in NSCs of 0 and 0.5 day differentiation is decreased only at 50 ng/ml while that in cells of 1 day differentiation is increased at 50 and 100 ng/ml, but not at other concentrations, which is not changed in cells of 3 days differentiation; and finally, the phosphorylation status of FAK and paxillin is closely related to the differentiation levels of NSCs subjected to VEGF. During the chemotactic migration carried out by Dunn chamber, VEGF promotes focal complex formation and increases the number of FAs with broad lamellipodium generation at the leading edge in cells of 0, 0.5, and 1 day differentiation, but not in cells of 3 days differentiation, and cells of 1 day differentiation show a maximal asymmetry of FAs between lamella (located inward from the lamellipodia) and cell rear. It is confirmed by Time-lapse video analysis, which also shows that FAs disassemble at cell rear and cell tails detach to facilitate cell body forward movement. More importantly, in NSCs of 1 day differentiation, FAs at the trailing edge slide transversally into cell body more quickly to induce a rapid sharp retraction of tail and a directional migration to VEGF. Taken together, these results indicate that dynamics of FAs and reorganization of F-actin in NSCs that undergo directional migration upon VEGF stimulation correlates closely with their differentiation states, contributing to the different chemotactic responses of NSCs of varying differentiation states.

MATERIALS AND METHODS

CELL CULTURE

C17.2 cells (provided by Dr. Evan Snyder, Burnham Institute for Medical Research) were cultured in high glucose-containing Dulbecco's modified Eagle's medium (H-DMEM), supplemented with fetal calf serum (FBS, 10%; Gibco, Grand Island, NY), horse serum (5%; Gibco), sodium pyruvate (1 mM; Invitrogen), 2 mM L-glutamine, and 1% penicillin/streptomycin. Cells were split at 1/5–1/10 when cells reached approximately 90% confluence. The cultures were maintained in a standard humidified incubator in 5% CO_2 at 37°C.

Cells were induced to differentiate by transferring into the induction medium (DMEM/F12 (Gibco) supplemented with 1% N_2 (Gibco), 2 mM glutamine, and 1% (penicillin/streptomycin) when cells reached approximately 50% confluence [Liu et al., 2007, 2011], and cells at 0 day (undifferentiated), 0.5, 1, and 3 days after differentiation were used in the following experiments.

For Western blot and immunocytochemistry analysis, undifferentiated or differentiating NSCs were treated with VEGF of different concentrations (5, 25, 50, and 100 ng/ml) for 30 min or with 5 ng/ml

VEGF for different times (1, 5, 15, 30, and 60 min) after starvation in serum-free medium for 30 min.

All animal experiments were approved by the local government and were conducted according to the local guidelines and law. The subventricular zone (SVZ) was dissected from coronal slices of newborn rat brains, dissociated mechanically, and trypsinized as described previously [Liu et al., 2011]. SVZ progenitors were purified using Percoll (22%; Amersham Biosciences, Sweden) gradient centrifugation and seeded at a density of 5×10^4 cell/ml onto 1 μg/cm^2 laminin-coated (BD) coverslips. Isolated cells were allowed to grow in neurobasal medium (Gibco) supplemented with 2% B27 (Gibco), 2 mM glutamate, 1 mM sodium pyruvate, and 1% penicillin–streptomycin. Cultures were fed every 3 days on fresh medium.

IMMUNOCYTOCHEMISTRY

Immunostaining of cultures was performed as described previously [Zhang et al., 2003; Liu et al., 2011]. Briefly, NSCs were fixed in cold (4°C) 4% paraformaldehyde in 0.1 M phosphate buffer overnight, then washed three times with phosphate-buffered saline (PBS), pH 7.2, and incubated with rabbit polyclonal Ab paxillin (1:50 dilution; Santa Cruze Biotechnology, Inc.) for 1.5 h at room temperature or overnight at 4°C. Primary Abs were diluted in PBS/3% BSA/0.03% NaN_3/0.2% Triton X-100. After incubated with primary antibodies, cells were rinsed with PBS three times, for 5 min each, prior to secondary antibody application. Then cells were incubated in FITC-conjugated goat anti-rabbit IgG secondary Abs (1:200 diluted in PBS/3% BSA/0.03% NaN_3 solution; Proteintech) for 1 h at room temperature in the dark. Cells were rinsed with PBS three times, for 5 min each. For F-actin staining, cells were then incubated with TRITC-phalloidin (1:2,000; Invitrogen) for 40 min, washed with PBS and mounted in 80% glycerol/20% water mix immersion. Fluorescence was examined with Leica DMI 6000 B microscope. Controls treated with nonspecific mouse IgM or secondary Abs alone showed no staining.

WESTERN BLOT ANALYSIS

Cells were exposed to liquid nitrogen and then lysed with protein extraction reagent (25 mM Tris–HCl, pH 7.2, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM EDTA, 0.1% SDS, 1% PMSF, and 1 mM $NaVO_3$). Lysates were centrifuged at 12,000 rpm at 4°C for 5 min to remove cell debris. The cleared supernatants were transferred to fresh tubes, and protein concentrations were determined by BCA assay kit (Applygen). Identical amounts (20–30 μg) of protein lysates were separated using 10% SDS–PAGE gels and transferred to a 0.45-μm nitrocellulose membrane (Millipore, Bedford, MA) at a constant 2.5 mA/cm^2 for 30 min using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA). After blocking with 5% nonfat milk in TBST (100 mM Tris–HCl pH 7.4, 150 mM NaCl, with 0.1% Tween-20), the membrane was probed with primary antibodies for phospho- or nonphospho-protein kinases (rabbit antibody (Ab) antiphospho-paxillin (Y31/Y118), 1:1,000 dilution, rabbit Ab anti-paxillin, 1:1,000 dilution, Santa Cruze Biotechnology, Inc.; rabbit Ab antiphospho-FAK (Y397), 1:1,000 dilution, rabbit Ab anti-FAK, 1:1,000 dilution, Cell Signaling Technologies; mouse monoclonal antibody (mAb) anti-vinculin, 1:70 dilution, mouse mAb anti-β-actin, 1:200; Boster Bio-Engineering Limited Company) overnight at 4°C. Membranes were then washed three times, 10 min each, with TBST and then incubated for 1 h at room temperature with the appropriate horseradish peroxidase-linked secondary antibody (1:2,000 dilution; Cell Signaling Technologies). Membranes were again washed three times, 10 min each, and antigen–antibody complexes were visualized by ECL (Beit Haemek).

FORMATION OF FAs IN CELLS MIGRATING INTO THE WOUND AREA

Wound healing assay was performed as described previously [Yu et al., 2009]. Briefly, NSCs in varying differentiation states cultured on the glass coverslips in 35 mm dishes as a monolayer were scraped with the narrow end of a pipette tip to generate a denuded area (wound area) ~0.1 cm in width and washed two times with pre-warmed medium. Then cells were cultured as usual in serum-containing medium for 6 h and cells that migrated into the wound area were stained with antibodies (paxillin, the Y31 or Y118 phosphorylation of paxillin, FAK, the Y397 phosphorylation of FAK, and TRITC-phalloidin). Fluorescence was examined with Leica DMI 6000 B microscope.

CELL TRANSFECTION

Cell transfection was performed according to the manufacturer and as described before [Pasapera et al., 2010]. Cells were transfected with 3 μg of EGFP-paxillin plasmids (provided by Clare M. Waterman, Cell Biology and Physiology Center, National Heart, Lung, and Blood Institute, National Institutes of Health) mixed with 2 μl of Lipofectamine2000 Transfection Reagent (Invitrogen) in 1 ml serum-free H-DMEM. After transfection for 4 h, cells were cultured in serum-containing medium as usual for 24 h before experiment.

FORMATION OF FAs IN NSCs DURING CHEMOTACTIC MIGRATION USING DUNN CHAMBER

Chemotaxis of C17.2 cells was directly viewed and recorded in stable concentration gradients of VEGF using a Dunn chamber (Hawksley), which allowed for generation of a stable chemotactic gradient and observation of cell migration in the context of the gradient [Zhang et al., 2003; Liu et al., 2011]. This device is made from a Helber bacteria counting chamber by grinding a circular well in the central platform to leave a 1-mm-wide annular bridge between the inner and the outer wells. Chemoattractants added to the outer well of the Dunn chamber diffuse across the bridge to the inner well and form a linear, steady gradient within 30 min of setting up the chamber. The gradient remains stable for 30 h thereafter [Liu et al., 2011]. This apparatus allows for the direct monitoring of cell locomotion and the analysis of migration speed, turning behavior and directionality of migration. Coverslips with cells transfected by EGFP-target paxillin were inverted onto the chamber, and cell migration and turnover of paxillin was recorded through the annular bridge between the concentric inner and outer wells. In chemotaxis experiments, the outer well of the Dunn chamber was filled with induction medium containing 50 ng/ml VEGF and the concentric inner well with only medium.

MICROSCOPY AND IMAGE ANALYSIS

Fixed and immunolabeled cells were imaged on an inverted microscope system (Leica AF6000 LX) with a CoolSNAP HQ charge-coupled device (CCD, Leica) using a 63 × 1.3 Glycerol objective lens (Leica) for cells plated on glass. Time-lapse imaging of EGFP-tagged paxillin in living cells by Dunn chamber was performed at 37°C on the same microscope and images were captured at 30 s intervals for 45 min. The laser was supplied by EL6000 external light source. For EGFP images and FITC images, the 488-nm laser was used, and for TRITC images, the 561-nm laser was used.

STATISTICAL ANALYSIS

The number of periphery, intermediate or total FAs and the ratio of FAs at the periphery to FAs in cell center, which provided an assessment of the distribution of FAs, in VEGF-stimulated NSCs under varying differentiation states was obtained from an average of 20 cells in each condition from three independent experiments. Data are presented as the mean ± SEM. Analysis of one-way variance (ANOVA) with Bonferroni multiple comparison method was used to evaluate the statistical significance of data. $^{*}P < 0.05$; $^{**}P < 0.01$.

RESULTS

FORMATION OF FAs AND POLYMERIZATION OF F-ACTIN IN SPREADING NSCs

As cell spreading is an essential step for cell migration, we examined the formation of FAs and polymerization of F-actin during this process. Consistent with the events in fibroblasts spreading [Senju and Miyata, 2009], paxillin, which is a component of focal adhesions (FAs) and smaller focal complexes, was initially diffused the entire cell body, then concentrated to form focal complexes or FAs in lamellipodium or filopodium 15 min after plating. The number of FAs was sharply increased from 15 to 30 min and maintained a steady growth from 1 to 5 h (Fig. 1A,B). F-actin was disorderly arranged in round sphere-like NSCs at the beginning of cell spreading, and polymerized with tiny focal complexes present in the lamellipodium at cell periphery after spreading for 15 min. In NSCs that were allowed to spread for 30 min, filopodia formed around cells while lamellipodium also existed at the cell periphery with cavity lack of filaments, where new focal complexes formed. The actin subsequently organized circular bundles, as a bridge between filopodia. At 1–2 h after plating, cells became flat, polygonal and polarized with appearance of fibrillar-like FAs. The proportion of cells having circular bundles was decreased, whereas the number of cells with straight bundles of actin filaments was increased. FAs began to distribute at intermediate ventral surfaces of the cells. When NSCs were allowed to spread for 5 h, they were completely polarized and stress fibers anchored by FAs were present at the periphery and the dorsal and ventral surfaces of the cells.

As shown in Figure 1C, during NSC spreading, phosphorylation of FAK at Y397 increased from 5 to 30 min and reduced at 2 h. Phosphorylation of FAK at Y397 is critical for the induction of downstream effects, including phosphorylation of Y31-paxillin to assemble FAs [Nakamura et al., 2000]. In this process, an increased activation of Y31-paxillin was observed, but the phosphorylation decreased at 1 h, which reversed at 2 h.

DIFFERENTIATION OF NSCs INFLUENCES THE ASSEMBLY OF FAs AND ORGANIZATION OF F-ACTIN

Undifferentiated cells, grown in serum-containing H-DMEM, had a small, round, or fibroblastic morphology. When cultures were allowed to differentiate in induction medium, cells displayed a slim morphology with lamellipodia after 1 day, and typical neuron-like cells appeared after 3 days differentiation [Liu et al., 2011]. To examine the assembly of FAs and the actin cytoskeleton in cells in varying differentiation states in vitro, cells were double stained with antibodies against paxillin and TRITC-phalloidin. Results showed that stress fiber bundles were terminally anchored by paxillin-containing focal adhesions (Fig. 2A). Similar to fibroblasts [Nakamura et al., 2000], the distribution of paxillin in NSCs exhibited three distinct sub-cellular localizations as follows: localization along the cell periphery colocalized with circumferential actin meshworks, macroaggregation at FAs connected to actin stress fibers, and diffuse cytoplasmic distribution. However, immunofluorescence microscopy showed significant differences in size and distribution of FAs among NSCs in varying differentiation states. Unlike NSCs of 0 day (undifferentiation) and 0.5 days differentiation, in which there were many fibrillar-like FAs, NSCs of 1 day differentiation formed dot-like FAs at cell periphery (Fig. 2A) whereas small and short punctuate paxillin-containing adhesions at the turning point of dendrites and elongated FAs in cell center was observed NSCs of 3 days differentiation (Fig. 2A). We also observed FAs in primary neurons isolated from the subventricular zone (SVZ) of newborn rat brain cultured for 5–13 days and found dot-like vinculin- or paxillin-positive FAs at the turning point of dendrites with no visible FAs in cell center (Supplementary Fig. S1). The number of FAs and ratio of FAs at the periphery to FAs in the center, which provided an assessment of the distribution of FAs, were collected under each condition and quantified using SPSS software. Quantified data were obtained from an average of 20 cells in each differentiation state from three independent experiments. Quantitative analysis showed that the number of FAs in the center of NSCs of 3 days differentiation was more than that of undifferentiated NSCs with no change in FAs at the periphery, leading to a decreased ratio of FAs (Fig. 2B). In motile cells, many mature adhesions decrease in size or totally disassemble in the perinuclear region to promote cell migration [Broussard et al., 2008]. The number of FAs in NSCs of 1 day differentiation was the least among that in cells in varying differentiation states, suggesting that these cells might exhibit stronger cell motility, which was confirmed in a follow-up experiment (as shown in Fig. 8C and Supplementary Movie 6).

Cell protrusive activity and the formation of FAs are dynamically regulated by the actin cytoskeleton, and we observed marked differences in the actin cytoskeleton in NSCs in varying differentiation states. NSCs of 0.5 and 1 day differentiation typically exhibited major stress fiber bundles aligned to the long axis of the cell body (Fig. 2A), while undifferentiated cells showed a loose arrangement of F-actin. By contrast, actin polymerized at the tip of dendrites and stress fibers in the middle radiated from several foci in NSCs of 3 days differentiation.

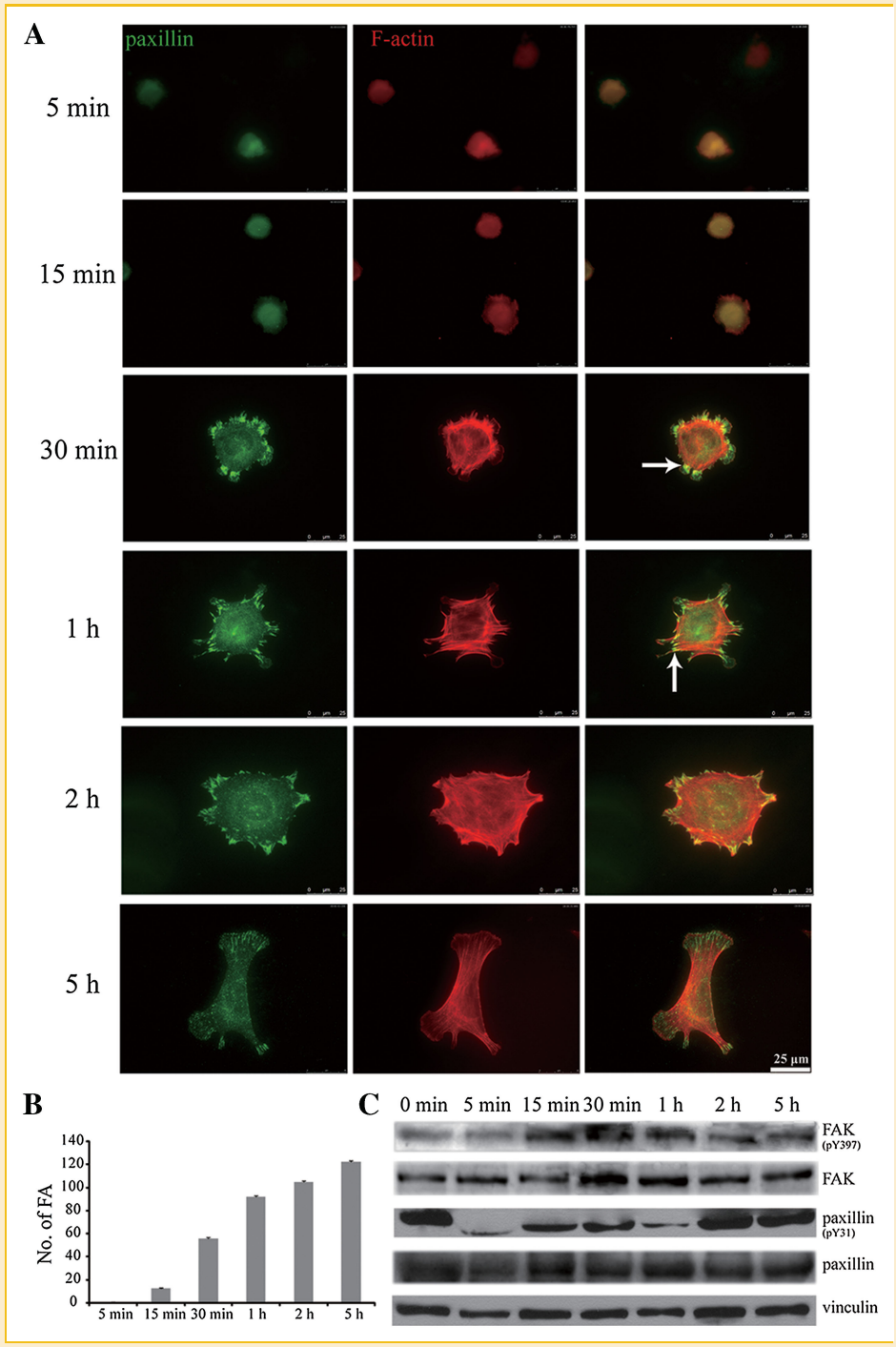

Fig. 1. Formation of FAs and organization of F-actin in spreading NSCs. A: NSCs were fixed at 5 min, 15 min, 30 min, 1 h, 2 h, or 5 h after plating, and double stained with paxillin (green) and TRITC-phalloidin (red). Paxillin was initially concentrated to focal complexes at 15 min after plating then enlarged to FAs. F-actin was polymerized with tiny focal complexes present in the lamellipodium at cell periphery after spreading for 15 min. In NSCs that were allowed to spread for 30 min, filopodia formed around cell co-localizing with fibrillar-like adhesions (as shown by arrow) and subsequently organized circular bundles, as a bridge between filopodia. B: The number of FAs during the process of NSCs spreading. Bar, 25 μm. C: NSCs in A were lysed and immunoprecipitated with primary antibodies for phospho- or nonphospho-protein (FAK and paxillin).

FORMATION OF FAs AND ORGANIZATION OF F-ACTIN IN NSCs MIGRATING INTO THE WOUND AREA UNDER VARYING DIFFERENTIATION STATES

Directed cell migration requires front-rear cell polarization and plays a fundamental role in neurogenesis and repair in the nervous system, wound repair as well as in tumor cell metastasis [Yu et al., 2009]. We examined the formation of FAs and organization of F-actin in NSCs, which were migrating to the wound area produced by scratching the confluent monolayers under varying differentiation states (Fig. 3A). Six hours after scratching, compared with

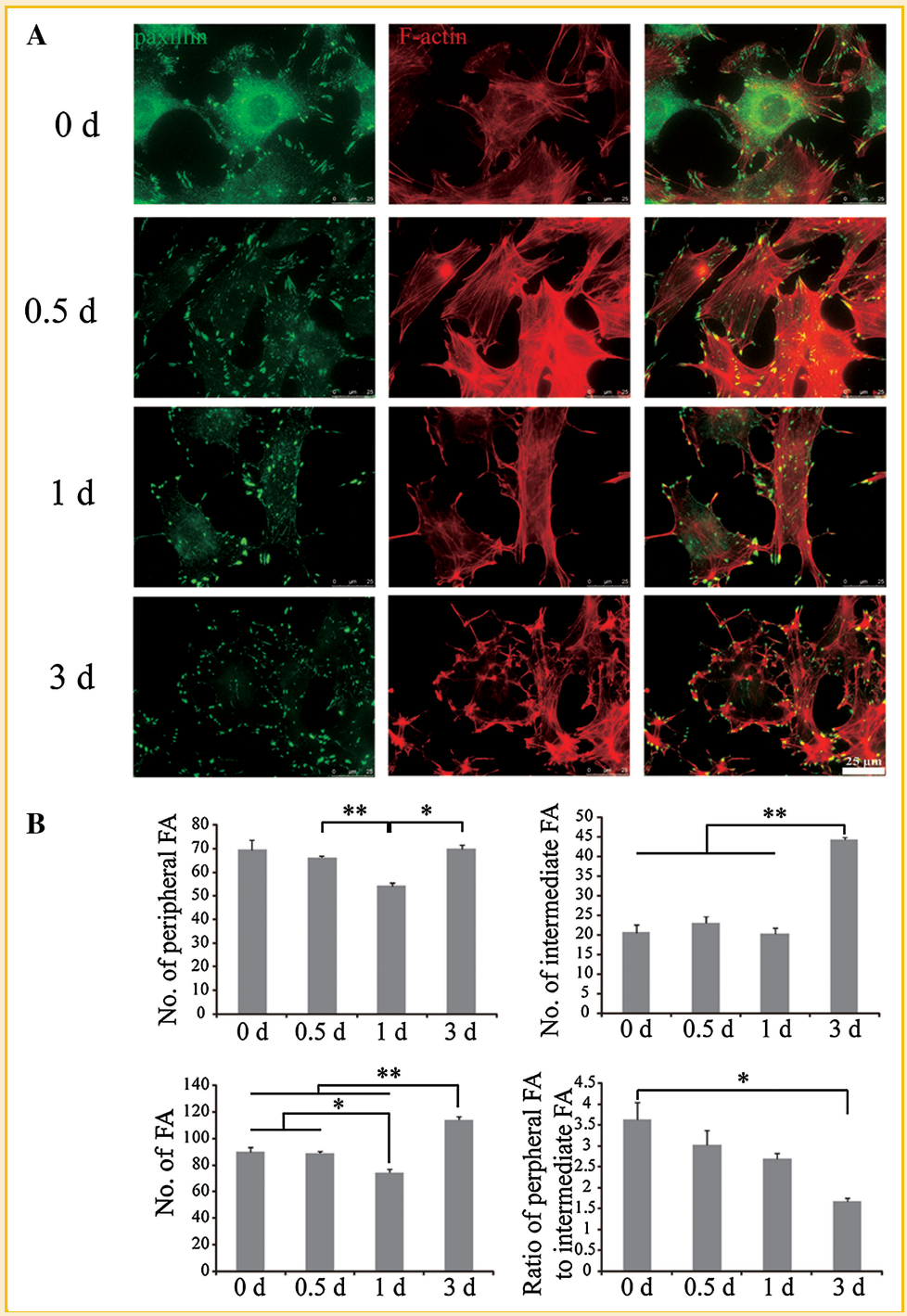

Fig. 2. Assembly of FAs and organization of F-actin in NSCs of varying differentiations. A: NSCs cultured in induction medium for 0 day (undifferentiated), 0.5, 1, and 3 days were double stained with paxillin (green) and TRITC-phalloidin (red). Compared with NSCs of 0 and 0.5 days differentiation, NSCs of 1 day differentiation formed large elongated or dot-like FAs at the periphery. NSCs of 3 days differentiation formed small and short punctuate adhesions at the turning point of dendrites and elongated FAs in cell center. FAs in NSCs of 3 days differentiation were diffuse and symmetric rather than accumulating at the cell periphery. Bar, 25 μm. B: The number of FAs and the ratio of FAs at the periphery to FAs in the center (short for the ratio of FAs) in cells (>20 cells, n = 3) under the conditions in A. FAs in the center of NSCs of 3 days differentiation were more than those in cells of 0, 0.5, and 1 days differentiation, leading to a decreased ratio of FAs. Data represent the mean ± SEM from at least three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

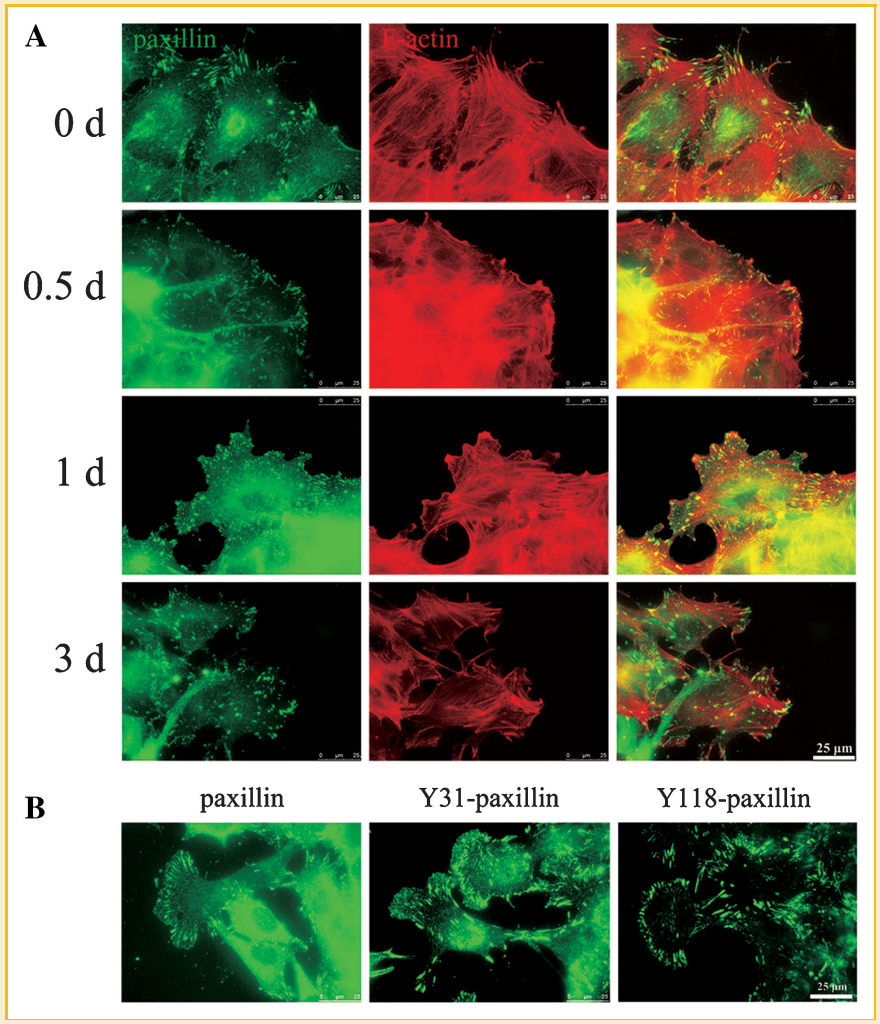

Fig. 3. The formation of FAs and organization of F-actin in migrating cells after wounding. A: NSCs, which were directed migrating from wounding area 6 h post-scratching, were double stained with paxillin (green) and TRITC-phalloidin (red). Cells of 0.5 and 1 day differentiation exhibited a characteristic polarized morphology with extension of flat lamellipodia at the leading edge and a slim trailing edge. Meanwhile, paxillin was recruited to focal complexes and FAs in the front of the lamellipodia, displaying an asymmetry distribution of FAs. Bar, 25 μm. B: Phosphorylation of paxillin in assembly of FAs in NSCs during directional migration. NSCs were stained with paxillin or Y31/118-paxillin (green) at 6 h after wounding. At the leading edge of NSCs, Y31-paxillin gathered in small FAs at cell periphery while Y118-paxillin localized in mature FAs closely to the center of cell body. Bar, 25 μm.

undifferentiated cells, in which showed a fibroblastic morphology, NSCs of 0.5 and 1 day differentiation exhibited a characteristic polarized morphology, with extension of flat membrane protrusion called lamellipodia at the leading edge and a slim trailing edge. In these cells, paxillin was recruited to focal complexes and FAs in the lamellipodium at the leading edge and there were more FAs in the front than those at cell rear, leading to an asymmetric distribution of FAs. NSCs of 3 days differentiation hardly migrated after wounding, and the distribution of FAs and patterns of F-actin arrangement were similar to untreated cells.

Phosphorylation of paxillin at Y31/Y118 and FAK at Y397 has been proved to take part in FA assembly and to regulate cell directional migration. Using phosphorylation site-specific antibodies, we detected that at the leading edge of NSCs, Y31-paxillin distally gathered in small focal complexes and stable FAs while Y118-paxillin localized at stable FAs close to the middle of cell body (Fig. 3B and Supplementary Fig. S2). We also examined the localization of Y397-FAK and found it colocalized with FAK at FAs in undifferentiated NSCs in this process (data not shown), consistent with previous result that the recruitment of FAK to FAs is essential for its activation and function [Scheswohl et al., 2008].

THE FORMATION OF FAs AND ORGANIZATION OF F-ACTIN IS CLOSELY RELATED TO THE DIFFERENTIATION LEVELS OF NSCs SUBJECTED TO VEGF

Because 5 ng/ml is a minimum concentration of VEGF that could induce chemotaxis of NSCs, which is correlated closely with their differentiation states [Liu et al., 2011], we used this concentration to analyze formation of FAs and rearrangement of F-actin in

differentiating NSCs in vitro. Results showed that VEGF-treated undifferentiated NSCs exhibited a characteristic polarized morphology, exhibiting a leading edge with extension of lamellipodia and filopodia and a slim trailing edge (Fig. 4A). Immunofluorescence staining of paxillin showed that the number of FAs in those cells was significantly increased after treatment with VEGF for 15 min relative to 1 and 5 min, meanwhile the ratio of FAs at the cell periphery to FAs in cell center was decreased by the treatment for 60 min compared to that in untreated cells, suggesting that VEGF could induce the remodeling of FAs in undifferentiated NSCs. After stimulation from 1 to 15 min, large and stable FAs were generally present in terminal of parallel stress fibers, while small focal complexes were mainly concentrated in lamellipodium.

As shown in Figures 4–6 and Supplementary Figures S3 and S4, the distribution and the number of FAs in differentiating NSCs exposing to VEGF at 5 ng/ml altered differently upon differentiation, illustrating that formation of FAs in VEGF-stimulated NSCs is correlated to their differentiation states. After stimulation of VEGF at 5 ng/ml, NSCs of 0.5 and 1 day differentiation exhibited an elongated cell morphology with the presence of a unique persistent lamellipodium and small adhesions (Figs. 5 and 6A), which was thought conducive to cell migration [Le Clainche and Carlier, 2008], especially at 15 min. Treatment with VEGF induced a significant increase in the number of FAs (Figs. 5 and 6C), especially at 15 min, while most of them localized at cell periphery. By contrast, the ratio of FAs in NSCs of 3 days differentiation was significantly increased by treatment with VEGF for 60 min although there was no change in the number of FAs (Supplementary Fig. S4A,C).

Previously, we showed that the chemotactic migration of NSCs to VEGF was in a dose-dependent manner [Liu et al., 2011]. The formation of FAs and reorganization of F-actin induced by VEGF at concentrations ranging from 5 to 100 ng/ml were then studied. As shown in Figures 4 and 5D, the number of peripheral FAs was decreased significantly and there were more small focal complexes, which turn over faster than FAs to promote cell migration [Broussard et al., 2008], localized at cell periphery when NSCs of 0 and 0.5 days differentiation were exposed to VEGF only at 50 ng/ml compared with other concentrations, indicating that VEGF at 50 ng/ml could induce the formation of focal complexes and prevent maturity of FAs. This is consistent with previous research that VEGF at 50 ng/ml induces significant migration of undifferentiated NSCs [Liu et al., 2011]. By contrast, VEGF at 50 and 100 ng/ml, but not other concentrations, significantly increased the number of peripheral FAs in NSCs of 1 day differentiation (Fig. 6B,D). The formation of FAs in VEGF-stimulated NSCs of 3 days differentiation (Supplementary Fig. S4B,D) was hardly altered, proving that the formation of FAs in those cells was not sensitive to stimulation.

TRITC-phalloidin staining revealed that unlike loose and random network of actin filaments in unstimulated cells, actin filaments in cell center changed into well-organized and clear stress fibers along the cell axis after the stimulation of VEGF (Figs. 4–6A,B and Supplementary Figs. S3 and S4). Meanwhile F-actin polymerized to form broad lamellipodium at the periphery of NSCs of 0, 0.5, and 1 day differentiation when they were treated with VEGF for 5 and 15 min (Figs. 4–6A,B).

PHOSPHORYLATION OF PAXILLIN AND FAK IN VEGF-STIMULATED NSCs

Cell attachment stimulates a series of tyrosine phosphorylation on adhesion proteins, which form phosphorylated SH2 and SH3 domains necessary for FA assembly [Mitra et al., 2005]. In particular, tyrosine phosphorylation of FAK on Y397 and paxillin on Y31/Y118 play an important role in FA formation [Sieg et al., 2000]. To check the activation of focal adhesion proteins in NSCs stimulated by VEGF, we monitored levels of Y397-FAK and Y31/Y118-paxillin by Western blot and found that activation of FAK and paxillin in NSCs subjected to VEGF was closely related to their differentiation states (Fig. 7). Five nanogrm pre milliliters of VEGF increased the level of Y397-FAK in undifferentiated and differentiating cells. Unlikely cells of 0.5 and 1 day differentiation (Fig. 7B,C), in which a maximal phosphorylation of FAK was observed at 5 min, cells of 0 and 3 days differentiation showed a maximal activation occurring at 30 and 15 min separately (Fig. 7A,D). Phosphorylation of FAK at Y397 in NSCs of 3 days differentiation (Fig. 7D) was significantly lower than that in cells at other differentiation states, which indicated the slowest turnover of FAs and increased the number of FAs in those cells (as shown in Figs. 2 and 8). Although exposing to 5 ng/ml VEGF induced an increased expression in undifferentiated NSCs (Fig. 7A), no obvious change of Y118-paxillin was detected in cells of 1 day differentiation (Fig. 7C). By contrast, VEGF firstly increased and then decreased the phosphorylation of paxillin at Y118 from 15 min in cells of 0.5 to 3 days differentiation (Fig. 7B,D). A sustained phosphorylation of Y31-paxillin was present in NSCs of undifferentiated and 3 days differentiation (Fig. 7A,D) whereas NSCs of 0.5 days differentiation showed a transient and maximal activation at 1 min which last to 5 min (Fig. 7B). Notably, decreases in expression of paxillin by VEGF (Fig. 7), which may be correlated with the ubiquitination regulated by Wnt signaling [Iioka et al., 2007] need to be studied further.

Responses of phosphorylation of FAK and paxillin to VEGF at concentrations ranging from 5 to 100 ng/ml were also tested and found that they were closely dependent on cell differentiation. As shown in Figure 7, in undifferentiated NSCs, VEGF at varying concentrations increased the activation of Y397-FAK and Y31/Y118-paxillin. However, the concentration of VEGF that induced the maximal activation was different, which was 5 ng/ml for Y397-FAK, 25 ng/ml for Y118-paxillin, and 100 ng/ml for Y31-paxillin. In NSCs of 0.5 days differentiation, phosphorylation of FAK at Y397 was gradually decreased by the increase of VEGF concentration, while phosphorylation of paxillin at Y31 was suddenly increased by VEGF at 100 ng/ml and Y118 was decreased below basal level at 50 and 100 ng/ml. Phosphorylation of FAK at Y397 was significantly increased at 50 ng/ml while decreased to basal level at 100 ng/ml in NSCs of 1 day differentiation. Meanwhile, there was no significantly change in activation of Y118/Y31-paxillin. By contrast, VEGF of 100 ng/ml significantly increased the phosphorylation of FAK at Y397 while treatment with VEGF from 5 to 100 ng/ml for 30 min decreased the phosphorylation of paxillin at Y118/Y31 in NSCs of 3 days differentiation. Unlike the decrease of paxillin in NSCs stimulated by VEGF at 5 ng/ml for different times, an increase in the translation of paxillin, which was found in epithelial cell stimulated with hepatocyte growth factor [Hopkins et al., 2004],

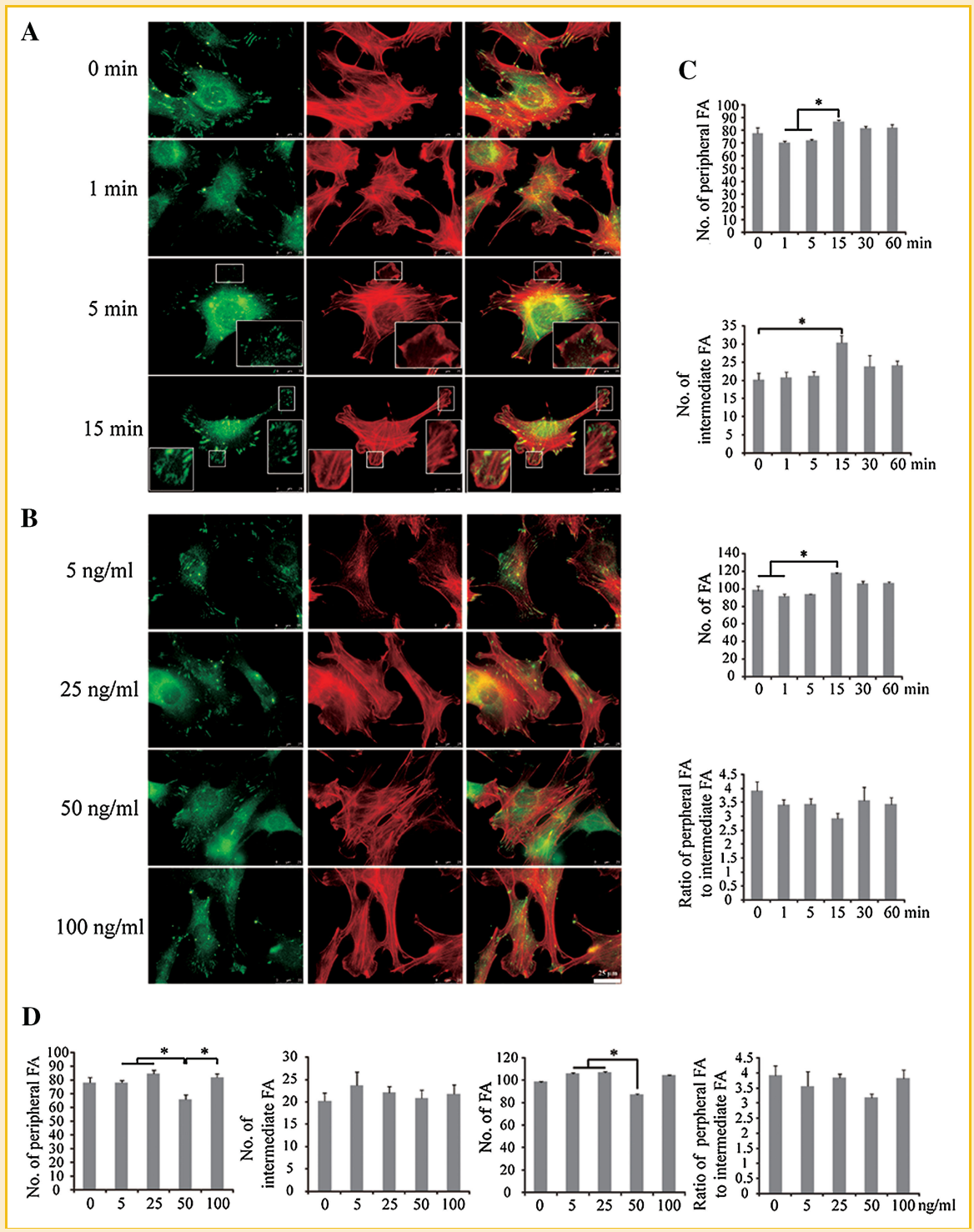

Fig. 4. VEGF induces differential patterns of FAs formation and F-actin organization in NSCs. NSCs were treated with VEGF at 5 ng/ml for different times (1, 5, and 15 min; A) or treated with VEGF at concentrations ranging from 5 to 100 ng/ml (5, 25, 50, and 100 ng/ml) for 30 min (B) after serum-starvation for 30 min. After stimulation, cells were double stained with paxillin (green) and TRITC-phalloidin (red). Bar, 25 μm. NSCs treated with VEGF at 5 ng/ml for 30 and 60 min were shown in Supplementary Figure S3A. C,D: The number of FAs and the ratio of FAs in cells (>20 cells, n = 3) under the conditions in A, B, and Supplementary Figure S3A. Data represent the mean ± SEM from at least three independent experiments. $^{*}P < 0.05$.

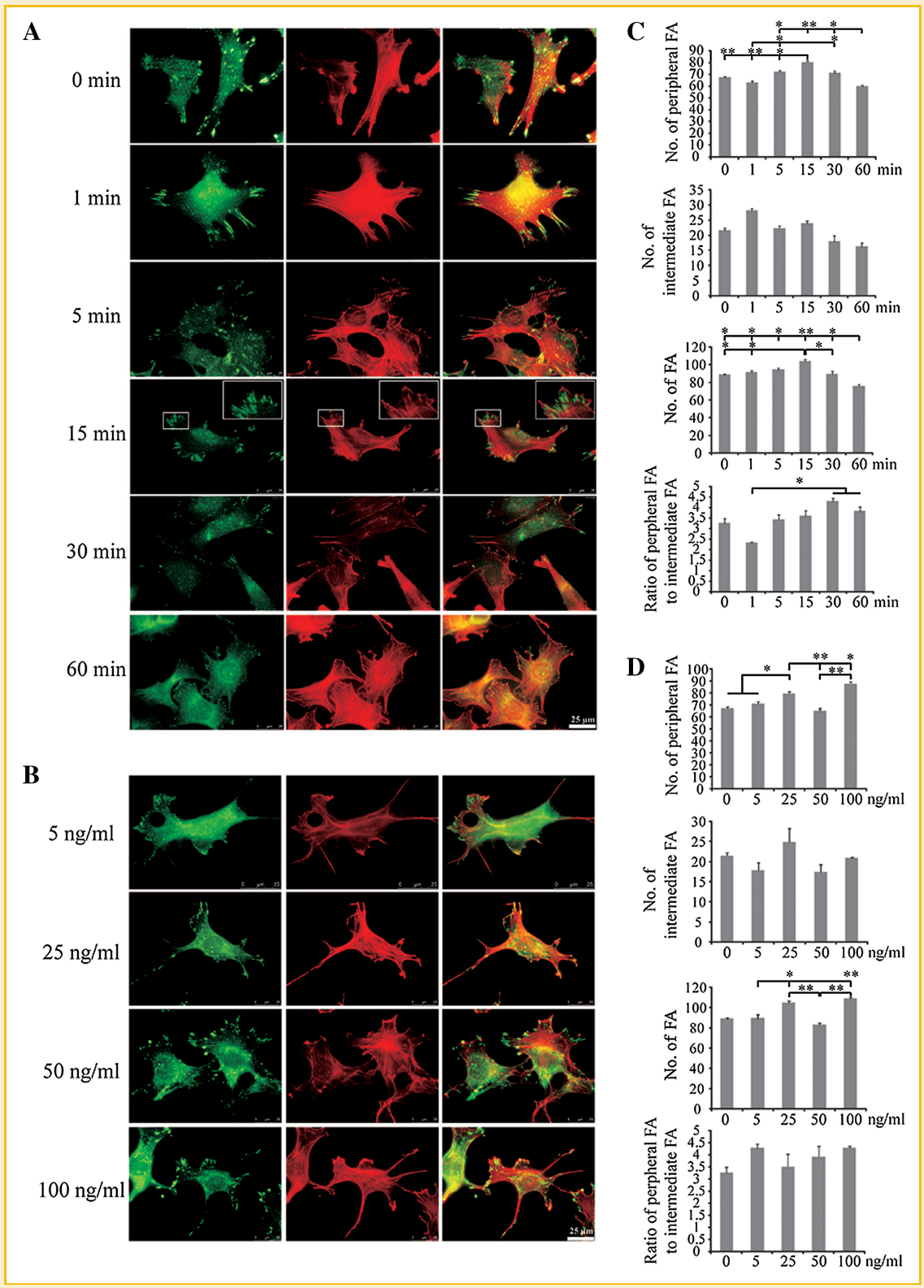

Fig. 5. VEGF induces differential patterns of FAs formation and F-actin organization in NSCs of 0.5 days differentiation. Cells of 0.5 days differentiation were treated with VEGF at 5 ng/ml for different times (1, 5, 15, 30, and 60 min) (A) or treated with VEGF at concentrations ranging from 5 to 100 ng/ml (5, 25, 50, and 100 ng/ml) for 30 min (B) after serum-starvation for 30 min. After stimulation, cells were double stained with paxillin (green) and TRITC-phalloidin (red). Bar, 25 μm. C,D: The number of FAs and the ratio of FAs in cells (>20 cells, n = 3) under the conditions in A and B. Data represent the mean ± SEM from at least three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

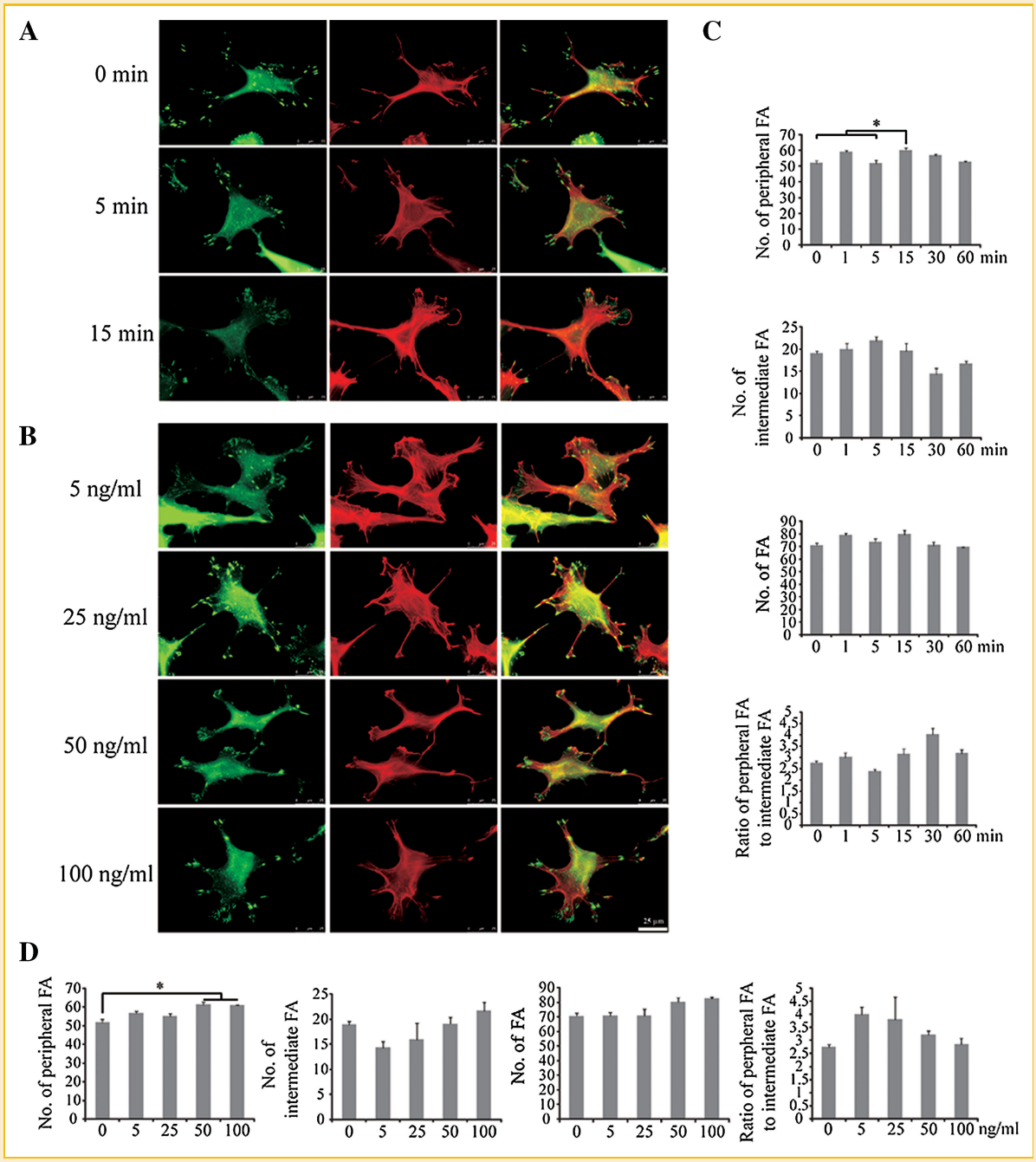

Fig. 6. VEGF induces differential patterns of FAs formation and F-actin organization in NSCs of 1 day differentiation. Cells of 1 day differentiation were treated with VEGF at 5 ng/ml for different times (5 and 15 min) (A) or treated with VEGF at concentrations ranging from 5 to 100 ng/ml (5, 25, 50, and 100 ng/ml) for 30 min (B) after serum-starvation for 30 min. After stimulation, cells were double stained with paxillin (green) and TRITC-phalloidin (red). Bar, 25 μm. NSCs treated with VEGF at 5 ng/ml for 1, 30, and 60 min were shown in Supplementary Figure S3B. C,D: The number of FAs and the ratio of FAs in cells (>20 cells, n = 3) under the conditions in A, B, and Supplementary Figure S3B. Data represent the mean ± SEM from at least three independent experiments. $^{*}P < 0.05$.

was observed when undifferentiated NSCs were treated by VEGF at 50 ng/ml.

DYNAMICS OF FAs IN NSCs DURING DIRECTIONAL MIGRATION

We further observed the distribution of FAs and arrangement of F-actin in NSCs during chemotactic migration to VEGF. To directly observe chemotactic migration and the assembly of FAs in this process, we took advantage of the direct-viewing Dunn chemotaxis chamber [Zhang et al., 2003; Liu et al., 2011]. VEGF at 50 ng/ml added to the outer well of the device will diffuse across the bridge to the inner blind the well of chamber and form gradient within 30 min [Liu et al., 2011]. During the chemotactic migration, NSCs showed a typical polarized morphology with more small FAs clustering and a unique persistent lamellipodium to VEGF compared to unstimulated

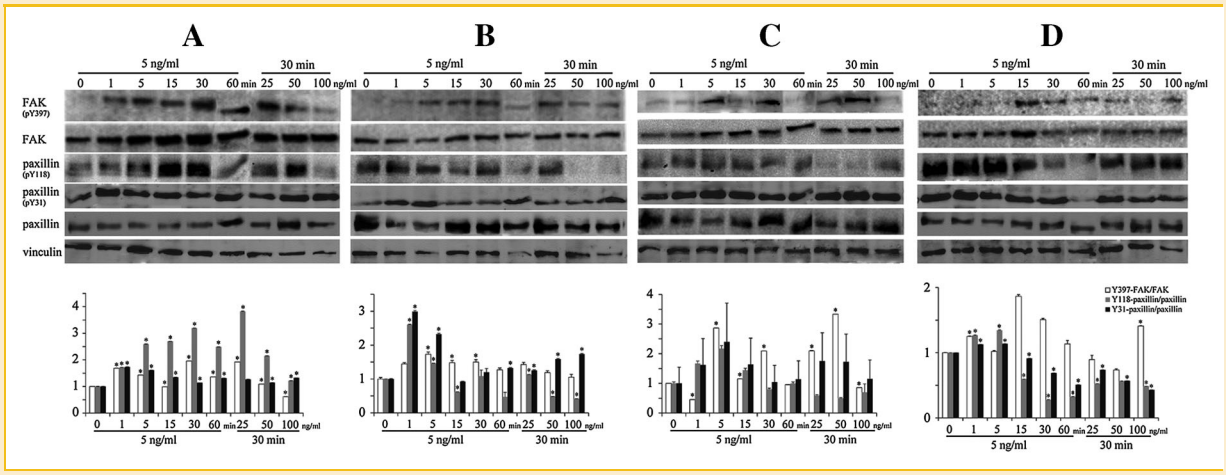

Fig. 7. The phosphorylation of paxillin and FAK in VEGF-stimulated NSCs. Cells of undifferentiation (A) and 0.5 day (B), 1 day (C), 3 days (D) differentiation were treated with VEGF at 5 ng/ml for different times (1, 5, 15, 30, and 60 min) or concentrations ranging from 5 to 100 ng/ml (5, 25, 50, and 100 ng/ml) for 30 min after serum-starvation for 30 min. Then cells were lysed and immunobloted with primary antibodies for phospho- or nonphospho-protein (FAK and paxillin). Levels of Y397-FAK/FAK, Y118-paxillin/paxillin and Y31-paxillin/paxillin were measured by densitometry of immunoreactive bands using ImageJ software. Values were expressed as a percentage of control, i.e, the value of cells in each state without VEGF. Shown are representative results of three separate experiments. Data were presented as mean ± SEM. $^{*}P < 0.05$ compared with the respective control cells of each state.

cells. In NSCs that directionally migrate toward VEGF, actin in lamellipodium was organized in a loose dendritic array of branched filaments with tiny FAs gathering at the leading edge, while in the back, prominent stress fibers were well arranged and aggregated at the trailing edge with fibrillar-like FAs (Fig. 8A). Furthermore, more FAs were formed in the front of cells than at cell rear, and this asymmetry was more prominent in cells of 1 day differentiation relative to cells in other differentiation states (Fig. 8B), implying that these cells exhibited a strongest chemotactic response to VEGF, as proved in our previous study [Liu et al., 2011].

Time-lapse video analysis revealed that new lamellipodium ruffled at cell periphery and paxillin was initially clustered into tiny focal complexes at the periphery and then enlarged at the same place to assemble mature FAs in NSCs (Fig. 8C). Once formed, individual focal complex matured into FA to anchor the lamellipodia at the same direction or disassembly with retraction of the protrusion. Rapid cell directional migration requires the efficient regulated formation and breakdown of adhesions and cycling of components from the rear to the front [Zaidel-Bar et al., 2003]. VEGF promoted the formation of focal complexes in lamellipodium and increased the number of mature FAs in lamella at the leading edge with the disassembly of FAs at the trailing edge in NSCs of 0, 0.5, and 1 day differentiation (Fig. 8C, b–d and Supplementary Movie 1, 3, 5), whereas it was not observed in matched control cells (Fig. 8C, a, Supplementary Fig. S5, and Movies 2, 4, 6). The lamella, characterized by a slow actin retrograde flow and behind the lamellipodium, plays a major role in protrusion to determine the direction of cell migration [Le Clainche and Carlier, 2008]. It was confirmed by the video showing that the asymmetry of mature FAs between lamella and rear in undifferentiated cells provided a persistent migratory direction to VEGF. Furthermore, in cells of 1 day differentiation compared to cells in other differentiation states (Fig. 8C and Supplementary Movie 1B, 3B, 7), FAs at the trailing edge slid forward more rapidly and cell tail retracted rapidly with a concurrent enlarged size of FAs at the leading edge to facilitate forward movement of cell body (Fig. 8C, d and Supplementary Movie 5B,C), which was consistent with the observations that NSCs of 1 day differentiation possessed the strongest chemotactic migration to VEGF [Liu et al., 2011]. By contrast, FA dynamics in cells of 3 days differentiation was not influenced by VEGF (data not shown).

DISCUSSION

In this study, using a neural stem cell line, C17.2 cells, we examined the formation of FAs and reorganization of F-actin in NSCs during the migration. We provide evidences that the formation of FAs and organization of F-actin in NSCs in varying differentiation states during cell spreading after seeding are different. By using a wound model in which a denuded area (wound area) was produced by scratching a monolayer of NSCs in varying differentiation states and cells migrated into the wound area, we found that more FAs at leading edge after wounding were formed in NSCs of 0.5 and 1 day differentiation, than cells of undifferentiation and 3 days differentiation. Furthermore, in the lamellipodium generated in the front of NSCs, Y31-paxillin distally gathers in small focal complexes and stable FAs while Y118-paxillin accumulates at stable FAs close to cell center. VEGF induces the remodeling of FAs in a temporal and spatial manner with stress fiber formation, the degree of which varies, depending on cell differentiation states: first, the number of FAs in NSCs is increased by 5 ng/ml VEGF, and the distribution of FAs remains unchanged; second, 5 ng/ml VEGF promotes focal complexes formation and increases the number of FAs at cell periphery in cells of 0.5 and 1 day differentiation; third, 50 ng/ml VEGF decreases FAs at the periphery in NSCs of 0 and 0.5 days

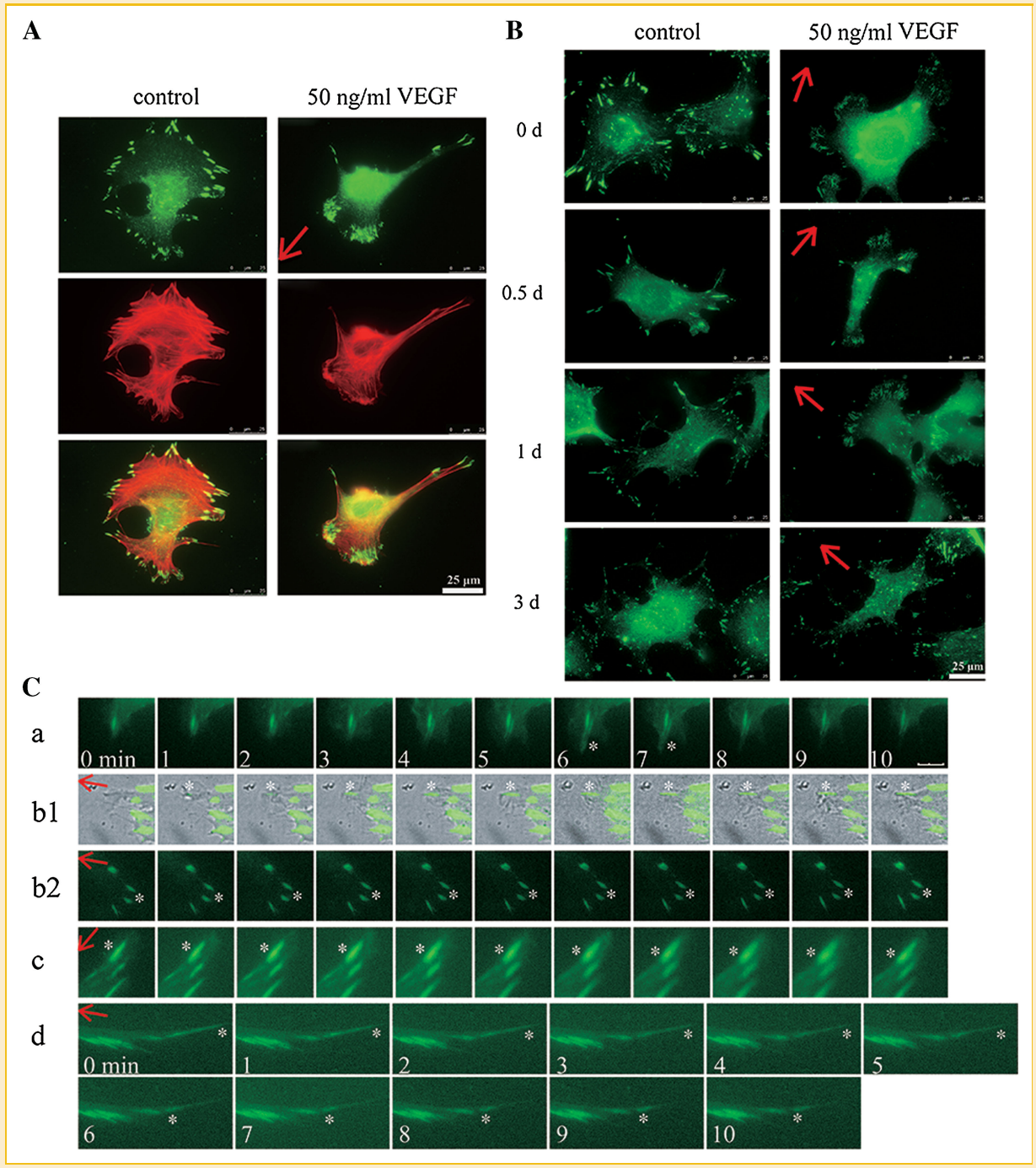

Fig. 8. Formation of FAs and reorganization of F-actin in NSCs during chemotactic migration to VEGF. Using Dunn chamber, NSCs were stimulated by VEGF at 50 ng/ml for 30 min, and were double stained with paxillin (green) and with TRITC-phalloidin (red). Bar, 25 μm. Arrow indicated the direction of VEGF and asterisks show the dynamics of FAs. A: During the chemotactic migration, F-actin in lamellipodium was organized in a loose bidimensional dendritic array of branched filaments with tiny focal complexes gathering at the leading edge, while in the back, prominent stress fibers were well arranged and aggregated at the trailing edge with fibrillar-like FAs. B: FAs in NSCs of varying differentiations during chemotactic migration to VEGF. Bar, 25 μm. Arrow indicated the direction of growth factor. A more asymmetric distribution of FAs in cells of 1 days differentiation suggested that those cells exhibited a strongest chemotactic response to VEGF. C: By using a Dunn chamber, NSCs transfected by pEGFP-paxillin were made at 1-min intervals between exposures, showing the differential dynamics of paxillin-containing FAs in cells of undifferentiation (a for control, b1 and b2 for VEGF-induced) and 0.5 days (c for VEGF-induced), 1 day (d for VEGF-induced) during the migration (see also Supplementary Fig. S5 and Movie 1–6). b1 was shown the formation of FAs at the leading edge of NSCs. b2, c, and d were shown the dynamics of FAs at the trailing edge. Arrow indicated the direction of the outer well of the chamber, in which contained 50 ng/ml VEGF. Bar, 5 μm. New lamellipodium ruffled at cell periphery and paxillin was initially clustered into tiny focal complexes near the leading edge of the lamellipodium and then enlarged at the same place to assemble mature FAs in NSCs. VEGF promoted the formation of focal complexes in lamellipodium and increased the number of mature FAs in lamella at the leading edge with the disassembly of FAs at the trailing edge. In cells of 1 day differentiation, FAs at the trailing edge slid forward more rapidly and cell tail retracted rapidly with a concurrent enlarged size of FAs at the leading edge to facilitate forward movement of cell body.

differentiation while increases those in cells of 1 day differentiation; and finally, cells of 3 days differentiation are hardly sensitive to the stimulation. Correlated with formation of FAs and reorganization of F-actin, FAK at Y397 and paxillin at Y31/118 are significantly activated by VEGF. In chemotactic migration carried out by Dunn chamber, VEGF induces a prominent directional migration of NSCs of 0, 0.5, and 1 day differentiation with generation of broad lamellipodium and formation of focal complexes at leading edge, and cells of 1 day differentiation display a more asymmetry of FAs distribution. Time-lapse video analysis demonstrates that VEGF induces asymmetric distribution of FAs between lamella and cell rear to orchestrate cell polarization and directional cell migration, with persistent formation of focal complexes at the leading edge and disassembly of FAs at the rear. Furthermore, cells of 1 day differentiation detach more rapidly at the rear to facilitate forward movement of cell body with a concurrent enlarged size of FAs at the leading edge, while cells of 3 days differentiation show the slowest turnover of FAs and are not sensitive to stimulation of VEGF. Taken together, these results indicate that dynamics of FAs and reorganization of F-actin in NSCs that undergo directional migration upon VEGF stimulation correlate closely with their differentiation states, contributing to the different chemotactic responses of NSCs of varying differentiation states.

Cell adhesion to extracellular matrix, which is also named FA and composed of many proteins, such as FAK and paxillin, defines a very important process in cell spreading and migration. During early stage of NSCs spreading, focal complexes are initially observed at cell periphery where F-actin polymerizes rapidly and transmit into FAs with translocation to cell center, which is similar to the process of fibroblasts spreading [Senju and Miyata, 2009]. F-actin organizes around these focal complexes to construct a cortical bridge structure connecting them. Finally, NSCs completely spread with FAs anchoring at the end of stress fibers and focal complexes present in lamellipodium. The phosphorylation of FAK and paxillin is proved to be involved in regulating FA formation and dynamics, and we found that the number of FAs increases with phosphorylation of FAK at Y397 and paxillin at Y31 during NSCs spreading, indicating this process requires FAs formation with activation of FAK and paxillin.

FAs do not only serve as the mechanical linkages to the ECM, but also concentrate intracellular signals from integrins as a biochemical sensor to remodeling FAs and modulate the dynamics of actin filaments for further membrane extension [Schoenwaelder and Burridge, 1999]. We proved that formation of paxillin-containing FAs in NSCs was regulated by their differentiation, and FAs are fewer and distribute at cell periphery in NSCs of 1 day differentiation while NSCs of 3 days differentiation have more FAs diffusing in cell body. Furthermore, we used the ratio of FAs (the ratio of FAs at the periphery to FAs in the center) to illustrate its distribution in cells, and large ratio suggested that FAs preferred to gather around cell periphery to promote cell migration [Totsukawa et al., 2004]. In a motile cell, FAs around nucleus disassemble and their number is decreased to facilitate migration [Broussard et al., 2008]. Not only the largest number of intermediated FAs but also the smallest ratio in NSCs of 3 days differentiation indicated that the motility of these cells was not as strong as that of cells in other differentiation states. Paxillin, as one component of FAs, binds regulators of actin to modulate the reorganization of actin filaments. Upon cell differentiation, loose and random F-actin in NSCs is changed into parallel stress fiber along cell body. NSCs of 0.5 and 1 day differentiation extend flat lamellipodia with a loose dendritic array of branched F-actin at the leading edge and a slim trailing edge, exhibiting a characteristic polarized morphology to promote migration.

Cell migration is influenced by both the intracellular and extracellular signals [Liu et al., 2011]. FA, as a sensor, integrates inside and outside signals to remodel itself and to reorganize F-actin [Burnette et al., 2011]; focal adhesion remodeling is critical for migration [Zhu et al., 2009]. In this study, by using a wound model in which a denuded area (wound area) was produced by scratching a monolayer of NSCs in varying differentiation states and cells migrated into the wound area, we found that more FAs at leading edge after wounding were formed in NSCs of 0.5 and 1 day differentiation, than cells of undifferentiation and 3 days differentiation. In the process of FA remodeling, FAK is activated at Y397 site, it is autophosphorylated, binds to Src, which in turn phosphorylates other sites on FAK and the FAK-binding proteins, such as paxillin [Mitra and Schlaepfer, 2006]. Paxillin has been implicated in the regulation of cell adhesion, spreading and motility, muscle differentiation and gene expression through its ability to directly interact with multiple structural and signaling proteins involved in coordinating these events, such as tubulin [Tumbarello et al., 2005]. After wounding, NSCs exhibit a polarized morphology with Y31-paxillin distally gathering in small FAs at cell periphery and Y118-paxillin localizing stable FAs, indicating that paxillin at Y31/118 is differently activated to take part in FAs assembly during cell migration. Y397-FAK colocalized with FAK at FAs in NSCs in this process (data not shown), consistent with previous result that the recruitment of FAK to FAs is essential for its activation and function [Scheswohl et al., 2008].

Our previous data showed that the chemotactic responses of NSCs to VEGF vary greatly, depending on the differentiation states of these cells [Liu et al., 2011]. There is evidence showing that VEGF reduces the number of smaller FAs and randomly forms enlarged FAs to remodel FAs in pulmonary endothelial cell [Birukova et al., 2009]. Mutation of FAK at Y397 fails to induce disassembly of FAs leading to a decrease of turnover [Hamadi et al., 2005] and suppression of the FAs turnover by deletion of FAK increases the stable and large FAs [Sieg et al., 1999]. In this study, we observed that VEGF temporally and spatially regulates formation of FAs with different phosphorylation levels of Y397-FAK and Y31/118-paxillin in NSCs under varying differentiation states. VEGF decreases the level of Y397-FAK and increases the number of FAs when NSCs were treated with VEGF at 5 ng/ml for 15 min. At the same time, the level of Y118-paxillin increased with phosphorylation of paxillin at Y31, consistent with previous study that tyrosine phosphorylation of paxillin at Y31/118 sties promotes focal complexes formation and translocation of FAs [Zaidel-Bar et al., 2007]. The level of phosphorylation of Y397-FAK was lower in cells of 3 days differentiation than that in cells of other states, indicating that FAs in those cells were stable with a slow turnover.

During chemotactic migration to VEGF, NSCs forms more FAs in broad lamellipodium at leading edge, displaying a prominent asymmetric distribution of FAs, especially in cells of 1 day differentiation. Directional migration requires polar protrusions at the leading edge containing many focal complexes, some of which develop into stable FAs. FAs, in turn, play a dual role in motility; they provide robust anchors to the ECM, allowing the contractile actomyosin system to pull the cell body and trailing edge forward and they may also restrain the migration process [Gupton and Waterman-Storer, 2006]. VEGF increases the assembly of focal complexes with a rapid turnover at the leading edge in NSCs during directional migration compared with control cells. However, more stable FAs assembled in lamella which was characterized by a slow actin turnover and localized behind the lamellipodium [Le Clainche and Carlier, 2008], anchored at the actin filaments to determine the direction of migration. Cell migration involves not only the assembly of FAs at the leading edge but also the disassembly of FAs at the rear end [Qin and Zhang, 2010]. When cell body translocates forward, FAs slide towards cell center and the tail of cell detaches at the rear to facilitate cell movement. Tails of cells of 1 day differentiation retracts more rapidly than cells in other differentiation states after stimulation with VEGF, with a concurrent enlarged size of FAs in lamella, demonstrating that NSCs of 1 day differentiation possess stronger chemotactic response than cells in other differentiation states [Liu et al., 2011].

Signal transduction events governing cell migration involve an ever-expanding number of molecules functioning in interconnected biochemical pathways regulating the turnover of adhesion complexes at the leading edge of migrating cells [Wagner et al., 2008]. FAK as downstream of integrins, has recently been proposed as a mediator of cell migration through the activation of mitogen activated protein kinases (MAPKs) including p38MAPK, extracellular signal-regulated kinase (ERK) and stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) pathways [Huang et al., 2008; Yoshizuka et al., 2012]. In this study, we prove that VEGF induces different phosphorylation of Y397-FAK and Y31/118-paxillin in NSCs under varying differentiation states. There is evidence showing that the complex of FAK with PI3K is formed in VEGF-stimulated endothelial cells [Qi and Claesson-Welsh, 2001]. It is also proved that ERK formed a complex with FAK and paxillin in a manner dependent on ERK activity during wound of human corneal epithelial (HCE) cell monolayers closure [Teranishi et al., 2009] and the directional migration is regulated by JNK/paxillin pathway through phosphorylation of paxillin on S178 and subsequent tyrosine phosphorylation of paxillin [Huang et al., 2003, 2008]. Our previous data show that various activation of PI3K and MAPKs is closely related to the differentiation levels of NSCs exposing to VEGF [Liu et al., 2011], suggesting a crosstalk between integrin-FAK pathway and PI3K, ERK or JNK pathway in the regulation of VEGF-induced directional migration of NSCs. How focal adhesion integrates various signals to govern the directional migration requires more intensive studies, thereby allowing to delineate the cellular and molecular mechanisms of chemotactic responses of NSCs to VEGF.

ACKNOWLEDGMENTS

We thank Dr. Evan Snyder for the C17.2 cell line, Clare M. Waterman and Ana M. Pasapera (Cell Biology and Physiology Center, National Heart, Lung, and Blood Institute, National Institutes of Health) for the plasmid of EGFP-paxillin.

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