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Fascin 1 is transiently expressed in mouse melanoblasts during development and promotes migration and proliferation

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SUMMARY
Fascins, a family of actin-bundling proteins, are expressed in a spatially and temporally restricted manner during development and often in cancer. Fascin 1 has a clear role in cell migration in vitro, but its role in vivo in mammals is not well understood. Here, we investigate the role of fascin 1 in the melanocytic lineage and in melanoma cells. Fascin 1 knockout causes hypopigmentation in adult mice owing to migration and cell cycle progression defects in melanoblasts, the melanocyte precursor cell. Study of live embryo skin explants reveals that E14.5 fascin 1-null melanoblasts migrate slower, and generate fewer and thinner pseudopods. By contrast, fascin 1 expression drives faster migration and lamellipodia protrusion in melanocytes in vitro. In addition, fascin 1 depletion retards melanoblast proliferation in vivo and melanoma cell growth in vitro. These data indicate that fascin 1 not only promotes cell migration in mouse melanoblasts but it also has a role in growth and cell cycle progression.

KEY WORDS: Fascin 1 (Fscn1), Melanoblast, Migration, Mouse

INTRODUCTION
Fascins are actin-bundling proteins involved in cytoskeletal dynamics and regulated by protein kinase C (Adams, 1997; Adams and Schwartz, 2000; Aratyn et al., 2007). Fascin 1 is enriched in filopodia (Adams, 1997; Adams and Schwartz, 2000; Aratyn et al., 2007) and in invadopodia (Li et al., 2010; Schoumacher et al., 2010). The expression level and actin-bundling activity of fascin 1 positively correlate with the length, number and lifetime of filopodia (Vignjevic et al., 2006; Hashimoto et al., 2007; Yamakita et al., 2009), as well as two-dimensional cell migration (Yamashiro et al., 1998). In addition, fascin 1 stabilizes invadopodia and protrusive pseudopods of mesenchymal-type cancer cells (Li et al., 2010). Fascin 1 expression is time and tissue specific during embryogenesis (De Arcangelis et al., 2004), and a high level of fascin 1 is often found in various motile embryonic cells, such as macrophage-like plasmocytes and border cells in Drosophila (Borghese et al., 2006; Zanet et al., 2009), and E10.5-12.5 hepatoblasts in rat (Hayashi et al., 2008).

Melanoblasts, which are melanocyte precursors, emigrate dorsolaterally from the neural tube after differentiating from neural crest cells. Mouse melanoblasts translocate from dermis to epidermis and accumulate at hair follicles before birth. Melanoblast differentiation, migration, proliferation and survival are crucial for early skin development and adult pigmentation (Baxter et al., 2004). Gene mutations or alterations causing melanoblast deficiencies often result in a “white-spot” phenotype and several genes encoding growth factors and respective ligands (Jordan and Jackson, 2000; Harris et al., 2008), transcription factors (Hornyak et al., 2001), secreted metalloproteinases (Silver et al., 2008), Rac1 and the Rac1 GEF pREX-1 (Li et al., 2011; Lindsay et al., 2011) affect melanoblast migration and survival (see also http://www.espcr.org/micemut/).

Here, we demonstrate that mice lacking fascin 1 (Yamakita et al., 2009) have pigment defects that result from melanoblast migration defects and delayed cell cycle progression. We validated these results in human melanoma cultured cells. Our studies suggest that fascin 1 is transiently upregulated when melanoblasts are migratory and proliferative during mouse embryogenesis, and that some melanoma cells may also depend on fascin for enhanced motility and normal cell cycle progression.

MATERIALS AND METHODS
Transgenic mice
All experiments were performed according to UK Home Office regulations. Fascin 1−/− mice in C57BL/6 background have been previously described (Yamakita et al., 2009). Z:EG (lacZ/EGFP) mice (Novak et al., 2000) and DCT (dopachrome tautomerase):lacZ mice (Mackenzie et al., 1997; Pla et al., 2004; Takemoto et al., 2006) have been previously described. Tyrosinase Cre (Tyr::Cre) mice have been previously described (Delmas et al., 2003). Mouse genotyping was performed by the company Transnetyx (Memphis, TN, USA).

Retroviral constructs
Stable cell lines expressing GFP-tagged human fascin 1 were generated by retroviral infection using the modified Retro-X retroviral expression system (Clontech Laboratories). A HindIII restriction site, followed by a Kozak consensus translation initiation site was introduced to the 5’ end of the coding sequence of GFP-fascin 1 (Li et al., 2010) by PCR (5’-AGAAAGCCTTATGGTGAGCAAGGGCGAGGAGCTGTTC-3’). In the same reaction, a ClaI restriction site was introduced to the 3’ end (5’-AGAATTCGATACTAGTACTCCCCAGAGCGAGGCGGGGTACGTC-3’). The HindIII-ClaI DNA fragments containing human fascin 1 were subcloned into...
**β-Galactosidase assay**

Timed embryos were dissected in ice-cold PBS and fixed in ice-cold 0.25% glutaraldehyde in PBS for 40 minutes. After 10 minutes of washing in ice-cold PBS, embryos were immersed in permeabilization solution (2 mM MgCl$_2$, 0.01% sodium deoxycholate and 0.02% NP-40 in PBS) for 30 minutes at room temperature and stained with X-gal staining solution [2 mM MgCl$_2$, 0.01% sodium deoxycholate, 0.02% NP-40, 5 mM K$_4$Fe(CN)$_6$, 5 mM K$_3$Fe(CN)$_6$, and 1 mg/ml X-gal (Invitrogen, Carlsbad, CA, USA) in PBS (pH 7.4)] as described previously (Loughna and Henderson, 2007) for 48 hours at 4°C. Embryos were maintained in 10% formalin after washes with PBS. The pictures of the forelimbs, trunks and entire embryos were taken at the same magnification for embryos at the same developmental stage under Zeiss Stemi-2000 dissection microscope (EOS utility, Edmund Optics, NJ, USA). For the quantitation of the melanoblasts in the trunks, an area of 1000×500 pixels$^2$ was cropped to cover most forelimb skin and gridded with an area per point of 20,000 pixels$^2$ (Image J). For melanoblast quantification in the forelimb, an area of 1100×450 pixels$^2$ was cropped to cover most forelimb skin and gridded with an area per point of 15,000 pixels$^2$. Cell number in sub-areas was counted for E13.5 and E15.5 compared between wild-type, fascin 1$^{−/−}$ and fascin 1$^{+/−}$ embryos.

**Immunohistochemistry (IHC) and immunofluorescence (IF)**

Formalin-fixed, paraffin wax-embedded embryos or skin samples were transversely sectioned and de-waxed, rehydrated in xylene and decreasing concentrations of ethanol. Antigen-retrieval process was performed with sections immersed in citrate buffer (pH 6; Lab Vision, Fremont, CA, USA) in a boiling water bath and the samples were blocked in 5% normal donkey serum (NDS) in PBS and stained sequentially with primary antibodies in PBS containing 5% NDS overnight at 4°C and secondary antibodies in PBS containing 5% NDS for 1 hour at room temperature. The following primary antibodies were used: goat anti-TRP2 antibody (DCT) (D-18, 1:200, Santa Cruz), mouse anti-BrdU antibody (DCT) (D-18, 1:200, Santa Cruz) combined with rabbit anti-cleaved caspase 3 (Asp175) (SA1E, 1:100, NEB) or rabbit anti-phospho-histone H3 (ser10) (1:100, Cell Signaling), mouse anti-BrdU (5-bromo-2′-deoxyuridine) antibody (1:100, DAKO), rabbit anti-cyclin D1 antibody (EP12, 1:100, DAKO) and rabbit anti-fascin 1 (1:100, Sigma-Aldrich). The secondary antibodies (1:500, Invitrogen) were used as follows: Alexa 594-conjugated donkey anti-rabbit or mouse, Alexa 488-conjugated donkey anti-goat, and Alexa 488-conjugated donkey anti-goat. Prolong Gold antifade reagent with DAPI (Invitrogen) was used to counter-stain and mount. For DCT immunohistochemistry stains, the sections were blocked in TBS-T (tris-buffered saline and Tween 20) containing 5% rabbit serum and 5% NDS overnight at 4°C and stained with 1:100 diluted primary antibody (anti-TRP2) and goat anti-Rac1 polyclonal antibody (1:50 dilution) combined with rabbit anti-goat biotinylated secondary antibody followed with Vectastain ABC-AP kit (Vector Laboratories) and vector red alkaline phosphatase substrate kit (Vector Laboratories). Haematoxylin was used to counterstain. TUNEL assay was performed with in situ cell death detection kit (Fluorescein, Roche), as suggested by the manufacturer’s manual and Alexa 555-conjugated donkey anti-goat was used as secondary antibody to target goat anti-TRP2 antibody (DCT).

**Cell culture distribution**

Human melanoma cell line MV3 in six-well plates was transfected with siRNAs (100 nM, sifascin1-1: on-target plus siRNA fascin1 oligo3 from Thermo Scientific or sifascin1-2: Hs-FSCN1 2HP siRNA from Qiagen) with HiPerfect transfection reagent (Qiagen) twice on two sequential days. Cells were re-seeded and the experiments were performed 48 hours post-transfection. Asynchronously growing cells were collected after 1 hour BrdU pulse (20 μM) to measure cell in S phase or challenged with 100 ng/ml nodocazole in presence of cumulative BrdU for 2 hours, 4 hours, 6 hours, 8 hours, 10 hours and 12 hours. This is a modified method of the nodocazole trapping experiment used previously (Gillespie and Walker, 2006). For BrdU/PI analysis, cells were treated with 2 N HCl (room temperature, 15 minutes) prior to 1 hour primary antibody incubation with mouse anti-BrdU antibody (1:40, DAKO). After washing with PBT, cells were then incubated in PBT with Alexa 488-conjugated anti-mouse antibody (1:60; Invitrogen) and resuspended in PBS with 250 μg/ml RNaseA and 10 μg/ml propidium iodide for 30 minutes prior to FACS analysis on BD FACScalibur using CellQuest software (BD). The results were analysed and plotted with FlowJo (Tree Star).

**Melanoblast migration and pseudopod dynamics**

Embryos (E14.5) were dissected from Tyr::Cre Z/EG WT, and fascin 1$^{−/−}$ female mice and skin in the trunk area of embryos was carefully removed under a Zeiss Stemi-2000 dissection microscope (Edmund Optics, NJ). Embryonic skins were immediately mounted and cultured in Greiner Luxom culture dish (Greiner Bio-One) as described previously (Mort et al., 2010; Li et al., 2011; Lindsay et al., 2011). Ex vivo time lapse images were captured on a Nikon AR and Olympus FV1000 (20× objective, 1024×1024) for 4 hours with an interval of 5 minutes per frame in a 37°C humid chamber of 5% CO$_2$. Cell tracks and speeds were determined with ImageJ manual tracking and chemotaxis tool plug-in. The criteria for pseudopod generation rate, long or short pseudopods and numbers were determined as reported previously (Li et al., 2011). Skin samples were fixed after image capture and detailed cell proliferation picture were taken as a z-series on an Olympus FV1000 confocal microscope at 1 μm intervals (60× objective, 1024×1024). The diameter of a cross-section through the middle of a pseudopod is expressed as pixels measured with the straight-line tool (ImageJ).

**Melanocyte migration and lamellipodial kymograph**

Imortalized melanocytes from Tyr::CreERT2$^{−/−}$ Ink4a$^{−/−}$ Rac1$^{−/−}$ mice (M4) were established (Li et al., 2011) and stably expressed GFP-fascin 1 with pLHCX vector as above (M4F). Although these cells are immortal, because of the Ink4a loss, they are not transformed and do not form tumours in nude mice or form invadopodia in vitro (Li et al., 2011) (data not shown). Time-lapse images were captured on Nikon TE 2000 Timelapse microscope (20× objective) at 10-minute intervals for 4 hours. Cell speed was measured with ImageJ plug-in manual tracking and chemotaxis tool. For lamellipodial kymographs, 300 images were captured at 1-second intervals on Nikon TE 2000 Timelapse microscope systems with PFS (20× objective, x1.5). Lamellipodial kymographs show pixel densities over time (z-axis) on a single pixel-wide straight line (y-axis) in the direction of lamellipod protrusion (ImageJ plug-in multiple kymograph). Distance extended (d) was measured against lamellipodial persistence (p), defined as the amount of time that the cell spends persistently extending a lamellipod before pausing or entering a retraction phase. Speed of protrusion is shown as a broken line in Fig. 5C and represents the distance protruded (d) divided by the time of protrusion (p).
and detected with ECL chemiluminescence detection kits (Pierce). Primary antibodies are: rabbit anti-fascin 1 (Sigma-Aldrich), rabbit anti-phospho-AKT (Ser473) (Cell Signaling), rabbit anti-p44/42 MAPK (Erk1/2) (Cell Signaling), rabbit anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (Cell Signaling), rabbit anti-pY397FAK (Invitrogen) and rabbit anti-total FAK (Cell Signaling).

BrdU incorporation
Timed matings were set up and pregnant females were injected with 250 μl BrdU (10 mg/ml, BD Pharmingen) at E13.5. After 24 hours, mice were sacrificed and embryos were fixed for histology in 10% neutral-buffered formalin.

Eosin staining
X-gal-stained, formalin-fixed and paraffin-embedded fascin 1 DCT::lacZ embryos (E13.5) were transversely sectioned (10 μm depth). Eosin staining on the sections was performed in a standard fashion. Pictures were taken on Olympus BX51 FL microscope. DCT-positive cells in the epidermis and dermis (E13.5) were counted.

Statistical analysis
To test whether groups of large datasets (e.g. wild type versus −/−) come from a Gaussian distribution, the D’Agostino and Pearson omnibus normality test was performed. If the data failed to pass the normality test, a Mann-Whitney test was used to assess statistical significance. Otherwise, two-tailed, unpaired Student’s t-test was used to assess statistical significance (GraphPad Prism 5). To compare three groups of data (wild type, −/+ and −/−), Kruskal-Wallis one-way ANOVA test and Mann-Whitney test were used (GraphPad Prism 5). *P<0.05, **P<0.01 and ***P<0.001 are statistically significant. n.s. indicates not significant.

RESULTS

Fascin 1-null mice have melanocyte deficient patches
Fascin 1-null pups were weaned at less than Mendelian ratios (Yamakita et al., 2009), but otherwise were healthy and fertile. Fascin 1 was undetectable at the protein level in fascin 1−/− mice and reduced in fascin 1−/+ mice (Fig. 1A; supplementary material Fig. S1A). Fascin 1−/− mice in C57BL/6 background displayed a white patch near the midline of the abdomen, varying from a few white hairs or a thin white line (63% of 30 animals) to a big white patch (37% of 30 animals, Fig. 1B). White abdominal patches have been previously correlated with the absence of melanocytes (Baxter et al., 2004; Silver et al., 2008; Li et al., 2011; Lindsay et al., 2011) and suggest possible melanoblast migration and/or proliferation defects. Immunofluorescence staining of embryonic skin (E14.5) indicated that melanoblasts (DCT positive) had relatively high cytoplasmic fascin 1 staining, which appeared to be enriched in protrusions (Fig. 1C, yellow arrowheads). Fascin 1 is also expressed by dermal stromal cells (Fig. 1C, lower white dotted line marked with Ep (epidermis) and De (dermis)) and weakly expressed by basal keratinocytes (Fig. 1C, white arrowheads). Furthermore, fascin 1 mRNA was robustly expressed in E15.5 melanoblasts at levels around threefold higher than in keratinocytes (Lionel Larue, personal communication) (Colombo et al., 2012). Histological analysis revealed an absence of melanocytes in the white patch of abdominal skin from fascin 1−/− mouse (P14, hair follicle in anagen stage) whereas the black dorsal skin of fascin 1−/− mice had normal...
Fascin 1 deficiency reduces melanoblast number during embryogenesis

To further understand the pigmentation defect, we crossed fascin 1−/− mice with mice harbouring DCT::lacZ, which is a reporter for melanocyte lineage cells (Mackenzie et al., 1997). DCT::lacZ fascin 1−/− mice generally displayed large white patches on the abdomen (supplementary material Fig. S1C). We performed a β-galactosidase assay to determine the number and distribution of DCT::lacZ positive melanoblasts. At E11.5, when the melanoblasts have just emerged from the neural tube, the number and position of melanoblasts was indistinguishable between wild-type, fascin 1+/− and fascin 1−/− embryos, e.g. melanoblasts appeared to have emerged from the neural tube and were situated in the migration staging area adjacent to the neural tube (Fig. 2A,B). However, fascin 1−/− displayed fewer melanoblasts on the forelimbs and trunks at E13.5 (Fig. 2D,G) and E15.5 (Fig. 2E,F,H), and significantly fewer melanoblasts in the distal areas (Fig. 2C-H). In most cases, fascin 1−/− embryos had an intermediate phenotype. Thus, fascin 1 is not required for neural crest cell to melanoblast differentiation or for emigration from the neural tube; however, fascin 1 rapidly becomes important for cell numbers to increase and for migration in the dermis/epidermis.

Fascin 1 deficiency delays melanoblast and melanoma cell cycle progression

We examined whether fascin 1 contributed to proliferation in melanoblasts and melanoma cell lines that express high levels of fascin 1 (Li et al., 2010). Melanoblasts proliferate with a doubling rate of ~16 hours at E10.5-E15.5 (Luciani et al., 2011). No apoptotic melanoblasts were detected from co-stains of TUNEL assay (supplementary material Fig. S2A) or cleaved caspase 3 (apoptosis marker, data not shown) and DCT in wild-type or fascin 1+/− embryos (E14.5). By contrast, BrdU incorporation into fascin 1−/− melanoblasts was significantly reduced following a 24-hour BrdU pulse (Fig. 3A; supplementary material Fig. S2B), indicating that the cell cycle is delayed in fascin 1−/− embryos. Furthermore, we observed fewer phospho-histone H3 (pH3, mitosis marker) positive melanoblasts in fascin 1+/− embryos (Fig. 3B; supplementary material Fig. S2C). Cyclin D1 acts to promote G1- to S-phase transition and cell proliferation, and high cyclin D1 expression marks cells entering S phase of the cell cycle (Sherr, 1995). Cyclin D1-positive melanoblasts were significantly reduced in fascin 1−/− embryos (Fig. 3C; supplementary material Fig. S2D). To further test the role of fascin 1 in proliferation, we examined melanoma cell proliferation in 3D. Although fascin 1-depleted melanoma cells proliferated more slowly in 3D collagen I matrix (supplementary material Fig. S3A), they displayed normal FAK-P13K-AKT pathway and MAPK/ERK pathway activation (supplementary material Fig. S3B), in contrast to a previous report in hepatic stellate cells, where these pathways were reduced in fascin-depleted cells (Uyama et al., 2012). Asynchronous fascin 1 knockdown MV3 cells exhibit more G1-phase cells and fewer S-phase cells (Fig. 3D). To further validate this, MV3 melanoma cells were trapped with nocodazole, a mitotic inhibitor. From the time of the nocodazole addition, we measured the cumulative BrdU incorporation as cells progressed through S phase before being blocked in M phase (Gillespie and Walker, 2006). We observed substantially fewer sifascin (fascin 1 siRNAs) cells in S phase, indicating that fascin depletion slowed progression through G1-phase cells and fewer S-phase cells (Fig. 3D).
**Fascin 1 deficiency reduces melanoblast migration and pseudopod generation rate**

Fascin 1 (singed in *Drosophila*) is important for *Drosophila* haemocyte migration (Zanet et al., 2009), migration of mature dendritic cells to lymph nodes in mice (Yamakita et al., 2011) and for melanoma tumour cell invasion in 3D Matrigel/collagen matrix (Li et al., 2010). We investigated the migration of melanoblasts in live embryo skin to better understand the role of fascin in *in vivo* Mice carrying a Z/EG double reporter transgene (Novak et al., 2000) and Tyr::Cre (Delmas et al., 2003) were crossed into the fascin 1−/− background to drive GFP expression in the melanoblast lineage. The skin from E14.5 embryonic trunk was imaged as previously described (Mort et al., 2010; Li et al., 2011). Melanoblasts migrate individually within the developing mouse embryo epidermis using two types of protrusions: short stubs and long pseudopods (Li et al., 2011). Melanoblasts in E14.5 wild-type and fascin 1−/− skin explants maintained a similar elongated cell shape with both long and short protrusions (Fig. 4A,G; supplementary material Movies 1, 2). Fascin 1−/− melanoblasts were slightly smaller than wild type (Fig. 4C). In addition, cell migration speed and euclidean distance travelled was significantly reduced for melanoblasts in fascin 1−/− explants compared with wild type (Fig. 4D-F). Around 30% of fascin 1−/− melanoblasts migrated with a speed slower than 0.5 μm/minute compared with fewer than 10% of melanoblasts in wild-type explants (Fig. 4B,E). However, directionality (the ratio between the euclidean distance and total distance travelled) was unaffected by fascin 1 loss (supplementary material Fig. S5A).

In addition, the rate of pseudopod generation in fascin 1−/− melanoblasts was modestly reduced (Fig. 4G,H) but the lifetime of pseudopods was increased (supplementary material Fig. S5B). These two parameters roughly balanced out so that at any one time, fascin 1−/− melanoblasts and wild type had a similar number of pseudopods (supplementary material Fig. S5C); however, this number was quite variable and ranged between zero and eight pseudopods. Fascin 1−/− melanoblasts also extended thinner protrusions with a smaller cross-sectional width than wild type (Fig. 4G,I). This difference may be due to the proportionally smaller area of the fascin 1−/− cells in embryo skin (Fig. 4C). Thus, fascin 1 is required for efficient melanoblast migration and formation of thick robust pseudopods. *In vitro* fascin 1 is important for filopodia extension (Vignjevic et al., 2006; Hashimoto et al., 2007) and may be important for melanoblasts to probe the local environment, including contact with keratinocytes and the BM (basement membrane). Although we found that loss of fascin1 reduced the rate of generation of pseudopods, in agreement with these *in vitro* studies, it is possible that very small filopodia will not be detected at the resolution we are capable of imaging explants, so we cannot comment on those. Overall, we find that fascin 1 is required for efficient pseudopod generation rates, and for normal pseudopod dynamics, but lack of fascin causes only subtle changes overall in pseudopod dynamics.

Mouse melanoblasts migrate across the developing epidermal BM from dermis to epidermis between E11.5 and E13.5. As fascin 1−/− is implicated in invadopodia formation and invasion of melanoma cells (Li et al., 2010), we examined the effect of fascin 1 deletion on BM crossing. However, the proportion of melanoblasts crossing into the epidermis of Det::lacZ wild-type, fascin 1−/− and fascin 1−/− embryos was similar at E13.5 (supplementary material Fig. S6). Although it seems surprising that fascin 1 is not important for migration across the BM, these data agree with our previous study of Rac1 and N-WASP (Li et al., 2011) and further confirm that the relocation from dermis to epidermis probably does not require matrix degradation by invadopod-like structures.

**Fascin 1 regulates melanocyte migration and lamellipodial dynamics**

To further explore the migratory role of fascin 1, we established an early passage melanocyte cell line from 1-day-old mouse pups with a genetic deletion of Ink4a, which we term M4 (see Materials and methods). M4 represents line 4, as described in our recent study (Li et al., 2012). Fascin 1 protein was undetectable in M4 cells (supplementary material Fig. S1B), perhaps reflecting that murine melanocytes reside mainly in hair follicles and may have lost fascin expression when they are not migratory (Jordan and Jackson, 2000). Fascin 1 protein was not detected in other mouse melanocyte (with *Ink4a<sup>−/−</sup>*) or melanoma (mutant NRas<sup><i>p61K/R</i></sup>/Ink4a<sup>−/−</sup>) background) cell lines that we derived from 1-day-old pups (data not shown) (Ackermann et al., 2005). However, fascin 1 expression was detected in mouse melanoma cell lines B16F1 and B16F10 (supplementary material Fig. S1B) (Vignjevic et al., 2006; Kim et al., 2009), which indicates that the expression of fascin 1 might be dependent on signals from the microenvironment or on mutations other than loss of Ink4a and activation of NRas. Because many human and murine melanoma cell lines express fascin 1 (Li et al., 2010), we stably overexpressed GFP-tagged human fascin 1 in M4
melanocytes (M4F cells). M4 and M4F showed the same sizes in 2D cell culture (supplementary material Fig. S5D) and migrated randomly with similar directionality (supplementary material Fig. S5E). GFP-fascin 1 was cytoplasmic and also concentrated at filopodia and microspikes (Fig. 5A). Fascin 1 expression increased migration speed (Fig. 5B), which is consistent with other studies (Yamashiro et al., 1998; Hashimoto et al., 2005a). Melanocytes displayed broad fan-like lamellipodia towards the direction of migration on a 2D surface. Fascin 1-enriched actin bundles (filopodia and microspikes) are interspersed within the lamellipodia of motile melanoma cells (Pelosi et al., 2003; Vignjevic et al., 2006). We further analysed four parameters of lamellipodial protrusions: protrusion rate, protrusion frequency, protrusion persistence and distance as shown in the lamellipodial kymographs (Fig. 5C). Expression of fascin 1 increased the speed of advancement of lamellipodial protrusions and the generation frequency. Cells expressing GFP-fascin 1 protruded less persistently, although protrusion distance was not affected (Fig. 5D-G). Thus, fascin 1 expression increases melanocyte migration and promotes lamellipodial dynamics in a 2D environment.

**DISCUSSION**

We demonstrate an important role for fascin in the migration and cell cycle progression of mouse embryo melanoblasts that gives rise to a white abdominal patch on fascin 1-null animals. The white patch arises because melanoblasts have not populated the areas most distal to their sites of origin near the neural tube during embryogenesis and once they differentiate into melanocytes after birth, they are no longer migratory in the mouse skin. Evidence for this comes from showing that expression of Kit-L in the skin can allow repopulation of new hair follicles after birth (Luciani et al., 2011). The role of fascin in migration in 2D in vitro is well documented (Jayo and Parsons, 2010; Machesky and Li, 2010) and recent reports show a requirement in 3D matrix degradation (Li et al., 2010), as well as in the normal embryonic migration and inflammatory responses of Drosophila haemocytes (Zanet et al., 2009). We find that loss of fascin 1 impairs pseudopod generation rate and fascin 1-null cells generate thinner pseudopodia that are less dynamic (longer-lived), leading to slower migration. We were surprised that pseudopods generated by fascin 1 nulls had a slightly increased lifetime, but we also found that expressing fascin 1 in M4 melanocytes in culture decreased lamellipodial persistence, which also indicates that fascin 1 promotes dynamics. This agrees with studies showing increased lamellipodial dynamics of fascin 1-expressing Drosophila haemocytes (Zanet et al., 2009) and is interesting in light of a recent study showing that actin bundles containing fascin 1 are more susceptible to turnover by coflin (Breitsprecher et al., 2011). Furthermore, fascin 1 can interact with LIMK, a coflin regulatory kinase (Jayo et al., 2012) and thus fascin...
Mtorc1 regulation of transcription

interspersed in the lamellipodium. Scale bar: 5 μm. (***P<0.001; *P<0.05; n.s., not significant.

DEVELOPMENT

Fig. 5. Fascin 1 overexpression increases melanocyte lamellipodium dynamics. (A) GFP fluorescence and transmitted light photo of M4F melanocytes indicate that fascin 1-enriched filopodia (white arrows) are interspersed in the lamellipodium. Scale bar: 5 μm. (B) Cell migration speed generated from three independent experiments. Data are generated from 98 M4 cells and 135 M4F cells. (C) Representative kymograph pictures of M4 (top) and M4F (bottom) showing M4F cells have higher rates of lamellipodia protrusions. Lamellipodial kymographs are analysed by drawing a one pixel wide line (white line in top right box) at a 90° angle across a lamellipodium in the direction of protrusion. Pixel densities along this pixel-wide line are recorded at 1-second intervals for a period of 300 seconds, resulting in a time-space image. Scale bars for time and space are shown in the top image. Within the kymograph, a steeper slope of a protrusion corresponds to higher rates for lamellipodia protrusion. Distance extended (d) was measured against lamellipodial space are shown in the top image. Within the kymograph, a steeper slope of a protrusion corresponds to higher rates for lamellipodia protrusion. Distance extended (d) was measured against lamellipodial persistence (p), defined as the amount of time that the cell spends persistently extending a lamellipod before pausing or entering a retraction phase. Speed of protrusion is shown as a broken line and represents the distance protruded (d) divided by the time of protrusion (p). Frequency is measured by counting the number of lamellipodia per minute. (D) Frequency of lamellipodial protrusion events in M4 and M4F cells. (E-G) Speed (E), persistence (F) and distance (G) of individual lamellipodial protrusions. Data for M4 cells are from 273 protrusion events and for M4F cells from 144 protrusion events. In the box and whisker plots, horizontal line indicates median, box indicates interquartile range and whiskers indicate maximum value to minimum value. Mann-Whitney test; ***P<0.001; *P<0.05; n.s., not significant.

An interesting difference between the role of fascin 1 in Drosophila haemocytes and fascin 1 in mouse melanoblasts seems to be that fascin 1 loss in Drosophila haemocytes dramatically impairs directed migration and guidance (Zanet et al., 2009). Drosophila embryos mutant for fascin 1 showed an accumulation of haemocytes in the anterioventral regions where they originate and a failure to disperse during development or in response to wounding. By contrast, fascin 1-null melanoblasts were able to home to hair follicles and to reach most areas of the mouse epidermis during embryogenesis. Fascin 1-null haemocytes failed to polarize their actin cytoskeletons (Zanet et al., 2009), whereas melanoblasts devoid of fascin 1 showed relatively normal shape and polarization of pseudopods. This indicates that the requirements for fascin 1 in migration during development are cell type specific, similar to requirements for Rac1, which vary depending on the cell type and tissue context (Migeotte et al., 2010; Wang et al., 2010; Li et al., 2011).

The role of fascin 1 in cell proliferation and cell cycle progression is not entirely clear and has only previously been seen in tumour tissues or tissue culture cells. Quantification of the cell numbers at different stages of embryogenesis and subsequent staining for markers of apoptosis and cell proliferation revealed that fascin-null melanoblasts proliferate slower than normal cells and this phenotype was recapitulated in fascin 1-expressing melanoma cell lines in culture. The embryonic requirement for fascin 1 during melanoblast proliferation might reflect a possible mechanism by which cancer cells could gain not only a motility advantage but also a proliferation advantage by expressing fascin 1. In our studies, fewer fascin 1 knockout melanoblasts were BrdU positive (Fig. 3E), indicating that cells lacking fascin 1 were proliferating slower than controls. In agreement with our knockdown studies, enhanced proliferation was seen in fascin 1-transfected colon carcinoma cells (Jawhari et al., 2003) and oesophageal squamous carcinoma cells (Xie et al., 2005; Xie et al., 2010). However, as highlighted by Hashimoto et al. (Hashimoto et al., 2005b), there is some controversy over whether high fascin 1 levels correlate with high or low proliferation rates in tumours (Pelosi et al., 2003; Hashimoto et al., 2005a). We did not see any significant changes in levels of ERK/MAPK or FAK signalling by western blot detection of phospho-epitopes in cell lysates from fascin knockdown melanoma cells, so the mechanism of involvement of fascin 1 in cell proliferation remains unknown. Although the role of fascin 1 in cell cycle progression is not known, fascin 1 might indirectly affect microtubule spindle dynamics and cell mitosis through the interaction of a myosin X/fascin/actin and actin-microtubule network (Woolner et al., 2008; Nagy and Rock, 2010).

In summary, fascin 1 loss results in hypopigmentation defects in mice that arise from a reduced migration and proliferation of melanoblasts during embryogenesis. Melanoblasts devoid of fascin can still differentiate and migrate in the dermis and epidermis, but with reduced efficiency. Melanoblasts and melanoma cells show...
proliferation defects when fascin is depleted, suggesting a dependence of these cells on fascin for normal cell cycle progression. Melanoma tumour cells undergo changes associated with de-differentiation towards a more migratory and embryonic state, and this is thought to aid metastatic dissemination. Fascin1 expression benefits melanoma and melanocyte cells in migration, invasion and proliferation; such as expression, fascin 1 likely provides advantages in tumours.

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Competing interests statement
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