FULL PAPER

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Interplay between cadherins and α2β1 integrin differentially regulates melanoma cell invasion

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Background: Malignant transformation of melanocytes frequently coincides with an alteration in the expression of cell–cell adhesion molecules (cadherins) and cell-extracellular matrix proteins (integrins). How these two adhesion systems interplay to impact on cell invasion remains to be described in melanoma.

Methods: Cell adhesion networks were localised by immunofluorescence in human primary cutaneous melanoma, metastatic melanoma in the lymph nodes, and melanoma cell lines. The role of these cell adhesion networks was assessed both *in vivo*, by analysing their impact on tumour growth in mice, and *in vitro*, with the use of functional tests including cell aggregation and cell migration.

Results: We found that α2β1 integrin associates with both E-cadherin and N-cadherin to form two adhesive networks, distinguishable by the interaction—or not—of α2β1 integrin with type I collagen. N-cadherin/α2β1 integrin and E-cadherin/α2β1 integrin networks differently participated towards tumour growth in mice. The N-cadherin/α2β1 integrin network showed specific involvement in melanoma cell invasion and migration towards type I collagen. On the other hand, the E-cadherin/α2β1 network regulated cell–cell adhesion.

Conclusions: This suggests that different signalling environments can be generated, depending on the type and/or local concentration of cadherin present in the adhesion complex, which potentially leads to differential cell responses. Further clarification of how these adhesive networks are regulated is fundamental to understanding important physiological and pathological processes such as morphogenesis, wound healing, tumour invasion and metastasis.

Deregulated cell migration is a key feature of melanoma progression, and is required for metastasis. As melanoma progresses, tumour cells appear to undergo a series of molecular changes that allow them to travel through neighbouring cells, invade the extracellular matrix (ECM), and migrate until reaching the circulation, thus leading to metastasis (Haass and Herlyn, 2005). Altered cell–cell adhesion and cell–ECM interactions clearly have an important role therefore in this process (Johnson, 1999).

Integrins are heterodimeric transmembrane receptors composed of non-covalently linked α and β subunits, which are endowed with both structural and regulatory functions (Hynes, 2004). They are known to have a key role during cell migration by linking the ECM to the actin cytoskeleton at focal adhesion sites and by transmitting the forces required for migration (Wehrle-Haller, 2012). Changes in integrin repertoire have been shown to impact cancer development and progression (Seguin *et al*, 2015) and more specifically, increased levels of αv, α2, α3 and α4 integrin subunits found in primary and metastatic melanomas (Orimoto *et al*, 2008; Pinon and Wehrle-Haller, 2011; Kuphal and Bosserhoff, 2012). Furthermore, in some tumours including melanomas, α2β1 integrin expression is associated with cell migration and invasion (Gui *et al*, 1997; Girotti *et al*, 2011; Huang *et al*, 2011) and novel

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biomarkers based on integrin antagonists have been evaluated as important prognostic and diagnostic tools for cancer therapy (Cox *et al*, 2010).

Cadherins represent a superfamily of calcium-dependent cell–cell adhesion proteins (Nelson *et al*, 2013), which are differentially expressed throughout the body. These transmembrane glycoproteins establish homophilic adhesion between neighbouring cells through their extracellular domain and hence exert an organising function required for tissue integrity (Niessen *et al*, 2011). As observed for integrins, cadherins expression shows similar changes during tumour progression, depending on the environmental constraints encountered. In melanoma, a cadherin switch (a shift from E-cadherin and P-cadherin found on normal melanocytes, to N-cadherin on melanoma cells) has been described (Seline *et al*, 1996; Li *et al*, 2001; Kuphal and Bosserhoff, 2012). In this way, melanoma cells may escape from control by keratinocytes in the epidermis and interact with constituents of the dermis such as fibroblasts and endothelial cells. However, this switch is highly reversible, which explains why melanoma cells in primary melanomas and their metastases show heterogeneous and variable expression of cadherin (Kuphal and Bosserhoff, 2012). The use of N-cadherin antagonists in cancer treatment is in early stages of investigation (Blaschuk, 2015), although one clinical trial testing cadherin antagonists in combination with melphalan in the treatment of melanoma gave encouraging results (Beasley *et al*, 2009).

Accumulating data depict an adhesion machinery comprising an interdependent network of cell–cell and cell–ECM interactions that, together with their corresponding signalling responses, participate towards the regulation of cell migration (Borghi *et al*, 2010; Weber *et al*, 2011; Epifano and Perez-Moreno, 2012; Canel *et al*, 2013). What remains unclear is how these adhesion systems integrate to form such an interdependent adhesive network that can affect the response of cells within contextually appropriate and dynamic microenvironments (Canel *et al*, 2013). In this study, we aimed to decipher the role of the cadherin/$\alpha 2\beta 1$ integrin adhesive networks in melanoma cell invasion and cell–cell adhesion.

MATERIALS AND METHODS

Antibodies. The antibodies used in this report are listed in Supplementary Information.

Cells and tissues. The human melanoma IGR39 cell line was routinely cultured in DMEM (Invitrogen, Illkirch, France) containing 10% foetal calf serum (FCS, Invitrogen) as previously described, whereas human melanoma C8161 and WM278 cell lines were cultured in RPMI (Invitrogen) containing 10% FCS (Bregman *et al*, 1986; Lissitzky *et al*, 2009; Sarray *et al*, 2009). Integrin expression pattern of IGR39 cells is depicted in Supplementary Figure S1.

Formalin-fixed and paraffin-embedded melanomas (six primary tumours: Breslow 2.1 mm-Clark IV, Breslow 2.25 mm-Clark IV, Breslow 3.15 mm-Clark IV, Breslow 6.7 mm-Clark IV, Breslow 1.8 mm-Clark III, and Breslow 2.23 mm-Clark III; and three lymph node metastases) from adult patients were obtained from the Biobank of the canceropole in the PACA region of France. Tissues were obtained according to local ethical guidelines and approved by the local regulatory committee. All specimens were re-evaluated by an expert pathologist. A melanoma tissue array containing 12 cases of normal epithelium and naevus, 26 cases of primary melanomas and 10 cases of metastatic melanomas was purchased from Pantomics (Euromedex, Souffelweyersheim France).

Cadherin and integrin silencing. IGR39 cells were seeded at 150 000 cells per well in six-well plates. Cells were washed 24 h later with OptiMeM (Gibco, Illkirch, France) without FCS and transfected with Oligofectamine reagent (Invitrogen) and 50 nM of a mix of four different siRNAs targeting E-cadherin, N-cadherin, -$\alpha 1$ integrin, and $\alpha 2$ integrin; or siRNA control (ON-targetplus SMARTpool SiRNA; ThermoScientific, Illkirch, France). OptiMeM containing 20% FCS was then added 5 h later to each well and left for 48 h before experiments.

For cell injections in mice, E- and N-cadherin were stably knocked down in IGR39 cells by RNA interference using Mission shRNA lentiviral transduction particles (Sigma, St Quentin Fallavier, France).

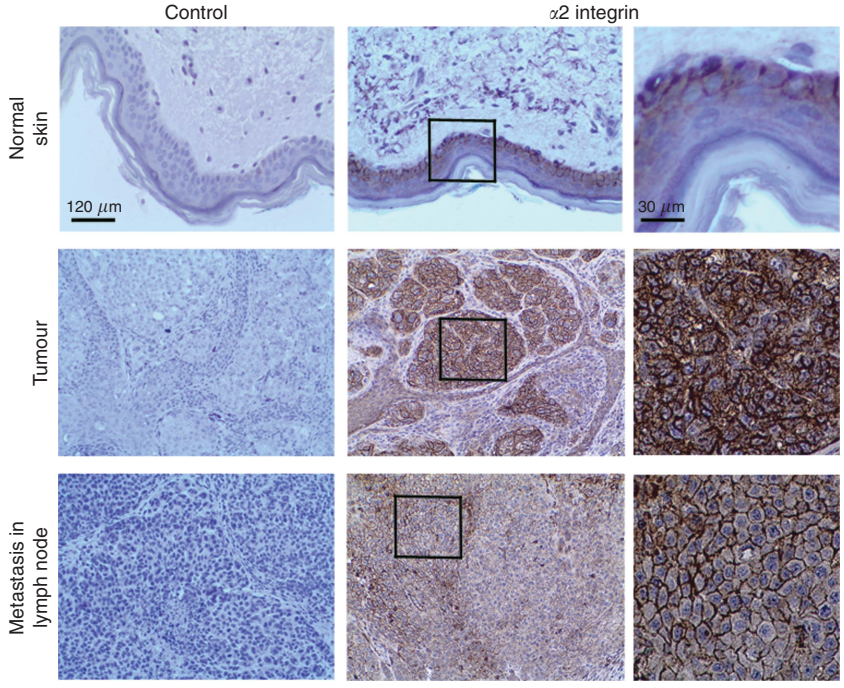

Figure 1. $\alpha 2\beta 1$ Integrin is detected at cell–cell contacts between melanoma cells. Paraffin sections of primary cutaneous melanoma and melanoma metastasis in lymph node were stained with a mouse anti-$\alpha 2$ integrin subunit or mouse IgG1 mAbs. The antigen/Ab complex was detected as mentioned in the Materials and Methods section. The mouse IgG1 was used as isotypic Ab control.

Two lentiviral transduction particles per cadherin were chosen (TRCN0000237840 and TRCN0000237843 for E-cadherin and TRCN0000312701 and TRCN0000327706 for N-cadherin) and a negative control. IGR39 cells were infected with lentiviral transduction particles according to the manufacturer's instructions. Small hairpin RNA-containing cells were selected with 5 μg ml^{-1} puromycin and cloned. Cells were harvested to assess cell surface cadherin expression by western blotting or immunofluorescence analysis (Supplementary Figures S2 and S3) and to perform functional assays. Cell clones obtained by infection with lentiviral particles TRCN0000237843 and TRCN0000312701 were inoculated into mice.

Table 1. Expression and localisation of alpha-2 integrin subunit, E-cadherin, N-cadherin and P-cadherin in sections of healthy skin, primary cutaneous melanoma and melanoma metastasis in the lymph node

	Alpha-2 int	E-cadh	N-cadh	P-cadh
Healthy skin ($n=4$)				
Mb	4	4	0	0
Cyt	0	0	0	0
Mb + Cyt	0	0	0	4
Naevus ($n=4$)				
Mb	4	4	1	0
Cyt	0	0	0	0
Mb + Cyt	0	0	0	4
Primary melanoma ($n=25$)				
Mb	20	23	19	0
Cyt	0	0	0	17
Mb + Cyt	0	0	0	1
Metastasis ($n=15$)				
Mb	15	11	9	0
Cyt	0	0	0	12
Mb + Cyt	0	0	0	0

Abbreviations: Cyt = exclusively cytoplasmic; Mb = exclusively membranous; Mb + Cyt = both membranous and cytoplasmic. Paraffin sections were stained with a rabbit anti-α2 integrin subunit (alpha-2 int), mouse anti-E-cadherin (E-cadh), mouse anti-N-cadherin (N-cadh) or rabbit anti-P-cadherin (P-cadh). The antigen/Ab complex was detected as mentioned in the Materials and Methods section.

Spheroid formation assay. Isolated IGR39 cells were seeded onto U-bottom non-treated tissue culture 96-well plates at a concentration of 5000 cells per well in 100 μl DMEM containing 10% FCS and 0.24% methylcellulose (Longati *et al*, 2013). Spheroids were grown under standard culture conditions for 24 h. The size of the spheroids was quantified by measuring the area occupied by cells using ImageJ software (rsb.info.nih.gov/ij/).

Cell invasion assay. *In vitro* invasion through type I collagen was assayed using transwell-based cell culture chamber systems (Millipore-Chemicon, Molsheim, France). Cells were suspended in DMEM/0.1% BSA before being added at a concentration of 20 000 cells per well to the upper chamber containing a polycarbonate membrane filter of 8 μm pore size and a type-I collagen layer. The lower chamber was filled with DMEM supplemented with 1% FCS. Plates were incubated under standard culture conditions for 24 h. Cells remaining on the upper side of the filter and type I collagen were removed using a cotton swab, and the invading cells on the lower side of the filter were stained with Coomasie Blue. Quantification of invading cells was

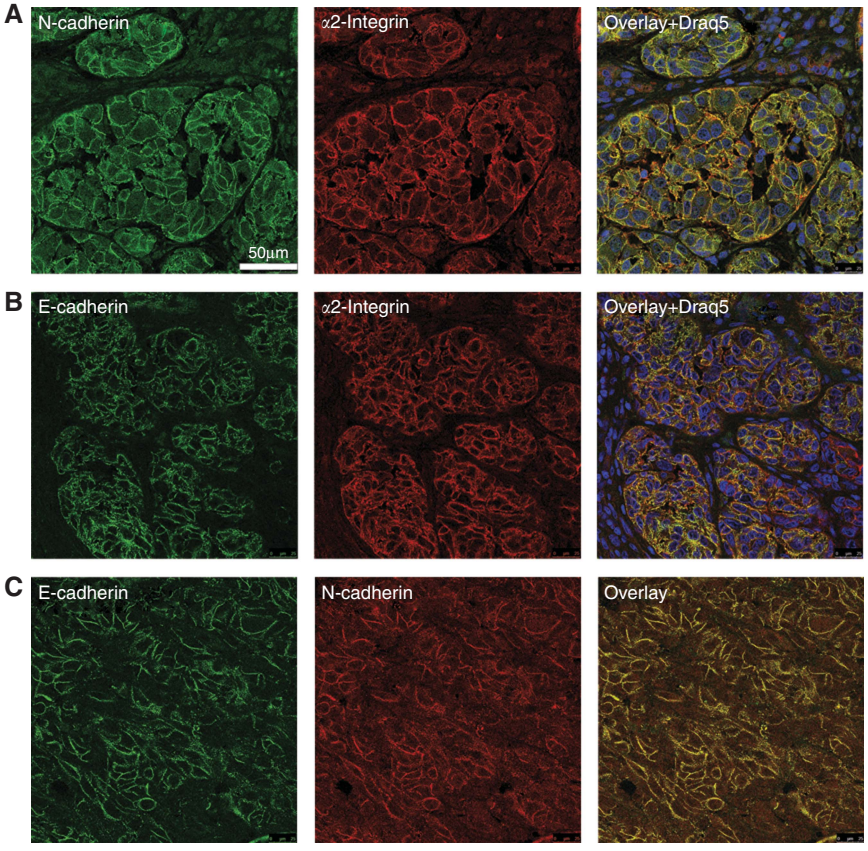

Figure 2. α2β1 Integrin and cadherins colocalise in melanomas. Sections of primary cutaneous melanoma were sequentially stained with (**A**) mouse anti-α2 integrin and rabbit anti-N-cadherin, (**B**) mouse anti-α2 integrin and rabbit anti-E-cadherin or (**C**) mouse anti-E-cadherin and rabbit anti-N-cadherin. Tissues were then incubated with Alexa 488-conjugated goat anti-mouse and Alexa 594-conjugated goat anti-rabbit Abs and were observed with a confocal microscope. Cell nuclei were stained with Draq 5.

performed in two independent experiments performed in triplicate. The invasion index was calculated as the mean number of migrated cells counted in 10 microscopic fields (magnification, $\times 200$) per well.

Cell migration assay. *In vitro* haptotaxis assays were performed as previously described for colon cancer cells (Defilles *et al*, 2011). Migration results were expressed as the average number of cells per microscopic field obtained from at least three separate experiments performed in triplicate.

Cell proliferation assay. Cells were seeded at a density of 4000 cells per well in 96-well plates and were incubated during 24 or 48 h. Cell proliferation was analysed using thiazolyl blue tetrazolium bromide (Sigma) staining according to the manufacturer's instructions.

Immunofluorescence. Cells plated on 10 $\mu g\,ml^{-1}$ type-I collagen I-coated glass coverslips (Upstate, Lake Placid, NY, USA) or cell aggregates were fixed for 20 min with 2% formaldehyde in PBS, permeabilised with 0.1% saponin in PBS for 20 min and then blocked for 30 min in PBS containing 4% (w/v) BSA. N-cadherin, E-cadherin and α2 integrin were detected by incubation with HECD-1, Ab 18203, and HAS-3 Abs, respectively. After three washes in PBS, cells were incubated with Alexa Fluor 488 or 594-conjugated goat Ig (20 $\mu g\,ml^{-1}$, Invitrogen), raised against mouse and rabbit Igs respectively, for 1 h, then washed and mounted in ProLong Gold (Invitrogen). Images were captured and analysed using a SP5 Leica (Nanterre, France) confocal microscope equipped with LAS AF Lite software (Nanterre, France).

Immunohistochemistry. After dewaxing and antigen retrieval, paraffin-embedded 4-μm sections were incubated with a mouse anti-α2 integrin mAb or mouse IgG1 isotype control for 2 h at room temperature. After washing, the sections were then processed in the En Vision G/2 system (DAKOCytomation, Trappes, France) with alkaline phosphatase-conjugated antibodies to mouse. Sections were counterstained in Mayer's haematoxylin solution, and mounted in aqueous permanent mounting medium. Images were captured using a BH-2 Olympus microscope with $\times 20$ objective (Rungis, France). For double immunostaining, tissues were treated and observed as described above.

Detection of tyrosine-phosphorylated FAK. Detection of tyrosine-phosphorylated FAK was performed as described in the Supplementary Information.

Subcutaneous xenografts of melanoma cells. All experimental procedures involving animals were performed in accordance with the French Guidelines and approved by the ethical committee of Marseille (agreement 50-31102012). Human melanoma cells were harvested by mild trypsinisation, washed twice in PBS, and then suspended in PBS mixed with Matrigel (1/1) at 5×10^6 cells per 100 μl. Cell suspension was injected subcutaneously into the flank of Female NMRI-Foxn1nu/Foxn1nu mice aged 6–8 weeks (Charles River Laboratories, L'Arbresle, France; six mice per group). The mice were monitored daily up to 4 weeks after inoculation when they were killed and their tumours removed and weighed.

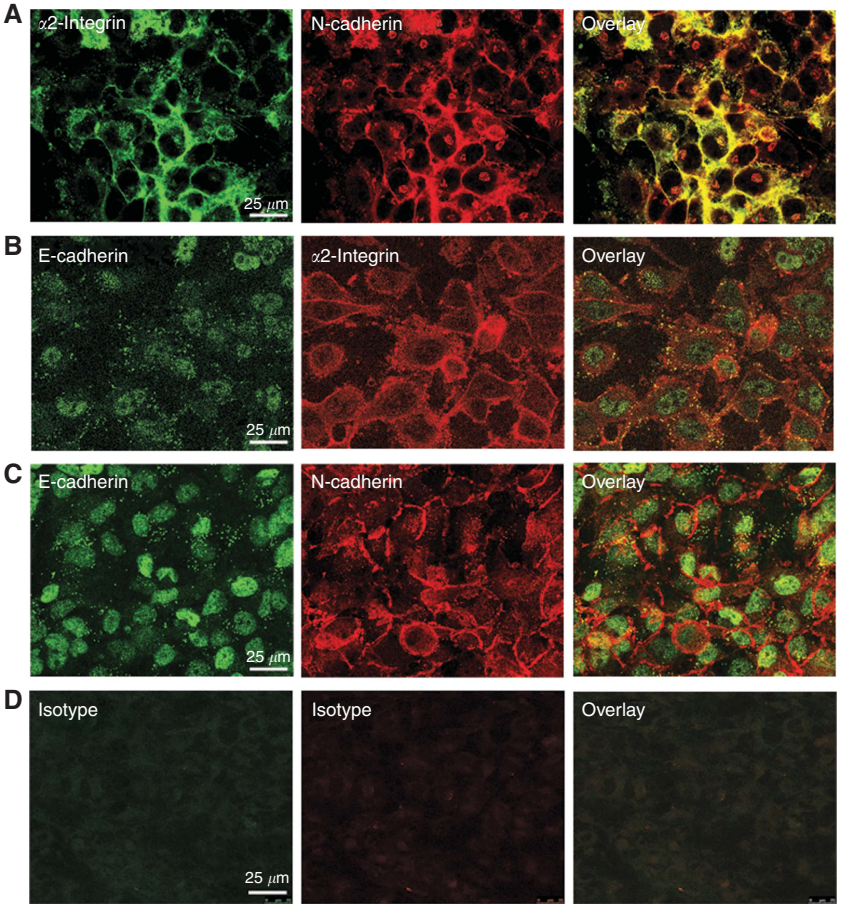

Figure 3. Localisation of α2β1 integrin and cadherins in melanoma cells (**A**–**C**) IGR39 cells cultured on type I collagen for 24 h were fixed, permeabilised and stained with a mixture of: (**A**) mouse anti-α2 integrin and rabbit anti-N-cadherin; (**B**) mouse anti-α2 integrin and rabbit anti-E-cadherin; (**C**) mouse anti-E-cadherin and rabbit anti-N-cadherin; or (**D**) mouse IgG1 and rabbit Igs. Cells were then incubated with Alexa 488-conjugated goat anti-mouse and Alexa 594-conjugated goat anti-rabbit Abs and were observed with a confocal microscope. The nuclear staining observed with rabbit anti-E-cadherin is non-specific.

Statistical analysis. Data are presented as the means ± s.d. for three independent experiments performed in triplicate. Comparison between the two conditions was made using the Mann–Whitney test. $P<0.05$ was considered statistically significant in all analyses and is indicated by '***' when <0.001, '**' when <0.01 and '*' when <0.05.

RESULTS

α2β1 Integrin localised with both N-cadherin and E-cadherin in primary and metastatic melanomas. We used immunostaining to assess the expression of cadherins and α2β1 integrin on a melanoma tissue microarray and on tissue sections from patients with cutaneous melanoma, both including primary and metastatic tumours. In normal epidermal layer, the α2β1 integrin staining was limited to the membrane of cells located at the dermal–epidermal junction (Figure 1), whereas in all naevi, primary and metastatic melanomas analysed, it was predominantly found not only at the cell–ECM interface but also at cell–cell contacts between tumour cells (Figure 1; Table 1). Concerning the cadherins, we observed an appropriate membranous expression of both E- and P-cadherin in healthy tissues. As expected, both primary and metastatic melanomas displayed a marked loss of membranous P-cadherin expression, whereas a large subset of them expressed N-cadherin. Interestingly, most melanomas found to express both membranous N-cadherin and α2β1 integrin, also displayed membranous E-cadherin immunoreactivity (Table 1). This strongly suggests that E-cadherin, N-cadherin and α2β1 integrin are co-expressed in a large number of primary and metastatic melanoma cells.

Double immunostaining confirmed that the majority of melanoma cells expressed these two cadherins (Figure 2C). Dual staining also showed that both E- and N-cadherin localised with α2β1 integrin at cell–cell contact sites (Figure 2A and B). Altogether, these results suggest that in primary and metastatic melanomas, N-cadherin and E-cadherin may elaborate adhesive networks with α2β1 integrin, unlike P-cadherin, which we did not detect at their cell surface.

N-cadherin and E-cadherin differentially localise with α2β1 integrin. To confirm this hypothesis, we studied IGR39 melanoma cells that express α2β1 integrin and both E- and N-cadherin, but not P-cadherin (not shown). These cells expressed α2β1 integrin not only at the cell–substratum interface but also at cell–cell contacts where it colocalised with N-cadherin (Figure 3A). This localisation appeared to be type-I collagen-specific, as plating of cells on laminin resulted in a weaker expression of α2β1 integrin at the cell–cell contacts (Supplementary Figure S4A), and could not be generalised to all integrins (Supplementary Figure S4B). The similarity of results we obtained using other melanoma cell lines indicates a generalised phenomenon in these cells (Supplementary Figure S5).

E-cadherin exhibited a punctated pattern of expression located in the vicinity of cell–cell contacts or at the cell periphery (Figure 3B). Double immunostaining indicated that E-cadherin and N-cadherin did not colocalise and were instead differentially distributed at the cell membrane (Figure 3C).

When cultured in suspension to abolish α2β1 integrin/type-I collagen interaction, IGR39 cells formed floating aggregates. In this cell organisation, E-cadherin, N-cadherin and α2β1 integrin colocalised at cell–cell contact sites (Supplementary Figure S6), suggesting that the α2β1 integrin/type-I collagen interaction induces E-cadherin relocalisation.

Both E-cadherin and N-cadherin regulate tumour growth. The different associations of α2β1 integrin with E-cadherin and N-cadherin suggest the existence of two adhesive networks that differ according to the embedded cadherin. We injected IGR39 melanoma cells into the flank of nude mice to assess the effect of altering adhesive networks on the resulting primary tumour growth. As observed in Figure 4A, stable depletion of N-cadherin or E-cadherin resulted in a reduction of tumour growth. Taken together, these results suggest that both N-cadherin/α2β1 integrin and E-cadherin/α2β1 integrin adhesive networks are involved in the tumour growth.

α2β1 Integrin modulates E-cadherin-mediated adhesion. We next investigated the effect of N-cadherin and E-cadherin silencing on the cell–cell adhesion properties of IGR39 cells. These cells spontaneously formed compact spheroids when cultured in suspension (Figure 4B). The spheroids formed by E-cadherin-depleted cells or stably knocked-down E-cadherin IGR39 cells were less compact (Supplementary Figure S7). N-cadherin depletion on the other hand weakly increased the size of the spheroids. Interestingly, silencing of α2β1 integrin, but not α1β1 integrin, modulated the cell–cell adhesion (Figure 4C). These results indicate that α2β1 integrin and E-cadherin are the major factors in cell–cell adhesion.

N-cadherin and E-cadherin differentially regulate α2β1 integrin-mediated invasion. IGR39 cells spontaneously invade type I collagen mainly through α2β1 integrin, although α1β1 integrin does seem to participate in this process (Figure 5A).

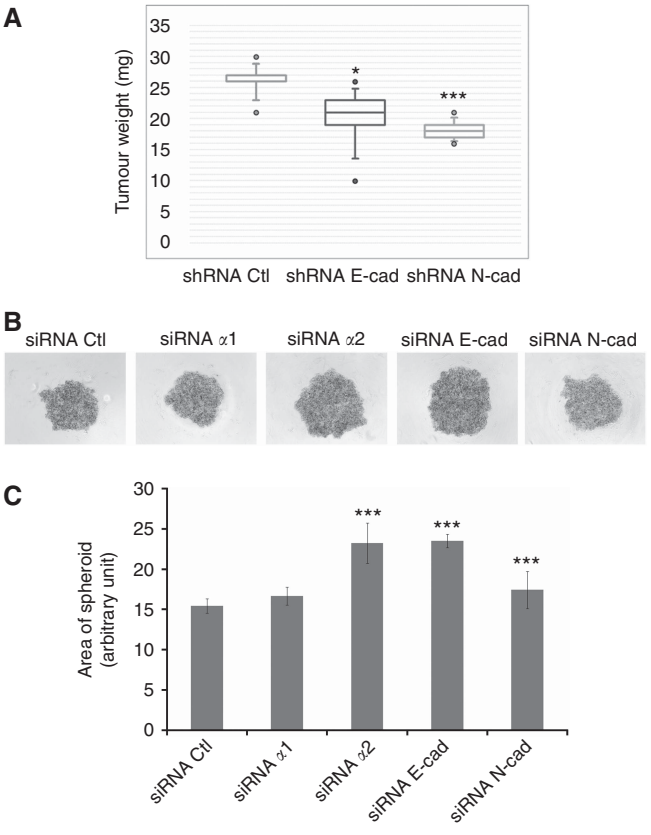

Figure 4. Role of adhesive network on tumour growth and cell–cell adhesion. (**A**) Effect of E- and N-cadherin silencing on tumour growth. Nude mice were injected in the flank with the different IGR39 cell lines generated. Box plot represents tumour weight 4 weeks after cell inoculation. (**B**) Effect of cadherin or integrin silencing on spheroid formation. IGR39 cells were transfected for 48 h with siRNAs against N-cadherin, E-cadherin, α1 integrin or α2 integrin, or scramble oligos (ctl) and allowed to form spheroids in suspension for 24 h. (**C**) The areas of the spheroids were measured by phase-contrast microscopy and analysed by ImageJ. Data represent the mean ± s.d. of three separate experiments performed in triplicate. *$P<0.05$; ***$P<0.001$.

E-cadherin silencing had no effect on this cell invasion, whereas N-cadherin knockdown inhibited it. This blockade was not due to an inhibition of cell proliferation, as neither E-cadherin nor N-cadherin modulated cell proliferation (Figure 5B). N-cadherin thus appeared here to be regulating the propensity of IGR39 to migrate. To confirm this hypothesis, IGR39 cells were allowed to migrate towards type I collagen (Figure 5C). The inhibition of N-cadherin, but not E-cadherin, via Ab blocking or siRNA knockdown approaches markedly suppressed $\alpha2\beta1$-integrin-dependent cell migration (Figure 5D and E). This blockade depended on the ECM protein used, as it did not occur when we used fibronectin as a chemoattractant (Figure 5F). These results suggest that N-cadherin exerts its proinvasive effect through activation of cell migration.

N-cadherin knockdown promoted relocalisation of $\alpha2\beta1$ integrin from cell–cell contact to cell–ECM contact sites (Figure 6A; Supplementary Figure S8). Furthermore, analysis of $\alpha2\beta1$ integrin detected at the cell–substratum interface revealed that N-cadherin silencing markedly increased both the density and the surface area of cell–ECM contact sites. However, E-cadherin knockdown failed to promote $\alpha2\beta1$ integrin redistribution to cell–ECM contact sites. Altogether, these data indicate that N-cadherin, not E-cadherin, regulates a pool of $\alpha2\beta1$ integrin at cell–cell adhesion sites and thus possibly modulates $\alpha2\beta1$ integrin-mediated cell function.

We then studied the impact of cadherin silencing on the phosphorylation status of FAK, a molecule involved in the $\alpha2\beta1$ integrin signalling pathway. Cell adhesion to type I collagen promoted phosphorylation of FAK on the Y397 residue, confirming activation of $\alpha2\beta1$ integrin. Interestingly, N-cadherin silencing induced an increase in the phosphorylation status of FAK, indicating that N-cadherin regulates the $\alpha2\beta1$ integrin-mediated signalling pathway (Figure 6C).

DISCUSSION

The aim of this study was to analyse the interplay between cadherins and $\alpha2\beta1$ integrin in melanoma cells. According to our findings, the role of cell adhesion molecules in melanoma cells can be summarised as follows: (i) E-cadherin, N-cadherin and $\alpha2\beta1$ integrin are co-expressed in a large number of primary and metastatic melanoma cells; (ii) a subset of $\alpha2\beta1$ integrin, localised at intercellular sites, can regulate cell–cell adhesion; (iii) $\alpha2\beta1$ integrin differentially localises with E-cadherin and N-cadherin, suggesting that at least two adhesive complexes coexist in melanoma cells; (iv) both $\alpha2\beta1$ integrin/N-cadherin and $\alpha2\beta1$ integrin/E-cadherin adhesive networks participate towards tumour growth in mice; (v) the $\alpha2\beta1$ integrin/N-cadherin adhesive network participates in the regulation of melanoma cell invasion

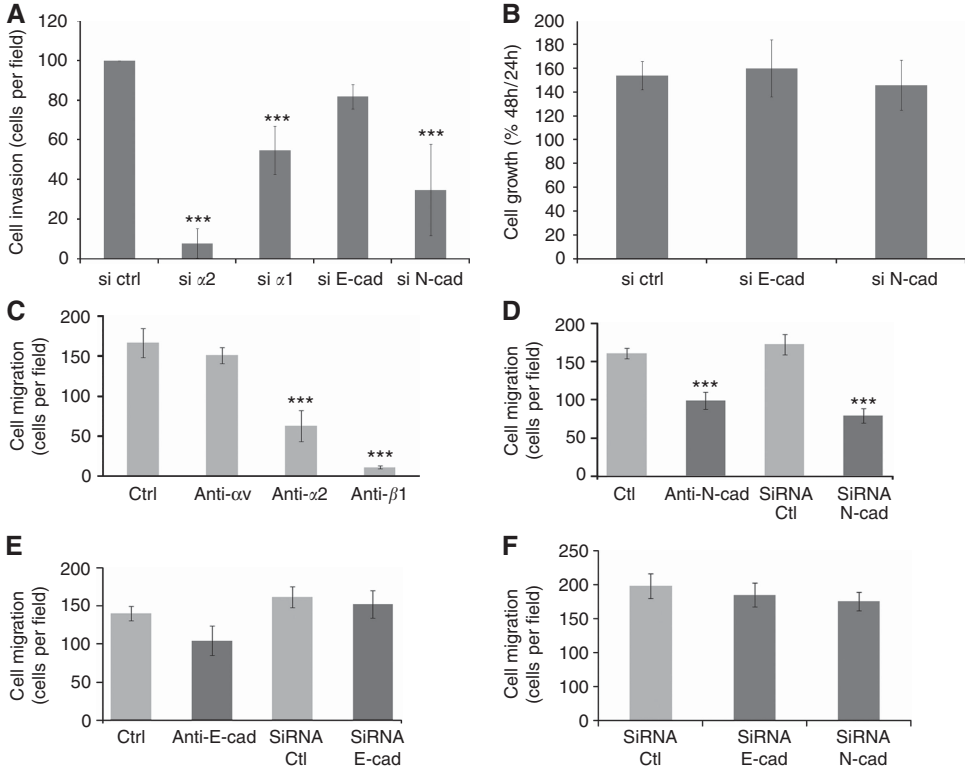

Figure 5. N-cadherin, but not E-cadherin, is required for type-I collagen-dependent cell invasion and migration. (**A**) IGR39 cells were transfected for 48 h with siRNA targeting N-cadherin, E-cadherin, α1 integrin or α2 integrin, or with scramble oligos (ctl) and allowed to invade a layer of type I collagen for 24 h. (**B**) IGR39 cells were transfected with siRNA targeting N-cadherin, E-cadherin or with scramble oligos (ctl). Cell proliferation was assessed using the thiazolyl blue tetrazolium bromide cell viability assay 24 and 72 h after transfection. Results are expressed as a percentage of cell proliferation between t24 and t48 h. (**C**–**E**) Migration assays were performed in modified Boyden chambers using filters coated with 3 $\mu g\,ml^{-1}$ type I collagen. (**C**) Isolated IGR39 cells were pretreated without or with anti-αv, -α2, or -β1 integrin subunit mAbs for 15 min, then allowed to migrate for 3.5 h. The specificity of the observed inhibitory effects was confirmed by the inability of αv integrin subunit to perturb cell migration. (**D** and **E**) Cadherin function or expression was abolished in IGR39 cells using neutralising anti-cadherin mAbs or siRNAs targeting N-cadherin or E-cadherin. Cells were then allowed to migrate for 3.5 h. Preliminary experiments showed that incubation of cells with 5 $\mu g\,ml^{-1}$ anti $\alpha2\beta1$ integrin Ab for 24 h did not affect cell viability. Data represent the mean ± s.d. of three separate experiments performed in triplicate. ***$P<0.001$. (**F**) Cadherin expression was abolished in IGR39 cells using siRNAs against either N-cadherin or E-cadherin. Cells were allowed to migrate 3.5 h in modified Boyden chambers using filters coated with 3 $\mu g\,ml^{-1}$ fibronectin.

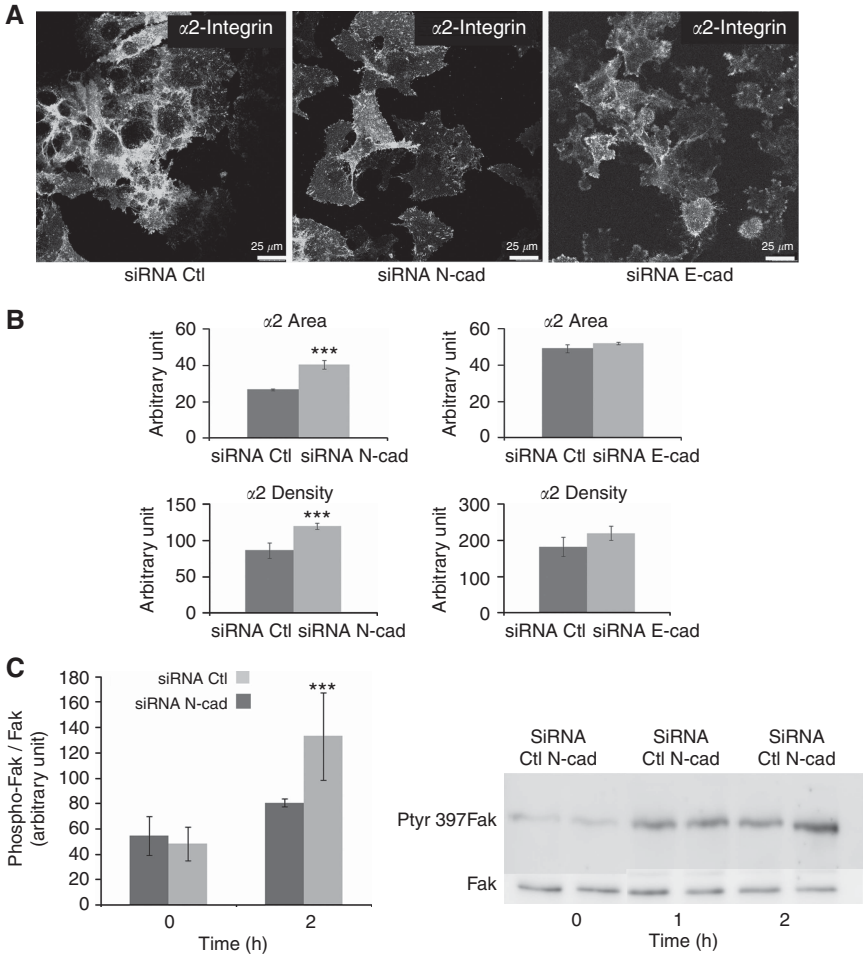

Figure 6. N-cadherin depletion promotes $\alpha 2\beta 1$ integrin redistribution in focal adhesions. (**A**) IGR39 cells were transfected for 48 h with siRNA targeting N-cadherin or E-cadherin or with scramble oligos (ctl), then plated for 24 h on type I collagen. Cells were stained with an anti-α2 integrin subunit Ab and observed using a confocal microscope. (**B**) α2 Integrin subunits associated with the ECM were quantified using ImageJ software as described in the Materials and Methods section. Both the area and density of each structure from all cells are reported. (**C**) Cells were transfected with siRNA against N-cadherin or with control siRNA for 48 h and then allowed to adhere on type I collagen. The phosphorylation of tyrosine residues in FAK (Tyr-397 FAK) was determined after cell lysis at the indicated times of cell adhesion. Samples were analysed by western blot analysis. Equal amounts of protein were analysed and loading amounts were verified by probing the blot with anti-FAK. Values presented correspond to the percentage of band intensity of tyr-397 FAK compared with FAK. ***$P<0.001$.

and migration, with N-cadherin silencing promoting the relocalisation of $\alpha 2\beta 1$ integrin in cell–ECM adhesion structures and modulation of $\alpha 2\beta 1$ integrin signalling leading to a decrease in cell migration; and finally (vi) in the absence of cell-type-I collagen interaction, $\alpha 2\beta 1$ integrin/E-cadherin complex regulates cell–cell adhesion.

Traditionally, integrins are thought to mediate cell–matrix adhesion. However, some integrins, including $\alpha 2\beta 1$, $\alpha 3\beta 1$ and $\alpha v\beta 5$ integrins, have also been detected at cell–cell contact sites both *in vitro* and *in vivo* (Yu *et al*, 2000; Chattopadhyay *et al*, 2003; Canonici *et al*, 2008; Canonici *et al*, 2011; Weber *et al*, 2011). Moreover, $\beta 1$ integrins have been shown to mediate cell–cell adhesion via homophilic or heterophilic integrin–integrin interactions (Sriramarao *et al*, 1993) (Sriramarao *et al*, 1993; Symington *et al*, 1993)(Sriramarao *et al*, 1993; Symington *et al*, 1993)(Sriramarao *et al*, 1993; Symington *et al*, 1993). In agreement with these studies, we have here reported that $\alpha 2\beta 1$ integrin may act as a functional member of the cell–cell adhesion complex in melanoma. Indeed, we detected $\alpha 2\beta 1$ integrin at cell–cell contacts of all tested primary and metastatic melanoma cells and of at least three of the melanoma cell lines; its downregulated or blocked expression abrogated cell–cell adhesion. The role of $\alpha 2\beta 1$ integrin at cell–cell contacts is unclear. One explanation may be that an association between $\alpha 2\beta 1$ integrin and cadherins could lead to a local increase in receptor density at cell–cell contacts. Such a local concentration increase could be crucial for the coupling between cadherin and $\alpha 2\beta 1$ integrin-mediated signalling pathways. Another possibility is a $\alpha 2\beta 1$ integrin sequestration outside focal adhesion sites. This could be of major importance in coordinating alterations in cell–ECM adhesion. In accordance with this theory, the downregulated expression of N-cadherin in our study induced $\alpha 2\beta 1$ integrin relocalisation at cell–ECM contact sites.

Some studies have shown that P-cadherin also strongly influences invasiveness of melanoma cells and that it should be considered as an invasion suppressor (Jacobs *et al*, 2010; Jacobs *et al*, 2011). Moreover, other studies, including our work, have shown that in most melanomas P-cadherin is shifted to the cytoplasm (Bachmann *et al*, 2005). Clearly, P-cadherin not localising at the melanoma cell membrane indicates it does not form part of the adhesive network with $\alpha 2\beta 1$ integrin at cell–cell contacts. For this reason, we chose not to analyse *in vitro* the consequence of the association between P-cadherin and $\alpha 2\beta 1$ integrin in this study. However, the impact that a cytoplasmic localisation of P-cadherin has in melanoma is unlikely to be neutral

and may be associated with increased tumour thickness, level of invasion and reduced overall survival (Bachmann *et al*, 2005).

The use of a melanoma cell line expressing both E- and N-cadherin enabled us to establish the hierarchy of crosstalk between cadherins and $\alpha2\beta1$ integrin. In our study, N-cadherin and E-cadherin differentially localised with $\alpha2\beta1$ integrin, suggesting the existence of two functional adhesive networks. Indeed, confocal immunofluorescence staining disclosed that both E- and N-cadherin localised with $\alpha2\beta1$ integrin. However, although we detected both cadherins at the cell surface, they exhibited distinct subcellular localisations. Indeed, E-cadherin displayed a mainly punctate distribution in the vicinity of cell–cell contacts where it did not localise with the there-localised N-cadherin. This E-cadherin pattern of expression requires further characterisation. Our unpublished data indicated that it is neither related to a recycling endosome compartment, as observed in some cells (Balzac *et al*, 2005), nor associated with the cell–ECM interface.

On the basis of the knowledge that both E- and N-cadherin are involved in tumour growth in mice, we postulated that different signalling environments could be generated according to the type of cadherin present in an adhesive network, which could lead to differential cell responses. In support of this hypothesis, we showed that E-cadherin is the major factor in melanoma intercellular adhesion. Moreover, we demonstrated that $\alpha2\beta1$-integrin-dependent cell invasion and migration are differentially regulated by cadherins. Our data showed that only N-cadherin regulates $\alpha2\beta1$-dependent melanoma cell invasion and migration towards type I collagen. Further studies using our cell model are now needed to characterise the impact of both E- and N-cadherin on particular parameters of cell migration.

It appears clear that N-cadherin is involved in invasion of various cancer cells including melanoma, although the mechanisms by which it participates in this process need to be explored in more detail (De Wever *et al*, 2004). Here we have reported that: (i) N-cadherin recruited a subset of $\alpha2\beta1$ integrin to cell–cell contacts, in a manner similar to the cadherin-dependent confinement in colon tissue and colon cancer cells we described previously (Canonici *et al*, 2008); (ii) silencing of N-cadherin promoted $\alpha2\beta1$ integrin relocalisation from cell–cell contacts to cell–ECM contact sites. This redistribution was associated with an increase in FAK tyrosine phosphorylation upon cell adhesion to type I collagen and a decrease in cell migration and invasion. FAK is a key mediator of intracellular signalling by integrins and may serve as a channel for the transmission of force necessary for cell migration and bidirectional signalling between the cell interior and its environment (Zaidel-Bar *et al*, 2007). Thus, we suggest that N-cadherin, by modulating FAK tyrosine phosphorylation, may regulate the strength of the $\alpha2\beta1$ integrin/collagen interaction, leading to a change in $\alpha2\beta1$ integrin-dependent melanoma migration. In accordance with this theory, FAK has previously been shown to regulate integrins/N-cadherin crosstalk (Yano *et al*, 2004). Moreover, the strength of cell–ECM adhesion has been shown to require different kinetics depending on the type of cadherin expressed by the cells (Martinez-Rico *et al*, 2010).

In conclusion, we have demonstrated that different signalling environments can be generated according to the type of cadherin present in the adhesion complex, which can lead to differential cell responses. Clarifying how these adhesive networks are regulated should improve our understanding of important physiological and pathological processes such as morphogenesis, wound healing, tumour invasion and metastasis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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