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Regulation of neural progenitor proliferation and survival by β1 integrins

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Summary

Neural stem cells give rise to undifferentiated nestin-positive progenitors that undergo extensive cell division before differentiating into neuronal and glial cells. The precise control of this process is likely to be, at least in part, controlled by instructive cues originating from the extracellular environment. Some of these cues are interpreted by the integrin family of extracellular matrix receptors. Using neurosphere cell cultures as a model system, we show that β1-integrin signalling plays a crucial role in the regulation of progenitor cell proliferation, survival and migration. Following conditional genetic ablation of the β1-integrin allele, and consequent loss of β1-integrin cell surface protein, mutant nestin-positive progenitor cells proliferate less and die in higher numbers than their wild-type counterparts. Mutant progenitor cell migration on different ECM substrates is also impaired. These effects can be partially compensated by the addition of exogenous growth factors. Thus, β1-integrin signalling and growth factor signalling tightly interact to control the number and migratory capacity of nestin-positive progenitor cells.

Key words: Integrins, Neural progenitors, Neurospheres, CNS

Introduction

During the development of the central nervous system (CNS) multipotent neural stem cells (NSCs) give rise to both neurons and glial progenitors in a sequential process during which first uncommitted, and later restricted progenitors are produced (McKay, 1997; Rao, 1999) (for a review, see Anderson, 2001). The differentiation of neural stem cells is controlled, in a context-dependent manner, by intrinsic factors and extracellular signalling molecules that act as positive or negative regulators. Positive regulators promote the commitment and differentiation of NSCs. For example, the synergy between the signalling molecule sonic hedgehog and the transcription factors olig-1 and olig-2 regulates oligodendrocyte differentiation in the ventral telencephelon (Tekki-Kessaris et al., 2001). Negative regulators maintain the self-renewal and the multipotent status of undifferentiated NSCs, and include members of the Notch signalling pathway (Gaiano and Fishell, 2002; Lutolf et al., 2002) and growth factors, such as EGF and FGF2 (Reynolds and Weiss, 1996; Weiss et al., 1996).

Neural stem cells can be obtained from perinatal forebrain germinal zones and grown in vitro in the presence of EGF and/or FGF-2 as multipotent neurospheres with self-renewing capacity (Ciccolini and Svendsen, 1998; Tropepe et al., 1999; Zhu et al., 1999b). However, the apparent lack of specific stem cell markers makes it difficult to identify these cells both in vivo and in vitro, and the ability to form clonal multipotent neurospheres over several passages remains the best functional assay for the presence and quantification of putative NSCs within a cell population (Imura et al., 2003). Neurospheres are intrinsically heterogeneous cellular entities almost entirely formed by a small fraction (1 to 5%) of slowly dividing neural stem cells and by their progeny, a population of fast-dividing nestin-positive progenitor cells (Campos et al., 2004; Lobo et al., 2003; Reynolds and Weiss, 1996; Zhu et al., 1999b). The total number of these progenitors determines the size of a neurosphere and, as a result, disparities in sphere size within different neurosphere populations may reflect alterations in the proliferation, survival and/or differentiation status of their neural progenitors.

Similar to other systems, the extracellular matrix constitutes an important source of instructive cues capable of regulating the behaviour of stem and progenitor cells in the developing CNS (Drago et al., 1991). The role of integrins in the recognition of such cues by cells of the CNS has been examined extensively (Clegg et al., 2003; Milner and Campbell, 2002). Integrins are a major group of cell-surface receptors for both extracellular matrix and cell-surface molecules (Montgomery et al., 1996; Ruppert et al., 1995). They are composed of two non-covalently associated

transmembrane glycoproteins, α and β, both of which participate in the binding of matrix proteins, and have been implicated in inside-outside signalling and in the coordination of the actin cytoskeleton and cellular response to growth factors (Hynes, 1992). Integrins regulate several fundamental processes such as proliferation, migration, cell survival and differentiation in a variety of tissues (Danen and Sonnenberg, 2003). They also play key instructive roles during the development of several embryonic regions (Adams and Watt, 1993; Hynes, 1996) including the developing CNS, where β1-integrins influence the development of laminae and folia in the cerebral and cerebellar cortex (Georges-Labouesse et al., 1998; Graus-Porta et al., 2001)

Signalling via β1 integrin also acts as a negative regulator of stem cell differentiation in various tissues, such as the skin (Brakebusch et al., 2000) and the prostate (Collins et al., 2001). Prompted by these findings, we have recently shown that β1-integrin signalling is likely to contribute to the maintenance of neural stem cells through the modulation of MAPK activity (Campos et al., 2004). Here, we use control and β1 integrin-deficient neurosphere cultures, to show that: (1) β1-integrin signalling is not an absolute requirement for the maintenance of neural stem cell self-renewal; (2) β1-integrin signalling, in cooperation with growth factor signalling, regulates the number of undifferentiated progenitor cells; (3) adhesion and migration of neurosphere-derived cells on ECM substrates is dependent upon β1 integrin.

Materials and Methods

Animals and genotyping

The generation of conditional β1-integrin mutant mice has been described previously (Brakebusch et al., 2000; Potocnik et al., 2000). β1-integrin lox/lox females were crossed with males that carry a heterozygous β1-integrin null allele (Fassler and Meyer, 1995) to obtain lox/0 and lox/wt litters. The β1-integrin lox/lox, the lox/wt and the lox/0 littermates, on a C57Bl6 background, were used for isolation of forebrain neural stem cells. Genotypes were determined by carrying out PCR on genomic DNA.

Neurosphere cell culture

Neural precursors were obtained from dissociated postnatal day 1 forebrains of β1-integrin lox/lox, lox/wt and lox/0 animals. Neurospheres were grown in DMEM/F12 supplemented with B27 (Invitrogen) using previously described methods (Reynolds et al., 1992; Reynolds and Weiss, 1996; Svendsen et al., 1995; Weiss et al., 1996; Zhu et al., 1999b) in the presence of 10 ng/ml EGF (human recombinant; Peprotech) and 20 ng/ml bFGF (human recombinant; Peprotech). Primary neurospheres were harvested after 7 days in culture, dissociated into single cells and plated at 6×10^4 cells/ml in the presence of an adenovirus expressing the Cre recombinase (Kalamarides et al., 2002) used at 50 virus particles per cell. Fresh medium was added to the cultures 3 days after infection and the medium was changed 6 days after infection. 10 days after infection, primary infected neurospheres (passage 1) were harvested and either stained for X-gal or trypsinized for later passages. Neurospheres were dissociated in 0.25% trypsin (Invitrogen) for 10 minutes and subsequent mechanical dissociation in L15 Medium (Sigma) supplemented with 1 mg/ml trypsin inhibitor (Sigma) and 0.8 mg/ml DNase I (Roche).

FACS sorting

For FACS sorting, neurospheres were dissociated into single cells, washed twice with FACS-PBS (1× PBS supplemented with 2% FCS), incubated with a FITC-conjugated hamster anti-β1-integrin antibody (1:100 in FACS-PBS; BD Biosciences) for 30 minutes on ice and washed three times with FACS-PBS. The cells were kept on ice and the cell suspension was passed through a 40 μm cell strainer (Falcon) before sorting. FACS analysis was performed using a FACStar PLUS (Becton Dickinson) connected to a Macintosh running CellQuest Software. Cells were analysed for forward scatter, side scatter and β1-integrin fluorescence using an Argon Laser (480 nm excitation, 520 nm emission). Dead cells and doublets were excluded by gating on forward and side scatter.

Short-term adhesion assays

Adhesion assays were performed as described previously (Milner et al., 2001). Briefly, small areas of bacteriological-grade plastic Petri dishes were coated with laminin-1, fibronectin or poly-D-lysine (PDL). After incubation at 37°C for 2 hours, substrates were washed twice with DMEM/F12. Cells derived from dissociated neurospheres were applied to the substrates in a 25 μl drop for between 5 and 60 minutes at 37°C. The adhesion assay was stopped by adding DMEM/F12 to the dishes, thereby washing off loosely attached cells. The attached cells were fixed, stained with Trypan Blue and washed twice with PBS. Adhesion was quantified by counting the attached, stained cells and results are expressed as a percentage of the number of cells adhering on PDL for each time point investigated. The experiments were done in duplicate and the results represent means±s.e. of three individual samples per genotype per substrate. Human laminin-1 (Chemicon) and fibronectin (Sigma) were diluted to the 10 μg/ml coating concentration in PBS; PDL (Sigma) was diluted to 5 μg/ml in PBS.

Immunocytochemistry

For whole mount X-gal staining, neurospheres were fixed in 0.2% glutaraldehyde in PBS for 30 minutes and stained in X-gal staining solution comprising 5 mM $K_3[Fe(CN)_6]$, 5 mM $K_4[Fe(CN)_6]$, 2 mM $MgCl_2$ and 2 mg/ml X-gal (Calbiochem) in PBS. After staining, neurospheres were post-fixed in 2% formaldehyde/PBS at 4°C. For immunostaining on sections, neurospheres were gently centrifuged (300 rpm for 5 minutes), embedded in O.C.T. compound (Tissue Tek) and 12 μm cryosections prepared. Sections and dissociated single cells were fixed in 4% paraformaldehyde/PBS for 20 minutes, permeabilized in 0.3% Triton X-100 for 15 minutes at room temperature and incubated overnight with primary antibodies. As primary antibodies, rabbit anti-phospho-histone H3 (1:200; Upstate), rabbit anti-β-galactosidase (1:200; Cappel), rabbit anti-β1 integrin (1:200), monoclonal anti-nestin (1:200; BD Biosciences), rabbit anti-GFAP (1:200; DAKO), monoclonal anti-β-III-tubulin (1:100; Sigma) and neurofilament (1:200; Sigma) were used. After incubation with the primary antibody, sections were washed three times for 5 minutes in PBS. Alexa 488-conjugated goat anti-rabbit (1:300; Molecular Probes) or Cy3-conjugated goat anti-mouse (1:300; Jackson Laboratories) were used as secondary antibodies. The TUNEL assay was carried out using indirect immunofluorescence to visualize nicked DNA according to the manufacturer's instructions (Apoptag Red, Intergen). Apoptotic cells were revealed by indirect immunofluorescence using an antibody recognizing endogenous levels of the large fragment (17/19 kDa) of activated caspase-3 (1:250, Cell Signaling) resulting from cleavage adjacent to Asp175.

Pictures were acquired using a Zeiss conventional or confocal fluorescence microscope equipped with a Zeiss Axiocam CCD camera connected to a Macintosh computer. Images were processed using Open Lab 3.0 software (Improvision).

Self renewal assays and characterization of neurospheres

After adenovirus infection, neurospheres were passaged every 10 days and replated each time at 6×10^4 cells/ml (clonal density). To calculate the percentage of recombined spheres in mutant and control cultures,

aliquots containing 150 to 250 neurospheres were obtained from individual cultures and stained for X-gal at different passages.

For measuring the size of the neurospheres, aliquots of the cultures were stained for X-gal and the diameter of the neurospheres was calculated from the cross-section of the neurospheres measured using Openlab 3.0 (Improvision) Software on a Macintosh. In total, the cross-section of 5001 neurospheres (1811 control and 3190 mutant spheres) from four independent experiments was measured. For determination of the cellular composition of the neurospheres, 8-day-old spheres were dissociated into single cells, plated onto PDL (Sigma)-coated chamber slides (Nunc) in DMEM/F12 supplemented with B27 for 3 hours, fixed and stained as described above. These experiments were done three times.

Maintenance and migration

Chamber slides (Nunc) were precoated with laminin-1, fibronectin or PDL. Intact, 9-day-old neurospheres were then plated onto the precoated chamber slides in DMEM/F12 supplemented with B27 with either no growth factors or with growth factors at limiting concentrations (3.3 ng/ml EGF, 2.0 ng/ml FGF, 10 ng/ml NGF). Cultures were left for 6 days to differentiate under the conditions given. The capacity of the individual clones to maintain progenitors and to generate neurons and glial cells was addressed by immunocytochemistry using antibodies against GFAP, nestin, β-III-tubulin and neurofilament as described above. Migration was quantified by measuring the extent of migration of cells from the neurospheres. The average of the four largest distances per neurosphere was divided by the core diameter of the neurosphere, thereby compensating for the different sizes of the individual neurospheres. Maintenance and migration experiments were done three times. In each experiment, at least 15 neurospheres for each ECM substrate were analysed.

Statistical analysis

The data show the mean±s.d. Statistical significance was determined using Student's *t*-test, unless indicated otherwise. Significance was set at $P<0.05$.

Results

Neurospheres, when clonally derived from neural stem cells (Reynolds and Weiss, 1992; Weiss et al., 1996), provide a valuable in vitro model system to investigate the mechanisms regulating neural stem cell maintenance and differentiation (Reynolds and Weiss, 1996). In the present work, we have studied the role(s) of β1-integrins in these processes by using neurosphere cultures where recombination and excision of a

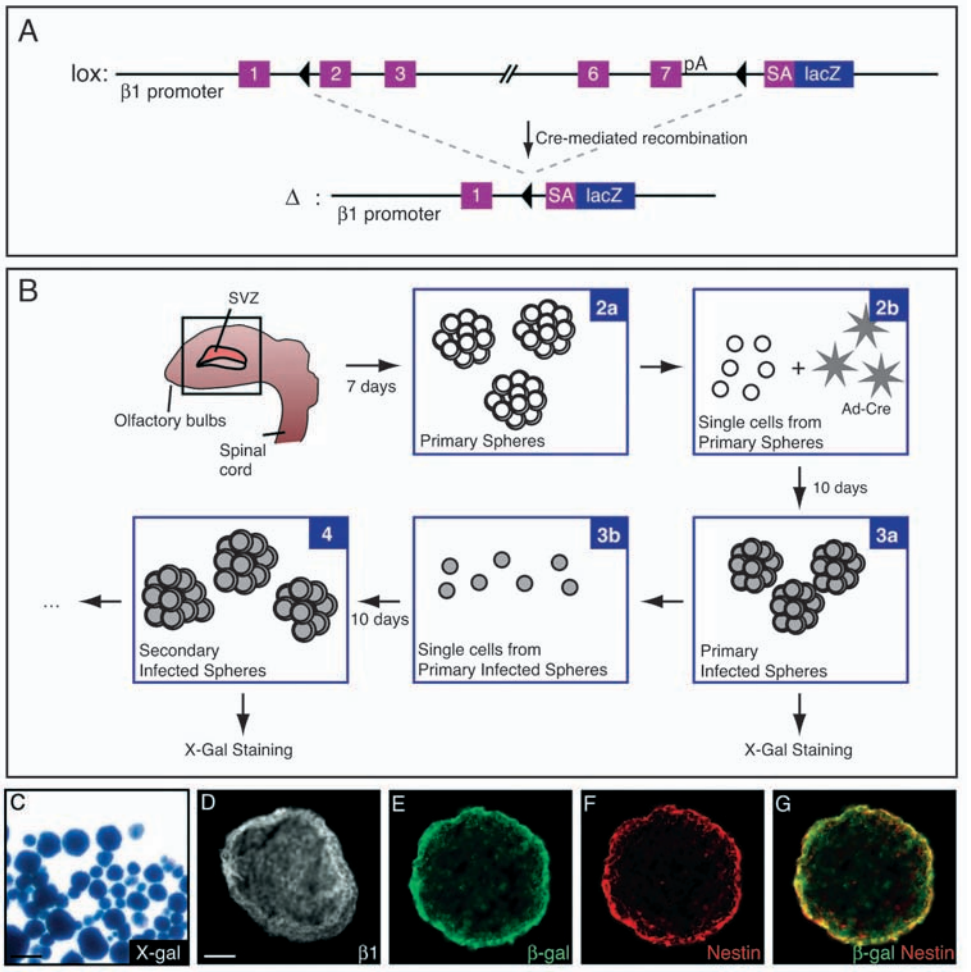

Fig. 1. Generation of β1-integrin null neurosphere cultures from the forebrain of neonatal mice. (A) Schematic representation of the conditional β1-integrin allele (lox) and Cre recombinase driven recombination leading to the recombined null (Δ) allele. Exons are indicated by purple boxes, *loxP* sites by black triangles. Note the promoterless *lacZ* gene trailing the conditional allele with a splice acceptor site (SA) derived from the intron upstream of exon 2 preceding it. This leads to the expression of β-gal in recombined cells under the control of the endogenous β1-integrin promoter. (B) Schematic drawing of the experimental set-up used in this work. Primary neurospheres (2a) were obtained from neonatal forebrains of conditional β1-integrin null mice. Neurospheres were trypsinized and the single cell suspension exposed to an adenovirus expressing the Cre recombinase (Ad-Cre; panel 2b). After an additional 10 days, primary infected neurospheres were harvested (3a) and either stained for X-gal or passaged (3b) to obtain further generations of infected neurospheres (4). (C) After infection with adeno Cre virus, the expression of β-gal, monitored by X-gal staining, indicates the recombination of the β1-integrin flox allele. Note that over 95% of the neurospheres are β-gal-positive. (D) 12 μm cryostat section of control wild-type neurospheres stained for β1-integrin. (E-G) Confocal microscopy analysis of 12 μm cryostat sections of recombined lox/wt spheres shows colocalization of β-gal (reporting β1-integrin promoter activity; green in E) and nestin (red in F) as yellow in G. Note that β1-integrin staining shown in D is topographically similar to that of β-gal in recombined lox/wt neurospheres. Bars, 150 μm (C); 50 μm (D-G).

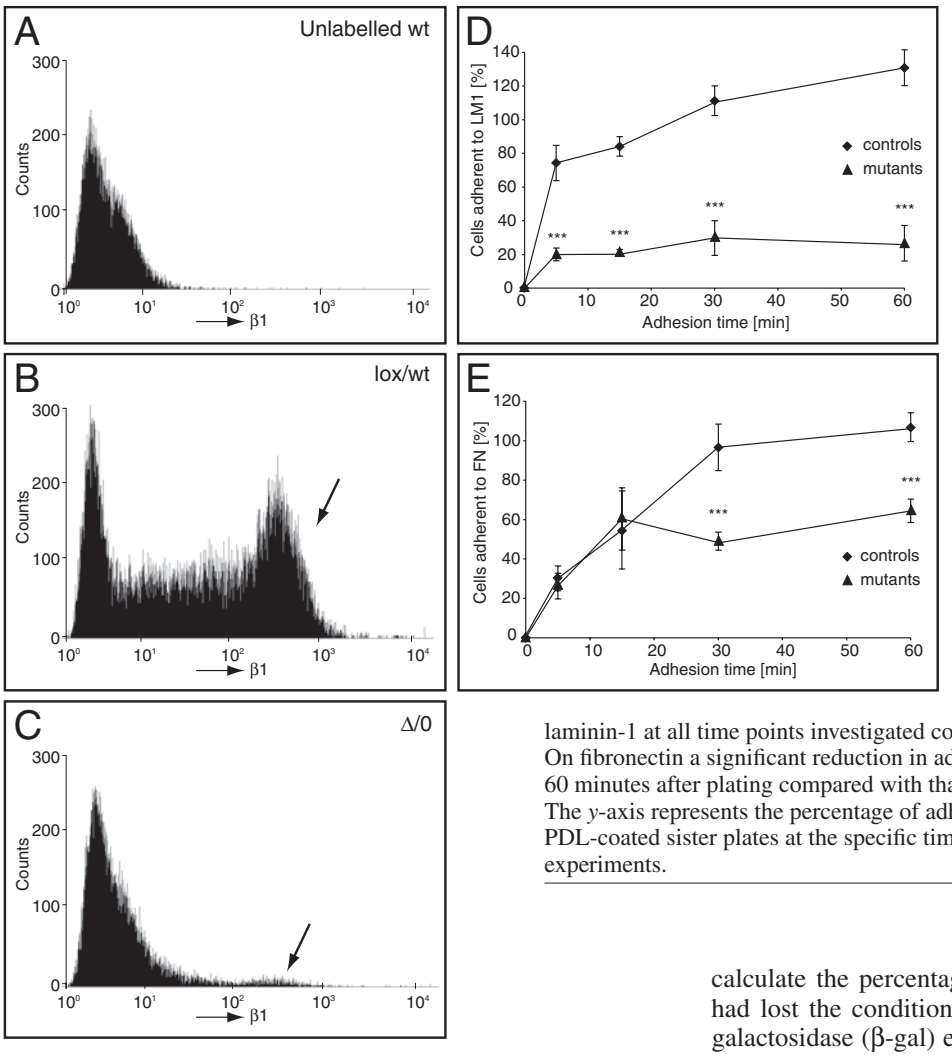

Fig. 2. Loss of β1-integrin surface expression of neurosphere-derived cells, confirmed by FACS analysis using a FITC-conjugated anti-β1-integrin antibody, reduces adhesion to fibronectin and laminin substrates. (A) Histogram of unlabelled wild-type neurospheres used as controls. (B) Histogram of non-infected, non-recombined neurospheres carrying the intact conditional β1-integrin allele showing β1-integrin surface expression (arrow). (C) Histogram of adeno Cre infected, recombined neurospheres demonstrating that the removal of the conditional β1-integrin allele leads to cell surface loss of β1-integrin expression (arrow). Two passages were needed for this dramatic reduction of β1-integrin expression (D-E). Absence of β1-integrin cell surface expression leads to reduced β1 integrin-mediated short term adhesion of mutant cells (triangles) compared with control cells (diamonds) on both laminin-1 (LM1, D) and fibronectin (FN, E) substrates. (D) A significantly reduced adhesion (***P<0.001) was found on laminin-1 at all time points investigated compared with adherence of control cells. (E) On fibronectin a significant reduction in adhesion (***P<0.001) was found at 30 and 60 minutes after plating compared with that in control cells at the same time points. The *y*-axis represents the percentage of adherent cells compared with adherent cells on PDL-coated sister plates at the specific time points. Results are the mean±s.d. of three experiments.

conditional β1-integrin allele (Fig. 1A) (Brakebusch et al., 2000; Fassler and Meyer, 1995; Potocnik et al., 2000) leads to loss of β1-integrin expression. The use of this system circumvents the problem posed by the early lethality of β1 integrin-deficient mice (Fassler and Meyer, 1995). Neurosphere cultures were derived from the forebrain of newborn mice having either the conditional β1-integrin lox/wt (control) or the conditional β1-integrin lox/0 (mutant) genotype. These cultures expressed β1 integrin and were maintained for over 20 passages, in the presence of EGF and FGF (Gritti et al., 1996; Reynolds et al., 1992; Svendsen et al., 1995; Vescovi et al., 1993; Zhu et al., 1999b), without apparent loss of neither self-renewal nor multipotentiality, as assessed by their continuous capacity to generate astrocytes (GFAP-positive cells), oligodendrocytes (O4-positive cells) and neurons (β-III-tubulin-positive cells) upon induction of differentiation (data not shown).

Recombination of the conditional β1-integrin allele was induced by infecting single cell suspensions, derived from 7-day-old primary neurospheres, with an adenovirus expressing the Cre recombinase (Kalamarides et al., 2002) (Fig. 1B). In these cells, recombination of the conditional allele activates the transcription of a *lacZ* reporter gene from the endogenous β1-integrin promoter (Fig. 1A). This feature allowed us to directly calculate the percentage of neurospheres in our cultures that had lost the conditional β1-integrin allele by determining β-galactosidase (β-gal) enzyme activity by X-gal staining. In all cultures, over 95% of the neurospheres were positive for X-gal (Fig. 1C) and β-gal was strongly expressed by cells on the rim of the neurospheres (Fig. 1E), a zone where β1 integrin (Fig. 1D) was also present. In this area, cells expressing β-gal also expressed the intermediate filament nestin, a known marker for undifferentiated neural progenitors (Frederiksen and McKay, 1988; Hockfield and McKay, 1985; Lendahl et al., 1990) (Fig. 1E-G). To confirm that the genetic ablation of the conditional β1-integrin allele led to loss of β1-integrin surface expression, we analysed neurosphere cells by FACS using a FITC-conjugated antibody that recognizes the extracellular domain of the β1-integrin subunit. In the second passage after adenovirus infection, the loss of β1 integrin was prominent on mutant neurosphere cells (Fig. 2C) but not on heterozygous control cells (Fig. 2B). Unless otherwise indicated, in the experiments described in this work, we have used neurosphere cultures at passage two or later, where the complete absence of β1-integrin surface expression was previously confirmed by FACS analysis.

Loss of β1-integrin surface expression disturbs adhesion of neurosphere cells to laminin and fibronectin substrates

Integrins are primarily extracellular matrix (ECM) adhesion

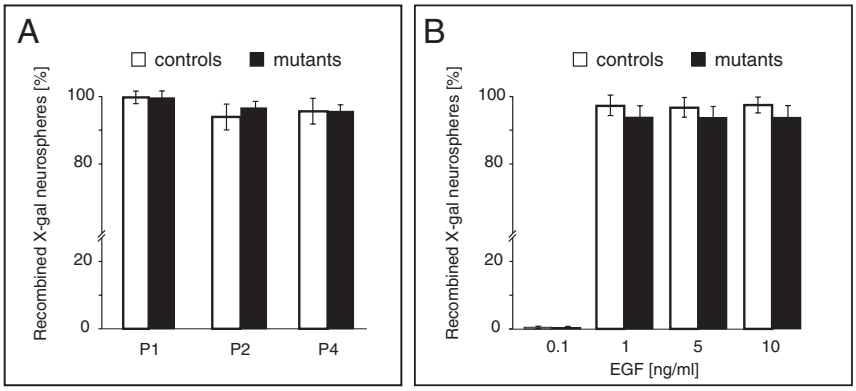

Fig. 3. Loss of β1 integrin does not impair neural stem cell self-renewal as assessed by neurosphere formation over several passages. (A) No difference was found in the percentage of X-gal-positive recombined neurospheres between mutants and controls at passages (P) 1, P2 and P4 in the presence of 10 ng/ml of EGF and 20 ng/ml of FGF-2. The absence of a significant genotype-specific reduction of recombined neurospheres over several passages suggests that β1 integrins are not essential for maintenance of neural stem cells. (B) The percentage of X-gal-positive neurospheres also did not significantly vary when the spherogenic assay was carried out at 1, 5 and 10 ng/ml EGF. This confirms that the loss of β1-integrin expression does not affect the capacity of neurosphere formation. Note that 0.1 ng/ml EGF was too low for both mutant and control cells to support the formation of neurospheres. Results are the mean±s.d. of four experiments in A and three experiments in B.

receptors. To determine if the loss of surface β1-integrin expression had a functional effect on cell adhesion, we compared the ability of recombined control (Δ/wt) and mutant (Δ/0) neurosphere cell populations to bind to the ECM substrates laminin-1 (LM1) and fibronectin (FN) in short term adhesion assays. Although the complete repertoire of integrin expression in mouse neurospheres has not been described, it is likely that it will not significantly differ from that of rat neurospheres; the major β1-containing integrins in rat neurospheres are the fibronectin receptor α5β1, the laminin receptors α6Aβ1 and, at lower levels, α6Bβ1, α1β1 and α3β1, and also the vitronectin receptor αvβ1, which shows some affinity for fibronectin substrates (Jacques et al., 1998). Thus, by eliminating β1 integrin from the surface of Δ/0 neurosphere cells, we predicted a reduction in their adhesion to both laminin-1 and fibronectin substrates. This was confirmed by our results, where we found that Δ/0 cells adhered significantly less to laminin-1 than Δ/wt control cells at all time points investigated (Fig. 2D), and to fibronectin substrates, at 30 and 60 minutes after plating (Fig. 2E). The effect on fibronectin was less pronounced than on laminin and may reflect the fact that other integrins capable of binding fibronectin, such as αvβ5 and αvβ8 may mediate binding to fibronectin, partially compensating for the lack of α5β1.

β1-integrin signalling is not an absolute requirement for neural stem cell maintenance

In a variety of tissues, β1-integrin signalling is a prerequisite for stem cell renewal (Brakebusch et al., 2000; Jones et al., 1995; Jones and Watt, 1993; Shinohara et al., 1999; Zhu et al., 1999a). Accordingly, we have suggested that β1-integrin signalling may be involved in neural stem cell maintenance through its role in the activation of the MAPK pathway (Campos et al., 2004). Here, we asked if β1-integrin signalling constitutes an absolute requirement for the maintenance of neural stem cells. To address this question, we tested the capacity of neural stem cell self-renewal in both mutant (Δ/0) and control (Δ/wt) neurosphere cell populations. We predicted that if β1-integrin signalling is indeed an absolute requirement for neural stem cell self-renewal, the percentage of recombined, β-gal-positive spheres formed by mutant (Δ/0) cells would decrease compared with their control (Δ/wt) counterparts over the course of several clonal passages. Concomitantly, the small percentage (less than 5%) of non-recombined, β-gal-negative spheres (retaining one functional β1-integrin allele), would gradually increase within the mutant (Δ/0) neurosphere cell cultures. Therefore, we carried out neurosphere-forming assays (see Materials and Methods) at passages one, two and four after adenoviral infection. At each passage, we determined the percentage of recombined neurospheres in both mutant (Δ/0) and control (Δ/wt) populations by X-gal staining. We found no significant difference in the percentages of recombined neurospheres between mutant and controls at passages one, two and four (Fig. 3A).

A synergy between growth factor receptor and integrin signalling has been described in various other systems (Aplin et al., 1999; Brooks et al., 1997). Hence, a possibility remained that the lack of a β1-integrin effect was masked by an increase in growth factor receptor signalling owing to the presumably high, non-physiological concentrations of EGF and FGF present in our assays. To address this point, we carried out neurosphere forming assays on passage ten of mutant and control populations in the absence of FGF and over a range of EGF concentrations. No significant difference was found between the percentage of mutant and control recombined spheres formed at 10, 5 and 1 ng/ml EGF (Fig. 3B). Note that the lowest EGF concentration investigated, 0.1 ng/ml, did not support either the formation of mutant or control neurospheres (Fig. 3B).

The percentage of small spheres is significantly increased in mutant β1-null neurosphere cultures

Although the loss of β1-integrin signalling does not significantly affect the capacity of mutant cells to form new neurospheres, it influences their size. In comparison with control (Δ/wt) neurosphere populations, a significantly higher percentage of small (10-30 μm diameter) spheres was present in the mutant (Δ/0) neurosphere populations (Fig. 4A). Concomitantly, the percentage of larger (40-70 μm diameter) spheres present in mutant populations was significantly lower than that found in control populations (Fig. 4A). This effect was also found in neurosphere cultures grown in EGF only (data not shown) and persisted over later passages. As the

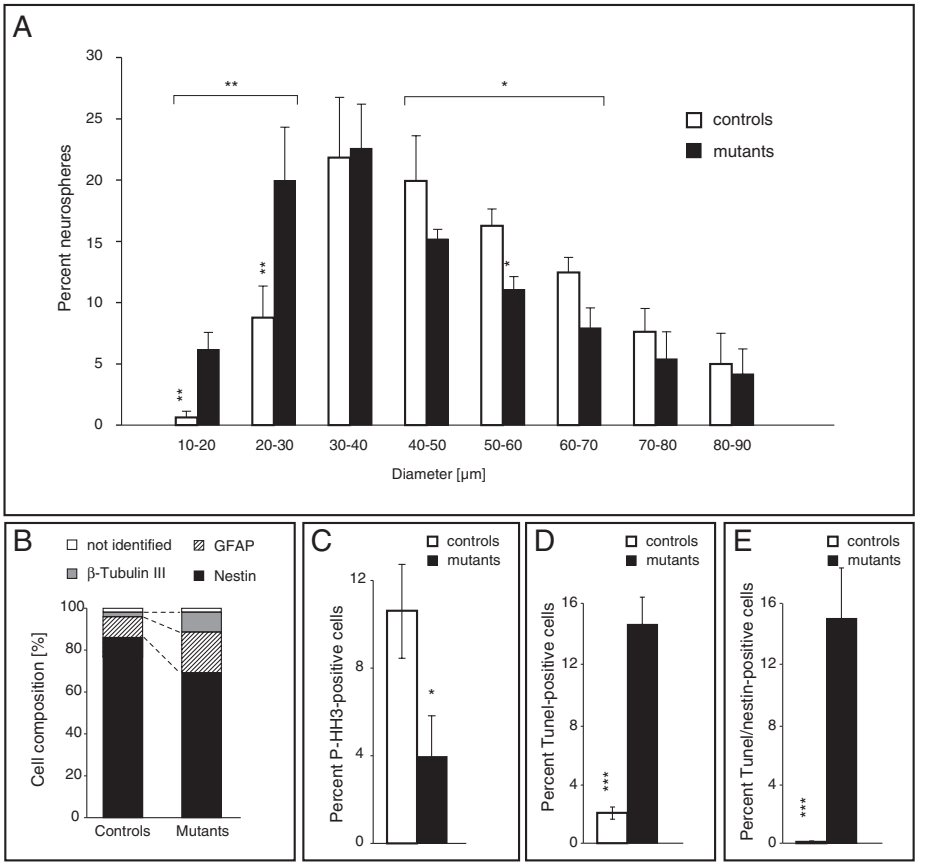

Fig. 4. A significant decrease in the diameter of mutant neurospheres is caused by a concomitant increase in cell death and a decrease in cell proliferation of nestin-positive progenitors. (A) The number of both mutant and control neurospheres was determined for each individual diameter ranging from 10 to 90 μm. The percentage of small (10-30 μm) neurospheres present in the mutant populations is significantly elevated in relation to control populations. In parallel, the mean percentage (±s.d.) of larger spheres (40-70 μm) is significantly reduced in β1 integrin-deficient neurospheres compared with the value in controls (n=4; *P<0.05; **P<0.01; ANOVA). (B) The cellular composition of mutant neurospheres is different from control populations. In mutant neurospheres, the percentages of both GFAP-positive astrocytes and β III-tubulin-positive neurons are increased whereas the number of nestin-positive progenitors is reduced. (C) The percentage of proliferating cells, stained with an antibody against phosphorylated histone H3, is significantly decreased in mutant neurospheres. (D) The number of apoptotic, TUNEL-positive cells is significantly increased in mutant neurospheres. (E) The percentage of apoptotic nestin-positive progenitors is significantly increased in the mutant neurospheres. Mean percentages±s.d. are shown for three separate experiments (D-E); *P<0.05, ***P<0.001 compared with respective values in control neurospheres (B-E).

nestin-positive, undifferentiated neural progenitor is the most abundant cell type present in a neurosphere (Jacques et al., 1998; Lobo et al., 2003; Reynolds and Weiss, 1996; Zhu et al., 1999b), it was likely that the size effect observed was caused by a decrease in the nestin-positive progenitors present in mutant neurospheres. To examine this hypothesis, we analysed the cellular composition of mutant and control neurospheres. Single cell suspensions, obtained by disaggregation of 8-day-old neurospheres from passage three or later, were plated on PDL-coated chamber slides for 8-12 hours, fixed and stained for nestin (a marker for undifferentiated neural progenitors), GFAP (an astrocyte marker) and β-tubulin (class III; a neuronal marker). These experiments were done three times. We found that in the mutant cell populations the percentage of nestin-positive cells was significantly reduced (73.4±3.2% compared with 90.1±3.4% in the control; Fig. 4B) whereas both the percentages of GFAP-positive cells (18.2±2.6% compared with 8.9±1.5% in the control; Fig. 4B) and β-III-tubulin-positive cells (8.3%±1.9% compared with 0.96±0.94% in the control; Fig. 4B) were significantly increased.

As the loss of integrin signalling can induce programmed cell death (Gary et al., 2003; Gilmore et al., 2000; Zhang et al., 1995) and affect proliferation in different cell types (Hirsch et al., 2002; Sastry et al., 1996), we next investigated if the reduction in the number of mutant nestin-positive cells was caused by increased cell death, reduction in cell proliferation, or by both processes acting simultaneously.

Increased cell death and reduced proliferation limit the growth of mutant neurospheres

To identify proliferating undifferentiated neural progenitors we carried out double immunofluorescence staining on single cells using antibodies against nestin and phosphorylated histone H3, a cell division marker (Gurley et al., 1978; Hendzel et al., 1997; Paulson and Taylor, 1982). We found a significant reduction in the number of proliferating, nestin-positive mutant cells (3.9±1.5%; n=3; Fig. 4C) when compared with their control counterparts (10.6±1.9%; Fig. 4C). In both mutant and control cell populations virtually all proliferating cells were nestin positive (data not shown). Neural progenitors undergoing programmed cell death were identified on sister cell cultures, by combining the TUNEL assay with nestin immunostaining. The percentage of TUNEL-positive cells in mutant cell populations was significantly higher (14.8±1.6%; Fig. 4D) than that in control cell populations (2.2±0.6%, Fig. 4D). In contrast to control populations, where virtually no apoptotic nestin progenitor cells were present, in mutant cultures virtually all TUNEL-positive cells were also positive for nestin (15.7±3.5%; Fig. 4E). These results were further confirmed by indirect immunofluorescence using an antibody recognizing the large fragment (17/19 kDa) of activated caspase-3. Here, apoptosis in the mutant cultures (22.7±4.2%) was also significantly higher than apoptosis in control cultures (4.4±0.92%; n=3, P<0.005). Our results suggest that because of the lack of β1-integrin signalling, more mutant nestin-

positive cells die and also proliferate less than their control counterparts. These two factors acting in conjunction probably reduce the absolute number of mutant nestin-positive cells, thereby limiting the growth of the mutant neurospheres.

β1-integrin signalling synergizes with growth factor signalling to regulate nestin progenitor cell survival

Although the mutant neurospheres displayed a significant phenotype in the high amounts of EGF (10 ng/ml) and FGF (20 ng/ml) present in our cultures, this did not exclude the possibility that the lack of β1 integrin was partially compensated by such high concentrations of growth factors. In order to investigate this issue, we quantified the number of nestin-positive progenitors present in mutant and control neurospheres after 6 days of differentiation on laminin-1 or fibronectin-coated plates, in the absence or presence of growth factor concentrations (3.3 ng/ml EGF, 2.0 ng/ml FGF, 10 ng/ml NGF) that promote neurosphere cell survival but keep proliferation to a minimum (Ohtsuka et al., 2001). In the absence of exogenous growth factors, 96.8±3.2% of control β1-integrin Δ/wt neurospheres plated on laminin-1 contained at least one nestin-positive cell per neurosphere explant. By contrast, in the mutant β1-integrin Δ/0 cultures, this percentage dropped to 7.5±2.0% (Fig. 5A). However, if these mutant cultures were grown in the presence of growth factors, 85.1±8.6% of the neurospheres contained nestin-positive cells (Fig. 5A), indicating that growth factor signalling could almost completely override the lack of β1 integrin. Similar results were found for neurospheres plated on fibronectin and on PDL (Fig. 5A). The results observed on PDL were probably owing to the fact that the ECM contains fibronectin and laminin, which are produced and deposited by the neurosphere itself (Campos et al., 2004). Taken together, our data suggest that integrin signalling may enable undifferentiated neural progenitors to 'read' the concentration of growth factors present in the extracellular environment with an increased sensitivity. This synergy between integrin and growth factor receptor signalling, also described in other systems (Schwartz and Ginsberg, 2002; Yamada and Even-Ram, 2002), is likely to become particularly important under physiological conditions when the availability of growth factors is limiting. Although the loss of β1-integrin expression reduced the numbers of nestin-positive cells, in the absence of growth factors, it did not influence cell fate choice as the number of mutant and control spheres containing differentiated GFAP- and β-III-tubulin-positive cells was not significantly different (data not shown).

Migration of neurosphere-derived mutant cells is significantly decreased on both laminin-1 and fibronectin substrates

Integrins also regulate the migration of several cell types both in vitro (Jacques et al., 1998; Milner et al., 1996) and in vivo (Bronner-Fraser, 1993; Galileo et al., 1992). To determine if the loss of β1 integrin could alter the migration capacities of cells derived from mutant and control neurospheres, we measured the extent of cell migration from intact neurospheres plated on PDL, laminin-1 and fibronectin substrates, in the absence or presence of growth factors (3.3 ng/ml EGF, 2.0 ng/ml FGF, 10 ng/ml NGF) (Ohtsuka et al., 2001). After 24 hours, it was apparent that the migration of mutant cells on laminin and fibronectin was impaired (data not shown). This was even more evident at later stages and we therefore quantified the extent of neurosphere cell migration after 5 days. On PDL, we did not find a significant difference in migration between β1 integrin-deficient and control cells. However, on laminin-1 we found a significantly reduced migration of mutant cells (migration index 0.7±0.2; Fig. 6A,F; see Materials and Methods for quantification) compared with controls (5.7±0.3; Fig. 6A,C). On fibronectin, we found impaired migration of mutant cells similar to that detected on laminin-1. Although control cells migrated up to 200 μm away from their plated neurospheres (migration index 3.1±0.4; Fig. 6A,B), in mutant cultures there was virtually no migration as reflected by the migration index of 0.4±0.2 (Fig. 6A,E).

Addition of exogenous growth factors significantly increased the migration on laminin of both control (7.2±0.6; Fig. 6A,I) and mutant neurosphere cells (2.2±0.5; Fig. 6A,L) but failed to do so on fibronectin (Fig. 6A,H,K) or on PDL substrates (Fig. 6A,J,M). This suggests that at least on fibronectin substrates, β1-integrin signalling is an absolute requirement for neurosphere cell migration.

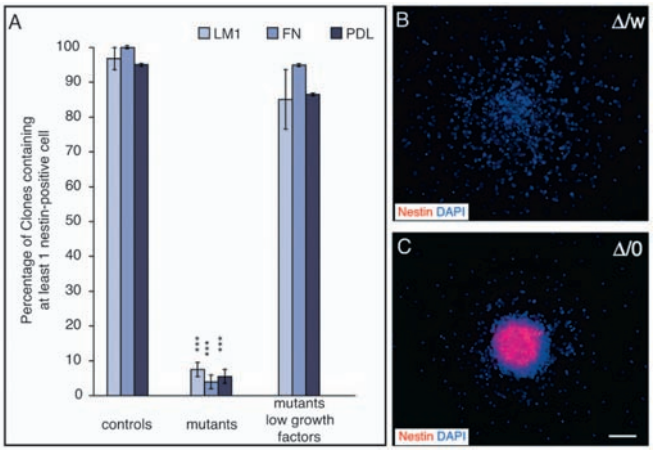

Fig. 5. The capacity to maintain nestin-positive progenitors within individual neurospheres is modulated by β1-integrin expression. (A) The presence of at least one nestin-positive cell was assessed for each plated β1-deficient neurosphere after 6 days of differentiation in the absence or in the presence of growth factors. In the absence of exogenous growth factors, the number of clones containing at least one nestin-positive cell is significantly decreased (***P<0.001) compared with control clones on laminin-1 (LM1), fibronectin (FN) or PDL substrates. By contrast, 85-95% of the mutant clones cultured in limiting growth factor conditions (3.3 ng/ml EGF, 2.0 ng/ml FGF, 10 ng/ml NGF) contained at least one nestin-positive progenitor cell. Results are the means±s.d. of three separate experiments. (B-C) Representative pictures of clones derived from control neurospheres (B) and from mutant neurospheres (C), cultured in the absence of growth factors for 6 days, and stained for nestin (red) and DAPI (blue). Bar, 70 μm.

Discussion

Neurosphere cell cultures have been instrumental in the study of several aspects of both neural stem and neural progenitor

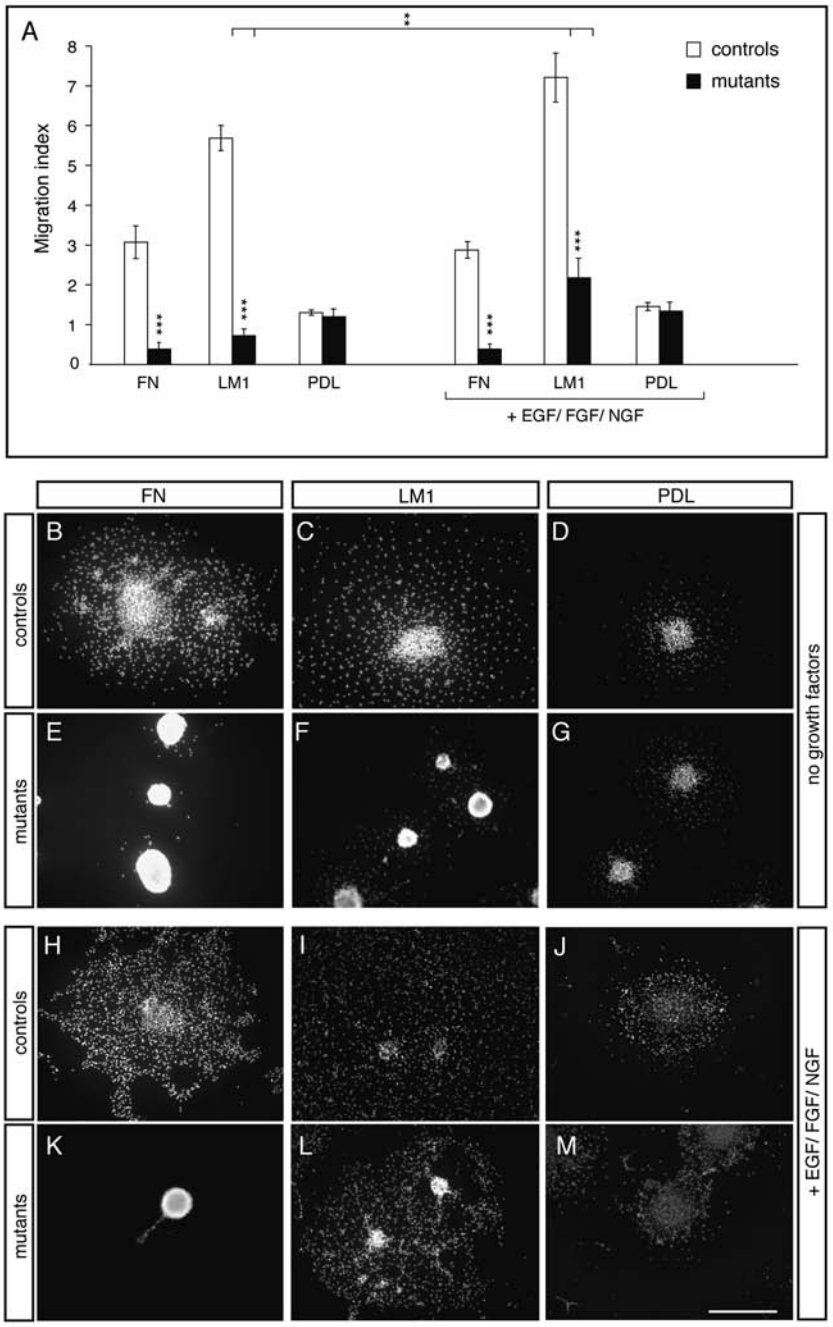

Fig. 6. β1 integrin-deficient cells show impaired migration on both fibronectin and laminin-1. Intact neurospheres were plated and cultured in the absence or presence of exogenous growth factors on either fibronectin (FN), laminin-1 (LM1) or PDL. After 5 days, cell migration from individual mutant and control neurospheres was measured as described in Materials and Methods. (A) A significant decrease in the migration capacity of mutant cells is found on both fibronectin and laminin-1, whereas no difference is detected on PDL. Addition of growth factors (3.3 ng/ml EGF, 2.0 ng/ml FGF, 10 ng/ml NGF) partially restores the migration capacity of neurosphere mutant cells on laminin, but not on fibronectin. Note that the increase in the number of migrating cells in the presence of added growth factors does not reflect an increase in the cell migration index (see Materials and Methods). Results are the mean migration index±s.d. of three separate experiments; $^{**}P<0.01$, $^{***}P<0.001$ when compared with the index in the relevant control cells.
(B-M) Representative images of cells migrating from neurospheres cultured in the absence of growth factors (B-G) and in the presence of growth factors (H-M). Note the striking absence of migrating cells in the mutants plated on fibronectin (E) and the increase in migration in the mutants cultured with growth factors (L) on laminin-1 compared with the same genotype in the absence of growth factors (F). Bar, 200 μm.

cell biology. Here, we have used these cultures as a model system to investigate the role of β1-integrin signalling in the behaviour of neurosphere cells. Neurospheres deficient in β1 integrin were generated from the perinatal forebrain germinal zones of mice carrying a conditional β1-integrin null mutation (Brakebusch et al., 2000; Fassler and Meyer, 1995; Potocnik et al., 2000). To excise the conditional allele and to induce a β1-integrin null genotype, we infected neurosphere cells with an adenovirus expressing the Cre recombinase. This method proved to be highly efficient and more than 95% of neurospheres expressing β-gal, indicating ablation of the β1 allele, were obtained in both null and control cell cultures. Alternative methods, such as retroviral infection, resulted only in 20-30% β-gal-positive spheres (results not shown). FACS analysis confirmed that the loss of the β1-integrin conditional allele was accompanied by a strong decrease of cell surface β1-integrin expression. However, this decrease was only observed after two passages in culture, possibly due to the slow turnover of the β1-containing integrin receptors present on the surface of these cells. As expected, the lack of β1-integrin expression significantly reduced the adhesion of β1-null cells to the ECM substrates fibronectin and laminin-1 in short-term adhesion assays, consistent with the absence of integrins containing the β1 chain expressed by neurospheres (Jacques et al., 1998) (our unpublished results).

We have suggested previously (Campos et al., 2004) that neural stem cell self-renewal is partially regulated by integrin and growth factor signalling through the MAPK pathway. We hypothesized that cross-talk between integrins and growth factors would allow neural stem cells to adjust better to changing local micro-environmental conditions during the development of the CNS. In the present study, we show that β1-integrin signalling is not an absolute requirement for neural stem cell renewal. Control and β1-deficient cells, plated at clonal density, showed no significant differences in the potential to form multipotent neurospheres over several passages. This was also true for limiting EGF concentrations. Furthermore, in assays where we followed the ratio of mutant (β-gal positive)/wild-type (β-gal negative) spheres within the same neurosphere culture over the course of at least five passages (more than 50 days in culture), no

significant variations were found. We conclude that, at least under these culture conditions, control neural stem cells did not have a competitive advantage over their β1 integrin-deficient counterparts.

In contrast to these results, the analysis of neurosphere size showed a significant increase in the percentage of small spheres and a decrease in large size spheres in β1-deficient cultures. This size difference was accompanied by a significant reduction in the percentage of nestin-positive progenitors in mutant spheres. In mutant spheres, the percentage of proliferating cells was also reduced and the percentage of apoptotic progenitor cells increased. This effect appeared to mainly affect the undifferentiated progenitors, as the relative contribution of nestin-negative cells to the total number of dying or proliferating cells was low. Integrin signalling and its close cooperation with growth factor signalling has been linked to the regulation of both proliferation and apoptosis in a variety of different cell types (Yamada and Even-Ram, 2002). For example, in epithelial cells, the integrin α5β1 mediates fibronectin-dependent cell proliferation through EGF receptor activation (Kuwada and Li, 2000), and in skin fibroblasts β1 integrins are able to activate the EGF receptor leading to ERK-1/MAPK induction and increased cell survival (Moro et al., 1998). Integrin-mediated attachment to the ECM is a general requirement for cell survival in a variety of cells (Frisch and Francis, 1994; Frisch and Ruoslahti, 1997). Integrins can activate survival pathways via the PI 3-kinase and MAPK pathways and act as essential factors for their stimulation by growth factors (Stupack and Cheresh, 2002). It seems conceivable therefore, that the increase in apoptotic progenitor cells in the mutant neurospheres is a direct consequence of the lack of β1 integrin-mediated growth factor signal integration. To test this hypothesis, we grew mutant and control spheres in the absence of exogenous growth-factors, FGF-2 and EGF, and predicted that in these conditions the difference in the number of nestin progenitors between control and mutant neurospheres would be even greater than that observed in the presence of growth factors. Mutant and control spheres were plated on adhesive substrates and the number of neurospheres containing nestin-positive cells quantified after 6 days. We did indeed find that the β1 integrin-deficient cells were much more sensitive than controls to the lack of exogenous growth factors as only 7.5% of the mutant neurosphere explants, in comparison with 96.8% of the control neurospheres, contained nestin-positive progenitors. It should be noted however, that the two neurosphere populations already showed differences in their cellular composition at the time of plating (see Fig. 4B). In the presence of low concentrations of exogenous growth factors, the number of mutant neurospheres containing nestin-positive progenitors increased to 85.1%, suggesting that in this context, β1-integrin signalling synergizes with growth factor signalling to promote neural progenitor cell survival.

There are two phases of cell death in the developing CNS. The first phase occurs within progenitor cell populations to control the numbers of precursors. Abnormalities in this control result in the production of supranumerary progeny and leads to severe brain malformations (Roth et al., 2000). The second phase occurs later within post-mitotic neurons and oligodendrocytes in order to match their final numbers to the size of their target fields (Barres et al., 1992; Colognato et al., 2002; Haydar et al., 1999; Kuan et al., 2000). These two regulatory steps have different mechanisms of apoptosis with proteins of the Bcl family being involved in the cell death pathway of post-mitotic cells but not in that of progenitor populations (Haydar et al., 1999; Kuan et al., 2000). Signalling via β1 integrin is known to play a role in target-dependent cell death (Colognato et al., 2002; Gary et al., 2003). Here, we show it also plays a role in the earlier phase of progenitor cell death. Our previous data on the distribution of ECM proteins within the subventricular zone (SVZ) (Campos et al., 2004) together with the present data, strongly implicates ECM-β1-integrin signalling as a regulator of this poorly understood mechanism of CNS size control.

Our data also provide evidence that β1 integrins are important for the migration of neurosphere-derived cells on ECM substrates. Two observations justify this conclusion: first, we detected a significantly enhanced migration of these cells on fibronectin and laminin-1 as compared with PDL suggesting an important role for integrin-ECM interaction in the migration of neurosphere-derived cells; second, we observed a significantly impaired migration of β1 integrin-deficient cells on both fibronectin and laminin-1 substrates. This effect was already visible 24 hours after plating and became more pronounced at later stages. Although on laminin-1 substrates, the addition of low amounts of growth factors elicited a modest increase in the extent of migration of mutant cells, it failed to do so on fibronectin. This indicates that expression of an integrin receptor containing a β1 chain, probably the integrin fibronectin receptor α5β1 expressed by neurospheres (Jacques et al., 1998), is an absolute requirement for the migration of neurosphere cells on fibronectin.

There are several reports concerning the role of β1 integrins in the migration of neuroepithelial cells in vivo. Galileo and colleagues (Galileo et al., 1992) reported that the radial migration of chicken tectal neuroepithelial cells is impaired following their infection with a retrovirus carrying the β1-integrin cDNA in antisense orientation. On the other hand, Graus-Porta and colleagues (Graus-Porta et al., 2001) have shown in an elegant loss-of-function study in vivo, that the lack of β1 integrin appears not to affect autonomous migration of neural progenitors in the developing cortex and cerebellum of the mouse. In other in vivo studies, β1-integrin signalling has been implicated in the regulation of tangential migration of olfactory interneuron precursors (Murase and Horwitz, 2002). Cell migration from neurosphere explants cultured on ECM substrates does not mimic radial migration. In contrast to radial migration, where neural precursors migrate along radial glial processes, neurosphere-derived cells migrate as single cells in direct contact with the substrate. In this context, and in line with our results, it has been shown that the extent of neurosphere cell migration is influenced by the composition of the ECM (Kearns et al., 2003) and that β1-integrin signalling also plays an important role in the autonomous cell migration of different CNS cell types (Milner and Campbell, 2002; Milner et al., 1996; Schmid and Anton, 2003).

In summary, by using neurosphere cultures, we were able to generate β1 integrin-deficient cells to address the role of this protein in stem and neural progenitor cell biology. This was instrumental to circumvent the problem we encountered when trying to eliminate β1 integrin from the cell surface of recombined cells during early embryonic development (our unpublished results). The slow turnover of β1 integrin

following recombination of the conditional allele prevented the loss of the protein in a time frame fitting with our in vivo experimental requirements. Our in vitro study is limited in that the composition of the ECM present within neurospheres might differ from the ECM composition in the ventricular zone. Nevertheless, neurospheres express some of the ECM molecules seen in vivo, and to a certain extent, the three-dimensional distribution of the ECM within the neurospheres reflects that of the ventricular zone (Campos et al., 2004). Therefore, it is conceivable that the β1 integrin-dependent regulation of undifferentiated progenitor cell behaviour might also operate in vivo during normal development or following injury to the CNS. In relation to CNS injury, Picard-Riera and colleagues have recently shown that during experimental autoimmune encephalomyelitis, where the ECM composition and growth factor availability go through dynamic changes, progenitor cells present in the SVZ increase their proliferation index before migrating into the periventricular white matter to give rise to oligodendrocytes and astrocytes (Picard-Riera et al., 2002). It will be interesting to determine if these processes are dependent upon β1 integrin.

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