

An angiogenic role for the $\alpha 5\beta 1$ integrin in promoting endothelial cell proliferation during cerebral hypoxia

Longxuan Li ^{a,1}, Jennifer Welser-Alves ^{a,1}, Arjan van der Flier ^b, Amin Boroujerdi ^a, Richard O. Hynes ^b, Richard Milner ^{a,*}

^a Department of Molecular and Experimental Medicine, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

^b Howard Hughes Medical Institute, Koch Institute for Integrative Cancer Research at the Massachusetts Institute of Technology, Cambridge, MA 02139, USA

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ABSTRACT

Fibronectin is a critical regulator of vascular modelling, both in development and in the adult. In the hypoxic adult central nervous system (CNS), fibronectin is induced on angiogenic vessels, and endothelial cells show strong induction of the two fibronectin receptors $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrins. In a previous study, we found that the $\alpha v\beta 3$ integrin is dispensable for hypoxic-induced cerebral angiogenesis, but a role for the endothelial $\alpha 5\beta 1$ integrin was suggested. To directly investigate the role of endothelial $\alpha 5$ integrin in cerebral angiogenesis, wild-type mice and mice lacking $\alpha 5$ integrin expression in endothelial cells ($\alpha 5$ -EC-KO) were subject to hypoxia (8% O₂) for 0, 2, 4, 7 or 14 days. Quantification of cerebral vessel density and endothelial-specific proteins claudin-5 and Glut-1 revealed that $\alpha 5$ -EC-KO mice displayed an attenuated angiogenic response, which correlated with delayed endothelial proliferation. $\alpha 5$ -EC-KO mice showed no defect in the ability to organize a cerebrovascular fibronectin matrix, and no compensatory increase in vascular $\alpha v\beta 3$ integrin expression. Consistent with these findings, primary $\alpha 5$ KO brain endothelial cells (BEC) in culture exhibited delayed growth and proliferation. Taken together, these studies demonstrate an important angiogenic role for the $\alpha 5\beta 1$ integrin in promoting BEC proliferation in response to cerebral hypoxia.

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Introduction

The blood supply to the central nervous system (CNS) is tightly regulated to maintain optimal delivery of oxygen and other nutrients (Attwell et al., 2010). Actually, the oxygen level in intact brain tissue is much lower than the 21% oxygen present in air at sea level; more in the range of 2–4% (Ndubuizu and LaManna, 2007). Cerebral hypoxia or ischemia trigger activation of several physiological adaptive responses, including increased ventilation rate, tachycardia, cerebral vasodilatation, cerebral angiogenesis, and raised hemotocrit (LaManna et al., 2004). Angiogenesis is defined as the active sprouting of new blood vessels (Folkman, 1995), and within days of an ischemic event, growing capillaries sprout specifically in the area bordering the ischemic core, also called the ischemic penumbra (Chen et al., 1994; Krupinski et al., 1994; Wei et al., 2001). Interestingly, experimental mice exposed to chronic hypoxia display a widespread, robust angiogenic response throughout the CNS, which acts to reduce the average distance between capillary and neuron (Kanaan et al., 2006; LaManna

et al., 1992). As animals that are hypoxic pre-conditioned are subsequently protected from ischemic insults (Dowden and Corbett, 1999; Miller et al., 2001), this raises the interesting possibility that the hypoxic-induced angiogenic remodeling may account for some of this protection.

To identify the molecular mechanisms that mediate this angiogenic response, much attention has focused on the transcription factor, hypoxia-inducible factor-1 α (HIF-1 α) (Chavez et al., 2000), and the associated growth factor signalling pathways, which include vascular endothelial growth factor (VEGF) (Kuo et al., 1999). However, the role of the extracellular matrix (ECM) in regulating cerebral angiogenesis has only recently come under the spotlight. This seems highly relevant in light of the evidence implicating a critical angiogenic role for the ECM, both during development and in adult tissues (Astrof and Hynes, 2009; del Zoppo and Milner, 2006; Hynes et al., 2002; Silva et al., 2008). In particular, the ECM protein fibronectin and its integrin receptors are essential for developmental angiogenesis, as shown by failure of angiogenesis in mutant mice lacking the fibronectin gene (George et al., 1993) or the fibronectin-specific $\alpha 5$ integrin (Yang et al., 1993).

Evidence suggests that fibronectin might also play an important role in cerebral angiogenesis. During development, cerebral vessels are surrounded by a fibronectin-rich ECM (Risau and Lemmon, 1988), and vascular cells within these vessels express high levels of fibronectin receptors, which are downregulated with vessel maturation (Milner

* Corresponding author at: Department of Molecular and Experimental Medicine, MEM-132, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA. Fax: +1 858 784 2174.

E-mail address: rmilner@scripps.edu (R. Milner).

¹ Longxuan Li and Jennifer Welser-Alves contributed equally to this work.

and Campbell, 2002b). This raised the question of whether this developmental expression pattern of fibronectin and its receptors is recapitulated on angiogenic vessels in the adult CNS. By examining this question in a model of hypoxia-induced cerebral angiogenesis, we demonstrated that angiogenic vessels in the hypoxic adult mouse CNS show parallel upregulation of fibronectin and endothelial expression of two BEC fibronectin receptors, $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrins (Li et al., 2010b; Milner et al., 2008a). To determine whether the $\alpha v\beta 3$ integrin is functionally important in the angiogenic response, we analyzed this process in $\beta 3$ integrin-null mice. This revealed that while the overall hypoxic increase in vessel density was unaffected by absence of the $\alpha v\beta 3$ integrin, surprisingly, endothelial proliferation in these mice was accelerated. Interestingly, this correlated closely with compensatory increase in BEC $\alpha 5\beta 1$ integrin expression (Li et al., 2010b), suggesting a role for the $\alpha 5\beta 1$ integrin in BEC proliferation. As the global knockout of the $\alpha 5$ integrin subunit has an embryonic lethal phenotype (Yang et al., 1993), it is not possible to test this hypothesis in these mice. To overcome this problem, in the current study, we used mice lacking $\alpha 5$ integrin expression in endothelial cells ($\alpha 5$ -EC-KO) (van der Flier et al., 2010) to directly investigate the role of endothelial $\alpha 5$ integrin in hypoxic-induced cerebral angiogenesis.

Materials and methods

Animals

The studies described have been reviewed and approved by The Scripps Research Institute Institutional Animal Care and Use Committee. The generation of the Tie2-Cre/ $\alpha 5$ integrin^{+/-} strain, and $\alpha 5$ integrin^{fllox/fllox} strains of mice have been described previously (Kisanuki et al., 2001; van der Flier et al., 2010; Yang et al., 1993). Both strains were backcrossed >6 times onto the C57BL/6 background and maintained under specific pathogen-free conditions in the closed breeding colony of The Scripps Research Institute (TSRI). Breeding of these two strains generated mice that lacked expression of the $\alpha 5$ integrin specifically in endothelial cells (referred to here as $\alpha 5$ endothelial cell knockout mice ($\alpha 5$ -EC-KO) mice; i.e. $\alpha 5$ ^{f/-}; Tie2-Cre), accounting for 25% of the offspring, as well as mice that were Tie2-Cre negative, having two copies of the $\alpha 5$ integrin gene ($\alpha 5$ ^{+f/+}), which were used as controls. Genotyping was performed using previously described protocols (Kisanuki et al., 2001; van der Flier et al., 2010; Yang et al., 1993).

Chronic hypoxia model

$\alpha 5$ -EC-KO mice or littermate controls ($\alpha 5$ ^{fllox/wt}), 8–10 weeks of age, were housed 4 to a cage, and placed into a hypoxic chamber (Biospherix, Redfield, NY), maintained at 8% oxygen for periods up to 14 days. Littermate controls of each strain were also kept in the same room under similar conditions except that they were kept at normal oxygen levels (normoxia, ~21% oxygen at sea level) for the duration of the experiment. Every few days, the chamber was opened for cage cleaning and food and water replacement as needed.

Immunohistochemistry and antibodies

Immunohistochemistry was performed as described previously (Milner and Campbell, 2002b) on 10 μ m frozen sections of cold saline-perfused brains taken from mice subject to either normoxia (control, ~21% O₂) or hypoxia for 2, 4, 7 or 14 days. The following monoclonal antibodies were obtained from BD Pharmingen (La Jolla, CA): rat monoclonal antibodies reactive for the integrin subunits $\alpha 5$ (clone 5 H10-27 (MFR5)), CD31 (PECAM-1, clone MEC13.3), the hamster monoclonal antibodies reactive for the $\beta 3$ integrin subunit (clone 2C9.G2) and isotype control antibodies; rat anti-KLH (A110-2) and hamster anti-TNP-KLH (G235-1). Rabbit polyclonal antibodies against

the following proteins were used in this study: fibronectin (Sigma), Ki67 (Vector Laboratories, Burlingame, CA), claudin-5 (Invitrogen, Carlsbad, CA), Glut-1 (Santa Cruz Technologies, Santa Cruz, CA), and beta-actin (Neo-marker, Fremont, CA). The Cy3-conjugated secondary antibodies against rat IgG, hamster IgG and rabbit IgG were all obtained from Jackson ImmunoResearch (West Grove, PA). Alexafluor 488-conjugated secondary anti-rat IgG was obtained from Invitrogen. Quantification of the number of capillaries positive for the different antigens was performed as previously described (Li et al., 2010b).

Western blotting

Western blotting was used to determine levels of claudin-5 or Glut-1 in brain lysates, as previously described (Milner et al., 2007). Within each sample, levels of these proteins were normalised to the level of β -actin, and expressed as the fold-increase over the level present within the normoxic brain ($n = 3$ mice per condition). Statistical significance was assessed by using Student's *t* test, in which $p < 0.05$ was defined as statistically significant.

Brain endothelial cell culture

Pure cultures of mouse brain endothelial cells (BEC) were obtained as described previously (Milner et al., 2008b), with the modification that puromycin (4 μ g/ml, Alexis GmbH, Grunberg, Germany) was included in culture media between days 1–3 to remove contaminating cell types. Endothelial cell purity was >99% as determined by CD31 in flow cytometry. BEC were used only for the first passage.

Cell adhesion assays

Adhesion assays were performed as previously described (Milner and Campbell, 2002a). Adhesion was quantified by phase microscopy by counting all attached cells within 5 fields of view per condition.

Flow cytometry

Integrin expression of $\alpha 5$ -EC-KO or littermate control BEC was examined as previously described (Milner et al., 2008b). The fluorescent intensity of labeled cells was analyzed with a Becton Dickinson FACScan machine (San Diego, CA), with 10,000 events recorded for each condition.

Proliferation assays

Freshly isolated primary BEC were cultured on fibronectin-coated (10 μ g/ml fibronectin (Sigma) for 2 hours at 37 °C) glass coverslips. After 3 days, BEC were cultured overnight in the presence of BrdU (Invitrogen, Carlsbad, CA), then fixed in acid/alcohol and analyzed for BrdU incorporation. BrdU-positive cells were expressed as the percentage of total cells (Hoechst staining), and the results presented as the mean \pm SEM of four experiments.

Migration assay

10 μ l drops containing 10,000 BEC were plated into the center of wells of a 24 well plate, on top of a small area coated with a 10 μ l drop containing 10 μ g/ml fibronectin. Cells were allowed to attach for 1 hour before the cell drop was surrounded by a 50 μ l drop of medium containing 10 μ g/ml fibronectin. Two hours later, the well was filled with 0.5 ml medium. At this time, the diameter of the cell mass (both vertical and horizontal) was measured using an eyepiece graticule. The diameter of the expanding cell mass was subsequently measured after 24 and 48 hours.

Immunofluorescence of primary BECs

Coverslips were coated with fibronectin or laminin (10 µg/ml) for 2 hours at 37 °C. BEC from $\alpha 5$ -EC-KO or control mice were plated onto the coverslips and cultured for 18–24 hours. After this time, cells were fixed for 5 minutes in acetone/methanol (1:1) and blocked in 5% normal goat serum in PBS. Cells were then incubated with primary antibodies against the $\alpha 5$ (clone 5H10-27 (MFR5)), αv (clone RMV-7) or $\beta 3$ (clone 2C9.G2) integrin subunits or fibronectin (polyclonal), for 1 hour, washed, then incubated with Cy3-conjugated secondary antibodies, washed then mounted on glass slides. The nuclear marker Hoechst (Sigma) was included in cells stained for fibronectin.

Quantification and statistical analysis

Quantification of the number of blood vessels positive for the different antigens was performed by capturing images of the two regions of interest (brainstem and cerebral cortex) with X 20 objective on a Zeiss Axio Observer A1 microscope and Zeiss AxioCam MRC digital camera. Multiple images (> 3) were taken of each section, the number of positive events per field of view quantified in the two different regions in each brain ($n = 3$ mice), and the results expressed as the mean \pm SEM of the number of antigen-positive events per field of view. In the *in vitro* assays of cell adhesion, proliferation and cell migration, all assays were performed in duplicate a minimum number of 4 times, and the results presented as the mean \pm SEM. In all experiments, statistical significance was assessed by using Student's *t* test, in which $p < 0.05$ was defined as statistically significant.

Results

Confirmation of the absence of $\alpha 5$ integrin expression in brain endothelial cells (BEC) of Tie2-Cre/ $\alpha 5$ integrin flox mice

To directly investigate the contribution of endothelial $\alpha 5$ integrin to hypoxic-induced cerebral angiogenesis, we used mice that lack $\alpha 5$ integrin expression in endothelial cells ($\alpha 5$ -EC-KO), as previously described (van der Flier et al., 2010). Transgenic mice expressing Cre recombinase under the control of the Tie2 promoter, (Tie2-Cre; $\alpha 5^{-/+}$) were crossed with mice in which the $\alpha 5$ integrin gene was flanked by LoxP sites ($\alpha 5^{f/f}$). From this breeding strategy, approximately 25% of the offspring were Tie2-Cre, $\alpha 5^{f/-}$ which lacked $\alpha 5$ integrin expression in endothelial cells (referred to as $\alpha 5$ -EC-KO mice). Littermate mice that had two copies of the $\alpha 5$ integrin gene ($\alpha 5^{+/+}$; Tie2-Cre negative) were used as controls. To confirm that this genetic approach was effective at deleting $\alpha 5$ integrin from BEC, we conducted two different analyses. First, using immunofluorescence (IF) staining, we examined $\alpha 5$ integrin expression in the brains of mice, maintained under normoxic conditions or after exposure to 8% hypoxia for 4 days, a stimulus we have previously shown promotes $\alpha 5$ integrin expression in angiogenic BEC (Milner et al., 2008a). As shown in Fig. 1A–B, cerebral blood vessels in control mice under normoxic conditions showed low levels of $\alpha 5$ integrin expression, and this expression was strongly increased after 4 days hypoxia. In contrast, $\alpha 5$ integrin was undetectable on cerebral blood vessels in $\alpha 5$ -EC-KO mice under any condition. This confirmed first, that the $\alpha 5$ integrin gene was totally deleted from BEC within $\alpha 5$ -EC-KO mice, and second, underlines our previous observation that BEC are the major cell type expressing $\alpha 5$ integrin in cerebral vessels (Milner and Campbell, 2002b; Milner et al., 2008a). Second, flow cytometry was used to examine $\alpha 5$ integrin expression on primary cultures of BEC derived from control littermates or $\alpha 5$ -EC-KO mice. As illustrated in Fig. 1C, whereas control BEC expressed high levels of the $\alpha 5$ integrin (greater than 82% of control BEC were $\alpha 5$ integrin-positive), BEC isolated from $\alpha 5$ -EC-KO mice showed total absence of this integrin.

$\alpha 5$ Integrin-deficient mice show a reduced angiogenic response to cerebral hypoxia

To examine directly the role of $\alpha 5\beta 1$ integrin in mediating an angiogenic response, we employed a mouse model of cerebral hypoxia, in which mice are subject to hypoxia (8% O₂) for 0, 2, 4, 7 or 14 days. This induces marked angiogenic remodelling within the CNS, culminating in increased vascular density in all areas of the CNS examined (LaManna et al., 1992; Milner et al., 2008a). The angiogenic response of $\alpha 5$ -EC-KO mice was compared with that of littermate controls. Measuring blood vessel density using CD31 labeling, cerebral hypoxia induced a strong time-dependent increase in cerebral blood vessel density. The hypoxic-induced changes in cerebral vessel density in $\alpha 5$ -EC-KO mice were significantly less than control mice (Fig. 2A–C). Quantification of this response revealed that under normoxic conditions, there was no difference in vessel density (vessels per field of view) between $\alpha 5$ -EC-KO and control mice, either in the brainstem (49.8 ± 2.8 compared with 50.2 ± 4.3) or cerebral cortex (43.6 ± 1.4 compared with 44.1 ± 2.8). However, following 14 days hypoxia, cerebral blood vessel density in the $\alpha 5$ -EC-KO mice was significantly less than in controls, both in the brainstem (73.8 ± 1.2 compared with 93.0 ± 3.8 , $p < 0.01$), and cerebral cortex (64.0 ± 2.3 compared with 81.7 ± 2.5 , $p < 0.01$). In addition, linear regression analysis, plotting vessel density against time in hypoxia, revealed a significantly reduced slope in $\alpha 5$ -EC-KO mice, both in the brainstem (1.62 ± 0.37 versus 3.01 ± 0.46 , $p = 0.027$) and cerebral cortex (1.37 ± 0.27 versus 2.71 ± 0.46 , $p = 0.015$). To seek confirmation of this difference, we performed western blotting to quantify expression levels of two proteins specifically expressed at high levels by BEC, the tight junction protein, claudin-5 and the glucose transporter protein, Glut-1. As shown in Fig. 2D and E, in control mice, 14 days hypoxia produced a strong increase in levels of claudin-5 and Glut-1 protein expression, reflecting the increased capillary density induced by hypoxia. Whereas levels of these two proteins were not significantly different in normoxic brain lysates made from $\alpha 5$ -EC-KO or control mice, clear differences became obvious after 14 days hypoxia. Compared to controls, 14-day hypoxic brain samples from $\alpha 5$ -EC-KO mice contained less claudin-5 ($64.5 \pm 12.8\%$ of the control level, $p < 0.05$), and less Glut-1 ($45.8 \pm 13.5\%$ of the control level, $p < 0.05$). In summary, using two different techniques (IF and western blot) and three different endothelial cell markers (CD31, claudin-5 and Glut-1), these studies demonstrate that mice lacking endothelial cell expression of the $\alpha 5$ integrin showed a significantly reduced angiogenic response to cerebral hypoxia.

$\alpha 5$ Integrin-deficient BEC show a delayed mitogenic response to cerebral hypoxia *in vivo*

Fibronectin drives angiogenesis, in part by promoting endothelial cell proliferation (Astrof and Hynes, 2009; Wang and Milner, 2006). As $\alpha 5$ -EC-KO mice displayed an attenuated angiogenic response to cerebral hypoxia, we next examined whether this might be a result of reduced endothelial proliferation in these mice, by performing dual-IF for the proliferation marker Ki67 and the endothelial marker CD31. As shown in Fig. 3, after 4 days hypoxia, the number of dual-positive Ki67+/CD31+ cells per field of view in the brain of $\alpha 5$ -EC-KO mice was significantly lower than in control mice, both in the brainstem (4.1 ± 0.3 compared with 9.8 ± 1.8 , $p < 0.05$) and the cerebral cortex (5.3 ± 0.6 compared with 9.4 ± 1.4 , $p < 0.05$). However, after 7 days hypoxia, this ranking had reversed, with the number of dual-positive Ki67+/CD31+ cells in the brain of $\alpha 5$ -EC-KO mice now significantly higher than in controls, both in the brainstem (6.6 ± 1.1 compared with 3.0 ± 0.6 , $p < 0.05$) and cerebral cortex (6.9 ± 1.0 compared with 3.8 ± 0.4 , $p < 0.05$). After 14 days hypoxia, levels of Ki67+/CD31+ positive events in both strains of mice were reduced to much lower levels with no significant difference detected at this time-point. This demonstrates that in keeping with the attenuated angiogenic response,

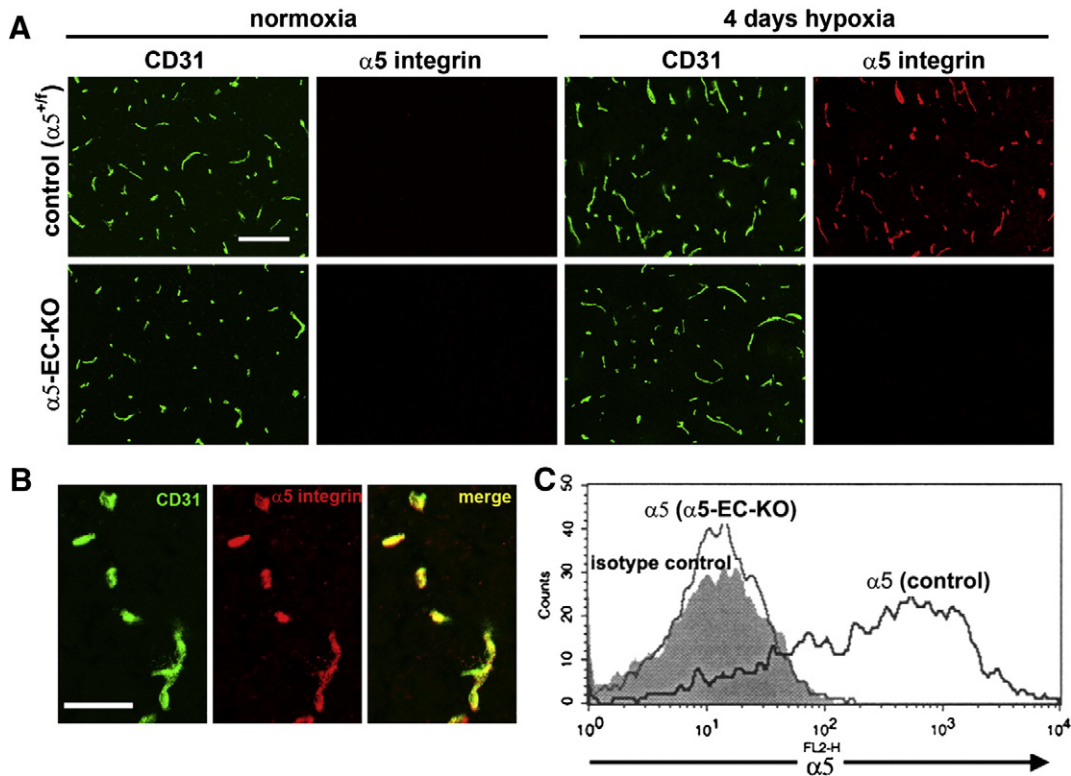


Fig. 1. Confirmation of the absence of $\alpha 5$ integrin expression in BEC of $\alpha 5$ -EC-KO mice. **A.** Frozen sections of brainstem taken from mice exposed to normoxia ($\sim 21\%$ O_2) or 4 days hypoxia were processed for dual-IF for CD31 and the $\alpha 5$ integrin. Scale bar = 100 μm . Note that in contrast to the control CNS, where strong hypoxic-induction of endothelial $\alpha 5$ integrin was observed, vessels in $\alpha 5$ -EC-KO mice showed a total lack of the $\alpha 5$ integrin. **B.** High power image of CD31/ $\alpha 5$ integrin dual-IF of 4 day hypoxia exposed brainstem. Scale bar = 50 μm . **C.** BEC from control or $\alpha 5$ -EC-KO mice were cultured on fibronectin and expression of the $\alpha 5$ integrin subunit analyzed by flow cytometry. Note that in contrast to control BEC, $\alpha 5$ integrin was totally absent on BEC obtained from $\alpha 5$ -EC-KO mice.

BEC in $\alpha 5$ -EC-KO mice show a delayed and reduced mitogenic response to cerebral hypoxia. It also shows that the $\alpha 5$ integrin is not absolutely essential for BEC to mount a mitogenic response. Another possibility that might account for these findings is that lack of the $\alpha 5$ integrin could lead to increased BEC apoptosis during angiogenic remodeling, which would contribute to the attenuated angiogenic response we observed in $\alpha 5$ -EC-KO mice. To investigate this, we performed TUNEL IF, which revealed a total absence of any TUNEL-positive cells during the hypoxic angiogenic response, in both control and $\alpha 5$ -EC-KO mice (not shown), demonstrating that absence of the $\alpha 5$ integrin does not lead to BEC cell death during the angiogenic response.

Mice deficient of endothelial $\alpha 5$ integrin show no defect in vascular fibronectin deposition upon cerebral hypoxia, and no compensatory increase in the expression of $\alpha v\beta 3$ integrin

Organization of a fibronectin matrix depends on cell surface fibronectin receptors (Wierzbicka-Patynowski and Schwartzbauer, 2003). Cerebral hypoxia promotes parallel increases in vascular expression of fibronectin and BEC expression of the $\alpha 5\beta 1$ integrin, which peak after 4 days hypoxia and then decline with time (Li et al., 2010a). As the $\alpha 5\beta 1$ integrin is one of the main fibronectin receptors expressed by BEC (Wang and Milner, 2006), and its demonstrated role in fibronectin assembly (Wierzbicka-Patynowski and Schwartzbauer, 2003), it is possible that absence of the $\alpha 5$ integrin might prevent fibronectin binding to BEC or result in diminished organization of a fibronectin matrix. To address this question, we used IF to examine the distribution of vascular fibronectin during the hypoxic response. This showed that in the brain of both control and $\alpha 5$ -EC-KO mice, 4 days hypoxia induced a marked increase in the level of fibronectin

expression in cerebral vessels (Fig. 4A), clearly demonstrating that lack of endothelial $\alpha 5$ integrin expression does not affect fibronectin distribution within cerebral vessels in the hypoxic CNS.

BEC express two fibronectin integrin receptors, $\alpha 5\beta 1$ and $\alpha v\beta 3$ (Wang and Milner, 2006), raising the possibility of redundancy in this system. In light of our recent finding that $\beta 3$ integrin-null mice show no defect in hypoxic-induced angiogenesis, but display compensatory upregulation of the $\alpha 5\beta 1$ integrin (Li et al., 2010b), we next examined whether compensation worked both ways, by comparing the number of $\beta 3$ integrin-positive vessels in $\alpha 5$ -EC-KO and littermate control mice. As shown in Fig. 4C, hypoxic brains of $\alpha 5$ -EC-KO mice contained marginally fewer $\beta 3$ integrin-positive vessels compared to littermate controls (which was significant after 7 days hypoxia, $p < 0.05$), consistent with the reduced vessel density seen in $\alpha 5$ -EC-KO mice, clearly demonstrating the absence of any compensatory upregulation of $\alpha v\beta 3$ integrin in $\alpha 5$ -EC-KO mice.

$\alpha 5$ Integrin-deficient BEC show delayed proliferation and growth in primary culture

Previous antibody-blocking studies suggest that both $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrins play functional roles in mediating BEC adhesion, survival and proliferation on fibronectin (Wang and Milner, 2006). To directly examine the role of the $\alpha 5$ integrin in mediating interactions with fibronectin, BEC were isolated from $\alpha 5$ -EC-KO mice, and their adhesion and growth on fibronectin compared with BEC from littermate controls. During the first few days of primary BEC culture, endothelial islands slowly disperse as BEC migrate out and proliferate. Compared with control cells, though similar numbers of $\alpha 5$ integrin-deficient BEC attached to fibronectin (Fig. 5A), they were much slower to disperse from

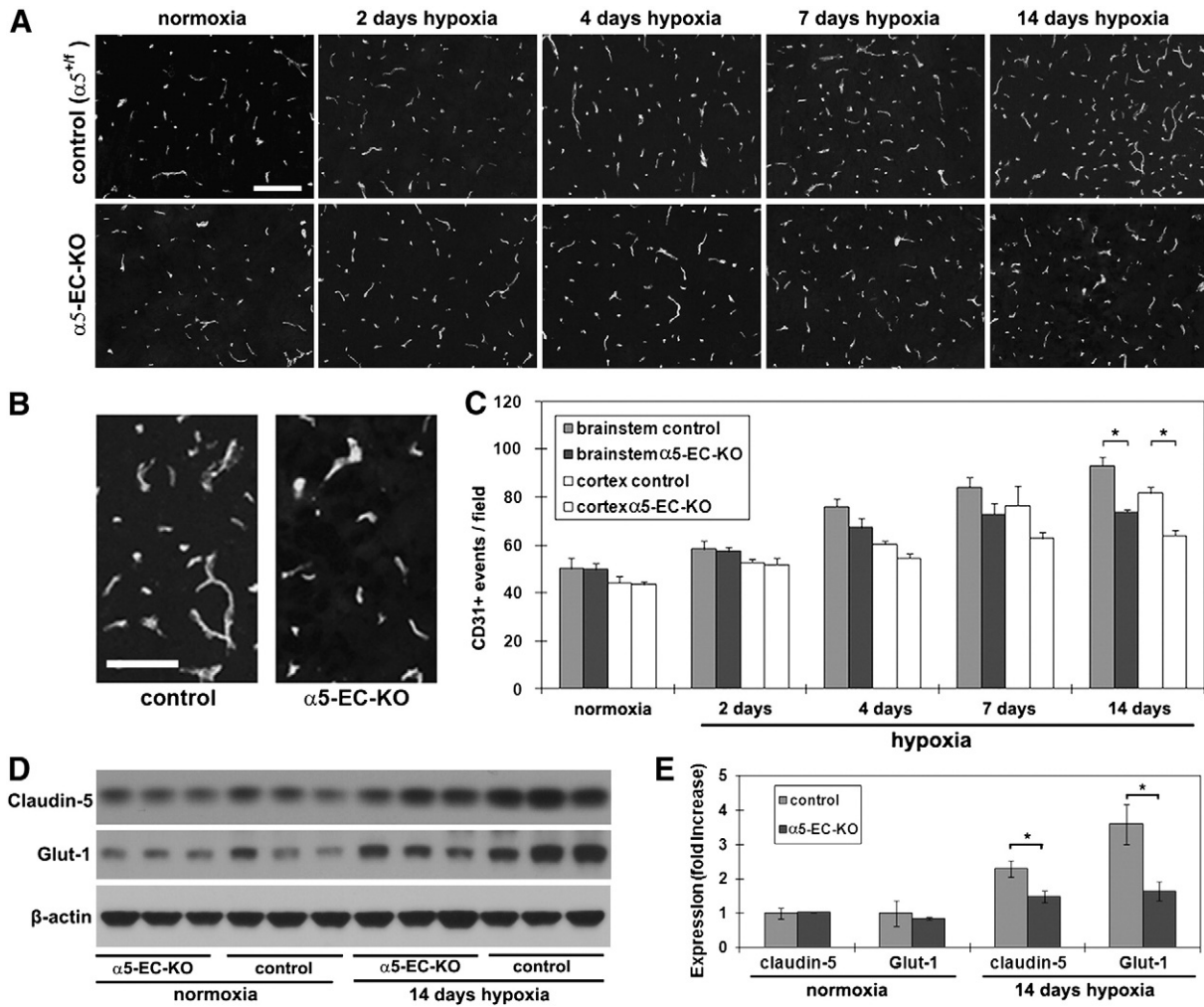


Fig. 2. Reduced hypoxia-induced cerebral angiogenic response in $\alpha 5$ -EC-KO mice. **A.** $\alpha 5$ -EC-KO mice and control littermates ($\alpha 5^{+/+}$) were maintained at normoxia (~21% O_2) or exposed to 8% hypoxia for 2, 4, 7 or 14 days before frozen brain sections were immunostained to assess blood vessel density (CD31) in the brainstem. Scale bar = 100 μ m. **B.** High power images of CD31 IF performed on brainstem of $\alpha 5$ -EC-KO and littermate control mice exposed to 14 day hypoxia. Scale bar = 50 μ m. **C.** Quantification of analyses. Brain sections were examined for the number of CD31+ vessels. Experiments were performed with three different animals per condition, and the results expressed as the mean \pm SEM of the number of CD31+ vessels per field of view. Note that after 14 days hypoxia, blood vessel density in $\alpha 5$ -EC-KO mice was significantly less than in control mice, both in the brainstem and cerebral cortex, * $p < 0.01$. **D.** Western analysis of brain expression of claudin-5 and Glut-1 during normoxia (~21% O_2) and after 14 days hypoxia. **E.** Quantification of western analysis. Note that after 14 days hypoxia, levels of the BEC proteins, claudin-5 and Glut-1 were significantly lower in the $\alpha 5$ -EC-KO mice relative to controls, * $p < 0.05$.

endothelial islands and displayed slower growth kinetics on fibronectin (Fig. 5B). BrdU incorporation studies showed that after 3 days primary culture, $\alpha 5$ integrin-deficient BEC displayed a significantly reduced proliferation rate (6.3 ± 3.5 cells/field compared with 22.2 ± 4.5 cells/field for control cells, $p < 0.05$; Fig. 5C). This demonstrates that while $\alpha 5$ integrin-deficient BEC can adhere to fibronectin, absence of this integrin results in delayed proliferation and growth of BEC.

Re-distribution of αv integrins to focal contacts in $\alpha 5$ integrin-deficient BEC

In a recent study, we demonstrated that αv integrin fibronectin receptors can functionally compensate for lack of $\alpha 5$ integrin by re-distributing into focal contacts normally occupied by $\alpha 5$ integrins (van der Flier et al., 2010). To address whether this shift also occurs in BEC, we compared the cellular distribution of the αv and $\beta 3$ integrin subunits on BEC derived from $\alpha 5$ -EC-KO mice and control mice plated on fibronectin (Fig. 6). In control BEC, the $\alpha 5$ and $\alpha v \beta 3$ integrins showed a clear difference in their sub-cellular localization. While the $\alpha v \beta 3$ integrin (demonstrated by αv and $\beta 3$ integrin IF) was predominantly

located in focal adhesions at the cell border, the $\alpha 5$ integrin was found in focal adhesions across the entire cell surface. Interestingly, in $\alpha 5$ integrin-deficient BEC, the $\alpha v \beta 3$ integrin shifted its distribution to a pattern similar to the $\alpha 5$ integrin in control cells. Thus, consistent with our studies on embryonic endothelial cells (van der Flier et al., 2010), αv integrins on adult $\alpha 5$ integrin-deficient BEC show functional compensation, re-locating to the position normally occupied by $\alpha 5$ integrins. In light of the distinct compartmentalization of $\alpha 5$ and $\alpha v \beta 3$ integrins demonstrated by our IF studies; $\alpha 5$ localized under the body of the cell, and $\alpha v \beta 3$ localized specifically at the cell border, we wondered whether these two integrins might play distinct roles in promoting BEC migration. To test this idea, and specifically examine the role of $\alpha 5$ integrin in driving BEC migration, we compared the migration rates of $\alpha 5$ integrin-deficient and control BEC. This revealed no obvious deficiency in the ability of $\alpha 5$ integrin KO cells to migrate on fibronectin (Fig. 5D). Next, we evaluated whether $\alpha 5$ integrin-deficient BEC could assemble a fibronectin fibrillar network. BEC were plated onto a laminin substrate, and fibronectin IF performed 24 hours later. As shown in Fig. 6, BEC lacking the $\alpha 5$ integrin were still capable of producing a fibronectin fibrillar network.

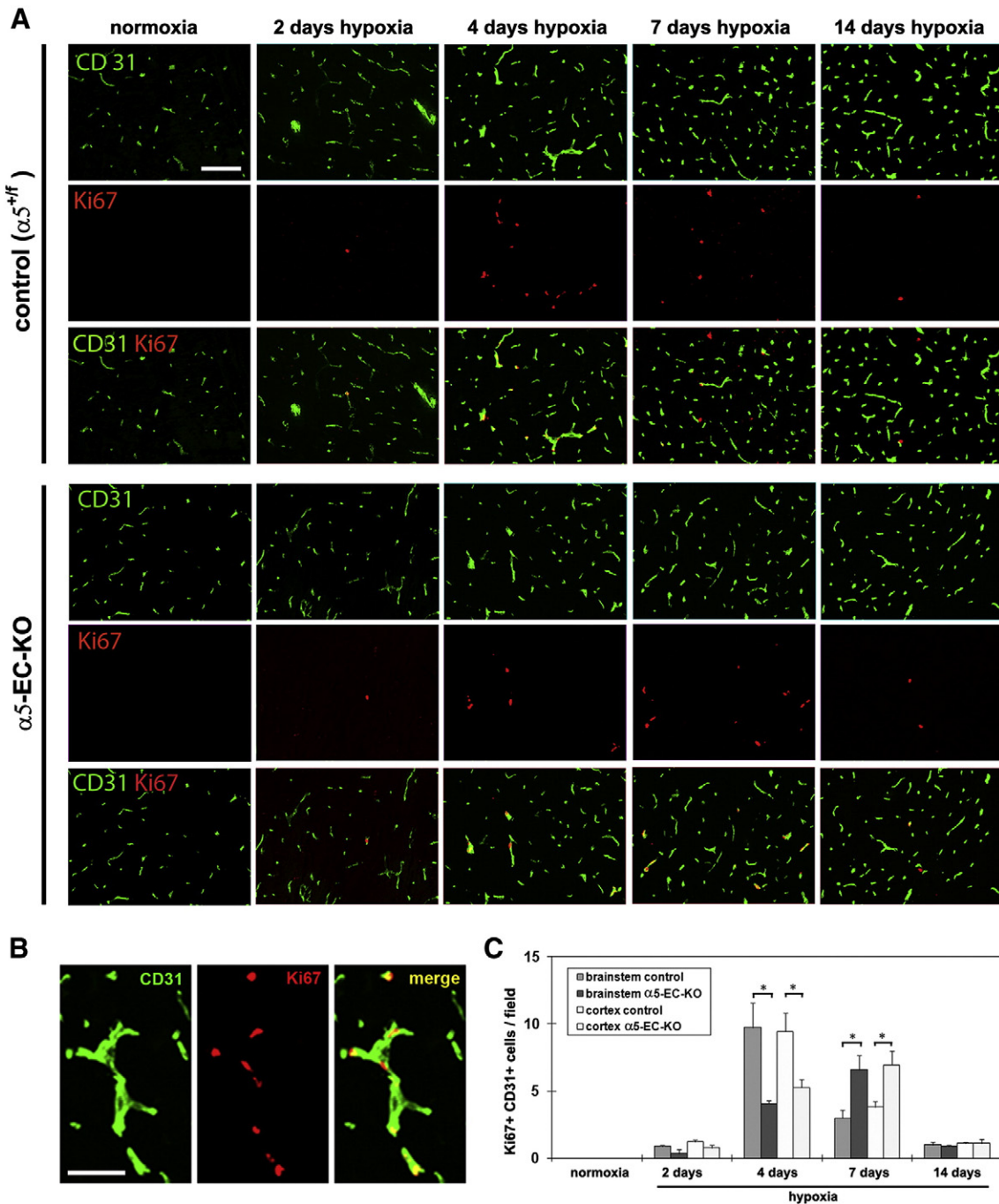


Fig. 3. Delayed and reduced BEC proliferation during the angiogenic response to cerebral hypoxia in $\alpha 5$ -EC-KO mice. **A.** Frozen sections of brainstem taken from control or $\alpha 5$ -EC-KO mice exposed to normoxia (~21% O_2) or 2, 4, 7 or 14 days hypoxia were examined for evidence of BEC proliferation, as assessed by CD31/Ki67 dual-IF. Scale bar = 100 μm . **B.** High power image of CD31/Ki67 dual-IF of 4 day hypoxia exposed brainstem of control mouse. Scale bar = 50 μm . **C.** Quantification of CD31+/Ki67+ proliferating BEC, in which each experiment was performed with three different animals per condition, and the results expressed as the mean \pm SEM of the number of CD31+/Ki67+ cells per field of view. Note that hypoxia promoted an increase in the number of proliferating BEC, though this response was delayed in $\alpha 5$ -EC-KO mice, * $p < 0.05$.

Discussion

Cerebral hypoxia stimulates a robust angiogenic response (LaManna et al., 1992), which is associated with strong upregulation of fibronectin and the fibronectin receptors $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrins on vascular endothelium (Li et al., 2010b; Milner et al., 2008a). A recent study from our laboratory using $\beta 3$ integrin-null mice, showed that the $\alpha v\beta 3$ integrin is dispensable for the angiogenic response, but suggested a role for the $\alpha 5\beta 1$ integrin (Li et al., 2010b). To address directly whether the $\alpha 5\beta 1$ integrin is required for the vascular remodeling response to

cerebral hypoxia, we examined this process in mice lacking $\alpha 5$ integrin expression in endothelial cells. This revealed that the angiogenic response to cerebral hypoxia was markedly attenuated in $\alpha 5$ -EC-KO mice, as these mice showed reduced hypoxia-induced increases in vessel density relative to control littermates. In keeping with the decreased angiogenic response, BEC in $\alpha 5$ -EC-KO mice showed a delayed mitogenic response to hypoxia, which was mirrored by delayed proliferation and growth of $\alpha 5$ -KO BEC in primary culture. Taken together, these studies demonstrate an important angiogenic role for the $\alpha 5\beta 1$ integrin in promoting BEC proliferation in response to cerebral hypoxia.

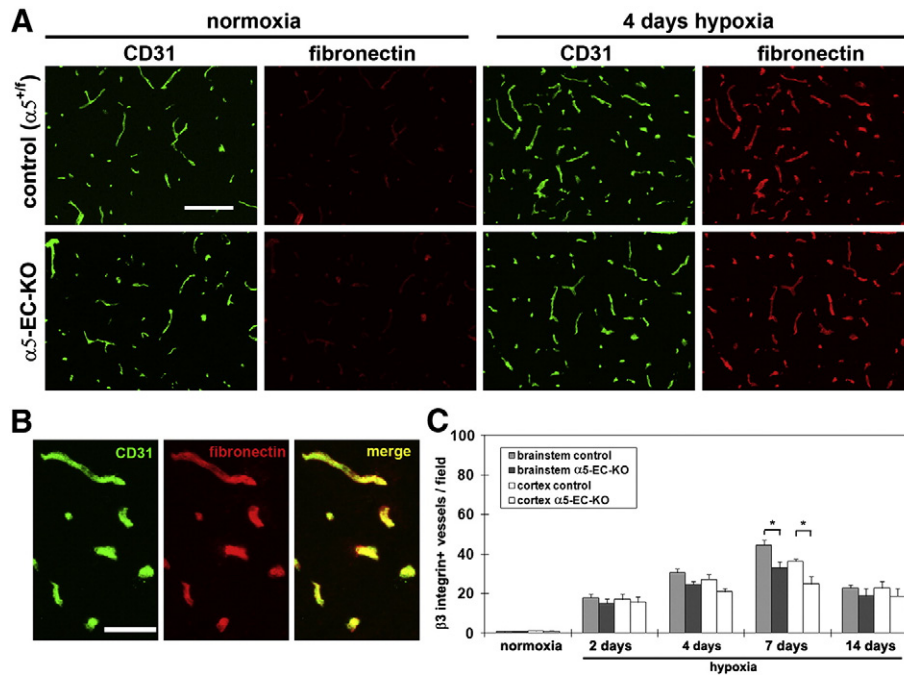


Fig. 4. Analysis of hypoxic-induced alterations in cerebrovascular expression of fibronectin and $\beta 3$ integrin. **A.** Frozen sections of cerebral cortex taken from control or $\alpha 5\text{-EC-KO}$ mice exposed to normoxia ($\sim 21\% \text{O}_2$), or 4 days hypoxia, underwent dual-IF for CD31 and fibronectin. Scale bar = 100 μm . Note that cerebral hypoxia promoted a strong increase in fibronectin expression in cerebral vessels of both control and $\alpha 5\text{-EC-KO}$ mice. **B.** High power image of CD31/fibronectin dual-IF of 4 day hypoxia exposed brainstem. Scale bar = 50 μm . **C.** Quantification of $\beta 3$ integrin-positive vessels in control or $\alpha 5\text{-EC-KO}$ mice exposed to hypoxia. Experiments were performed with three different animals per condition, and the results expressed as the mean \pm SEM of the number of $\beta 3$ integrin-positive vessels per field of view. Note that cerebral hypoxia increased the number of $\beta 3$ integrin-positive vessels (that peaked after 7 days hypoxia), and that this increase was significantly lower in $\alpha 5\text{-EC-KO}$ mice relative to controls, $p < 0.05$.

Chronic hypoxia drives an angiogenic response in the CNS

Relative to other tissues, the brain has extremely high demands for oxygen. This fact is well illustrated by the fact that the CNS

constitutes less than 2% of the body weight, but uses up to 20% of the total oxygen consumption in the body (Attwell et al., 2010). Interestingly, the oxygen levels in intact brain tissue of animals breathing 21% oxygen air at sea level are much lower than 21%, actually more in

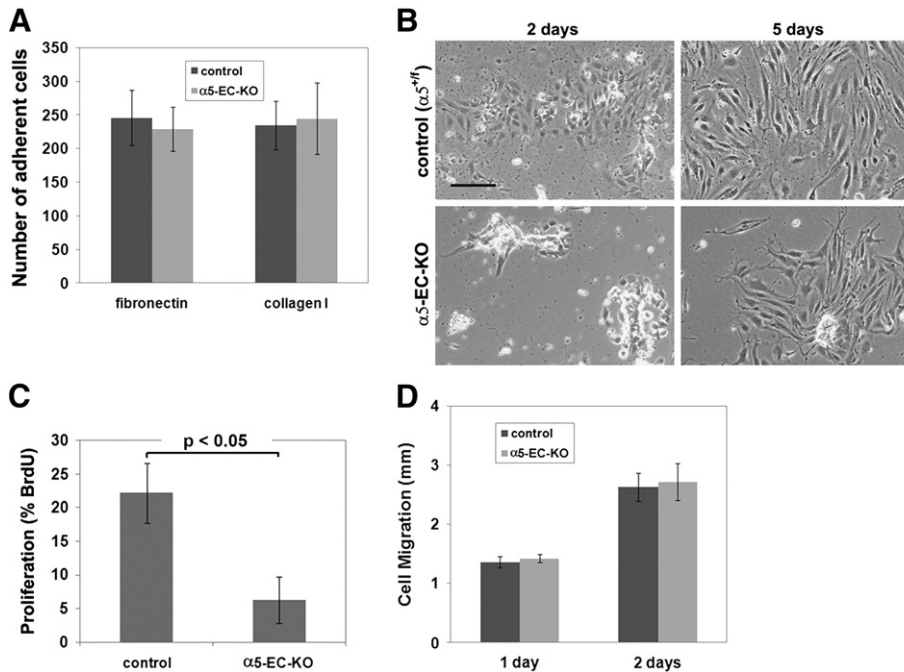


Fig. 5. Analysis of growth defects in primary $\alpha 5\text{-EC-KO}$ cultures. BEC from $\alpha 5\text{-EC-KO}$ or littermate control mice were isolated and cultured on fibronectin. **A.** Adhesion assays. Note that $\alpha 5$ integrin-deficient BEC showed no defect in adhesion to fibronectin or collagen I. **B.** Growth in primary culture. Note that after 2 days culture, while control BEC had dispersed from the endothelial islands, $\alpha 5$ integrin-deficient BEC were slow to disperse. After 5 days culture, BEC from both strains had dispersed and formed expanding colonies, though $\alpha 5$ integrin-deficient BEC were slower to grow, relative to control cells. Scale bar = 25 μm . **C.** BEC proliferation in 3-day old primary cultures cultured on fibronectin was examined over 16 hours, and expressed as the % of BEC that incorporated BrdU. All points represent the mean \pm SEM of four experiments. Note that the proliferation rate of $\alpha 5$ integrin-deficient BEC was significantly lower than control BEC ($p < 0.05$). **D.** Migration rates compared. All points represent the mean \pm SEM of four experiments. Note that $\alpha 5$ integrin-deficient BEC migrated on fibronectin at equivalent rates to control cells.

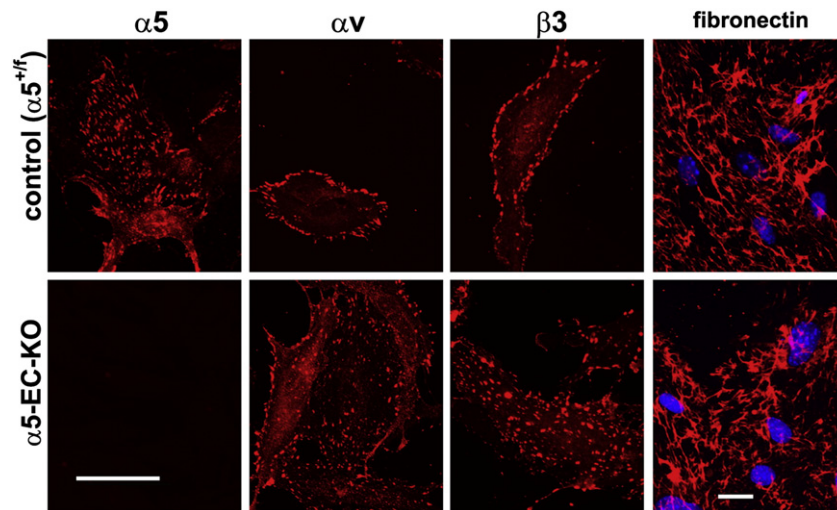


Fig. 6. Immunolocalization of $\alpha 5$ and $\alpha v\beta 3$ integrins on BEC. BEC from $\alpha 5$ -EC-KO and littermate control mice were cultured on fibronectin and integrin localization determined by IF. Scale bars = 25 μ m. Note that in control BEC, the $\alpha 5$ integrin was found in focal contacts throughout the body of the cell, while in contrast, the αv and $\beta 3$ subunits were specifically localized at the cell border. Interestingly, in $\alpha 5$ integrin-deficient BEC, the $\alpha v\beta 3$ integrin showed a re-distribution to focal contacts within the body of the cell. Fibronectin IF of cells plated onto laminin demonstrated that $\alpha 5$ integrin-deficient BEC were still capable of producing a fibronectin fibrillar network.

the range of 2–4% (15–30 torr) (Ndubuizu and LaManna, 2007). Thus, when animals breathe 8% oxygen, the oxygen concentration in the brain falls proportionally. This decline is detected by oxygen chemoreceptors, which trigger a number of physiological adaptive responses, including increased ventilation rate, tachycardia, cerebral vasodilatation, cerebral angiogenesis, and raised hematocrit (LaManna et al., 2004). All these mechanisms have a common aim: to raise the oxygen delivery to the brain, so as to compensate for the reduced oxygen availability in the atmospheric air.

The role of $\alpha 5\beta 1$ integrin in angiogenesis

Current data suggest that the $\alpha 5\beta 1$ integrin plays an important role in angiogenesis. Global deletion of the $\alpha 5$ integrin gene results in an embryonic lethal phenotype, with aberrant blood vessel formation in the embryo (Yang et al., 1993). Similar vascular defects are also apparent in $\alpha 5$ integrin-null embryoid bodies and teratoma cells (Taverna and Hynes, 2001). In addition, while quiescent endothelial cells express only low levels of $\alpha 5\beta 1$ integrin, angiogenic endothelial cells strongly upregulate this integrin, and functional blockade of the $\alpha 5\beta 1$ integrin in tumor-induced neovasculature inhibits angiogenesis and tumor growth in vivo (Kim et al., 2000). In the current study, mice lacking endothelial expression of the $\alpha 5$ integrin displayed a blunted angiogenic response to cerebral hypoxia. $\alpha 5$ -EC-KO mice showed reduced BEC proliferation at 4 days hypoxia, though interestingly, by 7 days hypoxia, BEC proliferation in these mice had caught up and was even slightly higher than littermate control mice. This demonstrates that in the adult CNS, $\alpha 5\beta 1$ integrin plays an important role in stimulating endothelial cell proliferation at an early step in the angiogenic process. Surprisingly, developmental angiogenesis is relatively unperturbed in $\alpha 5$ -EC-KO mice (van der Flier et al., 2010), implying that the mechanisms promoting angiogenesis in developmental and pathological situations might be different. Interestingly, the same situation has also been described for $\alpha 5\beta 1$ integrin function in lymphangiogenesis, in that developmental lymphangiogenesis appears unaffected by absence of the $\alpha 5$ integrin (van der Flier et al., 2010), but blockade of the $\alpha 5\beta 1$ integrin in the adult inhibits lymphangiogenesis (Okazaki et al., 2009). Why $\alpha 5\beta 1$ should play an important role in adult remodelling situations but not be essential during development remains to be determined. One possibility is that a higher level of plasticity is present during development and that with maturation, a more limited number of molecular

mechanisms are available. This suggests that developmental angiogenesis has considerably more redundancy and/or compensatory mechanisms compared to the adult situation.

The relative importance of $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrins in cerebral angiogenesis

BEC express two fibronectin receptors, the $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrins (Wang and Milner, 2006). As both these integrins show strong upregulation during hypoxia-induced cerebral angiogenesis, it is important to determine the relative importance of these two integrins. In a recent study with $\beta 3$ integrin-null mice, we showed that while $\beta 3$ was not essential for cerebral angiogenesis, lack of $\beta 3$ led to compensatory increases in BEC $\alpha 5$ integrin expression, and increased rate of BEC proliferation, suggesting a role for the $\alpha 5\beta 1$ integrin in driving BEC mitogenic responses during angiogenesis. Our current data support this idea, by showing that in response to hypoxia, $\alpha 5$ -EC-KO mice show attenuated angiogenesis and delayed BEC proliferation. This confirms that the $\alpha 5\beta 1$ integrin is important in driving BEC proliferation during the cerebral angiogenic response to hypoxia, and lends extra weight to the idea that of the two BEC fibronectin receptors, $\alpha 5\beta 1$ integrin is more important than $\alpha v\beta 3$. However, while the absence of BEC $\alpha 5\beta 1$ led to a delayed and weaker angiogenic response, it did not totally abrogate this response. Several explanations might account for this. First, in the absence of $\alpha 5\beta 1$ integrin, $\alpha v\beta 3$ may mediate the effect of fibronectin on BEC proliferation. In the current study, we did not observe increased vascular expression of the $\alpha v\beta 3$ integrin in $\alpha 5$ -EC-KO mice, though consistent with previous findings in embryonic endothelial cells (van der Flier et al., 2010), in $\alpha 5$ -KO BEC, $\alpha v\beta 3$ re-localized to cell surface focal adhesions where normally only $\alpha 5\beta 1$ integrin resides, suggesting a functional compensatory mechanism. It is important to note however, that while $\alpha v\beta 3$ integrin may compensate to some degree, our current findings clearly demonstrate that the $\alpha v\beta 3$ integrin is not as effective in promoting the angiogenic response as $\alpha 5\beta 1$ integrin. Second, other fibronectin receptors (integrins or non-integrins) may be upregulated in a compensatory manner. Third, other cell adhesion molecules may also promote BEC proliferation and angiogenesis, thus providing other redundant or compensatory mechanisms. For example, collagen I and its integrin receptors $\alpha 1\beta 1$ and $\alpha 2\beta 1$ have well described pro-angiogenic roles (Senger, 1996; Senger et al., 1997), making it feasible that in the absence of fibronectin or fibronectin receptors, collagen I

can still promote an angiogenic response. In the next phase of experiments, we plan to address the first two possibilities, by examining the hypoxic response in double KO mice lacking both $\alpha v\beta 3$ and $\alpha 5\beta 1$ in endothelial cells.

In summary, we have addressed the requirement of the $\alpha 5\beta 1$ integrin in mediating cerebral angiogenesis in response to cerebral hypoxia, by using mice lacking $\alpha 5$ integrin expression in endothelial cells ($\alpha 5$ -EC-KO). These mice showed a significantly attenuated angiogenic response, which was paralleled by a delayed endothelial mitogenic response. In vitro experiments confirmed the proliferation defect in $\alpha 5$ -KO BEC. Taken together, these studies demonstrate an important angiogenic role for the $\alpha 5\beta 1$ integrin in promoting BEC proliferation in response to cerebral hypoxia.

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