Supplement Material for Welser-Alves et al

METHODS

Animals

The generation of Tie2-Cre, nestin-Cre and β4 integrin$^{flox/flox}$ (β4 integrin$^{f/f}$) strains of mice and genotyping protocols have all been described previously [1-3]**.** All strains were backcrossed >10 times onto the C57BL/6 background and maintained under specific pathogen-free conditions in the closed breeding colony of The Scripps Research Institute (TSRI).

Chronic Hypoxia Model

β4-EC-KO mice or littermate controls (β4$^{flox/wt}$), 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) 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 on 10 µm frozen sections of cold phosphate buffer saline (PBS) perfused tissues as described previously [4]. Antibodies reactive for the following antigens were used in this study: CD31 (MEC13.3), α6 integrin (GoH3) and β4 integrin (346-11A) all from BD Pharmingen, La Jolla, CA; β1 integrin (MB1/2) from Chemicon, Temecula, CA; α-SMA-Cy3 conjugate (1A4), GFAP-Cy3 conjugate (G-A-5) and laminin (rabbit polyclonal) all from Sigma, St. Louis, MO; β-dystroglycan (43DAG/8D5) from Novocastra, Newcastle-upon-Tyne, United Kingdom, albumin (goat polyclonal) from Bethyl Labs, Montgomery, TX; Ki67 (rabbit polyclonal) from Vector Laboratories, Burlingame, CA; CD31 (2H8) from Abcam, Cambridge, MA; ALK1 (goat polyclonal) from R&D systems, Minneapolis, MN, and phospho-Smad 1/5/8 (rabbit polyclonal) from Cell Signaling, Danvers, MA. Secondary antibodies used included goat anti-Armenian hamster-DyLight 594 from Biolegend, San Diego, CA; Cy3-conjugated anti-rabbit, anti-rat and anti-goat from Jackson Immunoresearch, West Grove, PA; and anti-rat Alexa Fluor 488 and anti-mouse Alexa Fluor 488 from Invitrogen, Carlsbad, CA.

Image analysis

Images were taken using a 20X objective on a Zeiss Imager M1.m. Analysis was performed in the frontal lobe and medulla regions of the brain. Within these regions, and for each antigen, images of three randomly selected areas were taken at 20X magnification, and three sections per brain analyzed to calculate the mean for each subject. For each antigen in each experiment, exposure time was set to convey the maximum amount of information without saturating the image. Exposure time was maintained constant for analyzing the same antigen across the time-course of hypoxic exposure, and between the different strains of mice. All data analysis, including the vessel size distribution analysis and quantification of ALK1 expression levels was performed using Perkin Elmer Volocity software. The size distribution analysis is an area-based method, in which CD31 or β4 integrin/α-SMA-positive events were grouped into different size categories: 0-100 μm^2, 100-200 μm^2, 200-400 μm^2 and >400 μm^2. This analysis was performed over four different experiments, using one animal of each genotype per condition per experiment, and the results expressed as the mean ± SEM. Statistical significance was assessed by using the Student's t test, in which $p < 0.05$ was defined as statistically significant.

ALK1 expression levels on blood vessels in IF studies was measured by identifying CD31-positive structures (green channel), and quantifying the mean fluorescent intensity of vessels in the field using the red channel (ALK1). Data were presented as the % of ALK1 expression level under normoxic conditions (control).

Flow cytometry of acutely isolated brain endothelial cells

Brains were removed, digested in papain for one hour, then dissociated, and centrifuged through 22% BSA to obtain a myelin-free cell suspension as previously described [5]. The resulting single cell suspension was then subject to dual-color flow cytometry analysis to quantify expression of ALK1 and phospho-Smad1/5/8 by CD31-positive endothelial cells using fluorescent-conjugated monoclonal antibodies, as described previously [6]. For each antigen, the mean fluorescent intensity and percentage of CD31-positive cells expressing that antigen was quantified using a Becton Dickinson FACScan machine (San Diego, CA), with 10,000 events recorded for each condition. Each experiment was repeated three times and the data expressed as mean ± SEM. Statistical significance was assessed by using the Student's paired t test, in which $p < 0.05$ was defined as statistically significant.

Primary brain endothelial cell proliferation studies

Pure cultures of mouse brain endothelial cells (BEC) were obtained, and proliferation assays performed using the bromodeoxyuridine (BrdU) incorporation assay (Invitrogen, Carlsbad, CA), as previously described [7, 8]. Briefly, BEC were cultured on laminin under control conditions, or in the presence of two BEC mitogens, basic fibroblast growth factor (bFGF) or tumor necrosis factor (TNF), both 10 ng/ml. Mitotic rates were examined over 16 hours, and expressed as the percentage of BEC that incorporated BrdU; all points represent the mean ± SEM of four experiments.

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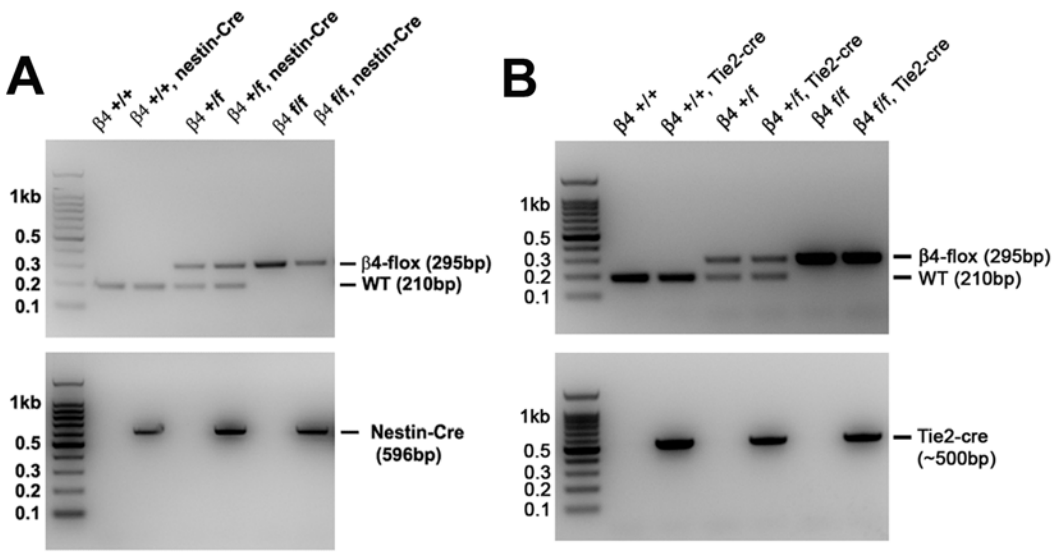
Supplemental figure legends

Supplemental Figure I.
PCR genotyping to identify β4-astro-KO (A) and β4-EC-KO (B) mice. Genotyping of mouse tail DNA to identify mice positive for the Tie2-Cre (500 bp), nestin-Cre (596 bp) and β4 integrin^{flox} (295 bp) transgenes was performed using previously described protocols. Mice were selected that carried one copy of the Tie2-Cre (endothelial cell-specific) or nestin-Cre (neural lineage-specific) and two copies of the flox β4 integrin transgene.



Supplemental Figure II.

Defining the relationship between α-SMA and β4 integrin on vessels in peripheral organs. Dual-IF was performed on frozen sections of heart, kidney and skeletal muscle from adult mice using antibodies specific for β4 integrin (AlexaFluor-488, green) and the smooth muscle cell (SMC) marker α-SMA (Cy3, red). Scale bar = 100 μm. Note that in all three organs, α-SMA and β4 integrin expression strongly co-localized in blood vessels, though in the kidney some non-endothelial β4 integrin expression was also observed, as seen in the β4-EC-KO kidney.