Materials and Methods

Reagents: Penicillin, streptomycin, DMEM, and FBS were obtained from Invitrogen Life Technologies (Carlsbad, CA). A protein assay kit was purchased from Bio-Rad (Hercules, CA). Integrin β3 antibodies were from Abcam (Cambridge, MA); antibodies against SMA-α-FITC, recombinant collagen I, vitronectin, and fibronectin were from Sigma-Aldrich (Louis, MO); anti-integrin β3 and N1ICD (Cleaved Notch1) antibodies were from Cell Signaling (Danvers, MA); Fluorescence-700 or -800 secondary antibodies were obtained from Invitrogen (Carlsbad, CA). Anti-CD42b, anti-CD41a, Gr-1-PE-Cy7, CD11b-APC, mouse hematopoietic lineage eFluor 450, and CD45-PerCP-Cy5.5 antibodies were purchased from eBioscience (San Diego, CA). The CD31 antibody, Sca I-FITC, CD41-FITC, C-Kit-APC, and CD144-APC were purchased from BD Pharmingen (San Jose, CA). Antibodies against Notch1, Jagged 1, and Hes1, Hes5, and αIIb integrin were from Santa Cruz Biotechnology (Santa Cruz, CA). A RBP-Jκ antibody was obtained from Millipore Corp (Billerica, MT); the adenovirus of soluble Jagged 1 was obtained from Dr. Post (Maastricht University, Netherlands). The recombinant TGF-β1, and γ-secretase inhibitor (DAPT) were purchased from R & D (Minneapolis, MN).

Mouse model of Venous Graft*:* All animal protocols were approved by IACUC of Baylor College of Medicine. Wild type C57/B6 and integrin $\beta3^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, Maine). AVG was performed as previously described.[1] In brief, the right common carotid artery of a male mouse was surgically exposed to place a cuff on both ends of the artery. The ends were then everted over the cuff and ligated with an 8.0 silk suture. Vena cava from donor mice was grafted by "sleeving" ends of the vein over the artery cuff and secured with 8.0 silk sutures. After 4 weeks, the AVG was dissected and vessel wall thickness measured as the area of the vessel minus that of the lumen using NIS-Elements BR 3.0 program.

CAC isolation and characterization by flow cytometry. Bone marrow cells were isolated from wild type or integrin $\beta3^{-/-}$ mice. Sca I/c-Kit double-positive CACs and Sca I/c-Kit double-positive/lineage negative CACs, which are capable of differentiating into endothelial cells, were purified by fluorescence-activated cell sorter with a purity of >90%. The enriched CACs were cultured on fibronectin-coated dishes in the EGM-2 medium supplemented with EGM SingleQuots (Cambrex, Inc., East Rutherford, NJ) for 1 week. Attached CACs were rinsed with a Hank's buffered saline solution, fixed with 4% paraformaldehyde for 10 min at 37°C, and stained for VE-cadherin or PECAM. These cells were visualized under a fluorescence microscope using FITC or rhodamine excitation/emission filters. The Di-LDL uptake was performed as described to sure that cultured cells were enrich in functional CACs.[2]

Blood collection and platelet preparation. Blood was collected from wild type and integrin $\beta3^{-/-}$ mice via interior vena cava and processed for platelets as described.[3] Briefly, platelet-rich plasma was obtained by centrifuging whole blood (anti-coagulated with 0.38% of sodium citrate) at 150 x g for 15 min at 24 °C. To obtain washed platelets, platelet-rich plasma was obtained from whole blood using acid-citrate dextrose as the anticoagulant (Cytosol Laboratories Inc., Braintree, MA) and centrifuged at 900 x g for

10 min. Platelet pellets were washed once with a CGS buffer (13 mM sodium citrate, 30 mM glucose and 120 mM sodium chloride, pH 7.0), supplemented with 1 μM PGE1 (Santa Cruz), and centrifuged at 900 x g for 10 min. The washed platelets were re-suspended in Ca^{2+}, Mg^{2+} Tyrode's buffer.

Platelet TGF-β1 detection: The immunologic enzyme-linked immunosorbent assay (ELISA) used an immobilized mAb specific for the active form of TGF-β1 (R & D systems). Active TGF-β1 was measured directly in untreated platelet, whereas total TGF-β1 (active + latent) was measured after pretreating the samples with 0.2 volume of 1N HCl for 20 min at room temperature to convert latent TGF-β1 to active TGF-β1. The platelets were stimulated with thrombin (0.125 U/ml) (Sigma Aldrich) or fibrillar type I collagen (5 μg/ml) (Sigma Aldrich) for 5 min at 37^0C. Platelet-free platelet releasates were prepared by centrifuging at 14 000 g for 20 min at 4^0C and the total TGF-β1 was determined.

Co-culture of platelets on CAC differentiation. Multiple-well glass slides were coated with 2 μg of fibrillar Type I collagen for 3 hrs at room temperature. Platelets isolated from wild type and integrin $\beta3^{-/-}$ mice were seeded onto collagen-coated coverslips. Isolated CACs were plated on top of the platelets and grown in EGM2 medium. CAC colonies were counted 7 days after plating. To detect CAC differentiation, randomly selected colonies were stained for the endothelial cell markers CD31 and VE-cadherin. CACs were also collected for real time RT-PCR.

Immunohistochemistry: AVGs were perfused through the left ventricle as described[11]. A graft was obtained by cutting the transplanted segment from the native vessel at the cuff end, fixed in 4% phosphate-buffered formaldehyde at 4 °C for 24 h, and processed for 5 μm sections as described.[1] Sections were blocked with 10% goat serum (Vector Laboratories, Burlingame, CA) for 30 min and then incubated with primary antibodies. They were then washed in 0.5% Tween 20 in PBS (PBST) and incubated with a biotinylated secondary antibody (Vector lab) at room temperature for 2 hrs. After washing with PBST, these sections were incubated with an *Elite*® ABC reagent (Vector Laboratories) according to the manufacturer's protocol and counterstained with hematoxylin. For double immunofluorescent staining of samples, fluorescent secondary antibodies were applied to sections; DAPI was used as a counter stain. Pictures were recorded using a Nikon Eclipse 80i fluorescence microscope (Melville, NY).

Real-time RT-PCR: Total RNA from freshly removed vena cava and AVGs was isolated using an RNeasy kit (Qiagen, Valencia, CA). Real-time RT-PCR was performed using an Opticon real-time RT-PCR instrument (MJ Research, Waltham, MA). The specificity of this real-time RT-PCR was confirmed by agarose gel electrophoresis and melting-curve analysis with GAPDH as the internal standard. Primers used for the amplification reaction were summarized in Supplemental Table 1.

Western Blot Analysis: Cells or AVGs were lysed in a RIPA buffer (25mM Tris-HCl (pH 7.6), 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) and ~30 μg of

proteins were separated by SDS-PAGE. After being transferred to a PVDF membrane, antibodies were used to probe molecules specified in the result section.

Bone marrow transplantation and flow cytometry analysis: The bone marrow (BM) transplant was performed as described.[4] Briefly, BM cells were harvested by flushing femurs and tibias of a donor mouse and infused (5 x 10^6 cells/mouse) into the lateral tail vein of a lethally irradiated (1100 rads) recipient mouse. For flow cytometry analysis, bone marrow and peripheral mononuclear cells from wild type and integrin $\beta3^{-/-}$ mice were isolated, incubated with antibodies, and analyzed by flow cytometry to identify neutrophils (Gr-1-PECy7), macrophages (CD11b-APC), monocytes (CD45-PerCP-Cy5.5) and stem cells (CD117-APC and Sca I-FITC; hematopoietic lineage eFluor 450).

Statistical analyses*.* All data are presented as the mean ± standard error of the mean. Comparison between groups was made using one-way ANOVA followed by pairwise comparisons with p value adjustment; $P < 0.05$ was considered to be statistically significant.

References:

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