



# HHS Public Access

Author manuscript

*Arterioscler Thromb Vasc Biol.* Author manuscript; available in PMC 2016 March 01.

Published in final edited form as:

*Arterioscler Thromb Vasc Biol.* 2015 March ; 35(3): 607–615. doi:10.1161/ATVBAHA.114.305089.

## Impaired Integrin $\beta 3$ Delays Endothelial Cell Regeneration and Contributes to Arteriovenous Graft Failure in mice

Ming Liang<sup>1,5</sup>, Yun Wang<sup>2,5</sup>, Anlin Liang<sup>5</sup>, Jin-Fei Dong<sup>3</sup>, Jie Du<sup>4</sup>, and Jizhong Cheng<sup>5</sup>

<sup>1</sup>Department of Nephrology, Guangzhou First People's Hospital, Guangzhou Medical University, China

<sup>2</sup>Department of Cell Biology, Third Military Medical University, Chongqing, China

<sup>3</sup>Puget Sound Blood Research Institute, Hematology Division, Department of Medicine, University of Washington

<sup>4</sup>Beijing Anzhen Hospital Affiliated to the Capital Medical University, Beijing Institute of Heart Lung and Blood Vessel Diseases, Beijing, China

<sup>5</sup>Nephrology Division, Baylor College of Medicine, Houston, Texas

### Abstract

**Objective**—Neointima formation is associated with stenosis and subsequent thrombosis in arteriovenous grafts (AVG). A role of integrin  $\beta 3$  in the neointima formation of AVGs remains poorly understood.

**Approach and Results**—In integrin  $\beta 3^{-/-}$  mice, we found significantly accelerated occlusion of AVGs compared to the wild type mice. This is caused by the development of neointima and lack of endothelial regeneration. The latter is a direct consequence of impaired functions of circulating angiogenic cells (CACs) and platelets in integrin  $\beta 3^{-/-}$  mice. Evidence suggests the involvement of platelet regulating CAC homing to and differentiation at graft sites via TGF- $\beta 1$  and Notch signaling pathway. First, CACs deficient of integrin  $\beta 3$  impaired adhesion activity toward exposed subendothelium. Second, platelets from integrin  $\beta 3^{-/-}$  mice failed to sufficiently stimulate CACs to differentiate into mature endothelial cells. Finally, we found that TGF- $\beta 1$  level was increased in platelets from integrin  $\beta 3^{-/-}$  mice and resulted in enhanced Notch1 activation in CACs in AVGs. These results demonstrate that integrin  $\beta 3$  is critical for endothelial cell homing and differentiation. The increased TGF- $\beta 1$  and Notch1 signaling mediates integrin  $\beta 3^{-/-}$ -induced AVG occlusion. This accelerated occlusion of AVGs was reversed in integrin  $\beta 3^{-/-}$  mice transplanted with the bone marrow from WT mice.

---

Corresponding Authors: Dr. Jizhong Cheng, Department of Medicine, Baylor College of Medicine, Houston, TX 77030, Tel: 713-798-2698, Fax: 713-798-5010, jizhongc@bcm.edu.

Contribution: ML, JC, YW carried out experiments, study design, and data analysis. JC, ML, YW searched the literature and interpreted the data. JC, JD, and JFD were involved in writing the paper. All authors had final approval of the submitted and published versions.

**Disclosures**  
None

**Conclusion**—Our results suggest that boosting integrin  $\beta 3$  function in the endothelial cells and platelets could prevent neointima and thrombosis in AVGs.

### Keywords

integrin  $\beta 3$ ; arteriovenous graft; platelets; bone marrow; endothelial progenitor cell; Notch

## Introduction

The creation of functional arteriovenous grafts (AVG) for patients who undergo coronary artery bypass or hemodialysis is a frequent and expensive surgical procedure. Even when initial surgery is successful, an AVG frequently fails. The primary patency rates of graft failure are 42% and 40–60% for coronary bypass surgery and hemodialysis, respectively,<sup>1, 2</sup> primarily due to neointima formation and AVG thrombosis.

The integrin  $\beta 3$  family consists of  $\alpha v\beta 3$  and  $\alpha I Ib\beta 3$ . The former is expressed in endothelial cells and selective inflammatory cells, while the latter is primarily on platelets. Platelets are involved in the recruitment and differentiation of bone marrow-derived terminal progenitor cells,<sup>3, 4</sup> mediated through the adhesive receptor integrin  $\alpha I Ib\beta 3$ .<sup>5</sup> Recent reports suggest that integrin  $\beta 3$  expressed on endothelial cell and platelets influences the function of bone marrow-derived progenitor cells.<sup>6</sup> Platelets from integrin  $\beta 3^{-/-}$  mice are also reported to secrete more chemokines and cytokines compared with the wild type mice, including TGF- $\beta 1$ .<sup>7</sup>

In a mouse AVG model, there was a massive loss of the endothelium 3 days after surgery. The subsequent endothelial regeneration plays a key role for vascular remodeling and maintaining a patent AVG.<sup>8</sup> Platelets influence the homing and differentiation of circulating angiogenic cells (CAC) into endothelial cells at sites of vascular injury by serving as carriers<sup>9, 10</sup> and by secreting SDF-1 $\alpha$ .<sup>11</sup> Here, we tested hypothesis that integrin  $\beta 3$  is critical for neointima formation and endothelial cell regeneration at sites of AVGs by studying the adhesion and differentiation of CACs *in vitro* and vascular repairs in a mouse AVG model. We demonstrate that CACs from integrin  $\beta 3^{-/-}$  mice homed poorly because of significant reduction in CAC adhesion and differentiation. The latter is caused by an altered TGF $\beta 1$ -Notch1 signaling mediated by  $\beta 3$  deficient platelets.

Materials and Methods are available in the online-only Data Supplement.

## Results

### AVG occlusion accelerated in integrin $\beta 3^{-/-}$ mice

Integrin  $\beta 3$  is expressed in artery, vein and heart tissues; there was no such expression in integrin  $\beta 3^{-/-}$  mice (Fig. 1A). In our model of AVGs in wild type mice, we used an enface analysis to study the endothelium of the vein of the AVG and a normal vena cava. Integrin  $\beta 3$  was expressed in endothelial cells in a clustered pattern (Fig. 1B). In a failed AVG from patients, integrin  $\beta 3$  expression was located in the endothelium and the neointima (Fig. 1C). At one month after surgery, similar results were found in AVGs created in wild type mice (Fig. 1D). Compared with results in a control vena cava, there was reduced integrin  $\beta 3$

expression in the endothelium of the AVG (Fig. 1D). Newly formed endothelial cells expressed integrin  $\beta 3$  on the basal side (Fig. 1D). In AVGs, there was marked thinning and attenuation of the endothelium compared to findings in normal vena cava.

Integrin  $\beta 3^{-/-}$  and wild type mice were subjected to AVG and evaluated one month after surgery. The rate of graft failure due to occlusion was 86.7% for  $\beta 3^{-/-}$  mice as compared to 5% for wild type mice (Fig. 2A & B). An intact vascular lining of CD31<sup>+</sup> endothelial cells was found in AVGs of WT mice, but not in integrin  $\beta 3^{-/-}$  mice (Fig. 2C). SMA- $\alpha$  was strongly expressed in neointima cells in WT AVGs but not in integrin  $\beta 3^{-/-}$  mice (Fig. 2C). When stained for markers for thrombosis (vWF, CD41, and CD42), we found much more expression of thrombotic positive markers in AVGs placed in integrin  $\beta 3$  KO mice vs. the expression in WT mice (Fig. 2D and E), suggesting that the integrin  $\beta 3$  deficiency prevents the formation of an intact endothelium and causes thrombosis, which could be the potential factor influencing the AVG patency.

### **Integrin $\beta 3^{-/-}$ delays endothelial regeneration in AVGs**

Endothelial cells became disorganized 3 hrs after AVG surgery and most were lost by 24 hours in wild type and integrin  $\beta 3^{-/-}$  mice (Fig. 3A & C). These results could not be claimed on surgeries as a cross section of AVG just after surgery revealed that the endothelium was intact (Fig. 3B). However, endothelial cells regenerated to form the new endothelium around 7 to 14 days after surgery (Fig. 3A & C). In contrast, endothelial cells were scarcely present at an AVG site of integrin  $\beta 3^{-/-}$  mouse during the same period (Fig. 3D). This result was further verified by enface analysis, which detected significantly less CD31 signals in AVGs from integrin  $\beta 3^{-/-}$  mice as compared to that from WT mice (Fig. 3E).

### **Integrin $\beta 3^{-/-}$ suppresses bone marrow-derived CAC adhesion *in vitro***

Alternatively, the reduced endothelial regeneration could be due to the impaired CAC homing capability. Reduced CAC homing could be caused by the lack of integrin  $\beta 3$ -mediated interaction between CACs and the subendothelial matrix. Consistent with the notion, CACs from integrin  $\beta 3^{-/-}$  mice adhered poorly to vitronectin, the ligand for integrin  $\beta 3$ . While the adherence of integrin  $\beta 3$ -deficiency CACs to laminin and collagen I were not affected (Fig. 4A & B). These results are further confirmed with experiments testing lineage negative/c-Kit/Sca I positive CACs isolated from the bone marrow of wild type and  $\beta 3^{-/-}$  mice (Fig. 4C). The absence of integrin  $\beta 3$  suppressed CAC adhesion to extracellular matrix. Notably, the extracellular matrix tested included vitronectin and fibronectin which are interacting with Integrin  $\beta 3$  (Fig. 4C). Together, these results suggest that  $\beta 3$  deficiency CACs adhere poorly to the subendothelium exposed at sites of AVGs.

This reduced CAC homing is not caused by a reduced number of CACs because integrin  $\beta 3^{-/-}$  mice had a comparable number of CACs in the bone marrow of WT mice (Fig. 4D and E). Moreover, the lineage negative CACs (lineage<sup>-</sup>/c-kit<sup>+</sup>/Sca I<sup>+</sup> cells) were also purified and analyzed. The results indicate that that  $\beta 3$  deficiency did not affect CAC numbers but impair their adhesion capability to vitronectin (Fig. 4F & G). Similar results were found from circulating Sca I<sup>+</sup>/c-Kit<sup>+</sup> cells (data not shown).

## Platelets from integrin $\beta 3^{-/-}$ mice delay homing and differentiation of bone marrow-derived CACs

Platelets are known to play a critical role in CAC homing<sup>10</sup>. We next examine whether integrin  $\beta 3$  deficiency in platelets contributes to the poor regeneration of endothelial cells at an AVG site. We found that there was significantly less ScaI<sup>+</sup>-CACs adhere to AVGs at day 5 in integrin  $\beta 3^{-/-}$  mice compared to controls (Fig. 5A and B). Moreover, CACs has less potential to differentiate into mature endothelial cells when co-cultured with platelets from integrin  $\beta 3^{-/-}$  mice compared with those on WT platelets (Fig. 5C). Furthermore, these  $\beta 3$  deficient platelets expressed a 2-fold higher level of TGF- $\beta 1$  (Fig. 5D). Using ELISA assay, we found that thrombin (0.125 U/ml) or collagen I (5  $\mu$ g/ml) treatments stimulated more TGF- $\beta 1$  release from platelets isolated from integrin  $\beta 3$  KO mice compared with that of WT mice (Fig. 5E). In AVGs, we found that TGF- $\beta 1$  colocalized with platelets and more TGF- $\beta 1$  level was detected in integrin  $\beta 3$  KO mice vs that in wild type mice (Fig. 5F).

To assess the impact of TGF- $\beta 1$ , we treated wild type CACs with TGF- $\beta 1$  and found impaired CAC differentiation into endothelial cells (Fig. 5G). Thus, high TGF- $\beta 1$  in platelets from integrin  $\beta 3$  KO mice suppresses CAC differentiation into mature endothelial cells and then reduced the recovery of the endothelium following endothelial cell losses in an AVG.

## Increased TGF- $\beta 1$ in $\beta 3$ -null platelets inhibits CAC differentiation by stimulating Notch signaling

Results presented so far have suggested that poor endothelial regeneration in integrin  $\beta 3$  KO mice at an AVG site may be caused by 1) lack of interaction between  $\beta 3^{-/-}$  CACs and components of subendothelial matrix and 2) increased TGF- $\beta 1$  in integrin  $\beta 3^{-/-}$  platelets. To explore the second possibility, we focus on Notch signaling in endothelial cells because TGF- $\beta 1$  activates Notch by upregulating the Notch ligand Jagged1.<sup>12</sup> This Notch signaling has also been linked to stem cell self-renewal and CAC differentiation. We detected enhanced expression of molecules critical for Notch signaling at the AVG site of integrin  $\beta 3^{-/-}$  mice as compared to WT (Fig. 6A). For example, the activated NICD (Notch 1 intracellular domain) was more prominently detected in AVGs isolated from  $\beta 3^{-/-}$  mice compared to WT mice (Fig. 6B & C). When treated with TGF- $\beta 1$ , CACs expressed the increasing levels of the Notch ligand Jagged 1 and its target Hes5 (Fig. 6D). In parallel with the increased Notch activation, CACs treated with -secretase inhibitor, DAPT, rescued the expressions of the endothelial cell markers VE-cadherin and CD31 that were reduced by treatment with TGF- $\beta 1$  or co-cultured with integrin  $\beta 3$ -null platelets (Fig. 6E). These results suggest that up-regulation of Notch signaling is associated with a poor differentiation of CACs at an AVG site. Consistent with the notion, when treated with soluble Jagged 1 (inhibits Notch signaling) or DAPT CACs from wild type and  $\beta 3$ -null mice increased the rate of differentiation as demonstrated by increasing expression of VE-cadherin (Fig. 6F & G).

## Bone marrow cells from wild type mice rescue AVG failure in integrin $\beta 3^{-/-}$ mice

The results so far have suggested two potential pathways through which integrin  $\beta 3$  deficiency results in poor homing of CACs and regeneration of the endothelium

(differentiation) at a site of AVG: decreased interaction between CACs and the subendothelium (directly mediated through platelets) and enhanced Notch signaling in CACs mediated by platelet-derived TGF- $\beta$ 1. These possibilities were further investigated by the transplantation of bone marrow from a wild type mouse donor to an integrin  $\beta$ 3<sup>-/-</sup> recipient. There was significant improvement in AVG patency in integrin  $\beta$ 3<sup>-/-</sup> mice after transplantation of WT bone marrow cells vs results from integrin  $\beta$ 3<sup>-/-</sup> mice (Fig. 7A). In a reciprocal experiment, AVGs in wild type mice transplanted with bone marrow cells from integrin  $\beta$ 3<sup>-/-</sup> mice were clogged (Fig. 7A). The AVG from transplanted integrin  $\beta$ 3<sup>-/-</sup> mice also had an intact vascular wall lined with mature CD31<sup>+</sup>-endothelial cells (Fig. 7B & C). There was ~75% of AVGs in the mice with the bone marrow transplantation had open lumens vs the AVGs generated in WT mice with bone marrow from integrin  $\beta$ 3<sup>-/-</sup> mice (Fig. 7D). To further compare the CAC homing capability, a mixture of bone marrow cells from WT (GFP<sup>+</sup>) and integrin  $\beta$ 3<sup>-/-</sup> mice were transplanted into integrin  $\beta$ 3<sup>-/-</sup> mice before AVGs. The results showed that more WT (GFP<sup>+</sup>)-derived CACs incorporated into the lumen area of the AVGs (Fig. 7E), indicating that deficiency of integrin  $\beta$ 3<sup>-/-</sup> limit CAC-mediated endothelium regeneration.

## Discussion

In our current study, we found that integrin  $\beta$ 3<sup>-/-</sup> causes AVG occlusion. Several lines of evidence support that the dysfunctions of platelets and CACs in integrin  $\beta$ 3<sup>-/-</sup> mice are the potential reasons for this failure. First, deficiency of integrin  $\beta$ 3 in CACs impairs their adhesion to the exposed extracellular matrix. Integrin  $\beta$ 3<sup>-/-</sup> platelets are deficient in mediating CAC homing and prevent CAC differentiation into mature endothelial cells due to an increased production of platelet-derived TGF- $\beta$ 1, which activates the Notch signaling pathway (Supplemental Fig. I).

AVG failure is a consequence of three related processes including early thrombosis formation, neointima formation, and graft atherosclerosis.<sup>13</sup> About 40 percent of AVGs experience such a failure within 18 months of the operation.<sup>14</sup> The failure of AVGs is attributable to adverse vascular remodeling, which remain poorly understood. Multiple events and cell types, including endothelial denude, platelet aggregation, smooth muscle cell proliferation, activated thrombocytes, and infiltration of monocytes, contributes to AVG failure. In mouse model of AVG, we found increased thrombosis and smooth muscle cell hyperplasia in AVGs at 2–4 weeks, and this process is accelerated in  $\beta$ 3-integrin KO mice. Deficiency of integrin  $\beta$ 3 delayed the endothelial cell regeneration in AVG in early time point, and led to AVG failure, which related with thrombosis. These findings are consistent with previous reports. The initial loss of venous endothelium after AVG is followed by platelet accumulation and the recruitment of CACs at a denuded AVG.<sup>15</sup> CACs are BM-derived cells that are mobilized to the systemic circulation in response to tissue injury and incorporate into sites of neovascularization.<sup>16, 17</sup> Following vascular injury, CACs have been shown to mobilize and home to the subendothelium at site of vessel injury.<sup>18</sup> Peripheral blood CACs transplanted into balloon-injured arteries rapidly endothelialize the denuded vessels.<sup>19</sup> In AVGs, the endothelium is denuded 72 hrs after surgery followed by on-site platelets aggregation and endothelial cell regeneration. There is approximately 30% of BM-derived CACs incorporation into newly formed endothelium.<sup>8, 15</sup> An integrin  $\beta$ 3

deficiency could potentially interfere with vascular repairs at an AVG site because the disruption of the integrin signal could result in a poor maturation of vessels<sup>6</sup>. Moreover, the neovascularization was restored in a DiYF  $\beta 3$ -integrin mutant mouse by transplantation with WT BM.<sup>20</sup>

Our data suggest an underlying mechanism for how integrin  $\beta 3^{-/-}$  could impair CAC homing and differentiation. First, integrin  $\beta 3$  deficient CACs failed to efficiently adhere to the denuded AVGs and to the subendothelial matrix proteins, vitronectin, and fibronectin (Fig. 3). There are no differences in a total number of lineage negative c-Kit/Sca I double positive CACs (Fig. 4G), suggesting that this loss-of-function phenotype is not caused by changes in the number of circulating CACs. This finding is consistent with a previous observation that the loss function of integrin  $\beta 3$  did not change the numbers of ScaI<sup>+</sup> cells.<sup>20</sup>

The activated platelets could be another factor that involved in CAC homing in AVGs.<sup>4, 10</sup> Following vascular insult, platelets first tether and adhere to subendothelium exposed by injury to not only seal the wound, but also promote P-selectin-mediated CAC homing as previously demonstrated.<sup>21,22</sup> We have recently shown that platelets are involved in trapping and recruiting BM-derived fibroblast specific protein 1 positive cells.<sup>23</sup> In current study, we further show that platelet aggregation was decreased in AVGs from integrin  $\beta 3^{-/-}$  mice, independent of platelet counts, which are normal in  $\beta 3$ -null mice.<sup>24</sup> The data is supported by previous finding that the integrin  $\beta 3$  partner,  $\alpha IIb$  integrin, is required for the recruitment of bone marrow-derived CD34<sup>+</sup> and Sca-1<sup>+</sup> progenitor cells into injured vessel walls.<sup>5, 9, 11</sup> More evidence showed that microparticles from Platelets promotes CAC function and tube formation through integrin  $\beta 3$ .<sup>25</sup>

Second,  $\beta 3$  deficiency may also result in defective differentiation of CACs at sites of AVGs in a platelet-dependent manner. Human CD34<sup>+</sup> CACs co-cultured with immobilized platelets form colonies and are capable of differentiating into mature endothelial cells.<sup>21, 26</sup> This regulatory activity of platelets towards CAC differentiation is likely mediated by platelet-derived growth factors (e.g., TGF- $\beta 1$ , platelet-derived growth factor, vascular endothelial growth factor, epidermal growth factor, and insulin-like growth factor).<sup>27, 28</sup> Among these factors, the TGF- $\beta 1$  suppresses reprogramming of other type of cells into endothelial cells.<sup>29</sup> A higher level of TGF- $\beta 1$  from integrin  $\beta 3$  deficient platelets adversely affected CAC regeneration (Fig. 5B). The result is consistent with our recent report that integrin  $\beta 3$  deficiency increases TGF- $\beta 1$  expression in muscle cells, impairing their capacity for muscle regeneration.<sup>30</sup> Since integrin regulates outside-in signal through cytoskeleton, and a recently report showed that Wiskott-Aldrich syndrome protein, an actin accessory protein, through CDC42 to regulate TGF- $\beta 1$  release.<sup>31</sup> So the integrin  $\beta 3$ -induced TGF- $\beta 1$  release could be associated with cytoskeleton changes.

We further show that the TGF- $\beta 1$ -induced activation of Notch signaling, may play a critical role in accelerated AVG occlusion (Fig. 6A–C). Because Notch signal maintains multipotential progenitor cells in an undifferentiated<sup>32</sup> and slows the differentiation of bone marrow-derived CAC.<sup>33, 34</sup> Increased Notch signaling in endothelial cells upregulates mesenchymal marker expression leading to endothelial barrier dysfunction in arteriovenous fistula.<sup>35</sup> TGF- $\beta 1$  is significantly increased in  $\beta 3$  null platelets and could result in enhanced

activation of Notch signaling through induction of Notch ligand Jagged1 (Fig. 6D). This finding was supported by an early report that endothelial repair is accelerated after arterial injury in B6 mice transplanted with the bone marrow cells from Notch1 knock down mice.<sup>33, 36</sup> We also find that platelet-mediated CAC differentiation was suppressed by the excess presence of TGF- $\beta$ 1, leading to delayed endothelium regeneration at sites of AVGs (Fig. 5D).

Finally, the influence of integrin  $\beta$ 3 was demonstrated by the drastic reduction in the rate of AVG failure by the transplantation of wild type bone marrow cells into integrin  $\beta$ 3<sup>-/-</sup> mice (Fig. 7). Our results differ from those found in models of arterial injury, where the inhibition of integrin  $\beta$ 3 reduces or blocks the neointima formation of vascular smooth muscle cells.<sup>37</sup> We found that deficiency of integrin  $\beta$ 3 delayed CAC regeneration, leading to thrombosis and adversely affect AVG function. Our results suggest that anti-platelet therapy in human should be careful considerate. For AVG, the  $\alpha$ IIb $\beta$ 3 based anti-platelet therapy could worsen the thrombosis due to the delayed endothelium repairmen, like the case in current animal model, in this case P2Y12-based anti-platelet therapy may be evaluated and considered.<sup>38</sup> For example, a report showed that a delayed anti-platelet therapy with clopidogrel and everolimus prevents progression of transplant arteriosclerosis in murine aortic allografts.<sup>39</sup>

It is known that in addition to a negative role of TGF  $\beta$ -signaling in CAC differentiation, other factors also regulate CAC adhesion and migration through  $\beta$ 3-integrin. Pula et al. found that proangiogenic factor thymidine phosphorylase (TP)-induced CAC migration can be blocked by RGD peptides and inhibitory antibodies to integrin  $\alpha$ V $\beta$ 3.<sup>40</sup> Without integrin  $\beta$ 3 also impairs its interaction with VEGFR2 and hypoxia-induced angiogenesis.<sup>41</sup> These results indicate that multiple factors could be involved in regulating integrin  $\beta$ 3-impaired CAC function or differentiation.

In summary, we have uncovered a novel mechanism for causing AVG occlusion and failure. In addition to poor adhesion of  $\beta$ 3 null endothelial progenitor cells to the subendothelium,  $\beta$ 3 deficiency in platelet contributes to AVG occlusion 1) by defective as a carrier for CAC homing and 2) activating Notch signaling to suppress endothelial cell differentiation. Our results, therefore, suggest that strategies that block Notch signaling or reduce TGF- $\beta$ 1 could improve the patency of an AVG.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We thank Dr. William E. Mitch for critical reading and editing of this manuscript. We thank Dr. Hongzhen Hu for helpful suggestions.

This project was supported by the Cytometry and Cell Sorting Core at Baylor College of Medicine with funding from the NIH (NIAID P30AI036211, NCI P30CA125123, and NCRR S10RR024574) and the assistance of Joel M. Sederstrom.

### Sources of Funding:

This study was supported by grants from NIH RO1DK095867 and American Heart Association Grant 10SDG2780009 (to J.C.), support from National Institutes of Health grants R37 DK37175 Grant, and a generous grant from Dr. and Mrs. Harold Selzman.

## Abbreviations

<b>AVG</b>	arteriovenous graft
<b>CAC</b>	circulating angiogenic cells
<b>NICD</b>	Notch intracellular domain
<b>DAPT</b>	N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine-t-butyl ester; $\gamma$ -secretase inhibitor

## References

1. Roger VL, Go AS, Lloyd-Jones DM, et al. on behalf of the American Heart Association Statistics Committee and Stroke Statistics S. Heart disease and stroke statistics–2011 update: A report from the American Heart Association. *Circulation*. 2011; 123:e18–e209. [PubMed: 21160056]
2. Group VAW. Clinical practice guidelines for vascular access. *Am J Kidney Dis*. 2006; 48(Suppl 1):S176–247. [PubMed: 16813989]
3. Nachman RL, Rafii S. Platelets, petechiae, and preservation of the vascular wall. *N Engl J Med*. 2008; 359:1261–1270. [PubMed: 18799560]
4. Stellos K, Gawaz M. Platelet interaction with progenitor cells: Potential implications for regenerative medicine. *Thromb Haemost*. 2007; 98:922–929. [PubMed: 18000594]
5. Massberg S, Schurzinger K, Lorenz M, Konrad I, Schulz C, Plesnila N, Kennerknecht E, Rudelius M, Sauer S, Braun S, Kremmer E, Emambokus NR, Frampton J, Gawaz M. Platelet adhesion via glycoprotein IIB integrin is critical for atheroprotection and focal cerebral ischemia: An in vivo study in mice lacking glycoprotein IIB. *Circulation*. 2005; 112:1180–1188. [PubMed: 16103235]
6. Watson AR, Pitchford SC, Reynolds LE, Direkze N, Brittan M, Alison MR, Rankin S, Wright NA, Hodivala-Dilke KM. Deficiency of bone marrow beta3-integrin enhances non-functional neovascularization. *J Pathol*. 2010; 220:435–445. [PubMed: 19967726]
7. Reynolds LE, Conti FJ, Lucas M, Grose R, Robinson S, Stone M, Saunders G, Dickson C, Hynes RO, Lacy-Hulbert A, Hodivala-Dilke K. Accelerated re-epithelialization in beta3-integrin-deficient mice is associated with enhanced TGF-beta1 signaling. *Nat Med*. 2005; 11:167–174. [PubMed: 15654327]
8. Xu Q, Zhang Z, Davison F, Hu Y. Circulating progenitor cells regenerate endothelium of vein graft atherosclerosis, which is diminished in ApoE-deficient mice. *Circ Res*. 2003; 93:e76–86. [PubMed: 14512446]
9. Langer H, May AE, Daub K, Heinzmann U, Lang P, Schumm M, Vestweber D, Massberg S, Schonberger T, Pfisterer I, Hatzopoulos AK, Gawaz M. Adherent platelets recruit and induce differentiation of murine embryonic endothelial progenitor cells to mature endothelial cells in vitro. *Circ Res*. 2006; 98:e2–10. [PubMed: 16373597]
10. Lev EI, Estrov Z, Aboulfatova K, Harris D, Granada JF, Alviar C, Kleiman NS, Dong JF. Potential role of activated platelets in homing of human endothelial progenitor cells to subendothelial matrix. *Thromb Haemost*. 2006; 96:498–504. [PubMed: 17003929]
11. Massberg S, Konrad I, Schurzinger K, Lorenz M, Schneider S, Zohlnhoefer D, Hoppe K, Schiemann M, Kennerknecht E, Sauer S, Schulz C, Kerstan S, Rudelius M, Seidl S, Sorge F, Langer H, Peluso M, Goyal P, Vestweber D, Emambokus NR, Busch DH, Frampton J, Gawaz M. Platelets secrete stromal cell-derived factor 1alpha and recruit bone marrow-derived progenitor cells to arterial thrombi in vivo. *J Exp Med*. 2006; 203:1221–1233. [PubMed: 16618794]
12. Zavadil J, Cermak L, Soto-Nieves N, Bottinger EP. Integration of TGF-beta/Smad and Jagged1/Notch signalling in epithelial-to-mesenchymal transition. *EMBO J*. 2004; 23:1155–1165. [PubMed: 14976548]



13. Loscalzo J. Vascular matrix and vein graft failure: Is the message in the medium? *Circulation*. 2000; 101:221–223. [PubMed: 10645913]
14. Cooley BC, Nevado J, Mellad J, Yang D, St Hilaire C, Negro A, Fang F, Chen G, San H, Walts AD, Schwartzbeck RL, Taylor B, Lanzer JD, Wragg A, Elagha A, Beltran LE, Berry C, Feil R, Virmani R, Ladich E, Kovacic JC, Boehm M. Tgf-beta signaling mediates endothelial-to-mesenchymal transition (endmt) during vein graft remodeling. *Sci Transl Med*. 2014; 6:227ra234.
15. Hu Y, Davison F, Zhang Z, Xu Q. Endothelial replacement and angiogenesis in arteriosclerotic lesions of allografts are contributed by circulating progenitor cells. *Circulation*. 2003; 108:3122–3127. [PubMed: 14656919]
16. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. *Science*. 1997; 275:964–966. [PubMed: 9020076]
17. Kawamoto A, Losordo DW. Endothelial progenitor cells for cardiovascular regeneration. *Trends Cardiovasc Med*. 2008; 18:33–37. [PubMed: 18206807]
18. Walter DH, Rittig K, Bahlmann FH, Kirchmair R, Silver M, Murayama T, Nishimura H, Losordo DW, Asahara T, Isner JM. Statin therapy accelerates reendothelialization: A novel effect involving mobilization and incorporation of bone marrow-derived endothelial progenitor cells. *Circulation*. 2002; 105:3017–3024. [PubMed: 12081997]
19. Werner N, Junk S, Laufs U, Link A, Walenta K, Bohm M, Nickenig G. Intravenous transfusion of endothelial progenitor cells reduces neointima formation after vascular injury. *Circ Res*. 2003; 93:e17–24. [PubMed: 12829619]
20. Feng W, McCabe NP, Mahabeleshwar GH, Somanath PR, Phillips DR, Byzova TV. The angiogenic response is dictated by beta3 integrin on bone marrow-derived cells. *J Cell Biol*. 2008; 183:1145–1157. [PubMed: 19075116]
21. Daub K, Langer H, Seizer P, Stellos K, May AE, Goyal P, Bigalke B, Schonberger T, Geisler T, Siegel-Axel D, Oostendorp RA, Lindemann S, Gawaz M. Platelets induce differentiation of human cd34+ progenitor cells into foam cells and endothelial cells. *Faseb J*. 2006; 20:2559–2561. [PubMed: 17077283]
22. Hidalgo A, Weiss LA, Frenette PS. Functional selectin ligands mediating human cd34(+) cell interactions with bone marrow endothelium are enhanced postnatally. *J Clin Invest*. 2002; 110:559–569. [PubMed: 12189250]
23. Cheng J, Wang Y, Liang A, Jia L, Du J. Fsp-1 silencing in bone marrow cells suppresses neointima formation in vein graft. *Circ Res*. 2012; 110:230–240. [PubMed: 22116816]
24. Hodivala-Dilke KM, McHugh KP, Tsakiris DA, Rayburn H, Crowley D, Ullman-Cullere M, Ross FP, Coller BS, Teitelbaum S, Hynes RO. Beta3-integrin-deficient mice are a model for glanzmann thrombasthenia showing placental defects and reduced survival. *J Clin Invest*. 1999; 103:229–238. [PubMed: 9916135]
25. Prokopi M, Pula G, Mayr U, Devue C, Gallagher J, Xiao Q, Boulanger CM, Westwood N, Urbich C, Willeit J, Steiner M, Breuss J, Xu Q, Kiechl S, Mayr M. Proteomic analysis reveals presence of platelet microparticles in endothelial progenitor cell cultures. *Blood*. 2009; 114:723–732. [PubMed: 19369228]
26. de Boer HC, Verseyden C, Ulfman LH, Zwaginga JJ, Bot I, Biessen EA, Rabelink TJ, van Zonneveld AJ. Fibrin and activated platelets cooperatively guide stem cells to a vascular injury and promote differentiation towards an endothelial cell phenotype. *Arterioscler Thromb Vasc Biol*. 2006; 26:1653–1659. [PubMed: 16627804]
27. Gawaz M, Langer H, May AE. Platelets in inflammation and atherogenesis. *J Clin Invest*. 2005; 115:3378–3384. [PubMed: 16322783]
28. Spencer EM, Tokunaga A, Hunt TK. Insulin-like growth factor binding protein-3 is present in the alpha-granules of platelets. *Endocrinology*. 1993; 132:996–1001. [PubMed: 7679986]
29. Ginsberg M, James D, Ding BS, Nolan D, Geng F, Butler JM, Schachterle W, Pulijaal VR, Mathew S, Chasen ST, Xiang J, Rosenwaks Z, Shido K, Elemento O, Rabbany SY, Rafii S. Efficient direct reprogramming of mature amniotic cells into endothelial cells by ets factors and tgf-beta suppression. *Cell*. 2012; 151:559–575. [PubMed: 23084400]

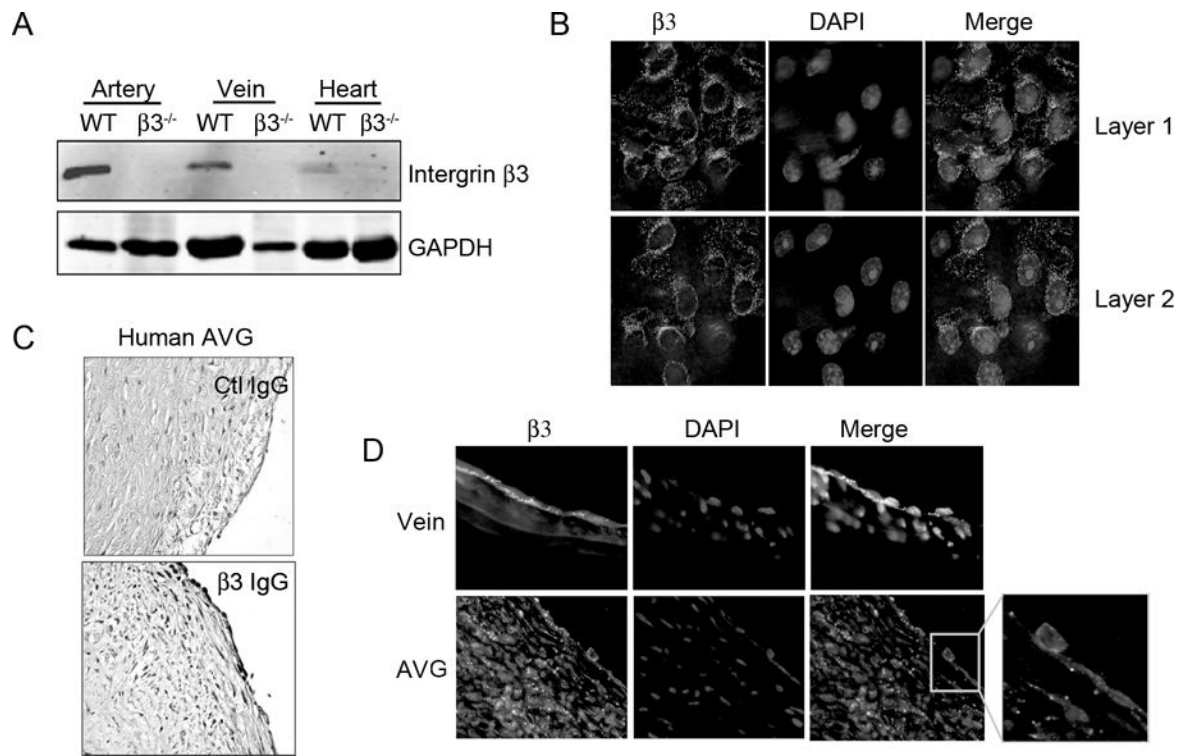
30. Zhang L, Dong Y, Dong Y, Cheng J, Du J. Role of integrin-beta3 protein in macrophage polarization and regeneration of injured muscle. *J Biol Chem.* 2012; 287:6177–6186. [PubMed: 22210777]
31. Kim H, Falet H, Hoffmeister KM, Hartwig JH. Wiskott-aldrich syndrome protein (wasp) controls the delivery of platelet transforming growth factor-beta1. *J Biol Chem.* 2013; 288:34352–34363. [PubMed: 24133214]
32. Hurlbut GD, Kankel MW, Lake RJ, Artavanis-Tsakonas S. Crossing paths with notch in the hyper-network. *Current opinion in cell biology.* 2007; 19:166–175. [PubMed: 17317139]
33. Ii M, Takeshita K, Ibusuki K, Luedemann C, Wecker A, Eaton E, Thorne T, Asahara T, Liao JK, Losordo DW. Notch signaling regulates endothelial progenitor cell activity during recovery from arterial injury in hypercholesterolemic mice. *Circulation.* 2010; 121:1104–1112. [PubMed: 20176991]
34. Kwon SM, Eguchi M, Wada M, Iwami Y, Hozumi K, Iwaguro H, Masuda H, Kawamoto A, Asahara T. Specific jagged-1 signal from bone marrow microenvironment is required for endothelial progenitor cell development for neovascularization. *Circulation.* 2008; 118:157–165. [PubMed: 18591437]
35. Wang Y, Liang A, Luo J, Liang M, Han G, Mitch WE, Cheng J. Blocking notch in endothelial cells prevents arteriovenous fistula failure despite ckd. *J Am Soc Nephrol.* 2014; 25:773–783. [PubMed: 24480830]
36. Vooijs M, Ong CT, Hadland B, Huppert S, Liu Z, Korving J, van den Born M, Stappenbeck T, Wu Y, Clevers H, Kopan R. Mapping the consequence of notch1 proteolysis in vivo with nip-cre. *Development.* 2007; 134:535–544. [PubMed: 17215306]
37. Choi ET, Khan MF, Leidenfrost JE, Collins ET, Boc KP, Villa BR, Novack DV, Parks WC, Abendschein DR. {beta}3-integrin mediates smooth muscle cell accumulation in neointima after carotid ligation in mice. *Circulation.* 2004; 109:1564–1569. [PubMed: 15007005]
38. Hollopeter G, Jantzen H-M, Vincent D, Li G, England L, Ramakrishnan V, Yang R-B, Nurden P, Nurden A, Julius D, Conley PB. Identification of the platelet adp receptor targeted by antithrombotic drugs. *Nature.* 2001; 409:202–207. [PubMed: 11196645]
39. Heim C, Eckl S, Preidl R, Ramsperger-Gleixner M, Koch N, Goldmann K, Spriewald BM, Weyand M, Ensminger SM. Delayed therapy with clopidogrel and everolimus prevents progression of transplant arteriosclerosis and impairs humoral alloimmunity in murine aortic allografts. *European journal of cardio-thoracic surgery: official journal of the European Association for Cardio-thoracic Surgery.* 2014
40. Puri TS, Shakaib MI, Chang A, Mathew L, Olayinka O, Minto AWM, Sarav M, Hack BK, Quigg RJ. Chronic kidney disease induced in mice by reversible unilateral ureteral obstruction is dependent on genetic background. *Am J Physiol Renal Physiol.* 2010; 298:F1024–F1032. [PubMed: 20089676]
41. Di Q, Cheng Z, Kim W, Liu Z, Song H, Li X, Nan Y, Wang C, Cheng X. Impaired cross-activation of beta3 integrin and vegfr-2 on endothelial progenitor cells with aging decreases angiogenesis in response to hypoxia. *Int J Cardiol.* 2013; 168:2167–2176. [PubMed: 23452889]

**Key Points**

1. Integrin  $\beta 3^{-/-}$  causes failure of arteriovenous graft.
2. Dysfunction of platelets in integrin  $\beta 3^{-/-}$  mice suppresses homing and differentiation of endothelial progenitor cells into endothelial cells in arteriovenous graft.

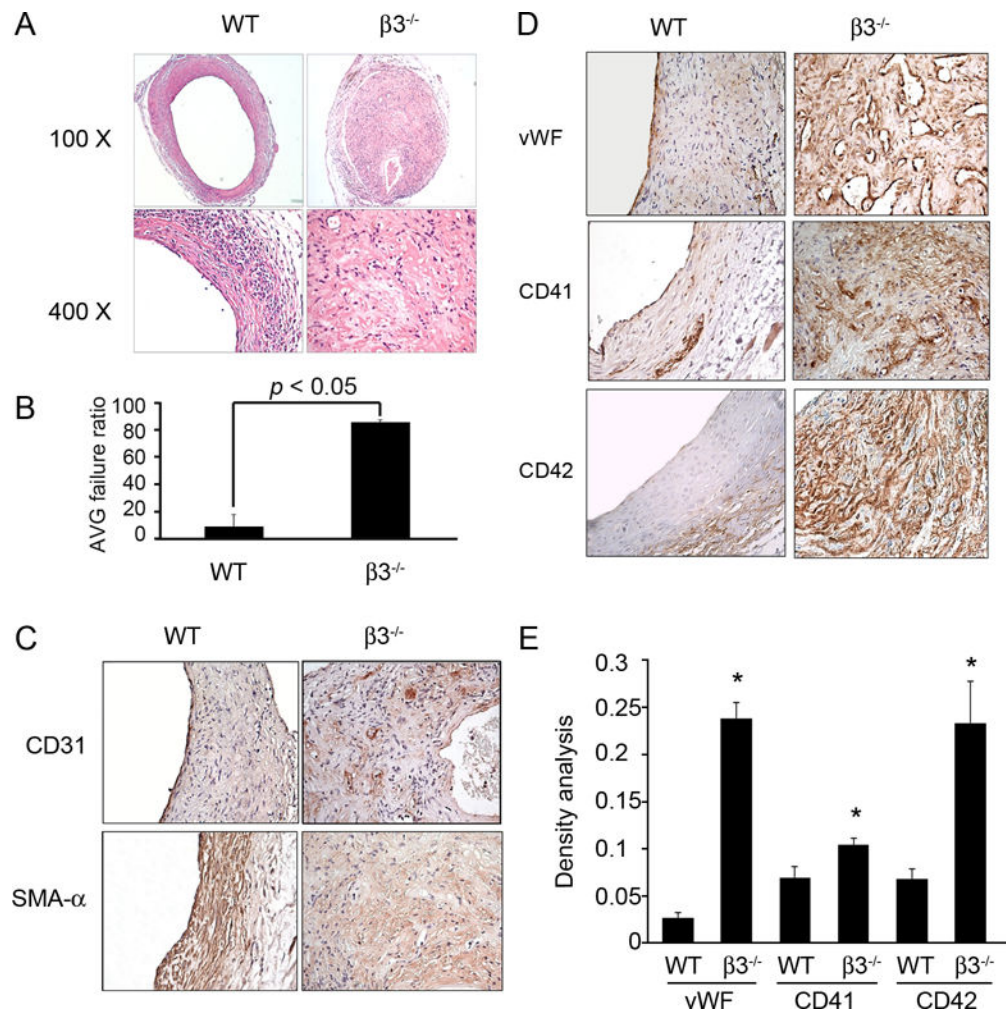
### Significance

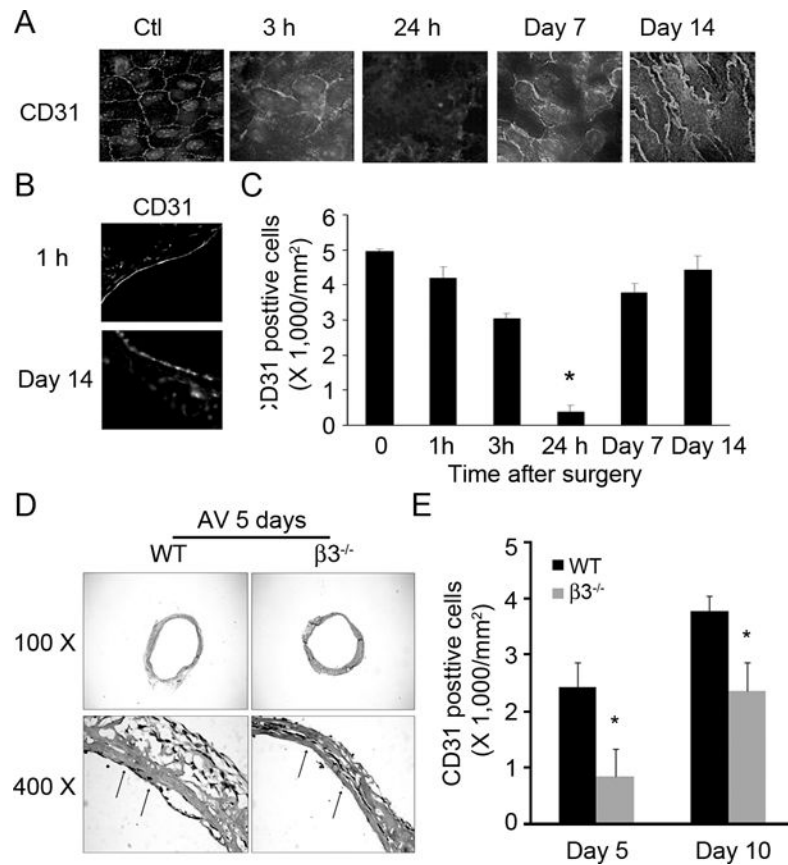
Neointima formation is associated with stenosis and subsequent thrombosis in arteriovenous grafts. Integrin  $\beta 3$  has been reported to regulate neointima formation in artery injury model. In a mouse model of arteriovenous graft, we found that deficiency of integrin  $\beta 3$  suppresses homing and differentiation of endothelial progenitor cells into mature endothelial cells in arteriovenous grafts, delays endothelial regeneration. These responses result in thrombosis and failure of arteriovenous graft. The increased TGF- $\beta 1$  in platelets and Notch1 signaling are the underlying molecular mechanisms that cause accelerated failure of the grafts in integrin  $\beta 3$  deficient mice. Wild type bone marrow transplantation can rescue the arteriovenous graft patency in integrin  $\beta 3$  deficient mice. Our results suggest that boosting integrin  $\beta 3$  function in the endothelial cells and platelets could prevent neointima and thrombosis in AVGs.



**Figure 1. Integrin  $\beta 3$  expression in arteriovenous graft (AVG)**

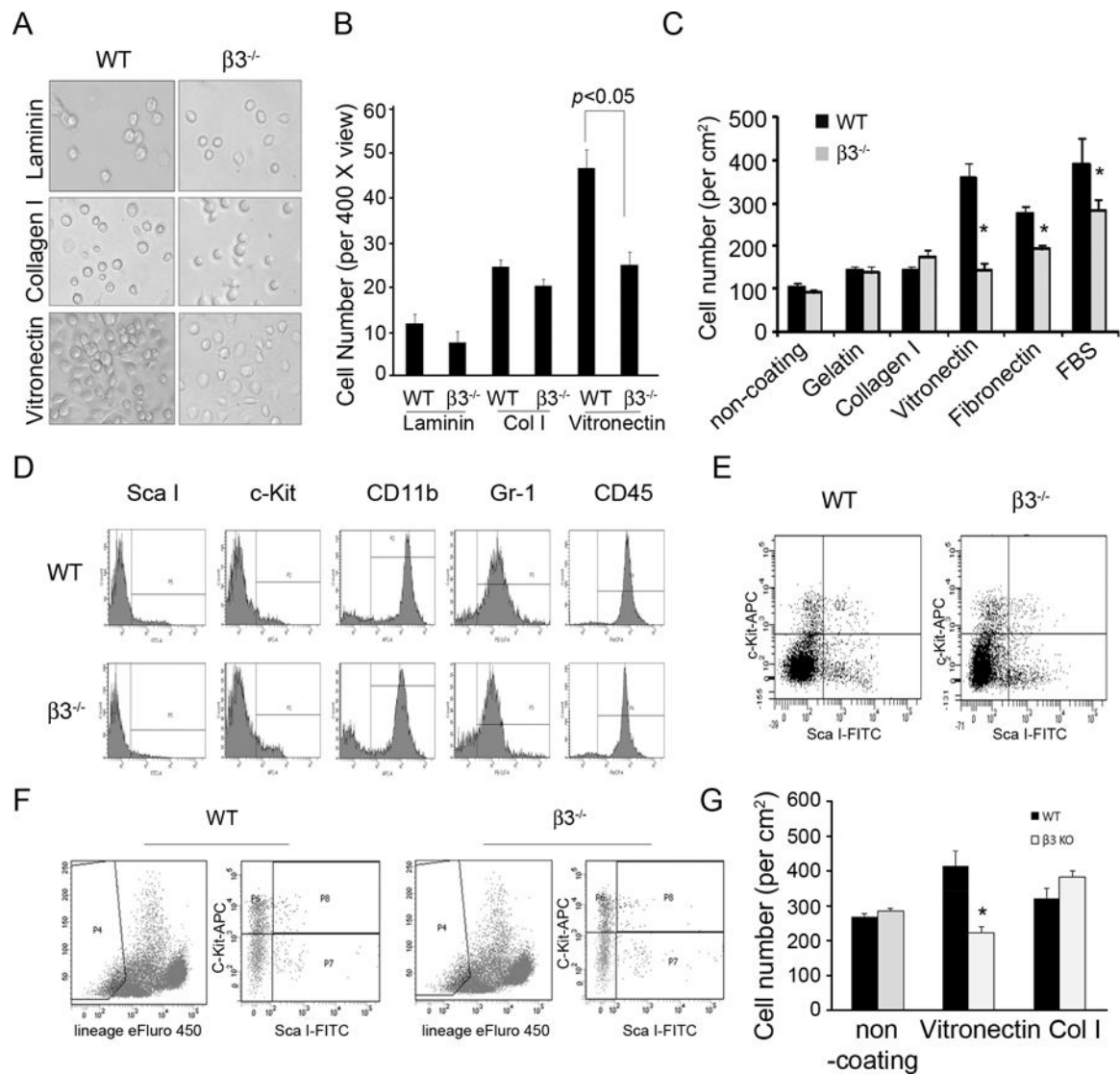
A. Integrin  $\beta 3$  expression in veins and arteries was detected by Western blot. The experiment was repeated for 3 times. B. Enface analysis of integrin  $\beta 3$  in vena cava. C. In AVGs from patient, immunostaining reveals integrin  $\beta 3$ . D. In endothelial cells in a mouse AVG integrin  $\beta 3$  expression was decreased, representative data from 5 AVGs.





**Figure 3. Integrin  $\beta 3$  KO delays endothelial regeneration in AVGs**

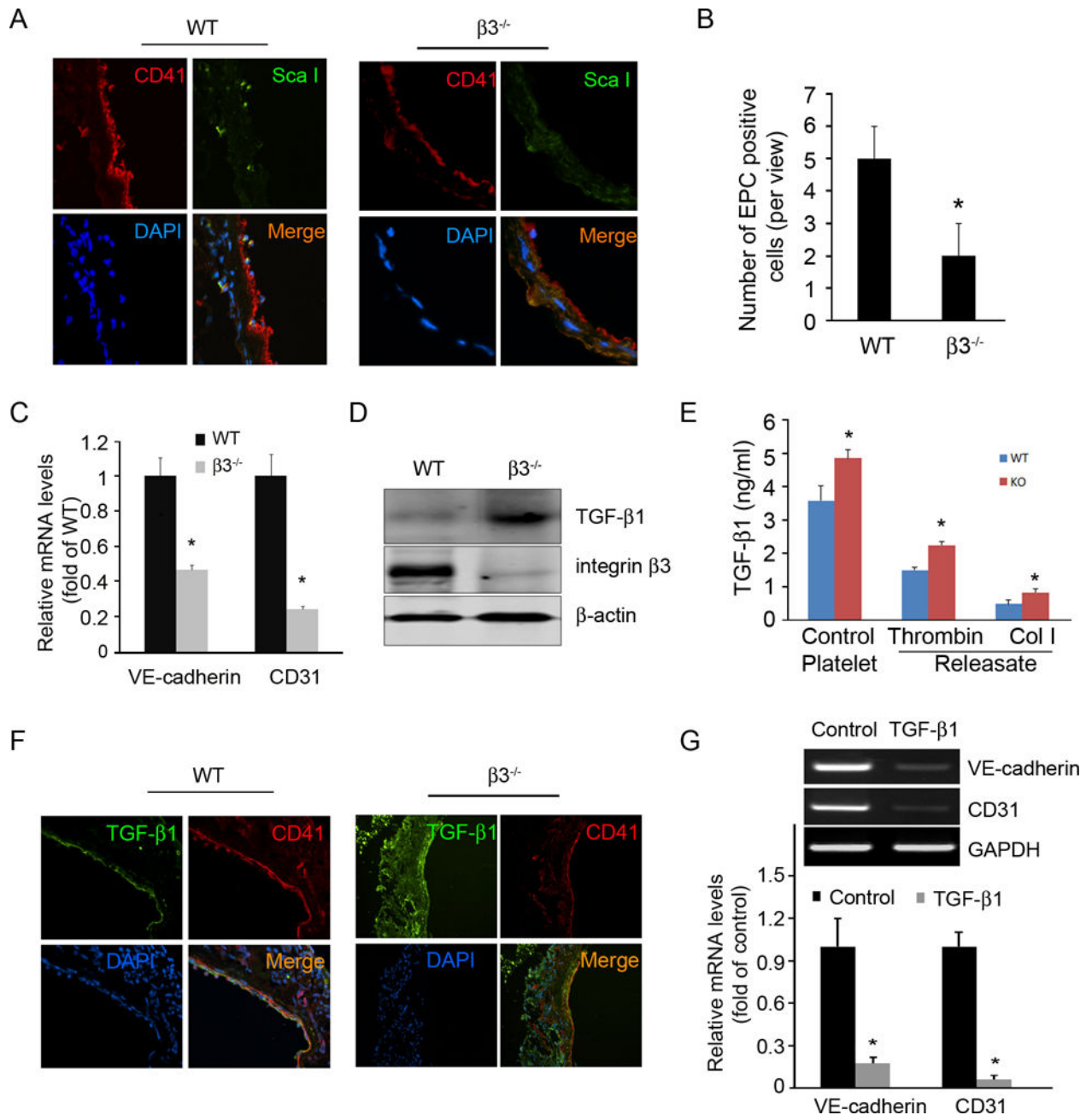
A. AVGs collected at different time points were examined by a deconvolution microscope after staining with the endothelial marker, CD31. B. Endothelial markers were present in cross sections of AVGs collected at different times from wild type mice. C. Statistical analysis of endothelial cell numbers shown in panel A. D. AVGs created in wild type and integrin  $\beta 3^{-/-}$  mice were collected after 5 days and examined by H & E staining. E. Statistical analysis of the endothelium in AVGs from wild type and integrin  $\beta 3^{-/-}$  mice after enface analysis, confirmed that the absence of integrin  $\beta 3$  delays regeneration. Representative data were from at least 3 mice.



**Figure 4. Integrin  $\beta 3^{-/-}$  inhibits CAC adhesion**

A and B. After 7 days, bone marrow cells from integrin  $\beta 3^{-/-}$  have limited attachment to extracellular matrices. C. Sca I and c-Kit positive CACs were isolated from bone marrow of wild type mice and the purity was confirmed. The purified cells from bone marrow and integrin  $\beta 3^{-/-}$  mice were seeded ( $\sim 1 \times 10^4$ ) on 48-well plates coated with varied extracellular cellular matrices. After 7 days culture, cell numbers were counted. D and E. Bone marrow cells from wild type and integrin  $\beta 3^{-/-}$  mice were labeled with different antibodies and subjected to flow cytometry. Monocyte (CD45), macrophage (CD11b), neutrophil (Gr-1) populations and Sca I/c-Kit double positive CACs were not different between the two types of mice. F and G. The CACs from lineage negative population in bone marrow cells were isolated from wild type and integrin  $\beta 3^{-/-}$  mice (F). The lineage negative population was gated for isolation of Sca I<sup>+</sup>/c-Kit<sup>+</sup> CACs and the adhesion analysis was performed (G). The data shown are representative of 2 experiments. \*,  $p < 0.05$  compared with WT.





**Figure 5. Integrin  $\beta 3^{-/-}$  affects CAC homing and differentiation**

A. Detection of CD41<sup>+</sup> platelets and Sca I<sup>+</sup> CACs in AVGs in wild type and integrin  $\beta 3$  KO mice. B. Statistical analysis of Sca I<sup>+</sup> cell accumulation in AVGs. n = 4 mice from both wild type and integrin  $\beta 3$  KO mice. C. Platelets isolated from wild type and integrin  $\beta 3$  KO mice were cocultured with CACs for 7 days. Endothelial markers (real time RT-PCR) revealed that platelets promote CAC differentiation. This was more prominent with wild type platelets. D. Integrin  $\beta 3$  null platelets express excess TGF- $\beta 1$  detected by western blot. E. Washed platelets isolated from wild type and integrin  $\beta 3$  KO mice were stimulated with 0.125 U/ml thrombin or 5  $\mu$ g/ml collagen I at 37°C for 5 minutes and centrifuged at 14 000 g for 20 minutes. The TGF- $\beta 1$  level in the platelets and releasates was measured by ELISA

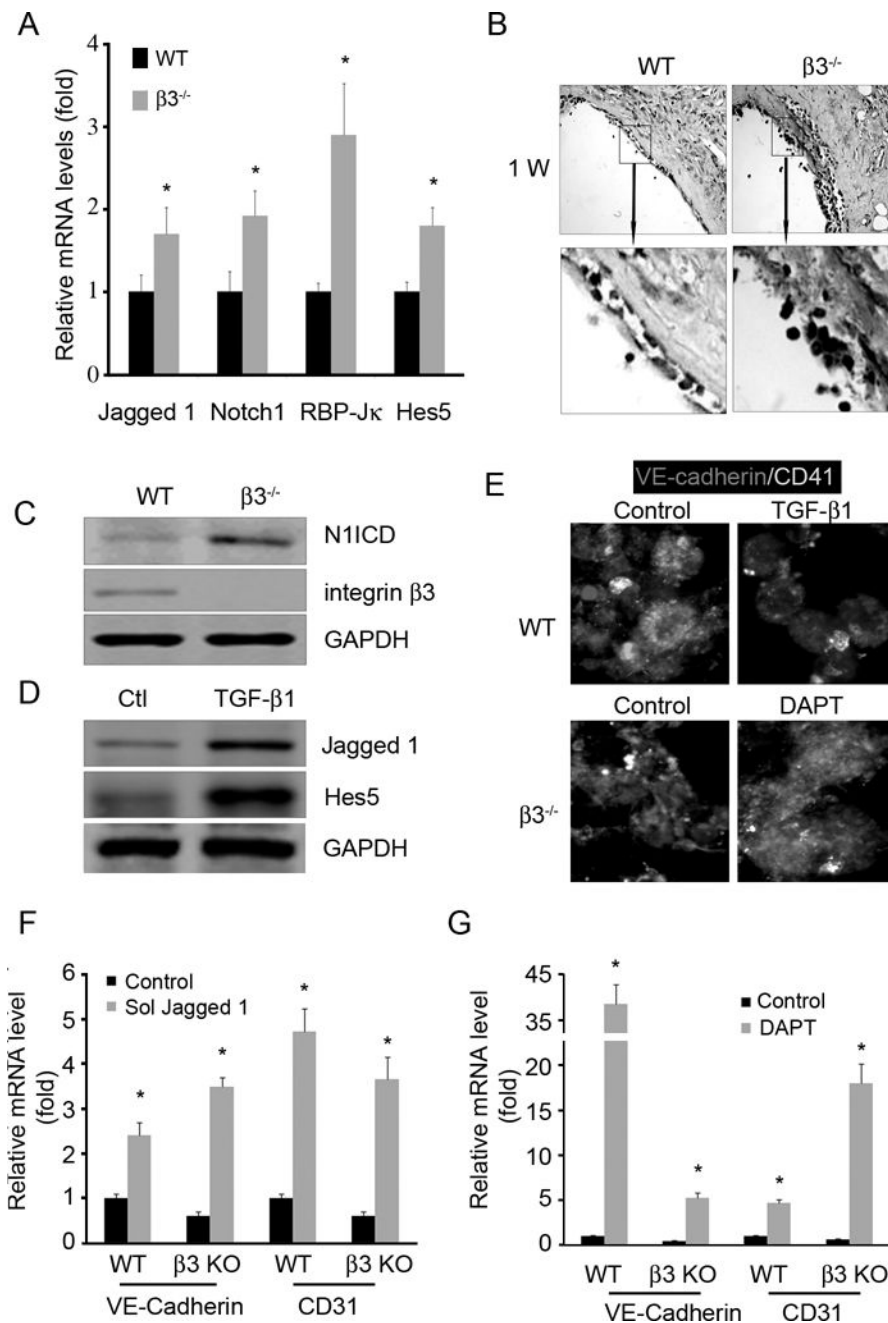
after acidification (n=3). F. In vivo, prominent TGF- $\beta$ 1 expression in AVGs from integrin  $\beta$ 3 KO mice colocalized with platelets. G. CACs isolated from bone marrow and cultured in EGM2 with or without incubation with TGF- $\beta$ 1 (3 ng/ml) for 7 days. Excess TGF- $\beta$ 1 inhibits express mature endothelial markers were detected by real time RT-PCR. Representative data are present from 3 independent experiments. \*,  $p < 0.05$  compared with WT.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript



**Figure 6. Integrin  $\beta 3$  KO inhibits CAC differentiation by increasing Notch signaling**

A and B. AVGs were collected from wild type and integrin  $\beta 3$  KO mice and subjected to real time RT-PCR (A), immunostaining of activated Notch, N1ICD, reveal an increase in Notch signaling in AVGs created in integrin  $\beta 3^{-/-}$  mice (B). C. Western blot confirmed an increase in Notch1 cleavage in AVGs from integrin  $\beta 3$  KO mice. D. CACs were treated with TGF- $\beta 1$  revealed an increase in Notch ligand and its target (Western blot). E–G. If Notch signaling is inhibited, integrin  $\beta 3$ -null platelets will improve CAC differentiation. Bone marrow-derived CACs were cocultured with wild type or integrin  $\beta 3$ -null platelets plus TGF- $\beta 1$  or DAPT. Markers of endothelial cells and platelets were detected by

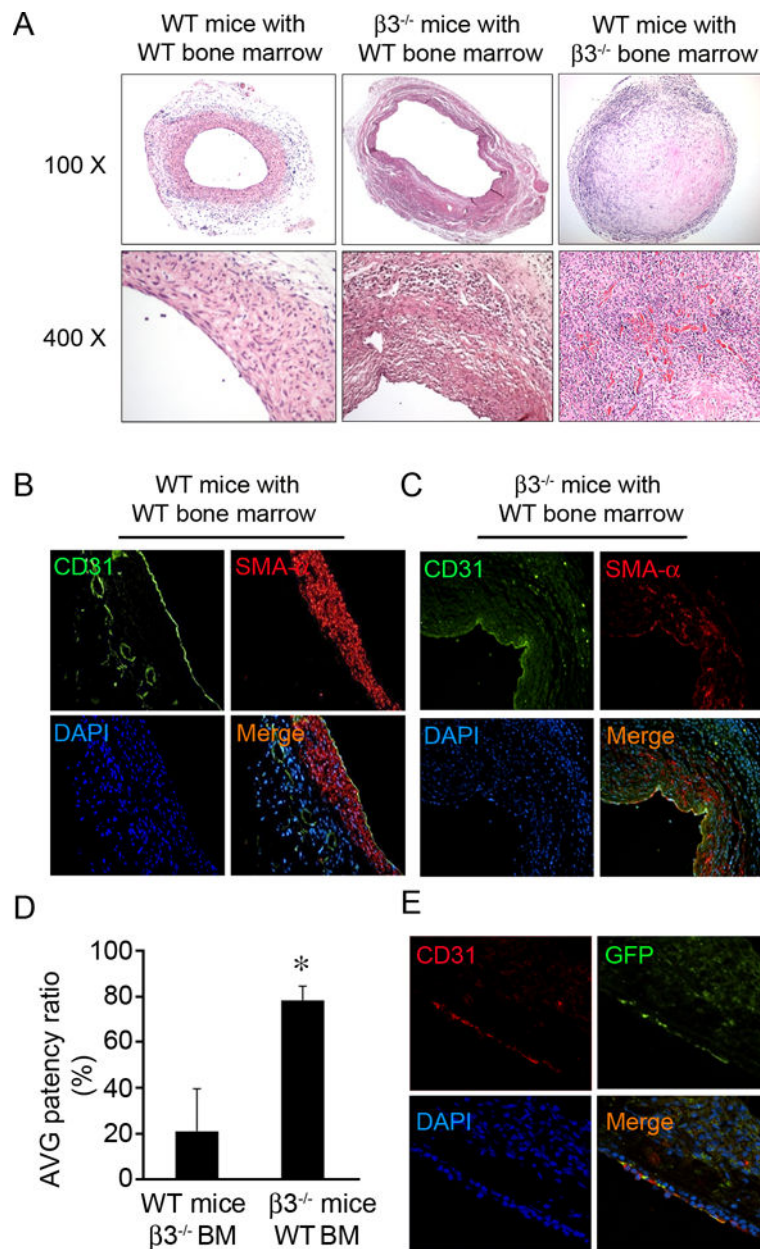
immunostaining, demonstrating that integrin  $\beta 3$  null platelets suppress CAC differentiation. This occurs via Notch activation (E). To inhibit Notch, CACs were infected with an adenovirus expressing soluble Jagged 1 (F) or with the DAPT (G), an increase in endothelial markers (VE-cadherin and CD31) were detected by real time RT-PCR followed inhibition of Notch, \*,  $p < 0.05$ , compared with control.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript



**Figure 7. AVG failure in integrin  $\beta 3$  KO was rescued by wild type bone marrow cells**

A. H & E staining of AVGs show the increased patency in integrin  $\beta 3$  KO mice. Each group had at least 6 mice for AVG surgery. B and C. Endothelial cell markers in AVGs of integrin  $\beta 3$  KO mice increased after transplantation with wild type bone marrow. D. Statistical analysis of the effect of bone marrow transplantation on AVG patency.  $n = 7$ , \*, compared with control WT mice,  $p < 0.01$ . E. Integrin  $\beta 3$  KO mice transplanted bone marrow cells from GFP transgenic mice demonstrate that cells from the transplanted bone marrow do contribute to regeneration of endothelial cells in an AVG. The GFP staining was detected after 5 days of AVGs. The endothelial marker and GFP costaining were shown.