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Age-Related Notch-4 Quiescence Is Associated with Altered Wall Remodeling During Vein Graft Adaptation

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Abstract

Background—The link of aging to specific mechanisms of vascular biology is not well understood. We have previously shown that aging is associated with increased vein graft wall thickness and that this process involves the VEGF-Delta/Notch-ephrin/Eph cascade. Therefore we examined whether Dll-4 or Notch-4 are differentially expressed, according to age, during vein graft adaptation.

Materials and Methods—Vein grafts were performed in 6-month and 24-month Fischer 344 rats. Gene expression was analyzed by quantitative real-time PCR, and the distribution of Dll-4 and Notch-4 was observed by immunofluorescence.

Results—The expression of Dll-4 and Notch-4 was reduced in vein grafts performed in aged rats compared to the expression in young adult rats. Both Dll-4 and Notch-4 were distributed in vein graft endothelium as well as the outer adventitia, with reduced amounts in the outer adventitia of aged vein grafts. Aged veins had reduced eNOS membrane targeting and colocalization with caveolin-1 as well as reduced eNOS protein expression in comparison to young adult veins. In an exchange model between young and aged animals, heterogeneous vein grafts (Yo^{Ag} and Ag^{Yo}) showed significantly thicker neointima compared to young (Yo^{Yo}) controls, and had Notch-4-positive cells, but not Dll-4-positive cells, diminished in the adventitia. Vein grafts that were air-denuded of endothelium did not show any adaptation to the arterial environment and also lacked both Dll-4 and Notch-4 expression at 3 weeks.

Conclusions—During vein graft adaptation to the arterial environment, both Dll-4 and Notch-4 expression are down-regulated in an aged, but not a young, background. Loss of Notch-4 is associated with loss of attenuation of neointima. The delta-Notch signaling pathway may be active during vein graft adaptation.

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Keywords

Notch-4; Delta like ligand-4; Vein graft adaptation; Aging

Aging is a well known risk factor for development of atherosclerotic vascular disease. The United States Administration on Aging reported that in 2009, 12.8% of the population was 65 years or older (40 million people) and the percentage of elderly people is expected to grow to 19.3% of the population in 2030 (72.1 million people) [1]. Therefore, an understanding of the effects of aging on the surgical management of vascular disease is required to improve the quality of life for this growing population of elderly patients.

Vein is the gold standard and most commonly used conduit for vascular reconstructive surgery. Vein grafts offer longer term patency compared to radial artery grafts in coronary artery bypass surgery, and compared to prosthetic grafts in lower limb arterial bypass surgery [2–4]. Veins demonstrate wall remodeling when transplanted from their normal venous milieu to an arterial environment, a process called vein graft adaptation [5, 6]. Vein graft adaptation, with smooth muscle cell accumulation and extracellular matrix deposition, is typically known as “neointimal hyperplasia”, and is part of the vein’s remodeling response to the high wall stretch force and shear stress found in the arterial circulation [6, 7]. Since excessive neointimal hyperplasia is responsible for approximately 50% of vein graft failures [8], the regulation of graft wall thickening – so that vein graft adaptation achieves the optimal wall thickness – is a critical mechanism to understand in order to determine targets that can potentially improve vein graft durability.

Aging is known to influence the process of vessel wall remodeling in response to injury or transplantation, as well as the ultimate clinical outcomes of vein grafts [9–12]. Recently, we have shown that aging causes increased vein graft wall inward remodeling and that this inward remodeling involves the VEGF - Delta/Notch - ephrin/Eph cascade [5]. This cascade is known to determine arterial and venous fate during embryonic vascular development [13, 14]. In embryonic tissues destined to become arteries, the transcription factor sonic hedgehog induces expression of VEGF, which subsequently stimulates the Delta/Notch signaling pathway. Delta/Notch continues to stimulate the arterial fate pathway in developing cells by causing increased ephrinB2 expression with simultaneous suppression of EphB4 expression, i.e. prevention of venous fate determination [15, 16]. In contrast to arterial development, in embryonic tissues destined to become veins, the transcription factor COUP-TFII down-regulates VEGF signaling, preventing Delta/Notch pathway activation, thereby relieving distal suppression to increase EphB4 expression without stimulation of ephrinB2 expression [13, 17].

Notch is a membrane receptor with 4 homologues in mammals, and is known to be involved in cell fate assignment and pattern formation during embryonic development in the central nervous system, somitogenesis, the endocrine system, immune cells, and cardiovascular development [18]. Notch is activated by the Delta, Serrate, and Lag-2 families of Notch ligands. After activation, Notch can be cleaved to its intracellular domain (NICD) by ADAM10, TACE and γ-secretase, allowing the NICD to become capable of target gene activation [19]. We have previously shown that vein grafts have increased neointimal hyperplasia in aged rats compared to little amounts that develop in young adult rats [5]. We hypothesized that the increased amounts of neointimal hyperplasia in aged rats is due to a defect in Delta-Notch signaling compared to signaling present in young adults rats. Therefore we examined the expression of Delta like ligand-4 (Dll-4) and Notch homologue-4 (Notch-4) in vein grafts implanted into young hosts, compared to those implanted in aged hosts. In addition, we examined the contribution of vein graft endothelial

cells (EC) to Dll-4/Notch-4 expression in order to examine whether quiescent Dll-4/Notch-4 expression is consistent with effects of aging on EC.

Materials and Methods

Animals

All experiments were approved by the Institutional Animal Care and Use Committee at Yale University School of Medicine.

6 month old (young adult) and 24 month old (aged) Fischer 344 rats were obtained from the National Institute of Aging (NIA) Rodent Colony (Bethesda, MD). Rats were raised in pathogen-free conditions at the NIA and housed locally at least 1 week prior to surgery. Rat vein graft surgery was performed as previously described [5]. Briefly, the right external jugular vein was exposed and harvested, and re-implanted into the right common carotid artery as an interposition graft under the general anesthesia. Arterial-graft anastomoses were performed with running 10-0 monofilament nylon suture. Vein grafts were harvested and examined 3 weeks postoperatively. After exsanguination under anesthesia, vein grafts were flushed with heparinized saline followed with pressure perfusion fixation with 10% formalin prior to excision and histological analysis. Control allografts (Yo^{Yo} and Ag^{Ag}) were constructed from different animals for vein harvest and subsequent vein graft implantation.

Endothelial denudation of vein grafts

Air denudation of the EC from the pre-implantion vein was performed as previously described [20]. After exposure of the external jugular vein, the proximal and distal ends, as well as the major side branches, were ligated with silk suture. All small branches were ligated completely to avoid air embolism. A pin hole for egress of air flow was made on the jugular vein distal end using a 30G needle, and a 30G butterfly needle for inflow of air was inserted from a major side branch of the vein (Figure 8A). Air was injected (15 ml/min) into the vein for 4 minutes, and injured EC, as well as infused air, was subsequently removed by saline flush via the 30G butterfly needle. The external jugular vein was harvested and implanted into the common carotid artery as above.

Real-time reverse transcription PCR

The harvested graft was immediately processed by snap freezing with liquid nitrogen, and transferred to −80°C storage. Individual grafts were analyzed individually without pooling. Total RNA was extracted by TRIzol Reagent (Invitrogen, Carlsbad, CA), and RNA clean up was performed with RNeasy mini-kit with DNase-I (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. Total RNA concentration and purity were measured by spectrophotometer with absorbance at 260 nm. RNA quality was confirmed by 260/280 nm absorbance ratio.

First strand cDNA was synthesized by Superscript III reverse transcriptase (Invitrogen). Quantitative PCR was performed with SYBR Green master mix (Bio-Rad). Primers for genes of interest and an internal standard (GAPDH) were used as previous described (Table) [21–24]. Amplification specificity was confirmed by 2% agarose gel electrophoresis, and primer efficiencies were determined by melting curve analysis. All data were normalized by GAPDH. The mean difference between aged and young transcript numbers was plotted in Figure 1A.

In situ whole mount staining

The vein graft, and associated vein, was harvested and fixed with cooled 4% paraformaldehyde, subsequently permeabilized with 0.3% Triton X-100 PBS, and blocked

with 3% normal goat serum containing PBS. The arteries were probed with rabbit anti-eNOS antibody, mouse anti-caveolin-1 antibody and/or mouse anti-GP130 antibody (BD Transduction Laboratories). Alexa Fluor 488 anti–rabbit IgG and 568 anti–mouse IgG (Invitrogen) were used as secondary antibodies. Stained veins and vein grafts were opened under the dissection microscope, and immediately captured images were viewed and saved with an Axioimager A1 (Carl Zeiss) under identical conditions.

Histological analysis

The harvested vein grafts were fixed in 10% formalin. Fixed tissues were embedded in paraffin; sections were taken from the mid-point of the graft. All sections were stained with Hematoxylin-Eosin (H&E) and Masson Tricrome (MT) for graft morphological analysis. For immunoflorescence staining, all sections were treated with Proteinase K solution for antigen retrieval, and incubated with primary antibody to protein of interest: Notch-4 goat polyclonal IgG, Dll-4 goat polyclonal IgG (N-19) (Santa Cruz Biotechnology, Santa Cruz, CA), Monoclonal Anti Alpha Smooth Muscle Actin (Sigma Aldrich, St. Louis, MO). Alexa Fluor-488 and -568 conjugated secondary antibodies (Invitrogen) were used for fluorescent labeling. All immunofluorescence sections were treated by Autofluorescent Eliminator Reagent (Millipore, Billerica, MA), and were mounted with SlowFade Gold antifade reagent with DAPI (Invitrogen) for cell counting. Images were captured by Axioimager A1 (Carl Zeiss, Thornwood, NY) under identical conditions. Morphological analysis and cell counts were measured by Image J software (NIH). Intimal thickening was measured in the combined intima and medial layers, as rats only have a single external elastic lamina [5]. Cells with a positive signal were counted per high power field, and this was repeated ten times for each sample.

Western Blotting

Jugular veins from young adult and aged animals were suspended in lysis buffer containing protease inhibitors. After mild sonication, each sample had the protein concentration determined using the Protein Assay Reagent (Bio-Rad Labotratories. Hercules, CA). For denaturing, samples were boiled with 5% beta-mercaptoethanol contained in Laemmli's sample buffer. Samples were applied equally protein amount on poly-acrylamide gels. Proteins were transferred to a nitrocellulose membrane and blocked with either BSA or casein (Bio-Rad Labotratories), and then probed with the first antibodies. Detection was performed with ECL detection reagent (GE Healthcare Life Sciences, Piscataway, NJ) using HRP conjugated antibodies (Vector), or Alexa Fluor 680 (Invitrogen, Carlsbad, CA) and IRDye 800 (Rockland, Gilbertsville, PA) conjugated antibodies with the Odyssey Infrared Imaging System (Li-Cor Bioscience, Lincolin, NE).

Statistical Analysis

Statistical analysis was carried out with StatView-J version 5.0. Statistical significance was determined using the analysis of variance (ANOVA). Post-hoc test were analyzed by Fisher's PSLD test. All values are expressed as mean ± standard error and value of $p < 0.05$ was considered to be statistically significant.

Results

Abnormal Dll-4 and Notch-4 gene expression in vein grafts in aged rats

Since aging influences the VEGF - Dll/Notch - ephrin/Eph cascade [5], we hypothesized that some aspect of this pathway is abnormal during vein graft adaptation in aged animals. The gene expression patterns of important members of this cascade were determined in young and aged autologous rat vein grafts. As shown in Figure 1A, the transcription level of

VEGF and its receptors did not show a significant difference between young adult and aged vein grafts after 3 weeks of adaptation, the time at which a mature neointimal layer is present. In contrast, Dll-4 mRNA demonstrated significantly low expression in vein grafts when implanted in aged animals, compared to the expression present in young adult vein grafts (Figure 1A, 6th lane). Other Notch ligands, Jagged-1 and -2, did not show significant differences between young and aged vein grafts. Interestingly, Notch-4 showed specifically significantly decreased expression in aged compared to young adult grafts, and compared to the similar expression present within the other Notch family members (Figure 1A, 11th lane). Downstream molecules ephrin-B2 and Eph-B4 exhibited consistently lower transcriptional levels in aged versus young adult vein grafts, and may reflect differences between embryonic and aged adult regulation. These results suggest that Dll-4 and Notch-4 expression are impaired during vein graft adaptation in aged animals, and subsequently the expression of downstream pathway members such as ephrin-B2 and Eph-B4 is also abnormal.

Since Dll-4-Notch-1/4 interaction is known to be important in signaling during specification of embryonic arterial EC fate [18], we examined the Dll-4 and Notch-4 distribution in preimplantation veins and vein grafts. As expected, the immunofluorescence pattern of staining of young adult veins confirmed Notch-4 expression in luminal endothelial cells (Figure 1B). Similarly, Notch-4 immunofluorescence was detected in the endothelial cells of aged veins, without any density differences between young adult and aged veins prior to implantation (Figure 1B, left panels). Notch-4 was also detectable in the endothelium of young adult vein grafts, and, interestingly, was also detectable in cells of the outer adventitia of the vein grafts (Fig 1B, right panel, white arrowheads). However, although Notch-4 was detectable in the vein graft endothelium of aged vein grafts, Notch-4-containing cells were not seen in the outer adventitia (Figure 1B). Similarly, Dll-4 protein expression was detectable in the preimplantation veins of both young adult and aged rats (data not shown); Dll-4 was also detectable in both the endothelium and outer adventitia of young adult vein grafts, with less adventitial staining detectable in aged vein grafts (Figure 2).

Abnormal venous endothelium with aging

Since our data shows that endothelial cells express Dll4 and Notch-4, in both young adult and aged endothelial cells during vein graft adaptation, we determined whether there were any other differences in morphology or gene expression in the endothelium, of young adult and aged endothelium, during vein graft adaptation. Aged endothelial cells and nuclei were slightly larger than those in young adult veins and vein grafts (Figures 3 and 4). In addition, in young adult veins and vein grafts, the distribution of eNOS and caveolin-1 was aligned to the direction of flow and preserved after vein graft adaptation (Figure 3A, upper rows). However, there were differences in aged veins and vein grafts; in preimplantation aged veins, the distribution of eNOS had an expression pattern consistent with high amounts of colocalization at the Golgi apparatus (Figure 4), and reduced distribution to the cell membrane (Figure 3A, left panels) and reduced colocalization with caveolin-1 (Figure 3A, middle and right columns). This altered pattern of eNOS expression was not aligned to the direction of flow, as it was in young endothelium, and similarly was not aligned after implantation (Figure 3A). Not surprisingly, the total amount of expressed eNOS protein was slightly reduced in aged veins in comparison to young adult veins (Figure 5).

To determine whether there were any differences in gene expression associated with aging endothelium during vein graft adaptation, the number of transcripts for PDGF-BB and VEGF-A were examined (Figure 3B, 3C). Different temporal patterns of mRNA transcript expression for both PDGF-BB (Figure 3B) and VEGF-A (Figure 3C) were found, confirming differences in aged vessels during vein graft adaptation.

Aging influences both graft and host factors during vein graft adaptation

Because Dll-4 and Notch-4 are expressed in the outer adventitia of the young adult but not the aged vein grafts, it is unclear whether host factors such as infiltrating cells, or graft factors, i.e. cells intrinsic to the vein graft, may be responsible for reduced Dll-4 and/or Notch-4 expression in aged vein grafts. In addition, we have previously shown that vein grafts implanted into aged rats develop thicker neointimal hyperplasia compared to the little amount that develops in young adult rats [5]. Therefore, to clarify whether graft or host environmental factors affect neointimal thickness in aged rats, we generated a syngeneic exchange vein graft model between young adult and aged animals. The young adult host/young adult graft (Yo^{Yo}) and the aged host/aged graft (Ag^{Ag}), were considered control phenotypes. As expected, the Ag^{Ag} group formed consistently thicker neointimal hyperplasia in the vein graft walls compared to the Yo^{Yo} group (Figure 6). Interestingly, both young adult host/aged graft (Yo^{Ag}) and aged host/young adult graft (Ag^{Yo}) showed significantly thicker neointimal formation than Yo^{Yo}, and similar to the larger amount present in the Ag^{Ag} group (Figure 6A, 6B). These results suggest that age-dependent neointimal formation is influenced by both factors present within the vein graft as well as factors that are endogenous to the host environment.

Notch-4 expression in the outer adventitia of vein grafts is influenced by age

To confirm the interaction between Dll-4/Notch-4 expression and age-dependent neointimal hyperplasia, we performed immunofluorescence staining of syngeneic exchanged vein grafts. Consistent with the gene expression pattern described earlier (Figure 1A), both Dll-4 and Notch-4 positive cells were seen in the outer adventitia of control young adult (Yo^{Yo}) grafts, and had a significantly decreased presence in aged (Ag^{Ag}) grafts (Figure 7). Dll-4 and Notch-4 positive cells were found equally distributed in luminal endothelial cells of both young adult and aged rat grafts (data not shown). In both heterogeneous vein grafts, i.e. the switched groups Yo^{Ag} and Ag^{Yo}, the number of Dll-4 positive cells was not significantly decreased compared to the young (Yo^{Yo}) control group (Figure 7A: upper panels, 7B). In comparison, the number of Notch-4 positive cells was diminished in the outer adventitia of both Yo^{Ag} and Ag^{Yo}, to the same degree as in aged (Ag^{Ag}) grafts (Figure 7A: lower panels, 7C). These results suggest that Notch-4 expression in the outer adventitia plays a role in limiting neointimal hyperplasia formation, but Dll-4 expression does not. In addition, this data also suggests that the appearance of Notch-4 positive cells during vein graft adaptation is influenced by both graft age as well as host age, consistent with our data (Figure 6).

Dll-4/Notch-4 expression depends on vein graft endothelial cells

Since EC are known to have a critical role in the development of vascular structures and patterning, and our data suggests a role for the adventitia in regulating the differential response of young adult and aged vein grafts (Figures 6 and 7), we wanted to test whether vein graft EC play a role in vein graft adaptation. To test our hypothesis, we generated an autologous vein graft model in which the EC were denuded with air, i.e. without a medial layer stretch injury (Figure 8A). Evans blue staining of denuded vein grafts confirmed the efficacy of the air denudation (Figure 8B). Control aged vein grafts demonstrated increased neointimal hyperplasia compared to control young adult grafts, as expected (Figure 8C: right upper panel, between yellow arrowheads). Surprisingly, both the young adult and aged vein grafts that were denuded of EC showed diminished neointimal thickening, with no endothelial cells observed in the mid-portion of the graft (Figure 8C, 8D). These results show the critical importance of vein graft EC in regulating vein graft adaptation, e.g. vein graft EC may regulate the normal adaptation response of the vein to the arterial environment.

Vein grafts that were denuded of EC also had both diminished Dll-4 and Notch-4 expression at 3 weeks (Figure 9); there was no detectable endothelial Dll-4 or Notch-4 as there were no detectable EC in the vein grafts, even at 3 weeks. In the young adult grafts (Figure 9B and 9D, gray bars), the number of Dll-4 and Notch-4 positive cells was reduced by approximately 50% in denuded vein grafts compared to control vein grafts with intact EC. In comparison, there was very little Dll-4 or Notch-4 expression in aged vein grafts (Figure 9B and 9D, black bars) and this minimal expression was not influenced by EC denudation. These results are consistent with Dll-4 and Notch-4 expression in the outer adventitia being dependent on the presence of an EC monolayer, with reduced Dll-4 and Notch-4 expression in aged vein grafts.

Discussion

Our results suggest that age influences Notch-4 expression in vein grafts, and specifically the loss of Notch-4 is associated with a loss of attenuation of neointima. Notch is known to be instrumental in the processes of vascular structuring and patterning in the embryo [25] as well as in bone marrow-derived cell differentiation to smooth muscle-like cells in vascular lesions [26]. Based on this knowledge, we hypothesized that dysfunction of Notch pathways in aged animals may lead to abnormal vein graft adaptation with thicker neointimal hyperplasia. Since our results show that aged vein grafts have impaired Dll-4/Notch-4 expression (Figure 1A) as well as a thicker neointima (Figure 6B) [5], our data supports the concept that Notch-4 expression may control the formation and/or regulation of neointimal thickening during vein graft adaptation to the arterial environment. Alternatively, excessive neointimal thickening in aged vein grafts may depend on the presence of a dysfunctional aged endothelium, such that neointima may not form in its absence (Figure 8); if such is the case, then aged endothelium may have alternative pathways to compensate for dysfunctional delta-Notch signaling. Since Notch signaling is likely to be impaired with aging, and aged vein grafts show exuberant vein graft thickening, we believe that these alternative pathways do play a role in vein graft adaptation.

Differential effects of arterial and venous endothelium

We believe that one of the most interesting pieces of data that we show is the complete lack of neointimal thickening in response to denudation of the venous endothelium with air (Figure 8C). The literature shows many examples of denudation of arterial endothelial cells leading to increased neointimal hyperplasia; however, most of these models use the balloon model of injury, a common experimental model that injures and stretches the media, and not surprisingly leads to increased neointimal thickening. We particularly use the air-denudation method to avoid medial stretch injury. Therefore, our finding that venous intimal hyperplasia does not occur in the absence of venous endothelium suggests that the response to injury in arterial and venous endothelium may be different. This is not surprising since arteries and veins are structurally and developmentally different. In addition, venous intimal hyperplasia depends on Eph-B4 expression [5], and arteries do not contain Eph-B4, implying different mechanisms of response to injury. However, since our specimens only examine the midpoint of the graft, it is possible that the anastomotic thickening may be regulated by a different mechanism. In addition, our findings may be specific to the Fischer 344 rat model with the air-injury method of EC denudation, described by Clowes et al [27, 28]. Nevertheless, our data is consistent with a critical role for endothelial cell regulation of vein graft adaptation to the arterial environment.

Effects of host environment age

It was surprising that synergenic exchange heterografts between young adult and aged rats presented similar phenotypes, i.e. a similar amount of neointimal thickening (Figure 6). In

contrast, in 2005, Conboy and colleagues described that a young environment rejuvenates aged tissue progenitor cells [29]. Interestingly, this group also showed that a Delta-Notch interaction functions in restoration of regenerative potential in aged tissue [29, 30]. Contrary to their experiments, our results revealed significantly lower Notch-4 expression levels in Ag^{Yo} heterografts compared to young adult controls; furthermore, Notch-4 expression was not rescued in Yo^{Ag} heterografts, although Dll-4 expression was rescued by the young environment (Figure 7). These results suggest that Dll-4 expression is dependent on the age of the vein graft, and a young environment is able to rescue impaired expression in aged grafts. In comparison, Notch-4 expression is dependent on both host environment age and vein graft age; advanced host age impairs Notch-4 expression, and advanced graft age impairs the participation of young adult host cells in potentially restoring a young phenotype. This finding may be related to our findings of differential eNOS localization in young adult and aged veins (Figures 3 and 4), or to the differential expression of growth factors (Figure 3). However, the effects of aging on interaction of the Notch and other intracellular signaling pathways is not currently well understood. We believe that the presence of a thick neointima when either the graft or host is aged is consistent with some aspect of Notch signaling being impaired with aging. It is also possible that upstream effects of VEGF or VEGF receptors, such as acquired resistance to VEGF signaling, may be responsible for the diminished Notch signaling in aged vein grafts. Changes in VEGF expression and signaling have been previously shown to be involved with the response to arterial injury [31], although the differential role of VEGF between young adult and aged animals during vein graft adaptation is not currently known. Our data is consistent with diminished or delayed down-regulation of VEGF-A expression in aged vein grafts (Figure 3C), although reduced PDGF-BB expression is also present (Figure 3B).

Aging effects on vein graft adaptation

In addition to their detection in the endothelium, Dll-4 and Notch-4 positive cells are found extensively in the outer adventitia of veins (Figure 1B, 7, 9). It is well known that the adventitia plays a critical role in the formation of neointimal thickening in vein grafts [6, 32, 33]. Monocytes that have migrated into the adventitia, and possibly progenitor cells resident in the adventitia, are involved in the inflammatory response characteristic of the early stages of vein graft adaptation. Additionally, Dll-4 activates Notch signaling, which is an important factor in inflammation [34]. Since quiescence of Dll-4 and Notch-4 expression in aged grafts is associated with the development of a thicker neointima (Figure 7), we believe that the effects of aging on the adventitia are relevant to vein graft wall remodeling. However, it is not yet clear whether Dll-4 or Notch-4 positive cells are derived from the graft vascular or perivascular cells, or from the host blood and/or bone marrow.

Our results also suggest that Dll-4/Notch-4 expression may be at least partially dependent on the presence of functional EC. Since vein grafts denuded of EC show loss of Dll-4/Notch-4 induction in young adult but not aged rats (Figure 9), these results suggest that Dll-4/Notch-4 expression is regulated by effects of aging on endothelial cells, i.e. EC senescence. EC are known to play both positive and negative roles during vascular wall remodeling. For instance, EC release of nitric oxide (NO), which prevents platelet activation, chemical mediator secretion, and adhesion molecule expression, all of which may play a role in the limitation of neointimal thickening (Figure 3) [6, 35–37]. On the other hand, EC secrete platelet-derived growth factor (PDGF) that leads to accelerated smooth muscle cell proliferation and accumulation during vein graft adaptation (Figure 3) [38, 39]. Accordingly, denudation of the vein grafts did not cause neointimal thickening in the aged grafts that have baseline Dll-4/Notch-4 quiescence. These results suggest that Dll-4/Notch-4 expression is a modifier of EC function during vascular remodeling. Finally, we believe that a full

elucidation of the role of the Dll-4/Notch-4 pathway is beyond the limits of this rat model and will depend on the better availability of suitable tools for the mouse model.

In conclusion, aging influences vein graft wall remodeling. Both aging of the host environment and aging of the graft itself are associated with relevant effects. Notch-4 expression is down-regulated in an aged background, and is dependent on endothelial cell function. Although aging is extensively studied in many fields, the link of aging to vascular biology is not well understood. Our results suggest that the Notch pathway is an important component of vein graft adaptation and may be a site of age-specific pharmacological modulation.

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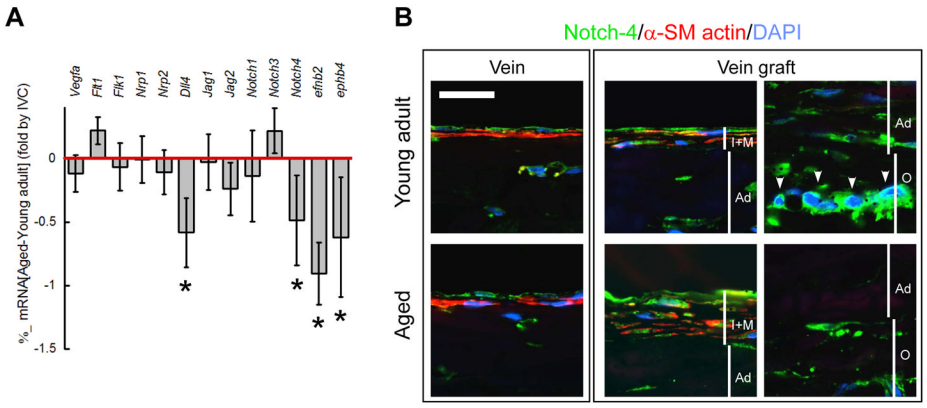

Figure 1.

(**A**) Real time quantitative RT-PCR shows impaired Dll-4, Notch-4, ephrin-B2, and Eph-B4 gene expression in aged rat vein grafts. Data is number of mRNA transcripts detectable by qPCR and normalized to GAPDH, with the bar graph showing the difference between aged and young adult vein graft expression, in n=4 distinct young adult and aged samples. *: $p<0.05$ of aged in comparison to young adult number of transcripts. (**B**) Representative immunofluorescence staining of Notch-4, from n=4 young adult and n=4 aged samples. White arrowheads indicate positive signal in the outer adventitia. I+M: intima and medial layer, Ad: adventitial layer, O: outer adventitia, SM: Smooth muscle. Scale bar: 20 micrometer.

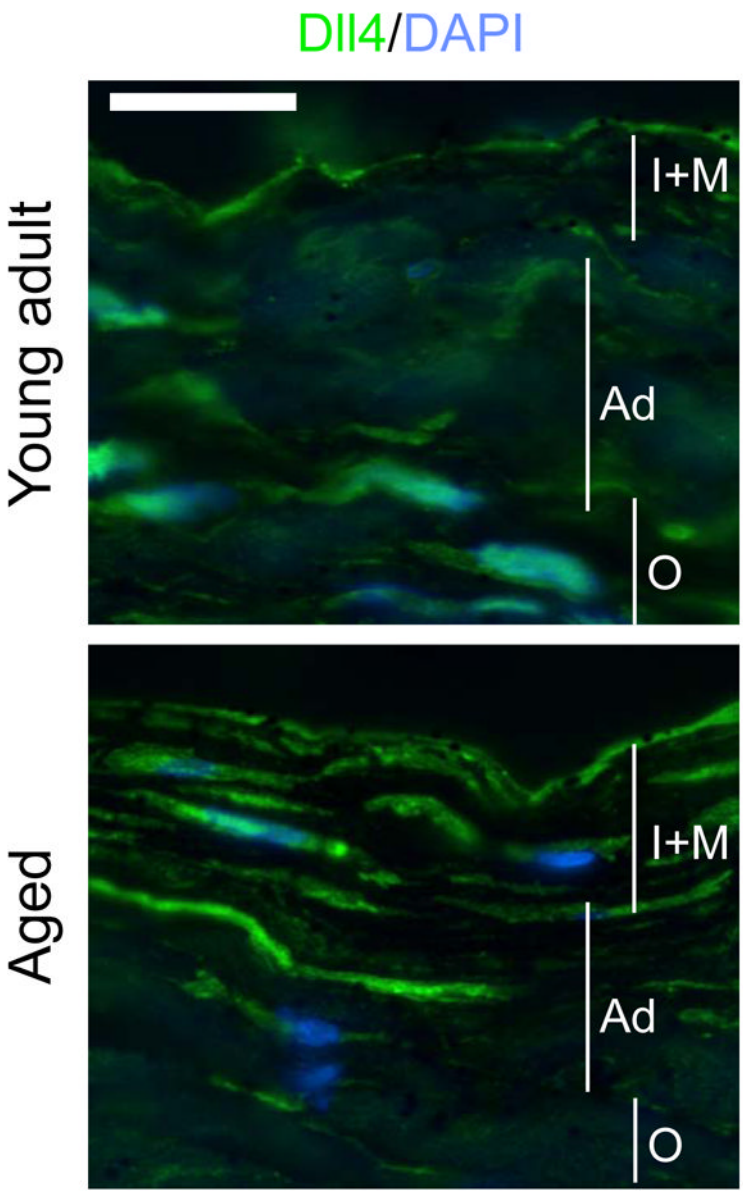

Figure 2.
Representative immunofluorescence staining of Dll-4, from n=4 young adult and n=4 aged vein graft samples. I+M: intima and medial layer, Ad: adventitial layer, O: outer adventitia. Scale bar: 20 micrometer.

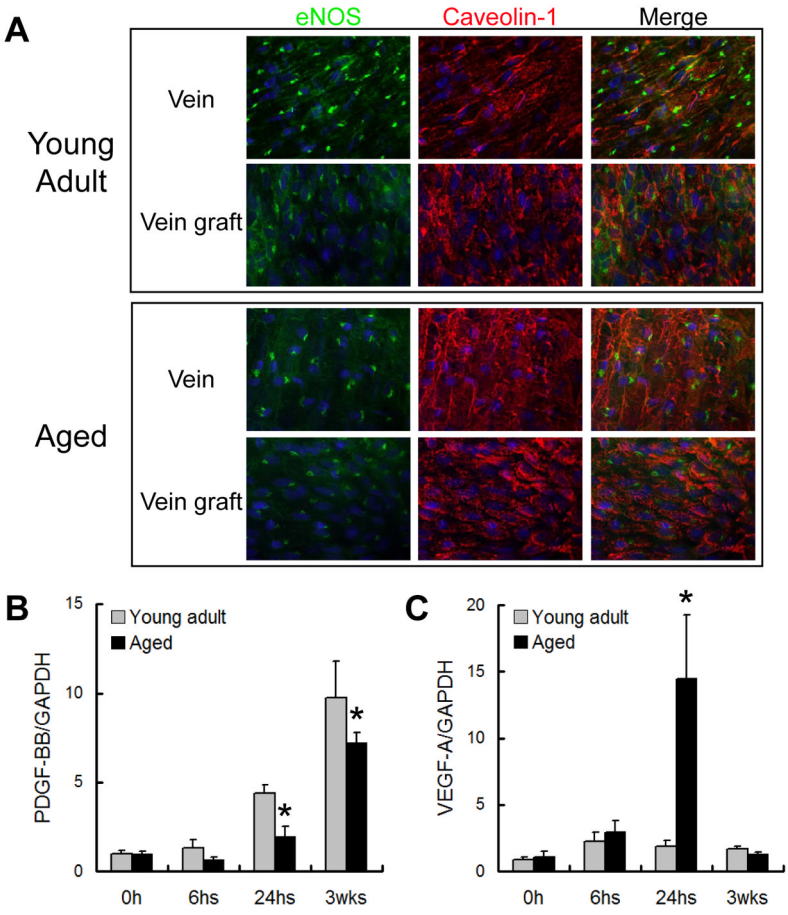

Figure 3.

(**A**) Representative immunofluorescence images of young adult (upper 2 rows) and aged (lower 2 rows) vein and vein grafts. eNOS, green signal; caveolin-1, red signal; merge, yellow signal. n=3 young adult and aged animals. (**B, C**) Bar graphs show mean number of transcripts detectable by qPCR at 0, 6 hr, 24 hr, and 3 wks after vein graft implantation, in both young adult and aged animals. Panel (**B**) shows PDGF-BB, and panel (**C**) shows VEGF-A, normalized to GAPDH. n=4. *, $p<0.05$ in comparison of young vs. aged data. Some the data from panel (**C**) has been previously published.[5]

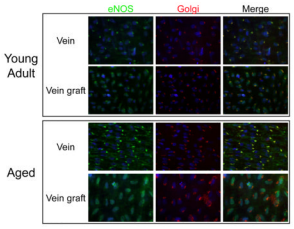

Figure 4.
Representative immunofluorescence images of young adult (upper 2 rows) and aged (lower 2 rows) vein and vein grafts. eNOS, green signal; Golgi, red signal; merge, yellow signal. n=3 young adult and aged animals.

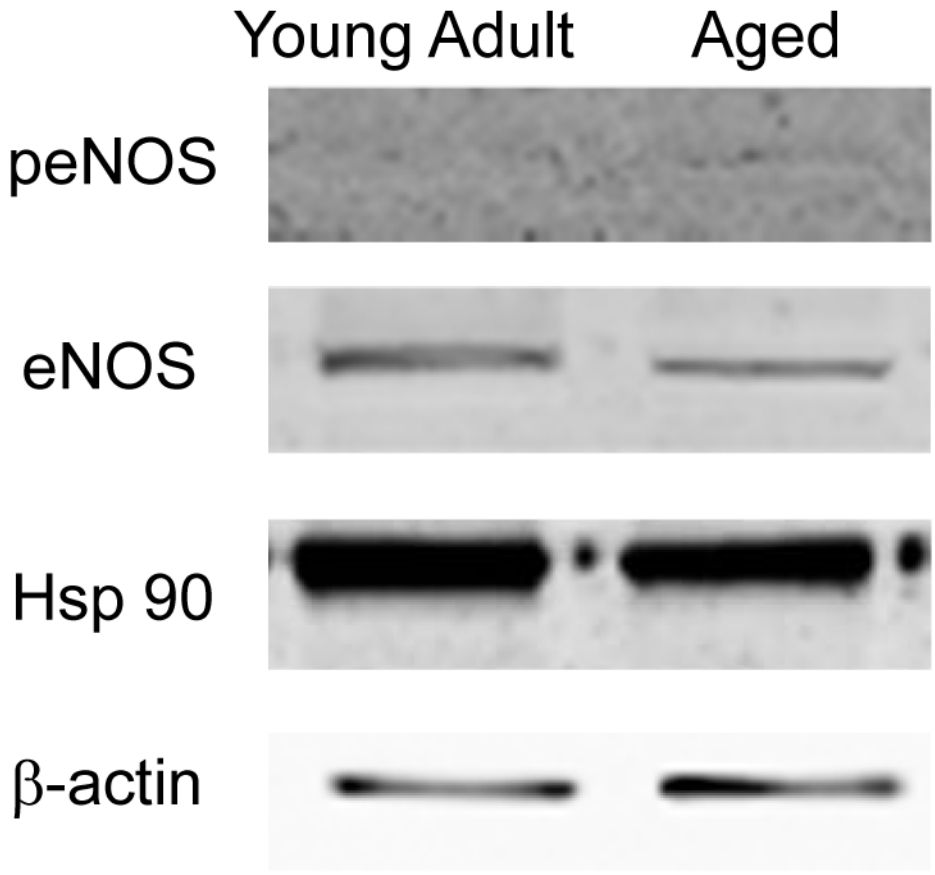

Figure 5.
Representative Western blot analysis from n=3 young adult and aged veins. There is reduced density of eNOS in the aged veins.

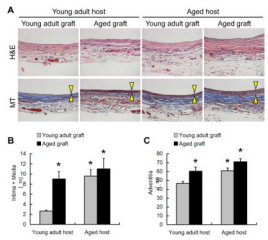

Figure 6.

Morphological analysis of syngeneic exchanged vein grafts between young adult and aged hosts. (**A**) Representative images of hematoxylin-eosin (H&E) and Masson's trichrome (MT) staining. Yellow arrowheads indicate neointimal hyperplasia formation. (**B**) Measurement of rat neointimal thickness as the intima+media layers (I+M) and (**C**) adventitial thickness. n=4 in each group. *: $p<0.05$ in comparison to the Yo^{Yo} group.

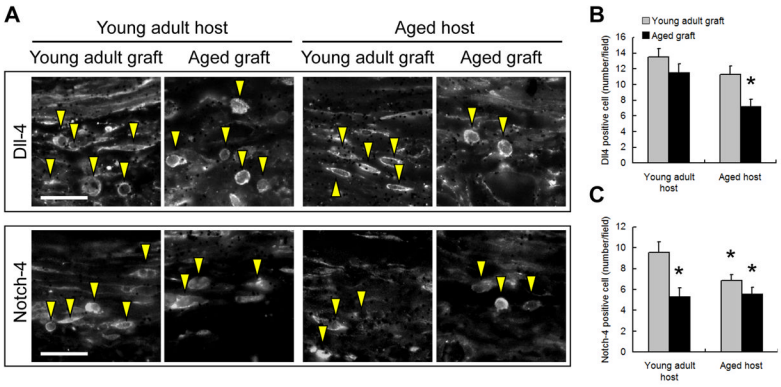

Figure 7.

(**A**) Representive immunofluorescence staining images of Dll-4 (upper panels) and Notch-4 (lower panels) in perivascular connective tissue (outer adventitia) of syngeneic switched rat vein grafts. Yellow arrowheads show positive cells. (**B**) Dll-4 and (**C**) Notch-4 positively stained cell counts. n=4 in each group. *: $p<0.05$ in comparison to the Yo^{Yo} group. Scale bar shows 20 micrometer.

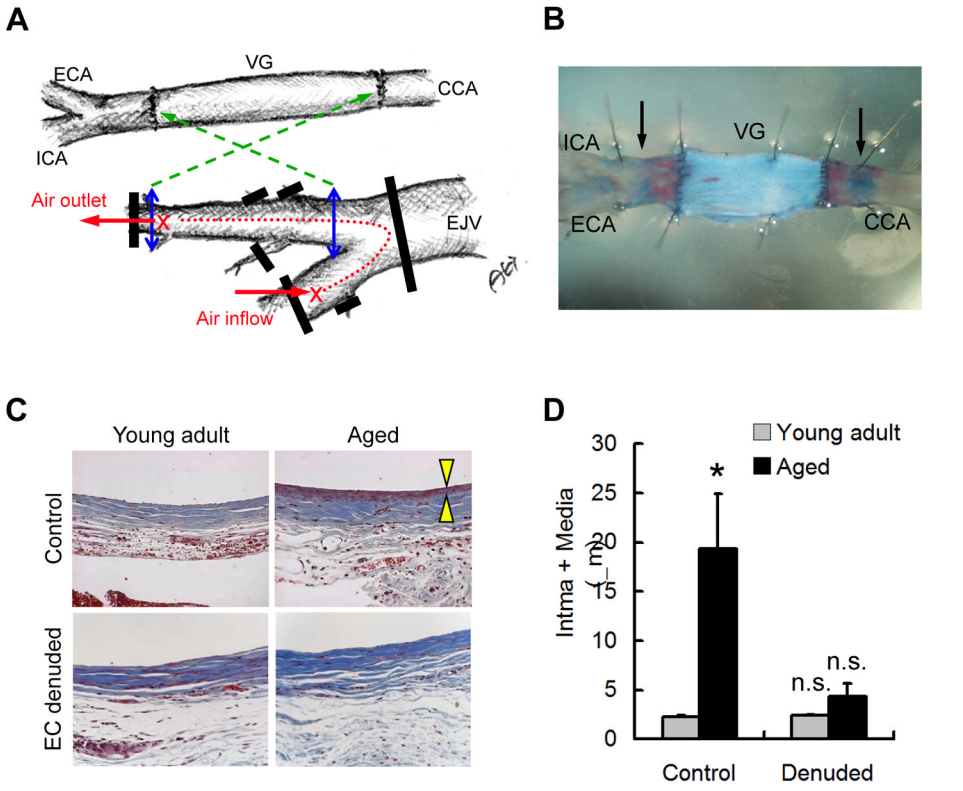

Figure 8.

EC denuded vein grafts. (**A**) Schema of EC denudation in vein grafts by air injury of the preimplantation vein. The external jugular vein was tied at three points: proximal end, distal end, and primary major branches. A pin hole for egress of air was made at the distal end, and a 30G needle was inserted at the primary major branch (red arrows). After air injury, the venous lumen was flushed with saline, and harvested at the blue arrows. Thick black lines indicate tie points. (**B**) Confirmation of EC denudation. Evans blue was injected 1 hour after vein graft surgery, and sacrificed 30 minutes after injection. Vein grafts were harvested with the proximal and distal carotid artery, and opened for observation of graft *en face*. Compared to the endothelial-intact artery, the vein graft luminal surface demonstrates blue staining due to EC denudation. Arrowheads show blue staining in artery luminal surface due to EC injury by clamping. CCA: common carotid artery, ICA: internal carotid artery, ECA: external carotid artery, EJV: external jugular vein, VG: vein graft. (**C**) Representive Masson's trichrome staining images of control and EC denuded vein grafts. No obvious neointimal hyperplasia was present in either denuded young adult or aged vein grafts. (**D**) Morphological analysis of EC denuded vein graft. Rat neointimal thickness is measured as the total of the intima + media layers. n=3 in each group. *: $p<0.05$ in comparison of young adult and aged groups. n. s.: not significant.

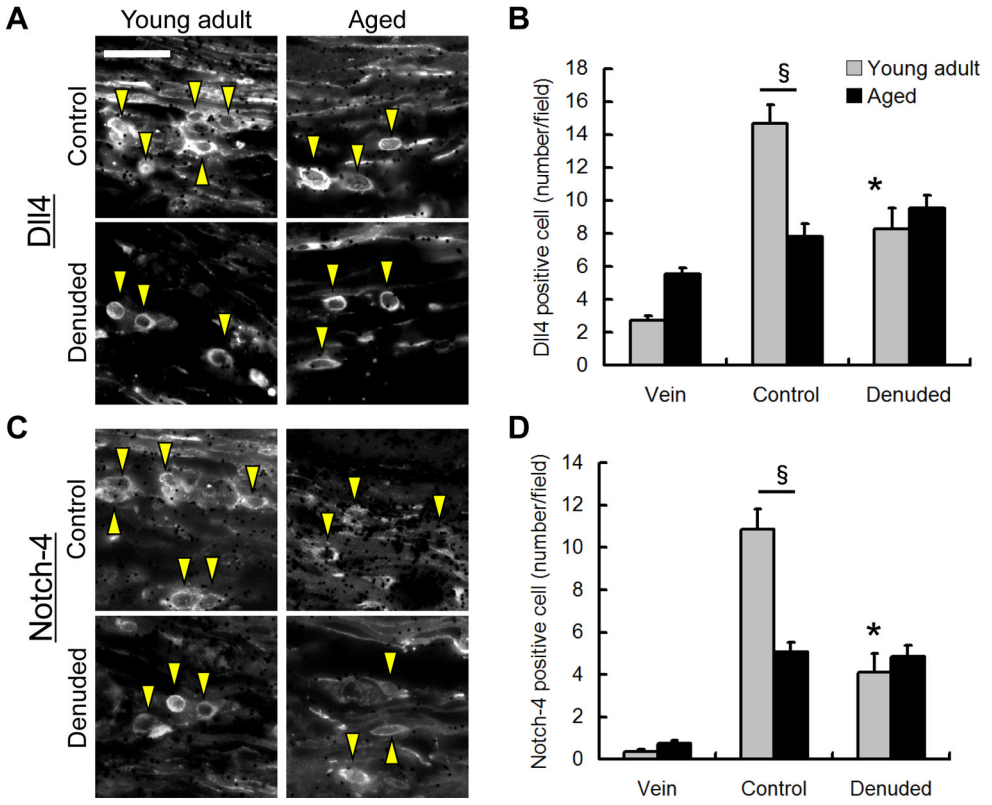

Figure 9.

Analysis of Dll-4 and Notch-4 positive cells in EC denuded vein grafts. (**A**) Representative images of Dll-4 immunofluorescence staining. (**B**) Dll-4 positive cell counts in pre-implantion vein (Vein), control vein graft (Control), and EC-denuded vein grafts (Denuded). (**C**) Representative images of Notch-4 immunofluorescence staining. (**D**) Notch-4 positive cell counts in pre-implantion vein, control VG, and EC-denuded VG. Yellow arrowheads indicate positive cells. Scale bar: 20 micrometer. n=3 in each group. *: $p<0.05$ between control vs. denuded vein graft. §: $p<0.05$ between young adult vs. aged.

Table

	Forward	Reverse
vegf-a	CTCACCAAAGCCAGCACATA	AAATGCTTTCTCCGCTCTGA
flt-1	TTCCGGACTTTCAACACCTC	CCGAATAGCGAGCAGATTTC
flk-1	CGATGTCTCCTCCATCGTTT	TTCCATCCGGAACAAATCTC
neuropilin-1	GGAGCTACTGGGCTGTGAAG	ATGTCGGGAACTCTGATTGG
neuropilin-2	ACACAAGGAGCCATTTCCAG	CGGATCCTGATGAAACGAGT
delta like ligand-4	AAGGTGCCACTTCGGTTACAC	AATGACACATTCGTTCCTCTCTT
jagged-1	GACCAGAATGGCAACAAAACCTGCATGGAA	TTGGTTTCACAGAGGCACTGCCAGGGTTCA
jagged-2	CGAGTTCCAGTGTGACGCCTAC	GGACCAGCAGGGCCTCGTGAAT
notch-1	GAGTCACCCCATGGCTAC	GTGGCTGCACCTGCTGGG
notch-3	GACCGTGTGGCCTCTTTCTATTGT	GCAGCTGAAGCCATTGACTCTATCCT
notch-4	CAGAACGCGGATCCCCTCAAGTT	TTCTGATTCCTTCCACCCGAGTTT
efnb2	ACCGCTAAGGACTGCAGACAG	GTCCAAGTGGGGATCTCCTAG
ephb4	CACCCAGCAGCTTGATCCTG	ACCAGGACCACACCCACAAC
pdgf-bb	TCCGCTCCTTTGATGACCTTC	TTGCGGTTATTGCAGCAGC
gapdh	AGACAGCCGCATCTTCTTGT	CCACAGTCTTCTGAGTGGCA