Overexpression of the Cell Cycle Inhibitor p16^{INK4a} Promotes a Prothrombotic Phenotype Following Vascular Injury in Mice

Jessica C. Cardenas, A. Phillip Owens III, Janakiraman Krishnamurthy, Norman E. Sharpless, Herbert C. Whinna, Frank C. Church

Objective—Age-associated cellular senescence is thought to promote vascular dysfunction. p16^{INK4a} is a cell cycle inhibitor that promotes senescence and is upregulated during normal aging. In this study, we examine the contribution of p16^{INK4a} overexpression to venous thrombosis.

Methods and Results—Mice overexpressing p16^{INK4a} were studied with 4 different vascular injury models: (1) ferric chloride (FeCl_3) and (2) Rose Bengal to induce saphenous vein thrombus formation; (3) FeCl_3 and vascular ligation to examine thrombus resolution; and (4) lipopolysaccharide administration to initiate inflammation-induced vascular dysfunction. p16^{INK4a} transgenic mice had accelerated occlusion times (13.1 ± 0.4 minutes) compared with normal controls (19.7 ± 1.1 minutes) in the FeCl_3 model and 12.7 ± 2.0 and 18.6 ± 1.9 minutes, respectively in the Rose Bengal model. Moreover, overexpression of p16^{INK4a} delayed thrombus resolution compared with normal controls. In response to lipopolysaccharide treatment, the p16^{INK4a} transgenic mice showed enhanced thrombin generation in plasma-based calibrated automated thrombography assays. Finally, bone marrow transplantation studies suggested increased p16^{INK4a} expression in hematopoietic cells contributes to thrombosis, demonstrating a role for p16^{INK4a} expression in venous thrombosis.

Conclusion—Venous thrombosis is augmented by overexpression of the cellular senescence gene p16^{INK4a}. (Arterioscler Thromb Vasc Biol, 2011;31:00-00.)

Key Words: aging ■ coagulation ■ pathology ■ thrombosis

Aging is an important risk factor for developing cardiovascular disease, and also the least understood. Vein thromboembolism (VTE) is characterized by the development of thrombi in the deep veins of the legs; these thrombi are prone to dislodging and embolizing to the lungs. This condition accounts for 140 000 to 200 000 deaths each year in the United States. The risk of developing VTE substantially increases with age, and individuals over the age of 55 years have an annual incidence 5 to 7 times higher than young adults. Although VTE in the younger population is often explained by mutations in hemostatic genes, mechanisms behind the increased risk of VTE in the elderly are less well understood.

Senescence is one cellular phenomenon known to be associated with aging. Cellular senescence is a stress-induced process that is controlled by cell cycle inhibitors and that promotes an irreversible growth arrest. p16^{INK4a}, a cell cycle inhibitor that promotes senescence, binds to cyclin-dependent kinases 4 and 6 to disrupt phosphorylation of the retinoblastoma protein, causing a G_1 cell cycle arrest. Expression of p16^{INK4a} increases with age in many tissues and is a biomarker of aging. Furthermore, p16^{INK4a} expression correlates with biomarkers of senescence, such as senescence-associated β-galactosidase expression, and expression is associated with gerontogenic activities, such as smoking, physical inactivity, and ad libitum feeding in humans or mice. In some tissues such as pancreatic β-cells, neural stem cells, and hematopoietic stem or progenitor cells, the age-induced increase in p16^{INK4a} expression is associated with reduced cellular proliferation coupled with an impaired tissue response to injury. Additionally, senescent cells are thought to contribute to aging pathology through the production of cytokines (interleukin-6) that further promote inflammation and cellular dysfunction.

The contribution of senescence to disease in the venous circulation and how it may be involved in age-related VTE or a possible prothrombotic phenotype remain largely uncharacterized. The aim of this study was to ascertain whether...
overexpression of p16INK4a modified venous thrombus formation in several well-defined animal models. Our results demonstrate that p16INK4a overexpression augments vascular occlusion and delayed thrombus resolution relative to wild-type controls. Furthermore, p16INK4a transgenic mice display enhanced thrombin generation, increased thrombin-antithrombin, and increased PAI-1 levels when exposed to enhanced thrombin generation, increased thrombin-occlusion and delayed thrombus resolution relative to wild-type and p16INK4a transgenic mice demonstrated a substantial contribution of hematopoietic cells to this phenotype. Overall, these results show that expression of p16INK4a is involved in promoting a prothrombotic environment in the venous vasculature.

Methods
A detailed description of the methods is presented in the Supplemental Materials, available online at http://atvb.ahajournals.org.

Mice
All animal procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of University of North Carolina–Chapel Hill. The bacterial artificial chromosome transgenic mice overexpressing p16INK4a used in this study have been described previously.15

Hemostasis Model
Hemostasis was assessed as previously described.19 Briefly, wild-type and p16INK4a transgenic mice at 8 weeks of age were anesthetized with 2.5% tribromoethanol (T48402, Sigma-Aldrich, St. Louis, MO) at 0.1 mL/g body weight. The saphenous vein of anesthetized mice was exposed and transected with a 23-gauge needle. Once bleeding stopped, a longitudinal cut was made in the vessel, and the blood was gently wiped away with Kimwipes (Kimberly-Clark, Roswell, GA) until it clotted. The blood clot was disrupted using a 30-gauge needle, and the blood was gently wiped away. Clot disruption was repeated every time hemostasis occurred, and each hemostatic event was recorded using Chariot software for 20 minutes.

FeCl3 Vascular Injury
The saphenous vein thrombosis model was performed as previously described.19 Briefly, the saphenous veins of anesthetized mice were exposed and dissected away from the saphenous artery. A 0.5×2-mm piece of filter paper was soaked in 2.5% tribromoethanol (T48402, Sigma-Aldrich, St. Louis, MO) for 1 minute. The time to flow restriction was defined as the time after injury at first cessation of blood flow.

Rose Bengal Photochemical Vascular Injury
Photochemical injury was performed as previously described.20 Briefly, both right and left saphenous veins of anesthetized mice were exposed. The left saphenous vein was catheterized using catheters made in-house using pulled PE-10 tubing (Braintree Scientific, Braintree, MA). Rose Bengal (R-3877, Sigma-Aldrich), diluted to 30 mg/mL in normal saline, was infused through the catheter at a dose of 75 mg/kg through a gastight syringe (Hamilton Co, Reno, NV). Before infusion of Rose Bengal, a 1.75-mW green light (540 nm) (Prizmatix, Southfield, MI) was directed 0.5 cm over the injury site on the right saphenous vein. Light was applied to the vessel until a stable thrombus (defined as the absence of blood flow for 1 minute) was achieved.

Results

Thrombus Resolution
We developed a new method to measure thrombus resolution using the saphenous vein. Wild-type and p16INK4a transgenic mice (n=3 per genotype at each time point) were subjected to 10% FeCl3 injury to the saphenous vein. The tissue was then washed 3 times with warm saline, a single ligature was placed upstream of the thrombus using a 8-0 monofilament polypropylene suture to prevent embolization, and the leg was sutured closed. Mice were euthanized at various time points, and the saphenous neurovascular bundle was removed and fixed overnight in 4% paraformaldehyde and paraffin embedded. Five-micrometer sections were cut and hematoxylin and eosin (H&E) stained to visualize the presence of a thrombus under light microscopy. Vessels were sectioned through, and those sections showing the greatest area of occlusion were chosen for analysis. Such sections typically occurred near the center of the injured vessel. Images were analyzed using ImageJ software to calculate the percentage of the vessel lumen that remained occluded by a thrombus.

Low-Dose LPS Treatment
Wild-type and p16INK4a transgenic mice were treated with 2 mg/kg intraperitoneal injection of LPS (L3012, Sigma-Aldrich). At various times (1, 3, and 5 hours), the mice (n=5 per genotype each time point) were anesthetized, and 1 mL of blood was collected from the inferior vena cava into 3.8% sodium citrate at a ratio of 1:9 using a 25-gauge needle. Whole blood was spun at 4000g for 15 minutes, and the platelet-poor plasma was collected and stored at −80°C until it was analyzed.

Bone Marrow Transplantation
This procedure was performed as described previously.21 Briefly, mice were irradiated using a cesium-137 irradiator (JL Shepherd, San Fernando, CA) with a total of 11 Gy (2 doses of 550 rad, with a 4-hour rest) to abolish endogenous hematopoietic cells. Bone marrow cells were isolated from donor mice, and 1×107 cells were injected into the retroorbital sinus. Four weeks after irradiation, recipient mice underwent FeCl3 injury to the saphenous vein to determine vascular occlusion times. At termination, recipient mouse bone marrow was genotyped to verify successful repopulation of donor cells by polymerase chain reaction. Expression of p16INK4a was compared with an interleukin-2 loading control.

Statistics
All statistical analyses were performed with GraphPad Prism. All measurements are represented as the mean±SEM. One-way ANOVA or Student’s t test was performed where indicated. Values of P<0.05 were considered statistically significant.

p16INK4a Transgenic and Wild-Type Mice Respond Similarly in a Hemostasis Model
To determine the contribution of p16INK4a overexpression to potential hemostatic defects, mice initially underwent a model of saphenous vein hemostasis. No difference was observed in the number of hemostatic clots formed over 20 minutes between transgenic mice (25.8±3.4) and wild-type mice (25.8±2.1, Figure 1A) or in the average time to hemostasis (33.5±3.7 and 36.6±2.6 seconds, respectively, Figure 1B). Furthermore, no significant differences were observed in body weight, venous blood flow velocity, plasma prothrombin time, and complete blood count between the 2 groups of mice (Supplemental Table I). These results suggest that there is no obvious physical or hematologic phenotype in the p16INK4a transgenic mice at the ages studied.
p16INK4a Transgenic Mice Display a Prothrombotic Phenotype in an FeCl3 Injury Model

FeCl3 injury is a well-established mechanism for inducing thrombus formation in vivo.22–24 We first demonstrated a dose-dependent effect of FeCl3 on the occlusion time in the saphenous vein, exposing wild-type mice to 2.5%, 5%, and 10% FeCl3 injuries to the saphenous vein (Figure 2A). Wild-type and p16INK4a transgenic mice were then subjected to FeCl3 (5%) injury to the saphenous vein. The p16INK4a transgenic mice showed a significantly shorter time to occlusion (13.1 ± 0.4 minutes) compared with wild-type mice (19.7 ± 1.1 minute, Figure 2B). Furthermore, the time to flow restriction was also measured. The p16INK4a transgenic mice demonstrated shorter times to flow restriction (6.4 ± 0.91) compared with wild-type controls (8.7 ± 0.54, P < 0.05, data not shown). These results indicate that overexpression of p16INK4a results in a prothrombotic phenotype following vascular injury.

p16INK4a Transgenic Mice Display a Prothrombotic Phenotype in a Photochemical Injury Model

The excitation of Rose Bengal to induce photochemical injury is another well-established mechanism for inducing thrombus formation in vivo.25–27 On photochemical injury to the saphenous vein, p16INK4a transgenic mice displayed a significantly shorter time to occlusion (12.7 ± 2.0 minutes) compared with wild-type mice (18.6 ± 1.9 minutes, Figure 3). These results suggest that the prothrombotic phenotype in mice overexpressing p16INK4a can be recapitulated in other vascular injury models.
overexpressing p16<sup>INK4a</sup> on thrombus resolution, thrombi formed post-FeCl<sub>3</sub> injury in wild-type and p16<sup>INK4a</sup> transgenic mice were monitored over time. Mice were euthanized from 1 hour to 15 days post-FeCl<sub>3</sub> injury and vascular ligation. No significant differences in thrombus resolution were observed until 7 days after vascular injury. By 10 days postinjury, all wild-type mice exhibited complete thrombus resolution, whereas p16<sup>INK4a</sup> transgenic mice maintained an average of 60% vessel occlusion. p16<sup>INK4a</sup> transgenic mice required additional time postinjury for thrombus resolution relative to wild-type controls (Figure 4A). Representative images show little difference in percentage of occlusion at 1 day (Figure 4B) between p16<sup>INK4a</sup> transgenic and wild-type mice. Black staining represents FeCl<sub>3</sub> trapped within the thrombus. At 10 days, we observed that residual FeCl<sub>3</sub> was mostly contained within inflammatory macrophages and was present in the perivascular space of wild-type mice. However, residual FeCl<sub>3</sub> contained within macrophages was still present in the intravascular space of p16<sup>INK4a</sup> transgenic mice at 10 days (Figure 4B). These results demonstrate a defect in thrombus resolution with p16<sup>INK4a</sup> overexpression.

**p16<sup>INK4a</sup> Transgenic Mice Display Enhanced Thrombin Generation in Response to LPS Challenge**

Chronic inflammation and endothelial dysfunction have been linked to enhanced thrombin generation and the risk of venous thrombosis. To study the effects of inflammation-induced coagulation, we exposed p16<sup>INK4a</sup> transgenic and wild-type mice to low-dose LPS. When analyzed by calibrated automated thrombography, plasma from the p16<sup>INK4a</sup> transgenic mice showed a significantly shorter lag time to initiation of thrombin generation and time to peak amount of thrombin generated at all time points post–LPS treatment (Table). The peak amount of thrombin generated was significantly higher in p16<sup>INK4a</sup> transgenic mice 3 and 5 hours after LPS treatment (Table). The observed differences in thrombin generation demonstrate p16<sup>INK4a</sup> transgenic mice are able to generate more thrombin and have a prothrombotic phenotype when challenged with LPS.

**p16<sup>INK4a</sup> Expression in Hematopoietic Cells Contributes to the Observed Prothrombotic Phenotype**

To determine the relative contribution of p16<sup>INK4a</sup> expression in the hematopoietic cell compartment to the observed prothrombotic phenotype, bone marrow transplants were performed between transgenic and wild-type mice. Following transplantation and recovery, mice were subjected to 10% FeCl<sub>3</sub> injury to the saphenous vein. Consistent with our previous results (Figure 2B), transgenic mice receiving transgenic bone marrow had retained their significantly reduced occlusion time (8.4 ± 0.48 minutes) when compared with

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**Table. Thrombin Generation in Wild-Type vs p16<sup>INK4a</sup> Transgenic Mouse Plasma after LPS Treatment**

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Lag Time (minutes)</th>
<th>Peak Height (nM)</th>
<th>Time to Peak (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>p16&lt;sup&gt;INK4a&lt;/sup&gt; Tg</td>
<td>WT</td>
</tr>
<tr>
<td>0</td>
<td>2.17 ± 0.19</td>
<td>2.33 ± 0.24</td>
<td>46.16 ± 0.53</td>
</tr>
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<td>3.4 ± 0.16</td>
<td>1.85 ± 0.12*</td>
<td>45.44 ± 9.45</td>
</tr>
<tr>
<td>3</td>
<td>2.85 ± 0.11</td>
<td>1.85 ± 0.09*</td>
<td>36.44 ± 0.43</td>
</tr>
<tr>
<td>5</td>
<td>2.01 ± 0.17</td>
<td>2.51 ± 0.19*</td>
<td>32.06 ± 0.46</td>
</tr>
</tbody>
</table>

Thrombin generation in plasma from mice treated with 2 mg/kg LPS was measured using calibrated automated thrombography, as described in the Supplemental Materials and Methods. Data represent experiments performed in duplicate with 5 mice per group per time point. WT indicates wild-type; Tg, transgenic.

*P<0.05.
Wild-type mouse receiving wild-type bone marrow (13.0 ± 0.79 minutes, Figure 5). Interestingly, transgenic mice receiving wild-type bone marrow displayed occlusion times similar to those of wild-type mice (12.8 ± 1.3 minutes), whereas wild-type mice receiving transgenic bone marrow displayed occlusion times similar to those of transgenic mice (9.5 ± 0.61 minutes, Figure 5). Polymerase chain reaction results confirmed successful reconstitution by donor bone marrow cells (Supplemental Figure III). These results demonstrate that the effects of p16INK4a overexpression are, at least in part, mediated by hematopoietic cells.

Discussion

Senescence is a complex process that is thought to contribute to cardiovascular pathologies associated with aging. Despite several reviews describing prothrombotic changes in senescent vascular endothelial cells, no studies have described a venous thrombotic phenotype in mice overexpressing senescence-promoting genes. In the current study, we examined parameters that define venous thrombotic potential in a mouse model of premature senescence through transgenic overexpression of the cell cycle inhibitor p16INK4a. As expected, the p16INK4a transgenic mouse exhibited increased expression of p16INK4a mRNA by real-time polymerase chain reaction analysis in all tissues tested. Mice overexpressing p16INK4a exhibit normal basal hemostatic parameters as tested by complete blood count, prothrombin time, and an in vivo hemostasis model. This suggests that in the absence of vascular injury, overexpression of p16INK4a has no overt hemostatic consequences. However, on challenge in various vascular injury models, the p16INK4a transgenic mice displayed an obvious prothrombotic response.

We have demonstrated a prothrombotic phenotype using 2 different vascular injury models. Exposure of the vessel to FeCl3, is a type of biochemical injury that results in endothelial denudation and exposure of the subendothelium following lipid peroxidation. This type of oxidative damage produces thrombi that are rich in platelets but also contain red blood cells both encased in a dense fibrin meshwork, indicating a role for soluble plasma factors driving thrombus formation.

The observation that p16INK4a transgenic mice exhibit shorter occlusion times in both of these models suggests that there is likely a contribution by both soluble plasma factors and circulating cells. The differences observed in the time to flow restriction may reflect altered rates of thrombus growth between wild-type and p16INK4a transgenic mice, which could be indicative of the potential to produce larger thrombi.

In addition to more rapid rates of venous occlusion, p16INK4a transgenic mice also display impaired thrombus resolution. The percentage of occlusion appears to be correlated with the sustaining of inflammatory infiltration. It is possible that the inability to clear residual FeCl3 from the intravascular space could be involved in further promoting thrombus formation. The increased production of PAI-1 observed in p16INK4a transgenic mice could also partly explain the thrombus resolution defect (Supplemental Figure III). Evidence in the literature suggests increased circulating PAI-1 could have a negative impact on wound healing and fibrinolysis. Originally, Farrehi et al demonstrated enhanced fibrinolysis in PAI-1-deficient mice. Eitzman et al found that transgenic mice overexpressing PAI-1 have more severe fibrosis following bleomycin-induced lung injury.

Zaman et al showed a profibrotic effect of PAI-1 overexpression in the heart following myocardial infarction. Recently, McDonald et al demonstrated that aged mice display impaired thrombus resolution following stasis induced by inferior vena cava ligation. In addition, they reported differences in various plasma and venous endothelium-associated proteins between aged and young wild-type mice. Although an exact mechanism to account for the observed thrombus resolution defect in the aged mice is not yet known, it is possible that changes in both the vessel wall and soluble plasma factors contribute, which may also be true of p16INK4a overexpressing mice.

To better understand differences in thrombus formation between p16INK4a transgenic and wild-type mice, coagulation parameters in mouse plasma samples were analyzed by calibrated automated thrombography after inducing endothelial dysfunction with LPS. Plasma analysis by calibrated automated thrombography was sensitive to changes in coagulation factor levels and able to detect differences in thrombin generation parameters following a thrombotic event in human patients. Our results show that p16INK4a transgenic mice are able to initiate thrombin generation faster, achieve a higher peak amount of thrombin, and peak at a faster rate than wild-type controls. Therefore, p16INK4a transgenic mice exhibit greater thrombin generation after LPS challenge compared with wild-type controls. To complement these data, p16INK4a transgenic mice also showed elevated plasma levels of thrombin-antithrombin and PAI-1 following LPS challenge. These markers are commonly used to measure activa-
tion of coagulation (thrombin-antithrombin) and endothelial activation (PAI-1).\textsuperscript{48,49} Yamamoto et al showed that aged mice had elevated induction of PAI-1 compared with young mice after LPS treatment, suggesting that PAI-1 is important in endotoxin-induced thrombosis.\textsuperscript{31} Because PAI-1 is both a marker of endothelial cell senescence and a potent fibrinolysis inhibitor,\textsuperscript{30} it could also participate in the delayed thrombus resolution seen in p16\textsuperscript{INK4a} transgenic mice.

To begin establishing a mechanism for the observed differences between wild-type and p16\textsuperscript{INK4a} transgenic mice, bone marrow transplants were performed to determine the contribution of hematopoietic cells to the prothrombotic phenotype. We found that wild-type mice given p16\textsuperscript{INK4a} transgenic bone marrow had occlusion times very similar to those of transgenic controls. Similarly, p16\textsuperscript{INK4a} transgenic mice given wild-type bone marrow had occlusion times very similar to those of wild-type controls. These data show that the prothrombotic phenotype observed in mice overexpressing this gene is attributed to p16\textsuperscript{INK4a} expression in hematopoietic cells.

A growing body of in vitro evidence suggests that senescence in the vascular endothelium may also participate in the transition to a procoagulant state during aging. Senescence in the vascular endothelium is associated with an array of phenotypic changes with pathological consequences.\textsuperscript{7,8} These changes include upregulation of PAI-1, inflammatory cytokines (including interleukin-1 and interleukin-6), matrix metalloproteinases, and the downregulation of endothelial nitric oxide synthase.\textsuperscript{35,36,51} Thus, a role for the endothelium cannot be discounted and may warrant further investigation in this model.

In contradistinction to the present data supporting a role for p16\textsuperscript{INK4a} in venous thrombosis, a differing role for the expression of p16\textsuperscript{INK4a} in arterial vascular diseases has been suggested. Through genome-wide association studies, several groups have found a link between single-nucleotide polymorphisms on chromosome 9p21.3 close to the p16\textsuperscript{INK4a} open-reading frame and several atherosclerotic diseases (coronary artery disease, ischemic stroke, abdominal aortic aneurysm).\textsuperscript{52} Liu et al have recently shown that individuals harboring the single-nucleotide polymorphism genotypes associated with increased atherosclerotic risk exhibit decreased expression of p16\textsuperscript{INK4a} and other INK4/ARF transcripts.\textsuperscript{53} Individuals at increased risk appear to differ in the expression and splicing of linear and circular forms of ANRIL, a long, noncoding RNA emanating from the INK4a/ARF locus thought to participate in INK4a/ARF expression.\textsuperscript{54} This observation suggests that decreased production of p16\textsuperscript{INK4a} is associated with an increased risk of atherosclerosis, likely through limiting aberrant or excess proliferation of cellular components of atheromatous plaques. This suggests that expression of antiproliferative molecules at the INK4a/ARF locus protects individuals from atherosclerosis.\textsuperscript{52,55} In accord with this view, mice lacking p16\textsuperscript{INK4a} have been shown to be more prone to vessel occlusion in a carotid artery injury model.\textsuperscript{56} Our current data, combined with prior work in the venous system,\textsuperscript{35,36} suggest the intriguing possibility that age-induced p16\textsuperscript{INK4a} expression and cellular senescence might play opposing roles with regard to thrombosis and atherosclerosis in the venous and arterial systems, respectively.

Characterizing the link between age-related genetic changes and age-related cardiovascular diseases, such as venous thrombosis, is of paramount importance. Overexpression of proteins such as p16\textsuperscript{INK4a}, which promote senescence and vascular dysfunction, could be the key age-related genetic change explaining cardiovascular maladies. Together, our results demonstrate that p16\textsuperscript{INK4a} overexpression and cellular senescence contribute to a prothrombotic phenotype and defective thrombus resolution. The results of this study provide the foundation for research on the effects of vascular senescence on venous thrombosis.

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**Disclosures**

None.

**References**

Role of p16\(\text{INK4a}\) Overexpression in Thrombosis


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Supplementary File

Overexpression of the Cell Cycle Inhibitor p16\textsuperscript{INK4a} Promotes a Prothrombotic Phenotype Following Vascular Injury in Mice

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Methods

Mice

These animals harbor a single copy integration of 60 kb of the murine p16\textsuperscript{INK4a} locus, and exhibit a 3-8 fold increase in p16\textsuperscript{INK4a} expression in all tissues examined to date (Supplemental Figure I). These animals do not overexpress other transcripts from the Ink4/Arf locus (i.e., p15\textsuperscript{INK4b} and Arf) (data not included). Animals were of the C57BL/6J genetic background and backcrossed as detailed previously\textsuperscript{1}. Experiments were done on male and female littermate progeny of indicated genotypes. In all cases the mice were 6-8 weeks of age. No differences were detected between males and females in the experiments performed.

Real-Time PCR

Quantitative Real-time PCR analysis was performed as previously described\textsuperscript{1}. Briefly, representative tissues were collected from transgenic and wild-type littermates used in thrombosis models for PCR analysis with the following primers. Forward primer sequence: CGGTCGTACCCCGATTCAG. Reverse primer sequence: GCACCGTAGTTGAGCAGAAGAG. Expression of ARF was also quantified as a control with
the following primers: Forward primer sequence: TGAGGCTAGAGAGGATCTTGAGAAG.
Reverse primer sequence: GTGAACGTTGCCCATCATCATC.

Immunochemistry

Tissues were extracted and fixed overnight in 4% paraformaldehyde. Tissue embedding and cutting was performed in the UNC Linberger Comprehensive Cancer Center Animal Histopathology Core Facility. Briefly, 5 micron sections were cut from paraffin blocks and antigen retrieval was performed in Target Retrieval Solution (Dako – Capinteria, CA, S1699) in a 95°C water bath. Slides were blocked for 1 hour in 1% BSA and stained with antibodies against p16\textsuperscript{INK4a} (Santa Cruz Biotechnology - Santa Cruz, CA, sc-1661, 1:200 dilution), Ki67 (Dako – M7249, 1:500 dilution), or PAI-1 (Santa Cruz Biotechnology – sc-8979, 1:250 dilution) for 1 hour at room temperature in a humidity-controlled chamber. Biotinylated secondary antibodies were obtained from Vector Laboratories. Tissue slides were developed using the avidin-biotin complex (ABC) method using reagents and protocols obtained from Dako. Negative control slides were stained simultaneously in the absence of primary antibody. Images were analyzed by taking a representative digital photograph of tissue from each mouse. A grid was laid over the images using ImageJ software and percent positive grid boxes were calculated by counting the number of grid boxes containing a positively stained cell and dividing it by the total number of grid boxes on the image.
**Complete Blood Count**

Whole blood collected by cardiac puncture into EDTA tubes was analyzed for complete blood counts (CBC) by the UNC-CH Division of Lab Animal Medicine. Blood samples were submitted from three mice per group.

**Prothrombin Time (PT)**

Prothrombin times were measured using a STart®4 semi-automated hemostasis analyzer (Diagnostica Stago – Parsippany, NJ) by mixing equal volumes platelet poor plasma, calcium chloride (25mM), and tissue factor (Innovin, 400 pM). Data represents pooled plasma from three mice per group.

**Thrombin Generation**

Thrombin generation was measured by calibrated automated thrombography (CAT) using Z-Gly-Gly-Arg-AMC fluorogenic substrate for thrombin (Diagnostica Stago, Parsippany, NJ) on a Fluoroskan Ascent fluorometer (ThermoLabsystem, Helsinki, Finland). Mouse plasma was analyzed as previously described\(^2\). Briefly, mouse plasma samples were pooled, diluted 1:4 in phosphate buffered saline and 80 µL of plasma was added to 20 µL low tissue factor (1 pM) reagent to initiate the reaction. Variations in plasma color were accounted for using a α2-macroglobulin/thrombin calibrator reagent (Diagnostica Stago, Parsippany, NJ). Parameters were calculated by Thrombinscope software version 3.0.0.29 (Thrombinscope BV, Maastricht, Netherlands).
**ELISAs**

TAT complexes were detected in mouse plasma using an Enzygnost TAT complex ELISA (Siemens – New York, NY, USA). Plasma samples were diluted 1:10 in Enzygnost ELISA sample buffer. PAI-1 was measured using an ELISA for mouse total PAI-1 from Molecular Innovations. Fibrinogen was measured using an ELISA for mouse fibrinogen from Molecular Innovations (Novi, MI, USA). Plasma samples from mice treated with LPS were diluted 1:40 and 1:160 for the PAI-1 and fibrinogen ELISAs, respectively. Plasma samples from control mice were diluted 1:5 for both ELISAs. All ELISAs were performed according to the company protocols and standard curves were generated using proteins supplied by the company.

**Statistics**

All statistical analyses were performed with Graphpad Prism. All measurements are represented as the mean ± standard error of the mean (SEM). One-way ANOVA or Students T-test was performed where indicated. One-way ANOVA was performed with a Tukey’s post-hoc test on measurements were indicated. For two group comparison of parametric data, a student’s t-test was performed where indicated. Values of p<0.05 were considered statistically significant.
**Results**

\(\text{p16}^{\text{INK4a}}\) transgenic mice show increased \(\text{p16}^{\text{INK4a}}\) expression and decreased cellular proliferation compared to wild-type.

In order to determine the utility of this model for vascular studies, saphenous veins and other tissues from mice were characterized by real-time PCR and immunohistochemistry as to \(\text{p16}^{\text{INK4a}}\) mRNA and protein expression, respectively. In accord with prior results, mRNA analysis showed between 2- and 6-fold increased expression of \(\text{p16}^{\text{INK4a}}\) over wild-type littermates in heart, lung and spleen (Supplemental Figure I A). Additionally, a six-fold increase in expression was noted in saphenous vein (Supplemental Figure I A). Immunohistochemical staining showed \(\sim\)30% positive staining for \(\text{p16}^{\text{INK4a}}\) in kidney cells of transgenic mice, and \(\sim\)3% positive staining for \(\text{p16}^{\text{INK4a}}\) in wild-type mice (Supplemental Figure I B). These results indicate that \(\text{p16}^{\text{INK4a}}\) expression is elevated in most tissues, including larger veins, of the transgenic mice compared to littermate controls, and that this increased expression is associated with decreased proliferation, as determined by staining for the proliferation marker Ki67 (data not shown).

\(\text{p16}^{\text{INK4a}}\) transgenic mice display enhanced thrombin generation in response to LPS challenge.

Mouse plasma was further analyzed to compare markers of coagulation and fibrinolysis. Fibrinogen was used as an acute phase reactant marker, no difference was detected by ELISA between wild-type and \(\text{p16}^{\text{INK4a}}\) transgenic mice (Supplemental Figure II A). Circulating TAT complexes peaked at 3 hours post LPS treatment and subsequently declined to untreated baseline
in both wild-type and p16\textsuperscript{INK4a} transgenic mice (Supplemental Figure IIB). While kinetic patterns were similar, transgenic mice had elevated TAT complex levels relative to wild-type at the 1 and 3 hour time points. Induction of PAI-1 following LPS treatment was similar in pattern to TAT complex formation between wild-type and transgenic mice. Peak PAI-1 levels were achieved at 3 hours post LPS treatment with a trending increase in PAI-1 in transgenic mice at this time point (Supplemental Figure IIC). Immunostaining of livers collected 3 hours post LPS treatment showed increased PAI-1 nuclear staining in p16\textsuperscript{INK4a} transgenic mice, but not in wild-type mice (Supplemental Figure IID). These results suggest the altered thrombin generation parameters seen in the transgenic mice are not due to general differences in liver function, but to enhanced activation of specific coagulation proteins in p16\textsuperscript{INK4a} transgenic mice.
References


### Supplemental Table I: Baseline Parameters in Wild-type vs. p16 Transgenic Mice*

<table>
<thead>
<tr>
<th>General Parameters</th>
<th>Wild-Type C57Bl/6</th>
<th>p16(^{INK4a}) Transgenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>22.9 ± 0.6</td>
<td>22.7 ± 1.7</td>
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<tr>
<td>Venous Flow Velocity (mm/s)</td>
<td>35.88 ± 4.11</td>
<td>38.85 ± 4.78</td>
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**Hemostasis Parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-Type C57Bl/6</th>
<th>p16(^{INK4a}) Transgenic</th>
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</thead>
<tbody>
<tr>
<td>Prothrombin Time (PT)</td>
<td>52.3 ± 0.5</td>
<td>52.4 ± 0.8</td>
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<tr>
<td>Fibrinogen (µg/ml)</td>
<td>29.2 ± 3.7</td>
<td>34.1 ± 10.4</td>
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<tr>
<td>PAI-1 (ng/ml)</td>
<td>0.012 ± 0.004</td>
<td>0.014 ± 0.004</td>
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<tr>
<td>TAT (ng/ml)</td>
<td>7.2 ± 3.0</td>
<td>9.2 ± 1.8</td>
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**Complete Blood Count**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-Type C57Bl/6</th>
<th>p16(^{INK4a}) Transgenic</th>
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</thead>
<tbody>
<tr>
<td>White Blood Cells (x 10(^6)/µL)</td>
<td>3.3 ± 1.3</td>
<td>2.1 ± 1.8</td>
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<tr>
<td>Red Blood Cells (x 10(^7)/µL)</td>
<td>8.82 ± 0.3</td>
<td>8.87 ± 1.1</td>
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<tr>
<td>Hemoglobin (g/dL)</td>
<td>13.7 ± 0.3</td>
<td>14.5 ± 0.9</td>
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<tr>
<td>Hematocrit (Vol %)</td>
<td>40.0 ± 1.3</td>
<td>42.2 ± 1.5</td>
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<tr>
<td>Mean Corpuscular Volume (fL)</td>
<td>45.3 ± 0.1</td>
<td>47.6 ± 1.0</td>
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<tr>
<td>Mean Corpuscular Hemoglobin (pg)</td>
<td>15.6 ± 0.2</td>
<td>16.3 ± 0.4</td>
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<tr>
<td>Mean Corpuscular Hemoglobin Concentration (g/dL)</td>
<td>34.3 ± 0.4</td>
<td>34.3 ± 0.8</td>
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<tr>
<td>Platelets (x 10(^7)/µL)</td>
<td>1294 ± 31.1</td>
<td>1406 ± 21.9</td>
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*Baseline parameters that could potentially affect thrombosis were compared in wild-type and p16\(^{INK4a}\) transgenic mice, as described in the Materials and Methods.*
Supplemental Figure Legends

Supplemental Figure I: p16$^{\text{INK4a}}$ mRNA and Protein Levels in Transgenic vs. Wild-type Mice. (A) Real-time quantitative PCR was performed on organs collected from transgenic and wild-type littermates. Data are expressed as fold change in transgenic mice over wild-type, as described in Materials and Methods. (B) p16$^{\text{INK4a}}$ protein was measured in kidneys of wild-type (top panel) and transgenic (middle panel) mice by immunostaining as described in the Supplemental Materials and Methods. Images were quantified in ImageJ and expressed as percent positive staining for p16$^{\text{INK4a}}$ (bottom panel). *denotes p<0.05 versus wild-type control by student t-test.

Supplemental Figure II: Plasma Analysis (A-C) and PAI-1 Levels in Liver (D) After LPS Treatment. Plasma collected from the IVC after LPS treatment was analyzed using ELISAs for fibrinogen (A) and circulating TAT complexes (B) and PAI-1 (C). *denotes p<0.05 versus respective wild-type control by student t-test. (D) PAI-1 protein levels were measured in wild-type (top panel) and transgenic (middle panel) livers by immunostaining as described in the Supplemental Materials and Methods. □ - Wild-Type, ■ - p16$^{\text{INK4a}}$ Transgenic

Supplemental Figure III: PCR Analysis of Bone Marrow Transplantation Recipients. Quantitation of PCR performed on bone marrow collected from transplantation recipient mice to confirm successful repopulation with donor cells. p16$^{\text{INK4a}}$ levels were compared to IL-2 and quantified using Image J software from a representative gel.
Supplemental Figures

Supplemental Figure I

A

B
Supplemental Figure II

A) Fibrinogen (µg/mL) vs. Treatment Time (Hrs)

B) TAT Level (ng/mL) vs. Treatment Time (Hrs)

C) PAI-1 ng/mL vs. Treatment Time (Hrs)

D) Images showing different staining patterns.
Supplemental Figure III

A

B

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<tr>
<th>Recipient</th>
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