**THROMBOSIS AND HEMOSTASIS**

**Acute and severe coagulopathy in adult mice following silencing of hepatic antithrombin and protein C production**

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**Key Points**

- RNA interference of *Serpinc1* and/or *Proc* allows for evaluation of the function of these genes, alone or in combination, in normal adult mice.
- RNA interference of *Serpinc1* and *Proc* provides a novel, controlled mouse model for spontaneous venous thrombosis.

Mice deficient in the anticoagulants antithrombin (*Serpinc1*) or protein C (*Proc*) display premature death due to thrombosis-related coagulopathy, thereby precluding their use in gene function studies and thrombosis models. We used RNA interference to silence *Serpinc1* and/or *Proc* in normal adult mice. The severe coagulopathy that followed combined “knockdown” of these genes is reported. Two days after siRNA injection, thrombi (occlusive) were observed in vessels (large and medium-sized) in multiple tissues, and hemorrhages were prominent in the ocular, mandibular, and maxillary areas. Tissue fibrin deposition and reduction of plasma fibrinogen accompanied this phenotype. The coagulopathy was prevented by dabigatran etexilate treatment. Silencing of *Serpinc1* alone yielded a comparable but milder phenotype with later onset. The phenotype was absent when *Proc* was targeted alone. We conclude that RNA interference of *Serpinc1* and/or *Proc* allows for evaluation of the function of these genes in vivo and provides a novel, controlled mouse model for spontaneous venous thrombosis. (*Blood* 2013;121(21):4413-4416)

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**Introduction**

Wild-type mice do not spontaneously develop venous thrombosis. Mice knockout for the natural anticoagulants antithrombin (*Serpinc1*) and protein C (*Proc*) feature spontaneous thrombosis-related coagulopathy,1,2 and succumb during embryogenesis and/or perinatally, precluding their use in studies on the function of these anticoagulants and as a model for thrombosis. To early death, we used RNA interference to silence *Serpinc1* and/or *Proc* production, alone or in combination, in livers of wild-type mice. Here, we report the spontaneous thrombotic phenotype that was observed shortly after this treatment.

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**Study design**

Effective synthetic siRNAs (Life Technologies, Carlsbad, CA) targeting *Serpinc1* and *Proc* were identified using mouse hepatocytes as described previously.3 Selected siSerpinc1, siProc (sequences, see supplemental Methods), and control siNEG (Life Technologies) complexed with Invivofectamine (Life Technologies) were injected into the tail veins of female C57Black/6J mice (Charles River, Maastricht, the Netherlands) age 8- to 10-weeks, alone or in combination. Mice were euthanized at different time points and subjected to necropsy according to international pathology guidelines.4 Liver *Serpinc1* and *Proc* transcript, plasma antithrombin, protein C, thrombin–antithrombin complexes, fibrinogen levels (prothrombin time [PT] and activated partial thromboplastin time [aPTT]), and tissue fibrin deposition were analyzed as described previously.3,5 Liver expression of antithrombin and protein C was analyzed as described previously.6 Leiden University’s animal welfare committee approved all experimental procedures.

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**Results and discussion**

Intravenous injection of siSerpinc1 or siProc (7 mg siRNA/kg body weight) resulted in a significant reduction in *Serpinc1* or *Proc* hepatic transcript (4 ± 0.3% and 11 ± 0.5% of siNEG-treated animals, respectively; n = 3) and plasma antigen level (1 ± 2.4% and 2 ± 1.8% of siNEG-treated animals, respectively; n = 3) after 2 days. It is important to note that consumption loss could have contributed to these low plasma levels. Consumptive loss, if present, did not coincide with detectable plasma thrombin–antithrombin complexes in siSerpinc1-treated animals (<2 ng/mL). After 2 days the animals appeared normal (observation in 6 animals per siRNA). A similar reduction in transcript and plasma antigen level was observed when animals were treated with a combination of siSerpinc1 and siProc (7 mg siRNA/kg body weight). The coagulopathy was prevented by dabigatran etexilate treatment. Silencing of *Serpinc1* alone yielded a comparable but milder phenotype with later onset. The phenotype was absent when *Proc* was targeted alone. We conclude that RNA interference of *Serpinc1* and/or *Proc* allows for evaluation of the function of these genes in vivo and provides a novel, controlled mouse model for spontaneous venous thrombosis. (*Blood* 2013;121(21):4413-4416)
Figure 1. Phenotypic appearance of mice following silencing of hepatic antithrombin and protein C production. (A) Effectiveness of silencing Serpinc1 and Proc in mouse liver. siRNAs targeting Serpinc1 and Proc were complexed, mixed, and intravenously injected into C57Black/6J mice at a dose of 3.5 mg/siRNA/kg (hatched bars, n = 13) or 7 mg/siRNA/kg (black bars, n = 6). At 2 days post siRNA injection, mice were removed from the experiment and euthanized; livers were subjected to Serpinc1 (left) or Proc (right) transcript analysis by quantitative polymerase chain reaction. β-actin was used as the internal control for quantification and normalization. The ΔCt values of the individual samples were related to the mean ΔCt of the reference group (siNEG-treated, 7 mg/kg, open bars, n = 11). (B) Plasma antithrombin (left) and protein C levels (right) in siNEG-injected mice (open circles), siSerpinc1/siProc-injected mice at 3.5 mg/siRNA/kg (open squares), and 7 mg/siRNA/kg (black squares). (C) Right eye of an siSerpinc1/siProc-injected animal. Unilateral severe exophthalmos and periorcular hemorrhages are shown. (D) Multifocal hemmorhages in the mandibular and submandibular area and masticator muscle. (E) Severe multifocal hemmorhages within the eye (Hematoxylin and Eosin [HE]-stained 5-μm section, magnification 40×). (F) Eye region: presence of harderian glands with multifocal hemmorhages (h) and thrombus (t) (HE-stained 5-μm section, magnification 100×). (G) Cyanosis of the right hind leg. (H) Hemmorhages (arrow) and thrombom (arrowheads) were present in the subcutis but also among muscular fibers of the tibial and femoral areas (HE-stained 5-μm section, magnification 200×). (I) Liver (formalin-fixed specimen) presenting focally extensive areas of necrosis (asterisk). (J) Liver section presenting severe multifocal to coalescing coagulative necrosis (cn) and thrombosis (t) in hepatic vein (HE-stained 5-μm section, magnification 100×). Liver fibrin (K) and plasma fibrinogen (L) in siNEG-treated mice (open circles), siSerpinc1/siProc-injected mice at 3.5 mg/siRNA/kg (open squares), and 7 mg/siRNA/kg (black squares). (M) Treatment of siSerpinc1/siProc-injected mice (3.5-mg/kg dose) with DE. DE was administered by oral gavage of 3 mg per mouse at 7 AM, 3 PM, and 11 PM for 5 days starting on the day before siRNA injection. Liver fibrin deposition in siSerpinc1/siProc-treated mice (3.5 mg/siRNA/kg) treated with DE (open squares) or vehicle (filled squares). Data were analyzed using the Instat software (GraphPad, San Diego, CA). Statistical differences between control siNEG and siSerpinc1, siProc, siSerpinc1/siProc were evaluated using a Mann-Whitney rank sum test. *P < .05, †P < .01, ‡P < .001.
In total, 19 of 19 siSerpinc1/siProc-treated animals (3.5 mg/siRNA/kg) demonstrated fibrin deposition (Figure 1K), coinciding with reduced plasma fibrinogen levels (Figure 1L). For the 7-mg/siRNA/kg dose, liver fibrin deposition was massive and plasma fibrinogen was virtually absent (Figure 1L), indicating a relationship with the siRNA dose. Liver fibrin deposition was at background levels in siNEG-treated animals (5.4 ± 2.7 ng/mg, n = 11) and siProc-treated animals (5.6 ± 2.7 ng/mg, n = 6). Remarkably, siSerpinc1-treated animals had low liver fibrin deposition (10.7 ± 3.1 ng/mg, n = 6). The siSerpinc1-treated animals displayed a later onset of the phenotype (reproduced in additional experiments; at both 3.5 and 7 mg/siSerpinc1/kg, n = 5 per dose) and unaffected plasma fibrinogen (data not shown), suggesting that a combination of siSerpinc1 and siProc results in a more severe phenotype than the sum of siSerpinc1 or siProc alone. Likely, combined loss of Serpin1 and Proc pushes the animals over a thrombotic threshold that cannot be reached with diminution of either one.

Tissue fibrin deposition was not restricted to the liver; lungs of siSerpinc1/siProc-treated mice demonstrated increased fibrin deposition (3.5-mg/siRNA/kg dose, 50 ± 38 ng/mg, n = 13 compared with 13 ± 7.3 ng/mg, n = 11 in siNEG; P = .0010), despite a minor incidence of microscopically visible thrombi in this tissue (1 of 12 animals).

The presence of thrombi (occlusive) and fibrin deposition in siSerpinc1/siProc-treated animals indicates that the observed coagulopathy is thrombotic in nature and that hemorrhages are likely secondary to consumption of fibrinogen and/or other coagulation factors. This is illustrated by prolonged PT and aPTT for 6 of 10 siSerpinc1/siProc-treated animals (>70 seconds and >120 seconds, respectively, compared with 11 ± 0.3 seconds and 27 ± 1.6 seconds for siNEG-treated animals; both P = .0039, Fisher test). To demonstrate that thrombin formation underlied this phenotype, siSerpinc1/siProc-treated mice (3.5 mg/siRNA/kg) were administered the thrombin inhibitor dabigatran etexilate (DE). DE prevented the clinical signs found in siSerpinc1/siProc-treated mice, including weight loss (−5.7 ± 5.2% compared with −11.2 ± 3.9% in vehicle-treated siSerpinc1/siProc animals; n = 16–17, P = .0011), exophthalmos, periocular contusion (0/17 compared with 16/16, P < .0001, Fisher test; Figure 1M), and largely suppressed liver fibrin deposition (Figure 1N).

We conclude that silencing hepatic Serpin1/Proc or Serpin1 alone acutely induces thrombotic coagulopathy. This study highlights the importance of protein C and antithrombin in animals under challenge-free conditions; points to synergism between these anticoagulant systems; will help to further unravel the in vivo function of these anticoagulants; and provides a novel, controlled model for venous thrombosis research.

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**Authorship**

Contribution: H.S., B.J.M.v.V., and P.H.R. designed the experiments; H.S., K.L.C., B.J.M.v.V., E.H.L., G.T.M.W., H.H.V., and...
D.S. performed experiments and analyzed data; B.J.M.v.V. wrote
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