**Phenotype of ribonuclease 1 deficiency in mice**

The MIT Faculty has made this article openly available. *Please share* how this access benefits you. Your story matters.

| Citation          | Garnett, Emily R. et al. "Phenotype of ribonuclease 1 deficiency in mice." RNA 25, 8 [May 2019]: 921-934 © 2019 Garnett et al |
|-------------------|----------------------------------------------------------------------------------------------------------------------------------|
| As Published      | http://dx.doi.org/10.1261/rna.070433.119                                                                                                                                 |
| Publisher         | Cold Spring Harbor Laboratory                                                                                                                                               |
| Version           | Final published version                                                                                                                                                     |
| Citable link      | https://hdl.handle.net/1721.1/123444                                                                                                                                         |
| Terms of Use      | Creative Commons Attribution NonCommercial License 4.0                                                                                                                    |
| Detailed Terms    | https://creativecommons.org/licenses/by-nc/4.0/                                                                                                                               |
Phenotype of ribonuclease 1 deficiency in mice

EMILY R. GARNETT,1,2 JO E. LOMAX,3 BASSEM M. MOHAMED,4 DAVID GAILANI,4 JOHN P. SHEEHAN,5 and RONALD T. RAINES2,6,7

1Graduate Program in Molecular and Cellular Pharmacology, University of Wisconsin–Madison, Madison, Wisconsin 53706, USA
2Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA
3Graduate Program Molecular and Cellular and Molecular Biology, University of Wisconsin–Madison, Madison, Wisconsin 53706, USA
4Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, Nashville, Tennessee 37232, USA
5Department of Medicine/Hematology–Oncology, University of Wisconsin–Madison, Madison, Wisconsin 53706, USA
6Department of Biochemistry, University of Wisconsin–Madison, Madison, Wisconsin 53706, USA
7Department of Chemistry, University of Wisconsin–Madison, Madison, Wisconsin 53706, USA

ABSTRACT

Biological roles for extracellular RNA (eRNA) have become apparent. For example, eRNA can induce contact activation in blood via activation of the plasma proteases factor XII (FXII) and factor XI (FXI). We sought to reveal the biological role of the secretory enzyme ribonuclease 1 (RNase 1) in an organismal context by generating and analyzing RNase 1 knockout (Rnase1–/–) mice. We found that these mice are viable, healthy, and fertile, though larger than Rnase1+/+ mice. Rnase1–/– plasma contains more RNA than the plasma of Rnase1+/+ mice. Moreover, the plasma of Rnase1–/– mice clots more rapidly than does wild-type plasma. This phenotype appeared to be due to increased levels of the active form of FXII (FXIIa) in the plasma of Rnase1–/– mice compared to Rnase1+/+ mice, and is consistent with the known effects of eRNA on FXII activation. The apparent activity of FXI in the plasma of Rnase1–/– mice was 1000-fold higher when measured in an assay triggered by a low concentration of tissue factor than in assays based on recalcification, consistent with eRNA enhancing FXI activation by thrombin. These findings suggest that one of the physiological functions of RNase 1 is to degrade eRNA in blood plasma. Loss of this function facilitates FXII and FXI activation, which could have effects on inflammation and blood coagulation. We anticipate that Rnase1–/– mice will be a useful tool for evaluating other hypotheses about the functions of RNase 1 and of eRNA in vivo.

Keywords: blood coagulation; eRNA; gene knockout; mouse; ribonuclease

INTRODUCTION

Ribonuclease 1 (RNase 1) is a vertebrate secretory protein with robust nonspecific ribonucleolytic activity. The physical and catalytic properties of this enzyme are well understood due to its similarity to RNase A, a bovine homolog of RNase 1 that served as a model protein for seminal studies in biological chemistry during the twentieth century (D’Alessio and Riordan 1997; Raines 1998; Marshall et al. 2008; Cuchillo et al. 2011). RNase 1 is a member of a large superfamily termed the pancreatic-type ribonucleases (ptRNases) or vertebrate secretory ribonucleases. ptRNases play diverse biological roles (Sorrentino 2010; Koczera et al. 2016; Lu et al. 2018) and might have evolved convergently in bacteria (Cuthbert et al. 2018).

RNase A is abundant in the pancreas of ruminants (Barnard 1969). This hypothesis is challenged by recent work demonstrating nondigestive activities when human RNase 1 variants and conjugates are administered exogenously in vitro and in vivo (Strong et al. 2012; D’Avino et al. 2016; Hoang et al. 2018).

RNase 1 has the highest catalytic activity and the widest expression pattern of the ptRNases, and is found in many bodily fluids (Morita et al. 1986; Su et al. 2004). In humans and mice, RNase 1 circulates in blood plasma at ~0.5 µg/mL (Weickmann et al. 1984). The major source of plasma RNase 1 is the vascular endothelium (Landré et al. 2002; Fischer et al. 2011), and the pH optimum for its enzymatic activity (7.3) is close to the pH of blood (Eller et al. 2014). These findings suggest that RNase 1 could be active within the vasculature.

© 2019 Garnett et al. This article is distributed exclusively by the RNA Society for the first 12 months after the full-issue publication date (see http://majournal.cshlp.org/site/misc/terms.xhtml). After 12 months, it is available under a Creative Commons License (Attribution-NonCommercial 4.0 International), as described at http://creativecommons.org/licenses/by-nc/4.0/.

Corresponding author: rtraines@mit.edu

Article is online at http://www.rnajournal.org/cgi/doi/10.1261/rna.070433.119.
Recent studies have highlighted the ability of RNase A to exert biological effects via degradation of extracellular RNA (eRNA), which is known to play roles in immunity and blood coagulation, as well as in cancer and atherosclerosis (Kannemeier et al. 2007; Fischer et al. 2012, 2013; Simsekylmaz et al. 2014). RNA enhances reciprocal activation of the blood plasma proteins factors XII (FXII) and prekallikrein to the active protease forms FXIIa and kallikrein by a process called contact activation that requires physical interaction between RNA and the enzymes (Kannemeier et al. 2007). Both FXIIa and kallikrein catalyze reactions that are proinflammatory. RNA also enhances activation of the plasma coagulation protease FXI by both FXIIa and thrombin, promoting blood clotting (Gajsiewicz et al. 2017). The importance of these reactions in vivo is suggested by the observations that RNase A produces a beneficial anticoagulant effect in murine models of arterial thrombosis (Kannemeier et al. 2007) and stroke (Walberer et al. 2009), and that RNase 1 is cardioprotective in humans (Cabrera-Fuentes et al. 2015). Here, we report on the first generation of an Rnase1 knockout (KO) mouse and the characterization of its phenotype, focusing on plasma RNA and blood coagulation.

RESULTS

Generation of Rnase1<sup>−/−</sup> mice by Cre–LoxP recombination

Prior to generating Rnase1<sup>−/−</sup> mice, we sought to validate the use of murine RNase 1 as a model for the human enzyme. Previously, we showed that the murine RI–RNase 1 complex is highly similar to its human homolog in its three-dimensional structure (Lomax et al. 2014). Further, murine RNase 1 exhibits ribonucleolytic activity similar to that of the human enzyme (Lomax et al. 2017). Given that human Rnase1 is expressed ubiquitously (Futami et al. 1997), we analyzed 18 mouse tissues for Rnase1 expression by qPCR and found the mRNA to be expressed ubiquitously (Fig. 1A). The in vitro structure–function data, coupled with a similarly broad expression pattern in vivo, instilled confidence in murine RNase 1 as a model for human RNase 1.

Given the unknown biological function of RNase 1, we chose a conditional KO approach to generate mice lacking Rnase1 expression. The entire Rnase1 protein-coding exon was flanked with loxP sites in the gene-targeting vector, allowing Cre-mediated recombination to excise this exon.
and result in an ablation of Rnase1 expression. Disruption of the Rnase1 gene was verified by a Southern blot (Supplemental Fig. S1).

**Rnase1**<sup>−/−</sup> mice express neither Rnase1 mRNA nor RNase 1 protein

qPCR was performed to verify loss of Rnase1 expression in Rnase1<sup>−/−</sup> mice. Eighteen tissue types were tested for Rnase1 expression in Rnase1<sup>−/−</sup> and Rnase1<sup>+/+</sup> mice, and levels were normalized to three reference genes. Samples from Rnase1<sup>−/−</sup> mice exhibited virtually undetectable Rnase1 expression (average C<sub>T</sub> = 34 ± 0.3 cycles in samples with amplification) (Fig. 2A). Any nonzero values are likely due to cross-reactivity of the qPCR primers with mRNA of RNase1 homologs.

We performed zymogram assays of plasma samples to detect RNase 1 by its ribonucleolytic activity and by its electrophoretic mobility. Ribonucleolytic activity as determined by the intensity of all bands on the zymogram was reduced significantly in Rnase1<sup>−/−</sup> samples relative to Rnase1<sup>+/+</sup> samples, representing a 69 ± 6% loss of ribonucleolytic activity (Fig. 2B). A comparable reduction was apparent in solution-phase assays of ribonucleolytic activity in plasma samples, with an estimated total ribonuclease loss of 52 ± 5% in males and 47 ± 2% in females (Fig. 2C).

We hypothesized that residual ribonucleolytic activity in Rnase1<sup>−/−</sup> plasma is due to other ptRNases. Members of this superfamily migrate at distinct rates during SDS-PAGE due to variation in molecular mass and charge. Given that RNase 1 and other ptRNases can be glycosylated in vivo (Beintema et al. 1984; Salazar et al. 2014; Ressler and Raines 2019), we treated plasma samples with PNGase F to reduce sample complexity, and measured mobility of the major ensuing band relative to an aglycosylated recombinant murine RNase 1 standard produced in E. coli. We found that whereas RNase1<sup>+/+</sup> plasma produced a band with mobility 101.2 ± 0.5% that of the RNase 1 standard, Rnase1<sup>−/−</sup> plasma does not, with the closest mobility being 103.5 ± 0.5% that of the standard (Fig. 2D). These results indicate that RNase 1 production was lost in Rnase1<sup>−/−</sup> mice, and confirm that residual ribonucleolytic activity in these mice arises from the activity of other ribonucleases. qPCR analysis of secretory ribonuclease genes in Rnase1<sup>−/−</sup> and Rnase1<sup>+/+</sup> showed the up-regulation of transcripts upon loss of Rnase1, notably those of Rnase4 and Ang1 (Fig. 3). qPCR analysis of Rnh1 mRNA showed less variation, except in skeletal muscle (Fig. 3).

**Rnase1**<sup>−/−</sup> mice are viable and appear physically normal

Genotyping of initial Rnase1<sup>fl/fox</sup> × B6.C-Tg(CMV-cre) 1Cgn/J progeny revealed that Rnase1<sup>−/−</sup> mice are viable. In heterozygote crosses, the Rnase1<sup>−</sup> allele was inherited according to expected Mendelian ratios, $\chi^2 (2, n = 243) = 9.62, P < 0.05$. Rnase1<sup>−/−</sup> males and females are both fertile, with crosses of Rnase1<sup>−/−</sup> mice producing litters of identical size and sex distribution ($7.4 \pm 1$ pups per litter, $39 \pm 9\%$ male, $n = 67$) to Rnase1<sup>+/+</sup> mice ($7.8 \pm 2$ pups per litter, $39 \pm 11\%$ male, $n = 39$).

Rnase1<sup>−/−</sup> mice of both sexes are significantly heavier than are Rnase1<sup>+/+</sup> mice (Fig. 4A,B), but do not exhibit any other obvious physical phenotype. Gross necropsy and histopathological analyses did not reveal differences between Rnase1<sup>−/−</sup> and Rnase1<sup>+/+</sup> littermates (data not shown). Rnase1<sup>−/−</sup> also exhibit similar longevity to Rnase1<sup>+/+</sup> mice, with a median survival time of 102 wk versus 126 wk (Fig. 4C).

**Loss of Rnase1 results in increased plasma RNA**

Given the reduced plasma ribonucleolytic activity in mice lacking Rnase1 (Fig. 2B–D), we anticipated elevated plasma RNA concentrations in Rnase1<sup>−/−</sup> mice. RNA was isolated from plasma and quantified by spectrophotometry and with a Bioanalyzer electrophoresis system. Whereas their plasma RNA does not appear to differ in size (Supplemental Fig. S2), Rnase1<sup>−/−</sup> mice have significantly elevated levels of RNA in their plasma compared to Rnase1<sup>+/+</sup> mice (Fig. 4D, E). The slightly higher level of RNA observed by spectrophotometry (Fig. 4D) is likely due to the Bioanalyzer only detecting RNA strands that contain >6 nt (Fig. 4E).

**Rnase1**<sup>−/−</sup> plasma clots more quickly than does Rnase1<sup>+/+</sup> plasma

Extracellular RNA can stimulate plasma coagulation in vitro through activation of the contact system (Kannemeier et al. 2007). To evaluate whether increased endogenous RNA in Rnase1<sup>−/−</sup> mice results in enhanced plasma coagulation, we carried out a series of kinetic coagulation studies.

Plasma collected into sodium citrate from Rnase1<sup>−/−</sup> mice formed fibrin clots significantly more quickly after recalcification than did plasma from Rnase1<sup>+/+</sup> mice. The respective values were 7.2 ± 1.0 min and 10.6 ± 2.0 min for males, and 5.9 ± 0.8 min and 23.9 ± 6.2 min for females (Fig. 5A,B). Induction of coagulation via the intrinsic pathway with Actin FSL activated partial thromboplastin time (aPTT) reagent reduced clotting time relative to unstimulated plasma, but did not reveal significant differences between clotting in plasma from Rnase1<sup>−/−</sup> and Rnase1<sup>+/+</sup> mice. This distinction is expected, as the aPTT reagent contains a charged substance that will substitute for RNA as an inducer of contact activation.

Inducing coagulation through the extrinsic pathway by addition of a tissue factor–containing reagent (Thromborel S) also resulted in significantly shorter clotting times in Rnase1<sup>−/−</sup> samples relative to Rnase1<sup>+/+</sup> in female mice.
FIGURE 2. Verification of the loss of RNase 1 in Rnase1−/− mice. (A) Bar graph showing the tissue-specific expression of Rnase 1 in Rnase1−/− mice relative to that in Rnase1+/+ littermates as measured by qPCR. Mice were matched for sex. n = 3 per tissue. Data were normalized to the geometric mean of three reference genes. (B) Zymogram of plasma samples (0.5 µL) from Rnase1+/+ or Rnase1−/− mice used to evaluate ribonucleolytic activity relative to recombinant murine RNase 1 (100 pmol; arrow). Graph showing total ribonucleolytic activity, which was quantified by integration of a densitometry map of each lane and found to be significantly lower in Rnase1−/− mice than in Rnase1+/+ mice. (C) Graph showing plasma ribonuclease concentration by genotype and sex as estimated from ribonucleolytic activity. Concentration was calculated with Equation 1 and $k_{cat}/K_M = 8.6 \times 10^6 \text{M}^{-1} \text{sec}^{-1}$. n ≥ 6. (D) Zymogram of plasma samples (0.5 µL) treated with PNGase F and of recombinant murine RNase 1 (100 pmol; arrow). The mobility was measured from the position of the well, and normalized to the mobility of recombinant murine RNase 1 (center lane). The position of the primary band present in Rnase1−/− plasma differs from that in wild-type plasma (arrow).
This effect was detectable at a high (saturating) concentration of tissue factor (3.52 ± 0.40 min for Rnase1−/− vs. 5.26 ± 0.28 min for Rnase1+/+) but was more pronounced at a limiting concentration of tissue factor (5.81 ± 0.83 min for Rnase1−/− vs. 12.9 ± 3.5 min for Rnase1+/+).

Additional experiments were performed to investigate potential causes for the differences in coagulation times unrelated to contact activation and the intrinsic pathway. Tissue factor expression is elevated in obesity (Samad and Ruf 2013), and Rnase1−/− mice are heavier than Rnase1+/+ mice. Tissue factor expression was, however, similar in Rnase1−/− and Rnase1+/+ mice (Fig. 5C). Fibrinogen levels, as measured by ELISA, were likewise similar in the two mouse lines (Fig. 5D).

**Apparent FXII and FXI activity are elevated in plasma from Rnase1−/− mice**

Coagulation induced with Actin FSL aPTT reagent was similar in Rnase1+/+ and Rnase1−/− mice (Fig. 6A), indicating that the intrinsic pathway of coagulation is intact in both lines. The specific activity of FXII appeared, however, to be increased by two- to threefold in Rnase1−/− mice when clotting was induced without an activator, and more than 100-fold in the presence of Actin FSL, relative to Rnase1+/+ mice. Furthermore, apparent FXII activity was increased more than 1000-fold in Rnase1−/− mice when coagulation was induced with a low (0.1 µM) concentration of soluble tissue factor. FIX activity was similar in the two genotypes (Fig. 6A).

The variations in FXII and FXI activity, depending on the method of inducing coagulation, cannot be explained by differences in plasma concentrations of FXII and FXI antigen in the two mouse lines. Indeed, antigen concentrations of these proteins were similar in Rnase1−/− and Rnase1+/+ mice on an immunoblot (Fig. 6B,C).
FXIIa activity contributes to several host defense mechanisms, including inflammation through the generation of kallikrein and coagulation through the conversion of the plasma protein FXI to the serine protease FXIIa. Although the latter reaction is not required for normal blood clot formation at a site of vessel injury (hemostasis), it is implicated in thrombosis and other pathologic processes (Maas and Renné 2018).

Our studies indicate that increased RNA in the plasma of Rnase1−/− mouse has effects on FXII and FXI. Specifically, RNA supports conversion of these proteins to their active forms. The shortened recalcification clotting times (clotting in the absence of aPTT reagent or tissue factor) indicate that levels of the activated forms of at least some coagulation factors are increased in the plasma of Rnase1−/− mice. The observations that plasma clotting times wherein coagulation was induced with tissue factor were shorter for Rnase1−/− mice than for Rnase1+/+ animals, whereas clotting times were similar in reactions with aPTT reagent, points to increased activity in the classic intrinsic pathway of coagulation in Rnase1−/− mice.

The intrinsic pathway is comprised of the components of the kallikrein–ki nin system, and the plasma clotting proteins factors IX and VIII. The activity levels of factors XII, XI, and IX were measured with one-stage clotting assays, in which an unknown plasma is tested for its capacity to restore clotting in a reagent plasma lacking the protein of interest. Results are compared to those of a standard plasma assigned an activity value of 100%. Values in excess of 100% indicate that the concentration of a protein is higher than that of the control plasma. This conclusion is based on the assumption that the clotting factor being measured is in its inactive precursor form at the start of the assay. If this assumption is valid, then results for different types of assays should agree on the activity level, and the activity level and antigen level should match. When these equivalences are not observed, then the active forms of one or more clotting factors are likely present in plasma prior to the start of the assay. This latter circumstance was observed for FXII and FXI in Rnase1−/− mice.

There was an apparent 100-fold discrepancy in FXII activity as determined by recalcification times and aPTT assays in plasma from Rnase1−/− mice, consistent with the presence of significant amounts of FXIIa in the plasma before the initiation of coagulation. The observation that the apparent increase in FXII activity was not accounted for by an increase in FXII antigen supports this conclusion. RNA induces FXII autoactivation and enhances PK activation by FXIIa (Kannemeier et al. 2007; Ivanov et al. 2017). Although FXIIa in plasma might be expected to result in increased FXIIa levels, that is not necessarily the case. In patients with deficiency of the FXIIa and kallikrein regulator C1 inhibitor, the consequence of unregulated FXIIa
activity is enhanced bradykinin generation and tissue swelling (angioedema), and not excessive coagulation (thrombosis). Increased FXIIa, then, does not necessarily translate into increased coagulation.

The results for FXI activity in this study are particularly intriguing. FXI levels based on recalcification times, aPTT assays, and coagulation induced with a saturating concentration of tissue factor roughly agreed with each other, and with results from \( \text{Rnase}^1/\text{+} \) mice, which indicated relatively little FXIa in plasma from \( \text{Rnase}^1/^\text{−} \) mice. Nonetheless, assays using a low concentration of tissue factor indicated a ∼10^3-fold higher level of FXI relative to plasma from \( \text{Rnase}^1/\text{+} \) mice. As with FXII, this increase was not accounted for by differences in the plasma FXI antigen levels. Polyanions, including RNA, enhance FXI activation by thrombin (Ivanov et al. 2017). This enhancement contributes little to clotting times in assays with saturating tissue factor because the initial burst of generated thrombin overwhelms contributions from the intrinsic pathway. At low tissue factor concentrations, however, the slower rate of thrombin generation allows feedback activation of FXI to FXIa by thrombin to affect the clotting time (Kravtsov et al. 2009). The large apparent increase in FXI activity, as determined by clotting in the presence of low tissue factor, is consistent with the presence of a polyanionic cofactor such as RNA in the plasma of \( \text{Rnase}^1/^\text{−} \) mice.

It is important to note that elevated RNA levels in \( \text{Rnase}^1/^\text{−} \) mice did not affect hemostatic or thrombotic responses appreciably in vivo. In the case of the hemostatic response to blood vessel injury, this result is not surprising, as a process that primarily affects FXII and FXI would not be expected to have much impact on hemostasis (Pauer et al. 2004). Although elevated plasma RNA levels in \( \text{Rnase}^1/^\text{−} \) mice might have rendered the animals more prone to injury-induced thrombosis, local tissue damage could have masked the effects of plasma RNA. It does seem reasonable to conclude, however, that the elevated RNA in \( \text{Rnase}^1/^\text{−} \) mice was not sufficient to induce thrombosis by itself. Still, our results indicate that elevated plasma RNA enhances the basal level of FXII activation, which

![FIGURE 5. Effect of Rnase1 on coagulation in vitro. (A) Graph showing the effect of activators Thromborel S (TF) and Actin FSL aPTT reagent (Actin) on the clotting of plasma from male mice. Rnase1^−/−^ plasma from mice clots significantly faster than does Rnase1^+/+^ plasma in the absence of added activator. n ≥ 3. (B) Graph as in panel A but with female mice. Rnase1^−/−^ plasma clots significantly faster than does Rnase1^+/+^ plasma in the absence of added activator and in the presence of Thromborel S. n ≥ 3. (C) Representative immunoblot to probe for tissue factor in the liver and graph showing the quantitation of band intensities in Rnase1^−/−^ and Rnase1^+/+^ mice. No significant difference was detected in the tissue factor level by genotype. n = 4. (D) Graph showing the fibrinogen level of plasma as assayed by ELISA in Rnase1^−/−^ and Rnase1^+/+^ mice. No significant difference was detected in fibrinogen level by genotype. n = 13. (*) P < 0.05.](https://www.rnajournal.org/927)
might predispose animals to inflammation or thrombosis in the context of injury, infection, or cancer (Leon et al. 1977; Garcia et al. 2008; Fischer et al. 2013). Differences in coagulation in vitro appear to be more robust by genotype in female mice than in male mice. This distinction could be due to hormonal differences between sexes, as hormonal contraceptive use and pregnancy are known to influence risk for aberrant blood clot formation (Artero et al. 2012; Bleker et al. 2014). Indeed, the FXII gene in humans is known to contain an estrogen-response element (Farsetti et al. 1995). Our analyses used nulliparous mice, but were performed in sexually mature mice and did not control for the estrus cycle in females, which could have contributed to trends in the data.

RNase 1 is one of ∼25 homologous ptRNases in mice (Cho et al. 2005). The mild phenotype resulting from the loss of RNase 1 could be due to functional redundancy, with RNase 1 homologs performing crucial RNA-regulatory functions (Cormier et al. 2001; Dyer and Rosenberg 2005). The importance of such functions is suggested by the increase in activity of other ptRNases in Rnase1−/− mice, which could mitigate the consequences of RNase 1 loss. RNase 4, which is up-regulated in Rnase1−/− mice, is a likely candidate to compensate, as it exhibits similar substrate preferences and is also expressed broadly (Sorrentino 2010).

The cause of the increased body weight of Rnase1−/− mice was not established by our studies but is not explicable by differences in food or water consumption, or body fat. Rnase1−/− mice do, however, have slightly longer bodies than Rnase1+/+ mice (data not shown). This difference suggests changes in growth factor activity in Rnase1−/− mice.

Given the proposed roles for RNA and RNase 1 in host defense, cancer, and blood coagulation (Sorrentino 2010; Koczera et al. 2016; Lu et al. 2018), our findings likely reveal only one of the biological functions of this enzyme, but do have implications for multiple processes. FXIIa promotes kallikrein formation, which in turn cleaves HK to liberate the vasoactive peptide bradykinin (Iwaki and Castellino 2006; Kaplan and Joseph 2014), an important mediator of inflammation and vascular permeability (Golas et al. 2007). Cancer patients are prone to coagulopathy (Falanga et al. 2014), and tumor-associated and circulating cancer cells express tissue factor (Falanga et al. 2015; Mitrugno et al. 2016) and activate FXII (Nickel et al. 2016). Moreover, RNA stimulates proinflammatory cytokine release, including tumor necrosis factor α (TNFα) and vascular endothelial growth factor (VEGF), promoting tumor cell growth and immune cell recruitment (Fischer et al. 2012, 2013). Plasma RNA could affect these biological processes, and a major function of RNase 1 could be to regulate such effects. We note, too, that eRNA secondary structure is thought to be important for its procoagulant activity (Gansler et al. 2012). Human RNase 1, which has higher activity against double-stranded RNA than does either mouse RNase 1 or RNase A (Eller et al. 2014; Lomax et al. 2017), might be better adapted to regulate endogenous eRNA alone.

## MATERIALS AND METHODS

### Materials

Murine ribonuclease 1 (RNase 1) and ribonuclease A were produced in *E. coli* as described previously (delCardayré et al. 1995; Lomax et al. 2014). PNGase F was from New England BioLabs. Human FXI-, FXII-, and PK-deficient plasma was from George King Bio-Medical. Murine FXI- and FXII-deficient plasma (Gailani et al. 1997; Pauer et al. 2004), and FIX-deficient plasma (Lin et al. 1997) were obtained as described. Soluble
tissue factor was a gift from B.S. Schwartz (University of Wisconsin–Madison).

Phosphatidylserine (PS), phosphatidylcholine (PC), and cholesterol were from Avanti Polar Lipids. PC:PS:C (molar ratio, 75:25:20) phospholipid vesicles were prepared by extrusion through a 100-nm polycarbonate filter. Primers, DNA constructs, and the 6-FAM–dArUdAdA–6-TAMRA ribonuclease substrate were from Integrated DNA Technologies. Poly(cytidylic acid)–the 6-FAM–6-TAMRA ribonuclease substrate (dArUdAdA) was from Sigma–Aldrich.

Equipment

Fluorescence measurements were made with a Tecan M1000 fluorescence plate reader.

Conditions

All procedures were performed at ambient temperature (≈22°C) and pressure (1.0 atm) unless noted otherwise.

**Generation of Rnase1 knockout mice**

All experiments with animals were conducted in accord with an Institution on Animal Care and Use Committee-approved protocol at the University of Wisconsin–Madison. Ribonuclease 1 KO mice (Rnase1<sup>/−/−</sup>) were generated by using recombinering methods (Liu et al. 2003). The Rnase1 targeting vector was generated via homologous recombination in *E. coli*. To target the mouse Rnase1 gene for deletion in vivo, loxP sites were engineered within intronic sequences to flank exon 2 (which contains the entire coding sequence of the gene), marking this region for eventual excision by Cre recombinase. A neomycin-resistance (Neo) cassette, flanked by FRT sites, was inserted for positive selection, and thymidine kinase (TK) was included for negative selection. The targeting vector was introduced into 129Sv/Ev murine embryonic stem (ES) cells, and positively selected clones were verified with a Southern blot. Correctly targeted clones were sequenced to confirm that all loxP and FRT sites were present and intact. Male germline chimeras were mated to wild-type C57Bl/6 females, transmitting the targeted Rnase1 allele. Karyotype analysis and chromosome counting was performed at the Molecular Cytogenetics Laboratory at Yale University. Blastocyst injection was performed by the University of Wisconsin–Madison Transgenic Animal Facility. Ribonuclease 1-deficient mice (Rnase1<sup>/−/−</sup>) were generated with assistance from the University of Wisconsin–Madison Transgenic Animal Facility. The KO mice were produced in a C57Bl6J (Stock 000664, Jackson Laboratories)/129SvJ (embryonic stem cells, Genome Systems) background. Neomycin-resistance cassettes used for selection were removed by crossing transgenic mice with FLP deleter mice (Stock 009086, Jackson Laboratories), and constitutive KO of Rnase1 was achieved by crossing Rnase1<sup>floox/floox</sup> mice with ubiquitously driven CMV-Cre mice (Stock 009086, Jackson Laboratories) (Fig. 1B). The genotypic analysis of Rnase1<sup>/−/−</sup> mice was performed by using a universal primer set for the Rnase1 locus: forward 5′–TGCAGGGACTAGGGTAGTG-3′ and reverse 5′–CATGACACAGGAGCAGAAG-3′. This primer set produces a 797-bp band for the wild-type locus, a 2.77-kb band for the transgenic locus + neomycin-resistance cassette, a 2.08-kb band for the KO locus + neomycin-resistance cassette, a 973-bp band for the transgenic locus–neomycin-resistance cassette, and a 286-bp band for the KO locus–neomycin-resistance cassette (Fig. 1C,D). The phenotypic analysis of Rnase1<sup>/−/−</sup> mice was carried out prior to the completion of backcrossing, and the experimental cohorts have a mixed C57Bl6/129SvJ background. To control for this mixed genetic background, all phenotypic analyses were
conducted with Rs nase1+/+ littermates of Rs nase1−/− mice serving as age- and sex-matched controls.

**Quantitative PCR**

Quantitative real-time PCR (qPCR) was conducted to identify gene expression changes in Rs nase1−/− mice. RNA was isolated from tissue samples homogenized in TRIzol Reagent (Invitrogen) using a Benchmark D1000 homogenizer, purified according to the manufacturer’s protocol, DNase-treated using the TURBO DNA-free Kit (Thermo Fisher Scientific), and subjected to reverse transcription–PCR using the qScript CDNA Synthesis Kit (Quanta Biosciences). cDNA preparations were probed for Rs nase1, Rs nase4, Ang1, and Rnh1 message using primer pairs shown in Table 1. Reactions were run in duplicate using SYBR Green master mix (Quanta Biosciences) with an Applied Biosystems ABI 7500 Fast Real-Time PCR system. Cycling conditions were 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, repeated for 30 cycles and monitored by melting curves. Cycle threshold (Ct) values were determined by setting a constant threshold at 0.6; fold changes in gene expression were determined by the comparative Ct method (Schmittgen and Livak 2008). Expression was normalized to that of Gapdh, Rpl13a, and Hprt1.

**Gross phenotypic analysis**

The viability of Rs nase1−/− mice was determined with a χ²-squared test. Fertility was assessed by pairing Rs nase1−/− male × Rs nase1+/− female, Rs nase1−/− male × Rs nase1−/− female, and Rs nase1−/− × Rs nase1+/− mice for mating. In longevity studies, aged mice were killed upon exhibiting signs of illness or distress (e.g., significant weight loss, tumors, reduced movement, or responsiveness).

Gross physical analysis and histopathological analysis of mice were performed by the University of Wisconsin–Madison Research Animal Resource Center Comparative Pathology Laboratory.

**Preparation of plasma samples**

Whole blood was collected by cardiac puncture into citrate-rinsed syringes immediately prior to mixing with 3.2% w/v sodium citrate (citrate/blood 1:9). Blood was subjected to centrifugation at 2400g for 10 min at 4°C to prepare platelet-poor plasma for subsequent assays. Plasma samples were aliquoted and snap-frozen, then stored at −80°C until the time of analysis. For mixing tests, blood was drawn from the inferior vena cava and prepared similarly.

**Ribonucleolytic activity assay**

As a proxy for protein concentration, solution-phase assays of ribonucleolytic activity were performed on plasma samples, using a fluorogenic substrate described previously (Kelemen et al. 1999). The background fluorescence intensity (I₀) of 100 mM Tris–HCl buffer, pH 7.0, containing 6-FAM–dArUdAdA–6-TAMRA (10 µM), NaCl (100 mM), and acetylated bovine serum albumin (0.1 mg/mL) was measured. A 1-µL aliquot of plasma was added, and the change in fluorescence intensity (ΔI) over time was monitored with λₑₓ = 490 nm and λₑₘ = 525 nm. Excess RNase A was added, and maximum fluorescence intensity (Iₘ₉₉) was measured. Recombinant murine RNase 1 was used to measure kₐₙ/Kₘ = 8.6 × 10⁵ M⁻¹ sec⁻¹, which is indistinguishable from the value at pH 6.4 reported previously (Lomax et al. 2017). This value was, in turn, used to estimate the concentration of ribonuclease in mouse plasma with Equation 1:

\[
\text{[ribonuclease]} = \frac{\Delta I}{k_{\text{cat}} I_{\text{max}}} = \frac{I_{\text{max}} - I_{\text{0}}}{k_{\text{cat}} K_M}.
\]

The ensuing value of [ribonuclease] is only an estimate because other plasma ribonucleases could differ in their kₐₙ/Kₘ values for the cleavage of 6-FAM–dArUdAdA–6-TAMRA.

**Ribonuclease zymogram assay**

Zymograms were performed on plasma samples to verify the loss of RNase 1 in Rs nase1−/− mice, using an assay similar to one reported previously (Bravo et al. 1994). Briefly, a polyacrylamide gel containing SDS (0.1% w/v), Tris (375 mM), and poly(cytidylic acid) (4.5 mg, incubated at 50°C prior to addition) was cast. Samples containing recombinant murine RNase 1 (100 pmol) or murine plasma (0.5 µL) were prepared with 2× Laemmli sample buffer (without dithiothreitol or β-mercaptoethanol, and without boiling). Some samples were prepared from murine plasma pretreated by incubating 2 µL of plasma with 1000 U of PNGase F (New England BioLabs) for 48 h to remove N-linked glycans.
from RNase 1 (Ressler and Raines 2019). After electrophoresis, the gel was washed twice in 10 mM Tris–HCl buffer, pH 7.5, containing isopropanol (20% v/v), then once in 10 mM Tris–HCl buffer, pH 7.5, before incubation overnight in 100 mM Tris–HCl buffer, pH 7.5 to allow RNase 1 to refold within the gel. During the next day, the gel was washed in 10 mM Tris–HCl buffer, pH 7.5 before staining in an aqueous solution of toluidine (0.2% w/v) for 10 min. The gel was washed with deionized water and de-stained in 10 mM Tris–HCl buffer, pH 7.5. Band intensity was evaluated with ImageJ software (Schneider et al. 2012).

**Quantitation of RNA in plasma**

The size and amount of RNA in mouse plasma was evaluated independently by using either a NanoVue Plus Spectrophotometer (GE Healthcare) or a 2100 Bioanalyzer electrophoresis system and associated RNA kit (Agilent). RNA was isolated from plasma with the TRIzol Reagent and TURBO DNA-free Kit as described above. For spectrophotometry, RNA (A260 nm/A280 nm ~ 2.0) was quantified by absorbance using the Beer–Lambert law and $\varepsilon = 0.025$ mg$^{-1}$·cm$^{-1}$·mL at 260 nm. RNA concentrations were normalized to the volume of plasma.

**Plasma coagulation assays**

Coagulation assays were performed on mouse plasma using a variation on published procedures (Jagadeeswaran et al. 2000). Briefly, mixtures contained plasma (10 µL), human fibrinogen (100 µg; Sigma–Aldrich), and PC:PS:C vesicles (4 µM), were induced to clot with CaCl$_2$ (6 mM). The reaction volume was increased to 100 µL by adding 20 mM HEPES–HCl buffer, pH 7.35, containing NaCl (20 mM) and BSA (1% w/v). A variation of the prothrombin time (PT) assay was conducted by adding 5 µL of Actin FSL Activated PTT reagent (Dade), at full strength or diluted 1:50. Clot formation was followed by monitoring $A_405$ nm over a period of ≤1 h. Clotting times are reported as the time at which the value of $A_{405}$ nm was half-maximal.

**Coagulation factor activity assays**

Activity assays for plasma factor IX, FXI, FXII, and prekallikrein were carried out by a mixing test using a variation of the kinetic coagulation assay described above. Briefly, 10 µL of factor-deficient human plasma was incubated with 10 µL of sample plasma to complement factor activity absent in the deficient plasma. (Sample was diluted in factor-deficient plasma in ratios ranging from 1:5 to 1:100, yielding samples with 20% to 1% of the normal factor activity.)

Some mixing tests were also performed with a Start 4 Hemostasis Analyzer (Diagnostica Stago). Briefly, 50 µL of factor-deficient human plasma was incubated with 25 µL of sample plasma (diluted in factor-deficient plasma) and 50 µL of Actin FSL Activated PTT reagent for 3 min at 37°C. An aqueous solution of CaCl$_2$ (17 mM) was added to initiate coagulation, and the time to clot formation was recorded.

Standard curves of factor activity as a function of plasma clotting time were generated by testing a pooled plasma set of five $\text{Rnase}^+/−$ mice and plotting clotting time (y) against plasma dilution factor (expressed as percent activity of undiluted plasma) on a log-linear chart, and the y-intercept (b) and slope (m) were calculated. Factor activity in $\text{Rnase}^+/−$ plasma relative to $\text{Rnase}^+/+$ plasma was calculated with Equation 2:

$$\text{activity} = 10^{b+y/m}. \quad (2)$$

**ELISA and immunoblotting**

An ELISA was performed to evaluate plasma fibrinogen content. Assays were performed with a Mouse Fibrinogen ELISA Kit (Abcam) according to the manufacturer’s instructions. Relative plasma concentrations for FXI, FXII, PK, and high molecular weight kininogen (HK) were determined by nonreducing immunoblotting using chemiluminescence. FXI was detected with biotinylated anti-mouse FXI IgG 14E11 and streptavidin-HRP (Cheng et al. 2010). FXII and PK were detected with polyclonal goat antihuman FXII or anti-human PK IgG conjugated to HRP (Affinity Biologicals). HK was detected with polyclonal rabbit IgG raised against mHK1-specific sequence, and donkey anti-rabbit IgG-HRP (Merkulov et al. 2008). Blots were imaged using a c600 system (Azure Biosystem) and band strength was assessed with ImageJ software (Schneider et al. 2012).

The relative expression of tissue factor in liver was determined by immunoblotting. Briefly, tissue samples were homogenized with a Benchmark D1000 homogenizer in T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific). Homogenized tissue and plasma samples were tested for protein content by using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific) and prepared with denaturing sample buffer prior to SDS–PAGE. Samples were transferred to a membrane using the iBlot 2 Dry Blotting System (Thermo Fisher Scientific) for 1 min at 20 V, 2 min at 23 V, and 1 min at 25 V. Membranes were blocked for 1 h in 5% w/v dry milk in Tris-buffered saline with Tween (TBST), which contained 19 mM Tris base, 137 mM NaCl, 2.7 mM KCl, and 0.1% v/v Tween 20, and incubated overnight at 4°C with antibody. Tissue factor was detected with a rabbit polyclonal IgG (Bioss Antibodies), and β-actin with a rabbit monoclonal IgG (Cell Signaling Technology). The membrane was washed in TBST before a 1-h incubation at room temperature with a secondary antibody, diluted with an aqueous solution of dry milk (5% w/v) in TBST, then washed in TBST again before imaging. The blot was treated with SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher Scientific) and imaged with an ImageQuant LAS 4000 imager (GE Healthcare), and band intensity was evaluated with ImageJ software (Schneider et al. 2012). Blots were stripped by using Restore Western Blot Stripping Buffer (Thermo Fisher Scientific) and probed again.

**Thrombin–antithrombin complex formation assay**

Thrombin–antithrombin (TAT) complex formation was measured as a marker of clot formation in response to LPS using a protocol adapted from one reported previously (Sommeijer et al. 2005). Briefly, mice were injected intraperitoneally with LPS (2 mg/kg...
of the lysate was used as a quantitative measure of blood loss.

considered significant.

MSTAT software (Michigan State University), with malin for fixation and staining.

rotid artery sections were collected into 4% neutral buffered for- rate. Mice were killed at the conclusion of the experiment, and ca-

have formed when blood flow dropped to <10% of its initial

and measure blood flow rate for 30 min. A clot was judged to

rebleeding. This procedure was repeated for 30 min while record-

lodged by wiping the vein in the direction of blood flow to allow

Time-to-cessation of bleeding was recorded, and the clot was dis-

sected free of membranes and punctured with a 27 g needle.

Briefly, the left saphenous vein of an anesthetized mouse was dis-

col adapted from one reported previously (Buyue et al. 2008).

Hemostasis in the saphenous vein was assessed by using a proto-

col adapted from one reported previously (Buyue et al. 2008).

Tail-bleeding assays were performed as described previously (Liu et al. 2012). Briefly, mice were anesthetized with isoflurane and placed on a warming pad, and a 2-mm section of distal tail was am-

After removal of the FeCl3 solution, an ultrasound gel was applied

FeCl3-induced thrombosis assay

Ferric-chloride induced arterial thrombosis assays were carried

out with assistance from the University of Wisconsin–Madison

Cardiovascular Physiology Core Laboratory. The left carotid artery

of anesthetized mice was dissected free of membranes and punctured with a 27 g needle. Time-to-cessation of bleeding was recorded, and the clot was dis-

lodged by wiping the vein in the direction of blood flow to allow

Statistical analyses

A minimum of three biological replicates were performed for ev-

ery experiment. All values are expressed as the mean ± SEM, and statistical evaluation was performed by Wilcoxon rank-sum test, log-rank test, and χ² test using Prism software (GraphPad) and MSTAT software (Michigan State University), with P < 0.05 being considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

ACKNOWLEDGMENTS

We are grateful to M.C. Haiges for initiating this project. We thank T.A. Hacker and the Cardiovascular Physiology Core at the University of Wisconsin–Madison for technical assistance with thrombo-

sis assays, and R. Sullivan for surgical and dissection training. We also thank C. Feldman and K.E. Tippins for technical assistance with the maintenance of mouse colonies and sample preparation. This work was supported by grant R01 CA073808 (National Institutes of Health). E.R.G. was supported by Molecular and Cellular Pharmacology Training Grant T32 GM008688 (NIH) and by a William H. Peterson Fellowship in Biochemistry (Department of Biochemistry, University of Wisconsin–Madison). J.E.L. was sup-

ported by a National Science Foundation Graduate Research Fellowship.

Received January 19, 2019; accepted April 27, 2019.

REFERENCES

Artero A, Tarín JJ, Cano A. 2012. The adverse effects of estrogen and selective estrogen receptor modulators on hemostasis and throm-

osis. Semin Thromb Hemost 38: 797–807. doi:10.1055/s-0032-

328883

Barnard EA. 1969. Biological function of pancreatic ribonuclease. Nature 221: 340–344. doi:10.1038/221340a0

Beintema JJ, Wietzes P, Weickmann JL, Glitz DG. 1984. The amino acid sequence of human pancreatic ribonuclease. Anal Biochem 136: 48–64. doi:10.1016/0003-2697(84)90306-3

Bleker SM, Coppens M, Middeldorp S. 2014. Sex, thrombosis and in-

herited thrombophilia. Blood Rev 28: 123–133. doi:10.1016/j.bre.

2014.03.005

Bravo J, Fernández E, Ribó M, de Llorens R, Cuchillo CM. 1994. A versatile negative-staining ribonuclease zymogram. Anal Biochem 219: 82–86. doi:10.1006/abio.1994.1234

Buyue Y, Whinna HC, Sheehan JP. 2008. The heparrin-binding exosite

of factor IXa is a critical regulator of plasma thrombin generation and venous thrombosis. Blood 112: 3234–3241. doi:10.1182/

blood-2008-01-136820

Cabrera-Fuentes HA, Niemann B, Grieshaber P, Wollbrueck M, Gehron J, Preisssner KT, Böning A. 2015. RNase1 as a potential medi-

ator of remote ischaemic preconditioning for cardioprotection. Eur J Cardiothorac Surg 48: 732–737. doi:10.1093/ejcts/ezu519

Cheng Q, Tucker EI, Pine MS, Sisler I, Matafonov A, Sun MF, White-

Adams TC, Smith SA, Hanson SR, McCarty OJ, et al. 2010. A role for factor XIIa-mediated factor XI activation in thrombus for-

mation in vivo. Blood 116: 3981–3989. doi:10.1182/blood-2010-

02-270918

Cho S, Beintema JJ, Zhang J. 2005. The ribonuclease A superfamily of mammals and birds: identifying new members and tracing evolu-

tionary histories. Genomics 85: 208–220. doi:10.1016/j.ygeno.

2004.10.008

Cormier SA, Larson KA, Yuan S, Mitchell TL, Lindengerker B, Carrigan P, Lee NA, Lee JJ. 2001. Mouse eosinophil-associated ribo-

ucleases: a unique subfamily expressed during hematopoiesis. Mamm Genome 12: 352–361. doi:10.1007/s003350020007

Cuchillo CM, Nogués MV, Raines RT. 2011. Bovine pancreatic ribonuc-

lease: fifty years of the first enzymatic reaction mechanism. Biochemistry 50: 7835–7841. doi:10.1021/bi201075b

Cuthbert BJ, Burley KH, Goulding CW. 2018. Introducing the new

nomenclature of the RNase A superfamily. RNA Biol 15: 9–12. doi:10.1080/15476286.2017.1387710

D’Alessio G, Riordan JF. 1997. Ribonucleases: structures and func-

tions. Academic Press, New York.
D’Avino C, Palmieri D, Braddock A, Zanesi N, James C, Cole S, Salvatore F, Croce CM, De Lorenzo C. 2016. A novel fully human anti-NCL immunoRNAse for triple-negative breast cancer therapy. Oncotarget 7: 87016–87030. doi:10.18632/oncotarget.13522

delCarmel AB, Ribó M, Yokel EM, Quirk DJ, Rutter WJ, Raines RT. 1995. Engineering ribonuclease A: production, purification, and characterization of wild-type enzyme and mutants at Gln11. Protein Eng 8: 261–273. doi:10.1093/protein/8.3.261

Dyer KD, Rosenberg HF. 2005. The mouse RNase 4 and RNase S/ang 1 locus utilizes dual promoters for tissue-specific expression. Nucleic Acids Res 33: 1077–1086. doi:10.1093/nar/gki250

Eller CH, Lomax JE, Raines RT. 2014. Bovine brain ribonuclease is the functional homolog of human ribonuclease 1. J Biol Chem 289: 25996–26006. doi:10.1074/jbc.M114.566166

Falanga A, Russo L, Milesi V. 2014. The coagulopathy of cancer. Curr Opin Hematol 21: 423–429. doi:10.1097/MOH.0000000000000072

Falanga A, Marchetti M, Russo L. 2015. The mechanisms of cancer-associated thrombosis. Thromb Res 135: S8–S11. doi:10.1016/S0049-3849(15)50432-5

Fasetti A, Misiti S, Citarella F, Felici A, Andreoli M, Fantoni A, Sacchi A, Pontecorvi A. 1995. Molecular basis of estrogen regulation of Hageman factor XII gene expression. Endocrinology 136: 5076–5083. doi:10.1210/endo.136.11.57588244

Fischer S, Nishio M, Dadkhahi S, Gansler J, Jaax M, Leiting S, Appel B, Greinacher A, Fischer S, Gailani D, Lasky NM, Broze GJ Jr. 1997. A murine model of factor XI deficiency. Blood Coagul Fibrinolysis 8: 413–419. doi:10.1089/dna.1997.16.413

Gailani D, Lasky NM, Broze GJ Jr. 1997. A murine model of factor XI deficiency. Blood Coagul Fibrinolysis 8: 134–144. doi:10.1097/00001721-199703000-00008

Gajewska WJ, Ribó M, Yokel EM, Quirk DJ, Rutter WJ, Raines RT. 1999. Hypersensitive substrate for ribonucleases. J Biol Chem 274: 1808–1814. doi:10.1074/jbc.116.754325

Gansler J, Jaax M, Leiting S, Appel B, Greinacher A, Fischer S, Preissner KT. 2012. Structural requirements for the procoagulant activity of nucleic acids. PLoS One 7: e50399. doi:10.1371/journal.pone.0050399

Garcia JM, Garcia V, Pena C, Domínguez G, Silva J, Díaz R, Espinosa P, Citeros MJ, Collado M, Bonilla F. 2008. Extracellular plasma RNA from colon cancer patients is confined in a vesicle-like structure and is mRNA-enriched. RNA 14: 1424–1432. doi:10.1261/rna.755908

Golias C, Charalabopoulos A, Stagkas D, Charalabopoulos K, Batistatou A. 2007. The kinase system-bradykinin: biological effects and clinical implications. Multiple role of the kinase system-bradykinin. Hippokratia 11: 124–128.

Hoang TT, Tamnikulu IC, Watland QA, Hoang TM, Raines RT. 2018. A human ribonuclease variant and ERK-pathway inhibitors exhibit highly synergistic toxicity for cancer cells. Mol Cancer Ther 17: 2622–2632. doi:10.1158/1535-7163.MCT-18-0724

Ivanov I, Shakkhatov RB, Sun MF, Dickeson SK, Puy C, McCart OJT, Gruber A, Matafonov A, Gailani D. 2017. Nucleic acids as cofactors for Factor XI and prekallikrein activation: different roles for high-molecular-weight kinogen. Thromb Haemost 117: 671–681. doi:10.1160/TH16-09-0691

Iwaki T, Castellino FJ. 2006. Plasma levels of bradykinin are suppressed in factor XII-deficient mice. Thromb Haemost 95: 1003–1110. doi:10.1160/TH06-09-0128

Jagadeeswaran P, Gregory M, Johnson S, Thankavel B. 2000. Haemospecific screening and identification of zebrafish mutants with coagulation pathway defects: an approach to identifying novel haemostatic genes in man. Br J Haematol 110: 946–956. doi:10.1046/j.1365-2141.2000.02284.x

Kannemeier C, Shibamiya A, Nakazawa F, Trusheim H, Ruppert C, Market P, Song Y, Tzima E, Kennerknecht E, Nienpenn M, et al. 2007. Extracellular RNA constitutes a natural procoagulant cofactor in blood coagulation. Proc Natl Acad Sci 104: 6388–6393. doi:10.1073/pnas.0608647104

Kaplan AP, Joseph K. 2014. Pathogenic mechanisms of bradykinin mediated diseases: dysregulation of an innate inflammatory pathway. Adv Immunol 121: 41–89. doi:10.1016/B978-0-12-800100-4.00002-7

Kelemen BR, Klink TA, Behlke MA, Eubanks SR, Leland PA, Raines RT. 1999. Hypersensitive substrate for ribonuclease. Nucleic Acids Res 27: 3696–3701. doi:10.1093/nar/27.18.3696

Koczera P, Martin L, Marx G, Schuerholz T. 2016. The ribonuclease A superfamily in humans: canonical RNases as the buttress of innate immunity. Int J Mol Sci 17: 1278. doi:10.3390/ijms17081278

Krstovs KV, Matafonov A, Tucker EI, Sun M-F, Walsh PN, Gruber A, Gailani D. 2009. Factor XI contributes to thrombin generation in the absence of factor XII. Blood 114: 452–458. doi:10.1182/blood-2009-02-203604

Landré JB, Hewett PW, Olivot JM, Friedl P, Ko Y, Sachinidis A, Lin H-F, Maeda N, Smithies O, Straight DL, Stafford DW. 1997. A coagulation-deficiency phenotype of ribonuclease 1 deficiency in mice. J Biol Chem 272: 650–657. doi:10.1074/jbc.272.419.650

Leon SA, Shapiro B, Sklaroff DM, Yaros MJ. 1977. Free DNA in the serum of cancer patients and the effect of therapy. Cancer Res 37: 646–650.

Lin H-F, Maeda N, Smithies O, Straight DL, Stafford DW. 1997. A coagulation factor IX-deficient mouse model for human hemophilia B. Blood 90: 3962–3966.

Liu P, Jenkins NA, Copeland NG. 2003. A highly efficient recombination-based method for generating conditional knockout mutations. Genome Res 13: 476–484. doi:10.1101/gr.749203

Liu Y, Jennings NL, Dart AM, Du XJ. 2012. Standardizing a simpler, more sensitive and accurate tail bleeding assay in mice. World J Exp Med 2: 30–36. doi:10.5493/wjem.v2.i2.30

Lomax JE, Bianchetti CM, Chang A, Phillips GN Jr, Fox BG, Raines RT. 2014. Functional evolution of ribonuclease inhibitor: insights from birds and reptiles. J Mol Biol 426: 3041–3056. doi:10.1016/j.jmb.2014.06.007

Lomax JE, Eiller CH, Raines RT. 2017. Comparative functional analysis of ribonuclease 1 homologs: molecular insights into evolving vertebrate physiology. Biochem J 474: 2219–2233. doi:10.1042/BCJ20170173

Lu L, Li J, Moussaiou M, Boix E. 2018. Immune modulation by human secreted RNases at the extracellular space. Front Immunol 9: 1012. doi:10.3389/fimmu.2018.01012

Maier C, René T. 2018. Coagulation factor XII in thrombosis and inflammation. Blood 131: 1903–1909. doi:10.1182/blood-2017-04-569111
Phenotype of ribonuclease 1 deficiency in mice

Emily R. Garnett, Jo E. Lomax, Bassem M. Mohammed, et al.

RNA 2019 25: 921-934 originally published online May 3, 2019
Access the most recent version at doi:10.1261/rna.070433.119

Supplemental Material
http://rnajournal.cshlp.org/content/suppl/2019/05/03/rna.070433.119.DC1

References
This article cites 68 articles, 19 of which can be accessed free at:
http://rnajournal.cshlp.org/content/25/8/921.full.html#ref-list-1

Creative Commons License
This article is distributed exclusively by the RNA Society for the first 12 months after the full-issue publication date (see http://rnajournal.cshlp.org/site/misc/terms.xhtml). After 12 months, it is available under a Creative Commons License (Attribution-NonCommercial 4.0 International), as described at http://creativecommons.org/licenses/by-nc/4.0/.

Email Alerting Service
Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or click here.