

# Targeted Gene Disruption of Natural Anticoagulant Proteins in Mice

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## Abstract

The blood coagulation system is a complicated cascade of reactions and feedback regulations that executes a rapid response to vascular injury, yet avoids occlusion of the vessel. There are several key components of this system in the regulation of blood clot propagation, such as antithrombin (AT), tissue factor pathway inhibitor (TFPI), thrombomodulin (TM) and protein C (PC), of which defect causes thromboembolic diseases. In recent years, targeted gene disruption technique by homologous recombination has been introduced to investigate the physiological roles of those natural anticoagulant molecules, not only in thrombogenesis but also in embryogenesis. We have studied the natural anticoagulation system in a decade, and recently established AT knockout mice as well as ryudocan (syndecan-4) knockout mice. Ryudocan is a cell surface heparan sulfate proteoglycan, which bears heparin-like glycosaminoglycan (heparan sulfate) chains, originally cloned from rat microvascular endothelial cells. We have demonstrated that ryudocan deficiency impairs the control of coagulation in fetal vessels of the placenta in mice. We have also reported that complete antithrombin deficiency in mice results in embryonic lethality, with severe fibrin deposition in the myocardium and the liver, accompanied with extensive subcutaneous hemorrhage. In this presentation, recent advances in understanding roles of natural anticoagulant molecules through the researches of targeted gene-knockout mice, including our experiences in antithrombin deficient mice and ryudocan deficient mice, will be discussed.

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## 1. Introduction

The blood coagulation system is a complicated cascade of reactions and feedback regulations that executes a rapid response to vascular injury, yet avoids thrombus occlusion of the vessel [1]. There are several key components of this system in the regulation of blood clot propagation, such as antithrombin (AT), tissue factor pathway inhibitor (TFPI), thrombomodulin (TM) and protein C (PC), of which defect causes thromboembolic diseases.

Recent advances in gene technology allow us to clarify the nature of anticoagulant proteins, in terms of not only anticoagulation but also other biological functions to participate in normal development and homeostasis [2-4] (Table 1). Targeting gene disruption technique by homologous recombination is powerful tool to elucidate biological functions of the specific molecule.

In this session, knockout mice of natural anticoagulant proteins, especially our experiences such as AT [5,6]

and ryudocan (Ryud; syndecan-4), heparin-like molecule, heparan sulfate proteoglycan [7-10], will be discussed.

## 2. Natural Anticoagulants

Many molecules participate to maintain blood fluidity through the natural anticoagulant reactions of the endothelial cells. It is well known that AT is one of the major natural anticoagulants, and AT deficiency in human causes deep vein thrombosis (DVT). It has been also reported that many cases of PC deficiency in human develop DVT. TM and TFPI are anticoagulant proteins synthesized in endothelial cells.

In 1995, Dr. Hearly reported that TM deficiency showed embryonic lethality at 9.5 gd due to dysfunction of maternal-embryonic interaction [2]. In 1996, Dr. Huang demonstrated that TFPI deficiency showed embryonic lethality, 60% have died at 9.5-11.5 gd with Yolk sac bleeding and 40% have died at late gestation with hemorrhage in the central nerve system and tail

[3]. In 1998, Dr. Jalbert described that PC deficiency also showed lethality at 17.5 gd-birth (within 24hrs) with signs of bleeding and thrombosis (Micro thrombosis and bleeding in the brain) [4]. Recently, we showed that complete AT deficiency causes embryonic lethality at 15.5-16.5 gd with fibrin deposition in the myocardium and the liver, accompanied with extensive subcutaneous hemorrhage [5] (Table 1). We also established ryudocan deficient mice and demonstrated its anticoagulant property *in vivo* [8].

### 3. Antithrombin Deficient Mice

Antithrombin (AT) is a typical molecule of serine protease inhibitors, serpins, which inhibits thrombin, activated factors IXa, Xa, XIa, XIIa and kallikrein, and individuals with AT deficiency are susceptible to venous thromboembolic diseases [11]. Complete AT deficiency is speculated to cause embryonic lethality similar to that of other anticoagulant proteins, because no case-report of quantitative homozygous AT deficiency has been published. It was not known, however, when or in what way AT is necessary in normal embryogenesis.

To further investigate the physiological roles of AT, especially those in embryogenesis, we generated AT deficient mice by gene targeting [5]. Exon 2 of AT gene was replaced with neomycin-resistance gene by homologous recombination. Disruption of AT gene was confirmed by genomic PCR, southern blot analysis, RT-PCR and Western blot analysis. Genotype distribution of embryos and newborns, derived through timed matings of AT deficient heterozygous mice, showed that the frequency of alive homozygous embryos matched "Mendelian rate" until 14.5 gd, and their appearance was indistinguishable from that of wild-type embryos. However, at 15.5 gd, the frequency was much less than 25 %, and none of alive homozygous embryos and newborns was observed after 16.5 gd. Because approximately 70 % of the homozygous AT deficient embryos at 15.5 gd, and 100 % at 16.5 gd, had died with severe fibrin deposition in the myocardium and the liver, accompanied with extensive subcutaneous hemorrhage. No other tissues, such as lung and kidney, showed any fibrin deposition, suggesting that fibrinogen

in plasma may have been decreased by consumptive coagulopathy and/or damaged-liver dysfunction.

On the other hand, heterozygous AT deficient mice were born and grew normally, and indistinguishable from wild type mice. Heterozygous AT deficient mice possess about 50% antigen and activity of AT, and they had no spontaneous thromboembolic episodes during the longest follow-up period of 20 months. However, LPS challenge markedly induced fibrin deposition in various tissues, such as kidney, myocardium, and liver in the heterozygous mice compared with the wild type mice. In addition, prophylactic administration of human AT concentrates into heterozygous AT deficient mice prevented the LPS-induced fibrin deposition effectively. Thus, heterozygous AT deficient mice are at risk of thrombosis, but additional thrombogenic stimulations are required for developing the disease [6]. Heterozygous AT deficient mouse will be a useful model for studying the effect of genetic or environmental risk factors upon thrombogenesis.

### 4. Ryudocan (Syndecan-4) Deficient Mice

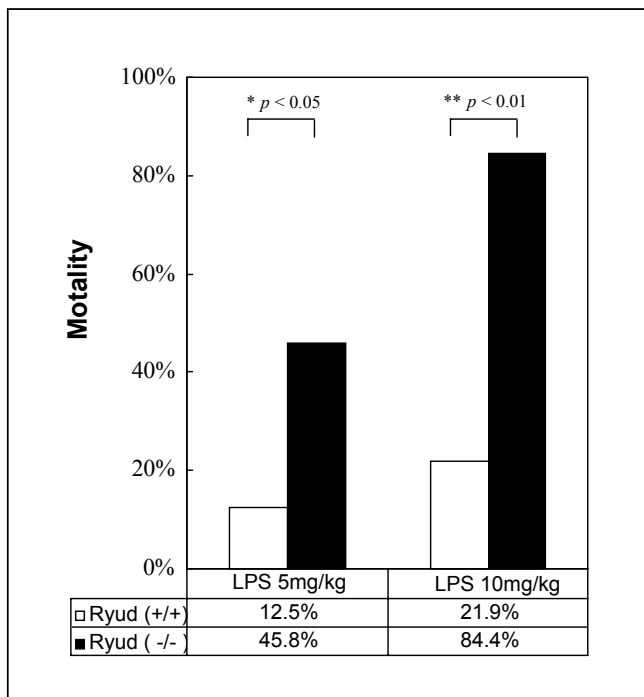
The inhibition of thrombin by AT is relatively slow, but is dramatically enhanced in the presence of the glycosaminoglycan, heparin or heparan sulfate [12]. Ryudocan (Ryud; syndecan-4), which is a member of the syndecan family [13], was originally cloned from rat microvascular endothelial cells as an anticoagulant heparan sulfate proteoglycan [14,15]. To investigate the *in vivo* functions of Ryud, we have made Ryud deficient mice replacing exon 2, 3 and a part of exon 4 with neomycin resistance gene by homologous recombination [7]. So far, complete Ryud deficient mice were born with expected frequency and reproduced normally, and the mode of their growth is not different from that of the wild type mice. No gross abnormality was found in Ryud deficient mice by histological examination. However, Ryud deficiency impairs the control of coagulation in fetal vessels of the placental labyrinth in mice described as following [8].

The degenerated vessels were observed in the placental labyrinth of the both type embryos, but they were more extensive and severe in the Ryud deficient

**Table 1.**

Phenotypes of Gene-targeted Natural Anticoagulant Mice.

Anticoagulant Protein	Protein	Reference
Thrombomodulin (TM)	Embryonic Lethality at 9.5 gd Dysfunction of maternal-embryonic interaction.	2
Tissue Factor Pathway Inhibitor (TFPI)	60% have died at 9.5-11.5 gd with Yolk sac hemorrhage, and 40% have died at late gestation with hemorrhage in the central nerve system and tail.	3
Protein C (PC)	Lethal at 17.5 gd-birth (within 24hrs) with signs of bleeding and thrombosis. Micro thrombosis and bleeding in the brain.	4
Antithrombin (AT)	Embryonic lethality at 15.5-16.5 gd. Fibrin deposition in the myocardium and the liver, accompanied with extensive subcutaneous hemorrhage.	5



**Figure 1.** Mortality of mice 7 days after LPS injection.

embryo. Ryud is expressed in the endothelium of vessels originated from an embryo containing nucleated red blood cells. The degenerated vessel also contains the nucleated red blood cell, indicating that degenerated vessels correspond, at least partially, to the fetal vessels. The placental labyrinth of the Ryud deficient embryo showed also more extensive calcium deposition and fibrin deposition, compared to that of the wild type. Thus, Ryud may function as a crucial anticoagulant molecule in the embryonic vessels of the mouse placental labyrinth [8]. Never the less an extensive degeneration of vessels with severe fibrin deposition was observed in placental labyrinth of the Ryud deficient embryos, their intrauterine growths were not affected as compared with the wild type controls. When the thrombogenic stress was added such as LPS challenge, however, the growth of Ryud deficient embryos was significantly retarded.

We also observed that the Ryud deficient mice exhibited significantly higher mortality than the wild-type controls after intraperitoneal injection of a relatively high dose of LPS (5 and 10 mg/kg) [10] (Fig. 1.). We could not find, however, any differences of fibrin deposition in both type mice tissues, while we observed that systolic blood pressure and left ventricular fractional shortening were significantly lower in the deficient mice at 9 h after LPS. Furthermore, we found that the TGF- $\beta$ 1-dependent suppression of excessive increase of IL-1 $\beta$  was impaired in Ryud deficient mice. We also observed that TGF- $\beta$ 1 bound to Ryud via its heparan sulfate chains. It is well known that TGF- $\beta$ 1 participates in preventing endotoxin shock mainly by inhibiting

the production of proinflammatory cytokines [16,17]. Taken together, Ryud deficient mice could be more susceptible to endotoxin shock, due to impaired suppression of an excess increase of IL-1 $\beta$  by TGF- $\beta$ 1 bound to Ryud [10]. In other words, Ryud is involved in a mechanism that prevents septic shock.

## 5. Conclusion

Targeting gene disruption technique is powerful tool to elucidate the *in vivo* functions of the natural anticoagulants, not only in anticoagulation but also in other biological phenomena. It has been reported that AT has an anti-inflammatory property. In this article, we also demonstrated that Ryud may have anti-inflammatory activity participating in host defense mechanisms. We believe that these gene-modified animals will be utilized to clarify the nature of the natural anticoagulant molecules and to elucidate the puzzling biological *in vivo* phenomena.

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