Depressed Corin Levels Indicate Early Systolic Dysfunction Before Increases of Atrial Natriuretic Peptide/B-Type Natriuretic Peptide and Heart Failure Development

Ranjana Tripathi, Dong Wang, Ryan Sullivan, Tai-Hwang M. Fan, Inna P. Gladysheva, Guy L. Reed

Abstract—Dilated cardiomyopathy is a major cause of heart failure (HF) that affects millions. Corin cleaves and biologically activates pro-atrial natriuretic peptide (pro-ANP) and pro–B-type natriuretic peptide (pro-BNP). High corin levels reduce the development of systolic dysfunction and HF in experimental dilated cardiomyopathy. Yet, patients with significant HF unexpectedly show low corin levels with high plasma ANP/BNP levels. Therefore, we examined the relationship between cardiac corin expression, ANP/BNP levels, and the stages of HF. We used a well-established, dilated cardiomyopathy model to evaluate gene and protein expression as mice longitudinally developed Stages A–D HF. Cardiac systolic function (ejection fraction) continuously declined over time (P<0.001). Cardiac corin transcripts were decreased at early Stage B HF and remained low through Stages C and D (P<0.001). Cardiac corin levels were positively correlated with systolic function (r=0.96, P=0.003) and inversely with lung water (r=−0.92, P=0.001). In contrast, cardiac pro-ANP/BNP transcripts increased later (Stages C and D) and plasma levels rose only with terminal HF (Stage D, P<0.001). Immunoreactive plasma ANP and BNP levels were positively associated with plasma cyclic guanosine monophosphate levels (r=0.82, P=0.01 and r=0.8, P=0.02, respectively). In experimental dilated cardiomyopathy, corin levels declined early with progressive systolic dysfunction before the development of HF, whereas significant increases in plasma ANP, BNP, and cyclic guanosine monophosphate levels were found only in later stage (C and D) HF. This dyssynchrony in expression of corin versus ANP/BNP may impair cleavage activation of pro-natriuretic peptides, and thereby promote the transition from earlier to later stage HF. (Hypertension. 2016;67:362-367. DOI: 10.1161/HYPERTENSIONAHA.115.06300.)

Key Words: atrial natriuretic peptide ■ corin protein ■ dilated cardiomyopathy ■ heart failure ■ natriuretic peptides

Dilated cardiomyopathy (DCM) is characterized by progressive enlargement of the heart ventricles and depressed systolic function. DCM is one of the major causes of severe heart failure (HF) worldwide.1 HF is associated with pathological accumulations of extracellular salt and water leading to lung edema, pleural effusions, and other types of edema. Four clinical stages of development of HF have been recognized.1 Stage A occurs in individuals at risk, but without structural heart disease or HF symptoms. Stage B includes evidence of structural heart disease, but no findings of HF. Stage C is associated with HF, and Stage D includes severe end-stage HF.1 HF is associated with increased blood levels of atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP).2–5 Pro-ANP and pro-BNP are cleaved to their active forms by corin, a cardiac transmembrane serine protease.6 ANP and BNP activate the guanylyl cyclase/natriuretic peptide receptor A–signaling cascade to generate cyclic guanosine monophosphate (cGMP).7,8 In experimental DCM, increased corin expression has protective effects, positively modulating plasma cGMP levels, systolic function, myocardial fibrosis, HF, and survival.9 Recent experimental data show that ANP also prevents HF and the progression of experimental DCM.10 Although the components of the corin–NP–natriuretic peptide receptor A pathway seem to protect against experimental HF,2 recent data suggest that this pathway is dysregulated in overt HF.9,11 Levels of circulating immunoreactive ANP and BNP rise,2,3 whereas circulating levels of corin unexpectedly are depressed in patients with severe HF.1,11 In addition, an inverse association between cardiac corin and pro-ANP/pro-BNP transcript levels has been found in severe systolic HF in humans12 and in experimental mouse models of HF.9,17

In this study, we examined if dyssynchrony in corin and ANP/BNP expression develops in experimental DCM with advancing systolic dysfunction because animals progress through all 4 stages of HF development. Our data indicate that there is an early, progressive decline in corin levels, which is a sensitive marker of early systolic dysfunction, that seems before the development of ventricular dilation, increases in NP levels, and the onset of HF. Although corin levels may prove to be an early biomarker of cardiac dysfunction, this
early dyssynchrony in the expression of NP system components provides further evidence of NP system dysregulation, which may affect the progression of cardiomyopathy and the development of HF.

**Methods**

We analyzed DCM and wild-type (WT) control mice of age 4, 7, 13, and 20 weeks in vivo and ex vivo. Experimental details are available in the online-only Data Supplement.

**Statistical Analysis**

Statistical analyses were performed using 2-way ANOVA (unless otherwise indicated). Differences were considered to be significant if the 2-tailed \( P \leq 0.05 \). The number of animals (n) is indicated in the figure legends. Data are reported as means±SE or means±SD.

**Results**

**Stages of DCM With Progressive Declines in Systolic Function and HF**

To evaluate longitudinal changes in the NP system, we used a well-characterized, translationally relevant experimental mouse model of DCM, in which progressive systolic dysfunction leads to HF in the setting of preserved kidney function.\(^9,10,18,19\) Ejection fraction (EF%) and lung edema (a criterion of human HF)\(^9,10,18,19\) were assessed in DCM mice and control WT littermates. Ejection fraction (EF%) and lung edema (a criterion of human HF)\(^9,10,18,19\) were assessed in DCM mice and control WT littermates.

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**Stages of DCM With Progressive Declines in Systolic Function and HF**

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The development of HF was associated with pleural effusions, increased lung water and histological evidence of alveolar edema. There was no evidence of HF in DCM mice at 4 and 7 weeks of age, but DCM mice aged 13 and 20 weeks showed large pleural effusions and lung edema. Consistent with this observation, lung/body weight ratios (%) and the wet–dry lung weights in DCM mice were significantly increased at 13 and 20 weeks of age (Figures 1C and 1D). Histological analysis of 20-week DCM mice showed lung congestion, alveolar and intra-alveolar edema (pink area), and accumulation of red blood cells and leakage of fluid into alveoli (solid arrow, Figure S1). Alveolar wall thickness seemed increased (broken arrow) in DCM mice when compared with WT controls (Figure S1). Circulating levels of cGMP are useful biomarkers of HF;\(^21\) Plasma cGMP levels were unchanged at 4 and 7 weeks in DCM and WT controls (Figure 1E). However, at 13 and 20 weeks, DCM mice showed significant elevations of cGMP by comparison with WT controls (Figure 1E; \( P < 0.001 \)).

The progressive systolic dysfunction and the development of lung edema observed in these DCM mice correspond roughly to the 4 stages of HF. Stage A (4 weeks) corresponds to the risk of DCM without a decline in systolic function. Stage B (7 weeks) was associated with development of systolic dysfunction without signs of HF. Stage C (13 weeks) was associated with systolic dysfunction and HF, whereas Stage D (20 weeks) was associated with end-stage HF and mortality.

**Cardiac Corin Levels Are Linked to Declining Contractile Function in DCM Mice**

Although corin expression seems to affect the development of HF,\(^9\) there is little known about how corin expression changes during the progression of cardiomyopathy. Corin transcripts were significantly decreased from Stage B (7 weeks; \( −46±6%, P < 0.001 \)) to Stage C (13 weeks; \( −60±10%, P < 0.001 \)) to Stage D (20 weeks; \( −69±5%, P < 0.001 \); Figure 2A). Corin cardiac transcript levels (Stages B–D) showed a linear association with contractile function as assessed by EF% (\( r = 0.96, P = 0.003 \); Figure 2B) or fractional shortening (\% \( r = 0.97, P = 0.001 \); Figure 2C). There was also a negative association between corin transcripts and the severity of lung edema assessed by the lung/body weight ratio (\( r = −0.92, P = 0.001 \); Figure 2D). Cardiac corin protein expression was assessed by immunofluorescence staining of paraffin embedded heart sections with anti-cerin IgG\(^22\) (Figure 2E). Corin protein expression was significantly reduced in the atria and ventricles of DCM mice at Stage D (\( P < 0.01 \)) versus WT (Figure 2F). Corin plasma levels in DCM were significantly decreased at Stage A (4 weeks, \( −45±28%, P < 0.001 \)), and they were consistently lower (\( −37±20% \)) than WT mice through all the stages of HF (\( P < 0.0001 \)).

**Changes in ANP and BNP Levels With Progressive Declines in Systolic Function and HF**

Cardiac pro-ANP and pro-BNP are natural substrates of corin.\(^6\) Pro-ANP and pro-BNP cardiac transcript levels were not significantly altered at Stage A (4 weeks) or Stage B (7 weeks) in DCM mice versus controls (Figures 3A and 3B). However, at Stage C (13 weeks) and Stage D (20 weeks), pro-ANP transcript levels were significantly increased by 178±76% (\( P < 0.001 \)) and 136±63% (\( P < 0.05 \)) in DCM mice (Figure 3A). In a similar fashion, pro-BNP transcript levels were increased by 209±99% (\( P < 0.001 \)) at Stage C and 149±58% (\( P < 0.01 \)) at Stage D in DCM mice versus WT controls.

Consistent with transcript levels, ANP and BNP plasma levels were unchanged in DCM and WT controls at Stages A and B but were significantly elevated at Stage D (\( P < 0.001 \)) by comparison with littermate controls (Figure 3C). Plasma ANP levels and cardiac pro-ANP transcripts (\( r = 0.5, P = 0.01 \); Figure 3D) were positively correlated, as were plasma BNP levels and cardiac pro-BNP transcripts (\( r = 0.4, P = 0.01 \); Figure 3E). ANP but not BNP plasma levels were directly related to edema as assessed by lung/body weight (\% \( r = 0.73, P = 0.04 \); Figure 3F). Unlike corin levels, there was not a strong relationship between ANP levels and declining systolic function (\( r = 0.04, P = 0.08 \)).

Plasma levels of cGMP in all experimental groups were positively associated with total levels of immunoreactive ANP (\( r = 0.82, P = 0.01 \); Figure 3G) and BNP (\( r = 0.8, P = 0.02 \); Figure 3H). There was a significant negative relationship...
between corin transcript levels and cGMP levels ($r=−0.7$, $P=0.05$).

### Discussion

Recent studies show that cardiac corin and ANP are protective against HF development in experimental DCM. Thus, it is reasonable to hypothesize that corin and the pro-NPs are coordinately regulated to forestall the development of HF as systolic function declines. However, when measured at a single time point during overt systolic HF in humans and in mice, cardiac and plasma levels of corin were diminished and ANP/BNP were paradoxically increased. In this study, we examined the longitudinal relationship between corin and NP expression because systolic dysfunction progressed in DCM. Corin expression was correlated with systolic function. Corin levels were depressed in Stage B in association with the decline in systolic function, even before the onset of HF or increases in levels of NPs. Corin levels remained depressed in Stages C and D, whereas cGMP and circulating levels of NPs rose only with the development of HF.

This study used a well-characterized mouse model of progressive DCM as an experimental tool to examine the longitudinal relationships between changes in contractile function, HF development and expression of corin, pro-ANP and pro-BNP. This DCM model shows significant similarities to the stages of HF development in humans. At 4 weeks of age, animals are at the risk for developing HF, but systolic function is normal and corin levels are not yet significantly different from age-matched animals; this is compatible with Stage A in humans, who are at risk for HF, but have not yet developed signs or symptoms. DCM mice at 7 weeks show findings similar to Stage B, with objective evidence of a decline in their EF% by a 1.5-fold, but without other findings. Animals at 13 weeks develop overt HF, consistent with Stage C. Animals at 20 weeks show Stage D HF with a nearly 3-fold decline in systolic function, limitation of activity and imminent demise.

Cardiac corin transcript levels were closely linked to contractile function and showed a $≈50\%$ decrease at Stage B. There was a further, but less significant ($≈60\%$ to $70\%$) decline in corin levels with Stages C and D. In contrast, transcripts for pro-ANP and pro-BNP showed different patterns of expression. Pro-ANP and pro-BNP transcripts were unchanged during Stages A and B but rose significantly with the onset of systolic dysfunction, during early incipient cardiomyopathy when immunoreactive ANP and BNP measurements...
are not changed. Cardiac corin was positively correlated with physiological markers of cardiomyopathy (impaired contractile or systolic function) and negatively associated with lung edema. However, ANP plasma levels, which reflected cardiac pro-ANP expression, were correlated only with lung edema and not with the cardiac contractile function.

Corin protein expression was significantly reduced in both the atria and the ventricles of DCM mice at terminal stage of HF; this finding is consistent with reports of reduced cardiac corin transcript levels in hearts from humans with end-stage HF undergoing transplantation. Insufficient cardiac corin protein levels might be responsible for impaired pro-ANP/pro-BNP cleavage reported at acute decompen-sated HF with diminished systolic functions in human and may, in part, explain the fact that the highest levels of circulating NPs are found in patients with the most severe HF. Although these studies were conducted in male DCM mice on C57/B6 background, they are consistent with our previous findings in female DCM mice on a CD1 background, suggesting that the observed effects are not sex or strain specific. Restoration of cardiac corin levels in DCM mice was shown to increase plasma cGMP levels, improve contractile function, reduce HF, and prolong survival. Still, it remains unclear how cardiac corin deficiency plays such critical role in HF development. Corin-knockout mice generated by several laboratories showed a mild hypertensive phenotype, but HF has not been reported in these mice. However, corin-deficient KitW-sh/W-sh mice developed rapidly progressive cardiac dilation and systolic dysfunction after aortic banding.

In circulation, biologically active ANP and BNP execute their cardiological and vascular effects through the cGMP signaling cascade after binding with the receptor natriuretic peptide receptor A. Plasma ANP and BNP levels were significantly elevated at terminal Stage D HF corresponding to 20 weeks. Consistent with clinical reports for overt HF and cGMP levels were elevated at Stages C and D of HF in DCM. Although plasma cGMP levels are positively associated with plasma immunoreactive ANP or BNP, NP system activation seems insufficient to attenuate the decline in systolic function and transition to terminal HF. NP signaling is an important, but not sole determinant of circulating cGMP. cGMP is an intracellular second messenger for nitric oxide, which is also elevated in chronic human HF. It has been suggested that cGMP is produced mainly by nitric oxide in humans with chronic HF requiring hospitalization, but as HF improves and the patient nears discharge, nitric oxide is primarily produced by ANP and BNP.

Figure 2. Relationship between corin cardiac expression and systolic function. A, Percent change (%Δ) of corin cardiac transcript levels in dilated cardiomyopathy (DCM) mice to wild-type (WT) littermates determined by quantitative reverse transcription polymerase chain reaction analysis. B–D, Linear regression analysis in DCM and WT mice showed a significant association between ejection fraction (EF%; B), fractional shortening (FS%; C), lung/body weight ratio (LW/BW%; D), and corin cardiac transcripts (arbitrary units); n=7 to 14 per group. E, Representative immunofluorescence images stained with anti-corin IgG (scale bars, 2 mm) of 20-week-old WT and DCM hearts with magnified views of atrium and ventricle (100 μm) from areas indicated by red and yellow boxes, respectively. F, Bar graph showing total immunofluorescence intensity/total myocardium area (intensity/MA) in WT and DCM mice (n=5 per group). WT (green), DCM (blue). Data represent mean±SE. **P<0.01, ***P<0.001.
To our knowledge, this is the first longitudinal study to demonstrate lack of coordination, or dyssynchrony, in cardiac corin and pro-ANP/pro-BNP expression because systolic function declines and DCM progresses from Stages A to D HF. A failure of coordinated expression of cardiac levels of corin and the pro-NPs may lead to reduced activation of pro-NPs and to the apparent functional dysregulation of the NP system in HF. This may contribute to the decreased cleavage of pro-NPs reported in human HF and lending support to the idea that HF is a corin-deficient state in which low corin levels may have a causative role. Still, the mechanisms responsible for the dyssynchronous regulation of corin and the NPs are unknown. It will be important to determine how dysynchronous expression of the NP system affects the activity of the renin–angiotensin–aldosterone system, which also modulates HF in DCM. Although we and others have found a similar pattern of corin and NPs expression in human Stage D HF, it is necessary to replicate these longitudinal findings in humans and define their relevance to cardiomyopathies caused by ischemia and other causes. Nevertheless, these data suggest that corin levels may be valuable as a potential marker of systolic dysfunction, before the development of symptomatic HF.

Perspectives

There is a critical need to identify patients at risk who may benefit from early interventions to prevent progressive systolic dysfunction and the development of HF. Corin levels are correlated with systolic function and low corin levels may be an early indicator of systolic dysfunction that precedes increases in ANP and BNP, and the development of overt HF.

Acknowledgments

We thankfully acknowledge the technical assistance of Ailylean K. Houng and Nelson Houng.

Sources of Funding

This work was supported by National Institutes of Health grants (HL58496, NS089707 to G.L. Reed and HL115036 to I.P. Gladysheva); and American Heart Association grant (14SDG20510068 to D. Wang).

Disclosures

None.

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_Hypertension_. 2016;67:362-367; originally published online December 14, 2015; doi: 10.1161/HYPERTENSIONAHA.115.06300

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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DEPRESSED CORIN LEVELS INDICATE EARLY SYSTOLIC DYSFUNCTION BEFORE INCREASES OF ANP/BNP AND HEART FAILURE DEVELOPMENT

Running Title: Corin expression and systolic dysfunction


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ONLINE SUPPLEMENT

Methods

Mice. DCM mice express a dominant negative CREB transcription factor in cardiomyocytes and develop progressive dilated cardiomyopathy and heart failure.1-4 DCM and C57BL/6 littermates male mice of 4, 7, 13 and 20 weeks of age were used for this study. Mice were housed in the same cage racks in AAALAC accredited facilities and fed a normal salt chow (0.4% NaCl, Harlan Teklad). Experiments were approved by the Animal Care and Use Committees of University of Tennessee Health Science Center and were performed in accordance with National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Real-time polymerase chain reaction (RT-PCR). Total RNA was extracted from snap frozen heart tissue using the RNeasy® Mini Kit (Qiagen). First strand cDNA synthesis was performed with 1 µg of total RNA (Transcriptor First Strand cDNA Synthesis Kit, Roche). Quantitative real-time PCR (qRT-PCR) was performed using the LightCycler® 480 System following the manufacturer's protocol. Specific primers were: ctggaaggattgtttggag and acgctcctgtctgctctca for corin (NM_016869.3); cacagatctgatggatttcaaga and cctcatcttctaccggcatc for ANP (NM_008725.2); tccatcaggggtcacac and gccctgtgaagggtgatta for BNP (AB039044.1). PCR was performed at: 95°C for 5 min, followed by 40 cycles of 95°C (10 s), 60°C (30 s), and 72°C (10 s). PCR products were confirmed by melting curve analysis using the Lightcycler Software 4.0 and samples normalized to a β-actin control for heart. Experiments were performed in triplicate and the qRT-PCR was subjected to log transformation as recommended to achieve a normal distribution3.

Immunofluorescence staining and analysis

Formalin fixed paraffin embedded heart sections (5 µm) were cut on slides and used for immunofluorescence staining after deparaffinization (Safeclear II, Fisher Diagnostics, MI) as described previously.5 Antigen retrieval was performed by heat-induced epitope retrieval method by heating the sections at 98º C for 20 min followed by 20 min cooling at room temperature. Sections were blocked with 10% normal donkey serum in phosphate buffer saline (PBS) for 1 h at room temperature and incubated with rabbit anti-mouse protease domain corin as primary antibody6 in 2% normal donkey serum overnight at 4 ºC. Rabbit preimmune serum was used for the negative controls staining. Donkey anti rabbit AlexaFluor® 488 (Life technologies, CA) was used as a secondary antibody. The cell nuclei were counterstained with DAPI using Vecta shield hardset mounting media (Vector Lab., CA). Slides were scanned for fluorescence images with Aperio image fluorescence scanner (Aperio ScanScope CS2, Vista, CA) and images were taken using ImageScope software (MAN-0001, revision G) at 1x and 20x magnification. Total gross fluorescence of both atria and ventricles of each heart section was measured using Image Pro Plus 6.2 (Media Cybernetics, Bethesda, MD) and expressed as ratio of total fluorescence intensity to total heart myocardium area (Intensity/MA) in DCM and WT controls.
**Lung Edema and Lung Water Retention Analysis.** For lung histology analysis, formalin-fixed lung sections were stained by hematoxylin and eosin as described previously. Slides were scanned by Aperio ScanScope CS2 scanner (Aperio) and images were taken using ImageScope software (MAN-0001, revision G) and 40X images were used as representative images to show lung edema and lung hemorrhage. Lung edema was also assessed by lung weight/body weight ratios (%) and the difference between wet and dry lung weight. In brief, right and left lungs were excised and immediately weighed followed by another weight for completely dried lungs. And then the lung weight/body ratios (%) were calculated as right plus left lung wet weight divided by body weight, while the difference between total wet and dry lung weight was also calculated.

**Echocardiography.** Transthoracic echocardiograms were performed by an echocardiographer blinded to the mice genotype using a Vevo 2100 Imaging System (Visual Sonic Inc., Toronto, Canada) as we previously described. Hair from the ventral thorax were removed by chemical depilatory (Nair) one day before the echocardiographic studies. Briefly, mice were sedated with 1.5% inhaled isoflurane. Two-dimensional and M-mode images of the LV were obtained from the parasternal long-axis and short axis acoustic windows. The 2D-guided M-mode recordings were analyzed using VisualSonics Vevo® software; left ventricular internal dimension in diastole (LVIDd), left ventricular internal dimension in systole (LVIDs), interventricular septal wall thickness (IVS) and left ventricular posterior wall thickness (LVPW) were measured on at least 3 cardiac cycles and averaged for each mouse. All measurements were performed using the leading-edge-to-leading-edge convention. The fractional shortening (FS, %) and ejection fraction (EF, %) were calculated according to standard equations.

**Enzyme immunoassay for corin, N-ANP, C-BNP, cGMP in plasma.** Plasma corin, N-ANP, C-BNP, cGMP levels were measured by enzyme immunoassays according to the manufacturer’s protocols (USCN Life Science Inc., China; Phoenix Pharmaceuticals, Inc., Burlingame, CA; Enzo Life Science Inc. Farmingdale, NY).

**Statistical Analysis.** Statistical analysis was performed in Graph Pad Prism 5.0 software (San Diego, CA). Survival was analyzed by the Kaplan-Meier method and comparison of two groups was assessed by log-rank test. Age related percent change of corin, ANP, BNP, cGMP level in DCM vs. WT littermates were calculated as $(\text{Level}_{\text{DCM}} - \text{Level}_{\text{WT}}) / \text{Level}_{\text{WT}} \times 100$. The standard errors for the percent change were calculated as $[(\text{Level}_{\text{DCM}} / \text{Level}_{\text{WT}}) \sqrt{(\text{SE}^2_{\text{DCM}} / \text{Level}^2_{\text{DCM}}) + (\text{SE}^2_{\text{WT}} / \text{Level}^2_{\text{WT}})} \times 100]$. Other statistical analyses were performed using nonparametric methods (unless otherwise indicated). Age and genotype factors between groups were analyzed by two-way ANOVA. Differences between genotypes (DCM vs. WT controls) were also analyzed by two-way ANOVA using bonferroni post test and analysis. Differences were considered to be significant if the 2-tailed $P \leq 0.05$. The number of animals (n or N) is indicated in the figure legends or results. All values were expressed as mean ± SEM for RT-PCR measurements and as mean ± SDM for ELISA measurements. Linear regression ($r$) or
Pearson correlation ($r_p$) analysis was used to analyze possible association between components of NP system and systolic functions.

**Supplementary References:**

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Supplementary Figure S1.

Figure S1. **Alveolar edema in DCM mice.** Representative H&E images (scale bars, 60 µm) showing severe interstitial and intra-alveolar edema (pink area, asterisk) in 20 weeks old DCM mice (n=5 per group) vs. control, WT mice. Solid arrows- lung hemorrhage; broken arrows- thickened alveolar walls.