國 立 交 通 大 學

生 物 科 技 學 院

分子醫學與生物工程研究所

碩士論文

第二型血管收縮素轉換酶和基質金屬蛋白酶的活性與肺部疾病之相關性

Activities of Angiotensin Conversion Enzyme II and Matrix Metalloproteinases Associated with Pulmonary Diseases

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中 華 民 國 102 年 7 月

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中文摘要

在腎素-血管收縮素系統中（Renin-angiotensin system; RAS），已知血管收縮素轉化酶（angiotensin-converting enzyme; ACE）/血管收縮素II（angiotensin II; Ang II）途徑（ACE/Ang II axis）與許多肺部疾病有關，但在RAS系統中的另一個ACE類似酵素，第二型血管收縮素轉化酶（angiotensin-converting enzyme II; ACE2）的生理功能卻較少被探討，ACE2可將Ang II水解成血管收縮素1-7（angiotensin 1-7; Ang 1-7），而Ang 1-7被認為有拮抗Ang II的功能。在我們過去研究結果中顯示，ACE2/Ang 1-7 axis的異常與心臟組織纖維化病程有關，而此與基質金屬蛋白酶（matrix metalloproteinases; MMPs）和基質金屬蛋白酶組織抑制因子（tissue inhibitors of MMPs; TIMPs）的調控失衡有關。

肺部疾病中常見的肋膜積液（pleural effusion）是指肋膜腔中積存過量的液體，為一種臨床上常見的病徵，其形成機制主要為心臟衰竭、炎症、惡性腫瘤、肺結核等病症所造成不同程度的病程。過去的研究中顯示肋膜積液病程中所誘發免疫反應的發炎機制，會進而導致肋膜腔纖維化。另外肺纖維化，臨床上稱之為原發性肺纖維化（idiopathic pulmonary fibrosis），是一種起因不明，長期漸進發展且不可逆轉的致命性肺部疾病。

在本研究中，我們欲探討肺部ACE2和MMPs/TIMPs平衡調控與肺部疾病和肺部組織纖維化的關係性。本研究的第一部分為探討ACE/ACE2和MMPs的變化，是否與特定之肋膜積液病變有關，進而發展可用於臨床診斷的生物性指標；研究的第二部分為利用博萊酶素（bleomycin）的胸腔注射誘發小鼠肺纖維化，用以探討肺臟組織纖維化病程中，

ACE/ACE2與MMPs/TIMPs活性表現的差異性。另外本研究也利用ACE2基因剔除小鼠（ACE2 knockout (KO) mice）探討ACE2在肺纖維化病變病程中所扮演的角色。

在第一部分的臨床肋膜積液研究中，相較於濾出型積液（transudates effusion），在滲出型積液（exudates effusion）中，可測得顯著較高ACE/ACE2之活性比值與MMPs活性。再則，在滲出型積液中，肺結核患者肋膜積液中的ACE/ACE2比值、腺核苷去氨酶（adenosine deaminase; ADA）活性及MMP-9活性均顯著高於肺炎與腺癌病患肋膜積液中所測得之值。本項研究之重要結論為RAS和MMP活性與肋膜積液病變有關，而肋膜積液中ACE/ACE2比值、ADA活性及MMP-9活性可做為肺結核患者之診斷生物標誌。

在第二部分的肺纖維化研究中，我們成功建立胸腔注射1 U/kg bleomycin方式誘發實驗小鼠產生肺部纖維化病症之模式，而在小鼠誘發肺部疾病病程中（bleomycin處理後第3、7及28天），小鼠肺臟組織中所測得的ACE/ACE2與MMP-9/TIMP-1活性有顯著改變，此即實驗小鼠在注射bleomycin後第7天時，其肺部組織中的ACE、MMP-9和TIMP-1皆有顯著上升，且組織切片觀察有顯著白血球的浸潤和膠原蛋白累積的病變；再則於heterozygous、homozygous及hemizygous ACE2 KO小鼠實驗中，結果顯示在缺乏ACE2的小鼠中，於注射bleomycin的早期（第3天）就會使肺組織中的MMP-9活性有顯著表現，以及TIMP-1含量顯著下降。本項研究之重要結論為ACE2在bleomycin誘發肺臟纖維化病程中，扮演的功能與MMP-9/ TIMP-1的表現調節有關。

【關鍵詞】 肺部疾病、肋膜積液、肺纖維化、第二型基質金屬蛋白酶、第九型基質金屬蛋白酶、第二型血管收縮素轉換酶

Activities of Angiotensin Conversion Enzyme II and Matrix Metalloproteinases Associated with Pulmonary Diseases

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Abstract

Angiotensin converting enzyme (ACE)/angiotensin II (Ang II) axis in renin-angiotensin system (RAS) is associated with the development of several pulmonary diseases; however, much less is known about the functions of angiotensin converting enzyme II (ACE2), an ACE homologue that hydrolyses Ang II to angiotensin 1-7 (Ang 1-7), a peptide that exerts the actions opposite to those of Ang II. In our previous studies, we showed that ACE2 dysregulation and unbalanced matrix metalloproteinases (MMPs)/tissue inhibitors of MMPs (TIMPs) are highly associated with the fibrotic damage in cardiovascular diseases. In this project, we aimed to study the molecular mechanism of ACE2 regulation on pulmonary fibrosis by experimental mouse models. We also attempted to elucidate the roles of ACE2/Ang 1-7 axis in pulmonary function and the hypothesis whether part of the antifibrotic effects of ACE2/Ang 1-7 axis is via the balancing regulation of MMPs/TIMPs?

Pleural effusion is a common medical problem in the chest and involves accumulation of an abnormal amount of pleural fluid in the pleural space. Several diseases, such as congestive heart failure, liver cirrhosis, tuberculosis, adenocarcinoma and pneumonia, are common diseases that cause pleural effusions, but there is less rapid and accurate diagnostic method for pleural effusion. Recent studies showed that the inflammatory response induced by these chest diseases would cause pleural fibrosis. The major enzymes, ACE and ACE2 in RAS, may be involved in the mechanism of pleural fibrosis and pleural effusion. Another lung disease such as idiopathic pulmonary fibrosis has been known as a chronic, progressive, irreversible, and usually lethal lung disease of unknown cause.

In this study, we proposed the goals to explore the relationships between ACE2 and

MMPs/TIMPs balance in pulmonary disease and pulmonary fibrosis. The first part of the present studies was to detect ACE/ACE2 and MMPs activities in pleural effusions. It can identify diagnosis based on clinical variables to differentiate from pleural effusions. For the second part of the present studies, we took mice to induce pulmonary fibrosis with bleomycin in lung cavity to investigate the pathogenesis of pulmonary fibrosis, and the balancing activity ACE/ACE2 as well as MMPs/TIMPs. In addition, we used ACE2 knockout (KO) mice to investigate the role of ACE2 in the pathogenesis of pulmonary fibrosis.

In the first part, the study of clinical pleural effusions, the ACE/ACE2 ratios and MMPs activity in exudate effusions were significantly higher than in transudate effusions. Furthermore, in exudative effusion, ACE/ACE2 ratio, adenosine deaminase (ADA) and MMP-9 activities in tuberculosis effusions were significantly higher than those of pneumonia effusions and adenocarcinoma effusions. The major results of the part I are that the RAS and MMPs activity are important in abnormal pleural fluid. ACE/ACE2 ratio, ADA activity and MMP-9 activity can be used as biomarkers in the diagnosis of tuberculosis patients.

In the second part, we have successfully established the mouse model of pulmonary inflammation and fibrosis induced by 1 U/kg bleomycin injection. The profiling of lung ACE/ACE2 and MMP-9/TIMP-1 activity in the mice challenged with bleomycin at 3-day, 7-day and 28-day after the treatments have been performed. The data showed that ACE, MMP-9 activities and TIMP-1 concentration in the lung tissue were significantly increased in wild-type (WT) mice injected bleomycin at 7-day, and the histological sections showed significant leukocyte infiltration and collagen accumulation. Furthermore, in heterozygous, homozygous and hemizygous ACE2 KO mice, the results showed that the lung tissue MMP-9 activity is significantly higher than Control in early stage (3-day), and TIMP-1 concentration is significantly decreased. The major results of the part II indicate that the important role of ACE2 on bleomycin-induced pulmonary fibrosis may be via the regulation of MMP-9/TIMP-1 expression.

Keywords: pulmonary disease, pleural effusion, pleural fibrosis, matrix metalloproteinase 2, matrix metalloproteinase 9, angiotensin-converting enzyme II

Abbreviation

ACE	Angiotensin-converting enzyme
ACE2	Angiotensin-converting enzyme II
ACEIs	Angiotensin-converting enzyme inhibitors
Ad	Adenocarcinoma
ADA	Adenosine deaminase
Ang II	Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu)
Ang-(1-7)	Angiotensin-(1-7) (Asp-Arg-Val-Tyr-Ile-His-Pro)
Ang-(1-9)	Angiotensin-(1-9) (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His)
AT1R	Angiotensin II type I receptor
AT2R	Angiotensin II type II receptor
Ble	Bleomycin
CRP	C-reactive protein
ECM	Extracellular matrix
IPF	Idiopathic pulmonary fibrosis
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated/ERK kinase
MMP-2	Matrix metalloproteinase 2
MMP-9	Matrix metalloproteinase 9
MMPs	Matrix metalloproteinases
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-kappa B
PE	Pleural effusion
Pn	Pneumonia
RAS	Renin-angiotensin system
ROS	Reactive oxygen species
TB	Tuberculosis
Tet	Tetracycline
TGF-β1	Transforming growth factor-beta 1
TIMPs	Tissue inhibitors of metalloproteinases
TNF-α	Tumor necrosis factor-alpha

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I. Literature Review

1-1. Pleural effusions

1-1-1. Physiology of the pleural space

The pleural space is the coupling system between the lung and the chest wall; accordingly, it is a crucial feature of the breathing apparatus. The pressure within the pleural space (the pleural pressure) is important in cardiopulmonary physiology, because it is the pressure at the outer surface of the lung and the heart and the inner surface of the thoracic cavity. The pleural mesothelium is a monolayer of cells that vary from a flattened ovoid shape to columnar or cuboidal cells that lie loosely over the underlying substructure. The connective tissue of the pleural basement membrane is a complex structure that underlies the surface layer of mesothelial cells and is involved in inflammation of the pleural space (Jantz et al., 2008). Fluid that enters the pleural space can originate in the pleural capillaries, the interstitial spaces of the lung, the intrathoracic lymphatics, the intrathoracic blood vessels, or the peritoneal cavity. It is believed that normal fluid enters the pleural space originates in the capillaries in the parietal pleura (Nahid et al., 2003). The normal pleural fluid production is approximately 15 mL in 50 kg individual human beings.

*1-1-2.*Pathogenesis of pleural effusions

Pleural fluid accumulates when the rate of pleural fluid formation exceeds the rate of pleural fluid absorption. The main factors that lead to increased pleural fluid formation or decreased pleural fluid absorption are tabulated in **Table 1-1.** Normally, a small amount (0.01 mL/kg/hour) of fluid constantly enters the pleural space from the capillaries in the parietal pleura. Almost all of this fluid is removed by the lymphatics in the parietal pleura, which have a capacity to remove at least 0.20 mL/kg/hour. Note that the capacity of the lymphatics to remove fluid exceeds the normal rate of fluid formation by a factor of 20.

*1-1-3.*Clinical manifestations and diagnose tests

The presence of moderate to large amounts of pleural fluid produces symptoms and characteristic changes on physical examination. The symptoms of a patient with a pleural effusion are mainly dictated by the underlying process causing the effusion. Many patients have no symptoms referable to the effusion. When symptoms are related to the effusion, they arise either from inflammation of the pleura, from compromise of pulmonary mechanics, from interference with gas exchange, or on rare occasions, from decreased cardiac output.

The accumulation of clinically detectable quantities of pleural fluid is distinctly abnormal. Pleural effusions have classically been divided into transudates and exudates (Light et al., 1972). A transudative pleural effusion develops when the systemic factors influencing the formation or absorption of pleural fluid are altered so that pleural fluid accumulates. The pleural fluid is a transudate. The fluid may originate in the lung, the pleura, or the peritoneal cavity (Broaddus et al., 1992). The permeability of the capillaries to proteins is normal in the area where the fluid is formed. In contrast, an exudative pleural effusion develops when the pleural surfaces or the capillaries in the location where the fluid originates are altered such that fluid accumulates. The pleural fluid is an exudate. The most common causes of exudative pleural effusions are pleural malignancy, parapneumonic effusions, and pulmonary embolism.

An accurate diagnosis of the cause of the effusion, transudate versus exudate, relies on a comparison of the chemistries in the pleural fluid to those in the blood, using Light's criteria. According to Light's criteria (Light et al., 1972), a pleural effusion is likely exudative if at least one of the following exists. Also see **Table 1-2**.

1. The ratio of pleural fluid protein to serum protein is greater than 0.5

2. The ratio of pleural fluid LDH and serum LDH is greater than 0.6

3. Pleural fluid LDH is greater than 0.6 or 2/3 times the normal upper limit for serum. Different laboratories have different values for the upper limit of serum LDH, but examples include 200 and 300 IU/L.

The sensitivity and specificity of Light's criteria for detection of exudates have been measured in many studies and are usually reported to be around 98% and 80%, respectively (Romero et al., 2000; Joseph et al., 2002).

1-2. Pleural inflammation and fibrosis

1-2-1. Mesothelium and regulation of pleural inflammation

Pleura is lined by a monolayer of mesothelial cells which secrete glycosaminoglycans and other surfactant-like molecules to lubricate the pleural surface (Roth, 1973; Beavis et al., 1994). The mesothelium has a number of other important functions including movement of fluid, particulates and cells across the pleural cavity; release of pro- and anti-inflammatory and other immunomodulatory mediators; antigen presentation; secretion of factors that promote both deposition and clearance of fibrin; and synthesis of growth factors and ECM proteins to aid in serosal repair (Mutsaers 2002; Mutsaers et al., 2004).

The onset of pleural infections and injury is characterized by a massive influx of leukocytes from the vascular compartment into the pleural space (Brauner et al., 1993). This is likely to be initiated on the surface of the mesothelial cell. For example, bacteria, talc and asbestos fibres are phagocytosed by mesothelial cells which induce cell activation and release of chemokines such as interleukin (IL)-8 (Tanaka et al., 2000). Furthermore, mediators released from activated macrophages also stimulate mesothelial cells to release potent inducers of neutrophil and monocyte chemokines including IL-8, growth-related oncogene (GRO)-α, interferoninducible protein (IP)-10, monocyte chemoattractant protein (MCP)-1 **(**Betjes et al., 1993; Nasreen et al., 2001). Secretion of these chemokines is polarised toward the cell apical surface, creating a chemotactic gradient from the basolateral to the apical side of the mesothelial cell (Li et al., 1998). By secreting chemokines in a polarized manner, mesothelial cells promote directed transmesothelial migration of both neutrophils and monocytes.

Movement of leukocytes from the circulation to the site of inflammation is facilitated by the expression of integrins and adhesion molecules. Mesothelial cells express several cell adhesion molecules including intercellular adhesion molecule (ICAM)-1, vascular cellular adhesion molecule (VCAM)-1, E-cadherin, Ncadherin, CD49a, CD49b and CD29 and can be induced by IL-1b (Rossen et al., 2001), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) *in vitro* (Hausmann et al., 2000). Leukocytes express the β2 integrin family members, lymphocyte function associated antigen (LFA)-1 (CD11a/CD18), and Mac-1 (CD11b/CD18) on their surface, which are counter receptors for ICAM-1. Interaction between LFA-1/ Mac-1

and ICAM-1 leads to cell-cell adherence and results in transmigration of leukocytes across mesothelial cell monolayers (Zeillemaker et al., 1996).

Resolution of inflammation and repair of the pleura without fibrosis requires a down-regulation of the inflammatory response, including leukocyte clearance and inhibition of fibroblast proliferation and collagen production. Mesothelial cells are likely to contribute to controlling inflammation both in normal and inflamed tissue as they have cyclooxygenase (Cox) activity, expressing both Cox-1 and Cox-2 mRNA and metabolise arachidonic acid to release prostaglandins and prostacyclin (Hott et al., 1994). Mesothelial cells also participate in regulating efflux of inflammatory cells from serosal cavities via stomata and the draining lymphatics. Recently we demonstrated that macrophage emigration from inflamed peritoneum is controlled through specific integrin mediated regulation of macrophage-mesothelial cell interactions involving very late antigen (VLA)-4 andVLA5 (Bellingan et al., 2002).

1-2-2. Pleural fibrosis

During the process of wound healing, formation of a transitional fibrin neomatrix contributes to tissue organization and fibrotic repair (Dvorak et al., 1986). In a similar context, disordered pathways of coagulation and fibrinolysis that favor intrapleural fibrin deposition are characteristic of pleural inflammation and pleurodesis (Idell et al., 1995). The observation of intrapleural fibrin in association with pleural inflammation lends further support to the notion that the fibrinous neomatrix is involved in the evolution of pleural repair and location (Bignon et al., 2001). The rapid appearance of intrapleural fibrin resembles that of intrapulmonary fibrin deposition in the progression of accelerated pulmonary fibrosis, as may occur in severe cases of acute respiratory distress syndrome (ARDS) (Idell et al., 1995). Disordered local fibrin turnover, therefore, occurs in both settings and represents an early tissue response that sets the stage for progressive remodeling and may lead to fibrotic repair in the face of protracted injury. Extravascular tissue fibrin deposition, as occurs in the setting of pleural injury, follows a progression recapitulating that which occurs in the setting of wound healing and the desmoplastic response associated with solid forms of neoplasia. As a function of increased microvascular permeability, plasma is extravasated and enters the parenchyma of inflamed tissues, where it encounters tissue factor (TF). TF forms a complex with activated factor VII to initiate coagulation at these extravascular sites, thereby initiating the formation of transitional fibrin, which may undergo progressive remodeling subject to the

influence of locally elaborated mediators of inflammation and the products of inflammatory and parenchymal cells. A wide array of myeloid and parenchymal cells, including mesothelial cells, can produce components of the fibrinolytic response as well as inhibitors of the fibrinolytic system, including urokinase (uPA), its receptor (uPAR), and plasminogen activator inhibitor (PAI)-1. The responses of these cells may thereby influence whether transitional fibrin is cleared or maintained, based on the balance of the expression of the components of the fibrinolytic system and their net influence on local fibrinolytic activity. As reviewed elsewhere, there is considerable interplay between the coagulation and fibrinolytic systems and other inflammatory pathways that may potentiate local inflammation and reparative responses (Idell et al., 2001; Ingelfinger et al., 2003).

Resident pleural cells express uPA, uPAR and PAI-1, as well as tPA, and all of these components can be detected in pleural fluid. Plasminogen, a substrate for both tPA and uPA, is likewise present in pleural fluids, and as such plasmin can be generated locally (Idell et al., 1991). Pleural fluids harvested from patients with congestive heart failure demonstrate detectable fibrinolytic activity, but the fibrinolytic activity of patients with exudative pleural effusions is subject to inhibition by PAI, in particular PAI-1, and antiplasmins. In these situations, much of the uPA expressed in pleural fluids is bound to PAI-1 (Idell et al., 1991). The decrement in fibrinolytic capacity associated with these disorders underlies the deposition of fibrin between the visceral and parietal pleural surfaces in these conditions. While inhibition of fibrin clearance is implicated in the development of accelerated pleural organization and fibrosis, the alterations are also observed in exudative effusions that do not locate. It is, therefore, likely that other alterations act in synergy to promote intrapleural organization in conditions such as evolving pleurodesis and complicated parapneumonic effusions or organizing haemothoraces. For example, cytokines implicated in the pathogenesis of pleural injury, including TNF-α, can upregulate uPAR expression at the surface of cell types involved in pleural injury and thereby influence local remodeling of transitional fibrin. Exposure of mesothelial cells to asbestos can also influence uPAR expression (Perkins et al., 1999). The control of the fibrinolytic system may occur at multiple regulatory levels, such as at gene transcription and post-transcriptionally, with respect to mRNA stability and translational control.

Over years, a large number of locally produced peptides have been identified and shown to play a role in the maintenance and regulation of cell and tissue function. These peptides,

including cytokines and polypeptide growth factors, are mediators produced by one cell that have the capacity to govern either their own cellular function (autocrine) or the function of other cells (paracrine). Cytokines and growth factors have been implicated in the regulation of intracellular and intercellular signaling events responsible for the maintenance of normal healthy pleural tissue and in the induction and progression of pathogenic states of pleural disease. The involvement of these proteins in the processes of pleural injury, repair and fibrosis is the subject of ongoing investigations. Mesothelial cells initiate the healing process by attracting inflammatory cells, fibroblasts, and other cells to the site of injury. These cells, in turn, release factors which regulate cell proliferation, migration, differentiation, and ECM production (**Table 1-3**).

1-3. Pulmonary fibrosis

Pulmonary fibrosis is a lung disease that is refractory to treatment and carries a high mortality rate. It includes a heterogeneous group of lung disorders characterized by the progressive and irreversible destruction of lung architecture caused by scar formation that ultimately leads to organ malfunction, disruption of gas exchange, and death from respiratory failure (Wynn, 2011).

Idiopathic pulmonary fibrosis (IPF) may induce by chronic inflammation of the alveolar lung injury or alveolar epithelial cell damaged continuously to become the structural remodeling and lung tissue fibrosis. Pathology and lung fibrosis related diseases about the type of sarcoma, radiation pneumonitis, chronic obstructive pulmonary disease (COPD) and ARDS. Generally, pulmonary fibrosis is the final phase of many severe lung injuries , characterized by lung cells damaged, the invasion of immune cells, releasing the promote fibrosis factor, fibroblast proliferation, differentiation and activation, and increased extracellular matrix production (Kuwano et al., 2001).

Repair of damaged tissues is a fundamental biological mechanism that allows the ordered replacement of dead or damaged cells after injury, a process critically important for survival (Wynn, 2007). However, if this process becomes dysregulated, it can lead to the development of a permanent fibrotic “scar,” which is characterized by the excess accumulation of ECM components (e.g., hyaluronic acid, fibronectin, proteoglycans, and interstitial collagens) at the site of tissue injury.

Although the relative importance of inflammation in the progression of pulmonary fibrosis has been debated, it was induced to become a strong inflammatory response by many forms of the disease (Crystal et al., 2002). Even if some types of pulmonary fibrosis maintain a significant inflammatory component throughout the course of the disease, other forms like IPF are often characterized as exhibiting highly progressive fibrotic disease in the absence of detectable inflammation (Thannickal et al., 2004).

The fibrosis inducing side effect associated with bleomycin therapy observer in the human population has been exploited by researchers attempting to develop murine models of human interstitial pneumonias (Chua et al., 2005). Nevertheless, many important advances have been generated from rodent models, which have been dominated by transgenic and knockout mice that display either enhanced or decreased susceptibility to pulmonary fibrosis.

A variety of experimental models have been generated to study the mechanisms of pulmonary fibrosis (Moore et al., 2008). However, the mouse bleomycin model has garnered the most attention, perhaps because it is a well- characterized and clinically relevant model of pulmonary fibrosis. Nevertheless, although it successfully models the early proinflammatory stages of the disease, because of the transient nature of the bleomycin response and the reversibility of the fibrosis, it is unclear whether this model can truly replicate the chronic and progressive forms of the disease seen in humans.

1-4. Renin angiotensin system

The renin-angiotensin system (RAS) is a classically hormonal system consists of endocrine, paracrine and intracrine system (Fyhrquist et al., 2008). The manly function of RAS involved the balance of salt and water, blood pressure and natriuresis, it also plays an important local role to regulate regional blood flow and nutrition in several target organs such as heart (Guimarães et al., 2012), blood vessels (Khakoo et al., 2008), and lungs (Imai et al., 2008; Shrikrishna et al., 2012; Wong et al., 2012).

Recently, a local tissue-based RAS has also been described and appears to play a key role in injury/repair responses (Marshall, 2003; Wösten-van et al., 2008). The expression of RAS components and the elevation of angiotensin converting enzyme (ACE) in a number of lung diseases suggest the existence of a pulmonary RAS and that Ang II could mediate, at least in part, the response to lung injury. The central role of ACE is to generate Ang II from

Ang I. Ang II is a key effector peptide of RAS that causes vasoconstriction and exerts multiple biological functions. Ang II can also cause the development of interstitial fibrosis via physiological regulation of MMPs expression and/or activation (Chen et al., 2008).

The angiotensin pathway is involved in the pathogenesis of many fibrotic diseases and in idiopathic pulmonary fibrosis an innate overexpression of Ang II, a potent TGF-β1 inductor has been demonstrated. Ang II therapeutic blockade could be therefore a promising antifibrotic approach (Jeffery et al., 2001; Mandegara et al., 2004; Antoniu, 2008). The molecular mechanism may be one of the parameters of pulmonary fibrosis. There are a lot of unknown about the pathway of Ang II/AT1R axis duration of pulmonary fibrosis (Budinger, 2011). Ang II has another signaling pathway to active Smads in cell (Rodríguez-Vita et al., 2005; Yang et al., 2009). Moreover, ROS (Reactive Oxygen Species) may be induced via Ang II to promote tissue fibrosis (Okada et al., 2009).

1-5. Angiotensin converting enzyme II

There is abundant expression of RAS components in lung, including ACE and ACE2. ACE2 was cloned as a first homolog of human ACE and mapped to the X chromosome by two independent research groups in 2000 (Donoghue et al., 2000; Tipnis et al., 2000). The ACE2 is an 805 amino acid zinc-metallopeptidase and type I integral membrane glycoprotein encoded from 18 exons with a molecular weight of approximately 120 kDa (Turner et al., 2002), it is predominantly observed in the heart, kidneys and testes (Tipnis et al., 2000) such as cardiomyocytes (Gallagher et al., 2008), luminal surface of tubular epithelial cells (Donoghue et al., 2000; Tipnis et al., 2000) and adult Leydig cells (Douglas et al., 2004). In addition, ACE2 also had been confined at a lower level in a wide variety of tissues including the brain (Xia et al., 2008; Xu et al., 2011), liver (Lambert et al., 2008; Pereira et al., 2009) and lung (Kuba et al., 2006; Imai et al., 2008).

In molecular structure, the human *ace2* gene comprise 18 exons, the first 12 exons of *ace2* is similar to the first 11 exons of the ace gene. Moreover, the zinc-binding motif (HEMGH) of ACE2 is located within exon 9, compared to exon 8 of the *ace* gene (Donoghue et al., 2000; Tipnis et al., 2000). As like ACE, ACE2 has two domains of the amino-terminal catalytic domain and the carboxy terminal domain, shares 42% sequence identity and 61% sequence similarity with the catalytic-domain of ACE (Donoghue et al., 2000; Tipnis et al.,

2000; Douglas et al., 2004). Unlike somatic ACE, ACE2 only contains a single catalytic site with the prototypical zinc-binding HEMGH motif, and functions as a carboxymonopeptidase removing a single C-terminal residue from peptide substrates whereas ACE acts as a carboxydipeptidase (peptidyldipeptidase), removing a C-terminal dipeptide (Clarke et al., 2012). In addition, the carboxy-terminal domain of ACE2 shows 48% sequence identity with collectrin, which was a non-catalytic protein that has a critical role in amino acid absorption in the kidney (Danilczyk et al., 2006; Malakauskas et al., 2007), pancreatic beta-cell proliferation (Akpinar et al., 2005) and insulin exocytosis (Fukui et al., 2005) (**Fig. 1-1**).

ACE2, a close homologue of ACE, functions as a negative regulator of the angiotensin system and was identified as a key receptor for severe acute respiratory syndrome coronavirus infections (Kuba et al., 2005; Imai et al., 2010). ACE2 reduces the generation of Ang II by catalyzing the conversion of Ang I to Ang 1-9 and facilitating hydrolysis of Ang II to Ang 1-7. Ang 1-7 has been recognized as a potential endogenous inhibitor of the classical RAS cascade (Mercure et al., 2008) (**Fig. 1-2**). Hence, the ACE2/Ang 1-7 axis may be an important negative modulator of Ang II bioactivity, counteracting the effects of ACE in determining net tissue Ang II levels. Abnormally elevated ACE combined with decreased ACE2 expression may be involved in fibrotic processes *in vitro* and *in vivo*, and the mechanism may involve expression and activation of specific MMPs (Pan et al., 2008).

In recent years, ACE2/Ang 1-7 / Mas axis have been explored in pulmonary hypertension disease (Ferreira et al., 2009; Yamazato et al., 2009). Moreover, the first research was found that ACE2 associated with pulmonary fibrosis directly in 2008 at the AJP-Lung Cell Mol Physiol (Li et al., 2008). Li et al have found ACE2 mRNA, protein and enzymatic activity were severely decreased in human IPF and in both rat and mouse models of experimental lung fibrosis induced by bleomycin. In addition, the mice with lung fibrosis were given recombinant ACE2 protein and reduced lung fibrosis. (Shenoy et al., 2010). ACE2 KO mice do not have lung abnormalities when compared with their wild-type littermates (Turner et al., 2002). However, it was recently shown that loss of ACE2 expression precipitates severe acute lung failure. Moreover, injection of recombinant human ACE2 attenuates acute lung failure in Ace2 KO as well as in wild-type mice (Fourrier et al., 1985).

With respect to the possible role of ACE2 in the lung in relation to acute lung injury in particular, the relationship to the ACE2 expression at the alveolar capillary interface is of interest. It is tempting to speculate that increased ACE2 may play a role in reducing the initial

leakage over the alveolar capillary interface. This would then slow down the vicious circle that often occurs after a damaging effect to this interface and leads to the clinical pathological picture of diffuse alveolar damage with intra-alveolar oedema and fibrin deposits. These results support a critical role for the intrapulmonary RAS in the pathogenesis of acute lung injury and show that ACE2 is a key molecule involved in the development and progression of acute lung failure (Inge et al., 2007).

1-6. Matrix metalloproteinase

The tissue fibrosis is caused by excessive accumulation of ECM components, especially types I and III collagen, in various pathological manifestation diseases (LeRoy et al., 1974; Uitto et al., 1979). The balance of ECM components is maintained by MMPs and TIMPs (Clutterbuck et al., 2009). MMPs are essential components for various normal biological processes such as embryonic development, morphogenesis, reproduction tissue resorption and remodeling (Szarvas et al., 2011), they also implicated in a number of key pathologic processes including inflammation, fibrosis, arthritis, pulmonary diseases and cancer (Amălinei et al., 2010), because of the abnormally ECM deposition that imbalance MMPs and the TIMPs caused.

Compared to the cardiovascular disease, it reported less about the balance of MMPs and TIMPs activity in respiratory diseases less. However, the research results showed MMPs and TIMPs in some respiratory disease in the past (Gaggar et al., 2011). Researchers can measure higher activity of MMP-3 (Yamashita et al., 2011) and MMP-7 (Fujishima et al., 2010) in patients with pulmonary fibrosis. The results of experimental animals pulmonary fibrosis induced by bleomycin, TGF-ß1 or radiation irradiation, showed abnormal activity of MMP-3 (Yamashita et al., 2011), MMP-12 (Kang et al., 2007.), MMP-13 (Flechsig et al., 2010) related to the course of pulmonary fibrosis. Furthermore, it was also detected the abnormal performance of TIMPs or the imbalance activity of MMPs/TIMPs in experimental animal pulmonary fibrosis (Manoury et al., 2007). Interestingly, a lot of research about MMPs gelatinase (MMP-2 and MMP-9) has been reported in cardiovascular research but less in the lung tissue and pulmonary fibrosis.

MMPs had been discovered by Gross and Lapiere in 1962, are a group of Zn^{2+} and calcium dependent endopeptidases of common significant peptide chain sections, however

glycosylated in different amount and different locations (Sternlicht et al., 2001). MMPs comprise a large family of protease and share several similarities in terms of their structure, regulation and function (Nagase et al., 1999; Bode et al., 2001). Up to now, 28 types of MMPs have been identified, and they are further divided into six major subfamilies based on structure and substrate specificity, including collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs and other MMPs (**Table 1-4**; Vargová et al., 2012).

The major structure of all MMPs consists of three domains: N-terminal hydrophobic signal sequence, a propeptide domain region and a catalytic domain (Nagase, 1997; Visse and Nagase, 2003). The N-terminal hydrophobic signal sequence decides the MMPs which been released out or maintained in the cell membrane. Furinrecognition motifs are seen in multiple MMP isoforms' prodomain regions, and type II fibronectin repeats are seen in gelatinase (MMP-2 and -9) catalytic domains. These regions seem to have important implications for the interaction of the MMPs with its substrate. The molecular structure of different type of MMPs was present in **Fig. 1-3.**

MMPs have the combined ability to degrade essentially all connective tissue components. MMPs are involved in many normal homeostatic mechanisms, the expression and activity is commonly elevated in conditions where inflammation and tissue remodeling/repair are operative (McCaw et al., 2007).

In addition to regulation of activation, there are numerous inhibitors of MMPs. Although TIMPs are often thought of as the predominant group of inhibitors for MMPs, they are really the most specific endogenous MMP inhibitors. The majority of MMP-related inhibition *in vivo* occurs through relatively nonspecific MMP inhibitors such as α2 macroglobulin (McCaw et al., 2007). TIMPs are a group of four small (20–24 kDa) MMP-specific inhibitors that bind to MMPs in a 1:1 stoichiometric relationship (Zucker et al., 1998). All four mammalian TIMPs have many basic similarities, but they exhibit distinctively structural features, biochemical properties and expression patterns (**Table 1-5**). Animal studies, in addition to human studies in adults, support a role for MMPs and an imbalance between MMPs and TIMPs in the pathogenesis of several well-recognised pulmonary disorders, such as COPD (Finlay et al., 1997) and asthma (Tanaka et al., 2000).

The ECM decreased by MMPs is mainly impressed by TIMPs, MMPs and TIMPs play a critical role in maintaining the balance between ECM deposition and degradation in physiological processes (Hulboy et al., 1997; Vu et al., 2000). Four TIMPs, TIMP-1, -2, -3,

and -4, have been identified (Cruz-Munoz et al., 2008), these TIMPs are secreted by a variety of cell lines such as smooth muscle cells and macrophages. TIMPs also involved in the process of inflammation and fibrosis, their activity is increased by PDGF and TGF-β and either increased or decreased by different ILs (Jones et al., 2003). In addition, evidences suggest that fibrotic livers have high expression of the TIMP-1 and TIMP-2, and thus the combination of low expression of MMPs and high TIMPs may prevent the degradation of the fibrillar collagens.

Recently, abnormal pleural MMP levels, MMP-2 and MMP-9, have been reported, and the association between MMPs and development of pleurisy has been investigated (Iglesias et al., 2005; Oikonomidi S et al., 2010).

1-6-1. Gelatinase A (MMP-2, Type II collagenase)

In 1978, Sellers et al. were first to separate a gelatinase activity from collagenase and stromelysin in the culture medium from rabbit bone (Sellers et al., 1978). A similar enzyme, acting on basement membrane type IV collagen was reported by Liotta et al. (1979) in the following year. Gelatinase was purified from human skin, mouse tumor cells, rabbit bone, and human gingival. The completed sequence of the human MMP-2 except for the signal peptide was reported by Collier et al. (2001). Gelainase A has a triple repeat of fibronectin type I domains inserted in the catalytic domain; this domain participates in binding to the gelatin substrates of the enzyme. MMP-2 is ubiquitously expressed in the cells which comprise the heart and is found in normal cardiomyocytes, as well as in endothelium, vascular smooth muscle cells and fibroblasts (Coker et al., 1999).

MMP-2 (EC 3.4.24.24) is a protease with gelatinolytic activity (hence its alternate name, gelatinase A), which is found to be expressed constitutively in various cell types found in the lungs. This enzyme has a broad spectrum of substrates and is involved in modulating diverse cellular functions, including angiogenesis (Brown et al., 2003), tissue remodelling and potentiation of inflammatory response (Kumagai et al., 1999). MMP-2 is activated in a unique membrane-type MMP-dependent manner, demonstrating a classic example of MMP-to-MMP activation. MMP-2 is thought to contribute to the pathogenesis of a variety of pulmonary disorders, including COPD, asthma, lung cancer and interstitial pulmonary fibrosis (Chakrabarti et al., 2005).

1-6-2. Gelatinase B (MMP-9, Type V collagenase)

In 1972, Harrwas and Krane detected a gelatinase activity in rheumatoid synovial fluid. Sopata et al. described a gelatinase from human polymorphonuclear leukocytes. Rabbit macrophages produce a very similar enzyme which is able to digest type V collagen (Horwitz et al., 1977). The neutrophil collagenase and gelatinase were resolved in 1980 (Murphy et al., 1980). Purification of MMP-9 protein was achieved in 1983 and sequencing of the cDNA was completed in 1989. An interesting phenomenon, still not fully understood, is the binding of TIMP-1 to proMMP-9 to form a complex (Stetler-Stevenson et al., 1989). Human neutrophil MMP-9 commonly occurs as a complex with lipocalin. A series of papers concerned a 95 kDa protein in plasma that binds to gelatin culminated in the identification of this protein as MMP-9 (Makowski et al., 1998).

MMP-9 (EC 3.4.24.35), or gelatinase B, is broadly expressed in a variety of cells in the lung, including inflammatory (PMNs, macrophages), epithelial and endothelial cells. This observed redundancy belies important location-specific functions of MMP-9, some of which seem in opposition to other MMP-9 functions. For example, MMP-9 has observed important pro-inflammatory effects by generating PGP (Gaggar et al., 2008) and increasing the chemokine potency of IL-8 (Van den Steen et al., 2000), but MMP-9 also plays an important role in the regulation of granuloma formation in tuberculosis (Volkman et al., 2010). Similarly, MMP-9 had been thought to lead to matrix breakdown, but recently it has been suggested that MMP-9 may have a role in matrix repair (Bove et al., 2007; Gagger et al., 2011).

1-7. ACE2 and gelatinase (MMP-2 and MMP-9)

ACE2 is a newly identified component of RAS and plays a negative regulator of Ang II in the RAS. Most of published papers reveal that Ang II could break the balance of MMPs expression in heart and induce heart remodeling (Brassard et al., 2005; Yaghooti et al., 2011), but the relative between ACE2 and MMPs are still unknown. In 2009, Kassiri's group utilized the left anterior descending artery ligation and ACE2 KO mice to investigate the role of ACE2 in MI (Kassiri et al., 2009). In wild-type mice, ACE2 was persistent increased in the infarct zone of heart, ACE2-deficient was increased interferon-γ, interleukin-6, phosphorylation of

ERK1/2 and JNK1/2 signaling pathways and MMP-2 and MMP-9 levels in response to MI. Loss of ACE2 also associated with the increased expression and phosphorylation of $p47^{phox}$, Ang II levels, NADPH oxidase activity, and superoxide generation, which could lead to enhanced MMP-mediated degradation of the extracellular matrix in ACE2-deficient myocardium and eccentric remodeling, increased pathological hypertrophy, and worsening of systolic performance (Bodiga et al., 2011; Patel et al., 2012).

Interesting, ACE2 overexpression inhibited cell growth, MMP-2 and MMP-9 expression, VEGFa production, and ACE and AT1R expression in human lung cancer xenografts and A549 cells in vitro (Feng et al., 2011). These evidences reveal that Ang II mediated AT1R to induce NADPH oxidase and MMP activation, AT1R blocker and Ang 1-7 supplementation inhibited NADPH oxidase and MMP activation (Kassiri et al., 2009; Bodiga et al., 2011). Furthermore, these results suggest that ACE2 serves as a protective mechanism and associated with MMPs expression, especially MMP-2 and MMP-9, but the detail signal pathway still not clear.

Table 1-1. General causes of pleural effusions

Processes occurring	Caused	References
Increased pleural fluid formation		
Increased interstitial fluid in the lung	Left ventricular failure, pneumonia, and pulmonary embolus	Wiener et al., 1993
Increased intravascular pressure in pleura	Right or left ventricular failure, superior vena caval syndrome	Light et al., 1980
Increased permeability of the capillaries in the pleura	Pleural inflammation Increased levels of vascular endothelial growth factor	Cheng et al., 1999; Thickett et al., 1999
Increased pleural fluid protein level		
Decreased pleural pressure	Lung atelectasis or increased elastic recoil of the lung	Eid et al., 1999
Increased fluid in peritoneal cavity	Ascites or peritoneal dialysis	Kirschner et al., 1998
Disruption of the thoracic duct		
Disruption of blood vessels in the thorax		
Decreased pleural fluid formation		
Obstruction of the lymphatics draining the parietal pleura		Stephen, 2004
Elevation of systemic vascular pressures	Superior vena caval syndrome or right ventricular failure	Allen et al., 1988

Table 1-2. Diagnose of transudates and exsudates

	Transudate	Exsudate	Reference
Main causes	Increased hydrostatic pressure, ecreased colloid osmotic pressure	Inflammation	Marel et al., 1993
Appearance	Clear	Cloudy	
Specific gravity	< 1.012	> 1.020	Light et al., 1979
Protein content	< 25 g/L	> 35 g/L	Heffner et al., 1997
fluid protein / serum protein	< 0.5	> 0.5	Light et al., 1972
Difference of albumin content with blood albumin	> 1.2 g/dL	< 1.2 g/dL	Roth et al., 1990
fluid LDH / upper limit for serum	< 0.6 or < 2/3 of normal	> 0.6 or > 2/3 of normal	Light et al., 1972
Cholesterol content	< 45 mg/dL	> 45 mg/dL	Heffner et al., 1997

Table 1-3. The cellular responses of cytokines and growth factors implicated in the progression of pleural fibrosis

Cytokine/ Growth Factor	Action	Cellular Response
TGF-β	Pro-fibrotic	Increased ECM production; collagen, laminin, fibronectin, thrombospondin, tenascin, biglycan. Decreased matrix degradation; decreased MMP-1, MMP-3 and PA production and increased TIMP production
	EMT	Induces fibroblastic changes, i.e. increase in collagen I and III production and decreased cytokeratin expression
PDGF	Pro-fibrotic	Stimulates collagen and hyaluronan production. Stimulates the expression of TGF-β
	Mitogen	Promotes fibroblast proliferation: Induces cell motility and chemotaxis
FGF	Mitogen	Enhances proliferation of fibroblasts and mesothelial cells. Stimulates PA synthesis
	Angiogenic	Implicated in tumour growth and metastasis. FGF production stimulated by IL-1 and TGF-β
IGF	Profibrotic	Stimulates collagen synthesis
	Mitogen	Induces proliferation of fibroblasts and mesothelial cells
Endothelin-1	Profibrotic	Stimulates collagen synthesis
	Mitogen	Promotes fibroblast proliferation; Induces cell motility and chemotaxis
EGF	Mitogenic	Induces DNA synthesis and cell proliferation
	EMT	Induces fibroblastic changes, i.e. increase in collagen I and III production and decreased cytokeratin reactivity
TNF-α	Profibrotic	Fibroblast proliferation and collagen production and enhances PAI-1 and tPA production
	Mitogenic	Promotes fibroblast cell proliferation
IL-1	Profibrotic	Simulates TGF-β production of collagen and fibronectin. Upregulates FGF synthesis

(Mutsaers et al., 2004)

Table 1-4. Types of different matrix metalloproteinases and their substrate specificity

Subgroups	MMPs	Name	Substrate
Collagenases	MMP-1	Collagenase-1	Collagen I, II, III, VII, VIII, X, and gelatin
	MMP-8	Collagenase-2	
	MMP-13	Collagenase-3	
Gelatinases	MMP-2	Gelatinase A	Collagen I, IV, V, VII, X, XI, XIV, and gelatin
	MMP-9	Gelatinase B	
Stromelysins	MMP-3	Stromelysin-1	Collagen II, IV, IX, X, and gelatin, α-casein, β-casein
	MMP-10	Stromelysin-2	
	MMP-11	Stromelysin-3	
Matrilysins	MMP-7	Matrilysin-1	Collagen I, II, III, V, IV, X and casein
	MMP-26	Matrilysin-2	
Membrane-type MMPs	MMP-14	MT1-MMP	Gelatin, fibronectin and laminin
	MMP-15	MT2-MMP	Gelatin, fibronectin and laminin
	MMP-16	MT3-MMP	Gelatin, fibronectin and laminin
	MMP-17	MT4-MMP	Fibrinogen and fibrin
	MMP-24	MT5-MMP	Gelatin, fibronectin and laminin
	MMP-25	MT6-MMP	Gelatin
Other MMPs	MMP-12	Metalloelastase	Collagen IV, elastin and gelatin
	MMP-19	RASI-1	Collagen I, IV and gelatin
	MMP-20	Enamelysin	Collagen I, IV, and gelatin
	MMP-23	CA-MMP	Gelatin
	MMP-26	Matrilysin-2, endometase	Collagen IV and gelatin
	MMP-28	Epilysin	Gelatin

(Swarnakar et al., 2011)

Table 1-5. Functional properties of the tissue inhibitors of metalloproteinases (TIMPs)

	TIMP-1	**TIMP-2**	**TIMP-3**	**TIMP-4**
Protein kDa	28	21	24/27	22
N-glycosylation sites	2	0	1	0
Protein localization	Soluble	Soluble/cell surface	ECM	Soluble/cell surface
Pro-MMP association	pro-MMP-9	pro-MMP-2	pro-MMP-2/-9	pro-MMP-2
MMPs poorly inhibited	MT1-MMP MT2-MMP MT3-MMP MT5-MMP MMP-19	None	None	None
ADAM inhibition	ADAM 10	None	ADAM 12 ADAM 17 ADAM 19 (ADAM 10) ADAMTS-4, TS-5	None
Cell proliferation	Erythroid precursors Tumour cells	Erythroid precursors Tumour cells Fibroblasts Smooth muscle cells Endothelial cells	Smooth muscle cells and cancer cells	Mammary tumour cells Wilm's tumour cells
Apoptosis	Burkitt's lymphoma cells	Colorectal cancer cells Melanoma	Smooth muscle cells Tumour cells Retinal pigmented epithelial cells	Cardiac fibroblasts
Tumour angiogenesis	Mammary Liver	Melanoma Mammary	Melanoma	
Angiogenesis in 3D collagen/fibrin gels	No effect	Inhibits	Inhibits	Inhibits
Tumourigenesis effects	Inhibits	Inhibits	Inhibits	Inhibits
Metastasis effects	Stimulates			Stimulates

MT-MMP, membrane-type matrix metalloproteinase; ADAM, a disintegrin and metalloproteinase; ADAMTS, a disintegrin and metalloproteinase thrombospondin type.(Bokarewa et al., 2005)

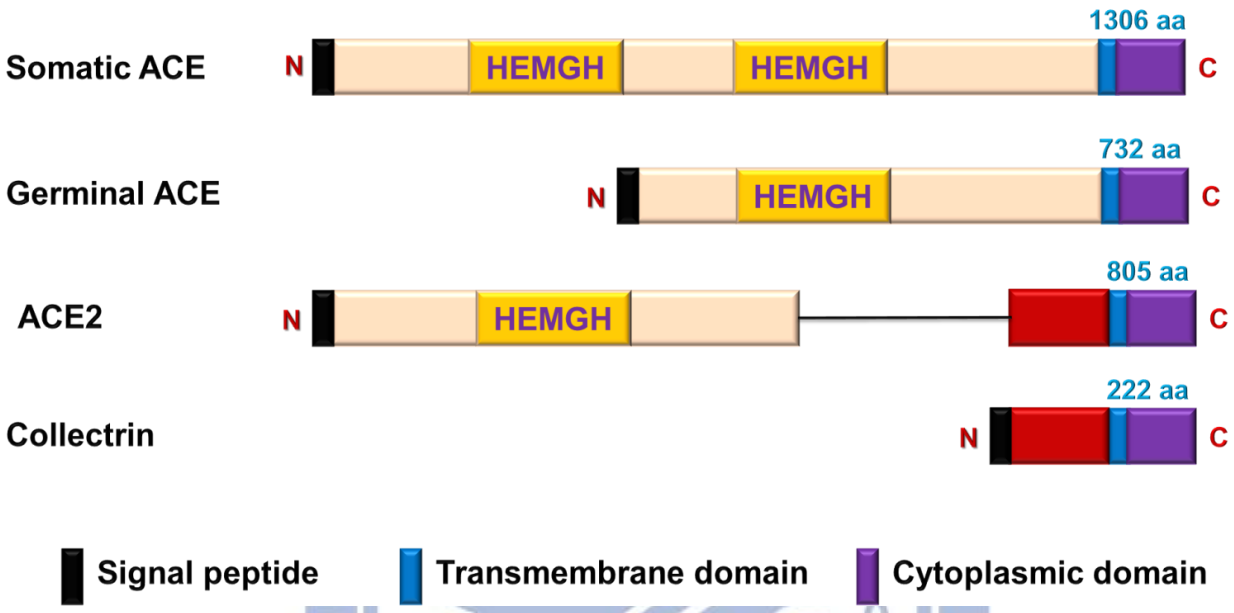

Fig. 1-1. Family of enzymes and proteins belonging to the ACE family of proteins. The schematic present the molecular structure of ACE, ACE2 and collectrin. HEMGH is a set of conserved amino acid residues critical for the activity of the zinc binding catalytic site. ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme II. (Wang et al., 2012)

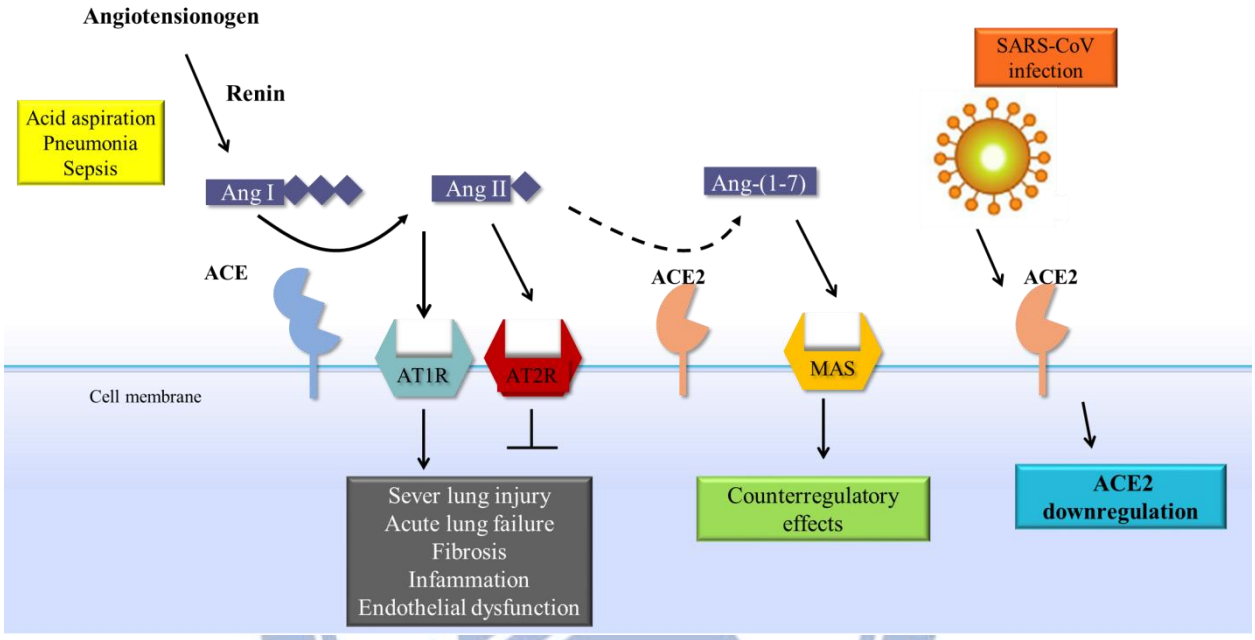

Fig. 1-2. Schematic diagram of the role of the RAS in acute lung failure and the proposed action of the SARS-coronavirus (SARS-CoV). In acute lung injury, such as acid aspiration, pneumonia or sepsis, the generation of angiotensin II (Ang II) from angiotensin I (Ang I) is enhanced by angiotensin-converting enzyme (ACE), and Ang II induces ALI through stimulation of the AngII type 1 receptor (AT1R), whereas ACE2 and AngII type 2 receptor (AT2R) negatively regulate this pathway and are protective. On the other hand, SARS-CoV infection is mediated through binding of the SARS–spike protein to ACE2 ,which downregulates the protective molecule ACE2, and thus leads to severe lung injury and acute lung failure. (Imai et al., 2010)

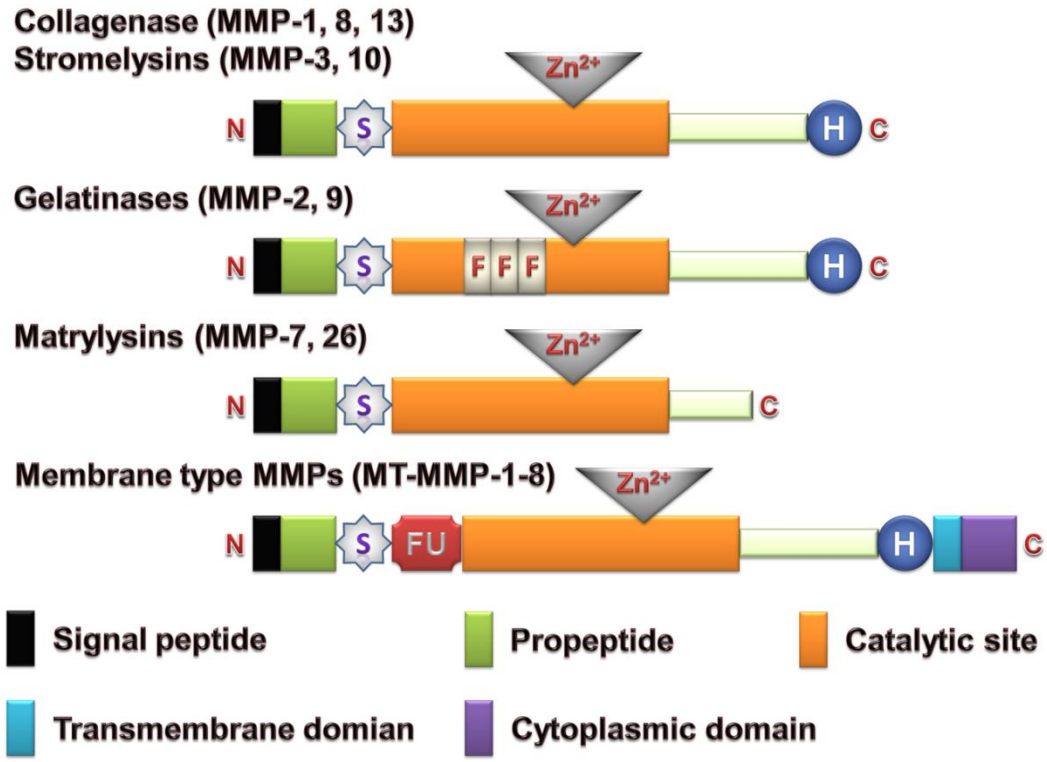

Fig. 1-3. Family of enzymes and proteins belonging to the MMPs family of proteins. The schematic present the mainly molecular structure of collagenases, stromelysins, gelatinases, matrylysins and membrane type MMPs. S, cysteine switch; FU, intracellular furin-like serine proteinases; F, collagen-binding type II repeats of fibronectin; Zn^{2+}, zinc-binding site; H, hemopexin domain. (Vargová et al., 2012)

II. Research Purpose and Strategy

Remodeling of the lung architecture is a hallmark of many lung diseases, for example, loss of alveolar walls in emphysema, subepithelial fibrosis in asthmatic airways, IPF, cavity formation in tuberculosis, and bronchiectasis in cystic fibrosis. ACE2 is a novel element in RAS and plays a significant role against the harmful effect of Ang II induced. The Ang II has been degraded by ACE2 and form Ang 1-7 to suppress the tissue remodeling that Ang II induced. All of these pathologic changes involve extensive alterations of lung ECM. MMPs or TIMPs have been proposed to be key in causing these changes because of their capacity to cleave structural proteins such as collagens and elastin.

The aim of this study is investigating the regulatory mechanism of ACE/ACE2 and MMPs/TIMPs in pulmonary diseases, and the association between ACE2 and gelatinase (MMP-2 and MMP-9) in the process of lung fibrosis. This study includes two parts. The first part aimed to identify diagnosis based on clinical variables to differentiate TB, Pn and Ad from pleural effusions. The Pleural effusions from 125 patients were processed in our laboratory from Mackay Memorial Hospital among August 2010 to December 2011.We investigated the ratio of ACE/ACE2 and MMPs in the pathogenesis of pleural effusions. The second part aimed to establish the animal model of pulmonary fibrosis, wild-type mice and ACE2 knockout ($ACE2^{+/-}$, $ACE2^{-/y}$ and $ACE2^{-/-}$) were received 1 U/kg bleomycin (Rey-Parra et al., 2012) and 20 mg/kg tetracycline (Dryzer et al., 1993) in chest cavity to induce inflammatory and fibrosis response and sacrificed after 3-, 7- and 28-day, respectively. Through the detection of MMP activity and the level of fibrosis by Histological section, we can understand the ACE2 regulation associated with the balanced expression and activity of MMPs/TIMPs.

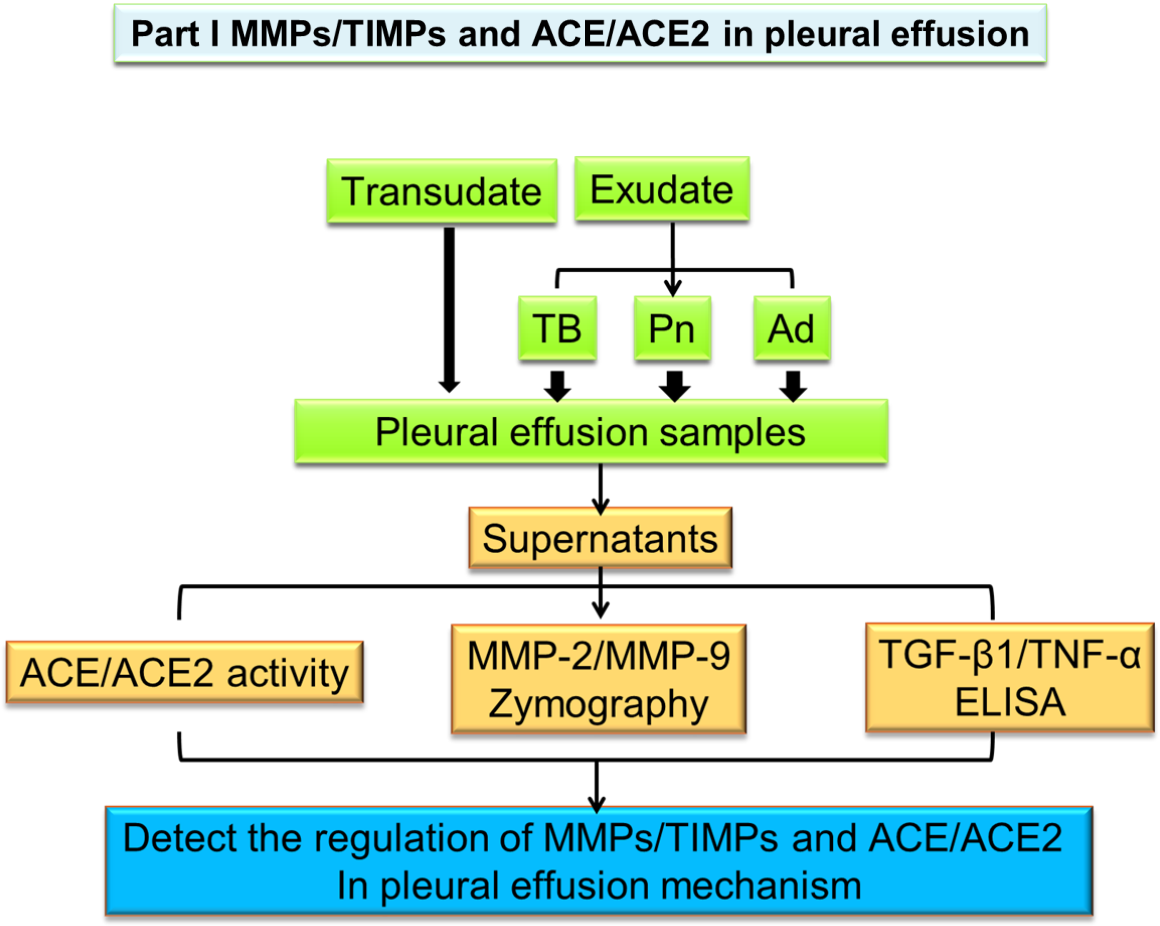

Fig. 2-1. The flowchart of research strategy of Part I. The purpose of this study is to identify the association of two key enzymes in RAS, ACE and ACE2, with MMP-2 and MMP-9 in the pleural fluid of patients. The Pleural effusions from 125 patients were processed in our laboratory from Mackay Memorial Hospital among August 2010 to December 2011.We also detail discussed whether abnormal RAS, accompanied with high levels of ACE/ACE2, might be a cause of elevated MMP-9 activity in tuberculous pleural effusion.

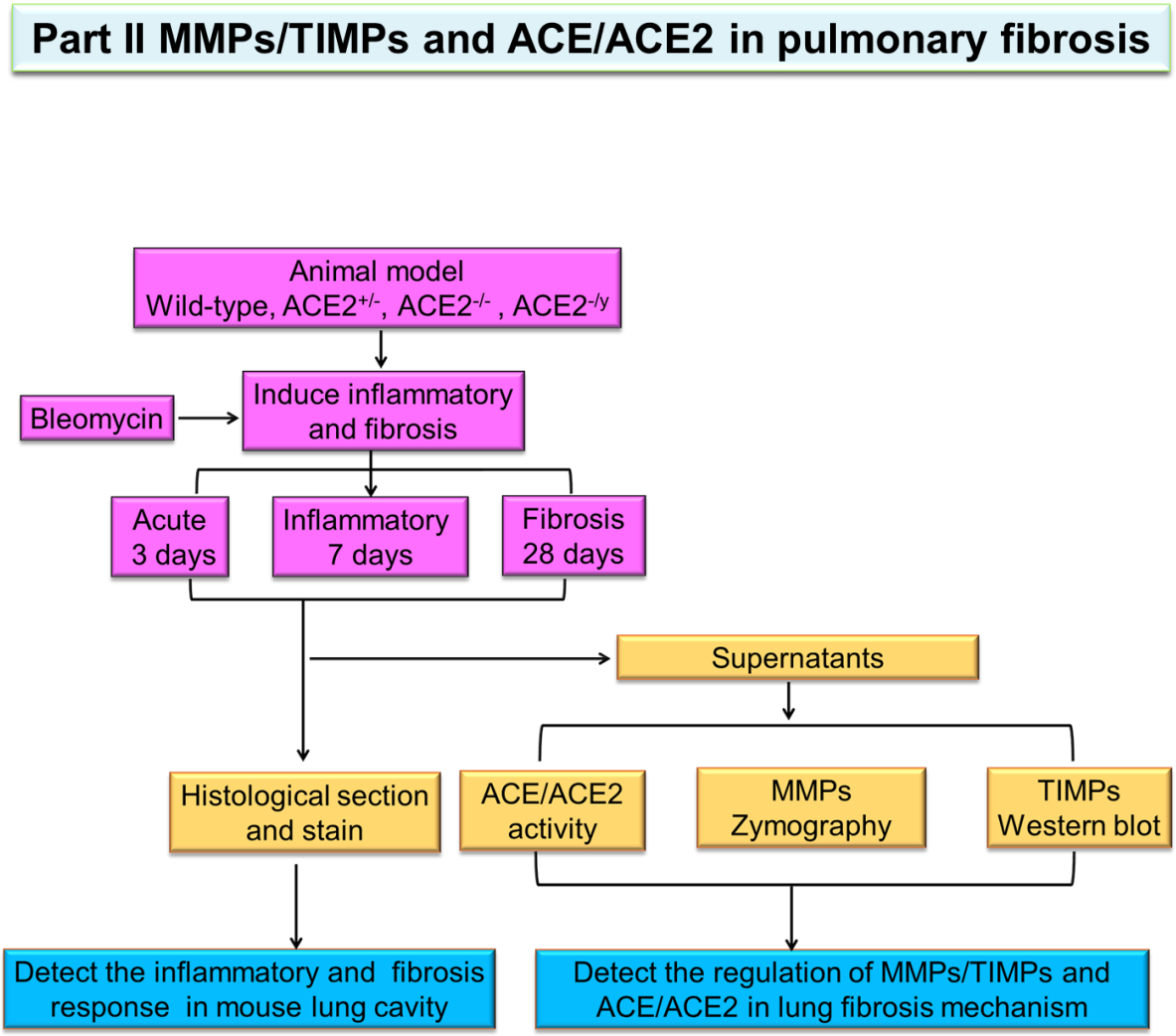

Fig. 2-2. The flowchart of research strategy of Part II. The purpose of this study is to identify the association of two key enzymes in RAS, ACE and ACE2, with MMP-2 and MMP-9 in pulmonary fibrosis. We establish a pulmonary fibrosis animal model and ACE2 knockout ($ACE2^{+/-}$, $ACE2^{-/y}$ and $ACE2^{-/-}$) mice by injecting 1 U/kg bleomycin and 20 mg/kg tetracycline, an antibiotic, that is generally used to induce inflammatory and fibrosis response to understand the association between ACE2 and MMPs.

III. Materials and Methods

3-1. Chemicals and reagents

The Monoclonal Anti-b-Actin IgG, glyceraldehyde-3-phosphate-dehydrogenase antibody (AC-15; #A5441), horseradish peroxidase (HRP) labeled secondary antibodies (donkey anti-goat IgG and goat anti-rabbit IgG; #sc2020 and #sc2004), and the rabbit polyclonal IgG, Ikaros antibody (H-100; #sc13039), were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-ACE2 (#ab59351) antibodies were purchased from Abcam (Cambridge, MA, USA). Bleomycin and Tetracycline were purchased from Sigma-Aldrich (St.Louis, MO, USA). The ACE2 overexpression lentiviruses were purchased from Vectorite Biomedica Inc. (Vectorite Biomedica, Taipei, Taiwan). The ACE2 inhibitor, DX600, ACE and ACE2 fluorescence substrate, Mca-APK (Dnp), were purchased from Ana Spec (Fremont, CA, USA). All other reagents were obtained from Sigma-Aldrich.

3-2. Pleural effusion samples collection and analyses

Pleural effusions from 125 patients were processed in our laboratory from August 2010 to December 2011. All pleural effusions were designated as transudates or exudates according to Light's criteria. Definitive diagnosis of tuberculous, pneumonia, or adenocarcinoma effusions for the exudate was verified by examining effusion biochemistry, cytology, acid-fast staining, and clinical follow-up. The study was performed with the approval of the Institutional Review Board of Mackay Memorial Hospital. Informed consent was obtained from all patients.

According to Light's criteria, pleural effusions were divided into 45 transudative pleural effusions and 80 exudative pleural effusions. The exudative pleural effusions were further divided into tuberculous pleural effusions (20 patients), pneumonia pleural effusions (32 patients), and malignant pleural effusions (28 patients). Fresh pleural fluid was collected in sterile tubes without anticoagulant reagents to prevent the release of gelatinases during platelet activation, and the tubes were immediately centrifuged at 3,000 × g for 30 min to separate the supernatants and cell pellets. The supernatants were aliquoted and stored with the cell pellets at −80°C until further use.

For each pleural effusion sample, the routine pleural analyses included total protein, lactate dehydrogenase (LDH), glucose, and white blood cell (WBC) count. In addition, the activities of ACE, ACE2, ADA , gelatinases (MMP-2 and MMP-9), TGF-β1 and TNF-α in the pleural effusions were measured.

3-3. ADA activity

ADA activity was measured or determined in the pleural fluids with a commercial colorimetric assay kit (Bio Quant, San Diego, CA, USA) (Valdés et al., 1996). Adenosine deaminase (ADA) isoenzyme analysis in pleural effusions: Diagnostic role and relevance to the origin of increased ADA in tuberculous pleurisy. The absorbance of the quinone dye generated in the last step of the ADA reaction series was monitored by absorbance at 550 nm with an ELISA reader (Bio-Rad model 550; Hercules, CA, USA).

3-4. ACE and ACE2 activity assay

ACE and ACE2 activities were assayed with the fluorogenic substrates Mca-YVADAPK and Mca-APK-Dnp (AnaSpec, San Jose, CA, USA), according to Vickers et al. with slight modifications (Vickers et al., 2002). The assay was performed in a microquartz cuvette with 20 μL pleural fluid, 50 μM fluorogenic substrate and protease inhibitor cocktail (1: 200; Sigma-Aldrich) in a final volume of 100 μL in ACE or ACE2 assay buffer. The reaction was followed kinetically for 1 hour using a fluorescence reader at an excitation wavelength of 330 nm and an emission wavelength of 390 nm. All samples were fitted and plotted using Grafit v. 4.0 (Sigma-Aldrich), and enzyme activity was expressed as RFU/hour/mL. Parallel samples were incubated with the above mentioned reaction mixture in the presence of 1 μM captopril (Sigma-Aldrich), a specific ACE inhibitor for determining specific ACE activity, or 1 μM DX600 (AnaSpec), a specific ACE2 inhibitor for determining specific ACE2 activity.

3-5. Gelatin zymography assay

The MMP-2 and -9 activities were detected by gelatin zymography utilized gelatin-containing gels as our previous report (Chang et al., 2011). Plasma was mixed with 2x

zymography sample buffer (0.125 M Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, and 0.005% bromophenol blue) incubated for 10 min at room temperature, and then loaded into SDS-PAGE which was performed in 8% acrylamide gels containing 0.1% (w/v) gelatin (Sigma-Aldrich). After electrophoresis under power supply of 100 V, the gel was washed twice for 30 min in zymography renaturing buffer (2.5% Triton X-100) with gentle shake at room temperature to remove SDS, then incubated 18 hour at 37℃ in reaction buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 5 mM $CaCl_2$, and 0.02% Brij35). The gels were then stained with Coomossie blue for 30 min prior to destain with destain buffer (50% methanol, 10% acetic acid, and 40% ddH_2O). The presence of enzyme activity was evident by clear or unstained zones, indicating the action of the enzyme on the gelatin substrate (Stawowy et al., 2004). Gelatinase activities in the gel slabs were quantified by Scion Image software (NIH, Bethesda, MD, USA), which quantifies the area of bands hydrolyzed by gelatinase. A MMP-2 or MMP-9 positive controls (Chemicon, Temecula, CA, USA) was contained in each gel as a standard intensity value to normalize sample intensity and express in arbitrary units.

3-6. Enzyme-linked immunosorbent assay (ELISA)

Pleural fluids were analyzed for TGF-β1 or TNF-α using sandwich ELISA. Each recombinant human TGF-β1 or recombinant human TNF-α were used as a standard. Pleural fluids were incubated in ELISA plates in which wells had been coated with anti-human TGF-β1 or anti-human TNF-α primary antibodies. Following addition of biotinylated antibodies, the plates were washed and reacted with HRP-conjugated streptavidin. Tetramethylbenzidine (TMB) one- step substrate tablets were used to detect TGF-β1 or TNF-α activity and the product was measured at 450 nm using a micro-plate reader (Bio-Rad Laboratories).

3-7. ACE2 knockout mice

ACE2 knockout mice were established by Gurley et al. (2006). The *ace2* gene consists of 18 exons, and the exon 1 was targeted by homologous recombination. The exon containing nucleotides +1069 to +1299 encoding the active site of the ACE2 enzyme (including the Zn-binding signature motif, HEMGH) was replaced with a NEO/URA3 cassette to obtain the targeting vector which disrupted *ace2* gene (**Fig. 3-1**). The targeting construct was

electroporated into MPI1-12D ES cells that had been derived from 129/SvEvfBRTac mice and then injected into C57BL/6H blastocysts to generate chimeras.

The male chimeras were crossed with C57BL/6J female mice to obtain male hemizygous mutants and female heterozygous and homozygous females mutants. The ACE2 KO mice utilized in this study was named B6;129S5-Ace2tm1Lex/Mmcd (MMRRC:31665) and obtain from Mutant Mouse Regional Resource Centers (MMRRC). The first generation of ACE2 KO mice we obtained from MMRRC was bred in National laboratory animal center (NLAC) and distinguished between hemizygous, heterozygous and homozygous mutants by DNA genotyping.

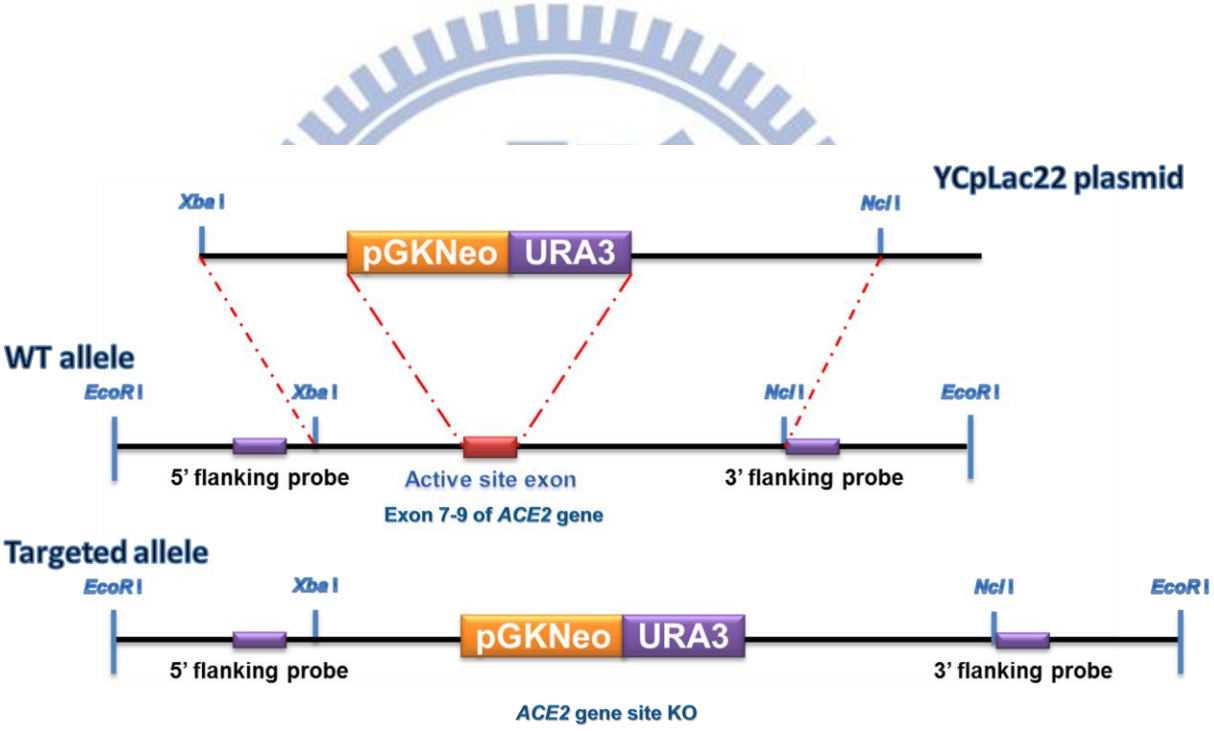

Fig. 3-1. Strategy for producing targeted disruption of the *ace2* gene. Strategy for producing targeted disruption of the *ace2* gene. In the targeting vector, the exon containing nucleotides +1069 to +1299 encoding the active site of the ACE2 enzyme (including the Zn-binding signature motif, HEMGH) was replaced with a NEO/URA3 cassette from YCpLac22 plasmid. (Gurley et al, 2006)

3-8. RNA isolation and quantification

Total tissue RNA was extracted using TRIzol Plus RNA Purification System (Invitrogen) following the manufacturer's recommendations and procedures reported by Pan et al. (Pan et al., 2008). Briefly, 1 mL of TRIzol reagent was added to one part of lung tissue. The mixture was vigorously agitated for 30 sec and incubated at room temperature for 5 min. Next, 200 μL chloroform was added and the solution was centrifuged at 12,000 x g for 15 min. The aqueous phase was transferred to a clean tube, precipitated with 500 μL of isopropyl alcohol, and centrifuged at 12,000 x g for 15 min. The resulting RNA pellet was washed with 1 mL of 75% cold ethanol (−20°C) and centrifuged at 12,000 x g at 4℃ for 5 min. The pellet was dried at room temperature, resuspended in 25 μL of diethylpyrocarbonate (DEPC)-treated water, and stored at −80°C. RNA was quantified by measuring the absorbance at 260 nm and 280 nm, and was electrophoresed on a denaturing 1% agarose gel.

3-9. Reverse transcription-polymerase chain reaction (RT-PCR) and Real time polymerase chain reaction

The cDNA was synthesized using ReverTra Ace Set (Toyobo, Osaka, Japan). For cDNA synthesis, 3 μg of RNA was reverse transcribed in a total reaction volume of 20 μL with 1 x reverse transcription buffer, 0.5 mM of dNTPs, 2.5 μM of oligo-dT (TOYOBO, Osaka, Japan), 1 U/μL of RNase inhibitor (TOYOBO), and 5 U/μL of ReverTra AceTM reverse transcriptase (TOYOBO). After incubation for 60 min at 42℃, the mixture was incubated for 5 min at 95 ℃ to denature the products. The PCR reactions contained 2 μL of cDNA, 2 μL of each primer (10 μM), 5 μL of 10 x PCR buffer, 2 μL of 10 mM of dNTPs, 1 μL of 5 U/μL Taq polymerase (Promega, Madison, WI, USA), and 36 μL distilled water in a total volume of 50 μL. Thermal cycler (MiniCyclerTM; MJ Research, Waltham, MA, USA) conditions were 5 min at 94℃ followed by 18-36 cycles of denaturation (94℃ for 30 sec), annealing (55℃ for 30 sec), and elongation (72℃ for 45 sec). The resulting PCR products were visualized on 2% agarose gels stained with ethidium bromide. The stained image was recorded by an image analyzer (Kodak DC290 Digital Camera SystemTM; Eastman Kodak, Rochester, NY, USA). Band intensity was quantified using densitometric analysis by ImageJ^{TM}. The relative mRNA expression of the determined gene was normalized as a ratio to GAPDH expression.

Semi-quantitative real-time (RT) PCR was performed using SYBR Green Realtime PCR

Master Mix Plus (Toyobo) with 20 pM of each primer and 5 μL cDNA, in a total volume of 25 μL and monitored using Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations. Specificity of the real-time PCR was confirmed by routine agarose gel electrophoresis and melting-curve analysis, according to a published method (Livak et al., 2001). Expression of the GAPDH (GenBank ID: NM_002046.3) gene was used as an internal standard. The primers for ACE2 (GenBank ID: AF291820 and NM002046.3), were: ACE2 forward, hACE2-F, 5'-CATTGGAGCAAGTGTTGGATCTT-3', and, ACE2 reverse, hACE2-R, 5´-GAGCTAATGCATGCCATTCTCA-3´; GAPDH forward, hGAPDH-F, 5´-ACAGTCAGCCGCATCTTCTT-3´, and, GAPDH reverse, hGAPDH-R, 5´-GTTAAAAGCAGCCCTGGTGA-3´.

3-10. Animal model, induction of lung fibrosis and treatment of mice with Lenti-ACE2

Control animals were wild-type B6;129S5 and ACE2 KO mice ($ACE2^{-/y}$, $ACE2^{+/-}$ and$ACE2^{-/-}$) weighing 20-25 g obtained from the National laboratory animal center (NLAC), and mice were randomly assigned into 9 groups of 3-5 animals per group. They were house in a plastic suspended cage placed in a well-ventilated mice house, provided mice pellets and water ad libitum, and subject to a natural photoperiod of 12 hour light and 12 hour dark cycle. After the animals were anaesthetised by the intraperitoneal injection of 40mg/kg pentobarbital, the right chest was cleansed with an Et-OH solution and a 25-gauge needle attached to a 1-mL syringe containing the solution was inserted through the skin and chest muscles, 1 cm lateral to the right parasternal line. The plunger of the syringe was removed and the needle was slowly advanced until it reached the pleural space, where the subatmospheric intrapleural pressure allowed the fluid to enter the pleural cavity spontaneously. Received 0.1 mL of intrapleural 1 U/kg bleomycin or 20 mg/kg tetracycline. A Control animals group received PBS. The mice were monitored after the procedure until they completely awakened. Mice treated with PBS, bleomycin and tetracycline were sacrificed after 3 days, 7 days and 28 days. The animals were injected of pentobarbital and the thorax was dissected in order excise the right lung. Empty virus (Control), lenti-ACE2 viral particles (3×10^6 U in 100 μL of phosphate-buffered saline) were injected through the tail vein of ACE2 KO mice, using a

27-gauge syringe needle. One week after lentiviral treatment, animals were subjected to bleomycin or tetracycline administration. All experimental procedures were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of National Chiao Tung University, Every effort was made to minimize the suffering of the animals and the number of animals used.

3-11. Sample preparation

3-11-1. Protein extraction

The organ samples were supplied by B6;129S5 and ACE2 KO mice ($ACE2^{-/y}$, $ACE2^{+/-}$ and$ACE2^{-/-}$) mice and used for further analyses was prepared as described. The organ samples were collected and were weighted 100 mg, and then homogenous for 3 to 5 times by lysis buffer PRO-PREP^{TM} Protein Extraction Solution (iNtRON Biotechnology, Inc., Kyungki-Do, Korea). Samples were centrifuged at 13,000 x g for 10 min to separate the supernatants and pellets. The total amount of protein in homogeneous extract was measured by the Bradford dye binding assay (Bio-Rad Laboratories, Hercules, CA, USA) and bovine serum albumin as the standard. The supernatants were aliquoted and stored at - 80℃ until further use.

3-11-2. Histological determination of fibrosis

The organ samples were supplied by B6;129S5 and ACE2 KO mice ($ACE2^{-/y}$, $ACE2^{+/-}$ and$ACE2^{-/-}$) mice and a part of right lung were excised and encased in 10% formaldehyde prepared for Masson's Trichrome and hematoxylin-eosin staining. Inflation fixed lungs were stained with Masson's Trichrome; muscles and cells are stained red, nuclei black and collagen blue. The stained sections were photographed using a digital camera mounted on a microscope. Manual planimetry was performed on the microscope using PALM RoboSoftware v2.2 using hematoxylin-eosin-stained slices. A computerized microscope equipped with a high-resolution video camera (BX 51, Olympus, Tokyo, Japan, magnification 100 x) was used for morphometric analysis.

3-12. Western blotting

The Western blot for TIMP1 and ß-actin was carried out as our previous report. Aliquots containing 20 μg protein were electrophoresed on 12% SDS-PAGE gels and then transferred electrophoretically to polyvinylidene fluoride membranes (Immobilon-PTM; Millipore, Bedford, MA, USA) by semi-dry electro-blotting (HoeferTM). Briefly, nonspecific binding sites were blocked by incubating the membranes in 5% non-fat milk in Tris-buffered saline. Primary antibodies against proteins were diluted 1:1,000 for TIMP1 and ß-actin. The secondary antibodies were applied using a dilution of 1:2,000. Substrates were visualized using enhanced chemiluminescence detection (Western Lightning Plus-ECL, Enhanced Chemiluminescence Substrate; PerkinElmer, Boston, MA, USA) and exposing the membranes to X-ray film (Fujifilm). The bands on the film were detected at the anticipated location, based on size. Band intensity was quantified by densitometric analysis using Scion Image software (Scion, Frederick, MD, USA). The amounts of TIMP-1 were expressed relative to the amount of ß-actin (as the internal standard) in each sample.

3-13. Statistical analysis

All values were expressed as mean ± standard deviation (SD). Data were compared with one-way analysis of variance (ANOVA) test to evaluate differences among multiple groups. The Student's t-test was used for comparisons involving two groups. All results are expressed as the mean ± standard deviation (SD). Differences were considered statistically significant when $p < 0.05$. Statistical analysis was performed using statistical software (SPSS, Chicago, IL, USA).

IV. Results

Part I: MMPs and ACE/ACE2 in pleural effusion

4-1-1. General characteristics of pleural effusions

The pleural fluid characteristics of 125 patient s included in the study are presented in **Table 4-1-1** and **Table 4-1-2**. Generally, total protein, LDH, and the number of WBC in exudative effusions (n = 80) were significantly higher than those in transudative effusions (n = 45) ($p < 0.001$), whereas a lower glucose level was detected in the pleural fluid of exudates compared with that in transudates ($p < 0.001$).

4-1-2. ACE and ACE2 activities in transudates and exudates

ACE and ACE2 activities in the pleural effusions of all patients were determined. ACE activity in exudative effusions was higher than that in transudative effusions (0.74 (0.43–1.47) RFU/hour/mL vs. 0.57 (0.32–0.9) RFU/hour/mL, respectively, $p < 0.01$) (**Fig. 4-1-2A**). On the contrary, ACE2 activity in exudative effusions was lower than that in transudative effusions (1.58 (1.02–2.54) RFU/hour/mL vs. 1.98 (1.27–3.12) RFU/hour/mL, respectively) (**Fig. 4-1-2B**), but this difference was not statistically significant ($p = 0.485$). The ratio of ACE/ACE2 activity in the pleural effusions was significantly higher in exudative effusions than in transudative effusions (median 0.49 (0.27–0.91) vs. 0.27 (0.19–0.43), respectively, $p < 0.001$) (**Fig. 4-1-2C**). In transudates, a significant positive correlation was found between ACE and ACE2 activities ($r = 0.456$, $p < 0.001$) (**Fig. 4-1-3A**). However, the ACE level was not correlated with the ACE2 level in exudative effusions ($r = 0.020$, $p = 0.214$) (**Fig. 4-1-3B**).

4-1-3. MMP-2 and MMP-9 activities in transudates and exudates

The MMP-2 and MMP-9 activities in the pleural effusions of all patients were determined with gelatin zymography. The activity level of MMP-2 in exudative effusions was comparable with that in transudative effusions (median, 1375 (944–1841) ng/mL vs. 1148 (806–1730) ng/mL, respectively) (**Fig. 4-1-4A**). However, MMP-9 activity in exudative

effusions was significantly higher than that in transudative effusions (median, 30 (19–52) ng/mL vs. 11 (8–23) ng/mL, respectively, $p < 0.001$) (**Fig. 4-1-4B**). In transudates, a significant positive correlation was found between MMP-2 and MMP-9 activities (r = 0.544, $p < 0.001$). However, no strong correlation was found between MMP-2 and MMP-9 activities in exudates (r = 0.125, $p < 0.01$).

4-1-4. ACE and ACE2 levels in exudates from patients with different diseases

Elevated ACE activity and an elevated ACE/ACE2 ratio were observed in exudative effusions. We then differentiated both ACE and ACE2 enzyme levels in the exudates according to different diseases, including tuberculosis, pneumonia, and adenocarcinoma. The level of ACE activity in the tuberculous pleural effusions was significantly higher than in pneumonia and adenocarcinoma effusions, by 2.89 ($p < 0.001$) and 2.62 ($p < 0.001$) folds, respectively (**Fig. 4-1-5A**). In contrast, ACE2 activity in the tuberculous effusions was significantly lower than in pneumonia and adenocarcinoma effusions, by 0.68 ($p < 0.05$) and 0.58 ($p < 0.05$) folds, respectively (**Fig. 4-1-5B**). According to the changes we detected in ACE and ACE2, a significantly higher difference in the ACE/ACE2 ratio in tuberculous effusions (median, 1.72 (0.15–2.52)) compared with ratios in pneumonia (0.32 (0.20–0.50)) and adenocarcinoma (0.43 (0.26–0.67)) effusions was also observed ($p < 0.001$) (**Fig. 4-1-5C**).

4-1-5. MMP-2, MMP-9, and ADA levels in exudates from patients with different diseases

In tuberculous pleural effusions, MMP-2 activity was 1.48 ($p < 0.01$) and 1.36 fold ($p < 0.05$) higher than MMP-2 activity in pneumonia and adenocarcinoma effusions, respectively (**Fig. 4-1-6A**). Similar to MMP-2, MMP-9 activity in tuberculosis effusions was significantly higher than MMP-9 activity in pneumonia and adenocarcinoma effusions by 1.62 ($p < 0.01$) and 1.77 fold ($p < 0.01$), respectively (**Fig. 4-1-6B**). In tuberculous effusions, significantly higher ADA activity was also detected compared with ADA activity in pneumonia and adenocarcinoma effusions ($p < 0.001$) (**Fig. 4-1-6C**).

4-1-6. ELISA for TGF-β1 concentration in pleural transudative and exudative effusions

The concentration of TGF-β1 in pleural transudative and exudative effusion (including TB, Pn and Ad effusions) was determined with ELISA. The concentration level of TGF-β1in exudative effusions was comparable with that in transudative effusions (median, 0.103 (0.007–1.113) ng/mL vs. 0.155 (0.007–1.314) ng/mL, respectively) (**Fig. 4-1-7A**). The concentration of TGF-β1 was no differentiation between transudative and exudative effusions. Even the concentration of TGF-β1 in different exudative effusions was no significant difference in each other's.

4-1-7. ELISA for TNF-α concentration in pleural transudative and exudative effusions

The concentration of TNF-α in pleural transudative and exudative effusion (including TB, Pn and Ad effusions) was determined with ELISA. The concentration level of TNF-α in exudative effusions was comparable with that in transudative effusions (median, 35.3 (6.3–98.6) pg/mL vs. 30.3 (2.3–138.3) pg/mL, respectively) (**Fig. 4-1-8A**). The concentration of TNF-α was no differentiation between transudative and exudative effusions. However, a significantly higher difference in the concentration of TNF-α in pneumonia (39.9 (2.3–138.3)) compared with the concentration in adenocarcinoma (24.6 (6.3–76.6)) effusions was also observed ($p < 0.01$) (**Fig. 4-1-8B**).

Table 4-1-1. Pleural fluid characteristics of the study population

	Transudates (n = 45)	**Exudates (n = 80)**
Age (years)	74 ± 12	66 ± 18
Male/Female	29/16	49/31
Pleural fluid		
White blood cells (cell/mm^3)	305 ± 326	927 ± 1090 ***
Glucose (mmol/L)	176 ± 77	130 ± 58 ***
Total protein (g/L)	2.11 ± 1.02	4.01 ± 1.02 ***
Lactate dehydrogenase (U/L)	86 ± 32	265 ± 222 ***

Transudates were from patients with heart failure or liver cirrhosis.

Exudates were from patients with tuberculous, pneumonia, or adenocarcinoma effusions.

Data are the means ± SD. *** indicates $p < 0.001$ compared with transudates.

Table 4-1-2. Pleural fluid characteristics of tuberculous, pneumonia and adenocarcinoma effusions.

Pleural fluid	Tuberculosis (n = 20)	Pneumonia (n = 32)	Adenocarcinoma (n = 28)
White blood (cell/mm^3)	1,201 ± 1,284	825 ± 905	848 ± 1,099
Glucose (mmol/L)	126 ± 62	135 ± 53	126 ± 59
Total protein (g/L)	4.56 ± 1.25	3.75 ± 0.86*	4.14 ± 0.74
Lactate dehydrogenase (U/L)	313 ± 255	237 ± 239	264 ±164

Data are the means ±SD, * indicates $p < 0.05$.

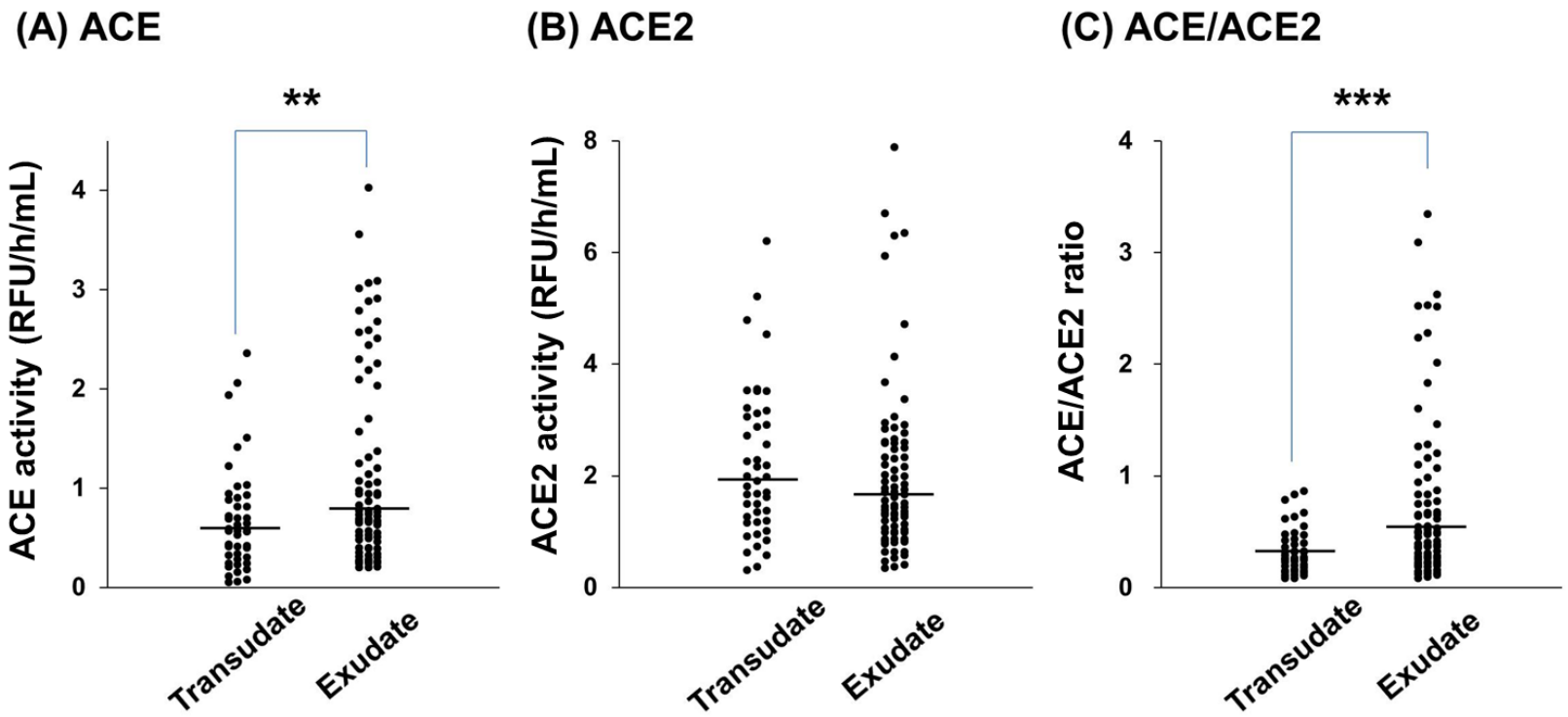

Fig. 4-1-2. Enzymatic activity of ACE and ACE2 in pleural transudative and exudates effusions. The specific activities of ACE **(A)** and ACE2 **(B)** in pleural transudative (n = 45) and exudative (n = 80) effusions from 125 patients. The ratio of ACE/ACE2 in the pleural effusions **(C)**. Pleural effusion (20 μL) was assayed for the ability to cleave the fluorescent substrate at 37℃ for 1 hour with a specific ACE inhibitor or a specific ACE2 inhibitor. Each symbol represents one individual, and horizontal bars represent median values. ** and *** indicate $p < 0.01$ and $p < 0.001$, respectively, compared with transudates.

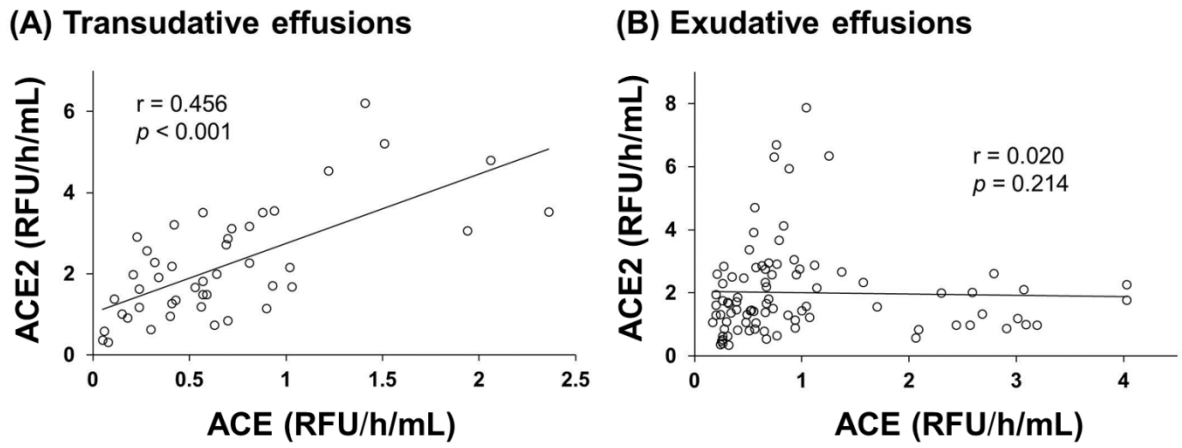

Fig. 4-1-3. Correlations between ACE and ACE2 activity in pleural effusion. ACE and ACE2 activities were measured in each sample of pleural transudative effusions (**A**; n = 45) and exudative effusions (**B**; n = 80). For correlation analysis, Pearson's correlation analysis (SPSS statistics package, Chicago, IL) was applied. Statistically significant differences were established at $p < 0.05$. ACE and ACE2 positively correlate with each other in the group of transudative effusions ($F(1,43) = 36.052$, $r = 0.456$, $p < 0.001$) **(A)**, but not in the group of exudative effusions ($F(1,78) = 1.567$, $r = 0.020$, $p > 0.05$) **(B)**.

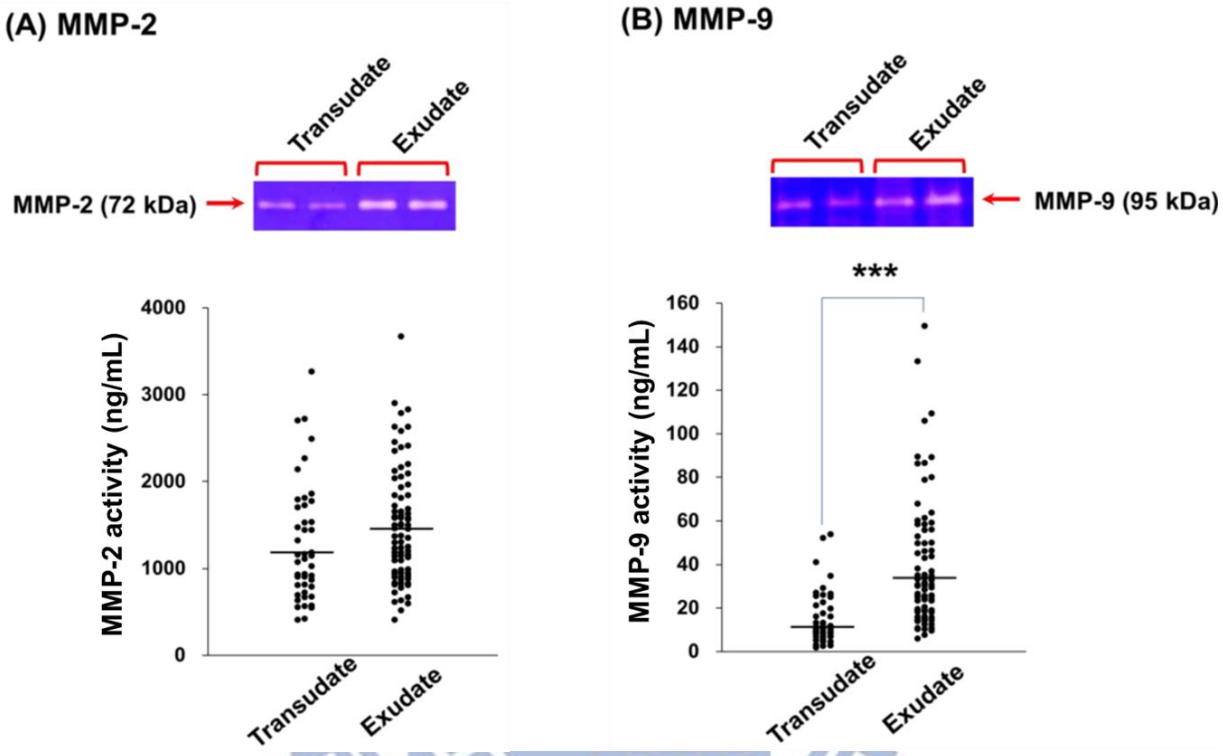

Fig. 4-1-4. MMP-2 and MMP-9 activities in pleural transudative and exudative effusions. The activities of MMP-2 **(A)** and MMP-9 **(B)** in pleural transudative and exudative effusions from 125 patients were determined with zymography. The gelatinase activities detected in this study were based on pro-MMP-2 (72 kDa) and pro-MMP-9 (95 kDa). Each symbol represents one individual, and horizontal bars represent median values. *** indicates $p < 0.001$ compared with transudates.

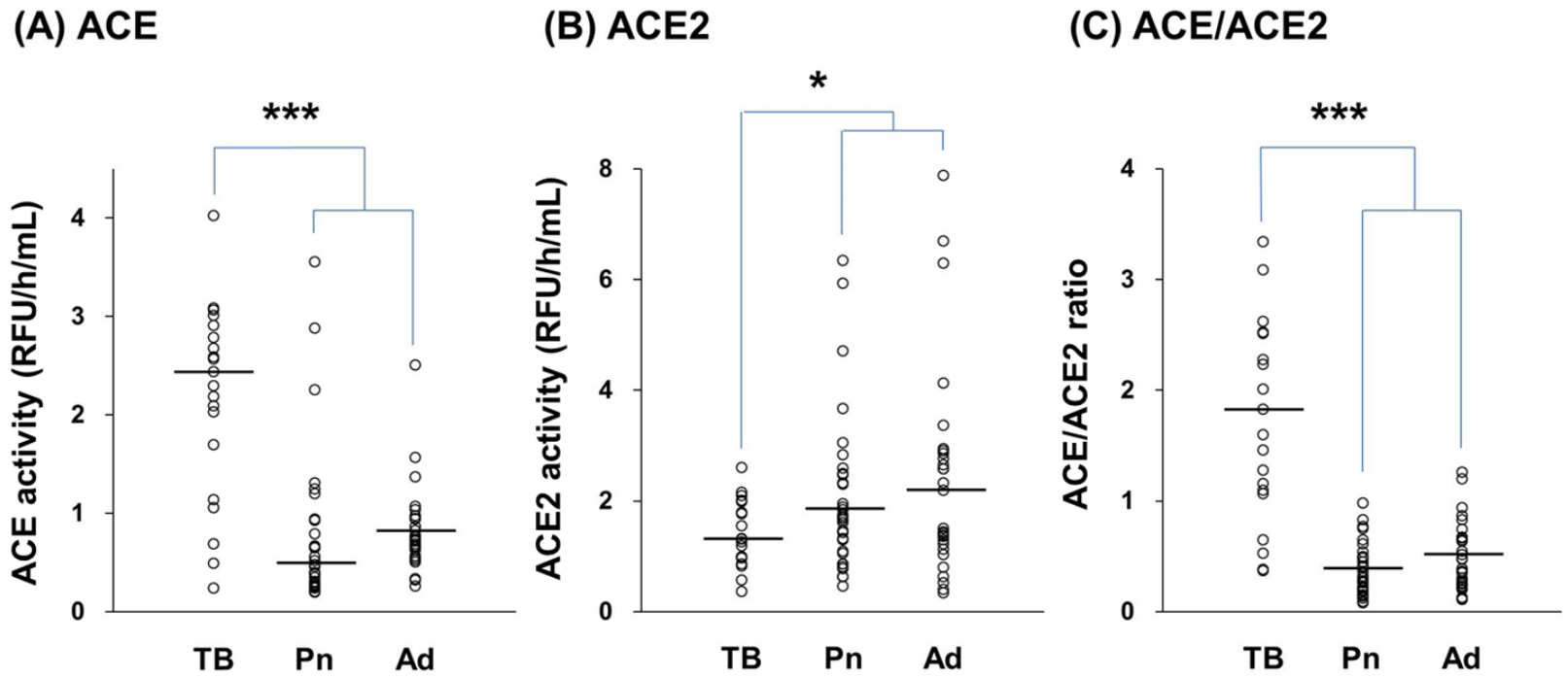

Fig. 4-1-5. ACE and ACE2 activities in exudative effusions from patients with tuberculosis (TB), pneumonia (Pn), and adenocarcinoma (Ad). The activities of ACE **(A)**, ACE2 **(B)**, and the ratio of ACE/ACE2 **(C)** in TB (n = 20), Pn (n = 32), and Ad (n = 28) effusions. Each symbol represents one individual, and horizontal bars represent median values. * and *** indicate $p < 0.05$ and $p < 0.001$, respectively, compared with values measured in Pn and/or Ad effusions.

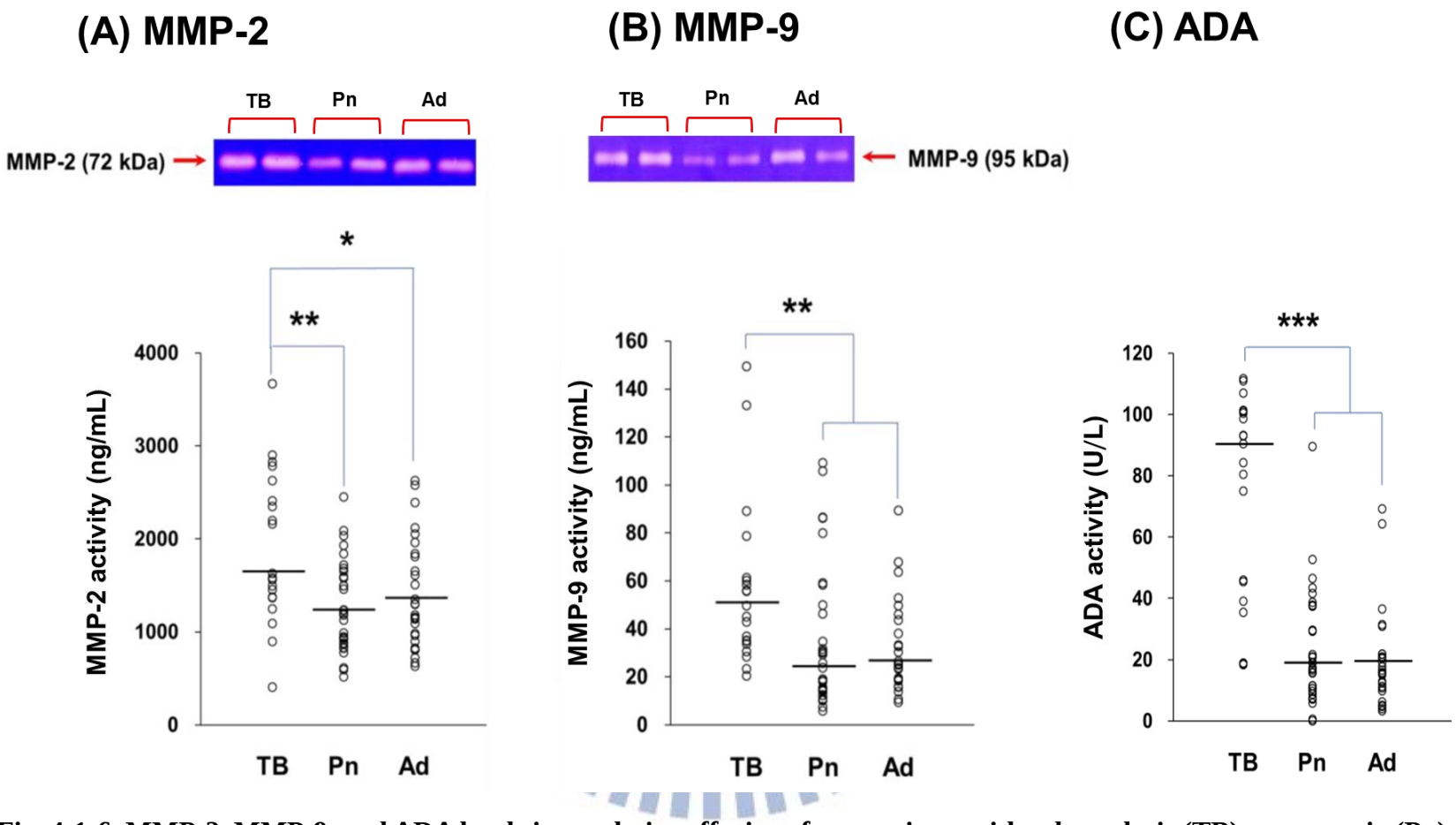

Fig. 4-1-6. MMP-2, MMP-9, and ADA levels in exudative effusions from patients with tuberculosis (TB), pneumonia (Pn), and adenocarcinoma (Ad). The activities of MMP-2 **(A)**, MMP-9 **(B)**, and ADA **(C)** in TB (n=20), Pn (n=32), and Ad (n=28) effusions. Each symbol represents one individual, and horizontal bars represent median values. *, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, compared with values measured in Pn and/or Ad effusions.

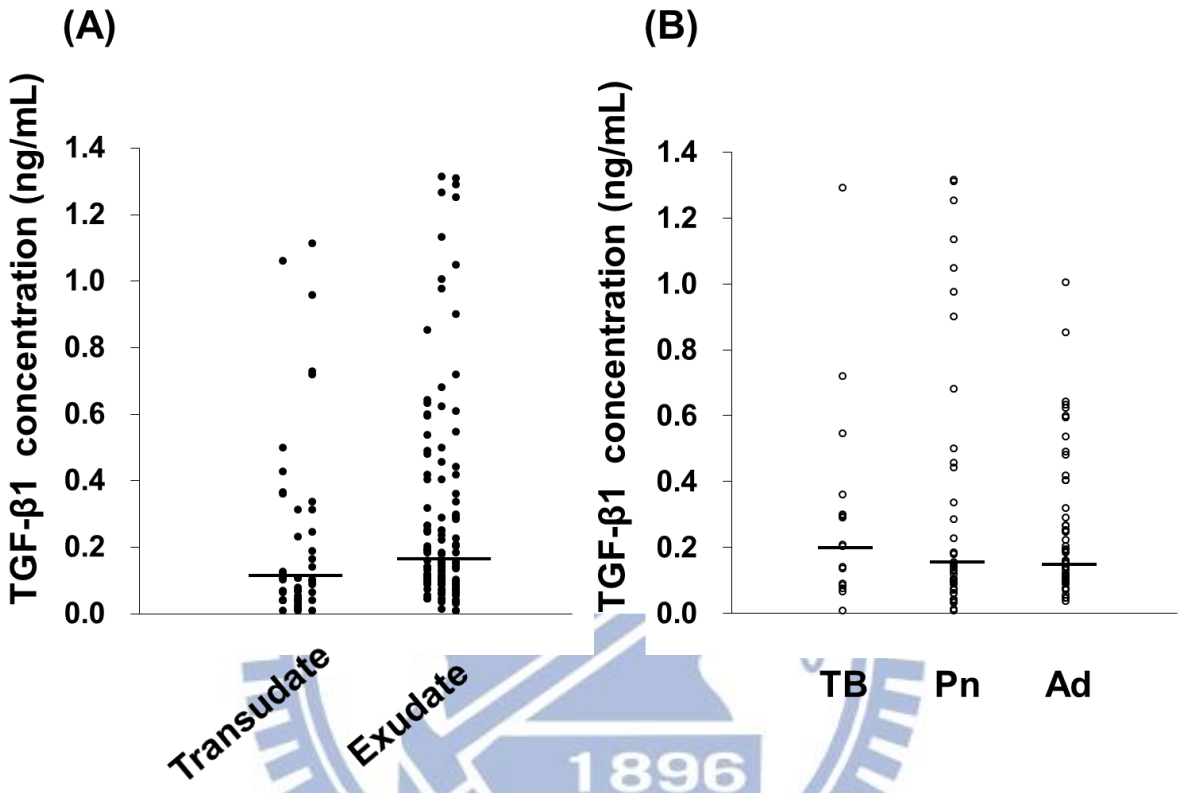

Fig. 4-1-7. ELISA for TGF-β1 concentration in pleural transudative and exudative effusions. The activities of TGF-β1 in pleural transudative and exudative effusions **(A)** and in TB, Pn and Ad effusions **(B)** were determined with ELISA. The concentration of TGF-β1 was no differentiation between transudative and exudative effusions. Each symbol represents one individual, and horizontal bars represent median values.

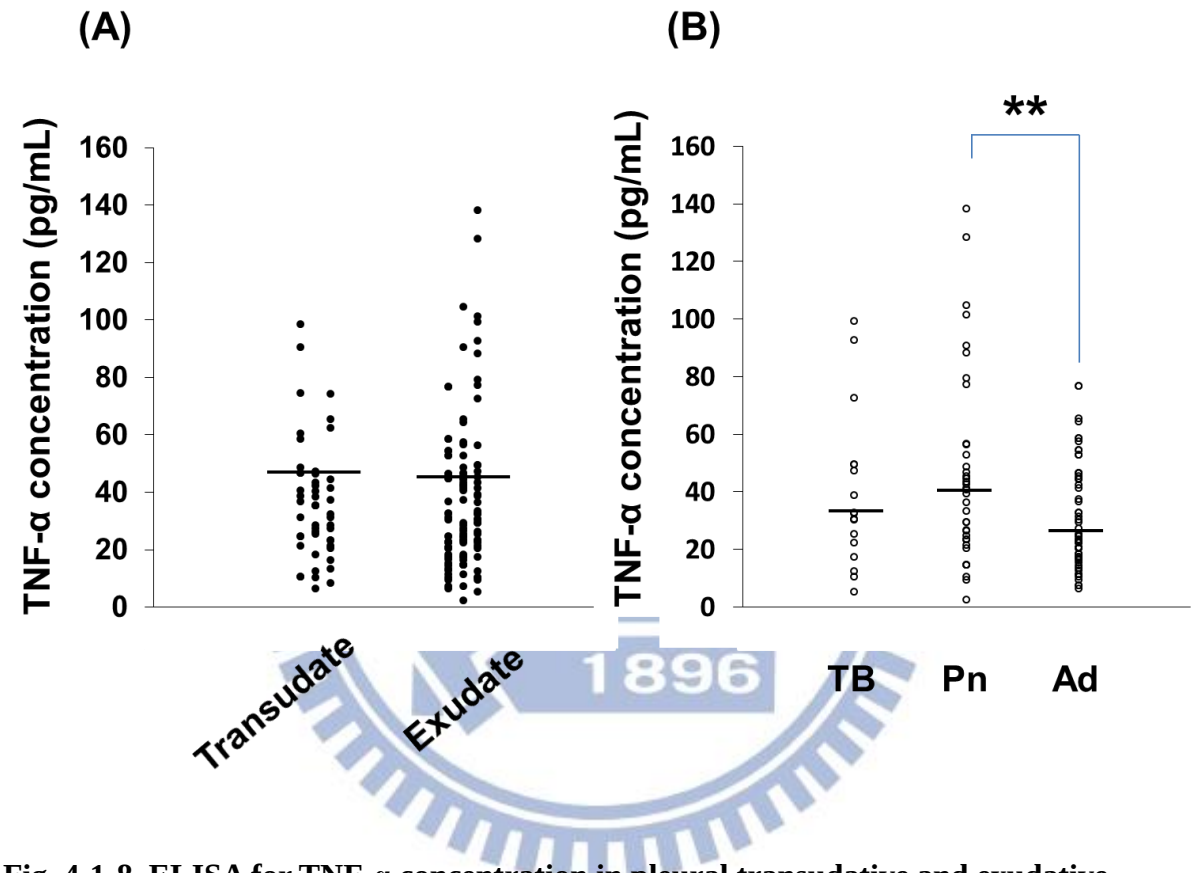

Fig. 4-1-8. ELISA for TNF-α concentration in pleural transudative and exudative effusions. The activities of TNF-α in pleural transudative and exudative effusions **(A)** and in TB, Pn and Ad effusions **(B)** were determined with ELISA. Each symbol represents one individual, and horizontal bars represent median values. Each symbol represents one individual, and horizontal bars represent median values. ** indicates $p < 0.01$, Pn compared with values measured in Ad effusions.

Part II MMPs/TIMPs and ACE/ACE2 in pulmonary fibrosis

4-2-1. ACE2 knockout mice

In this study, our laboratory obtained the ACE2 KO mice, B6;129S5-Ace2tm1Lex/Mmcd (MMRRC:31665) from Mutant Mouse Regional Resource Centers (MMRRC) and bred in National laboratory animal center (NLAC). The hemizygous, heterozygous and homozygous mutants of ACE2 KO mice were distinguished by DNA genotyping and sex, the information of primer pairs that DNA genotyping utilized were listed in **Fig. 4-2-1A**. Because of *ace2* is an X-linked gene, there are three different patterns of DNA genotyping in ACE2 KO mice. In WT mice, only the WT allele (500 bp) was present, oppositely, the KO allele (468 bp) only present in hemizygous and homozygous mutants of ACE2 KO mice. Specially, the heterozygous mutants of ACE2 KO mice were present WT and KO alleles, simultaneously **(Fig. 4-2-1B)**.

The mRNA and protein expression of ACE2 and ACE2 activity were evidenced in ACE2 KO mice. The protein extracts of lung was prepared from wild type, hemizygous, heterozygous and homozygous mutants mice, and then detected by real time PCR, Western blot and ACE2 activity assay. All results evidenced that the lung tissue of ACE2 KO mice were deficiency of ACE2 expression in mRNA, protein and ACE2 activity. Compared to WT mice, the mRNA expression of heterozygous, homozygous and hemizygous mutants mice were reduced to 60%, 2% and 3% (**Fig. 4-2-2A**). The protein expression also reduced to 42%, 25% and 29% in heterozygous, homozygous and hemizygous mutants mice compared to WT mice (**Fig. 4-2-2B**). As like the trend of mRNA and protein expression, ACE2 activity were reduced to 29%, 2% and 1% compared to WT mice (**Fig. 4-2-2C**).

4-2-2. ACE and ACE2 activity in WT mouse lung

The ACE and ACE2 activity in wild-type (WT) mice injected with bleomycin or tetracycline were compared to the Control (PBS injection). Lung tissue ACE activity was significantly lower compared to the Control group at 3 days after bleomycin injection of the chest and higher compared to the Control group at 7 days (**Fig. 4-2-3A**). ACE2 activities significantly reduced to 37% at 28 days after bleomycin injection (**Fig. 4-2-3B**). Another treatment with tetracycline injection, the lung tissue ACE activity was similar to the Control

groups at 3-, 7- and 28-day (**Fig. 4-2-4A**). ACE2 activities significantly reduced to 45% at 28 days after tetracycline injection (**Fig. 4-2-4B**). According to these results, bleomycin and tetracycline treatment of mouse lung tissue had similar regulation on ACE2 activity, but the regulation on ACE activity was different.

4-2-3. MMP-9 and TIMP-1 activities in WT mouse lung

The MMP-9 activity and TIMP-1 concentration in the lung homogenate of mice treated with PBS (Control) or bleomycin were determined by gelatin Zymography and Western blotting, respectively. The MMP-9 and TIMP-1 activity in wild-type mice injected with bleomycin or tetracycline were compared to those in the Control (PBS injection). The MMP-9 activity in lung tissue was similar to the Control group at 3 days and 28 days. The MMP-9 activity which induces extents up to 3.5 to 4 folds can be measured significant higher than the Control at 7 days (**Fig. 4-2-5A** and **Fig. 4-2-6A**).

There were significantly different on bleomycin induced to 138% TIMP-1 concentration compared with the Control at 7-day, but insignificant different in the 3-day and 28-day group. Whereas, there were insignificantly different on TIMP-1 concentration among the groups of tetracyclin treated and Control at 3-, 7- and 28-day after treatments (**Fig. 4-2-5B** and **Fig. 4-2-6B**).

4-2-4. ACE activity in ACE2 KO mouse lung

The ACE activities in the ACE2 KO heterozygous ($ACE2^{-/+}$), homozygous ($ACE2^{-/-}$) and hemizygous ($ACE2^{-/y}$) mouse lungs treated with PBS, bleomycin or tetracycline after 3-, 7- and 28- day were determined by the ability to cleave the fluorescent substrate at 37℃ for 1 hour with a specific ACE inhibitor. Heterozygous mice lung tissue ACE activity significantly reduced to 69% and 61% compared to the Control group at 7 days after bleomycin or tetracycline injection of the chest (**Fig. 4-2-7A** and **Fig. 4-2-8A**). The ACE performance measurements in homozygous relative the Control mouse ACE activity were no significant differences in different treatment groups (**Fig. 4-2-7B** and **Fig. 4-2-8B**). Hemizygous mice lung tissue ACE activity significantly lower compared to the Control group at 3 days (**Fig. 4-2-8C**)

4-2-5. MMP-9 activities in ACE2 KO mouse lung

The MMP-9 activity in ACE2 KO mice injected with bleomycin or tetracycline was compared to the Control. To investigate the functions of ACE2 in lung fibrosis, ACE2 KO heterozygous, hemizygous and homozygous mice were induced lung fibrosis by bleomycin or tetracycline injected to breast cavity. First, we explore the MMP-9 activity of ACE2 KO mice lung tissue. Heterozygous and homozygous mice injected to chest cavity with bleomycin and the MMP-9 activity in lung tissue was significantly induced after 3 days and 7 days; But in the hemizygous mice, only the MMP-9 of lung tissure was significantly induced in 3 days group (**Fig. 4-2-9**). In tetracycline treatment group, the measurements in the heterozygous, homozygous hemizygous relative to the Control mice, MMP-9 expressions were no significant differences among different treatment groups (**Fig. 4-2-10**).

4-2-6. TIMP-1 concentration in ACE2 KO mouse lung

TIMP-1 concentration in ACE2 KO mice injected with bleomycin or tetracycline was compared to the Control. To investigate the functions of TIMP-1concentration in lung fibrosis, ACE2 KO heterozygous, hemizygous and homozygous mice were induced lung fibrosis by bleomycin or tetracycline injected to breast cavity. Heterozygous mice injected to chest with bleomycin and the TIMP-1 concentration in lung tissue was significantly reduced after 3-day and 7-day (**Fig. 4-2-11A**); but in the hemizygous and homozygous mice, both the TIMP-1 concentration of lung tissure was significantly reduced after 3-, 7- and 28-day (**Fig. 4-2-11B** and **C**). Heterozygous and homozygous mice injected to chest with tetracycline and the TIMP-1 concentration in lung tissue was significantly reduced after 7 days (**Fig. 4-2-12A** and **B**); whereas in the hemizygous mice, the TIMP-1 concentration of lung tissure was significantly reduced after 3-, 7- and 28-day (**Fig. 4-2-12C**).

4-2-7. Histological section staining of mouse lung tissue

We injected with bleomycin or tetracycline in mice chest induced pulmonary fibrosis. The mice were sacrificed in the 3-, 7-, 28-day after treatment then collected their lungs organizations to do histological section by the HE and Masson trichrome staining. The figures show a significantly loss of lung architecture, leukocyte infiltration and collagen

accumulation and other diseases, including leukocyte infiltrates in the lungs of mice after bleomycin (**Fig. 4-2-13**) or tetracycline (**Fig. 4-2-14**) treatment. There were lung tissue collagen accumulations significantly after injection 3-day, the most significant in the 7-day. The loss of lung architecture was significant in the 28-day. Based on the above results, we have successfully established pulmonary fibrosis experimental animal model with bleomycin. There were evidenced by the loss of lung architecture and the blue sections indicated collagen deposition.

4-2-8. ACE2 activity in ACE2 KO mice after lenti-ACE2 injection

The ACE2 activities in the ACE2 KO homozygous ($ACE2^{-/-}$) and hemizygous ($ACE2^{-/y}$) mouse lungs treated with empty virus (Control), lenti-ACE2 viral particles (3×10^6 TU in 100 μL of phosphate-buffered saline) through the tail vein using a 27-gauge syringe needle after 1-, 3- and 7-day were determined by the ability to cleave the fluorescent substrate at 37℃ for 1 hour with a specific ACE2 inhibitor. Both ACE2 activities in homozygous and hemizygous significantly induced at 1 and 3 days in lung and liver after lentivirus injection (**Fig. 4-2-15**).

PCR Genotyping

3	5' – GATGTCCAGCTCCTCCTGG
37	5' –GCTCAATAACGACTTAGAACAT
Neo3A	5' –GCAGCGCATCGCCTTCTATC

Wild type-specific (absent in targeted allele)	
5' Primer Name:	3
3' Primer Name:	37
Predicted Wild-type Band (bp):	500
Predicted mutant band (bp)	none

Mutation-specific product (absent in wt)	
5' Primer Name:	Neo3A
3' Primer Name:	37
Predicted Wild-type Band (bp):	none
Predicted mutant band (bp)	468

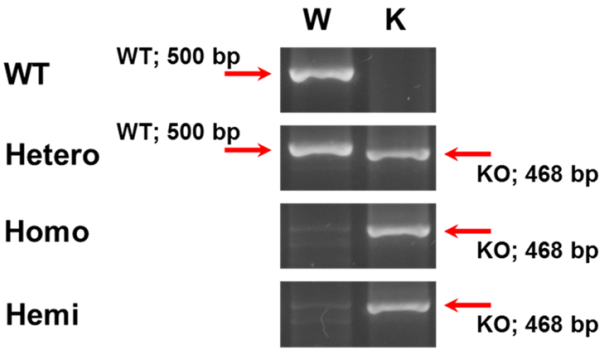

Fig. 4-2-1. PCR genotyping protocol for ACE2 knockout mouse. (A) The primer sequences, the specificity of each primer pair, and the expected PCR product size information (adapted from MMRRC). **(B)** The genotyping result from wild type and ACE2 knockout mouse (B6;129S5-Ace2^{tm1Lex}/Mmcd). After PCR amplifying, the WT DNA region was 500 bp and the KO DNA region was 468 bp. Genomic DNA templates were prepared from tail tissues, performed PCR reactions, and the result showed only WT allele (WT mice), WT allele with KO allele (ACE2 KO, hetero), and only KO allele (ACE2 KO, homo and hemi) was present.

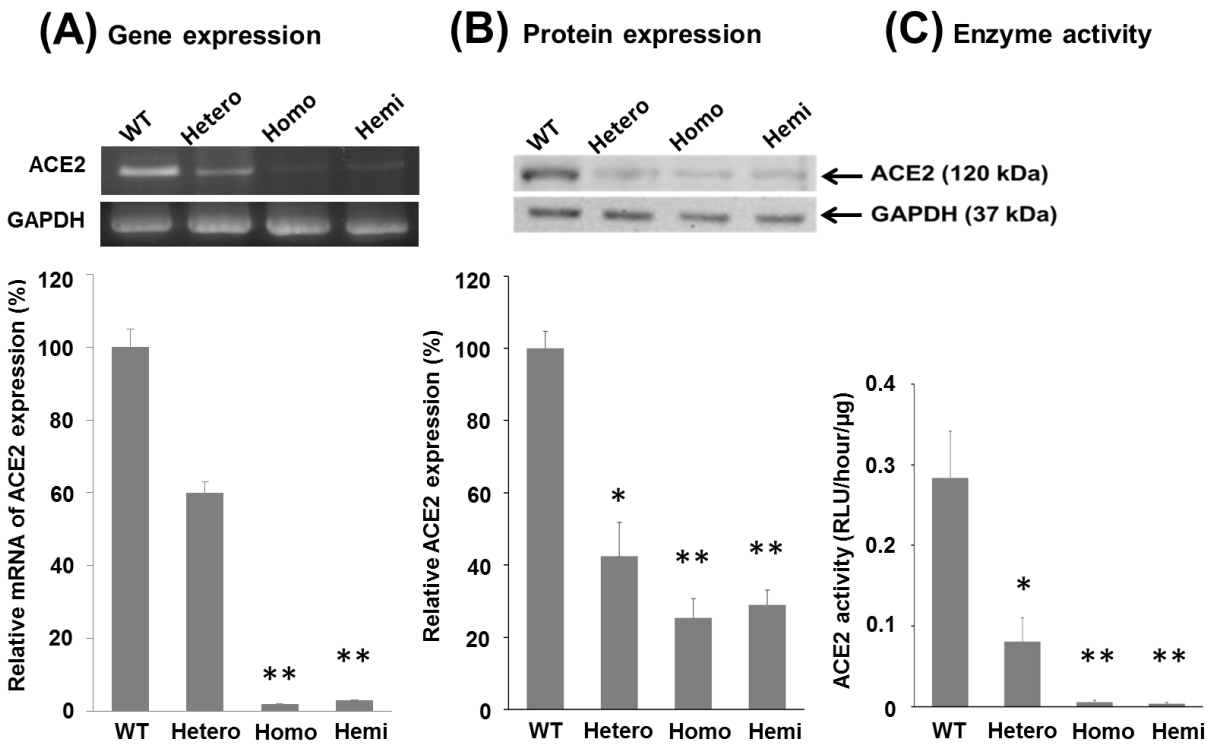

Fig. 4-2-2. ACE2 expression and activity in the ACE2 knockout (KO) mice. Wild-type (WT) and ACE2 KO mice (B6;129S5-Ace2tm1Lex/Mmcd; heterozygous ($ACE^{-/+}$), hemizygous ($ACE^{-/y}$) and homozygous ($ACE2^{-/-}$) genotypes) were sacrificed and the RNA and protein extracts of the lung was prepared by homogenizer. The ACE2 gene and protein expression of the lung extracts were detected by real-time PCR **(A)** and Western blot **(B)**, respectively. ACE2 expression was normalized using by GAPDH expression, and the relative expression of ACE2 was calculated using the Control group as 100%. The ACE2 enzyme activity of the lung extracts was detected by fluorescence ACE2 activity assay (**C**). ACE2 activity was detected by the ability to cleave the fluorescent substrate at 37℃ for 1 hour with a specific ACE2 inhibitor. All values were expressed as the mean ± SD from three animals of each group.

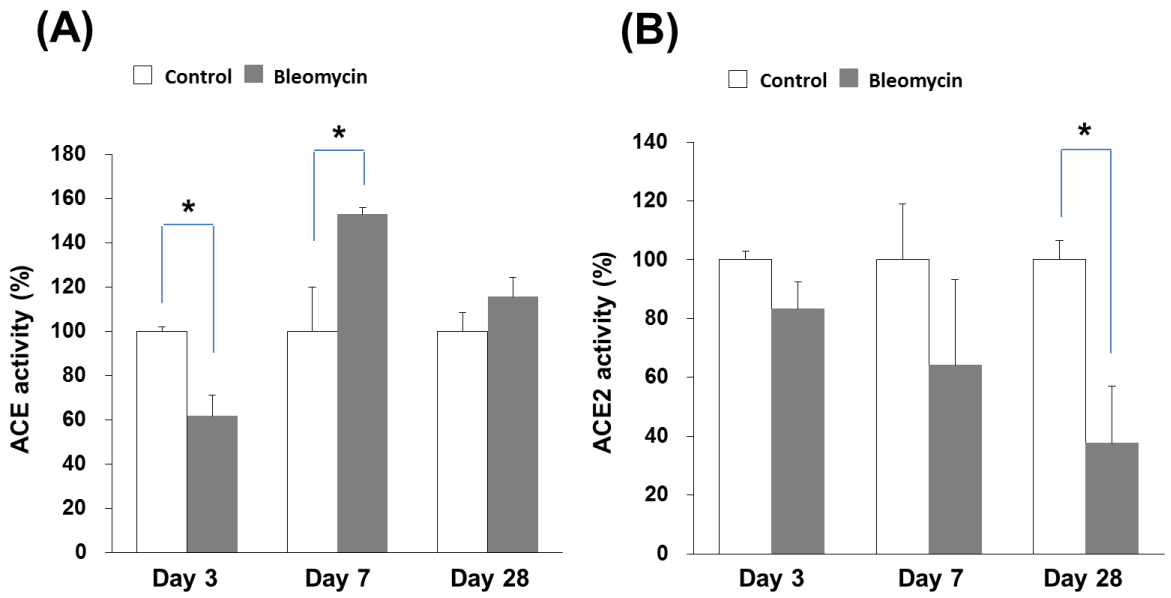

Fig. 4-2-3. ACE and ACE2 activity in the lung tissue of mice challenged with bleomycin. In the lung tissues obtained from the WT mice at 3-, 7- and 28-day after given intrapleural injection of PBS (as the Control) or 1 U/kg bleomycin, the ACE **(A)** and ACE2 **(B)** activity in the lung homogenates were detected and compared using fluorescence ACE and ACE2 activity assay, respectively. All of the values were expressed as the mean ± SD from three mice of each group. * indicates $p < 0.05$ compared to the Control, respectively.

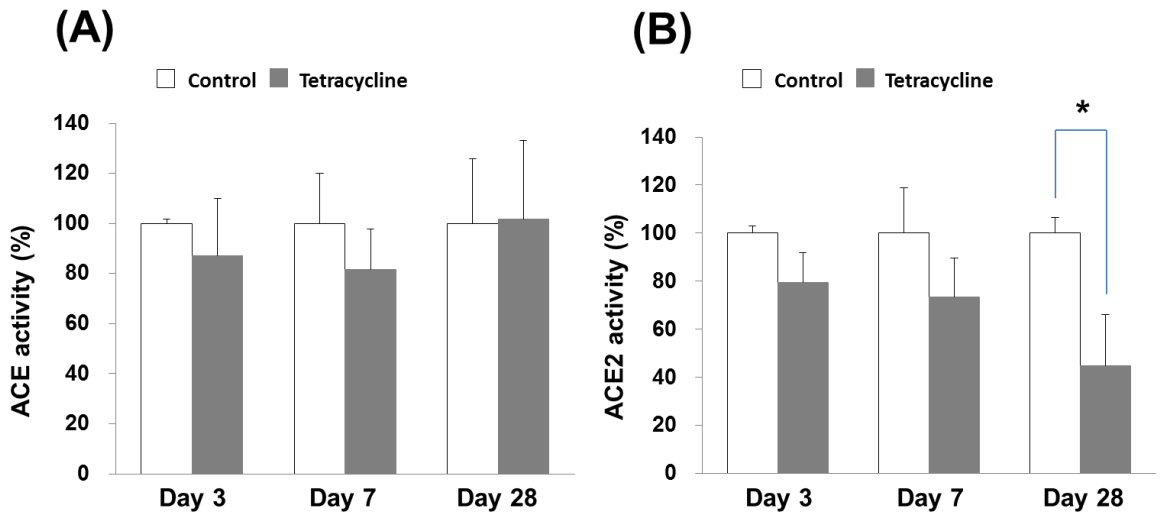

Fig. 4-2-4. ACE and ACE2 activity in the lung tissue of mice challenged with tetracycline. In the lung tissues obtained from the WT mice at 3-, 7- and 28-day after given intrapleural injection of PBS (as the Control) or 20mg/kg tetracycline, the ACE **(A)** and ACE2 **(B)** activity in the lung homogenates were detected and compared using fluorescence ACE and ACE2 activity assay, respectively. All of the values were expressed as the mean ± SD from three mice of each group. * indicates $p < 0.05$ compared to the Control, respectively.

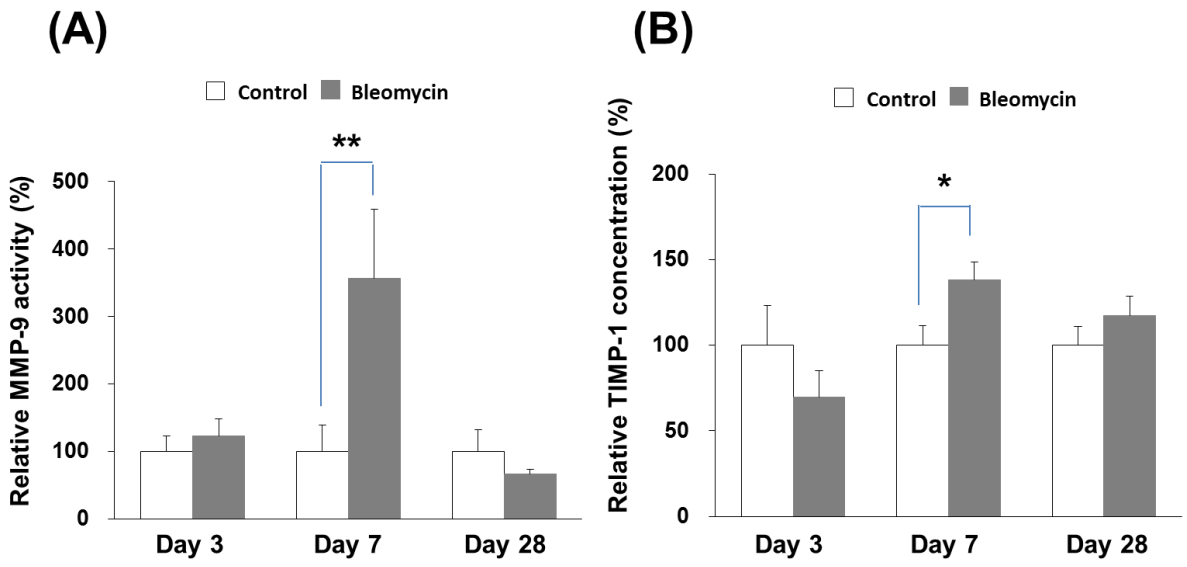

Fig. 4-2-5. MMP-9 and TIMP-1 in the lung tissue of mice challenged with bleomycin. The MMP-9 activity and TIMP-1 concentration in the lung homogenate of mice treated with PBS (Control) or 1 U/kg bleomycin were determined by gelatin Zymography and Western blotting , respectively. MMP-9 activity showed no significant difference in the 3-day and 28-day group, and the MMP-9 activity in mice treated with bleomycin was significantly higher than the Control at 7-day group **(A)**. On the other hand, there were significantly different on TIMP-1 concentration among the groups of bleomycin-treated and the Control at 7- day after the treatments but insignificant different in the 3-day and 28-day group **(B)**. All of the values were expressed as the mean ± SD from three animals of each group. * and ** indicate $p < 0.05$ and $p < 0.01$ compared to the Control.

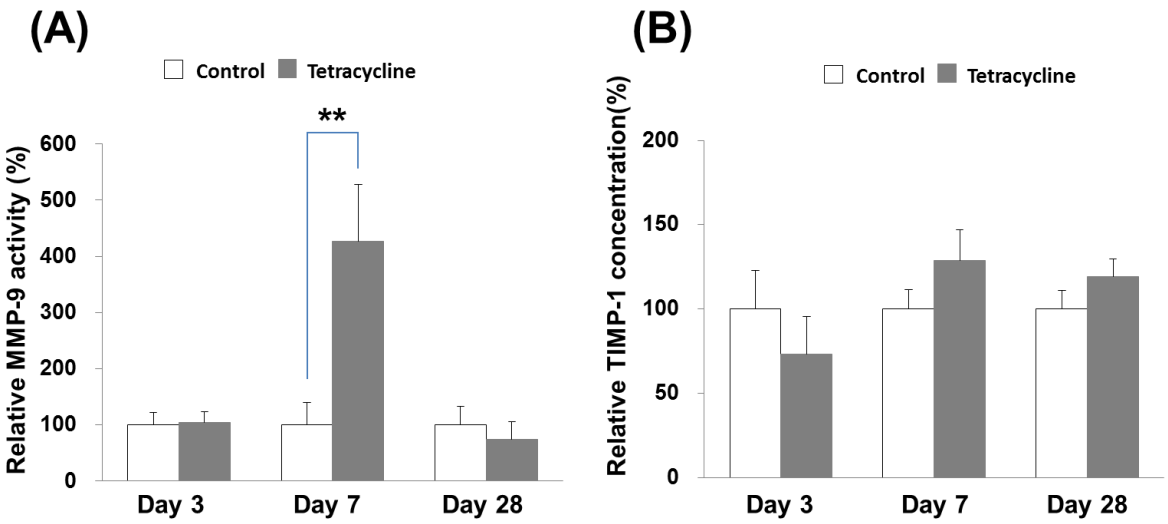

Fig. 4-2-6. MMP-9 and TIMP-1 in the lung tissue of mice challenged with tetracycline. The MMP-9 activity and TIMP-1 concentration in the lung homogenate of mice treated with PBS (Control) or 20mg/kg tetracycline were determined by gelatin Zymography and Western blotting, respectively. MMP-9 activity showed no significant difference in the 3-day and 28-day group, and the MMP-9 activity in mice treated with bleomycin was significantly higher than the Control at 7-day group **(A)**. Whereas, there were insignificantly different on TIMP-1 concentration among the groups of tetracyclin-treated and the Control at 3-, 7- and 28-day after treatments **(B)**. All of the values were expressed as the mean ± SD from three animals. ** indicates $p < 0.01$ compared to the Control.

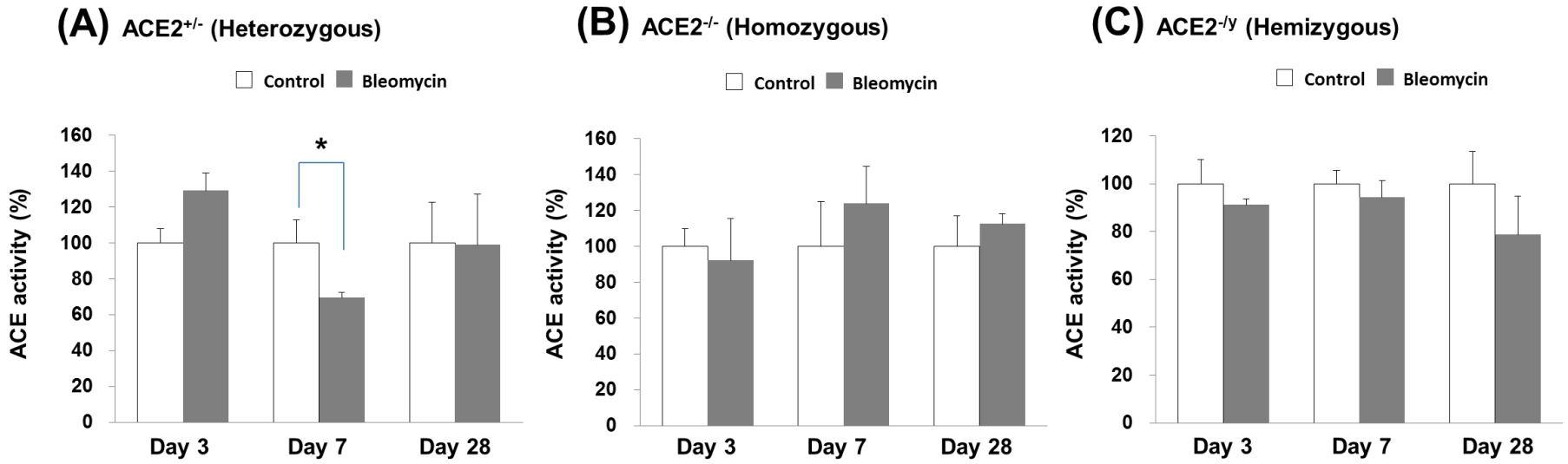

Fig. 4-2-7. ACE activity in the lung tissue of ACE2 KO mice challenged with bleomycin. The ACE activities in the ACE2 KO heterozygous ($ACE^{-/+}$) **(A)**, homozygous ($ACE^{-/-}$) **(B)** and hemizygous ($ACE^{-/y}$) **(C)** mouse lungs treated with PBS or 1 U/kg bleomycin after 3-, 7- and 28-day were determined by the ability to cleave the fluorescent substrate at 37℃ for 1 hour with a specific ACE inhibitor. Heterozygous mice injected with bleomycin and the ACE activity in lung tissue was significantly induced after 7 days. All of the values were expressed as the mean ± SD from three mice of each group. * indicates $p < 0.05$ compared to the Control, respectively.

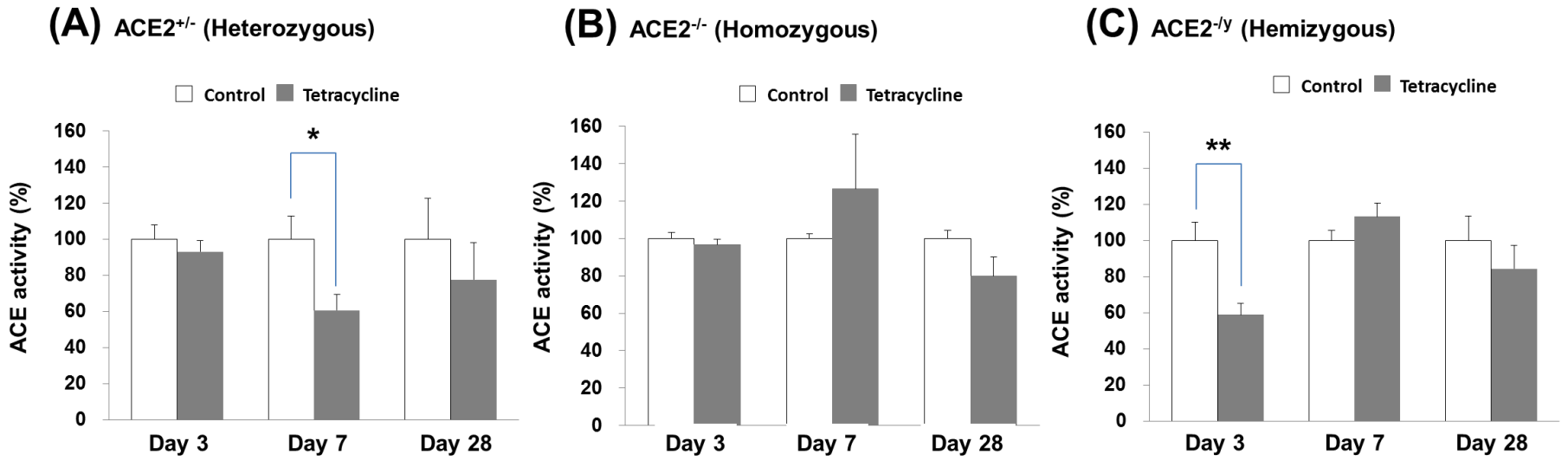

Fig. 4-2-8. ACE activity in the lung tissue of ACE2 KO mice challenged with tetracycline. The ACE activities in the ACE2 KO heterozygous ($ACE^{-/+}$) **(A)**, homozygous ($ACE^{-/-}$) **(B)** and hemizygous ($ACE^{-/y}$) **(C)** mouse lungs treated with PBS or 20mg/kg tetracycline after 3-, 7- and 28-day were determined by the ability to cleave the fluorescent substrate at 37℃ for 1 hour with a specific ACE inhibitor. The ACE activity in lung tissue was significantly induced heterozygous mice injected with tetracycline at 7-day group and hemizygous mice injected with tetracycline at 3-day group. All of the values were expressed as the mean ± SD from three mice of each group. * and ** indicates $p < 0.05$ and $p < 0.01$ compared to the Control.

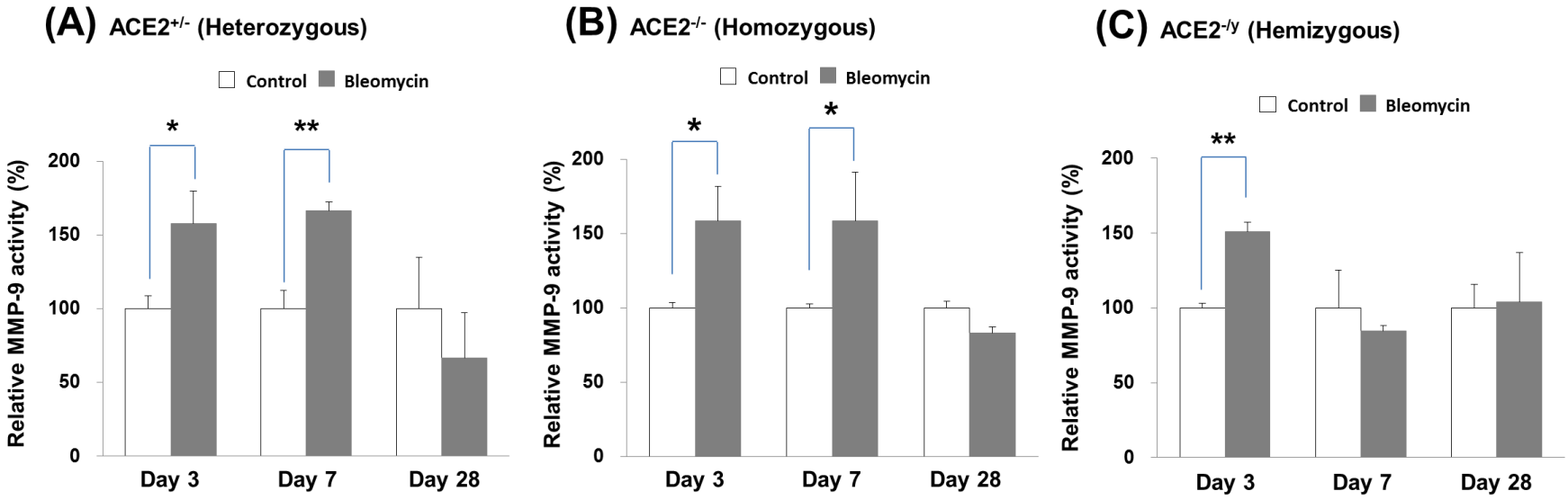

Fig. 4-2-9. MMP-9 activity in the lung of ACE2 KO mice treated with bleomycin. The MMP-9 activities in the ACE2 KO heterozygous ($ACE^{+/-}$) **(A)**, homozygous ($ACE^{-/-}$) **(B)** and hemizygous ($ACE^{-/y}$) **(C)** mouse lungs treated with PBS or 1 U/kg bleomycin were determined by gelatin Zymography at 3-, 7- and 28-day after the treatments. All of the values were expressed as the mean ± SD from three mice of each group. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, compared to the Control.

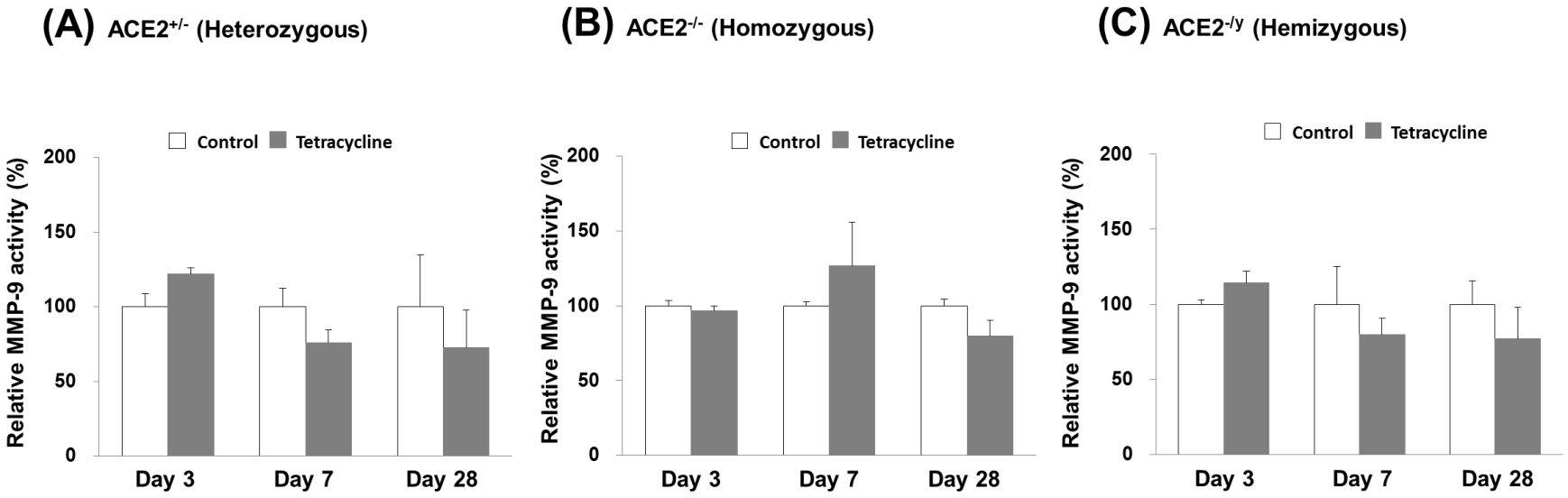

Fig. 4-2-10. MMP-9 activity in the lung of ACE2 KO mice treated with tetracycline. The The MMP-9 activities in the ACE2 KO heterozygous ($ACE^{+/-}$) **(A)**, homozygous ($ACE^{-/-}$) **(B)** and hemizygous ($ACE^{-/y}$) **(C)** mouse lungs treated with PBS or 20mg/kg tetracycline were determined by gelatin Zymography at 3-, 7- and 28-day after the treatments. All of the values were expressed as the mean ± SD from three mice of each group.

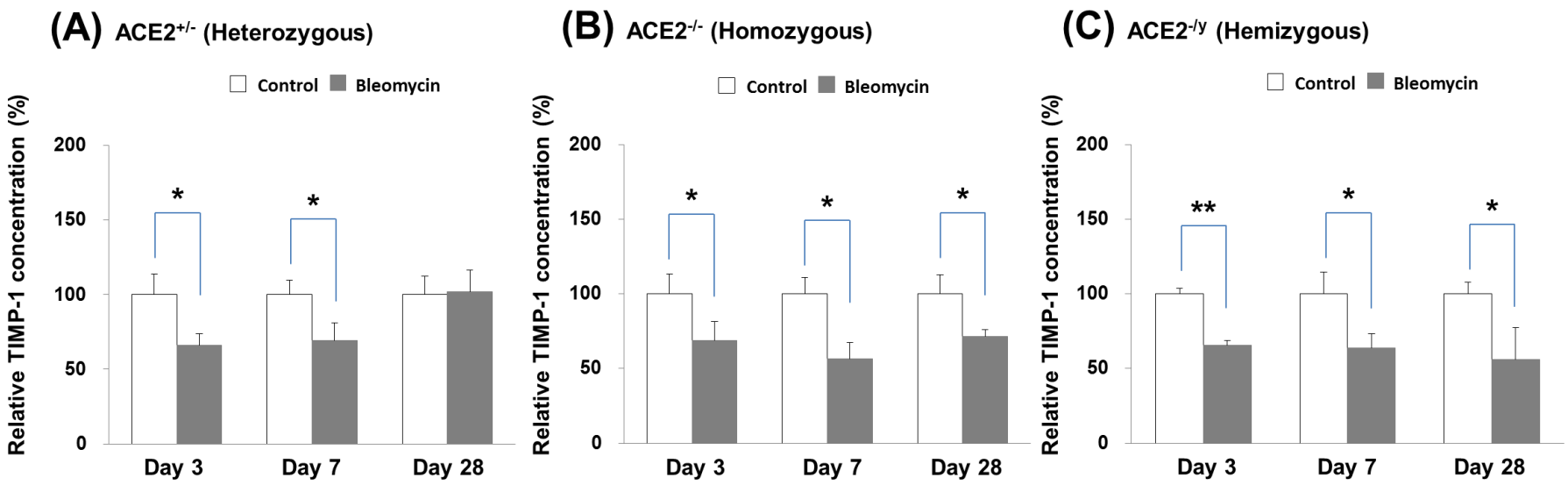

Fig. 4-2-11.TIMP-1 concentration in the lung of ACE2 KO mice treated with bleomycin. The TIMP-1 concentration in the ACE2 KO heterozygous ($ACE^{+/-}$) **(A)**, homozygous ($ACE^{-/-}$) **(B)** and hemizygous ($ACE^{-/y}$) **(C)** mouse lungs treated with PBS or 1 U/kg bleomycin were determined by Western blotting at 3-, 7- and 28-day after the treatments. Heterozygous mice injected to chest with bleomycin and the TIMP-1 concentration in lung tissue was significantly reduced after 3 days and 7 days; but in the hemizygous and homozygous mice, both the TIMP-1 concentration of lung tissure was significantly reduced after 3-, 7- and 28-day. All of the values were expressed as the mean ± SD from three mice of each group. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, compared to the Control.

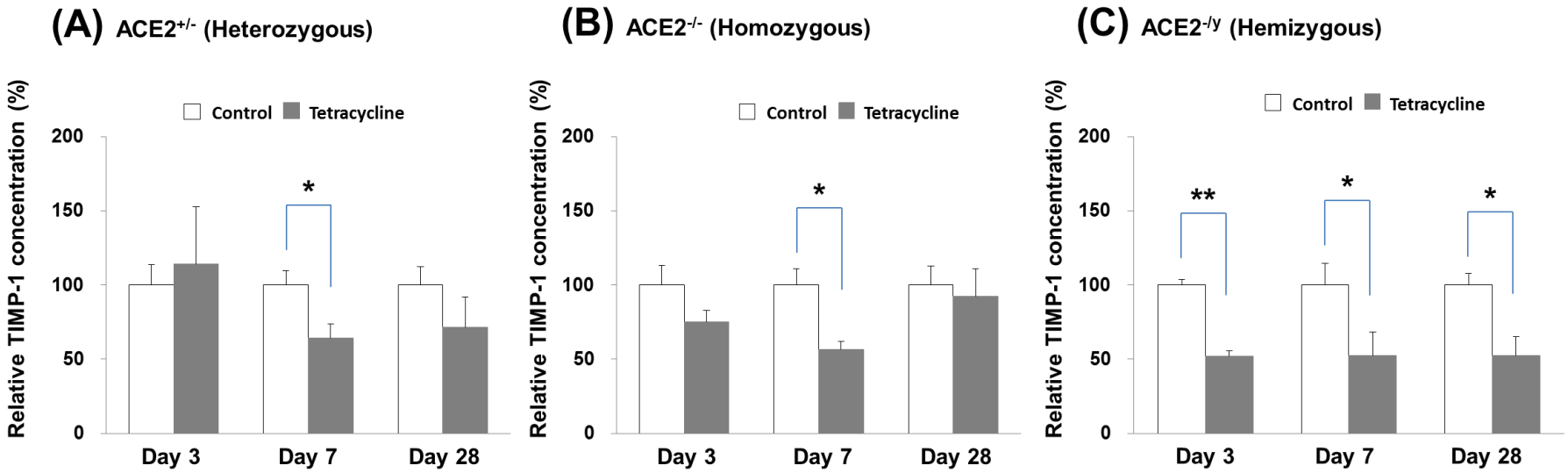

Fig. 4-2-12. TIMP-1 concentration in the lung of ACE2 KO mice treated with tetracycline. The TIMP-1 concentration in the ACE2 KO heterozygous ($ACE^{+/-}$) **(A)**, homozygous ($ACE^{-/-}$) **(B)** and hemizygous ($ACE^{-/y}$) **(C)** mouse lungs treated with PBS or 20mg/kg tetracycline were determined by Western blotting at 3-, 7- and 28-day after the treatments. Heterozygous and homozygous mice injected to chest with tetracycline and the TIMP-1 concentration in lung tissue were significantly reduced after 7-day; but in the hemizygous mice, the TIMP-1 concentration of lung tissure was significantly reduced after 3-, 7- and 28-day. All of the values were expressed as the mean ± SD from three mice of each group. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, compared to the Control.

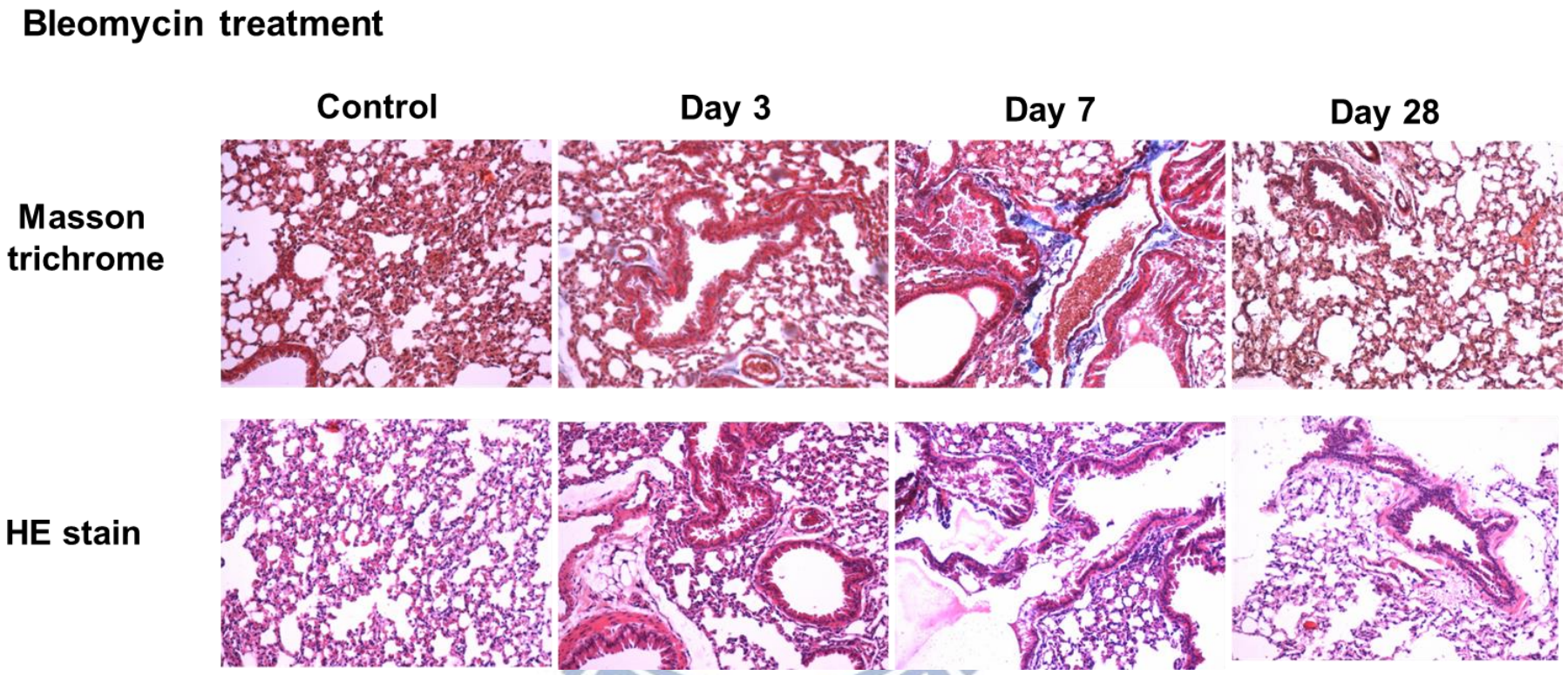

Fig. 4-2-13. Histological examinations of the lung tissue of wild-type mice treated with bleomycin. The mice were challenged with 1 unit/kg bleomycin by intrapleural injection, and then the animals were sacrificed to isolate the lung tissues for pathological examinations after 3 days, 7 days and 28 days of the treatments. The Masson trichrome and Hematoxylin-Eosin (HE) stain of the tissue sections were performed, and there were evidenced the pathological features of loss of lung architecture, white blood cells infiltration and blue stained area indicating collagen deposition in the bleomycin treated lung tissues.

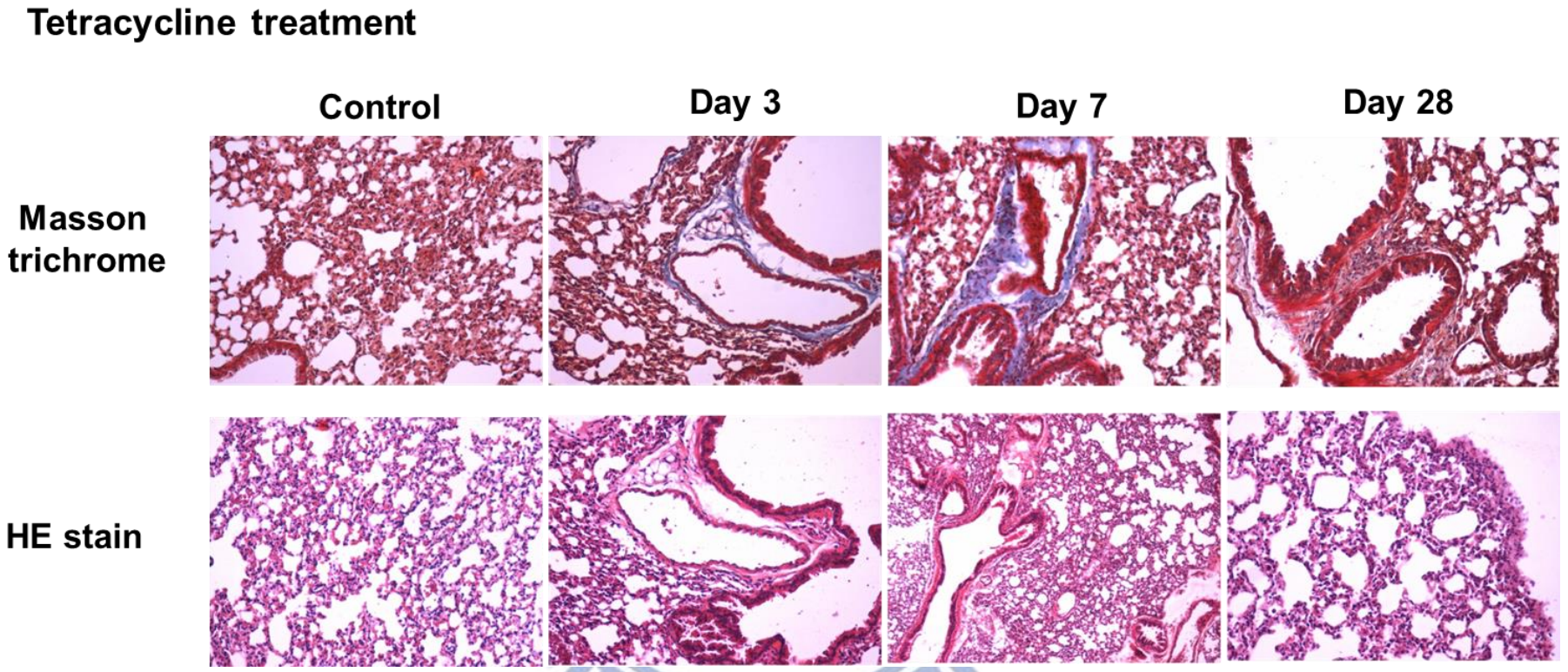

Fig. 4-2-14. Histological examinations of the lung tissue of wild-type mice treated with tetracycline. The mice were challenged with 20 mg/kg tetracycline by intrapleural injection, and then the animals were sacrificed to isolate the lung tissues for pathological examinations after 3 days, 7 days and 28 days of the treatments. The Masson trichrome and Hematoxylin-Eosin (HE) stain of the tissue sections were performed, and there were evidenced the pathological features of loss of lung architecture, white blood cells infiltration and blue stained area indicating collagen deposition in the tetracycline treated lung tissues.

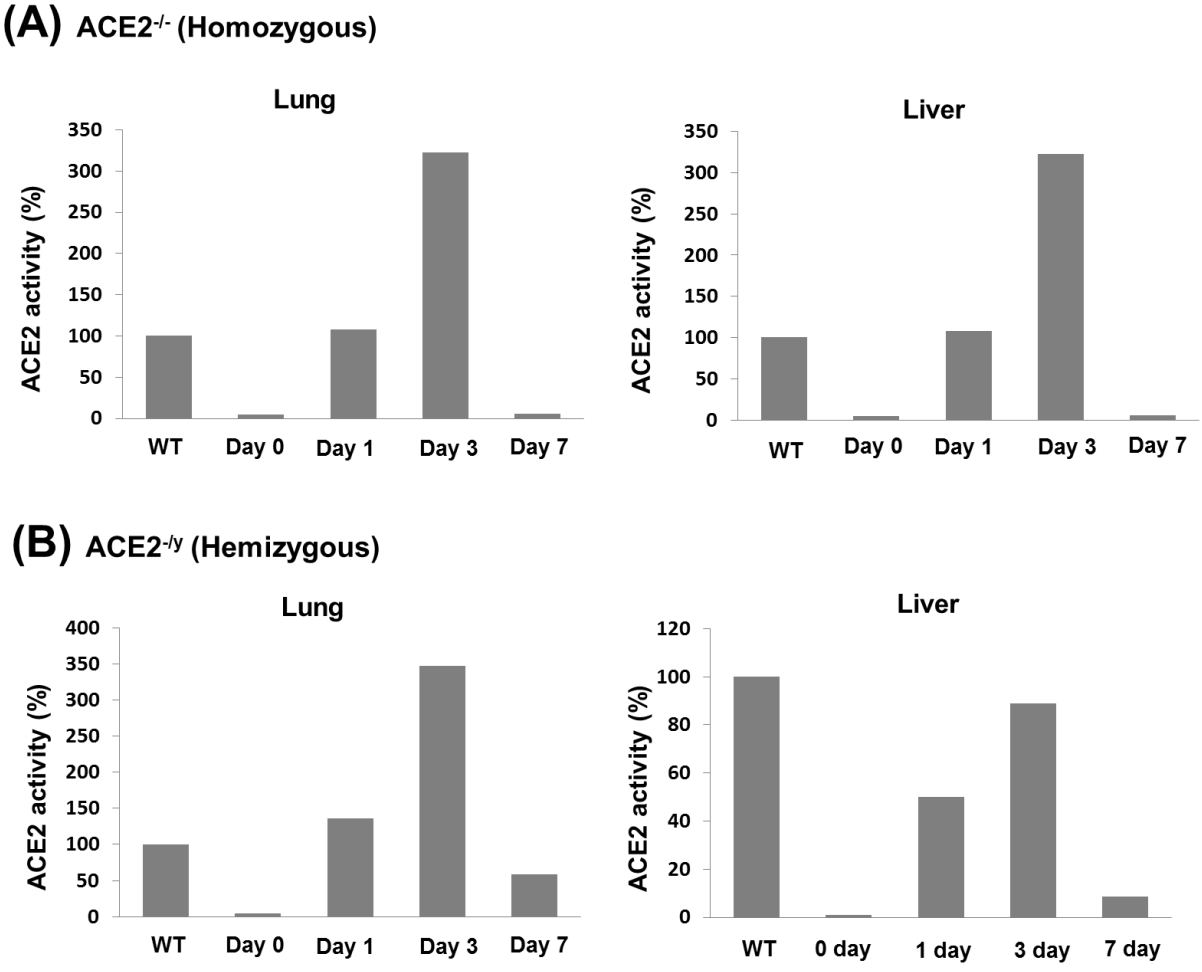

Fig. 4-2-15. ACE2 activity in ACE2 KO mice after lenti-ACE2 injection. The ACE2 activities in the ACE2 KO homozygous (ACE2 $^{-/-}$) **(A)** and hemizygous (ACE2 $^{-/y}$) **(B)** mouse lungs treated with empty virus (Control), lenti-ACE2 viral particles (3×10^6 U in 100 μL of phosphate-buffered saline) through the tail vein using a 27-gauge syringe needle after 1, 3 and 7 days were determined by the ability to cleave the fluorescent substrate at 37℃ for 1 hour with a specific ACE2 inhibitor. Both ACE2 activities in homozygous and hemizygous significantly induced at 1 and 3 days in lung and liver after lentivirus injection.

V. Discussion

In the present study, major components of RAS and ECM metabolism, pleural ACE, ACE2, MMP-2, and MMP-9, were measured in patients with pleural effusions. Our main findings are: (1) significantly higher the ACE, MMP-9 activity and ACE/ACE2 ratio in exudative effusions; (2) in tuberculous effusions, significantly higher ADA activity combined with elevated MMP-9 levels compared with these values in pneumonia and adenocarcinoma effusions; (3) the population of ACE2 KO mice, hemizygous ($ACE2^{-/y}$, male), heterozygous ($ACE2^{+/-}$, female) and homozygous ($ACE2^{-/-}$, female), have been successfully bred. (4) The mouse model of pulmonary inflammation and fibrosis has been established by intrapleural injection of bleomycin. The profiling of lung ACE/ACE2 and MMP-9/TIMP-1 activity in the mice challenged with bleomycin at 3-day, 7-day and 28-day after the treatments have been performed.

Only a few reports have been published on the importance of ACE in pleural fluid. ACE could be detected in pleural fluid, but the ACE level cannot be used to discriminate cancer from non-cancer patients (Bedrossian et al., 1981; Rømer et al., 1982). Söderblom et al. had also measured ACE in pleural effusions and sera of 364 patients and showed that tuberculous effusions contain higher ACE concentrations than any other type of nonrheumatoid effusions. These results indicate that ACE determinations may aid in differentiating rheumatoid and tuberculous pleurisy from other types of pleural disease. Some of the physiological functions of ACE2 are opposite to those of ACE. The significant positive correlation between ACE and ACE2 activities in the transudates suggests that ACE and ACE2 maintain a normal physiological balance. This loss of balance in exudates was detected because of increased ACE. Although the importance of the dysregulation in ACE in exudative effusions has not been explored, a significant role for RAS in the pathophysiological process of exudate development is possible.

ACE activity was increased ($p < 0.001$) and ACE2 was decreased ($p < 0.05$) in tuberculosis specifically, but the ACE and ACE2 activities were no difference between transudate, pneumonia and adenocarcinoma effusions. These results indicated that ACE and ACE2 maybe a biomarker for tuberculosis diagnosis in pleural effusion and it could be emphasized that the increased ACE activity and ratio of ACE/ACE2 in the exudates are mainly contributed from a higher ACE level and lower ACE2 enzyme activities in the

tuberculous pleural effusion. However, more patients should be further included in order to examine the proposition.

MMPs have been implicated in the pathogenesis of various lung diseases, including pleural effusions (McKeown et al., 2009). The activity of MMPs within the pleural space may play a role in the formation of pleural effusions by altering the integrity of the mesothelial and endothelial cell layers and by increasing vascular permeability (Park et al., 2005). Proteolysis by MMPs may be involved in the formation of pleural effusions by increasing vascular permeability, and thus by facilitating fluid influx into the pleural space (Zucker et al., 1998). Therefore, the presence and enzymatic activities of MMPs have been identified in pleural effusions (Sheen et al., 2009). Previous studies have shown that the expression of MMPs in the pleural space is altered in a variety of inflammatory and malignant diseases, suggesting that certain members of the MMP family may participate in the formation of pleural effusions (Bodiga et al., 2011). Animal model showed that MMP-9 activity has no significant change in acute immune response. However, there is an increased activity of MMP-9 and tissue fibrosis around the blood vascular in inflammatory response. Cytokine, was induced during inflammatory response, may be powerful stimulus to MMP-9 induction.

Tissue damage is a characteristic manifestation of mycobacterium tuberculosis infection. Proteolysis by macrophage secreted proteases has been implicated in such destructive processes. In this regard, the proteolytic action of MMPs may be involved in the pathogenesis of tuberculosis, like many other diseases associated with tissue destruction. Several studies have reported that macrophages and monocytes release MMP-9 in response to tuberculosis or its cellular components. Studies have evaluated MMPs in tuberculous pleural effusions and found that the MMP levels in pleural fluid are higher in patients with tuberculosis compared with patients with transudative effusions. Our results are in agreement with the above findings, as the level of MMP-9 was highest in tuberculous effusions among the exudates we examined. However, more patients should be further included in order to examine the proposition.

It was reported that overexpression of ACE2 may inhibit MMP-9 activity (Dong et al., 2008), and ACE2 deficiency leads to increased MMP-9 levels (Bodiga et al., 2011). In inflammatory signalling pathways study, Ang II could stimulate human monocytic U-937 cells to increase MMP-9 expression and activity significantly via activated NF-κB, JNK, and p38 (Yaghooti et al., 2011). The ACE inhibitor, captopril, was administrated to isoprenaline-induced left ventricular fibrosis rats and showed that captopril significantly

enhanced the isoprenaline-induced myocardial fibrosis and augmented the isoprenaline-induced MMP-9 expression (Okada et al., 2010). Our observations of decreased ACE2 and increased ADA, MMP-9 activity in tuberculous effusions confirm this conclusion.

A number of studies have indicated that the ACE/Ang II axis and ACE2/Ang 1-7 axis not only regulate the metabolism of ECM proteins, but also modulate MMP expression and activity levels (Pan et al., 2008). MMPs have been implicated in the pathogenesis of various lung diseases, including pleural effusions (McKeown et al., 2009). The activity of MMPs within the pleural space may play a role in the formation of pleural effusions by altering the integrity of the mesothelial and endothelial cell layers and by increasing vascular permeability.

The population of ACE2 KO mice including hemizygous ($ACE2^{-/y}$, male), heterozygous ($ACE2^{-/+}$, female) and homozygous ($ACE2^{-/-}$, female) have been successfully bred in our laboratory. Use of $ACE2^{-/-}$ mating with $ACE2^{-/y}$, all of the female offspring are homozygous $ACE2^{-/-}$ and male offspring are hemizygous $ACE2^{-/y}$ genotype. It is convenient to use ACE2 KO mice in variety of biomedical research. In fact, there were few researchers reported using ACE2 KO homozygous $ACE2^{-/-}$ mice (Rey-Parra et al., 2012). However, it is successfully established $ACE2^{-/-}$ mice, $ACE2^{-/-}$ mice like as WT mice in our laboratory but the number of births is low.

Recent evidence suggests that the RAS has important functions outside the cardiovascular system. Latest since ACE2 was identified as a key receptor for coronavirus infections responsible for the severe acute respiratory syndrome (Li et al., 2003) major attention has been drawn to the potential protective role of ACE2 in lung diseases. ACE2 knockout mice exhibit exacerbated lung injury compared with WT mice (Imai et al., 2005): loss of ACE2 caused enhanced vascular permeability, increased lung edema, neutrophil accumulation, and worsened lung function. Importantly, treatment with catalytically active recombinant ACE2 protein improved the symptoms of ALI in WT mice, as well as in ACE2 knockout mice (Imai et al., 2005). Furthermore, lung injury in experimental ARDS in mice can be attenuated by blocking the RAS (Imai et al., 2005). One complication of ARDS is lung fibrosis. Li et al. (2008) have demonstrated that ACE2 mRNA and activity are downregulated in human and experimental lung fibrosis and suggest that ACE2 limits the local accumulation of ANG II.

Bleomycin and tetracycline treated mice after 3, 7 and 28 days sacrificed to get the lung tissue. It showed ACE/ACE2 and MMP-9/TIMP-1 activity in the mouse lung tissue had little of differentiation. It presented that the course of lung fibrosis pathological is not similar in the two kinds of molecules. In bleomycin processing experiments, ACE activity was lower in 3 days but higher in 7 days significantly. Even ACE2 activity was decreased in later stage (28 days) significantly. The rise of ACE activity and the decrease of ACE2 will make RAS disorder then it will lead to Ang II increased. Hence, it affected the blood vessels and caused inflammation and regulation of MMPs in the molecular pathway. In tetracycline processing experiments, only the ACE2 activity significantly decreased in late stage (28 days). According to the result, ACE2 activity decreased when lung injury. RAS disorder can cause abnormal pulmonary vasoconstriction and vascular remodeling. It causes severe chronic obstructive lung disease or acute respiratory distress syndrome. It shows RAS is an important role in lung injury (Jeffery and Wanstall, 2001; Mandegar et al., 2004).

There were using minocycline and tetracycline on the rabbit pleura to induce early inflammatory response (Dryzer et al., 1993). Most papers reported there were using bleomycin-induced lung injury (Rey-Parra et al., 2011). We could find the difference effects in lung tissue between bleomycin and tetracycline. It was obvious pulmonary fibrosis reaction with bleomycin-induced. However it seems that tetracycline-induced were not work in pulmonary fibrosis in mice. Therefore we will use bleomycin-induced pulmonary fibrosis animal models to do the succeeding experiment.

In lung fibrosis suggests that MMP-9 could be rather linked to inflammation-induced tissue remodeling, while MMp-2 may be associated with an impaired tissue remodeling leading to pathological collagen deposition and interstitial fibrosis (Gueders et al., 2006). MMP-9 activity were induced highly only at 7 days after the treatment with bleomycin and tetracycline. The data correspond with collagen deposition in histological examinations of the lung tissue. Hence, the high performance of MMP-9 will deal with abnormal matrix proteins accumulation. It is not easy to measure the changes of MMPs or TIMPs activities significantly in pulmonary fibrosis in some research reports. Take TIMP-1 for example, Manoury et al. (2006) that TIMP-1 plays an important role in bleomycin induced pulmonary fibrosis in mice. However, Fattman et al. (2008) reported that there were no significant in TIMPs activity in lung epithelial tissue that induced pulmonary fibrosis in mice by bleomycin.

It is still to be confirmed the relationship MMPs or TIMPs activity in the course of pulmonary fibrosis.

Important increased TIMP-1 expression has been observerd in lung extracts after bleomycin administration and after the transfer of the active TGF-β gene to “fibrosis-prone” C57BL/6 mice (Madtes et al., 2001). In humans, increaseds level of TIMP protein and RNA were observed in the lung of patients with IPF, and TIMP expression there exceeds that of MMP (Selman et al., 2000). TIMPs and particularly TIMP-1 induction could lead to a “noncollagenolytic microenvironment”, building adequate conditions for further ECM deposition (Selman et al., 2001). In our study, there were significantly different on bleomycin induced to 138% TIMP-1 concentration compared with the Control at 7-day but insignificant different in the 3-day and 28-day group. However, heterozygous mice injected to chest with bleomycin and the TIMP-1 concentration in lung tissue was significantly reduced after 3-day and 7 day; but in the hemizygous and homozygous mice, both the TIMP-1 concentration of lung tissue was significantly reduced after 3, 7 and 28 days. Above the results, without ACE2 gene mice may decrease TIMP-1 expression when ACE2 KO mice got pulmonary fibrosis.

The bleomycin treatment of heterozygous, hemizygous and homozygous ACE2 KO mice showed MMP-9 activity increased significantly by approximately 1.6-1.7 fold in heterozygous and homozygous mice at 3 and 7 days treatment, but raised significantly only at 3 days of hemizygous mice (approximately 1.5-fold). In the above experiments, bleomycin treatment could regulate MMP-9 activity. In particular, WT mice with bleomycin processed at 7 days induced MMP-9 activity approximately 3.5 fold compared to the Control. Thus, ACE2 may play an important role with bleomycin induced lung fibrosis, but its compromise of pulmonary mechanism has yet understood. Kruit et al. (2005) the earliest mention of ACE2 may affect the evolution of pulmonary fibrosis. Therefore, ACE2 has also been proposed to associate to IPF. In IPF, ACE2 mRNA expression and enzyme activity were decreased to 92% and 74% and caused Ang II rised and collagen abnormally accumulated. It showed ACE2 served as a protective role in primary pulmonary fibrotic disease (Li et al., 2008). And then, some studies found that ACE2 decreased in pulmonary fibrosis and increased AT1R and TGF-β performance. When add ACE2 or Ang 1-7 can slow the evolution of pulmonary fibrosis (Shenoy et al., 2010).

Rey-Parra et al. (2012) found gender differences in $ACE2^{-/-}$mice in bleomycin- induced lung injury. Their data show significantly worse lung function and higher lung collagen

deposition in male ACE2 KO compared with females. This gender-based difference could suggest a hormonal involvement in the pathophysiology of bleomycin -induced lung injury. Men with idiopathic pulmonary fibrosis have decreased quality of life compared with women (Han et al., 2010). In rodents, castrated male mice exhibited a female-like response to bleomycin while female mice given exogenous androgen exhibited a male-like response, suggesting a detrimental role of androgens in pulmonary function in fibrosis (Voltz et al., 2008). However, in our data were not significantly difference between homozygous and hemizygous.

Lenti-ACE2 treatment, before the induction of pulmonary fibrosis, resulted in an almost complete prevention of increases in RVSP, RV hypertrophy, and attenuation of thickening of pulmonary vessels (Yamazato et al., 2009). It is conceivable that the animals were still in an adaptive phase at this time and the pathophysiological aspects would be manifested at a later time point. Targeting of ACE2 in the lungs appears to be a better strategy than the use of systemic administration of AT1R antagonists and ACE inhibitors, which have been found to have limited or no success in the prevention of PH (Mascitelli et al., 2007). Therefore, we will induce mice lung fibrosis to find the agents as ACE2, MMPs/TIMPs activator or inhibitor to control pulmonary fibrosis.

VI. Conclusions

Our data suggested that the interplay between ACE and ACE2, an essential function in RAS, and the change in gelatinase activity may be involved in the development of pleural effusions. Our findings suggest that increased ACE, MMP-9 and ADA activities and decreased ACE2 activity in pleural fluid are features of pleural space infection in patients with pleural tuberculosis. Such measurements may be helpful for diagnosing tuberculous pleurisy. However, the findings of the present study require further validation in a large prospective study examining the treatment and outcome of unselected patients with tuberculosis before the above indicators can become part of a clinically meaningful practice. Such measurements may be helpful for diagnosing tuberculous pleurisy.

The mouse model of pulmonary inflammation and fibrosis has been established by chest cavity injection of bleomycin. Our findings suggest that wild-type mice after bleomycin-induced fibrosis treatment which MMP-9, TIMP-1 and ACE activity higher than the Control at 7 day to cause pulmonary inflammation. The data correspond with collagen deposition in histological examinations of the lung tissue. Hence, the high performance of MMP-9 will deal with abnormal matrix proteins accumulation. However, ACE2 KO mice, heterozygous, homozygous and hemizygous were treated with bleomycin and then pulmonary MMP-9 activities were increasing at acute inflammation response (3 day). Even histological examinations of the lung tissue confirmed collagen deposition. TIMP-1 concentrations in ACE2 KO mice after treatment were reduced at early stage (3 and 7 day). The different activity patterns of MMP-9/ TIMP-1 were shown and indicated a role of ACE2 on the MMP-9/ TIMP-1 regulation in the pathogenesis of lung fibrosis.

Our animal model could help study the immune response and fibrosis mechanism of pulmonary more. However, the findings of the present study require further validation in a large prospective study examining the treatment and outcome of unselected patients with tuberculosis before the above indicators can become part of a clinically meaningful practice.

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