國 立 交 通 大 學

生 物 科 技 學 院

生 物 科 技 學 系

博士論文

血管收縮素對心臟細胞的第二型血管收縮素轉化酶表現調節與第二型基質金屬蛋白酶表現之影響

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時光向來都在不知不覺中，飄然流逝，六年半的時間，轉眼間就這麼過去了。在博士學位口試結束的那一刻，宣告了我博士班學程的結束，得到的，是本厚重的論文以及一紙畢業證書，留在心底的，是許多令人值得去細細品味的經歷與回憶。

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中文摘要

腎素-血管收縮素系統（renin-angiotensin system, RAS）為人體最重要的調節系統之一，不正常的 RAS 與心血管疾病的病程機制有很大的關聯性。血管收縮素 II（Angiotensin II, Ang II）、血管收縮素 1-7（angiotensin 1-7, Ang 1-7）、血管收縮素轉化酶（angiotensin converting enzyme），以及第二型血管收縮素轉化酶（angiotensin converting enzyme II, ACE2）為 RAS 中的主要因子。Ang II 會刺激發炎反應、纖維母細胞生長、心肌細胞凋零、細胞分化以及纖維化，進而導致組織修復與重塑，而 ACE2 會水解 Ang II 形成 Ang 1-7，以抑制 Ang II 所造成之不良反應。多數的文獻顯示 ACE2 於 RAS 與心臟疾病中扮演重要角色，但其於心臟細胞中之表現調節功能仍尚未被釐清。

本研究主要目的為探討心臟細胞中 ACE2 與 *ace2* 基因之表現調節，以及 ACE2 的表現調節與第二型金屬基質蛋白酶（matrix metalloproteinases-2, MMP-2）的關聯性。我們以初代人類心纖維細胞（human cardiac fibroblasts, HCFs）來探討 Ang II 和 Ang 1-7 對於 ACE2 的表現調節，以冷光報導分析法來探討 *ace2* 啟動子上與 ACE2 表現調控有關的序列。此外，我們也用慢病毒（lentivirus）方法促使細胞高量表現 ACE2 或抑制 ACE2 生成，探討 ACE2 表現受到調控時，其對 MMP-2 表現之影響性。

本研究的重要結果歸納如下：(1) Ang II 與 Ang 1-7 分別會經由 AT1R 及 Mas 活化 ERK-MAPK 訊息傳遞途徑以刺激 ACE2 表現; (2) *ace2* 基因啟動子的-516/-481 序列上之 5’-ATTTGGA-3’為特定轉錄因子的結合位置並可藉此調控 ACE2 表現; (3) Ang II 會經由

AT1R 與其下游 ERK-MAPK 訊息傳遞鏈刺激 *ace2* 基因啟動子的-516/+20 序列藉此調控 ACE2 表現; (4) TGF-β1 和 TNF-α 處理 HCFs 對於其 *ace2* 基因啟動子活性並無顯著影響; (5) ACE2 高表現會造成 MMP-2 活性提昇，且 Ang II-AT1R-ERK1/2 傳遞途徑可降低 MMP-2 活性表現; (6) 相對於 Ang II，Ang 1-7 會經由 Mas 抑制 ERK1/2 的活化，但對於 MMP-2 的活性表現並無明顯之影響性; (7) Ang II-AT1R-ERK1/2 及 Ang 1-7-Mas-ERK1/2 這兩條傳遞途徑也可調控腫瘤壞死因子-α 轉化酶（tumor necrosis factor-α-converting enzyme; TACE or ADAM17）的表現以改變細胞膜上 ACE2 的脫落效應（shedding ACE2, shed ACE2）; (8) 我們重新建立了 ACE2 基因剔除鼠（ACE2 knockout (KO) mice）群，包括 $ACE2^{+/-}$、$ACE2^{-/-}$ 及 $ACE2^{-/y}$ 基因型; (9) 相較於野生型小鼠，在 ACE2 KO 小鼠的心臟組織中，MMP-2 活性表現量會顯著提昇。

本研究顯示 ACE2 會受到 Ang II 及 Ang 1-7 之表現調節，Ang II 及 Ang 1-7 會提昇 HCFs 之 ACE2 表現，且 Ang II 會經由 *ace2* 啟動子中的-516/+20 序列區段調控 *ace2* 基因表現。於 HCFs，ACE2 的高表現會提昇 MMP-2 之活性表現，而 Ang II 會降低 HCFs/ACE2 的 ADAM17 mRNA 表現，也使 MMP-2 與 shed ACE2 的活性表現下降。這些結果皆暗示 ACE2 於心臟重塑病程中擔任保護的角色，Ang II-AT1R-ERK1/2 及 Ang 1-7-Mas-ERK1/2 這兩條傳遞途徑可調控 ADAM17 的 mRNA 表現，以及 ACE2 與 shed ACE2 之活性表現，且 Ang II-AT1R-ERK1/2 可調控 MMP-2 之活性表現。

關鍵詞：血管收縮素轉化酶 II、血管收縮素 1-7、血管收縮素 II、心臟細胞、ACE2 基因剔除鼠

The effects of angiotensin peptides on angiotensin converting enzyme II regulation and matrix metalloproteinase-2 expression in cardiac cells

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Abstract

Renin-angiotensin system (RAS) is an important regulation system in the human circulatory system. The abnormal RAS is associated with the pathogenesis of cardiovascular diseases. Angiotensin II (Ang II), angiotensin 1-7 (Ang 1-7), angiotensin converting enzyme (ACE) and angiotensin converting enzyme II (ACE2) are the mainly members in RAS. Ang II stimulates inflammation, fibroblasts growth, cardiac myocyte apoptosis, fibrogenesis, and differentiation to cause tissue repair/remodeling, and it has been hydrolyzed by ACE2 to obtain the Ang 1-7 to against the disadvantageous effect of Ang II. ACE2 plays a significant role in RAS and heart diseases, but the regulation mechanisms of ACE2 in the heart and cardiac cells are unclear.

This study demonstrates the regulation mechanism of ACE2 and *ace2* gene expression in cardiac cells, and the association between ACE2 and matrix metalloproteinases-2 (MMP-2) in the cells. The ACE2 expression in human cardiac fibroblasts (HCFs) treated with angiotensin peptides, Ang II and Ang 1-7, and analyzed the promoter activity of human *ace2* using luciferase report assay to identify regulatory elements of *ace2* gene. In addition, we also revealed the relationship with ACE2 and MMP-2 in HCFs utilized the technology of lentivirus to investigate the role of ACE2 regulation in cardiac cells.

The major results of this study are: (1) Ang II-AT1R and Ang 1-7-Mas axes were via ERK-MAPK signal pathway to regulate ACE2 expression, respectively; (2) the sequences of

5’-ATTTGGA-3’ within -516/-481 domain of the *ace2* promoter could regulate ACE2 expression and this sequences was the binding site of the transcription factor; (3) Ang II regulated ACE2 expression through AT1R to activate ERK-MAPK signal pathway to stimulate the -516/+20 domain within the *ace2* promoter; (4) the *ace2* promoter activity was no significant response when HCFs treated with TGF-β1 and TNF-α; (5) ACE2 overexpression enhanced MMP-2 activity and Ang II-AT1R-ERK1/2 axis decreased MMP-2 activity; (6) compared to Ang II, Ang 1-7 through Mas receptor inhibited the activation of ERK1/2, but no significant effect of MMP-2 activity; (7) the tumor necrosis factor-α-converting enzyme (TACE or ADAM17) expression could be regulated through Ang II-AT1R-ERK1/2 and Ang 1-7-Mas-ERK1/2 axes to alter ACE2 shedding; (8) the ACE2 knockout (KO) mice were re-established in our laboratory, including the mice with $ACE2^{+/-}$, $ACE2^{-/-}$ and $ACE2^{-/y}$ genotypes; (9) ACE2 deficiency enhances MMP-2 activity in heart tissue of ACE2 KO mice compared to WT mice.

This study reveals the ACE2 regulation by angiotensin peptides. Ang II and Ang 1-7 could enhance the ACE2 expression in HCFs and indicated that Ang II through the -516/+20 sequence domain within *ace2* promoter to regulate *ace2* gene expression. ACE2 overexpression enhances MMP-2 activity in HCFs, Ang II decrease the mRNA expression of ADAM17, MMP-2 activity and shed ACE2 activity in HCFs/ACE2. These results show ACE2 plays a protect role against the mechanism of heart remodeling by Ang II induction. Two mainly axes, Ang II-AT1R-ERK1/2 and Ang 1-7-Mas-ERK1/2, regulate the mRNA expression of ADAM17 and the activity of ACE2 and shed ACE2, and MMP-2 activity also could be regulated through Ang II-AT1R-ERK1/2 pathway.

Keywords: angiotensin-converting enzyme II, angiotensin II, angiotensin 1-7, cardiac cells, ACE2 knockout mice

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1. Research background and significance

1-1. Heart remodeling and cardiac fibrosis

Organ fibrosis is a complex process that is defined as excess ECM deposited and accumulated in the tissues including skin, heart, lung, kidney and vessels (Jinnin, 2010). Heart remodeling occurs in response to injury and an increase in wall stress plays a key role in the progressive deterioration of cardiac function that leads to heart failure (Pfeffer and Braunwald, 1990; Sharpe, 2000). Remodeling is characterized by cardiac hypertrophy and dilatation as well as conformational changes in the shape of the heart (**Fig. 1-1**). The remodeling process consists of a series of timed molecular events that include the inflammatory response to injury, proliferation of cardiac fibroblasts and differentiation to myofibroblasts, and formation of the fibrotic scar tissue in defected myocardium. Myocardium is comprised of a number of cell types such as cardiomyocytes, cardiofibroblasts, endothelial cells and smooth muscle cells, cardiac fibroblasts are also the highest cell population in the myocardium, accounting for about two-thirds of the cells (Camelliti et al., 2005). Cardiac fibroblasts are a critical element of myocardial repair that produce collagens, providing the tensile strength for cardiac tissue (Camelliti et al., 2005). The morphology of cardiac fibroblasts is the flat and spindle shaped cell in myocardium, it is the only cell population lack a basement membrane in the myocardium. Furthermore, cardiac fibroblasts has more function such as homeostasis and remodeling of the cardiac ECM, electrical activity, production of growth factors and cytokines, and intercellular signaling with cardiomyocytes, endothelial or smooth muscle cells to impact cellular angiogenesis, cell proliferation, cardiomyocyte hypertrophy or apoptosis, appear that play a key role during pathological remodeling of the heart by maintaining normal cardiac structure, function, biochemical and electrical features of the heart (Fan et al., 2012).

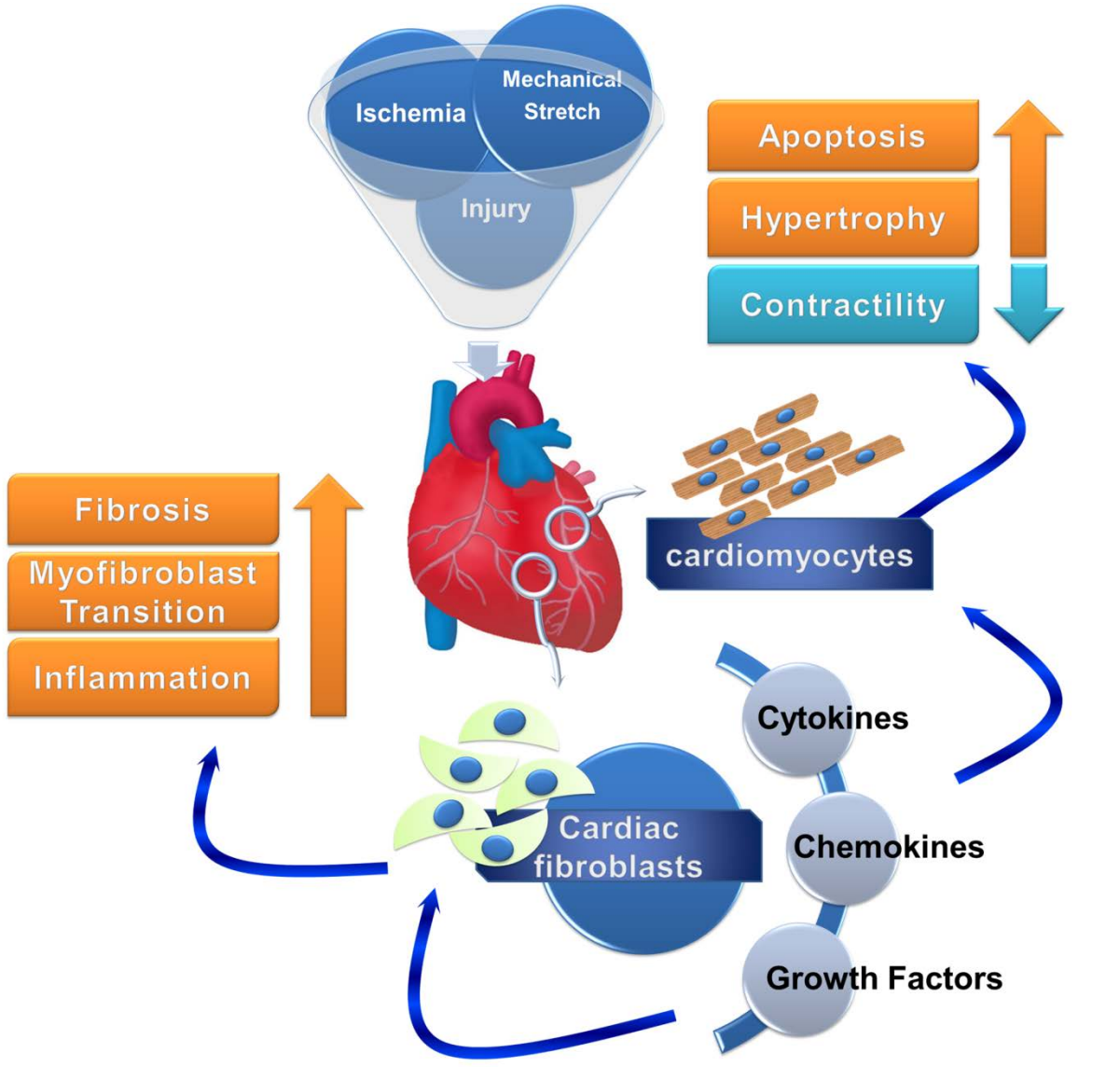


Fig. 1-1. Pathologic cardiac intercellular communication. In response to injury, mechanical stretch, and ischemia, the cardiac fibroblast undergoes a phenotypic transition to a myofibroblast, releasing a variety of growth factors, chemokines and cytokines that act both in an autocrine and paracrine fashion. Stimulation of cardiac fibroblast results in a positive feedback loop to further enhance their activation, collagen deposition, and cytokine release, resulting in fibrosis and chronic inflammation. Cytokine effects on cardiomyocytes leads to pathologic effects including hypertrophy, apoptosis, and impaired contractile responses. [Martin and Blaxall, 2012]

In response to cardiac injury or stress, cardiac fibroblasts have been triggered and differentiated into myofibroblasts, which have greater synthetic ability to produce ECM

proteins, chemokines and cytokines such as IL-1α, IL-1β, IL-6, IL-10, TGF-β1 and TNF-α (Petrov et al., 2002; Baum and Duffy, 2011). These ECM, chemokines and cytokines mediate migration and contractile of cardiac fibroblasts and myofibroblasts at the site of injury, and maintain the inflammatory response to injury (Eghbali, 1992; Baum and Duffy, 2011). In addition, the TGF-β1 that cardiac fibroblasts and myofibroblasts released accelerate differentiation of cardiac fibroblasts into myofibroblasts and increase collagen expression (Butt et al., 1995; Walker et al., 2004), the accumulation of fibrotic depositions that can interrupts the connection between the myocardial cells and blood vessels in the myocardium leading to overall impairment of cardiac function. These results reveal that cardiac fibroblasts and myofibroblasts have been demonstrated to play a key role in reparative fibrosis in the infarcted heart (Díez et al., 2002; Calderone et al., 2006).

Besides of ECM production, cardiac injury causes chronic cardiac fibroblasts and myofibroblasts activation to produce matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), leading to imbalanced collagen/MMP secretion. A number of growth factors, cytokines, and chemokines have been identified that can regulate production of MMPs and TIMPs to maintain ECM homeostasis by cardiac fibroblasts (Moore et al., 2012). In various MMPs that cardiac fibroblasts released, MMP-2 and MMP-9 have been shown to release the ECM-bound latent TGF-β1, thereby inducing collagen synthesis and further contribute to the adverse remodeling (Yu and Stamenkovic, 2000). In MI and unstable angina patients, MMP-2, MMP-9 and TIMP-1 in the serum were significantly elevated compared with healthy controls, suggesting that these MMPs and TIMP-1 and proinflammatory cytokines could play an important role in the pathophysiology of acute coronary syndrome (Tziakas et al., 2004). In addition, overexpression of MMP-2 led to severe myocardial fibrosis (Bergman et al., 2007) and MMP2-deficient mice showed the reduced myocardial hypertrophy and fibrosis (Matsusaka et al., 2006), while MMP-9 deficiency

partially improved myocardial hypertrophy and fibrosis following pressure overload (Heymans et al., 2005).

1-2. Renin angiotensin system

The renin-angiotensin system (RAS) is a classically hormonal system consists of endocrine, paracrine and intracrine system (Fyhrquist and Saijonmaa, 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 (Giani et al., 2012; Guimarães et al., 2012), blood vessels (Khakoo et al., 2008), and lungs (Imai et al., 2008; Shrikrishna et al., 2012; Wong et al., 2012). Furthermore, abnormal activation of the RAS is associated with the pathogenesis of cardiovascular and renal diseases such as hypertension (Jan Danser, 2012; Lo et al., 2012), myocardial infarction (Connelly et al., 2011; Burchil et al., 2012) and heart failure (Agarwal et al., 2012; Birner et al., 2012).

In a classical RAS, the glycoprotein angiotensinogen (AGT; Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile) released from the liver is degraded by the enzyme renin that originates in the kidney, generating the inactive angiotensin I (Ang I; Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) (Ferrario and Strawn, 2006). Subsequently, the dipeptide carboxypeptidase, angiotensin-converting enzyme (ACE) hydrolyzes the C-terminal dipeptide His-Leu of decapeptide Ang I to generate octapeptide angiotensin II (Ang II; Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) (Kokubu et al, 1979), and the C-terminal peptide Phe of Ang II is metabolised by the carboxypeptidase, ACE2 to produce the vasodilator, angiotensin (1-7) (Ang 1-7; Asp-Arg-Val-Tyr-Ile-His-Pro) (Donoghue et al., 2000; Turner and Hoope, 2002; Rice et al., 2004). Finally, the C-terminal dipeptide His-Pro of Ang 1-7 was been

hydrolyzed by ACE to obtain inactive peptide angiotensin (1-5) (Ang 1-5; Asp-Arg-Val-Tyr-Ile) (Ferreira et al., 2012).

Ang II is the main regulator of the RAS, has been revealed that stimulate inflammation, cell growth, apoptosis, fibrogenesis, and differentiation to cause tissue repair/remodeling (Ruiz-Ortega and Ortiz, 2005; Mehta and Griendling, 2007). It has a very short half-life and is quickly degraded to Ang III and Ang 1-7, a similar function peptide and an oppose function peptide, respectively (Sun, 2010). Ang II stimulates a wide variety of biological functions in the heart (Zheng et al., 2012), blood vessels (Wehlage et al., 2012), kidneys (Pinheiro et al., 2012), adipose tissue (Kalupahana and Moustaid-Moussa, 2012), pancreas (Lau and Leung, 2011; Chan and Leung, 2011) and brain (Chrissobolis et al., 2012; Vargas et al., 2012) mediated the specific receptors Ang II receptor type 1 (AT1R) and Ang II receptor type 2 (AT2R) (Skeggs et al., 1980; Corvol et al., 1995; Komatsu et al., 2009). The majority physiological and pathophysiological effects of Ang II are mediated by the AT1R. Compared with those effects through AT1R, Ang II binding to the AT2R generally causes opposite effects such as stimulated bradykinin and nitric oxide to induce a counterregulatory vasodilatation (Horiuchi et al., 1997; Touyz etal., 1999; Sun, 2010).

Ang 1-7 is an important regulator in the RAS activity acts as an endogenous inhibitor of Ang II. Ang 1-7 binding to the Mas receptor and triggering signal pathways to release of bradykinin (Isa et al., 2011; Gembardt et al., 2012), prostaglandins (Yousif et al., 2012; Costa et al., 2012), and endothelial nitric oxide (Ferrario et al., 2005; Shah et al., 2012) and induce opposite effects to those elicited by Ang II such as apoptosis (Santos et al., 2003; Wang et al., 2012), vasodilation (Savergnini et al., 2010; Pringle et al., 2011), anti-fibrosis (Grobe et al., 2007; Nadu et al., 2008; Ferreira et al., 2010), anti-hypertrophic (Santos et al., 2004; Mercure et al., 2008; Santiago et al., 2010) and anti-proliferative (McCollum et al., 2012; Ni et al., 2012). The schematic representation was present in **Fig. 1-2**.

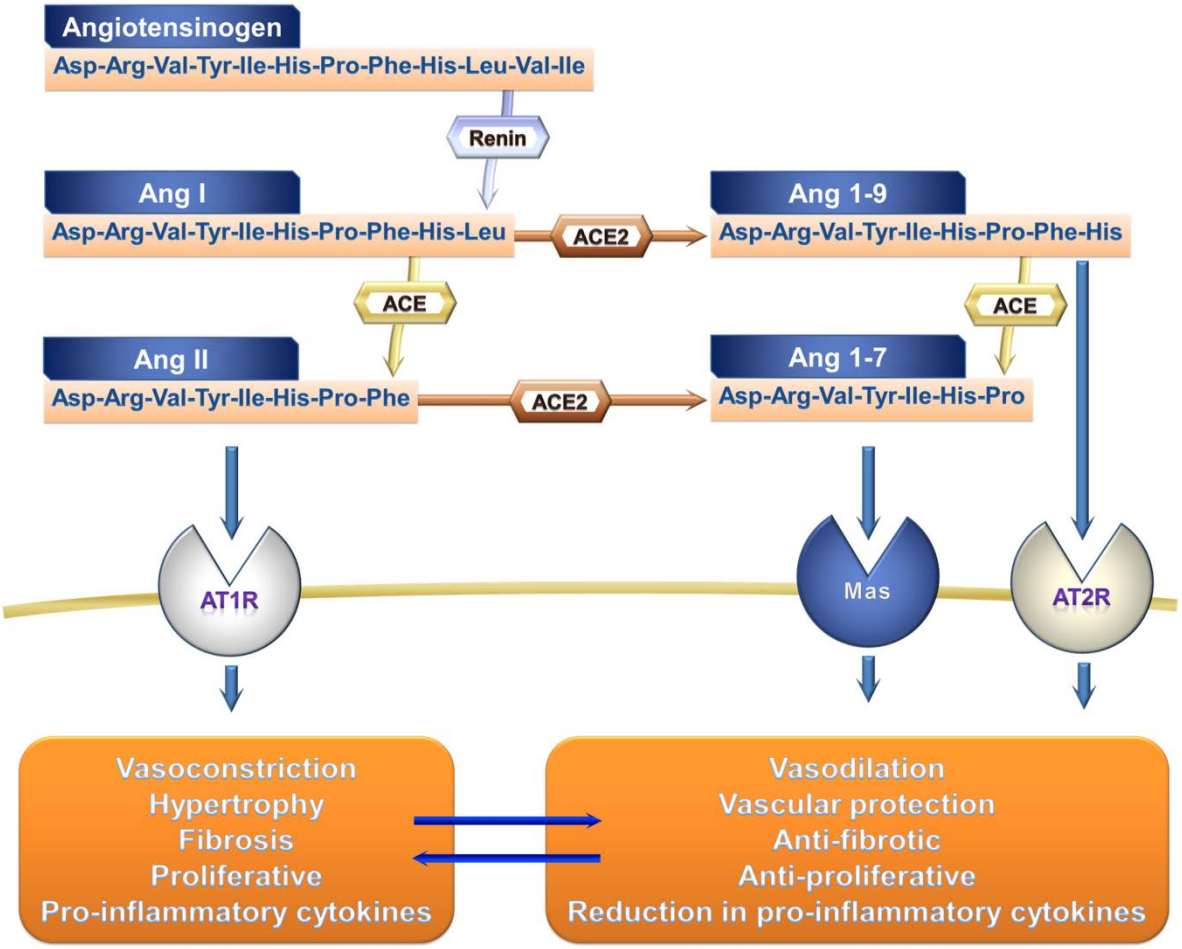


Fig. 1-2. Schematic representation of the renin-angiotensin system (RAS) cascade. The significant counterregulatory axes of the RAS are composed by ACE-Ang II-AT1R and ACE2-Ang 1-7-Mas. ACE, angiotensin-converting enzyme; Ang II, angiotensin II; AT1R, Ang II type 1 receptor; AT2R, Ang II type 2 receptor; ACE2, angiotensin-converting enzyme 2; Ang 1-7, angiotensin 1-7; Mas, Ang 1-7 receptor. [Wang et al., 2012]

1-3. Angiotensin converting enzyme II

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 and Hooper, 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 and Lazartigues, 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 2 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 carboxy-dipeptidase (peptidyldipeptidase), removing a C-terminal dipeptide (Clarke and Turner, 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). The molecular structure of ACE, ACE2 and collectrin was present in **Fig. 1-3**.

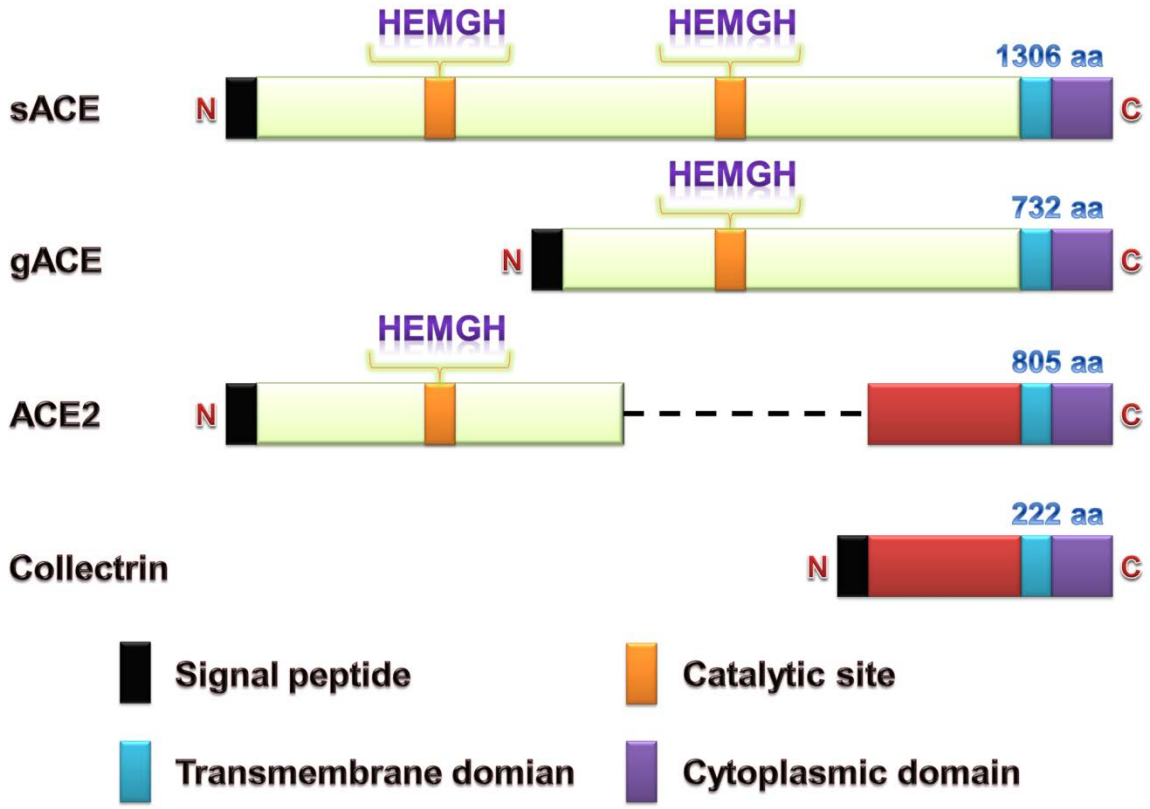


Fig. 1-3. Family of enzymes and proteins belonging to the ACE family of proteins. The schematic present the molecular structure of ACE, ACE2 and collectrin. HEMGH is a set of conserved amino acid residues critical for the activity of the zincbinding catalytic site. gACE, germinal angiotensin converting enzyme; sACE: somatic ACE; ACE2, angiotensin converting enzyme II. [Wang et al., 2012]

In RAS, the major substrate of ACE2 are Ang I and Ang II (Donoghue et al., 2000; Turner and Hooper, 2002; Rice et al., 2004), ACE2 efficiently cleaves a single residue phenylalanine from Ang II to generate Ang 1-7, with about 400-fold higher catalytic efficiency than the conversion of Ang I to Ang 1-9 by removing the C-terminal leucine residue (Vickers et al., 2002). Furthermore, ACE2 is also a multifunctional enzyme as a monocarboxypeptidase to degrade other biological substrates such as vasoactive bradykinin (1–8) (Donoghue et al., 2000), [des-Arg^{9}]-bradykinin (Vickers et al., 2002; Warner et al., 2004), Apelin-13 (Kalea and Batlle, 2010), Apelin-17 (Vickers et al., 2002; Oudit and

Penninger, 2011) and Apelin-36 (Kuba et al., 2007). ACE2 hydrolyzed apelin-13 and apelin-36 peptides with high catalytic efficiency (Vickers et al., 2002), and that apelin peptides were mediated APJ receptors to activate the G-protein coupled seven-transmembrane-domain receptor (GPCR) family predominantly expressed to regulate cardiovascular function and fluid homeostasis in the heart and lungs (Lee et al., 2000; Kleinz and Davenport, 2005; Pitkin et al., 2010).

Ang II-ACE2-Ang 1-7 and apelin-APJ are two important peptide systems with various and fundamental cardiovascular effects, and ACE2 may prevent or supress a variety of vascular and cardiac disorders (Kalea and Batlle, 2010; Oudit and Penninger, 2011; Wang et al., 2012). The major function of ACE2 is to counter-regulate ACE activity by reducing Ang II bioavailability and increasing the vasoprotective/antiproliferative peptide, Ang 1-7 formation. As a result, ACE2 plays a crucial role in maintaining the balance between the two axes ACE2-Ang 1-7-Mas and ACE-Ang II-AT1R of the RAS, chronic and sustained imbalance may lead to pathophysiology of the cardiovascular, renal, pulmonary and central nervous systems. ACE2 is effectively control fibrosis and structural remodeling in heart (Huentelman et al., 2005; Dong et al., 2012), lung (Shenoy et al., 2010; Rey-Parra et al., 2012) and liver (Paizis et al., 2005; Osterreicher et al., 2009), and extremely beneficial for pulmonary hypertension (Ferreira et al., 2009; Li et al., 2012).

1-4. ACE2 with heart diseases and heart remodeling

In the heart damage such as hypertension, myocardial infarction (MI) and chronic heart failure (CHF) (Cohn et al., 2000), cardiac myocytes were die and been replaced by fibroblasts and collagen to form fibrous tissue, these changes are referred to as “heart remodeling” (Opie et al., 2006). Ang II is a mainly factor of the RAS, activate cardiac fibroblast functions via

AT1R to increase the amount of ECM in the heart (Villarreal et al., 1993; Kim et al., 1995) to induce cardiomyocyte hypertrophy, cardiac remodeling and left ventricular dysfunction (Iwata et al., 2005; De et al., 2006; Whaley-Connell et al., 2007). In the failing heart, the local Ang II concentration is increased and related to the pathological signs of heart failure (Serneri et al., 2001). ACE2 is a significant regulator in RAS to degrade Ang II to suppress the heart dysfunction that Ang II stimulated (Bikkavilli et al., 2006).

In the heart, ACE2 had been certified a dramatic decrease with aging (Xie et al., 2006) and expressed in coronary microcirculation (Donoghue et al., 2000), macrophages (Burrell et al., 2005), myofibroblasts (Guy et al., 2008), cardiofibroblasts (Zhong et al., 2010), and cardiomyocytes (Gallagher et al., 2008). ACE2 polymorphism was also been reported that four single nucleotide polymorphisms were associated with higher left ventricular mass index, higher septal wall thickness and increased odds ratio for left ventricular hypertrophy (Lieb et al., 2006). In addition, ACE and ACE2 immunoreactivity were higher in cardiac tissue of patients with ischemic heart failure compared to normal subjects (Burrell et al., 2005), the ACE2 activity was also increased in failing human heart ventricles obtained from patients with either idiopathic dilated cardio-myopathy or primary pulmonary hypertension (Zisman et al., 2003). These results appear that ACE2 plays a significant role in cardiac diseases.

In MI rats, cardiac ACE2 mRNA expression and activity were decreased after 2-4 weeks ligation of the left coronary artery (Karram et al., 2005), increased at 4 weeks (Burrell et al., 2005) and down-regulation at 8 weeks post-MI (Ocaranza et al., 2006), these results suggest that regulation of cardiac ACE2 expression and activity varies depending on disease state and time point at which measurements are obtained.

ACE2 overexpression protects the heart from Ang II-induced hypertrophy (ez-Freire et al., 2006), MI (Der et al., 2008), fibrosis (Huentelman et al., 2005), and also improved left ventricular remodeling after experimental MI (D´ıez-Freire et al., 2006). In MI rat,

overexpression of ACE2 inhibited the development of early atherosclerotic lesions by suppressing the growth of vascular smooth muscle cells (Rentzsch et al., 2008) and ameliorated left ventricular remodeling and dysfunction (Zhao et al., 2010). In addition, cardiac fibroblasts infected with ACE2 lentivirus decreased the collagen production that acute hypoxic exposure induced (Grobe et al., 2007).

Loss of ACE2 worsened the pathological remodeling and progressive reduction in LV contractile function to cause systolic dysfunction and heart failure (Crackower et al., 2002; Yamamoto et al., 2006; Bodiga et al., 2011). In ACE2 null mice, transverse aortic constriction or exacerbated pressure overload stimulated cardiac Ang II and AT1R activation increased. This result reduced cardiac contractility and induced cardiac dysfunction and heart remodeling (Gurley et al., 2006; Yamamoto et al., 2006). Furthermore, the response of Ang II stimulated via hypoxia-activation was greater in cardiomyocytes isolated from $ACE2^{-/y}$ mice than isolated from WT mice (Keidar et al., 2007). In the absence of ACE2, $p47^{phox}$ NADPH oxidase subunit plays a critical role to activate myocardial NAPDH oxidase system to increase superoxide and activate MMPs leading to the severe adverse myocardial remodeling and dysfunction in ACE2 KO mice (Bodiga et al., 2011). Almost reports appear that ACE2 plays a protector in the heart and loss of ACE2 severely impaired cardiac function was probably related to the Ang II accumulation. The significant references were listed in **Appendix 8-1**.

1-5. Matrix metalloproteinases

The tissue fibrosis is caused by excessive accumulation of extracellular matrix (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

matrix metalloproteinases (MMPs) and MMPs inhibitors, tissue inhibitors of metalloproteinases (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.

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 and Werb 2001). MMPs comprise a large family of protease and share several similarities in terms of their structure, regulation and function (Nagase and Woessner, 1999; Bode and Maskos, 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 1-1**; Vargová et al., 2012).

Table 1-1. 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]

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. For example, membrane-type MMPs utilize transmembrane and cytosolic domains anchoring them to the cell membrane (Nagase and Woessner 1999; Stoker and Bode, 1995). The function of the propeptide domain is to maintain latency of the MMPs until a signal for activation is given. Catalytic domain contains two ions of zinc and at least one ion of calcium bound on various amino acid residues. The catalytic domain of all MMPs contains the consensus motif HExGHxxGxxH and three histidines that coordinate with the zinc ion in the active center. Second ion of zinc and calcium are bound in inactive part of catalytic domain with high affinity, but their role remains still unknown (van Wart and Hansen-Birkedal 1990; Nagase and Woessner 1999). The molecular structure of different type of MMPs was present in **Fig. 1-4**.

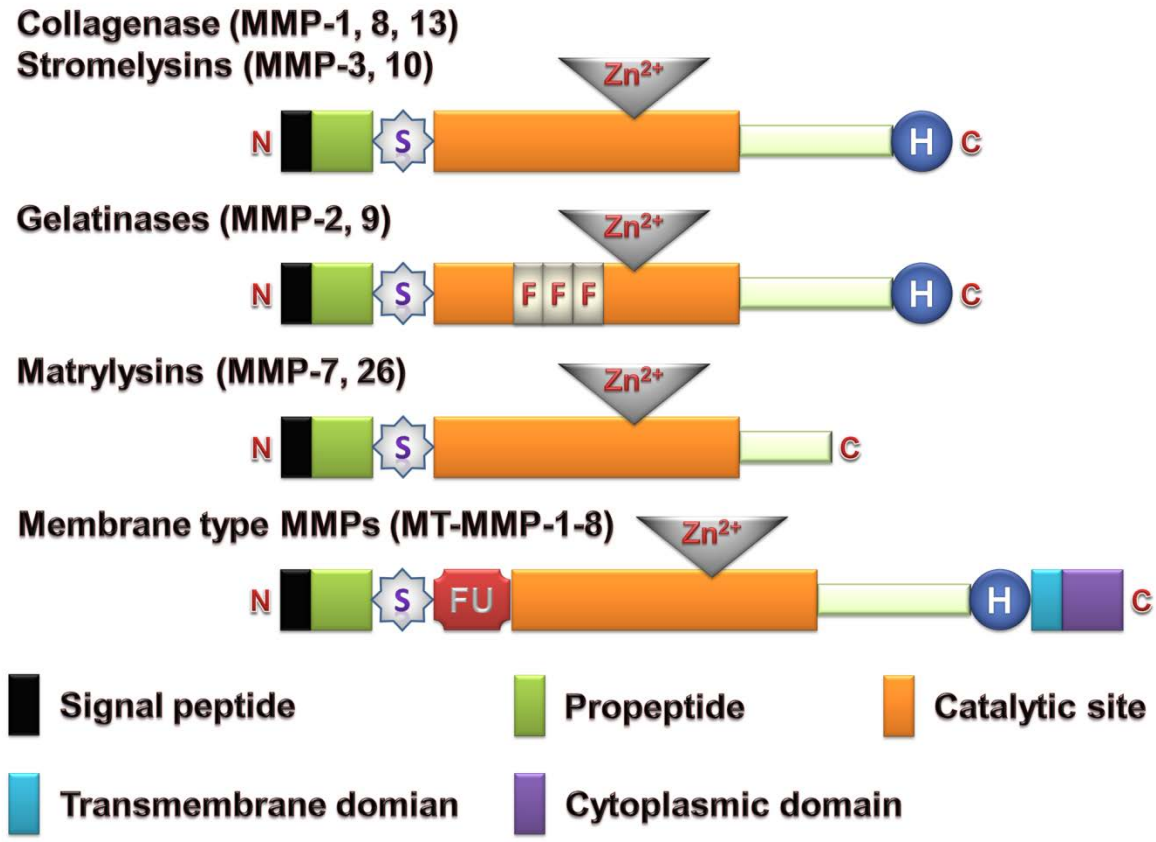


Fig. 1-4. Family of enzymes and proteins belonging to the MMPs family of proteins. The schematic present the mainly molecular structure of collagenases, stromelysins, gelatinases, matrylysins and membrane type MMPs. S, cysteine switch; FU, intracellular furin-like serine proteinases; F, collagen-binding type II repeats of fibronectin; Zn^{2+}, zinc-binding site; H, hemopexin domain. [Vargová et al., 2012]

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 and Werb, 2000). Four TIMPs, TIMP-1, -2, -3, and -4, have been identified (Cruz-Munoz and Khokha, 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.

1-6. 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. The significant references were listed in **Appendix 8-2**.

2. Research Approaches

Heart remodeling is causing of the process of heart repair and making heart structure change, which heart structure change forms heart remodeling and leads to most heart diseases such as cardiac hypertrophy, heart failure and atrial fibrillation. 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. Although ACE2 inhibit Ang II induced disadvantageous effect is well known, the regulation of ACE2 in the process of heart fibrosis is unclear.

The aim of this study is investigating the regulatory mechanism of ACE2 and *ace2* gene expression in cardiac cell, and the association between ACE2 and gelatinase (MMP-2 and MMP-9) in the process of heart remodeling. First, the condition and ACE2 expression in HCFs treated with angiotensin peptides, Ang II and Ang 1-7, have been detected. The signal pathway regulated ACE2 expression was also estimated. Second, the serial fragments of *ace2* promoter have constructed into pGL3-basic vector to drive luciferase expression. The luciferase expression has been detected and represents the *ace2* promoter activity. These constructs are the powerful tool to identify the regulatory element within *ace2* promoter and confirm the signal pathway that regulated ACE2 expression. Third, HCFs infect with the ACE2 lentivirus, TLC-ACE2, and shRNA to create ACE2 overexpression and knockout HCFs. The association between gelatinase, ACE2 overexpression and knockout has been indicated. The signal pathway and shed ACE2 activity that ACE2 overexpression and knockout HCFs treated with angiotensin peptides also estimate. The flowchart of research strategies was present in **Fig. 2-1**.

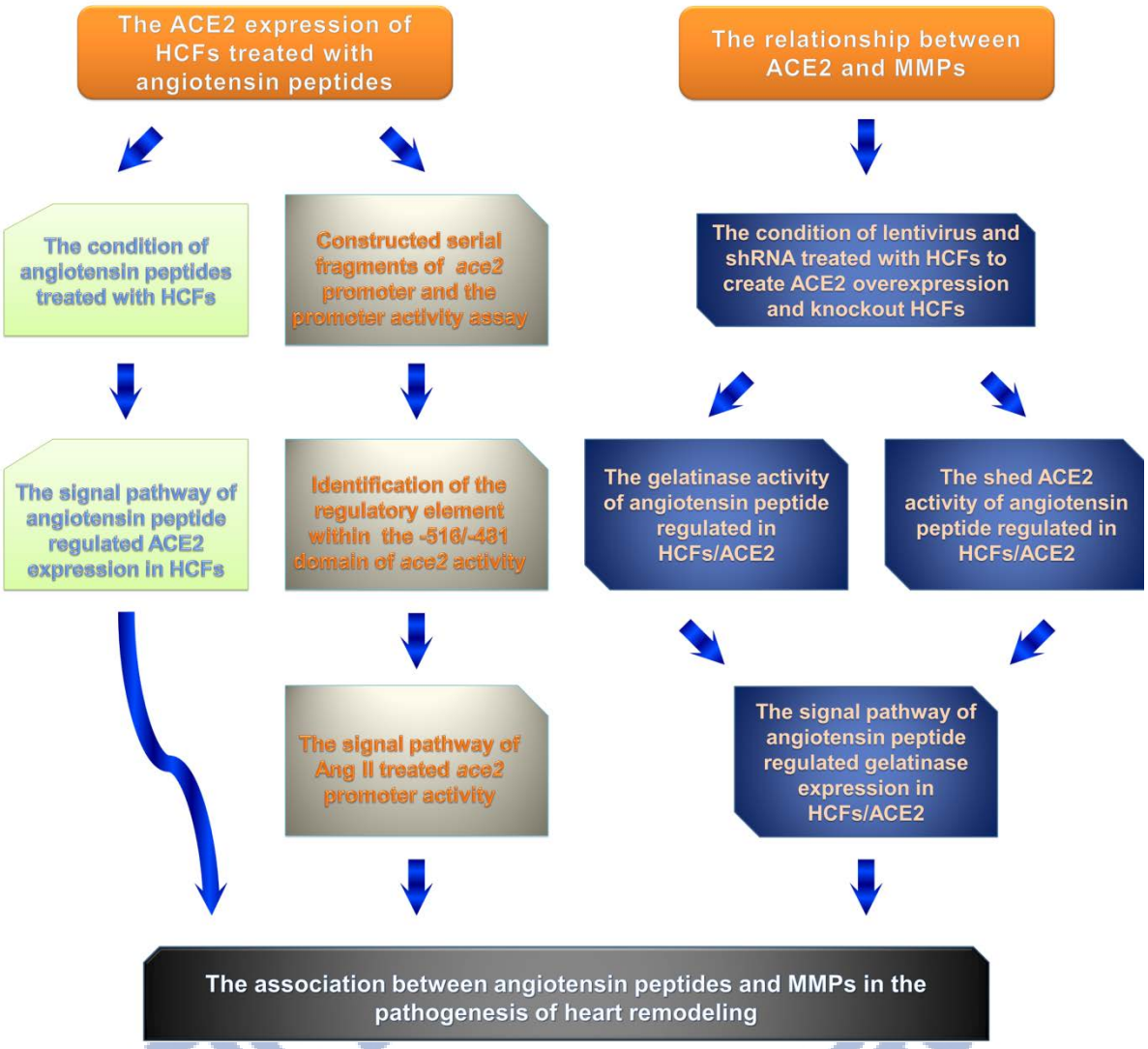


Fig. 2-1. The flowchart of research strategies. The purpose of this study is to identify the regulatory elements of ACE2 and the molecular mechanism of ACE2 regulation on heart remodeling using by angiotensin peptides, lentivirus and shRNA. First, we want to identify the regulation and the signal pathway of ACE2 expression by angiotensin peptides. Second, we using the constructs included serial fragments of ace2 promoter to identify the transcription factors which affect ACE2 promoter activity. Finally, we using lentivirus and shRNA to establish the ACE2 overexpression and knockout HCFs and estimate the association between ACE2 and MMPs.

3. Materials and Methods

3-1. Chemicals and reagents

The goat polyclonal IgG, glyceraldehyde-3-phosphate-dehydrogenase antibody (V-18; #sc20357), 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-phospho-MEK1/2 (#9121) and anti-phospho-ERK1/2 (#4370) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-ACE2 (#ab59351) and anti-AT1R (#ab9391) antibodies were purchased from Abcam (Cambridge, MA, USA). Alexa FluorTM 488-conjugated secondary antibody was obtained from Invitrogen (#A11034; Eugene, OR, USA). Ang II (#H1705), Ang 1-7 (#H1715) and Ang 1-7 Mas receptor blocker A779 (#H2888) were purchased from Bachem (Merseyside, United Kingdom). The Ang II type-1 receptor (AT1R) antagonist, valsartan (Val; #1708762), was obtained from U.S. Pharmacopeia (Rockville, MD, USA), and the mitogen-activated protein kinase kinase (MEK) inhibitor (PD98059; #P215), and poly-*L*-lysine (0.01% solution; #P4832) were obtained from Sigma-Aldrich (St. Louis, MO, USA). A commercial medium (#2301) was obtained from (ScienCell Research Laboratories, SanDiego, CA, USA) for cell culture. The luciferase reporter vectors, pGL3-Control Vector (#E1741) and pGL3-Basic Vector (which lacks a promoter; #E1751), and the Luciferase Assay System (#E1500) were purchased from Promega (Madison, WI, USA). The ACE2 overexpression lentivirus, TLC-hACE2, and ACE2 shRNA, TRCN-46697, were purchased from Vectorite Biomedica Inc. (Vectorite Biomedica, Taipei, Taiwan) and National RNAi Core Facility Platform (Institute of Molecular Biology/Genomic Research Center, Academia Sinica, Taipei, Taiwan), respectively. The ACE2 inhibitor, DX600 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. Cell culture and treatments

Primary human cardiac fibroblasts (HCFs; #6300; ScienCell Research Laboratories, San Diego, CA, USA) were cultured according to our published protocol (Lin et al., 2010). In brief, the HCFs were seeded in 100-mm Petri dishes (2 x 10^6 cells/dish) or 12-well plates (1 x 10^5 cells/well) that had been pre-coated with 0.01% poly-*L*-lysine (Sigma), and were cultured in Fibroblast Medium (#2301; ScienCell Research Laboratories), which included 2% fetal bovine serum (#0010; ScienCell Research Laboratories). The cells were incubated at 37°C in a humidified 5% CO_2 atmosphere and the culture medium as exchanged with fresh medium every 2 days. The cells at passages 3 or 4 were used in all experiments and were placed in serum-free medium for 24 h prior to their use in further experiments.

3-3. Human *ace2* constructs

Human genomic DNA was used as the template to obtain the upstream of *ace2* via polymerase chain reaction (PCR) and DNA cloning. A 2.1-kb DNA fragment was obtained by PCR with primers based on the sequence for human *ace2* (GenBank ID: AY217547). The sequences for the forward (Hace2-proF) and reverse (Hace2-proR) primers were 5′-AACCCTCGAGTTTCATTTAGGA-3′ and 5′-GAGCTAAGCTTCGTCCCCTGTG-3′, respectively; *Xho* I and *Hind* III sites are indicated by underlined nucleic acids in the forward and reverse primers, respectively.

The DNA fragment was then cloned into the pGL3-Basic luciferase reporter vector at the *Xho* I and *Hind* III sites to generate the –2069/+20 construct. A series of deleted DNA

fragments of the upstream region of *ace2* were obtained by PCR using the plasmid DNA of the –2069/+20 construct as template with the specific recognition primer pairs (**Table 3-1**). These deleted DNA fragments were also cloned into the pGL3-Basic vector at the *Xho* I and *Hind* III sites to generate a series of deletion constructs to test the promoter activity of *ace2*. All of the constructs generated in this study were checked by restriction-mapping and sequencing to confirm their authenticity.

Table 3-1. Sequences of the primer pairs used for human *ace2* promoter constructs

Constructs	Forward/Reverse primers (5'→3')	Promoter region	Ampliconlength (bp)
(-2069/+20)	F- AACCCTCGAGTTTCATTTAGGA R- GAGCTAAGCTTCGTCCCCTGTG	-2069 ~ +20	2089
(-1493/+20)	F- GTTTCTCGAGATGCTCAAATGA R- GAGCTAAGCTTCGTCCCCTGTG	-1493 ~ +20	1513
(-1110/+20)	F- TGACCTCGAGTGAGTTTTGAAT R- GAGCTAAGCTTCGTCCCCTGTG	-1110 ~ +20	1130
(-916/+20)	F- TAAAGACTCGAGCAAAGTCATG R- GAGCTAAGCTTCGTCCCCTGTG	-916 ~ +20	936
(-786/+20)	F- AACCCTCGAGTTTCATTTAGGA R- GAGCTAAGCTTCGTCCCCTGTG	-786~ +20	806
(-664/+20)	F- GTTTCTCGAGATGCTCAAATGA R- GAGCTAAGCTTCGTCCCCTGTG	-664 ~ +20	684
(-627/+20)	F- CTTGCAGTGACTCGAGATCG R- GAGCTAAGCTTCGTCCCCTGTG	-627 ~ +20	647
(-516/+20)	F- TAAAGACTCGAGCAAAGTCATG R- GAGCTAAGCTTCGTCCCCTGTG	-516 ~ +20	536
(-481/+20)	F- GTTGCCCAACTCGAGAGTTTC R- GAGCTAAGCTTCGTCCCCTGTG	-481~ +20	501
(-355/+20)	F- AGTTCTAGACCTCGAGGGTCAC R- GAGCTAAGCTTCGTCCCCTGTG	-355~ +20	375
(-253/+20)	F- AAGTGACTCGAGAGGTAAGG R- GAGCTAAGCTTCGTCCCCTGTG	-253~ +20	273
(-161/+20)	F-CTGTCCTCGAGAGGATGAAC R- GAGCTAAGCTTCGTCCCCTGTG	-161 ~ +20	181

The recognition sequences of restriction enzymes, CTCGAG for *Xho* I in the forward primers and AAGCTT for *Hind* III in the reverse primers, were shown in blue letters.

The promoter region was defined according to the position relative to the transcription start site (+1) in ACE2 mRNA sequence (GenBankno. AF_291820).

3-4. Transient transfection

Transient transfection was carried out according to our published protocol (Sun et al., 2005) with some minor modifications. Briefly, 2 x 10^5 HCFs were seeded in a 6-well culture plate one day before DNA transfection, and grown to approximately 70% confluence. The cells were washed with GIBCO Dulbecco's phosphate-buffered saline (D-PBS) (Invitrogen, Carlsbad, CA, USA) to remove the remaining medium, then 400 µl of cell growth medium containing 4 µg of plasmid DNA mixed with 6 µl of TurboFect Transfection Reagent (Thermo Fisher Scientific, Waltham, MA, USA) was added gently. The DNA-transfected cells were then incubated at 37°C and under 5% CO_2 in an incubator. After 24 h the cells were collected and lysed, and assayed for luciferase activity.

3-5. Lentivirus infection

The cloning of human ACE2 in lentiviral vector and production of lenti-hACE2 viral particles was according to Huentelman et al. with slight modifications (Huentelman et al., 2005). Homo sapiens angiotensin I converting enzyme (peptidyl-dipeptidase A) 2, mRNA (cDNA clone MGC:57146 IMAGE:5297380) was used as a template with primer pairs: Spe-ACE2-F, 5' - GAACCCACTGCTTACTGGCTTATCG - 3'; and Spe-ACE2-R, 5' - GCTGGCAACTAGAAGGCACAGTCG - 3' to carry out PCR amplification, the PCR production was cloned into pCR II-TOPO vector (Invitrogen, Carlsbad, CA) to obtain pACE2-TOPO. The complementary DNA encoding human ACE2 in pACE2-TOPO was subcloned into VBI-TLC vector using the SpeI sites to obtain TLC-ACE2 clone and produced lenti-hACE2 viral particles by Vectorite Biomedica Inc.

A pLKO.1-shRNA plasmid encoding a short hairpin RNA (shRNA) with sequences targeting human ACE2 was introduced into HEK293T cells with lentiviral packaging vectors

pMD.G and pCMV 8.91 by National RNAi Core Facility, Taiwan. The RNAi Consortium Numbers (TRCNs) and sequences of this shACE2 are 5'-GCCCTTATTTACCTGGCTGAA-3' (TRCN0000046693); 5'-GCCCAAATGTATCCACTACAA-3' (TRCN0000046694); 5'-GCAAAGTTGATGAATGCCTAT-3' (TRCN0000046695); 5'-GCTGGACAGAAACTGTTCAAT-3' (TRCN0000046696) and 5'-GCCGAAGACCTGTTCTATCAA-3' (TRCN0000046697).

The ACE2 overexpression and knockdown experiments were performed as previously described, with optimization (Lee et al., 2008; Lin et al., 2012), HCFs were infected with the collected viruses 24 h in the presence of polybrene (8 μg/ml) at different MOI. After virus infection, cells were cultured in fresh growth medium for 24 h prior to their use in further experiments.

3-6. 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-Ace2^{tm1Lex}/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.

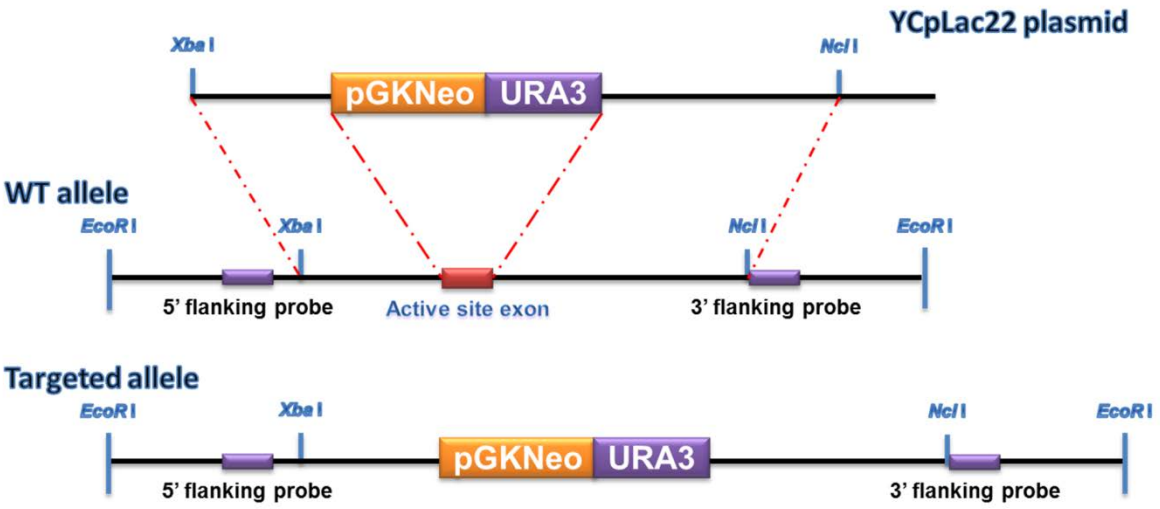


Fig. 3-1. Strategy for producing targeted disruption of the *ace2* gene. Strategy for producing targeted disruption of the *ace2* gene. In the targeting vector, the exon containing nucleotides +1069 to +1299 encoding the active site of the ACE2 enzyme (including the Zn-binding signature motif, HEMGH)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

3-7. Protein extraction

The protein extraction was performed as our previous report (Sun et al, 2008). HCFs washed twice by D-PBS and lysis by PRO-PREP^{TM} Protein Extraction Solution (iNtRON Biotechnology, Inc., Kyungki-Do, Korea) on ice for 10 min incubation. After incubation, the cell lysis solution was transfer to 1.5 ml eppendrof tube and sonicated 3 times for 5 s with interval 10 s on ice by ultrasonic processor (UP950A, Hansor, Taichung, Taiwan). Finally the protein extraction was isolated by 13,000 rpm centrifugation at 4°C for 5 min (Biofuge primo

R, Sorvall, Osterode, Germany). 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.

3-8. Luciferase reporter assay

The luciferase assay was performed according to the manufacturer's instructions of Luciferase Assay System (Promega). The DNA-transfected HCFs were rinsed twice with D-PBS (Invitrogen) and lysed with luciferase cell culture lysis reagent included in the kit (CCLR; Promega). Cell lysates were centrifuged at 4°C for 2 min, and the supernatants were removed and mixed with the luciferase assay reagent (Promega). Luciferase activity was measured using a single tube luminometer (Lumat LB9507, Brethold Technologies, Bad Wildbad, Germany).

3-9. Nuclear extraction

Nuclear protein was extracted using a Nuclear Extraction kit (P/N 13938; Panomics, Redwood City, CA, USA) according to the manufacturer's protocol. HCFs (1 x 10^7 cells) were collected and washed twice with D-PBS, then centrifuged at 500 x *g* for 5 min. The cells were resuspended in 1 ml of Working Reagent and the tubes were shaken at 200 rpm on ice for 10 min. The sample was centrifuged at 14,000 x *g* for 3 min at 4°C and the supernatant, consisting of cytoplasmic extract, was then removed. Forty µl of Buffer B Working Reagent was added to each pellet and then the sample was vortexed for 10 s. The mixture was incubated on ice for 60 min with gentle agitation by hand every 20 min. The nuclear extract was obtained as supernatant after centrifugation at 14,000 x *g* for 5 min at 4°C.

3-10. Electrophoretic mobility shift assay (EMSA)

EMSA was performed using an EMSA Gel Shift kit (P/N 13009; Panomics). The double-stranded oligonucleotides comprising the sequence –516/–481 of *ace2* were labeled with biotin. Nuclear extracts of HCFs were incubated in the Reaction Buffer for 5 min, before adding the biotin-labeled DNA probe. After incubating for 30 min at 15°C, the mixture was separated by electrophoresis in a 6% polyacrylamide gel operating at 120 V, with 0.5 x TBE as the running buffer, for 1 h. In competition assays, 66-fold molar excess of unlabeled double-stranded oligonucleotide was added to the binding reaction 5 min before the labeled oligonucleotides. After electrophoresis, the DNA-protein complexes were transferred to positively charged nylon membranes (BrightStar®-Plus; Ambion, Austin, TX, USA) by semi-dry electroblotting (Hoefer™; Amersham Biosciences, Uppsala, Sweden) and immobilized using a Spectroline Spectrolinker UV Crosslinker (Spectronics Corporation, New York, NY, USA). The membrane was blocked in 1 x Blocking Buffer, incubated with streptavidin-horseradish peroxidase for 15 min and incubated in 1 x Detection Buffer for 5 min. Working Substrate Solution (200 µl Solution I, 200 µl Solution II, and 1.6 ml Solution III) was added to develop the results (All of the aforementioned solutions were included in the Panomics Gel Shift kit). The developed bands were visualized by exposing the membrane to X-Ray film (Super Rx Medical X-Ray Film; Fujifilm, Kanagawa, Japan).

3-11. RNA isolation and quantification

Total cellular 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 5 x 10^6 cells. The

mixture was vigorously agitated for 30 s 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°C 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. The integrity and relative amounts of RNA were evaluated using ultraviolet visualization of ethidium bromide-stained RNA.

3-12. 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), 1U/μl of RNase inhibitor (TOYOBO), and 5 U/μl of ReverTra AceTM reverse transcriptase (TOYOBO). After incubation for 60 min at 42°C, the mixture was incubated for 5 min at 95 °C to denature the products. PCR primers for RT-PCR analysis were shown in **Table 3-2**. 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°C followed by 18-36 cycles of denaturation (94°C for 30 s), annealing (55°C for 30 s), and elongation (72°C for 45 s). 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 System™; Eastman Kodak, Rochester, NY, USA). Band intensity was quantified using densitometric analysis by ImageJ™. 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 and Schmittgen, 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), ADAM17 (GenBank ID: NM_003183.4) and MAPK1 (GenBank ID: NM_002745.4) were: ACE2 forward, hACE2-F, 5′-CATTGGAGCAAGTGTTGGATCTT-3′, and, ACE2 reverse, hACE2-R, 5′-GAGCTAATGCATGCCATTCTCA-3′; ADAM17 forward, hADAM17-F, 5′-CTTTCAGCATTCTTGTCCATTGTGTG-3′, and, ADAM17 reverse, hADAM17-R, 5′-GCTCAGCATTTCGACGTTACTGGG-3′; MAPK1 forward, hMAPK1-F, 5′-CAAGTCCATTGATATTTGGTCTGTAGGC-3′, and, MAPK1 reverse, hMAPK1-R, 5′-CAAGAATACCCAAAATGAGGTTCAGC-3′; GAPDH forward, hGAPDH-F, 5′-ACAGTCAGCCGCATCTTCTT-3′, and, GAPDH reverse, hGAPDH-R, 5′-GTTAAAAGCAGCCCTGGTGA-3′.

Table 3-2. Information of the PCR primers and condition performed in this study

Gene	GenBank accession no.	Sequence of forward primer (5'→3') Sequence of reverse primer (5'→3')	PCR condition (cycle number)	Size of PCR product (bp)
ACE2	NM021804	ACGACAATGAAATGTACCTGTTCCG TCCGATCTCTGATCCCAGTGAAG	94°C, 30 s → 55°C, 30 s → 72°C, 45 s (36 cycle)	399
AT1R	NM004835	CCAAAAGCCAAATCCCACTCAAACC TCTGACATTGTTCTTCGAGCAGCC	94°C, 30 s → 55°C, 30 s → 72°C, 45 s (26 cycle)	362
GAPDH	AF261085	GGTGATGCTGGTGCTGAGTA TTCAGCTCTGGGATGACCTT	94°C, 30 s → 55°C, 30 s → 72°C, 45 s (18 cycle)	413

ACE2, angiotensin converting enzyme II; AT1R, angiotensin II type 1 receptor;

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

3-13. Immunocytofluorescence assay

HCF cells were grown overnight on 0.01% poly-*L*-lysine-coated coverslips (1.2 mm diameter) and treated with Ang II or Ang 1-7 for 24 h. The cells were washed in PBS, fixed with 4% formaldehyde for 15 min, and permeabilized with 0.5% saponin (Sigma-Aldrich) for 15 min. Non-specific binding sites were blocked with 1% BSA for 30 min. The cells were then incubated with anti-ACE2 (1:100 dilu-tion) at 37°C for 1 h, followed by Alexa Fluor™ 488-conjugated secondary antibody (1:200 dilution) at 37°C for 1 h in a humidified chamber. The cells were also counterstained with DAPI (1:10,000 dilution) at 37°C for 5 min in a humidified chamber. After washing in PBS, the coverslips were mounted in DakoCytomation Fluorescent Mounting Medium (DakoCytomation, Denmark A/S, Denmark), and fluorescent signals were observed using the FluoView™ FV500 Confocal Microscope (Olympus, Tokyo, Japan).

3-14. Western blotting

The western blot for ACE2, ERK1/2 and GAPDH was carried out as our previous report (Kuan et al., 2011). Aliquots containing 30 μg protein were electrophoresed on 8% SDS-PAGE gels and then transferred electrophoretically to polyvinylidene fluoride membranes (Immobilon-P™; Millipore, Bedford, MA, USA) by semi-dry electro-blotting (Hoefer™). 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 ACE2, ERK1/2 and for GAPDH. 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 amount of ACE2 and ERK1/2 were expressed relative to the amount of GAPDH (as the internal standard) in each sample.

3-15. ACE2 activity assay

ACE2 activities was assayed with the fluorogenic substrates 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 micro-quartz cuvette with 20 μl cell protein, 50 μM fluorogenic substrate and protease inhibitor cocktail (1:200; Sigma-Aldrich) in a final volume of 100 μl in ACE2 assay buffer (75 mM Tris-HCl, 1 M NaCl, 5 mM $ZnCl_2$, pH 6.5). 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 presence of 1 μM captopril (Sigma-Aldrich), a specific ACE inhibitor, to avoid the effect of ACE in ACE2 activity assay. Parallel samples were incubated with the above-mentioned reaction mixture in the presence of 1 μM DX600 (AnaSpec), a specific ACE2 inhibitor for determining specific ACE2 activity.

3-16. Gelatin zymography

The MMP-2 and -9 activities were detected by gelatin zymography utilized gelatin-containing gels as our previous report (Chang et al., 2011). Briefly, 30 μg of cell homogenate was mixed with 6x zymography sample buffer (0.125 M Tris-HCl, pH = 6.8, 50% [v/v] glycerol, 4% [w/v] SDS, 0.005% bromophenol blue) and incubated for 10 min at room temperature, and then loaded into each lane of an 8% sodium dodecyl sulfate

polyacrylamide gel (SDS-PAGE) containing 1 mg/ml gelatin (Sigma). Following electrophoresis, the gel was washed twice for 30 min in zymogram renaturing buffer (2.5% Triton X-100) with gentle agitation at room temperature to remove SDS, then incubated overnight at 37°C in reaction buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 5 mM $CaCl_2$ and 0.02% Brij35). After Coomassie brilliant blue staining, gelatinase activities were identified as clear zones against a blue background.

3-17. Statistics

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 soft-ware (SPSS, Chicago, IL, USA).

4. Results

4-1. Ang II up-regulates ACE2 expression in HCF cells via the AT1R and the ERK-MAPK pathway

In HCF cells treated with Ang II, the amount of ACE2 mRNA was markedly increased ($p < 0.01$) in a concentration-dependent manner (**Fig. 4-1A**). When the HCF cells treated with 1 μM of Ang II, the relative ACE2 mRNA expression was approximately three fold higher than that of control ($p < 0.01$). Moreover, the increase in ACE2 mRNA was dependent on the duration of treatment (**Fig. 4-1B**).

The effect of Ang II on ACE2 expression in HCF cells was further demonstrated by blocking the downstream signaling pathway of Ang II by AT1R and ERK–MAPK cascades. HCF cells were treated with the AT1R inhibitor Val (1 μM) or MEK inhibitor PD98059 (10 μM) prior to treatment with Ang II. The cells pretreated with Val were used to confirm the receptor-specific effect of Ang II. The result shows that ACE2 mRNA increased by an AT1R-dependent effect that was abolished by Val pretreatment (Fig. 4-2A). Ang II-induced ACE2 expression was also abolished when HCF cells were treated with PD98059 prior to treatment with Ang II (**Fig. 4-2A**). The expression of signaling molecules in the ERK–MAPK pathway were explored by western blot (**Fig. 4-2B**). Following Ang II treatment in HCF cells, the results confirmed the up-regulation of ACE2, p-MEK1/2, and p-ERK1/2 protein expression (**Fig. 4-2C, D, E**). When HCF cells were pretreated with AT1R inhibitor Val or MEK inhibitor PD98059, the increases in ACE2, p-MEK1/2, and p-ERK1/2 expression were abolished (**Fig. 4-2C, D, E**). Control experiments confirmed that Val or PD98059 alone did not alter cardiac ACE2 mRNA or protein expression (data not shown).

ACE2 distribution and expression in Ang II-treated HCF cells were visualized and analyzed using immunocytofluorescence (**Fig. 4-3A**). ACE2 expression was significantly increased by Ang II treatment and was restored to the untreated control value by pretreatment

with the AT1R inhibitor Val (**Fig. 4-3B**). These immunocytofluorescent results were consistent with the changes in ACE2 expression detected by RT-PCR and western blotting (**Fig. 4-2**).

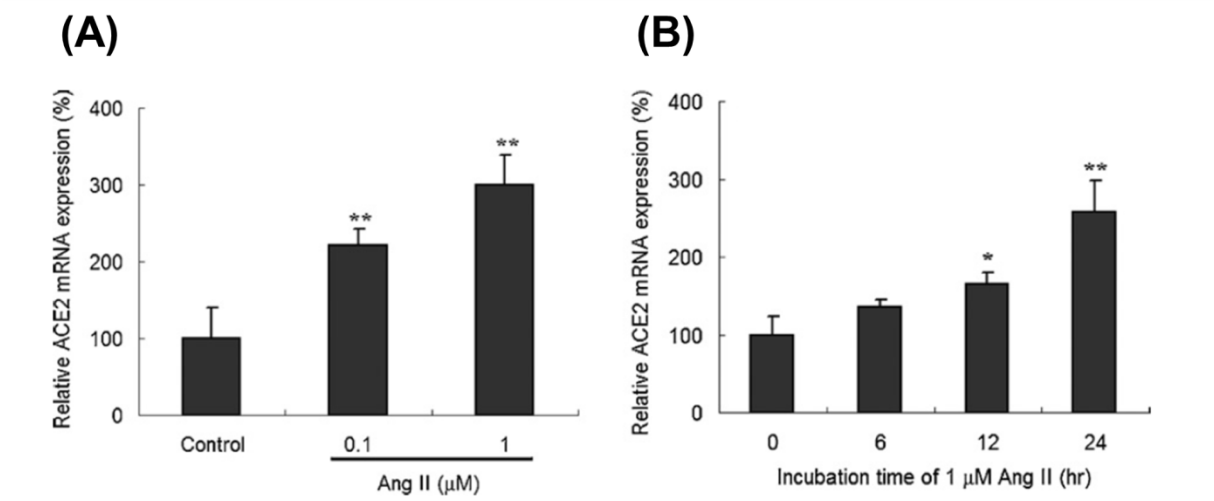


Fig. 4-1. Expression of ACE2 mRNA in Ang II-treated human cardiac fibroblast (HCF) cells. Treatment with Ang II produced a dose-dependent (A) and time-dependent (B) effect on ACE2 mRNA expression. ACE2 expression was normalized against GAPDH, and relative mRNA expression of ACE2 was calculated using the control group as 100%. Values are expressed as mean ± SD (n = 3). *, $p < 0.05$ vs. control; **, $p < 0.01$ vs. control.

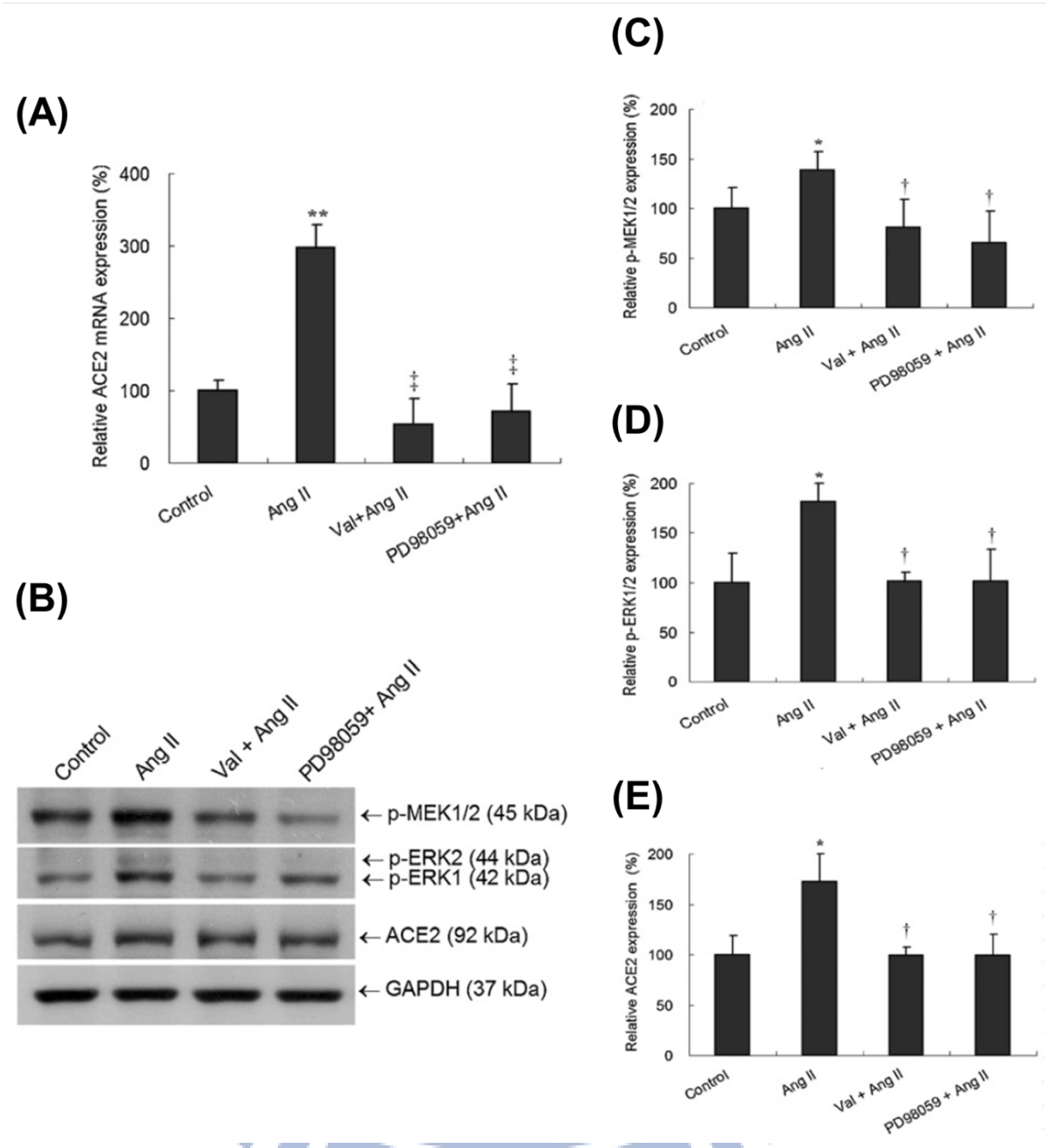


Fig. 4-2. Role of the ERK–MAPK signaling pathway of AT1R in Ang II-mediated ACE2 up-regulation. The effect of Ang II on ACE2 mRNA expression was determined in HCF cells pretreated with the AT1R blocker Val and the MEK1/2 inhibitor PD98059 to confirm the ACE2 expression being associated with Ang II treatment (A). The protein expression of phosphorylated MEK1/2 (p-MEK1/2), phosphorylated ERK1/2 (p-ERK1/2) and ACE2 was examined by western blotting (B). p-MEK1/2 (C), p-ERK1/2 (D) and ACE2 (E) expression was normalized using GAPDH expression, and the relative expression of p-MEK1/2, p-ERK1/2 and ACE2 was calculated using the control group as 100%. Values are expressed as mean ± SD (n = 3). *, $p < 0.05$ vs. control; **, $p < 0.01$ vs. control; †, $p < 0.05$ vs. Ang II treatment; ‡, $p < 0.01$ vs. Ang II treatment.

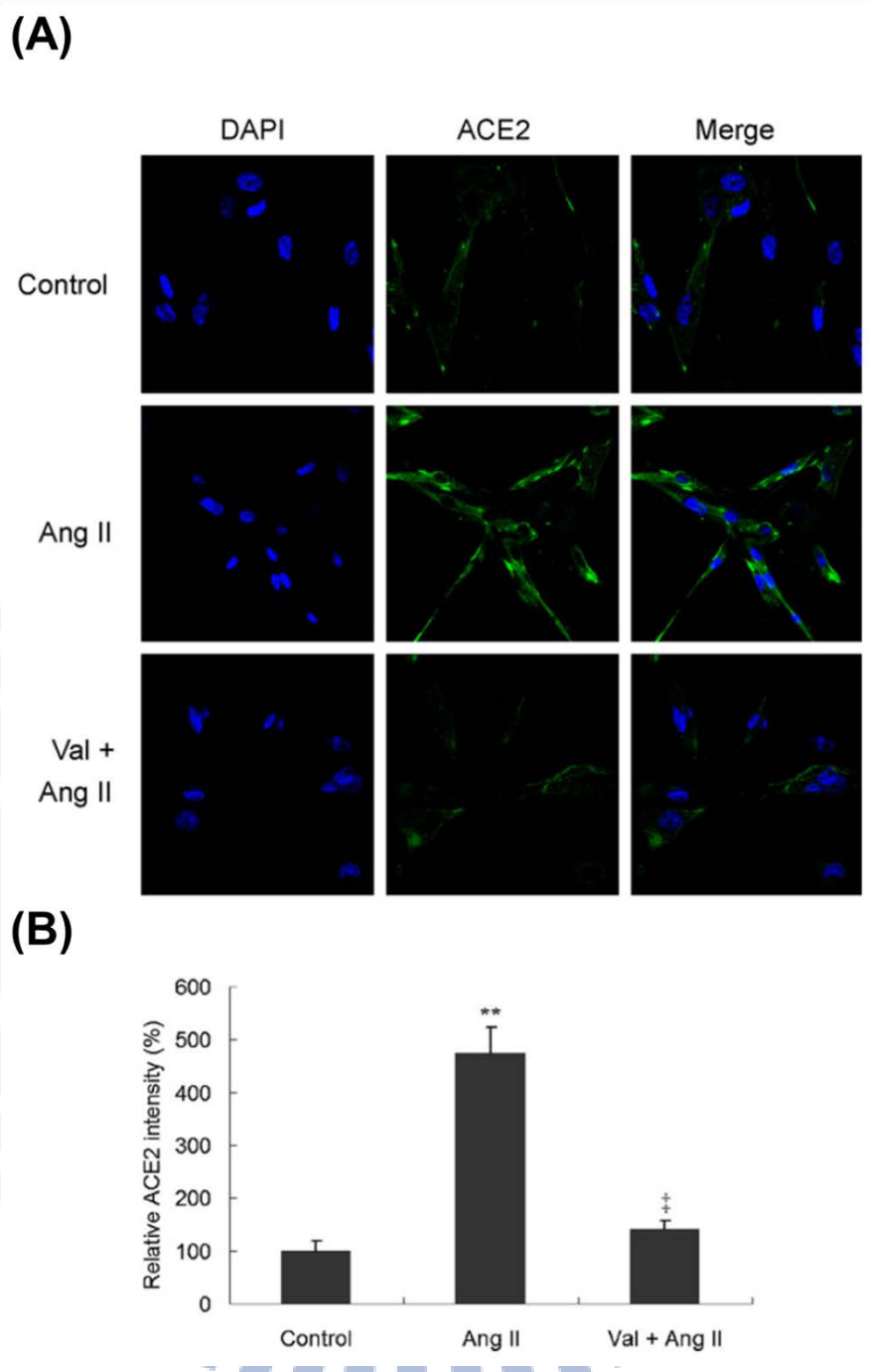


Fig. 4-3. Distribution and expression of ACE2 in Ang II-treated HCF cells. HCF cells grown on a coverslip were treated with Ang II with and without prior treatment with the AT1R inhibitor Val for 24 h. Treated cells were washed, fixed, and immunostained for ACE2 (green), and nuclei were counterstained with DAPI (blue). The localization of ACE2 protein was visualized by confocal microscopy (A) and the relative ACE2 fluorescence intensity was calculated using the control group as 100% (B). Values are expressed as mean ± SD (n = 3). **, $p < 0.01$ vs. control; ‡, $p < 0.01$ vs. Ang II treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

4-2. Ang 1-7 up-regulates ACE2 expression via the Mas receptor

The effect of Ang 1-7 on cardiac ACE2 expression was also evaluated in HCF cells. Ang 1-7 treatment (1 μM) significantly increased ACE2 mRNA and protein expression (**Fig. 4-4A, B**). To test the involvement of the Ang 1-7 Mas receptor, HCF cells were treated with the Mas receptor blocker A779 (1 μM) prior to Ang 1-7 treatment. The cells pretreated with A779 were used to confirm the receptor-specific effect of Ang 1-7. The up-regulation of ACE2 mRNA and protein expression by Ang 1-7 was depressed by A779 pretreatment. These results suggest that Ang 1-7 up-regulation of ACE2 expression is mediated via the Mas receptor. Treatment of HCF cells with A779 alone did not change ACE2 mRNA or protein levels (data not shown). The effects of Ang 1-7 on ACE2 expression were confirmed by immunocytofluorescence (**Fig. 4-5A**). ACE2 protein was markedly increased when HCF cells were treated with Ang 1-7, and cells pretreated with A779 showed significantly reduced ACE2 expression (**Fig. 4-5B**).

The regulation of p-ERK1/2 protein expression was analyzed in HCF cells treated with Ang 1-7 (**Fig. 4-6A**). Ang 1-7 caused an up-regulation of p-ERK1/2 that was abolished by the inhibitors A779 and PD98059 pretreatment. However, pretreatment with the AT1R blocker did not influence the Ang 1-7-mediated effect on ACE2 expression (**Fig. 4-6B**).

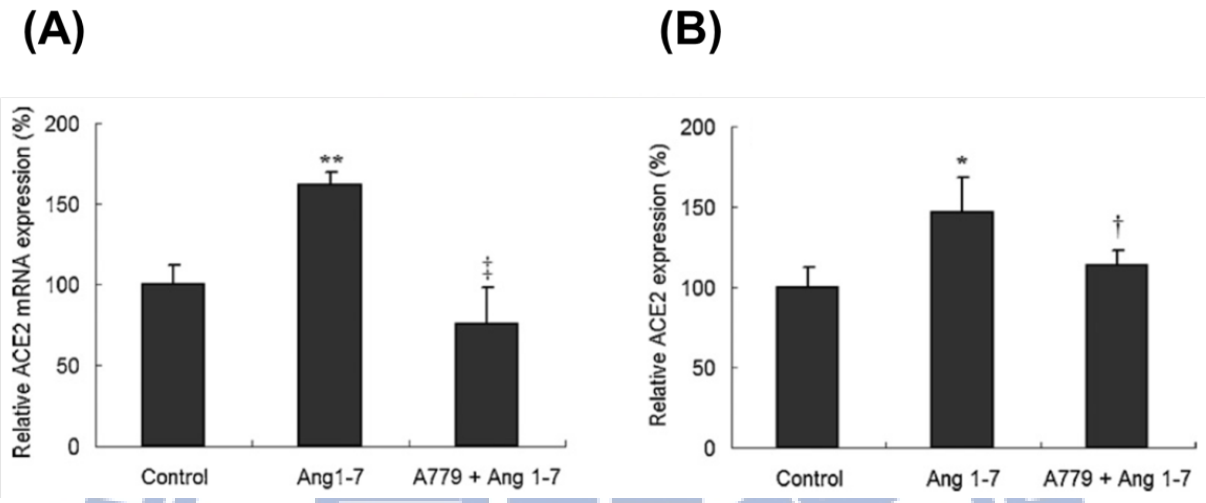


Fig. 4-4. Expression of ACE2 in HCF cells following Ang 1-7 treatment. The effect of Ang 1-7 on ACE2 mRNA and protein expression was examined by RT-PCR (A) and western blotting (B), respectively. Up-regulation of ACE2 was further confirmed using cells pretreated with the Ang 1-7 Mas receptor blocker A779. ACE2 expression was normalized using GAPDH expression, and the relative expression of ACE2 mRNA was calculated using the control group as 100%. Values are expressed as mean ± SD (n = 3). *, $p < 0.05$ vs. control; **, $p < 0.01$ vs. control; †, $p < 0.05$ vs. Ang 1-7 treatment; ‡, $p < 0.01$ vs. Ang 1-7 treatment.

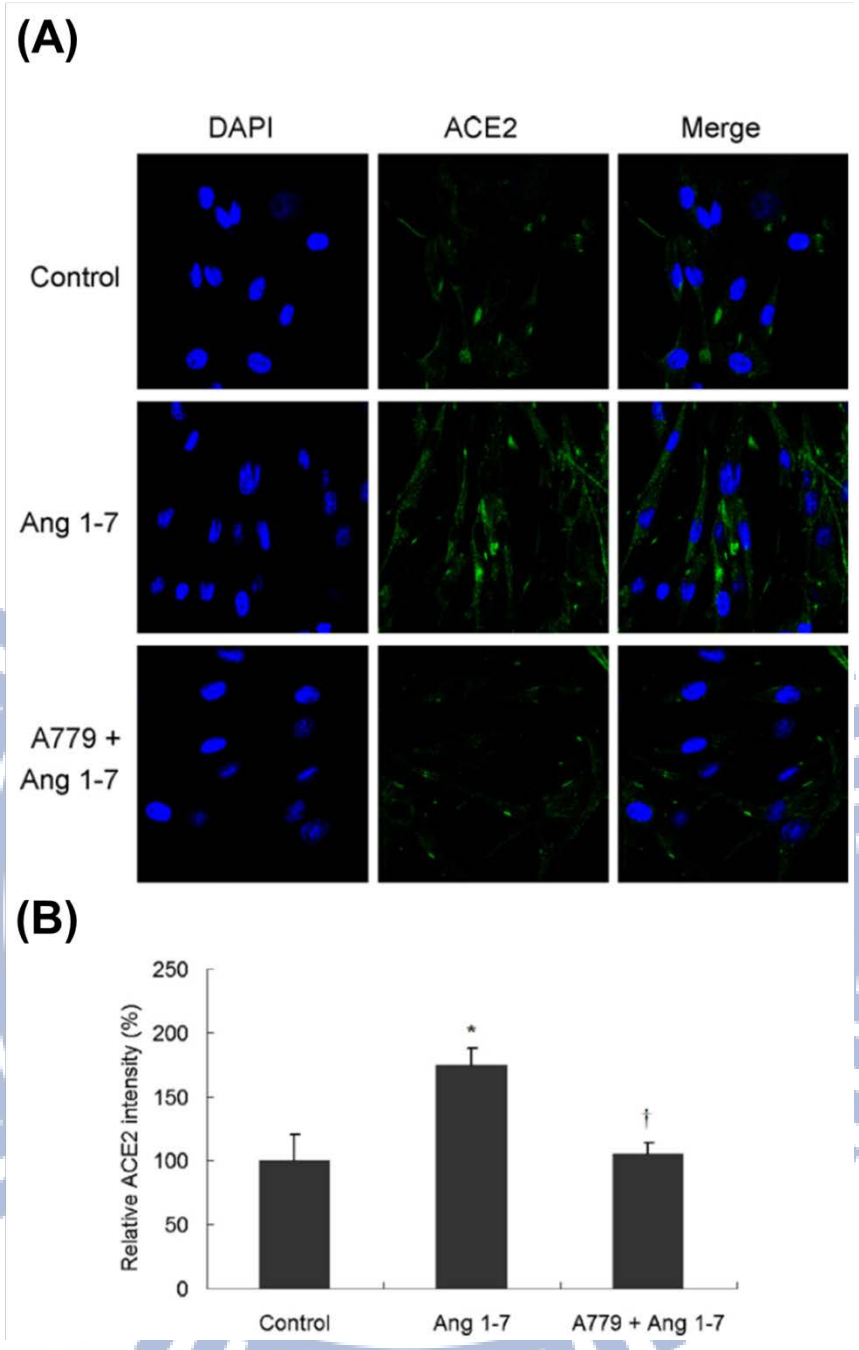


Fig. 4-5. Distribution and expression of ACE2 in HCF cells treated with Ang 1-7. HCF cells grown on a coverslip were treated with Ang 1-7 with or without prior treatment with the Mas receptor blocker A779 for 24 h. Treated cells were washed, fixed, and immunostained for ACE2 (green), and nuclei were counterstained with DAPI (blue). The localization of the ACE2 protein was visualized by confocal microscopy (A) and the relative ACE2 fluorescence intensity was calculated using the control group as 100% (B). Values are expressed as mean ± SD (n = 3). *, $p < 0.05$ vs. control; †, $p < 0.05$ vs. Ang 1-7 treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

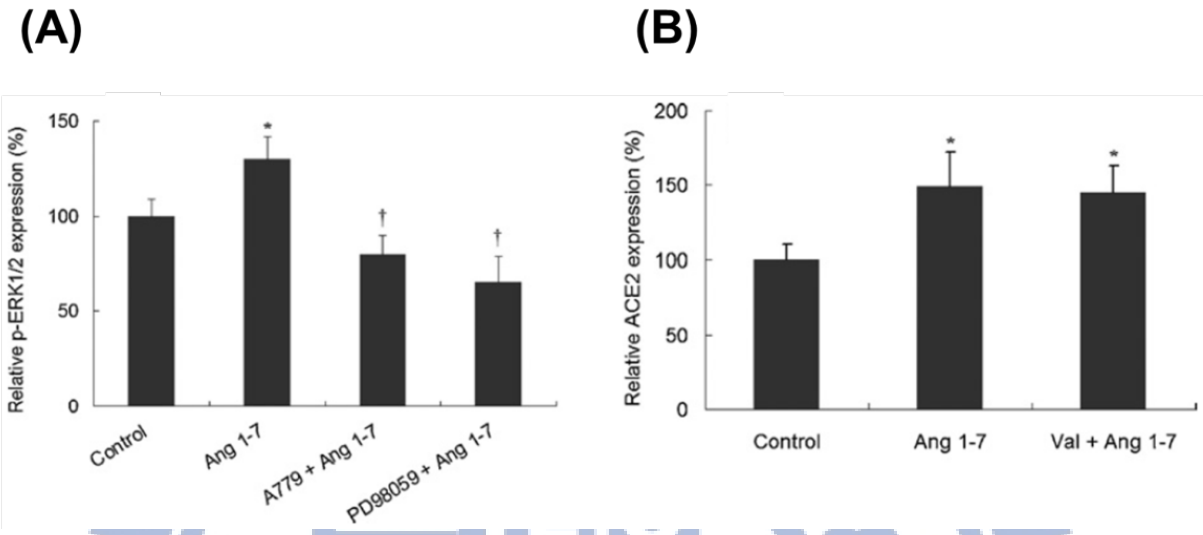


Fig. 4-6. Effect of blocking the AT1R-mediated signaling pathway on Ang 1-7-affected p-ERK1/2 and ACE2 protein expression in HCF cells. (A) Relative p-ERK1/2 protein expression was determined by western blotting in HCF cells treated with Ang 1-7 alone or following pretreatment with the Ang 1-7 Mas receptor blocker A779 or the MEK1/2 inhibitor PD98059. Expression of p-ERK1/2 was normalized using GAPDH expression, and the relative expression of p-ERK1/2 was calculated using the control group as 100%. (B) HCF cells were treated with Ang 1-7 with or without prior treatment with the AT1R blocker Val for 24 h. The extracted protein of the cells was analyzed by western blotting. ACE2 expression was normalized using GAPDH expression, and the relative expression of ACE2 was calculated using the control group as 100%. Values are expressed as mean ± SD (n = 3). *, $p < 0.05$ vs. control; †, $p < 0.05$ vs. Ang 1-7 treatment.

4-3. Expression levels of deletion constructs in the *ace2* promoter

To examine the transcriptional activity of *ace2*, a 2.1 kb fragment of the upstream region of human *ace2* was cloned into the upstream of the luciferase coding gene in the pGL3-Basic vector to generate the –2069/+20 construct. This construct was transiently transfected into HCFs, and the resulting expression of luciferase was monitored by measuring luciferase activity. Luciferase activities from HCFs transfected with the pGL3-Basic vector were compared with those transfected with the pGL3-Control vector, which was used to monitor DNA transfection efficiency. Transfection of the HCFs with the –2069/+20 construct showed a significant increase (8.9 ± 2.0 fold increase) in luciferase expression compared to the baseline levels for pGL3-Basic vector transfection.

Based on these results, we obtained eleven serially deleted constructs (starting at –1493, –1110, –916, –786, –664, –627, –516, –481, –355, –253, and –161) using the designed primer pairs (**Table 3-1**) and the plasmid DNA of the –2069/+20 construct as the template by PCR (**Fig. 4-7A**). These serial deletion fragments of the *ace2* promoter were used to drive the downstream gene expression of the reporter gene, luciferase, in order to determine which region contained critical regulatory activity of *ace2* expression. The results showed luciferase expression of the serial deletion constructs was essentially unchanged from position –2069 to position –627 within the *ace2* promoter. Deletion of the construct to position –516, however, resulted in a significant increase in promoter activity; a further 5′ deletion construct to position –481 resulted in markedly decreased promoter activity (**Fig. 4-7B**). These results indicate the presence of a significantly activating domain between position –516 and –481.

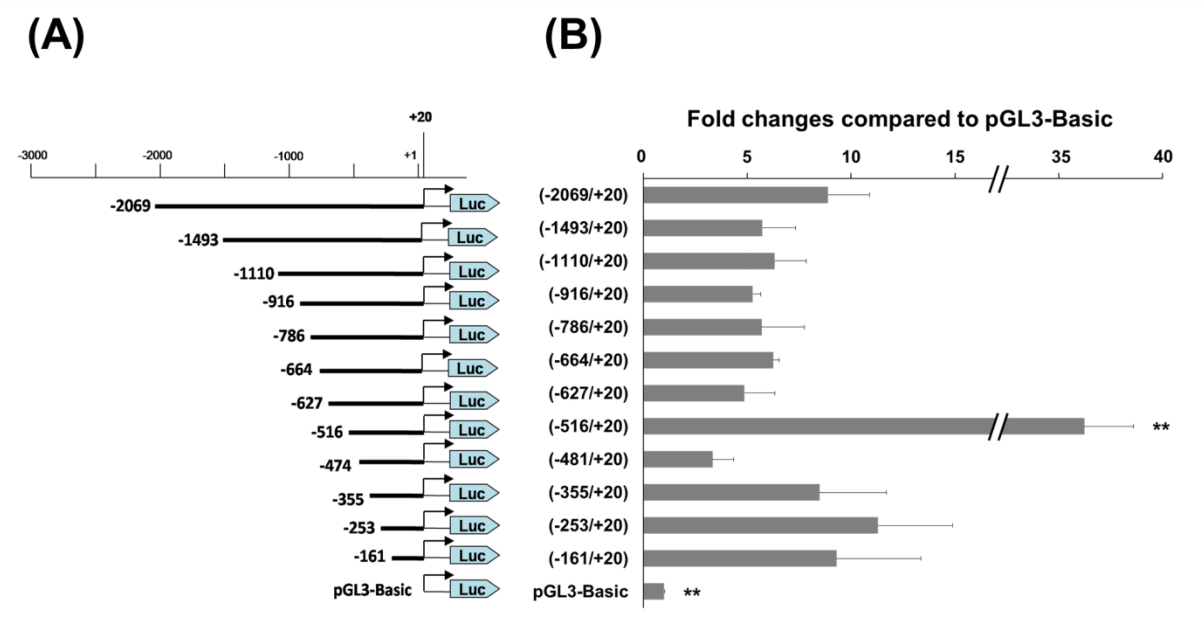


Fig. 4-7. Composition and promoter activity of the constructs on the expression of the reporter enzyme, luciferase, in HCFs. (A) The constructs were comprised of serially deleted portions of the upstream region of *ace2*, fused to firefly luciferase cDNA in the vector pGL3-Basic. The position of the promoter fragments relative to transcription start site (+1) is indicated. (B) The constructs were transfected into HCFs. Cells were lysed 24 h later and luciferase activities were measured. Relative luciferase activity of each construct (i.e., compared to that of the control, pGL3-Basic vector) is shown. All values are expressed as the mean ± SD from three independent experiments; ** indicates $p < 0.01$ compared to the –2069/+20 construct.

4-4. Identification of the regulatory domain within the *ace2* promoter

To further identify the regulatory sequences within the –516/–481 region that enhance *ace2* expression, two constructs were created from the –2069/+20 construct: one in which the –516/–481 domain was internally deleted and the other in which it was reversed (**Fig. 4-8A**). The –516/–481 deleted construct (–2069~–516/–481~+20) and the reversed construct (–2069~–481/–516~+20) were then transiently transfected into HCFs and the promoter activity

of *ace2* was assessed. The results showed both the deleted and the reversed sequence domain significantly reduced downstream luciferase expression (**Fig. 4-8B**).

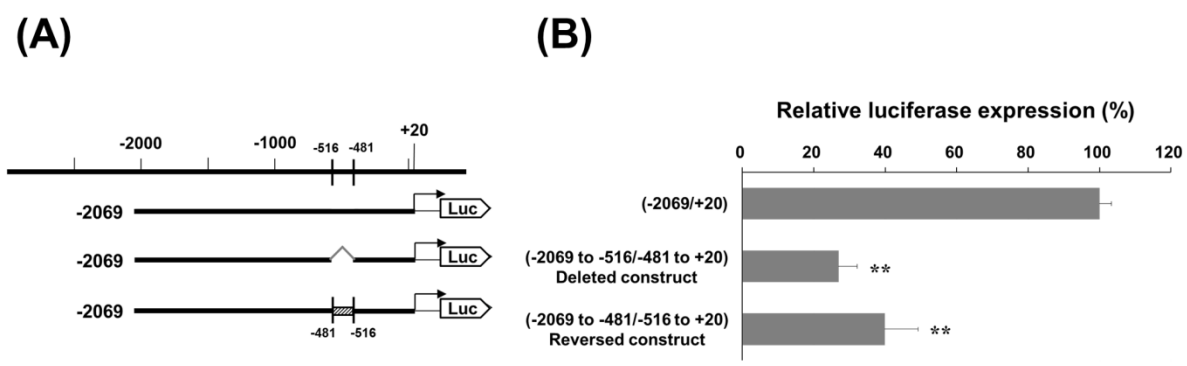


Fig. 4-8. Analyses of the promoter activity of the deleted and reversed domain within the upstream region of *ace2*. (A) Schematic representation of the deleted (–2069~–516/–481 ~+20) and reversed (–2069~-481/–516~+20) domain in the –2069/+20 construct. (B) The constructs were transfected into HCFs. Cells were lysed 24 h later and luciferase activities were measured. Relative luciferase activity of each construct (i.e., compared to that of the control –2069/+20 construct) is shown. All values are expressed as the mean ± SD from three independent experiments; ** indicates $p < 0.01$ compared to the –2069/+20 construct.

4-5. Identification of the regulatory element for *ace2*

We showed the –516/–481 domain of *ace2* contains major regulatory sequences, but the main regulatory element needed to be clarified. The nucleotide sequence of –516/–481 region was therefore analyzed using the database TFSEARCH (Vares et al., 2011) to find possible transcription factor binding elements. The results show a potential Ikaros binding site 5′-ATTTGGA–3′ with 95% calculated score. PCR site-directed mutagenesis was used to generate seven mutant sequences of ATTTGGA to further identify the regulatory element of *ace2* (**Fig. 4-9A**). The designed primer pairs used to PCR amplify and construct a series of site-directed mutant constructs are shown in **Table 4-1**. Compared to the original –516/+20 construct, luciferase expression was significantly decreased in all mutant constructs (**Fig. 4-9B**). This indicates that the sequence ATTTGGA is indeed a main regulatory element in the

–516/–481 domain of the *ace2* promoter.

To determine whether cellular regulatory factors are produced in HCFs that are capable of interacting with the –516/–481 domain, we used the synthetic and biotin-labeled double-stranded oligonucleotides of the –516/–481 sequences to react with the nuclear extracts prepared from HCFs by EMSA. As shown in **Fig. 4-10A**, one distinctive DNA-protein complex was observed when the –516/–481 double-stranded DNA was incubated with nuclear extracts of HCFs. This DNA-protein complex is specific to the –516/–481 sequences because it was readily eliminated by an excess of unlabeled competitor and was partially abolished when an Ikaros antibody was used to pretreat the nuclear extracts of HCFs.

For further confirmation that the sequence ATTTGGA within the –516/–481 domain of the *ace2* promoter was a significant binding element, seven mutant double-stranded oligonucleotides, M1 through M7, were synthesized and used for EMSA (**Fig. 4-10B**). The results show unlike the –516/–481 double-stranded oligonucleotides, the M1 through M7 double-stranded oligonucleotides could not form a DNA-protein complex with the nuclear extracts of HCFs (**Fig. 4-10B**). This result is consistent with the other results from the promoter activity.

Table 4-1. Sequences of the primer pairs used for generating the mutant constructs of -516/+20 construct

Constructs	Forward/Reverse primers (5'→3')
M1 (-516/+20)	F- TAAAGACTCGAGCAAAGTCATGTACTCGAAAGG R- GAGCTAAGCTTCGTCCCCTGTG
M2 (-516/+20)	F-TAAAGACTCGAGCAAAGTCATGTACTCGGAAGG R- GAGCTAAGCTTCGTCCCCTGTG
M3 (-516/+20)	F- TAAAGACTCGAGCAAAGTCATGTATTCGAAAGG R- GAGCTAAGCTTCGTCCCCTGTG
M4 (-516/+20)	F- TAAAGACTCGAGCAAAGTCATGTACTTGAAAGG R- GAGCTAAGCTTCGTCCCCTGTG
M5 (-516/+20)	F-TAAAGACTCGAGCAAAGTCATGTACTTGGAAGG R- GAGCTAAGCTTCGTCCCCTGTG
M6 (-516/+20)	F-TAAAGACTCGAGCAAAGTCATGTATTTGAAAGG R- GAGCTAAGCTTCGTCCCCTGTG
M7 (-516/+20)	F-TAAAGACTCGAGCAAAGTCATGTATTCGGAAGG R- GAGCTAAGCTTCGTCCCCTGTG

The method of PCR site-directed mutagenesis was used to generate seven mutant sequences of 5'-ATTTGGA-3' to clarify the regulatory element of *ace2*. According to the 5'-ATTTGGA-3 sequences, the constructs with mutant sequences were made by PCR site-directed mutagenesis and mutant sequences were shown in underline and red.

The recognition sequences of restriction enzymes, CTCGAG for *Xho* I in the forward primers and AAGCTT for *Hind* III in the reverse primers were shown in blue letters.

(A)

	Sequences (-516/-481)	Relative TF binding score
(-516/+20)	CAAAGTCATGTATTTGGAAGGGAAAATGTTGCCCAA	100
M1(-516/+20)	CAAAGTCATGTACTCGAAAGGGCAAATGTTGCCCAA	76.6
M2(-516/+20)	CAAAGTCATGTACTCGGAAGGGCAAATGTTGCCCAA	74.6
M3(-516/+20)	CAAAGTCATGTATTCGAAAGGGCAAATGTTGCCCAA	76.6
M4(-516/+20)	CAAAGTCATGTACTTGAAAGGGCAAATGTTGCCCAA	78.3
M5(-516/+20)	CAAAGTCATGTACTTGGAAGGGCAAATGTTGCCCAA	74.7
M6(-516/+20)	CAAAGTCATGTATTTGAAAGGGCAAATGTTGCCCAA	76.6
M7(-516/+20)	CAAAGTCATGTATTCGGAAGGGCAAATGTTGCCCAA	83.7

(B)

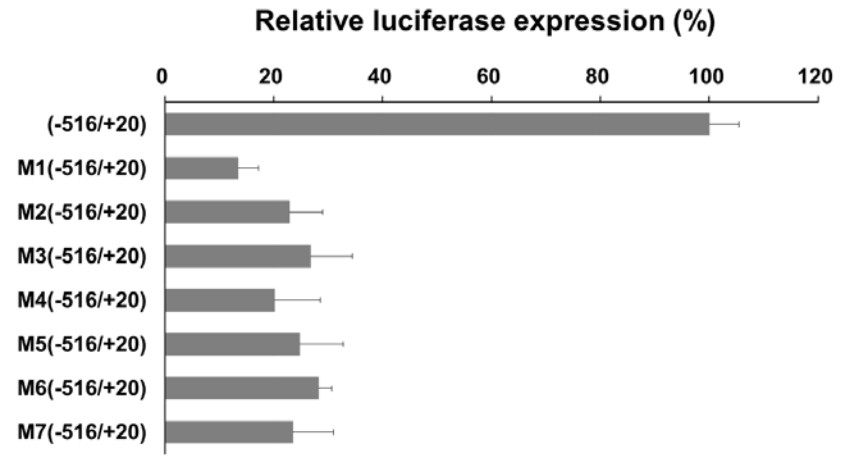


Fig. 4-9. Identification of the regulatory element within the –516/–481 domain. The full sequence, –516/–481, was analyzed for putative binding elements using TFSEARCH. The sequence, ATTTGGA, was identified as a potential binding element. (A) Using PCR site-directed mutagenesis at the ATTTGGA site, seven mutant constructs (M1 to M7) were generated. The location of the mutations is indicated in red typeface. The relative element binding score was calculated according to its TFSEARCH score, relative to a score of 100 for the full sequence, –516/–418. (B) The constructs were transfected into HCFs. Cells were lysed 24 h later and luciferase activities were measured. Relative luciferase activity of each construct (i.e., compared to that of the control –516/+20 construct) is shown. All values are expressed as the mean ± SD from three independent experiments.

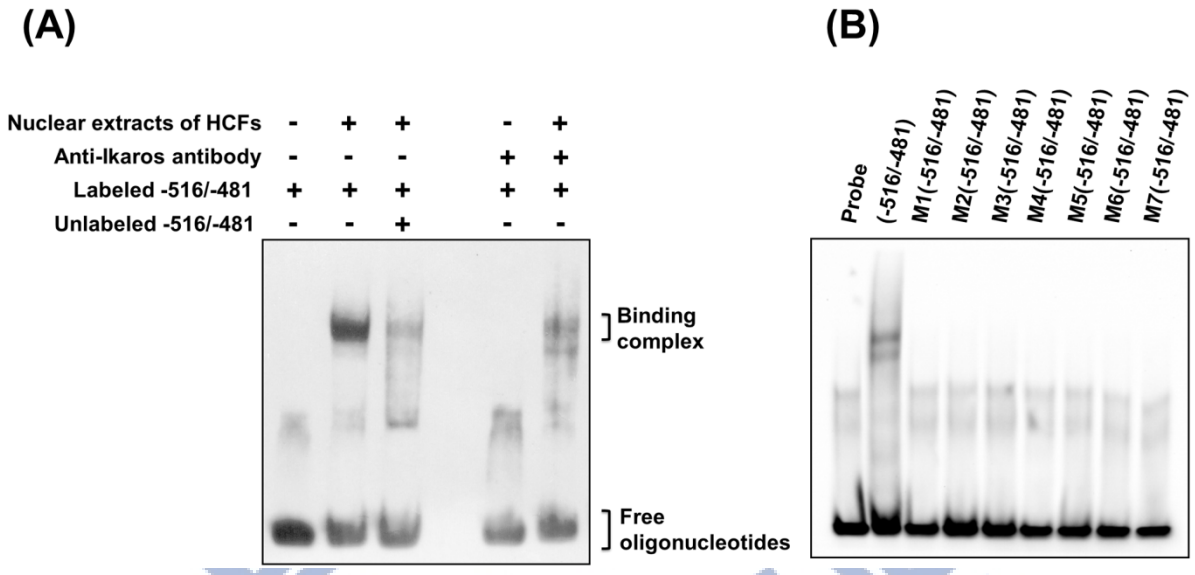


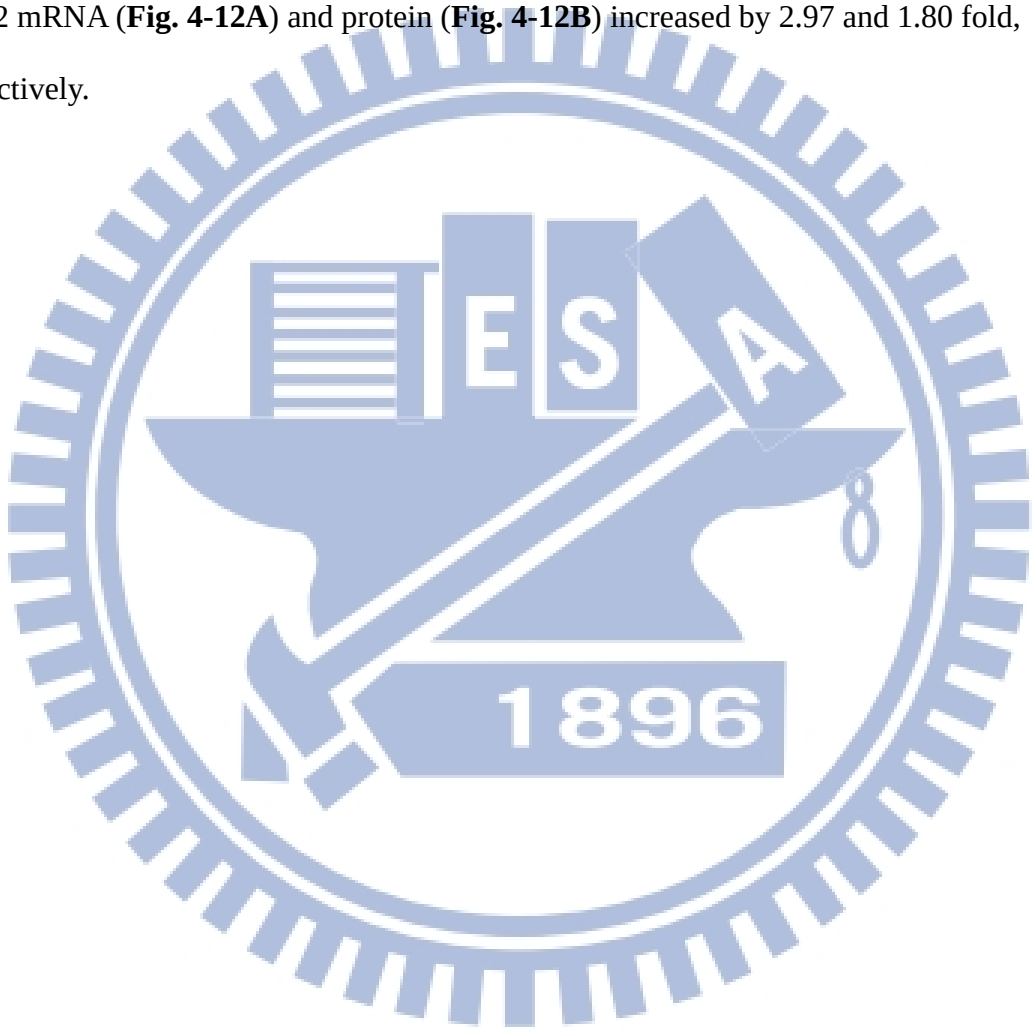
Fig. 4-10. Interaction of nuclear extracts from HCFs with (–516/–481) and mutant (M1 - M7) oligonucleotides by EMSA. Binding complexes were separated using 6% non-denaturing PAGE. (A) Unlabeled and labeled (biotinylated) double-stranded oligonucleotides, –516/–481, were mixed with nuclear extracts from HCFs. A 66 x molar excess of the unlabeled oligonucleotide, –516/–481, was used for competitive binding. (B) Nuclear extracts from HCFs were mixed with labeled oligonucleotides, (–516/–481) and labeled mutant oligonucleotides (M1 through M7). "Probe" indicates labeled oligonucleotides (–516/–481) alone, i.e., in the absence of nuclear extract.

4-6. Effect of Ang II on the transcriptional activation of *ace2*

The effect of Ang II on the transcriptional activation of *ace2* were investigated by transient transfection of HCFs with a –516/+20 construct, and a –481/–516/+20 construct (in which the sequence of the –516/–418 region was reversed) and treated with 0, 0.1, 1 and 10 μM of Ang II. The results show the relative luciferase expression from cells transfected with the –516/+20 construct was significantly increased by Ang II stimulation in a dose-dependent manner (**Fig. 4-11A**), and this increased luciferase expression could be abolished by pretreatment with Val (AT1R inhibitor) or PD98059 (MEK inhibitor) (**Fig. 4-11B**). In contrast, increased luciferase expression was not observed with Ang II treatment of HCFs transfected

with the –481/–516/+20 construct (**Fig. 4-11A**). These results indicate that Ang II can up-regulate the transcription of *ace2*.

To examine the expression regulation of endogenous ACE2 in HCFs, the expression of *ace2* and its protein production with and without treatment with 1 μM of Ang II was investigated. The results showed upon Ang II stimulation, the relative levels of expressed ACE2 mRNA (**Fig. 4-12A**) and protein (**Fig. 4-12B**) increased by 2.97 and 1.80 fold, respectively.



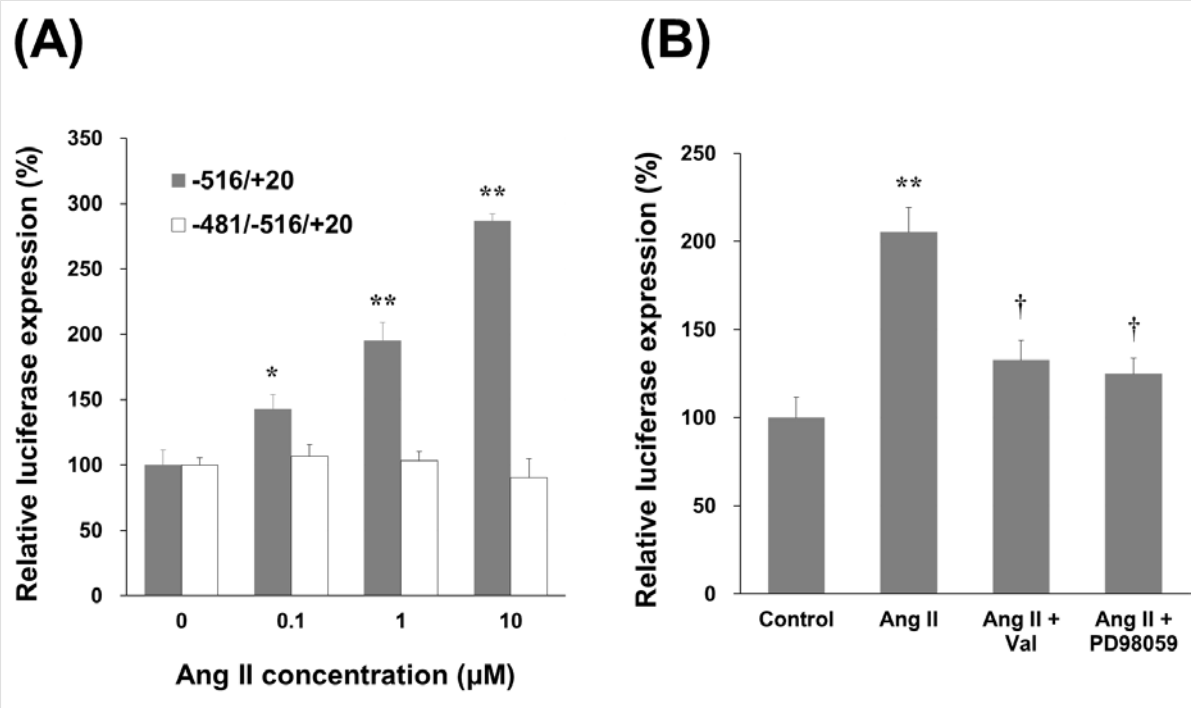


Fig. 4-11. The effects of Ang II stimulation on ACE2 expression in HCFs. (A) HCFs were transfected with the (–516/+20) and reversed (–481/–516/+20) constructs, then treated with various concentrations of Ang II. Cells were lysed 24h later and luciferase activity was measured. Relative luciferase activity (i.e., compared to luciferase activity in the absence of added Ang II) for each sample is shown. All values are expressed as the mean ± SD from three independent experiments; * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, compared to the group (control) with no added Ang II. (B) The signaling pathway of Ang II-induced ACE2 expression in HCFs was also investigated. HCFs transfected with the –516/+20 construct were pre-treated with 1 μg/ml of valsartan (AT1R inhibitor) or PD98059 (MEK inhibitor) for 1 h, then treated with 1 μg/ml of Ang II. The cells were lysed 24 h after addition of Ang II and luciferase activity was measured. Relative luciferase activity (i.e., compared to luciferase activity in the absence of added Ang II) for each sample is shown. All values are expressed as the mean ± SD from three independent experiments; ** indicates $p < 0.01$ compared to the group without added Ang II; † indicates $p < 0.01$ compared to the group with only Ang II added.

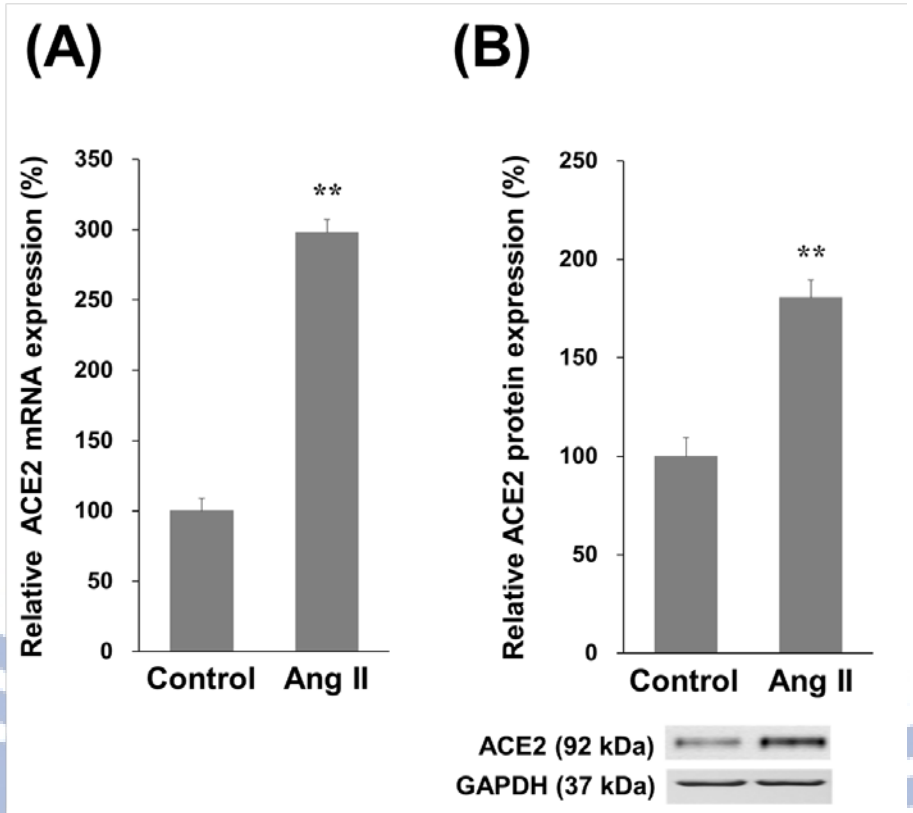


Fig. 4-12. The effect of Ang II stimulation on endogenous ACE2 expression in HCFs. HCFs were treated with 1 μM of Ang II for 24 h, and the cells were then analyzed for ACE2 mRNA using semi-quantitative RT-PCR (A), and for protein, using western blotting (B). Relative expression of ACE2 mRNA and protein (i.e., compared to expression without added Ang II) for each sample is shown. All values are expressed as the mean ± SD from three independent experiments; ** indicates $p < 0.01$ compared to the group without added Ang II.

4-7. Effect of pro-inflammatory factors on the transcriptional activation of *ace2*

We examined the effects of the pro-inflammatory cytokines, transforming growth factor-β1 (TGF-β1) and tumor necrosis factor-α (TNF-α) on the transcriptional activity of *ace2* in HCFs. The –516/+20 construct was transiently transfected into HCFs and the cells were treated with different dosages of TGF-β1 or TNF-α (0, 1, 5 and 10 ng/ml). Neither TGF-β1 (**Fig. 4-13A**) nor TNF-α (**Fig. 4-13B**) significantly affected luciferase expression: compared to expression levels in the absence of added pro-inflammatory factors, at the highest concentration of added cytokine (10 ng/ml), ACE2 mRNA expression and protein

expression decreased to 88% and 95%, respectively, with TGF-β1 treatment, and increased to 121% and 113%, respectively, with TNF-α treatment. These variations were not statistically significant.

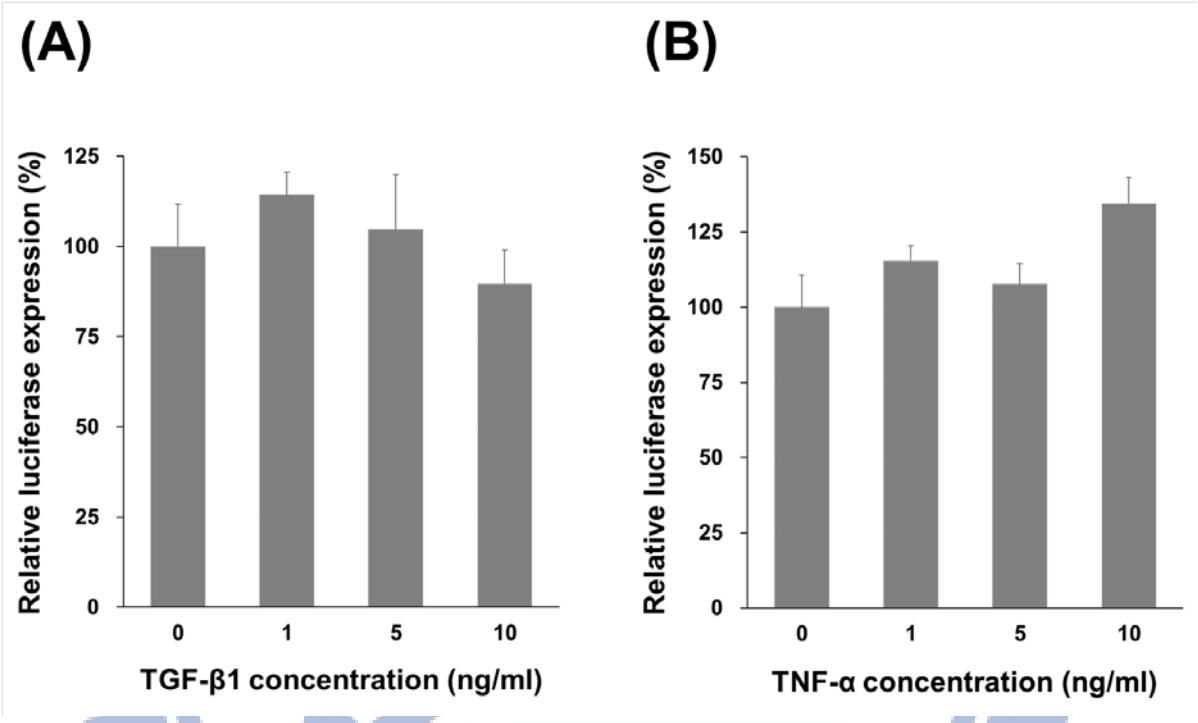


Fig. 4-13. The promoter activity of *ace2* in HCFs treated with pro-inflammatory factors. HCFs were transfected with the –516/+20 construct, and then treated with various concentrations of TGF-β1 (A), and TNF-α (B). The transfected HCFs were lysed 24 h after treatment and the luciferase activity was measured. Relative luciferase activity for each sample (i.e., compared to luciferase activity without added TGF-β1 or TNF-α) is shown. All values are expressed as the mean ± SD from three independent experiments.

4-8. The ACE2 activity of HCFs treated with Ang II and Ang 1-7

Previous results demonstrated that Ang II and Ang 1-7 were induced ACE2 expression of mRNA and protein in HCFs, respectively. This study also utilized fluorogenic substrates Mca-APK-Dnp to evidence the ACE2 activity of HCFs treated with Ang II and Ang 1-7. The results revealed that the dose dependent induction of ACE2 activity in HCFs by Ang II, the ACE2 activity of HCFs treated with 0.1 and 1 μM Ang II were induced 1.1 and 2.4 fold compared to non-treated HCFs (**Fig. 4-14A**). The HCFs treated with 1 μM Ang 1-7 also appeared the induced ACE2 activity, but there was no significant difference (**Fig. 4-14B**).

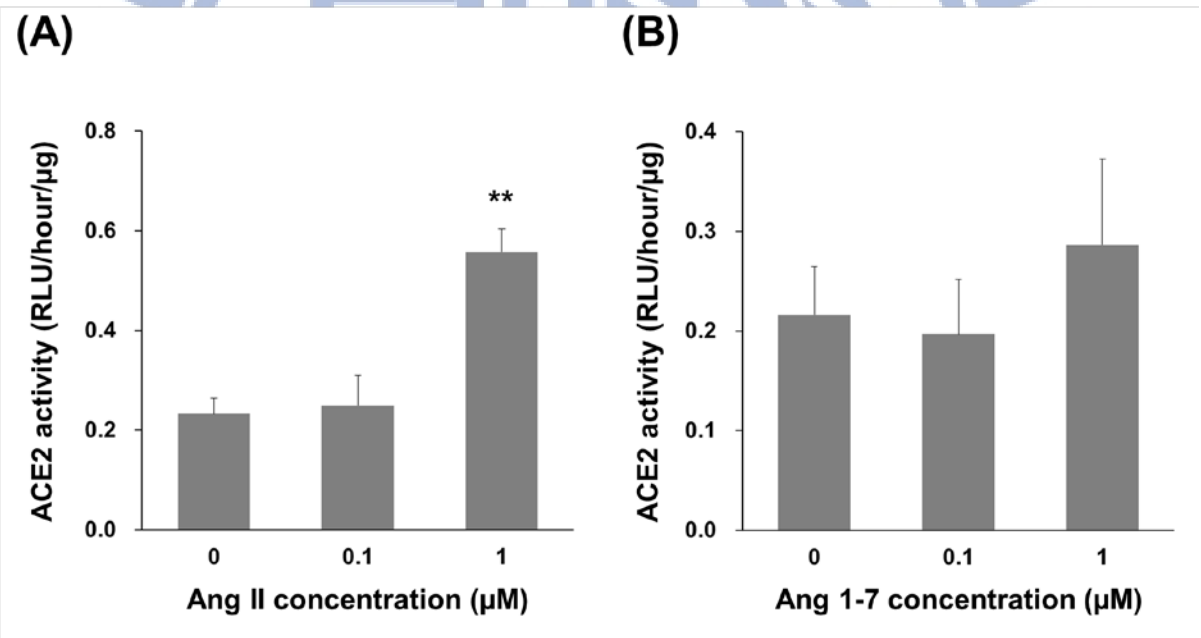


Fig. 4-14. The ACE2 activity of HCFs treated with Ang II and Ang 1-7. HCFs treated 0, 0.1 and 1 μM Ang II and Ang 1-7, respectively. The cell lysis of Ang II (A) and Ang 1-7 (B) treated HCFs was isolated to carry out the ACE2 activity assay, respectively. ACE2 activity was the ability to cleave the fluorescent substrate at 37°C for 1 hour with a specific ACE2 inhibitor. All values are expressed as the mean ± SD from three independent experiments; ** indicate $p < 0.01$ compared to the non-treated HCFs.

4-9. The ACE2 and MMP-2 activity of HCFs infected with ACE2 lentivirus

In this study, the association between ACE2 and MMP-2 was investigated in HCFs, especially ACE2 overexpression. ACE2 lentivirus, TLC-ACE2, was utilized to infect HCFs to create the ACE2 overexpressed HCFs, HCFs/ACE2. HCFs infected with TLC-ACE2 at different multiplicity of infection (MOI) and estimate the ACE2 and MMP-2 activity. The result showed ACE2 activity of HCFs/ACE2 was enhanced with the MOI. The ACE2 activity of HCFs/ACE2 infected at 1, 5, 10 and 20 MOI was 20, 78, 151 and 292-fold compared to non-infected HCFs, respectively (**Fig. 4-15A**).

Like as ACE2 activity, MMP-2 activity of HCFs/ACE2 also increased with the MOI and revealed the gentle trend at 5 MOI infected. The MMP-2 activity of HCFs/ACE2 infected at 1, 5, 10 and 20 MOI was 1.6-, 1.8-, 1.8- and 1.2-fold compared to non-infected HCFs, respectively (**Fig. 4-15B**).

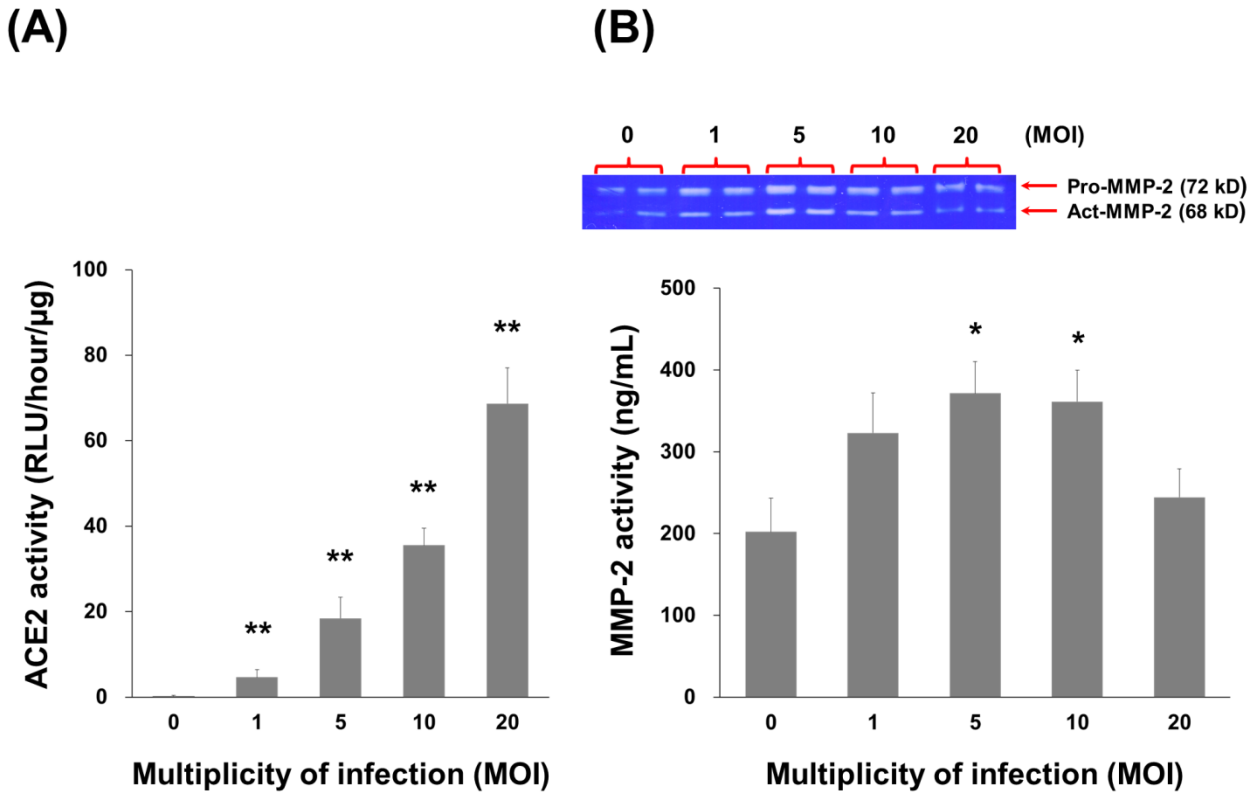


Fig. 4-15. The ACE2 and MMP-2 activity of HCFs infected with ACE2 lentivirus. HCFs infected with ACE2 lentivirus, TLC-ACE2, at 1, 5, 10 and 20 MOI to obtain HCFs/ACE2. The cells were lysed after 24 h infection at different MOI and the ACE2 (A) and MMP-2 (B) activity assay were measured, respectively. ACE2 activity was the ability to cleave the fluorescent substrate at 37°C for 1 hour with a specific ACE2 inhibitor. All values are expressed as the mean ± SD from three independent experiments; * and ** indicate $p < 0.01$ and $p < 0.05$, respectively, compared to the non-infected HCFs.

4-10. The ACE2 and MMP-2 activity of HCFs/ACE2 infected with ACE2 shRNA

The ACE2 shRNA was used to inhibit the ACE2 overexpression to evidence the effect of ACE2 in HCFs. The lentivirus contain different ACE2 shRNAs, TRCN-46693 ~ TRCN-46697, were used to infect HCFs/ACE2 at 5 MOI and measured the ACE2 activity to prove that which ACE2 shRNAs inhibited ACE2 overexpression effectively. The result revealed that all of the ACE2 shRNAs were suppressed the ACE2 expression in HCFs/ACE2,

but the efficiency was different. The TRCN-46694 and 46697 showed excellent ACE2 repression, the ACE2 activity of TRCN-46693 to 46697 were reduced to 53%, 7%, 25%, 17% and 5% compared to HCFs/ACE2, respectively (**Fig. 4-16A**).

In order to identify that ACE2 overexpression induced MMP-2 activity in HCFs, ACE2 shRNAs, TRCN-46697, utilized to suppress the ACE2 overexpression of HCFs/ACE2. The result illustrate that HCFs co-infected with TLC-ACE2 and TRCN-46697 was not affect MMP-2 activity different from HCFs infected with TLC-ACE2 (**Fig. 4-16B**). It appeared that used ACE2 lentivirus to infect HCFs induced ACE2 overexpression and the overexpressed ACE2 caused MMP-2 activity enhanced.

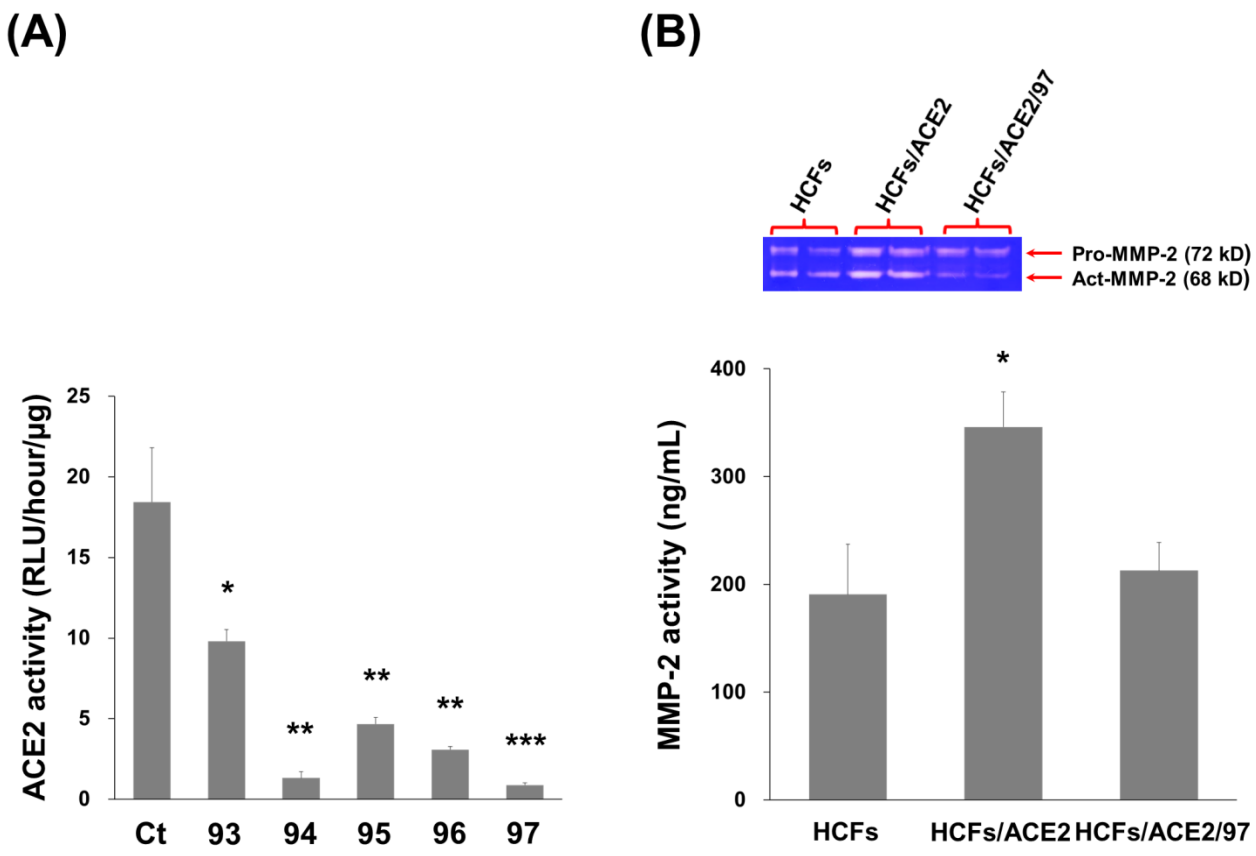


Fig. 4-16. The ACE2 and MMP-2 activity of HCFs/ACE2 infected with ACE2 shRNA. HCFs co-infected with TLC-ACE2 and different ACE2 shRNA, TRCN-46693 ~ 97, at 5 MOI. The cells were lysed 24 h after infection and the ACE2 (A) and MMP-2 (B) activity assay were measured, respectively. ACE2 activity was the ability to cleave the fluorescent substrate at 37°C for 1 hour with a specific ACE2 inhibitor. All values are expressed as the mean ± SD from three independent experiments; * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, compared to the HCFs/ACE2 in ACE2 activity assay; * indicate $p < 0.05$ compared to non-treated HCFs in MMP-2 activity assay.

4-11. The MMP-2 activities of HCFs/ACE2 treated with Ang II and Ang 1-7

ACE2 overexpression induced MMP-2 activity was indicated by TLC-ACE2 and TRCN-46697 co-infected HCFs in this study. For illustrating the role that ACE2 overexpression plays in HCFs and the associated with Ang II and Ang 1-7, the effect of MMP-2 activities in HCFs/ACE2 after Ang II and Ang 1-7 treating were detected. The results

showed MMP-2 activity was suppressed in HCFs/ACE2 treated with Ang II, and the suppressed MMP-2 activity by Ang II was been block by Val, AT1R inhibitor (**Fig. 4-17A**). The MMP-2 activity of HCFs/ACE2 treated with Ang II and HCFs/ACE2 co-treated with Ang II and Val compared to HCFs/ACE2 were decreased 44% and 9%, respectively, this result appeared that Ang II via AT1R inhibited MMP-2 activity in HCFs/ACE2.

Furthermore, the trend of MMP-2 activity while HCFs/ACE2 treated with Ang 1-7 is similar as HCFs/ACE2 treated with Ang II. The Ang 1-7 and the blocker of Mas receptor, A779, had been administrated in HCFs/ACE2. The results appeared that Ang 1-7 decreased MMP-2 activity of HCFs/ACE2, but the MMP-2 activity of HCFs/ACE2 that treated with Ang 1-7 and pre-treated with A779 and then treated with Ang 1-7 were no significantly different to HCFs/ACE2 (**Fig. 4-17B**). These results revealed that Ang 1-7 was no signigicant effect on MMP-2 activity of HCFs/ACE2.

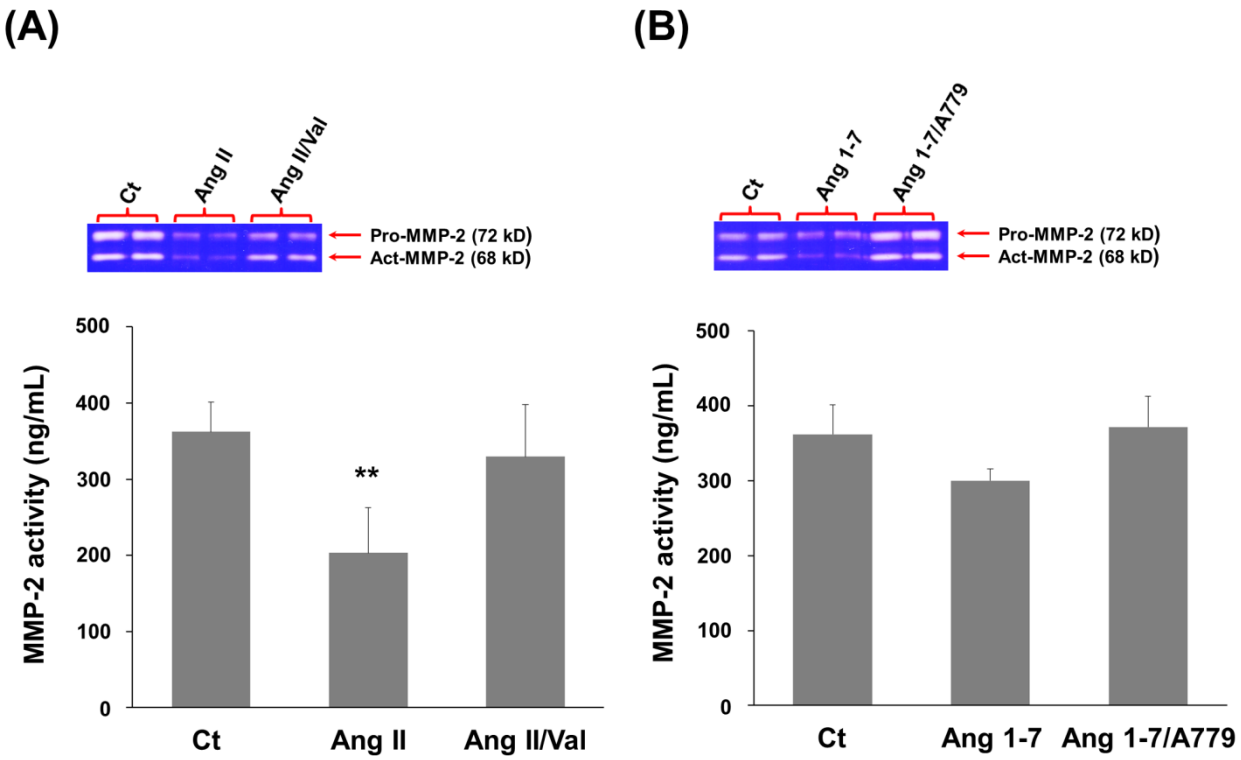


Fig. 4-17. The MMP-2 activities of HCFs/ACE2 treated with Ang II and Ang 1-7. HCFs/ACE2 pre-treated with 1 μM of valsartan (AT1R inhibitor) and A779 (Mas receptor inhibitor) for 1 h, and then treated with 1 μM of Ang II (A) and Ang 1-7 (B), respectively. The cells were lysed 24 h after Ang II and Ang 1-7 treated and then carried out the MMP-2 activity assay. All values are expressed as the mean ± SD from three independent experiments; ** indicate $p < 0.01$ compared to the non-treated HCFs/ACE2.

4-12. The ERK1/2 expression of HCFs/ACE2 treated with Ang II and Ang 1-7

Previous results appeared that Ang II and Ang 1-7 affected MMP-2 activity via AT1R and Mas receptor in HCFs/ACE2, respectively. This study further investigated the signal pathways of ACE2 associated with MMP-2 expression. The results revealed that the protein expression of ERK1/2 in HCFs/ACE2 treated with Ang II and Ang1-7 was similar as the trend of MMP-2 activity. The protein expression of ERK1/2 was reduced to 66% and 77% when HCFs/ACE2 treated with Ang II and Ang 1-7, respectively (**Fig. 4-18A, B**). However, compared to non-treated HCFs/ACE2, HCFs/ACE2 pre-treated with Val and A779 and then

treated with Ang II and Ang 1-7 were no significant difference in ERK1/2 expression, respectively. These results evidenced Ang II and Ang 1-7 affected the activation of ERK1/2 via AT1R and Mas receptor, respectively. In addition, Ang II-AT1R-ERK1/2 axis decreased the MMP-2 activity in HCFs/ACE2.

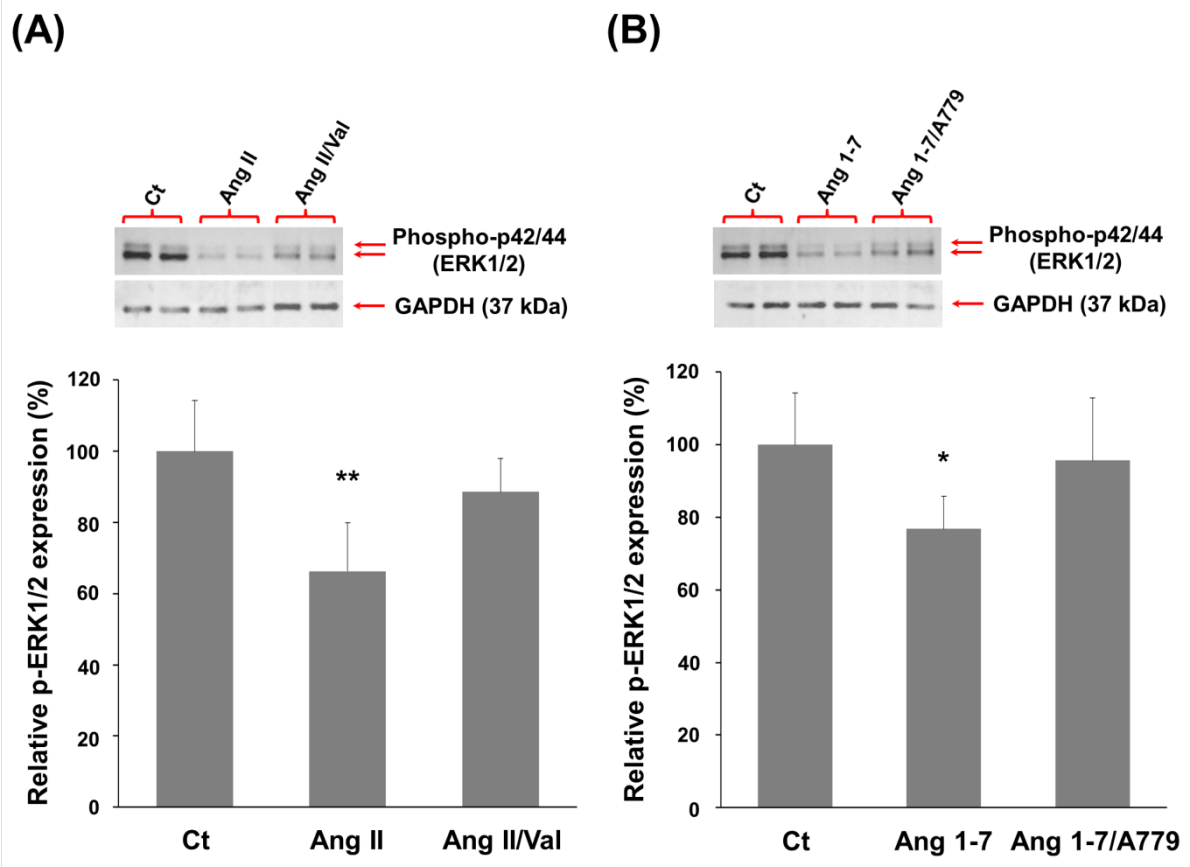


Fig. 4-18. The ERK1/2 expression of HCFs/ACE2 treated with Ang II and Ang 1-7. HCFs/ACE2 pre-treated with 1 μM of valsartan (AT1R inhibitor) and A779 (Mas receptor inhibitor) for 1 h, and then treated with 1 μM of Ang II (A) and Ang 1-7 (B), respectively. The cells were lysed 24 h after Ang II and Ang 1-7 treated and then detected the ERK1/2 and GAPDH expression by western blots. All values are expressed as the mean ± SD from three independent experiments; * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, compared to the non-treated HCFs/ACE2.

4-13. The shed ACE2 activity of HCFs/ACE2 treated with Ang II and Ang 1-7

This study investigated the relationship between MMP-2 activity and ACE2 activity, which consisted with cellular and shed ACE2 activity, to evidence the protect role of ACE2 in cardiac fibrosis. In HCFs, 1 μM Ang II and Ang 1-7 treatment were increased the cellular ACE2 activity, however, the shed ACE2 activity revealed different trends. HCFs/ACE2 treated with Ang II and Ang 1-7 were decreased shed ACE2 activity, the shed ACE2 activity

were reduced to 76% and 62% when HCFs/ACE2 treated with 0.1 and 1 μM Ang II compared to non-treated HCFs/ACE2 (**Fig. 4-19A**). As like Ang II treated HCFs/ACE2, the shed ACE2 activity of 0.1 and 1 μM Ang 1-7 treated HCFs/ACE2 reduced to 96% and 68% (**Fig. 4-19B**). In addition, Val and A779 also pressured the effect of Ang II and Ang 1-7 in shed ACE2 release. The shed ACE2 of HCFs/ACE2 pre-treated with Val and A779 and then treated with Ang II and Ang 1-7 were not difference with non-treated HCFs/ACE2 (**Fig. 4-20A, B**). These results appeared that Ang II and Ang 1-7 via AT1R and Mas receptor to induce cellular ACE2 activity and reduce shed ACE2 activity, respectively.

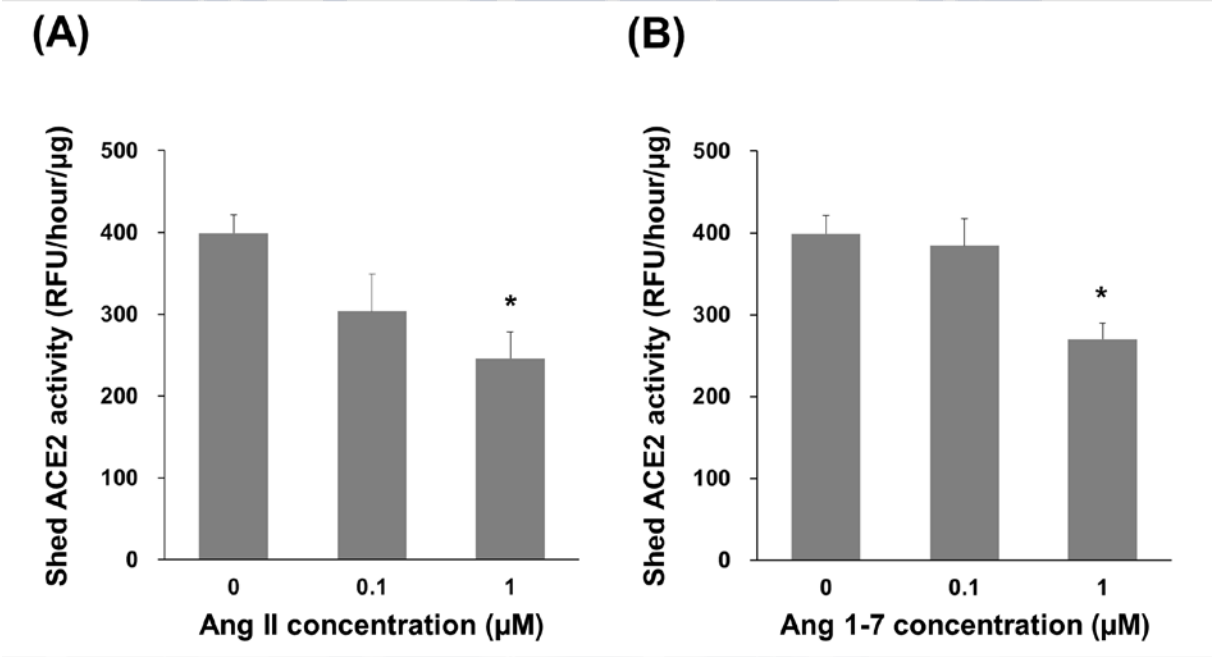


Fig. 4-19. The shed ACE2 activity of HCFs/ACE2 treated with various doses of Ang II and Ang 1-7. HCFs/ACE2 treated 0, 0.1 and 1 μM Ang II and Ang 1-7, respectively. The culture medium of Ang II (A) and Ang 1-7 (B) treated HCFs/ACE2 was isolated to carry out the ACE2 activity assay, respectively. ACE2 activity of medium was the ability to cleave the fluorescent substrate at 37°C for 1 hour with a specific ACE2 inhibitor. All values are expressed as the mean ± SD from three independent experiments; * indicate $p < 0.05$ compared to the non-treated HCFs.

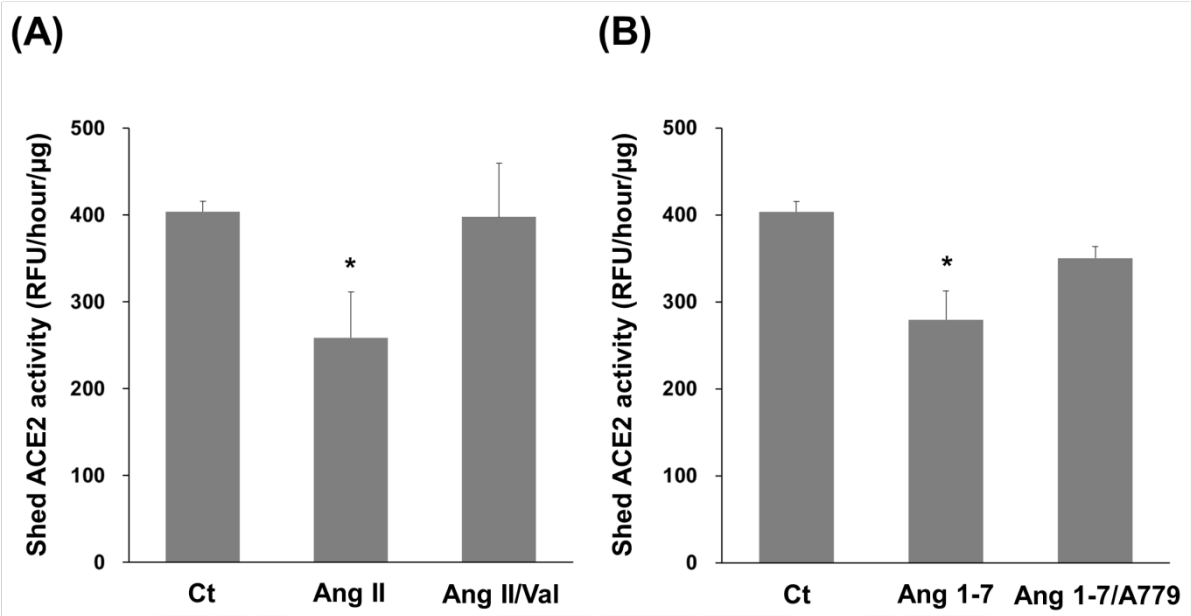


Fig. 4-20. The shed ACE2 activity of HCFs/ACE2 treated with Ang II and Ang 1-7. HCFs/ACE2 pre-treated with 1 μM of valsartan (AT1R inhibitor) and 1 μM A779 (Mas receptor inhibitor) for 1 h before 1 μM Ang II (A) and Ang 1-7 (B) treated, respectively. The culture medium of Ang II and Ang 1-7 treated HCFs/ACE2 for 24 h were isolated to carry out the ACE2 activity assay, respectively. ACE2 activity of medium was the ability to cleave the fluorescent substrate at 37°C for 1 hour with a specific ACE2 inhibitor. All values are expressed as the mean ± SD from three independent experiments; * indicate $p < 0.05$ compared to the non-treated HCFs/ACE2.

4-14. The mRNA expression of MAPK1 and ADAM17 in HCFs/ACE2 treated with Ang II and Ang 1-7

In previous experiment, the regulation of ACE2 shedding was been proved in HCFs/ACE2 via Ang II-AT1R and Ang 1-7-Mas axes. According to the published study of Lambert (2005), ADAM17 stimulated ACE2 shedding in HEK293 cells and Huh7 cells, the mRNA expression of ADAM17 and MAPK1 (ERK1/2) were been estimated to indicate the signal pathway of ACE2 shedding in this study. The results appeared that Ang II and Ang 1-7 inhibited ACE2 shedding in HCFs/ACE2, but co-treated with Ang II/Val and Ang1-7/A779 were no significant different. Compared to non-treated HCFs/ACE2, the mRNA expression of

ADAM17 in HCFs/ACE2 treated with Ang II and Ang 1-7 were reduced to 63% and 72%, respectively (**Fig. 4-21A**).

In addition, the mRNA expression of MAPK1 (ERK1/2) was been assessed to confirm and investigate the association between ERK1/2, ADAM17 and ACE2 shedding. The results showed the same trend of the mRNA expression of ADAM17 and the phospho-EKR1/2 protein expression in HCFs/ACE2 treated with Ang II and Ang 1-7. The mRNA expression of MAPK1 in HCFs/ACE2 treated with Ang II and Ang 1-7 reduced to 60% and 58% compared to non-treated HCFs/ACE2, respectively (**Fig. 4-21B**). Compared to non-treated HCFs/ACE2, the mRNA expression of MAPK1 in HCFs/ACE2 co-treated with Ang II/Val and Ang 1-7/A779 were no significant different. These results appeared that Ang II-AT1R-ERK1/2 and Ang 1-7-Mas-ERK1/2 axes inhibited the ADAM17 expression and caused to ACE2 shedding suppression, respectively.

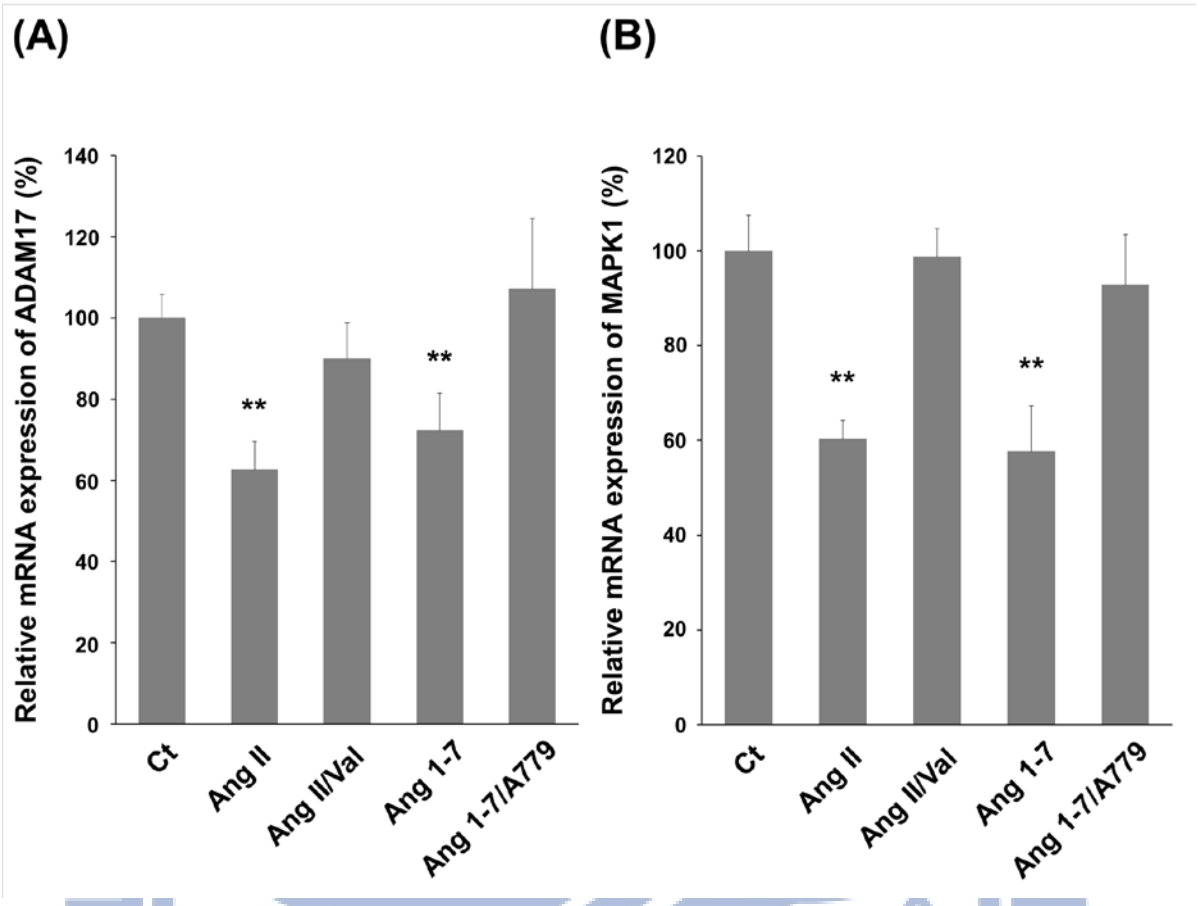


Fig. 4-21. The mRNA expression of ADAM17 and MAPK1 in HCFs/ACE2 treated with Ang II and Ang 1-7. HCFs/ACE2 pre-treated with 1 μM of valsartan (AT1R inhibitor) and 1 μM A779 (Mas receptor inhibitor) for 1 h before 1 μM Ang II and Ang 1-7 treated, respectively. After 24 h treatment, the mRNA of different groups was isolated and the mRNA expression of ADAM17 (A) and MAPK1 (B) were assay by real time PCR. ACE2 expression was normalized against GAPDH, and relative mRNA expression of ACE2 was calculated using the control group as 100%. Values are expressed as mean ± SD. ** indicate $p < 0.01$ compared to the non-treated HCFs/ACE2.

4-15. ACE2 knockout mice

In this study, our laboratory obtained the ACE2 KO mice, B6;129S5-Ace2^{tm1Lex}/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. 4-22A**. 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. 4-22B**).

The mRNA and protein expression of ACE2 and ACE2 activity were evidenced in ACE2 KO mice. The protein extracts of heart 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 heart 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 59%, 1% and 3% (**Fig. 4-23A**). The protein expression also reduced to 42%, 25% and 29% in heterozygous, homozygous and hemizygous mutants mice compared to WT mice (**Fig. 4-23B**). As like the trend of mRNA and protein expression, ACE2 activity were reduced to 29%, 2% and 1% compared to WT mice (**Fig. 4-24A**).

In addition, the gelatin zymography assay for the heart tissue of ACE2 KO mice also detected in this study. The gelatin zymography for heart tissue of ACE2 KO mice only revealed MMP-2 activity, ACE2 deficiency induced the MMP-2 activity in heterozygous, homozygous and hemizygous mutants mice. Compared to WT mice, the MMP-2 activity of heterozygous, homozygous and hemizygous mutants mice were increased 1.5, 1.8 and 1.7 fold (**Fig. 4-24B**). All data evidenced that the homozygous and hemizygous ACE2 KO mice appeared deficiency of ACE2 expression and activity, and the MMP-2 activity were increased compared to WT mice.

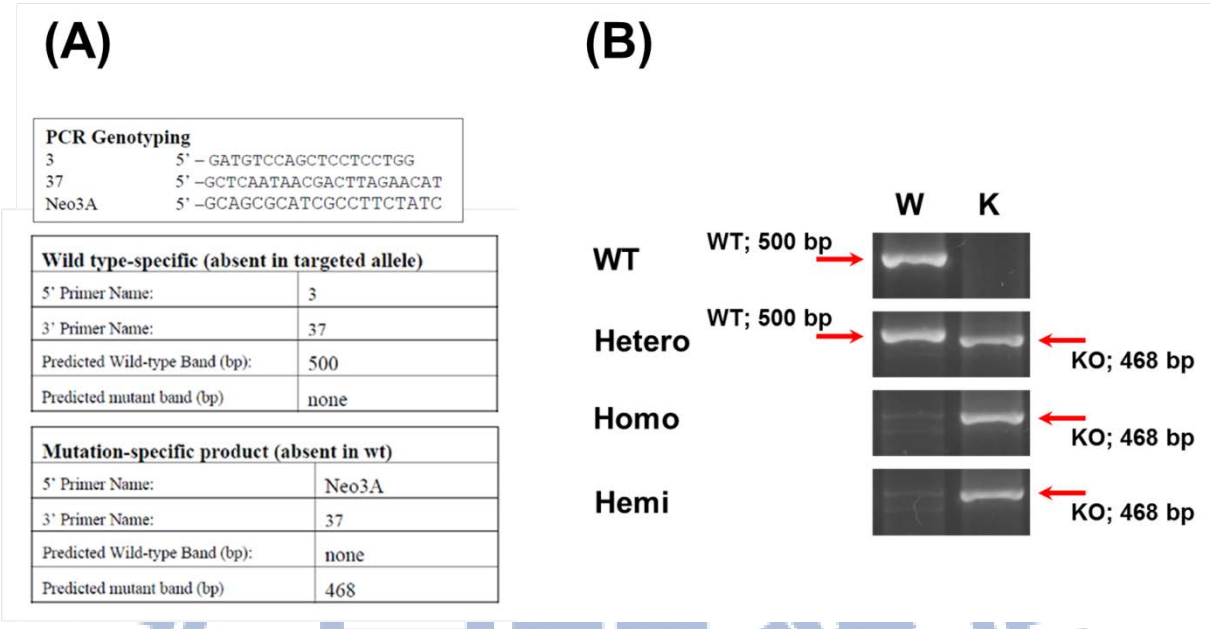


Fig. 4-22. PCR genotyping protocol for ACE2 knockout mouse. (A) The primer sequences, the specificity of each primer pair, and the expected PCR product size information (adapted from MMRRC). (B) The genotyping result from wild type and ACE2 knockout mouse (B6;129S5-Ace2^{tm1Lex}/Mmcd). Genomic DNA templates 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.

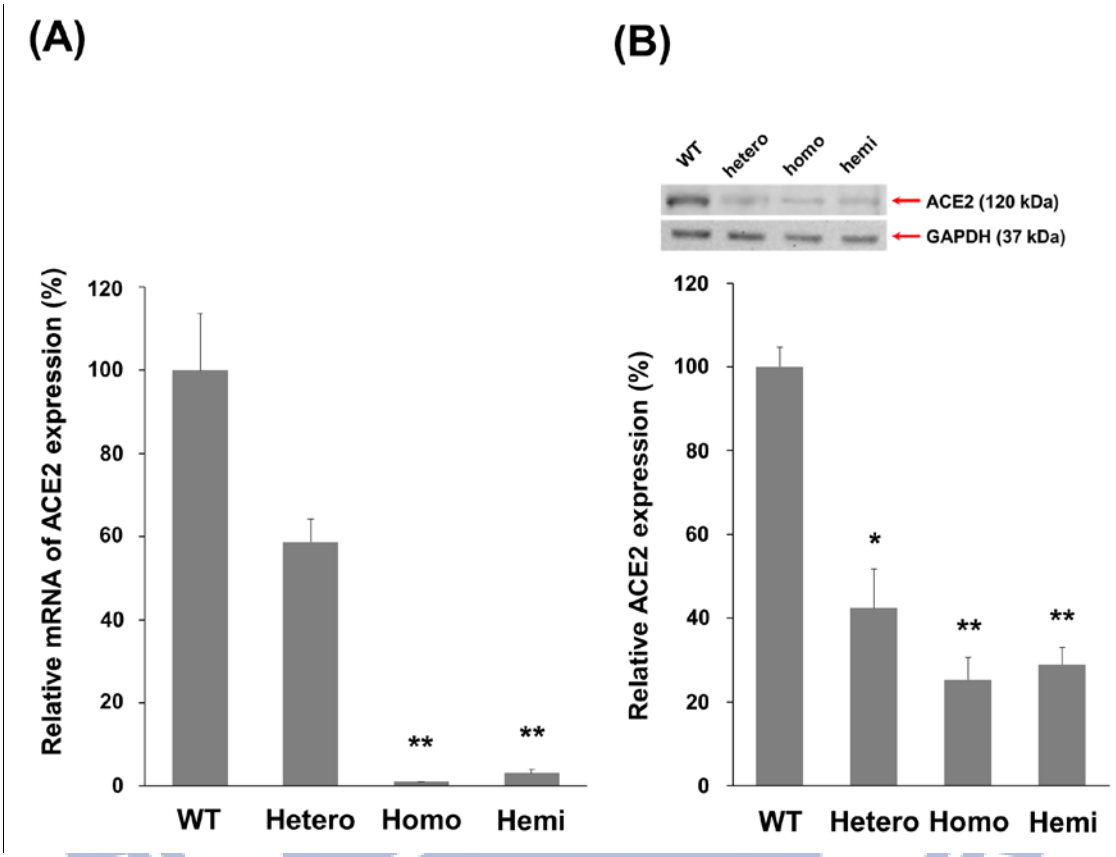


Fig. 4-23. The ACE2 expression of ACE2 knockout mice. Wild type and ACE2 knockout mice (B6;129S5-Ace2$^{tm1Lex/}$Mmcd; hetero, hemi and homo) were sacrificed and the protein extract of heart was performed by homogenizer. The ACE2 expression of heart extract was detected by real time PCR (A) and western blot (B). ACE2 expression was normalized using GAPDH expression, and the relative expression of ACE2 was calculated using the control group as 100%. All values are expressed as the mean ± SD from three independent experiments; * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, compared to the wild type mouse.

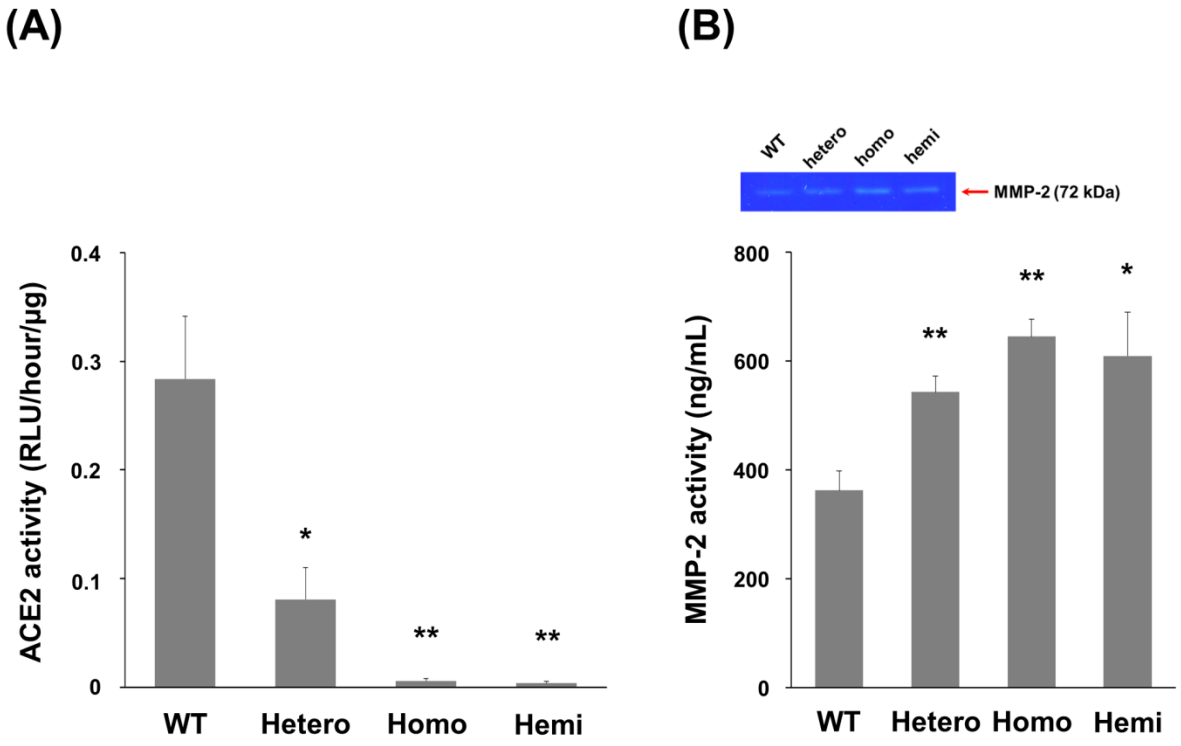


Fig. 4-24. The ACE2 and MMP-2 activity of ACE2 knockout mice. Wild type and ACE2 knockout mice (B6;129S5-Ace2$^{tm1Lex/}$Mmcd; hetero, hemi and homo) were sacrificed and the protein extract of heart was performed by homogenizer. The ACE2 (A) and MMP-2 (B) activity of heart extract were detected by fluorescence ACE2 activity assay and gelatin zymography assay, respectively. ACE2 activity of medium was the ability to cleave the fluorescent substrate at 37°C for 1 hour with a specific ACE2 inhibitor. All values are expressed as the mean ± SD from three independent experiments; * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, compared to the wild type mouse.

5. Discussion

5-1. Regulation of angiotensin converting enzyme II by angiotensin peptides

We reported that ACE2 expression could be detected in human cardiac myocytes and fibroblasts. This result is confirmed by a previous report (Guy et al., 2008), although other studies show a limited amount of ACE2 mRNA and no ACE2 enzyme activity in cardiac fibroblasts of neonatal rats (Grobe et al., 2007; Gallagher et al., 2008). In this study, we selected cardiac fibroblasts over cardiac myocytes to test the hypothesis of angiotenin peptides-regulated ACE2 expression based on the following three reasons. First, cardiac fibroblasts are the most abundant cell type present in the myocardium (estimated 2/3 of myocardial cells) (Grove et al., 1969). Second, AT1R are much more abundant on cardiac fibroblasts than on cardiac myocytes (Greenberg et al., 2005). Third, cardiac fibroblasts are mainly responsible for the deposition of extracellular matrix (ECM) in heart. Cardiac remodeling is characterized by the proliferation of cardiac fibroblasts and abnormal ECM metabolism leading to cardiac fibrosis (Cohn et al., 2000; Porter and Turner, 2009).

In the present study, we found that Ang II-induced ACE2 expression in HCF cells. This result contrasts with other studies indicating that Ang II reduces ACE2 expression (Gallagher et al., 2008; Koka et al., 2008) and that RAS blockade through ACE inhibitors (to inhibit Ang II synthesis) or AT1R antagonists (to reduce Ang II activity) induces ACE2 expression (Ishiyama et al., 2004; Ferrario et al., 2005; Kaiqiang et al., 2009). We do not challenge the concept that inhibition of Ang II synthesis or Ang II activity could induce ACE2 mRNA and protein expression *in vivo* or *in vitro* as reported previously. Instead, we emphasize that ACE2 regulation by angiotensin peptides may be largely dependent on the pathological or physiological process present in the study model, including disease state and species-specific variations.

Additionally, we do not exclude the possibility that the stage of cell differentiation could affect ACE2 gene regulation by Ang II. The primary HCF cells provided by ScienCell Research Laboratories may be isolated from fetal hearts. Gene regulation is altered in cardiac tissues at different stages of differentiation and in different diseased conditions (Razeghi et al., 2001). This is supported by the reported differential response of cardiac fibroblasts from young adult and senescent rats to Ang II (Shivakumar et al., 2003). Varied cell sources could explain some of the contradiction between our data and previous reports. However, cardiac fibroblasts are the most prevalent cell type in heart and play a key role in regulating normal myocardial function, this study provides valuable data on the effect of angiotensin peptides on cardiac fibroblast ACE2 expression.

We currently show increased ACE2 expression by Ang II stimulation could be abolished by both Val and PD98059 pretreatment. These data suggest that Ang II could stimulate ACE2 expression in HCF cells through the Ang II-AT1R signaling pathway. Our results suggest that up-regulated ACE2 may play a compensatory role in counteracting the effects of increased cardiac Ang II formation. This compensatory or protective role may serve as a means to maintain a steady state within RAS. We also demonstrated that ACE2 expression in HCF cells could be up-regulated by Ang 1-7. Ang 1-7, an angiotensin peptide of RAS, can be converted from Ang II by ACE2 enzyme catalysis. Several studies reveal that Ang 1-7 provides counter-regulatory effects to the deleterious effects of Ang II on cardiac function (Grobe et al., 2007; Pan et al., 2007; Mercure et al., 2008). The reported elevation of Ang 1-7 expression in failing heart tissue and in the ischemic zone following MI might result from increased ACE2 expression (Averill et al., 2003; Santos et al., 2005). Loss of ACE2 severely impairs cardiac function (Crackower et al., 2002), and Kassiri et al. hypothesize that loss of ACE2 would accelerate maladaptive left ventricular remodeling in response to MI (Kassiri et al., 2009).

ACE2 may modify AT1R expression by altering the balance between the Ang II and Ang

1-7 (Zucker et al., 2009). ACE2 regulation by angiotensin peptides may be influenced by the experimental model, age, species, cell type, state of health or type of cardiac disease (Shivakumar et al., 2003). In vascular smooth muscle cells, Clark et al. (Clark et al., 2003) indicated that Ang 1-7 down-regulates AT1R transcription and translation. In endothelial cells, Ang 1-7 reduces activation of AT1R-dependent c-Src protein and the downstream targets of ERK-MAPK via the Mas receptor (Sampaio et al., 2007). In the present study, both Ang 1-7 and Ang II comparably increased ACE2 expression in HCF cells. Additionally, Ang 1-7 increased the activation of p-ERK1/2 proteins (**Fig. 4-6A**). Furthermore, the increased ACE2 expression induced by Ang 1-7 could not be reversed by pretreatment with the AT1R inhibitor Val (**Fig. 4-6B**). These results suggest that the up-regulation of ACE2 expression by Ang 1-7 in cardiac fibroblasts may be independent of the Ang II-AT1R signaling pathway. This result is inconsistent with findings in vascular smooth muscle cells (Clark et al., 2001) and endothelial cells (Sampaio et al., 2007). The reasons for these differences are unclear, but we propose the existence of cell-type-specific differences in AT1R regulation among cardiofibroblasts, vascular smooth muscle cells and endothelial cells.

We report, for the first time, that Ang 1-7 increases ACE2 expression in HCF cells *in vitro*. Increased ACE2 expression could promote the conversion of Ang II to Ang 1-7 and thereby increase cardiac Ang 1-7. Furthermore, increased Ang 1-7 may up-regulate ACE2 expression in certain physiological conditions. This positive feedback loop may promote the Ang II conversion into Ang 1-7 to maintain a static state of cardiac Ang II concentration (**Fig. 5-1**). This proposed regulation of cardiac ACE2 expression maybe important for cardiac response to physiological stresses that would abnormally increase Ang II concentration. Furthermore, we propose that abnormal regulation on cardiac ACE2 expression may be related to cardiac pathophysiological processes such as hypertension (Koka et al., 2008), ischemia (Ishiyama et al., 2004) and atrial fibrillation (Pan et al., 2007).

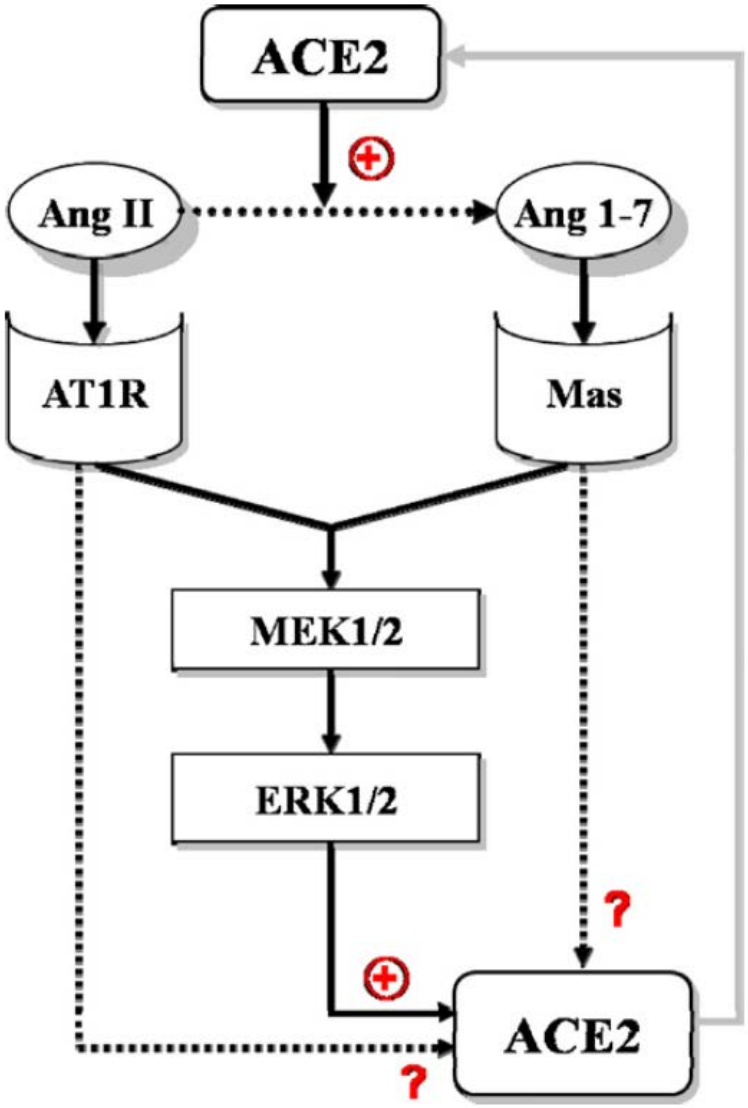


Fig. 5-1. Schematic diagram representing the interplay of Ang II and Ang 1-7 on cardiac ACE2 regulation. This scheme indicates that Ang II-up-regulated ACE2 may increase Ang 1-7 formation from Ang II, which can further increase ACE2 expression through a positive feedback loop. ACE2, angiotensin converting enzyme II; Ang II, angiotensin II; Ang 1-7, angiotensin 1–7; AT1R, angiotensin II type 1 receptor; ERK1/2, extracellular signal-regulated kinases 1/2; MEK1/2, mitogen-activated/ERK kinase 1/2.

5-2. Identifying the regulatory element for human angiotensin-converting enzyme 2 (ACE2) expression

To investigate the molecular mechanism by which Ang II regulates the expression of ACE2, we examined the promoter activity its gene, *ace2*. Using sequence deletion and site-directed mutation analyses, we identified a region upstream of *ace2*, at –516/–481 domain, that is required for Ang II-activated transcription. We also demonstrated that the sequence ATTTGGA is the Ang II responsive element.

From this study, the results of the promoter activity assay are consistent with those that show cardiac ACE2 was significantly up-regulated at both transcriptional and translational levels in HCFs after Ang II stimulation-presumably via the Ang II-AT1R signaling pathway. Several reports have shown that elevated Ang II levels were observed in conjunction with cardiac ACE2 up-regulation in subjects with cardiovascular disease, (e.g., MI, heart failure and atrial fibrillation) both in the clinic and in animal experiments (Zisman et al., 2003; Goulter et al., 2004; Burrell et al., 2005; Pan et al., 2007; Epelman et al., 2008). This raises the possibility that cardiac ACE2 up-regulation is associated with the modulation of the effect of Ang II, by an antagonist for example, which diminishes the effect of increased Ang II. Based on the results of Ang II-stimulated ACE2 up-regulation in HCFs, we suggest that the regulation of ACE2 by Ang II may be largely dependent on pathological and/or physiological conditions, and that up-regulated ACE2 may play a compensatory role in counteracting the effects from the increased ACE activity and Ang II production in the heart. This compensatory or protective role of ACE2 may serve to maintain homeostasis within the RAS.

In addition to the angiotensin peptides in the RAS, inflammation plays a key role in the initiation, progression, and clinical outcome of cardiovascular diseases. Substantial evidence suggests the involvement of the inflammatory and immune systems in adverse remodeling of cardiac failure and hypertrophy (Torre-Amione, 2005; Yndestad et al., 2007; Wynn, 2008). In

this study, we attempted to evaluate whether the expression of ACE2 could be modulated by pro-inflammatory factors in HCFs. We examined the effects of two pro-inflammatory cytokines, TGF-β1 and TNF-α on the expression ACE2. Increased doses of TGF-β1 and TNF-α did not cause significant change in ACE2 expression, however, nor in the promoter activity of *ace2*. This result confirms a previous report that ACE2 expression was not affected by TNF-α, IL-1 and chronic hypoxia in human cardiac myofibroblasts (Guy et al., 2008). It has been shown that Ang II can induce TGF-β1 and TNF-α expression in cardiac cells via the Ang II/AT1R signaling pathway (Kalra et al., 2002; Schultz Jel et al., 2002; Rosenkranz, 2004). We therefore suggest that Ang II-stimulated ACE2 up-regulation may occur via a TGF-β1/TNF-α independent pathway-although the results of ACE2 modulation by angiotensin and the cytokines reported here may be dependent on the specific experimental models used.

The sequence ATTTGGA is a potential binding domain for the transcriptional factor Ikaros. Ikaros was originally found to function as a key regulator of lymphocyte differentiation (Lo et al., 1991; Georgopoulos et al., 1992). Subsequent studies demonstrated the role of Ikaros in normal hematopoiesis (Lopez et al., 2002), and in the migration and invasion of extravillous trophoblasts in early placentation (Yamamoto et al., 2005). In a recent study, it was reported that Ikaros primes the lymphoid transcriptional program in hematopoietic stem cells, and that loss of Ikaros may confer aberrant self-renewing properties on myeloid progenitors (Yoshida et al., 2010); yet despite the clearly important biological role of Ikaros, its mechanism of action remains elusive. Consensus DNA recognition sequences for Ikaros have been unusually difficult to define because of several encoded Ikaros isoforms (Molnar and Georgopoulos, 1994) and because multiprotein complexes containing Ikaros family members have not been purified to homogeneity (Sridharan and Smale, 2007). From sequence analysis (using TFSEARCH) the potential binding domain of Ikaros was found in

the regulatory region of *ace2*, but was not found in *ace* gene. This may explain why some factors have been shown to regulate ace and *ace2* differently (Hamming et al., 2008; Koka et al., 2008; Zhang et al., 2009).

We report here for the first time the characterization of the regulatory element of human gene, *ace2*, and provide insight into the molecular mechanism controlling cardiac ACE2 expression in HCFs. We have identified the –516/–481 sequence domain within the upstream region of *ace2* as a putative protein binding domain for modulation of ACE2 expression, which is associated with the Ang II signaling pathway. Furthermore, a potential regulatory element, ATTTGGA, within the –516/–481 promoter region of *ace2* is responsible for Ang II stimulation, and this is unaffected by the pro-inflammatory cytokines, TGF-β1 and TNF-α. Our results suggest that the –516/–481 domain of *ace2* is involved in modulating ACE2 expression, and may be a binding domain for Ikaros, or other unidentified regulatory factor(s). Investigating the regulatory role of Ikaros on *ace2* and other potential regulatory factor(s) would lead to a greater understanding of the molecular mechanisms that regulate ACE2 expression.

5-3. The association between ACE2, MMP-2 and angiotensin peptides

In recent study, the relationship between Ang II and MMP-2 had been investigated in cell level. Human umbilical vein endothelial cells (HUVECs) treated with Ang II were induced TNF-α and MMP-2 release, and reduced the secretion of TIMP-2 via AT1R (Arenas et al., 2004). However, the association between ACE2 and MMP-2 is unclear. In this study, ACE2 lentivirus, TLC-ACE2 was used to infected HCFs to obtain HCFs/ACE2 and investigated the associated with ACE2 overexpression and MMP-2 activity. The results showed ACE2 overexpression enhanced MMP-2 activity and the Ang II and Ang 1-7 suppressed the

activation of ERK1/2 via AT1R and Mas receptor in HCFs/ACE2, respectively. Ang II was through AT1R to induce ERK1/2 activation and MMP-2 activity. Additionally, Ang II-AT1R-ERK1/2 and Ang 1-7-Mas-ERK1/2 axes also affected the ACE2 shedding from the cell membrane of HCFs/ACE2.

Cardiac fibroblasts and myocytes are the major cell type in heart tissue. The role of Ang II in modulating cardiac fibroblast activity is well accepted (Campbell et al., 1997; Kawano et al., 2000), but the actions of ACE2 in cardiac cells is unclear. Grobe and his group examined the effects of ACE2 gene delivery to cultured cardiac fibroblasts after acute hypoxic exposure. They utilized neonatal rat cardiac myocytes and cardiac fibroblasts to indicate that endogenous ACE2 activity is observed in cardiac myocytes, but not in cardiac fibroblasts (Grobe et al., 2007). Because of cardiac fibroblasts are the major cell type found in an infarct zone following a MI, lenti-ACE2 was been used to induce ACE2 expression of cardiac fibroblasts and revealed that ACE2 overexpression significant attenuated TGF-β and hypoxia/re-oxygenation-induced collagen production by the cardiac fibroblasts. In addition, the expression of ACE, ACE2 and AT1R were been detected in human cardiac myofibroblasts isolated from patients undergoing coronary artery bypass surgery (Guy et al., 2008). It also revealed that ACE2 can been released into extracellular medium and evidenced that ACE2 expression was not been regulated by TNF-α, IL-1β (Turner et al., 2007) or chronic hypoxia in human cardiac myofibroblasts. In addition, cardiac myocytes were isolated from diabetes rat and evidenced that ACE2 overexpression *in vitro* decreased high glucose (HG)-induced Ang II production, collagen accumulation and TGF-β expression in cardiac fibroblasts, and attenuated myocyte hypertrophy, myocardial fibrosis, and left ventricular (LV) remodeling (Dong et al., 2012). All results supported our results in HCFs and appeared that ACE2 plays a protect role in cardiac fibroblasts, myofibroblasts and myocytes to opposite to Ang II induced TGF-β expression and collagen accumulation, some difference of results maybe from the

different species and cell types (Guy et al., 2008).

Besides the discussion of cardiac cell, ACE2 also been investigated in animal model, especially the study of ACE2 overexpression and knockout. Huentelman (2005) and his coworker inserted a cDNA of mACE2 into the pTY.EF1.IRES.EGFP lentivirus cloning vector to construct the lenti-mACE2 and beginning the study of ACE2 overexpression. They injected the lenti-mACE2 into the left cardiac ventricular cavity to established ACE2 overexpression mice and infused Ang II to estimate the body weight and myocardial fibrosis. The results showed ACE2 overexpression were significant attenuation of the increased heart weight to body weight ratio and myocardial fibrosis induced by Ang II infusion (Huentelman et al., 2005). This lenti-mACE2 also used in spontaneously hypertensive rat (SHR) and coronary artery ligation (CAL) rat, the results demonstrated that ACE2 overexpression improved high blood pressure, left ventricular (LV) wall thickness and perivascular fibrosis in SHR mice (Díez-Freire et al., 2006; Der et al., 2008). Furthermore, the cardiac fibroblasts and cardiac myocytes of ACE2 overexpression mice were isolated and indicated that cardiac fibroblasts were not expressing ACE2, but ACE2 overexpression suppressed the hypoxia/re-oxygenation-induced collagen and TGF-β production in cardiac fibroblasts (Grobe et al., 2007).

In RAS and ECM, the further investigation indicated that ACE2 overexpression reduces Ang II levels and enhances Ang 1-7 to improve heart dysfunction (Dong et al., 2008; Guo et al., 2008). 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). In MI and SHR rat, ACE2 overexpression inhibiting ACE, Ang II and collagen expression, up-regulating Ang 1-7 and MMP-2 expression to attenuate LV fibrosis, and to improve LV remodeling and systolic function (Rentzsch et al., 2008; Zhao et al., 2010; Dong et al., 2012). 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). These results demonstrated that ACE2 overexpression regulates ACE-Ang II-AT1R axis and ACE2-Ang 1-7-Mas axis to affect the expression of pro-inflammatory cytokines, collagen and MMPs. These results also suggest that ACE2 serves as a protective mechanism and associated with MMPs expression, especially MMP-2 and MMP-9, to improve the hypertension, heart dysfunction and cardiac fibrosis. The significant references were listed in **Appendix 8-3**.

ACE2 knockout (KO) mice were also established in 2006 by Gurley and his group. They evidenced that ACE2-deficient mice are not any structural abnormalities and no effect on baseline blood pressures, moreover, acute Ang II infusion increased 3-fold higher Ang II concentration to cause hypertension in ACE2-deficient mice than in controls (Gurley et al., 2006). The further studies were using the different heart diseases model such as transverse aortic constriction (TAC) and left anterior descending artery ligation in ACE2 KO mice to investigate the effect of ACE2 deficient (Yamamoto et al., 2006; Kassiri et al., 2009). In MI which induced by left anterior descending artery ligation, ACE2 deficiency leads to increase phosphorylation of ERK1/2 and JNK1/2 signaling pathways, up-regulate MMP-2, MMP-9 and inflammatory cytokines and the chemokine such as interferon-gamma, interleukin-6, monocyte chemoattractant protein-1. Moreover, 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). These results support our data and appeared that loss of ACE2 facilitates adverse post-MI ventricular remodeling associated with MMP-2, MMP-9 and inflammatory factor

(Kassiri et al., 2009).

In addition, ACE2 KO mice received TAC was significant increased the Ang II concentration in heart and plasma compared to WT mice. The enhanced Ang II was induce mitogen-activated protein (MAP) kinases, MMPs and activation of NADPH oxidase, decrease cardiac contractility to develop cardiac hypertrophy and dilatation, revealed that Ang II is a major factor in development of cardiac hypertrophy and dilatation (Yamamoto et al., 2006; Patel et al., 2012). Therefore, some studies utilized Ang II infusion in WT and ACE2 KO mice to worse cardiac fibrosis and pathological hypertrophy. The WT and ACE2 KO mice infused Ang II and recombinant human ACE2 (rhACE2) revealed that Ang II induced collagen and MMP-2 expression, NADPH oxidase activity, hypertension, myocardial hypertrophy, fibrosis, and diastolic dysfunction were been attenuated by rhACE2 (Zhong et al., 2010; Alghamri et al., 2012). The significant references were listed in **Appendix 8-4**.

In this study, ACE2 shedding also been investigated in HCFs/ACE2 treated with angiotensin peptides to discuss the role that ACE2 plays in the cardiac fibrosis. In 2005, ADAM17 had been demostrated that involved with the regulated ectodomain shedding of ACE2. ADAM17 stimulated ACE2 shedding mediated phorbol ester-inducible ectodomain shedding in HEK293 cells and Huh7 cells, the ADAM17 overexpression and knockout were used to identify with this observation (Lambert et al., 2005). ADAM17 recognized in the site Arg(708) and Arg(710) within ACE2 peptides sequence and cleaved ACE2 peptide sequence between Arg(708) and Ser(709) (Lai et al., 2011). In addition, these results also had been affirmed in 3T3-L1 adipocytes and HeLa cells (Gupte et al., 2008; Jia et al., 2009). In RAS, ACE2 is homologous to one of the active sites of ACE and has 40% overall identity to ACE. However, as like captopril or other ‘classical’ ACE-inhibitors are not inhibiting the activity of ACE2, ADAM17 is not suitable to ACE shed (Zisman, 2005). Allinson and his group used antisense oligonucleotide of ADAM17 to reveal ADAM17 supressed was not effect ACE

shedding with the mercurial compound 4-aminophenylmercuric acetate (APMA) stimulated the shedding of ACE in human SH-SY5Y cells (Allinson et al., 2004).

The further study indicated that ACE2 plays a protector in severe acute respiratory syndrome (SARS) and the mechanism involved with the ectodomain shedding of ACE2. ACE2 is the receptor of SARS-CoV mediate infection of target cells (Li et al., 2003). The SARS-CoV induce ACE2 down-regulation and lead to the cell damage that Ang II induced in SARS-CoV infected cell (Oudit et al., 2009; Clarke and Turner, 2012), this situation because of SARS-CoV induce the activity of ADAM17 and to cause the shedding of ACE2 N-terminal domain and ACE2 down-regulation (Haga et al., 2008; Jia et al., 2009).

In molecular mechanism, our study revealed that Ang II and Ang 1-7 inhibited ERK1/2 activation and suppressed ADAM17 expression via AT1R and Mas receptor in HCFs/ACE2, respectively, and the suppressed ADAM17 expression caused to the ACE2 shedding decreased. Van Schaeybroeck and his group (2011) using isogenic Kras mutant HCT116 CRC cells to appear that ERk1/2 inhibition abrogated chemotherapy-induced ADAM17 activity and TGF-α shedding. These results indicated that ERK1/2 activation involved with ADAM17 activity and supported Ang II and Ang1-7 may inhibite ERK1/2 activation to reduce ADAM17 activity and inhibite ACE2 shedding in HCFs/ACE2. The suppressed ACE2 shedding may keep the ACE2 in the cell membrane against the damage that Ang II induced. In addition, Ang 1-7 suppress ACE2 shedding in HCFs/ACE2 conform to the positive feedback loop in our previous study (Lin et al., 2010).

6. Conclusions

In conclusion, ACE2 plays a protect role in hypertension, cardiac fibrosis and heart remodeling. This study investigated the associated with angiotensin peptides and MMPs, and the role of ACE2 in cardiac cells. The ACE2 expression and *ace2* promoter were study with angiotensin peptides in HCFs, the shed ACE2 activity and MMP-2 activity were also estimated in ACE2 overexpression HCFs, HCFs/ACE2. In addition, the signal pathway of ACE2, shed ACE2, ADAM17 and MMP-2 were investigated in this study.

The major results of this study are: (1) Ang II-AT1R and Ang 1-7-Mas signal pathways were via ERK-MAPK signal pathway to regulate ACE2 expression, respectively; (2) the sequences of 5'-ATTTGGA-3' within -516/-481 domain of the *ace2* promoter could regulate ACE2 expression and this sequences was the binding site of the transcription factor; (3) Ang II regulated ACE2 expression through AT1R to activate ERK-MAPK signal pathway to stimulate the -516/+20 domain within the *ace2* promoter; (4) the *ace2* promoter activity was no significant effect in HCFs treated with TGF-β1 and TNF-α; (5) in HCFs/ACE2, ACE2 overexpression enhanced MMP-2 activity and Ang II-AT1R-ERK1/2 axis decreased MMP-2 activity; (6) Ang 1-7 through Mas receptor enhanced ERK1/2 activation, but no significant effect on MMP-2 activity; (7) the ADAM17 expression could be regulated through Ang II-AT1R-ERK1/2 and Ang 1-7-Mas-ERK1/2 axes to alter ACE2 shedding; (8) the ACE2 knockout (KO) mice were re-established in our laboratory, including the mice with $ACE2^{+/-}$, $ACE2^{-/-}$ and $ACE2^{-/y}$ genotypes; (9) ACE2 deficiency enhances MMP-2 activity in heart tissue of ACE2 KO mice compared to WT mice.

The results in this study reveal angiotensin peptides regulate tha activation of ERK-MAPK and ACE2 expression via AT1R and Mas receptor, but the ACE2 expression is not be effect by TGF-β and TNF-α. Ang II also through -516/+20 sequence domain within *ace2* promoter to regulate *ace2* expression. ACE2 overexpression enhances MMP-2 activity

and Ang II decrease ADAM17 expression, shed ACE2 activity and MMP-2 activity in HCFs/ACE2. These results show the ACE2 regulation of angiotensin peptides and the association between ACE2 and the pathological heart remodeling. They also demonstrate that ACE2 regulation is an important protect mechanism against abnormal Ang II expression in the process of heart remodeling. In addition, we already introduce the ACE2 KO mice from MMRRC, this achievement is advantageous in ACE2 research development in future.

7. References

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8. Appendix

Appendix 8-1. The study of ACE2 and heart diseases

Authors	Year	Experimental model and study design	Key findings
Crackower et al.	2002	Showed *ace2* maps to a defined quantitative trait locus (QTL) on the X chromosome in three different rat models of hypertension.	In all hypertensive rat strains, ACE2 messenger RNA and protein expression were markedly reduced. Targeted disruption of ACE2 in mice results in a severe cardiac contractility defect, increased angiotensin II levels, and up-regulation of hypoxia-induced genes in the heart.
Donoghue et al.	2003	The hypothesis that cardiac ACE2 activity contributes to features of ventricular remodeling associated with the renin-angiotensin system by generating transgenic mice with increased cardiac ACE2 expression.	The gap junction proteins connexin40 and connexin43 were down-regulated in the transgenic hearts, indicating that ACE2-mediated gap junction remodeling may account for the observed electrophysiologic disturbances.
Goulter et al.	2004	Assessed changes in gene expression of ACE2, ACE, AT1R and renin in human ventricular myocardium from donors with non-diseased hearts, idiopathic dilated cardiomyopathy (IDC) and ischemic cardiomyopathy (ICM).	ACE and ACE2 is up-regulated in human IDC and ICM. ACE2 may be a relevant target for the treatment of heart failure and may have important functional consequences in heart failure.
Campbell et al.	2004	Ang I, Ang II, Ang 1-9 and Ang 1-7 were measured in arterial and coronary sinus blood of heart failure subjects receiving ACE inhibitor therapy and in normal subjects not receiving ACE inhibitor therapy.	The failure of Ang 1-9 levels to increase in response to increased Ang I levels indicated little role for ACE2 in Ang I metabolism. The levels of Ang 1-7 were more linked to those of Ang I than Ang II, consistent with its formation by endopeptidase-mediated metabolism of Ang I, rather than by ACE2-mediated metabolism of Ang II.

Ishiyama et al.	2004	Investigated in Lewis normotensive rats the effect of coronary artery ligation on the expression of ACE and ACE2 and AT1R 28 days after MI. Losartan, olmesartan, or the vehicle (isotonic saline) was administered via osmotic minipumps for 28 days after coronary artery ligation or sham operation.	Coronary artery ligation was associated with increased plasma concentrations of Ang I, Ang II, Ang 1-7, and serum aldosterone, and reduced AT1R mRNA. The effect of Ang II blockade on cardiac ACE2 mRNA that may be due to direct blockade of AT1R receptors or a modulatory effect of increased Ang 1-7.
Ferrario et al.	2005	Blood pressure, cardiac rate, and plasma and cardiac tissue levels of Ang II and Ang 1-7, together with cardiac ACE2, neprilysin, AT1R, and mas receptor mRNAs, were measured in Lewis rats 12 days after continuous administration of vehicle, lisinopril, losartan, or both drugs combined in their drinking water.	Selective blockade of either Ang II synthesis or activity induced increases in cardiac ACE2 gene expression and cardiac ACE2 activity, whereas the combination of losartan and lisinopril was associated with elevated cardiac ACE2 activity but not cardiac ACE2 mRNA. The antihypertensive action of AT1 antagonists may in part be due to increased Ang II metabolism by ACE2.
Karram et al.	2005	Examined the effects of experimental CHF induced by an aortocaval fistula (ACF) and of its treatment with Ang II and aldosterone inhibitors and antagonists on the relative levels of ACE and ACE2.	ACF increased the cardiac levels of ACE, local Ang II and aldosterone, and decreased those of ACE2. ACE isoform shift may represent an important component of the development of cardiac remodeling in response to hemodynamic overload, and its correction may contribute to the beneficial therapeutic effects of renin-angiotensin-aldosterone system inhibitors.
Burrell et al.	2005	Rats were killed at days 1, 3, and 28 after MI, or treated for 4 weeks with the ACE inhibitor ramipril. Cardiac gene and protein expression of ACE and ACE2 were assessed by quantitative real-time RT-PCR and immunohistochemistry/activity assays/in vitro autoradiography, respectively.	Both ACE and ACE2 mRNA increased in the border/infarct area compared with the viable area after MI. ACE2 protein localized to macrophages, vascular endothelium, smooth muscle, and myocytes. The increase in ACE2 after MI suggests that it plays an important role in the negative modulation of the RAS in the generation and degradation of angiotensin peptides after cardiac injury.

Huentelman et al	2005	Evaluate whether overexpression of ACE2 could protect the heart from Ang II-induced hypertrophy and fibrosis. Lentiviral vector encoding mouse ACE2 (lenti-mACE2) or GFP was injected intracardially in 5-day-old SD rats.	Transduction with lenti-mACE2 resulted in significant attenuation of the increased HW:BW and myocardial fibrosis induced by Ang II infusion. These observations demonstrate that ACE2 overexpression results in protective effects on Ang II-induced cardiac hypertrophy and fibrosis.
Díez-Freire et al.	2006	Determined whether ACE2 gene transfer could decrease high blood pressure (BP) and would improve cardiac dysfunctions induced by hypertension in the spontaneously hypertensive rat (SHR) model. Systolic BP, cardiac functions, and perivascular fibrosis were evaluated 4 mo after ACE2 gene transduction.	ACE2 gene transfer resulted in a significant attenuation of high BP and left ventricular wall thickness in the SHR. In addition, in lenti-ACE2-treated SHR, left ventricular end diastolic and end systolic diameters were increased. Finally, lenti-ACE2 treatment resulted in a significant attenuation of perivascular fibrosis in the SHR.
Gurley et al.	2006	To clarify the physiological roles of ACE2, generated mice with targeted disruption of the *ace2*. ACE2-deficient mice were viable, fertile, and lacked any gross structural abnormalities.	Acute Ang II infusion, plasma concentrations of Ang II increased almost 3-fold higher in ACE2-deficient mice than in controls, blood pressures were substantially higher in the ACE2-deficient mice than in WT. ACE2 is a functional component of the RAS and metabolizing Ang II and thereby contributing to regulation of blood pressure.
Yamamoto et al.	2006	Used $ACE2^{-/y}$ mice to analyze the role of ACE2 in the response to pressure overload, twelve-week-old $ACE2^{-/y}$ mice and wild-type (WT) mice received transverse aortic constriction (TAC) or sham operation.	In response to TAC, $ACE2^{-/y}$ mice developed cardiac hypertrophy and dilatation, displayed decreased cardiac contractility and increased fetal cardiac gene induction, compared with WT mice. Cardiac Ang II concentration and activity of mitogen-activated protein (MAP) kinases were markedly increased in $ACE2^{-/y}$ mice in response to TAC.

Ocaranza et al.	2006	The early and long-term effects of coronary artery ligation on the plasma and left ventricular angiotensin-converting enzyme (ACE and ACE2) activities, ACE and ACE2 mRNA levels, circulating angiotensin (Ang) levels [Ang I, Ang-(1-7), Ang-(1-9), and Ang II], and cardiac function were evaluated 1 and 8 weeks after experimental MI in adult SD rats.	At week 1, circulating Ang II and Ang 1-9 as well as left ventricular and plasma ACE and ACE2 activities increased in myocardial-infarcted rats as compared with controls. At 8 weeks post-MI, circulating ACE and Ang II remained higher, but plasma and left ventricular ACE2 and circulating Ang 1-9 were lower than in controls. The decrease in ACE2 expression and activity and circulating Ang 1-9 levels in late ventricular dysfunction post-MI were prevented with enalapril.
Batlle et al.	2006	Analyzed left ventricular biopsies from 30 patients with heart failure undergoing heart transplantation and 12 organ donors. The mRNA levels of ACE, ACE2, chymase and endothelial nitric oxide synthase (eNOS), were quantified by real-time PCR and mast cell density was assessed by immunohistochemistry.	There was higher ACE and chymase mRNA expression and mast cell density in failing than in control myocardium and no changes in ACE2 expression were detected. eNOS mRNA levels were lower in failing hearts. ACE2 mRNA expression is not altered in human end-stage HF.
Agata et al.	2006	Overexpression of ACE2 may be related to a reduction in Ang II level and the cardioprotective effect of olmesartan. Administration olmesartan for 4 weeks to 12-week-old stroke-prone spontaneously hypertensive rats (SHRSP) significantly reduced blood pressure and left ventricular weight compared to those in SHRSP given a vehicle.	Olmesartan significantly increased the cardiac ACE2 expression level compared to that in Wistar Kyoto rats and SHRSP treated with a vehicle. Olmesartan may exhibit an ACE inhibitory action in addition to an Ang II receptor blocking action, prevent an increase in Ang II, and protect cardiovascular remodeling through an increase in cardiac nitric oxide production and endogenous Ang 1-7 via overexpression of ACE2.

Grobe et al.	2007	Cardiac fibroblasts from SD rat hearts were grown to confluence and transduced with a lentiviral vector containing murine ACE2 cDNA under transcriptional control by the EF1α (elongation factor 1α) promoter (lenti-ACE2). Transduction of fibroblasts with lenti-ACE2 resulted in a viral dose-dependent increase in ACE2 activity.	The endogenous ACE2 activity is observed in cardiac myocytes, but not in cardiac fibroblasts. ACE2 overexpression was associated with a significant attenuation of basal and hypoxia/re-oxygenation-induced collagen production, and TGF-β expression by the fibroblasts.
Bäcklund et al.	2007	Male Wistar rats were randomised to receive either streptozotocin (diabetic group) or citrate buffer (control group) intravenously. MI was produced four weeks later by ligating the left descending coronary artery. ACE and ACE 2, AT1R, AT2R, and connective tissue growth factor (CTGF) mRNA expression were determined.	Myocardial ACE 2 and AT2R mRNA expression levels were significantly lower in diabetic compared to non-diabetic rats after MI. In contrast, AT1R, ACE and CTGF mRNA levels were up-regulated in diabetic as compared with non-diabetic rats after MI. This unbalanced activation of the RAS may influence the pathophysiology of myocardial injury in diabetes after MI.
Pan et al.	2007	Examined expression of ACE2 in the fibrillating atria of pigs and its involvement in fibrotic pathogenesis during AF. Nine adult pigs underwent continuous rapid atrial pacing to induce sustained AF and six pigs were sham controls (i.e., sinus rhythm; SR).	The relative amount of collagen type I and ACE activity in the atria with AF were significantly increased as compared with that in the SR, but ACE2 gene and protein expression in the AF subjects were significantly decreased compared with those in the SR subjects
Takeda et al.	2007	The blood pressure (BP) , plasma renin activity (PRA), plasma aldosterone concentration (PAC), heart weight, endothelium-dependent relaxation (EDR), and mRNA of collagen III, angiotensinogen, ACE, and ACE2 in the heart were measured in Dahl salt-sensitive hypertensive (DS) rats and in Dahl salt-resistant (DR) rats fed high or low salt diets.	A high salt diet increased BP (140%), heart/body weight (132%), and collagen III mRNA levels (146%) and decreased PRA and PAC concomitant with increased expression of cardiac angiotensinogen mRNA and decreased mRNA levels of ACE2 in DS rats. In DS rats, blockade of aldosterone or Ang II protects cardiac hypertrophy and fibrosis by inactivation of the local RAAS in the heart.

Grobe et al.	2007	Hypothesized that chronic infusion of Ang 1-7 would prevent cardiac remodeling induced by chronic infusion of Ang II. Infusion of Ang II into adult SD rats resulted in significantly increased blood pressure, myocyte hypertrophy, and midmyocardial interstitial fibrosis.	Chronic infusion of Ang II, co-infusion of Ang 1-7 resulted in significant attenuations of myocyte hypertrophy and interstitial fibrosis, without significant effects on blood pressure, but had no effect on AT1R or AT2R in cardiac tissue with or without co-infusion of Ang 1-7. Indicated an anti-remodeling role for Ang 1-7 in cardiac tissue maybe at least partially mediated through an Ang 1-7 receptor.
Trask et al.	2007	The enzyme participates in the cardiac processing of Ang II and Ang 1-7 is equivocal. Utilized the Langendorff preparation to characterize the ACE2 pathway in isolated hearts from male normotensive SD [$Tg^{(-)}$] and hypertensive [mRen2]27 [$Tg^{(+)}$] rats. During a 60-min recirculation period with 10 nM Ang II, the presence of Ang 1-7 was assessed in the cardiac effluent.	Ang 1-7 generation from Ang II was similar in both the normal and hypertensive hearts. ACE2 inhibition (MLN-4760, 1 μM) significantly reduced Ang 1-7 production in the $Tg^{(+)}$ rats, whereas the inhibitor had no significant effect in the $Tg^{(-)}$ rats. Predominant expression of cardiac ACE2 activity in the $Tg^{(+)}$ may be a compensatory response to the extensive cardiac remodeling.
Burchill et al.	2008	Assessed whether ACE2 plays a role in the cardiac remodeling that occurs in experimental acute kidney injury (AKI). SD rats had sham (control) or subtotal nephrectomy surgery (STNx). Control rats received vehicle, and STNx rats received the ACE inhibitor (ACEi) ramipril or vehicle orally after surgery. Rats with AKI had polyuria proteinuria and hypertension.	Cardiac structural changes were present and characterized by LVH, fibrosis and increased cardiac brain natriuretic peptide (BNP) and cardiac ACE2 mRNA. Ramipril decreased blood pressure, LVH, fibrosis and BNP mRNA, and reduced in cardiac ACE2 activity. ACE2 may have a cardioprotective role in AKI, particularly since amelioration of adverse cardiac effects with ACE inhibition was associated with normalization of cardiac ACE2 activity.

Wang et al.	2008	Coarctation of suprarenal abdominal aorta was reproduced in 8 week-old male SD rats and then randomized into 4 groups, including a sham group, a suprarenal aortic coarctation group, and suprarenal aortic coarctation with low and high-dose Telmisartan treatment groups. Changes in both protein quantity and gene expressions of Ang II, ACE2 and ACE were determined.	Suprarenal abdominal aortic coarctation induced a significant increase in the plasma and myocardium AngII concentration and expressions of gene and protein of ACE and ACE2. Telmisartan further increased the concentration of AngII in plasma and myocardium in a dose-dependent manner, and induced a dose-dependent increase in both protein and gene expression of ACE2.
Lovren et al.	2008	Hypothesized that ACE2 is a novel target to limit endothelial dysfunction and atherosclerosis. To perform in vitro gain and loss of function experiments in endothelial cells and evaluated *in vivo* angiogenesis and atherosclerosis in apolipoprotein E-knockout mice treated with Ad-ACE2.	Overexpression of ACE2 in human endothelial cells stimulated endothelial cell migration and tube formation, promoted capillary formation and neovessel maturation in vivo, reduced atherosclerosis in apolipoprotein E-knockout mice and attenuated Ang II-induced reactive oxygen species production in part through decreasing the expression of $p22^{phox}$.
Nakamura et al.	2008	Generated mice with targeted disruption of the *ace2* gene and compared the cardiovascular function of $ACE2^{-/y}$ mice with that of their wild-type littermates. ACE2-deficient mice were viable and fertile and lacked any gross structural abnormalities, displayed significantly enlarged hearts with impaired systolic and diastolic function.	The Ang II level was elevated in the plasma and heart of $ACE2^{-/y}$ mice. Pharmacological blockade of AT1R with candesartan attenuated the development of cardiac dysfunction in $ACE2^{-/y}$ mice. These results suggest that enhanced stimulation of AT1R may play a role in the development of cardiac dysfunction observed in ACE2-deficient mice.

Der et al.	2008	Used a gene overexpression approach to investigate the role of ACE2 in cardiac function and remodeling after MI. Rats received an intracardiac injection of lentivirus containing ACE2 cDNA, followed by permanent coronary artery ligation (CAL) of the left anterior descending artery. Cardiac functions, viability, and pathophysiology were assessed.	Lenti-ACE2-treated CAL rats showed a 60% reduction in delayed contrast-enhanced LV volume after gadodiamide injection, indicating early ischemic protection of myocardium by ACE2. Lenti-ACE2 rats demonstrated a complete rescue of cardiac output, a 41% rescue of ejection fraction, a 44% rescue in contractility, a 37% rescue in motion, and a 53% rescue in LV anterior (infracted) wall thinning compared with control CAL rats.
Epelman et al.	2008	Developed a sensitive and specific assay to measure sACE2 activity in human plasma and screened a heterogeneous group of patients suspected of having clinical HF.	Increasing sACE2 plasma activity strongly correlated with a clinical diagnosis of HF, worsening left ventricular ejection fraction, and increasing B-type natriuretic peptide levels. The sACE2 activity was increased in patients with both ischemic and nonischemic cardiomyopathies and also in patients with clinical HF.
Garabelli et al.	2008	Determined the metabolism of angiotensins in wild-type (WT), $ACE^{-/-}$ and ACE2 null mice ($ACE2^{-/-}$).	Ang II was converted almost exclusively to Ang 1-7 in the cardiac membranes of WT and $ACE^{-/-}$ strains, although generation of Ang 1-7 was greater in the $ACE^{-/-}$ mice. The ACE2 inhibitor MLN4760 significantly attenuated Ang II metabolism and the subsequent formation of Ang 1-7 in both strains. In the $ACE2^{-/-}$ hearts, Ang II metabolism and the generation of Ang 1-7 were significantly attenuated.

Qin et al.	2008	Suprarenal abdominal aortic coarctation was performed to create the pressure overload induced left ventricular hypertrophy model in rats. Rats were randomly divided into: (A) normal control group; (B) normal control group treated with atorvastatin; (C) sham group; (D) atorvastatin given orally by gastric gavage for 4 weeks; (E) vehicle group. ACE2 mRNA and its protein expression were detected by real-time RT-PCR and Western blot.	ACE2 mRNA and its protein expression increase significantly in hypertrophic myocardium in rats; atorvastatin can attenuate cardiac hypertrophy due to pressure overload in rats effectively, and part of this anti-hypertrophy effect may be attributed to decrease ACE2 mRNA and protein expression.
Guy et al.	2008	Revealed the functional expression of ACE2 in human cardiac myofibroblasts, cells that are essential to the maintenance of normal cardiac architecture and also play a key role in myocardial remodeling, and demonstrate the presence of ACE2 as an ectoenzyme and reveal that ACE2 undergoes phorbol-12-myristate-13-acetate-inducible ectodomain shedding from the membrane.	ACE2 to be expressed constitutively in cardiac myofibroblasts there were no detectable levels in either vascular smooth muscle cells or vascular endothelium, indicating that ACE2 expression is not ubiquitous. Reported co-expression of ACE and ACE2 in human cardiac myofibroblasts and may therefore present a model primary system for study of the comparative cell biology of ACE2 and ACE and their potentially opposing roles in myocardial remodeling.
Nadu et al.	2008	Investigated the expression of specific ECM proteins in cardiac hypertrophy induced by isoproterenol in TGR(A1-7)3292 rats. Additionally, changes in circulating and tissue RAS were analyzed. Left ventricles (LV) were used for quantification of collagen type I, III, and fibronectin.	TGR(A1-7)3292 presented lower Ang II levels and angiotensinogen expression and a higher ACE2 expression in LV. Isoproterenol treatment increased cardiac Ang II concentration only in normal rats, which was associated with an increase in ACE2 and a decrease in Mas expression.

Gallagher et al.	2008	Cardiac ACE2 is elevated following treatment of coronary artery-ligated rats with AT1R blockers (ARBs). Cardiac myocytes and fibroblasts were isolated from neonatal rats to determine the molecular mechanisms for the ACE2 up-regulation by ARB treatment.	Ang II significantly reduced ACE2 activity and down-regulated ACE2 mRNA in cardiac myocytes, effects blocked by the ARB losartan, indicating that Ang II regulates ACE2. Ang II also reduced ACE2 mRNA in cardiac fibroblasts. Ang II or Endothelin-1 (ET-1) activates extracellular signal-regulated kinase (ERK1/2) to reduce ACE2.
Hernández et al.	2008	To identify compounds that enhance ACE2 activity using a novel conformation-based rational drug discovery strategy and to evaluate whether such compounds reverse hypertension-induced pathophysiologies.	The xanthenone and resorcinolnaphthalein that enhanced ACE2 activity in a dose-dependent manner. Acute in vivo administration of the xanthenone resulted in a dose-dependent transient and robust decrease in blood pressure. Chronic infusion of the xanthenone resulted in a modest decrease in the spontaneously hypertensive rat blood pressure
Shenoy et al.	2009	Cardiac fibrosis and hypertrophy were examined in deoxycorticosterone acetate (DOCA)-salt treated rats. Bilaterally ovariectomized (Ovx) female SD rats were drank with 0.15M NaCl solution and randomly assigned to one of the following groups: Ovx-control; Ovx-DOCA; Ovx-DOCA+low-dose 17beta-estradiol (E2); or Ovx-DOCA+high-dose E2.	Increased cardiac levels of angiotensin converting enzyme (ACE) with DOCA treatment, which was attenuated by E2 replacement. Furthermore, increased levels of cardiac ACE2 protein were observed in animals receiving high-dose E2 replacement.
Jia et al.	2009	The present study was to investigate the effects of G-CSF on cardiac remodelling in Ang II-induced hypertrophy. Four groups of mice were investigated: control; injected with recombinant human G-CSF; pressor doses of Ang II or saline; infused with Ang II and G-CSF.	Ang II treatment significantly elevated blood pressure and ACE expression, down-regulation of ACE2 expression and caused cardiac hypertrophy and fibrosis in mice. G-CSF did not reduce the Ang II-induced increase in blood pressure, but ameliorated the development of cardiac fibrosis and hypertrophy, and reduced cardiac levels of ACE and increased ACE2 expression.

Kassiri et al.	2009	Hypothesized that ACE2 deficiency may compromise the cardiac response to MI (induced by left anterior descending artery ligation).	In ACE2-deficient hearts, elevated myocardial levels of Ang II, MMP-2 and MMP-9, and decreased levels of Ang 1-7 in the infarct-related zone was associated with increased production of reactive oxygen species. Treatment of $Ace2^{-/y}$-MI mice with irbesartan, an AT1R blocker, reduced nicotinamide-adenine dinucleotide phosphate oxidase activity, infarct size, MMP activation, and myocardial inflammation, ultimately resulting in improved post-MI ventricular function.
Masson et al.	2009	The role of ACE2 in the regulation of cardiac structure and function, as well as maintenance of systemic blood pressure, remains poorly understood. To assess ACE2 function *in vivo*, used a recombinant adeno-associated virus 6 delivery system to provide 11-week overexpression of ACE2 in the myocardium of stroke-prone spontaneously hypertensive rats.	ACE2, as well as the ACE inhibitor enalapril, significantly reduced systolic blood pressure. However, in the heart, ACE2 overexpression resulted in cardiac fibrosis. Furthermore, global gene expression profiling demonstrated the activation of profibrotic pathways in the heart mediated by ACE2 gene delivery. This study demonstrates that sustained overexpression of ACE2 in the heart in vivo leads to the onset of severe fibrosis.
Epelman et al.	2009	Measured sACE2 activity in 113 patients with chronic systolic heart failure (left ventricular ejection fraction [LVEF] ≤ 35%, New York Heart Association Class II-IV). Comprehensive echocardiography was performed at the time of blood sampling.	Patients who had higher sACE2 plasma activity were more likely to have a lower LVEF, greater right ventricular systolic dysfunction, higher estimated pulmonary artery systolic pressure, larger left ventricular end-diastolic diameter, and higher plasma NT-proBNP levels.
Calò et al.	2010	Evaluated the levels of ACE2 and Ang 1-7 in Bartter's/Gitelman's patients (BS/GS) who have elevated Ang II and endogenous blockade of AT1R signaling compared with healthy subjects and essential hypertensives (EH).	ACE2 and Ang 1-7 levels were significantly different between the three groups, ACE2 and Ang1-7 were significantly elevated in BS/GS compared with either C or EH. ACE2 and Ang 1-7 directly correlated only in BS/GS.

Ohtsuki et al.	2010	Evaluated the expression of the ACE2 gene by means of real-time RT-PCR in myocardium from 14 patients with end-stage heart failure.	The amount of ACE2 mRNA positively correlated with left ventricular (LV) end-diastolic diameter. The up-regulation of the ACE2 gene in the LV myocardium of patients with severe heart failure was associated with the degree of LV dilatation and may thereby constitute an important adaptive mechanism to retard the progression of adverse LV remodeling.
Varagic et al.	2010	Examined the changes in cardiac Ang 1-7, its forming enzyme ACE2 and receptor mas in response to a high salt diet in spontaneously hypertensive rats (SHR). Eight-week-old male spontaneously hypertensive rats (SHR) were given an 8% salt diet for 5 weeks.	Salt-induced left ventricular remodeling and diastolic dysfunction were associated with diminished levels of Ang 1-7 in the heart and no changes in cardiac Ang II levels. Exposure to high salt intake decreased cardiac ACE2 mRNA and protein level. There was no difference in the protein levels of AT1R and mas receptors between the two experimental groups.
Hu et al.	2010	Thirty 8-week-old male Wistar rats were randomly divided into control group, diabetic model group and irbesartan group. Diabetes was induced by a single intraperitoneal injection of STZ. The diabetes rats in irbesartan group were given irbesartan. ELISA was used to measure myocardial Ang II content in the rats, and myocardial ACE2 mRNA expression was determined by real-time PCR.	Myocardial Ang II level in the diabetic model group was significantly higher than that in the control group. Irbesartan administration significantly lowered cardiac Ang II levels in the diabetic rats. The rats in irbesartan group showed significantly increased myocardial ACE2 mRNA expression compared with those in the control and diabetic rat groups.
Kim et al.	2010	Treated adult male Sprague-Dawley rats with either placebo (PL) or C16, a selective ACE2 inhibitor, after permanent coronary artery ligation or sham operation.	Daily C16 administration from postoperative days 2 to 28 at a dose that inhibited myocardial ACE2 activity was associated with a significant increase in post-MI size and reduction in LV % fractional shortening. ACE2 exerts cardioprotective effects by preserving jeopardized cardiomyocytes in the border zone.

Guo et al.	2010	Models of acute myocardial infarction (AMI) were produced by ligation of left anterior descending coronary artery, 24 hours after operation the rats were randomly divided into control and experiment groups, then respectively administrated with NS, fosinopril and low, middle and high dosage of Panax notoginseng saponins (PNS).	Compared with the NS group, ACE2 increased and TNF-α significantly decreased in low-dose PNS group, middle and high-dose groups. PNS can stimulate ACE2 to inhibit the expression of TNF-α and enhance the antioxidance to reduce pathological injury of cardiac myocytes in myocardial ischemia and cardiac muscle, which can improve ventricular remodeling.
Trask et al.	2010	hypothesis that inhibition of ACE2 in the hearts of (mRen2)27 hypertensive rats may accelerate progression of cardiac hypertrophy and fibrosis by preventing conversion of Ang II into the antifibrotic peptide, Ang 1-7. Male (mRen2)27 transgenic hypertensive rats were administered either vehicle (0.9% saline) or the ACE2 inhibitor, MLN-4760, subcutaneously via mini-osmotic pumps.	Ang II levels were associated with significant increases in both LV anterior, posterior, and relative wall thicknesses, as well as interstitial collagen fraction area and cardiomyocyte hypertrophy in the transgenic animals chronically treated with the ACE2 inhibitor. Chronic inhibition of ACE2 causes an accumulation of cardiac Ang II, which exacerbates cardiac hypertrophy and fibrosis without having any further impact on blood pressure or cardiac function.
Lin et al.	2010	Human cardiac fibroblasts (HCF) were used to test the regulatory effects of Ang II and Ang 1-7 on ACE2 expression.	Ang II-mediated ACE2 up-regulation was blocked by antagonists of AT1R and ERK-MAPK signaling pathways. Additionally, Ang 1-7 increased ACE2 expression, and this up-regulation was inhibited by Mas receptor blockade. Ang II-up-regulated ACE2 may increase Ang 1-7 formation from Ang II, and that ACE2 expression is further enhanced by Ang 1-7 in a positive feedback loop.

Wysocki et al.	2010	Examined whether soluble human recombinant ACE2 (rACE2) can efficiently lower Ang II and increase Ang 1-7 and whether rACE2 can prevent hypertension caused by Ang II infusion as a result of systemic versus local mechanisms of ACE2 activity amplification. rACE2 was infused via osmotic minipumps for 3 days in conscious mice or acutely in anesthetized mice.	In acute studies, rACE2 prevented the rapid hypertensive effect of Ang II, and this was associated with both a decrease in Ang II and an increase in Ang 1-7 in plasma. Moreover, during infusion of Ang II, the effect of rACE2 on blood pressure was unaffected by a specific Ang 1-7 receptor blocker, A779, and infusing supraphysiologic levels of Ang 1-7 had no effect on blood pressure.
Johnson et al.	2011	Hypothesize that rhACE2 will improve RV function in a pressure overload model. rhACE2 administered improved RV systolic and diastolic function in pulmonary artery banded mice as measured by in vivo hemodynamics.	The rhACE2 increased RV ejection fraction and decreased RV end diastolic pressure and diastolic time constant. In pulmonary artery banded mice, rhACE2 increased Mas receptor expression and normalized connexin37 expression.
Ferreira et al.	2011	The XNT, an ACE2 activator, reverses hypertension-induced cardiac and renal fibrosis in spontaneously hypertensive rats (SHRs). Determined the mechanisms underlying the protective effects of XNT against cardiac fibrosis.	Chronic infusion of XNT significantly increased cardiac ACE2 activity in SHRs, the increased ACE2 was associated with decreased cardiac collagen content. Furthermore, the antifibrotic effect of XNT correlated with increased cardiac Ang 1-7 and reduced ERK phosphorylation, though no change in cardiac AT1R protein levels was observed.
Zheng et al.	2011	Investigated ACE2 and neuronal nitric oxide (NO) synthase (nNOS) expression within the paraventricular nucleus (PVN) of rats with chronic heart failure (CHF), then determined the effects of ACE2 gene transfer in the PVN on the contribution of NO-mediated sympathoinhibition in rats with CHF.	There were decreased expressions for ACE2, the Ang 1-7 receptor, and nNOS within the PVN of rats with CHF. AdACE2 transfection significantly increased nNOS protein levels in the PVN of CHF rats. The results highlight the importance of increased expression and subsequent interaction of ACE2 and nNOS within the PVN, leading to a reduction in sympathetic outflow in the CHF condition.

Xiao et al.	2011	Hypothesized that central overexpression of ACE2 decreases sympathetic outflow and enhances baroreflex function in chronic heart failure (CHF). Transgenic mice overexpressing human ACE2 selectively in the brain (SYN-hACE2 [SA]) and wild-type littermates (WT) were used. CHF was induced by permanent coronary artery ligation.	Compared with WT mice with CHF, brain-selective ACE2 overexpression attenuated left ventricular end-diastolic pressure; decreased urinary norepinephrine excretion; baseline renal sympathetic nerve activity; and enhanced baroreflex sensitivity. ACE2 overexpression exerts a potential protective effect in CHF involves Ang 1-7-Mas signaling, as well as a decrease in AT1R signaling in the medulla.
Sukumaran et al.	2011	Investigated the cardioprotective effects of telmisartan, a well-known angiotensin receptor blockers (ARBs) against experimental autoimmune myocarditis (EAM). EAM was induced in Lewis rats by immunization with porcine cardiac myosin.	Telmisartan lowered myocardial protein expressions of NADPH oxidase subunits 3-nitrotyrosine, $p47^{phox}$, $p67^{phox}$, Nox-4 and superoxide production significantly than vehicle-treated rats. In contrast myocardial protein levels of ACE2, Ang 1-7 Mas receptor were up-regulated in the telmisartan treated group compared with those of vehicle-treated rats.
Soro-Paavonen et al.	2011	Examined whether ACE2 activity is altered in patients with type 1 diabetes (T1D), with and without diabetic nephropathy. Quantitative ACE2 activity in serum was measured by a fluorometric assay in 859 patients with T1D in the Finnish Diabetic Nephropathy (FinnDiane) study and in 204 healthy controls.	ACE2 activity was increased in men with T1D and in male and female patients who were on ACE inhibitor (ACEi) treatment Male and female patients with coronary heart disease (CHD) had significantly increased ACE2 activity. ACE2 activity correlated positively with systolic blood pressure, AIx and diabetes duration, and negatively with estimated glomerular filtration rate among male T1D patients.
Yamazato et al.	2011	The hypothesis that ACE2 expression is decreased in the nucleus tractus solitarius (NTS) of spontaneously hypertensive rats (SHRs) and that its gene transfer in this nucleus would lead to beneficial effects on baroreflex function since this enzyme is key in the regulation of the vasoprotective axis of the RAS.	ACE2 protein levels and its activity were significantly decreased in the NTS of SHRs compared to normotensive Wistar-Kyoto (WKY) control rats. A 60% increase in heart rate baroreflex sensitivity in the lenti-ACE2 injected SHRs compared with the lenti-GFP injected control SHRs.

Tan et al.	2011	Eighteen male spontaneously hypertensive rats (SHR) and 20 normotensive Wistar-Kyoto rats were randomly allocated to four groups of rats each and received either Ang 1-7 in saline or saline alone. Tail-cuff systolic blood pressures were recorded and ACE2 and Mas expression was measured using quantitative real-time PCR (QRT-PCR) and Western blotting.	Cardiac and renal ACE2 mRNA was decreased in SHR. No effects on blood pressure, Ang 1-7 down-regulated cardiac ACE2 mRNA in normotensive rats but did not change renal ACE2. Ang 1-7 down-regulated cardiac Mas mRNA of Wistar rats only, and renal Mas mRNA of SHR receiving Ang 1-7 was decreased.
Inaba et al.	2011	Chimeric hypertensive mice that exhibit activation of the human RAS were produced by mating human renin (hRN) and human angiotensinogen (hANG) transgenic mice. Persistent NO inhibition with NG-nitro-L-arginine methyl ester (L-NAME) was administratied, blood pressure (BP) markedly increased in the chimeric mice (hRN/hANG-Tg), whereas wild-type mice (C57BL/6J) showed little increase in BP.	ACE2 mRNA expression and activity in cardiac tissue were markedly reduced in L-NAME-treated hRN/hANG-Tg. Co-administration of an AT1R blocker (ARB), olmesartan, inhibited L-NAME-induced cardiovascular remodeling and improved the reduction in cardiac ACE2. Cardiovascular remodeling induced by persistent NO inhibition was enhanced in hRN/hANG-Tg. An ARB, olmesartan, blunted cardiac remodeling induced by NO inhibition with RAS activation partially through the ACE2/Ang 1-7/Mas axis in addition to directly through its classical ACE/Ang II/AT1R axis-blocking action.
Sukumaran et al.	2012	Investigated the cardioprotective effects of telmisartan in rats with dilated cardiomyopathy [DCM] after experimental autoimmune myocarditis [EAM]. DCM was elicited in Lewis rats by immunization with cardiac myosin, the surviving Lewis rats were divided into two groups and treated with either telmisartan or vehicle.	Myocardial protein levels of ACE2 and Ang 1-7 Mas receptor were upregulated in the telmisartan-treated group compared with vehicle-treated rats. Telmisartan treatment significantly improved LV function and ameliorated the progression of cardiac remodeling through modulation of the ACE2/Ang 1-7/Mas receptor axis in rats with DCM after EAM.

Malfitano et al.	2012	Investigated the effects of angiotensin-converting enzyme inhibitor (ACEI), enalapril, on cardiac and autonomic functions in diabetic rats. Diabetes was induced by streptozotocin, and rats were treated with enalapril.	The ACEI improved diastolic cardiac responses to volume overload and total power of heart rate variability, reduced the ACE activity and protein expression and cardiac Ang II levels, and increased ACE2 activity, despite unchanged blood pressure.
Vasku et al.	2012	Determine whether invasively measured central pulse pressure (PP) in patients indicated for coronarography is associated with two common polymorphisms in the ACE2 region (rs4646156 and rs4646174).	In men, there was a higher incidence of previous MI in G0 genotype carriers of rs54646174. The AA genotype of rs4646156 had a 7.81× higher risk of severe angina pectoris in women. A significant difference in allelic frequency of ACE2 rs4646174 was found between women with and without significant stenoses of the circumflex branch of the left coronary artery.
Lo et al.	2012	Explored ACE2 is a negative regulator of Ang II-mediated pathological effects *in vivo*. The pressor response, NADPH oxidase activation and superoxide generation in the heart, kidney and blood vessels were been detected in Ang II-induced pressor response rat and SHR rat treated rhACE2.	Treatment with rhACE2 inhibited Ang II-mediated phosphorylation of the myocardial ERK1/2 pathway in WKY rats with congruent results seen in SHR hearts. In addition, rhACE2 also suppressed the pressor response, NADPH oxidase activation and superoxide generation in Ang II-induced pressor response rat and SHR rat.
Dong et al.	2012	Investigated ACE2 overexpression may inhibit myocardial collagen accumulation and improve left ventricular (LV) remodeling and function in diabetic cardiomyopathy. Diabetic rats (induced by a single intraperitoneal injection of streptozotocin) were further divided into adenovirus-ACE2, adenovirus-EGFP, losartan, and mock groups. LV volume; LV systolic and diastolic function; extent of myocardial fibrosis; ACE2 and MMP-2 activities were evaluated.	The adenovirus-ACE2 group showed increased ACE2, MMP-2 activities, and LV ejection fractions; decreased LV volumes, myocardial fibrosis, and ACE, Ang-II, and collagen expression in comparison with the adenovirus-EGFP and control groups. ACE2 was superior to losartan in improving LV remodeling and function and reducing collagen expression may through transforming growth factor-beta inhibited and enhance MMP-2 to degrade collagen.

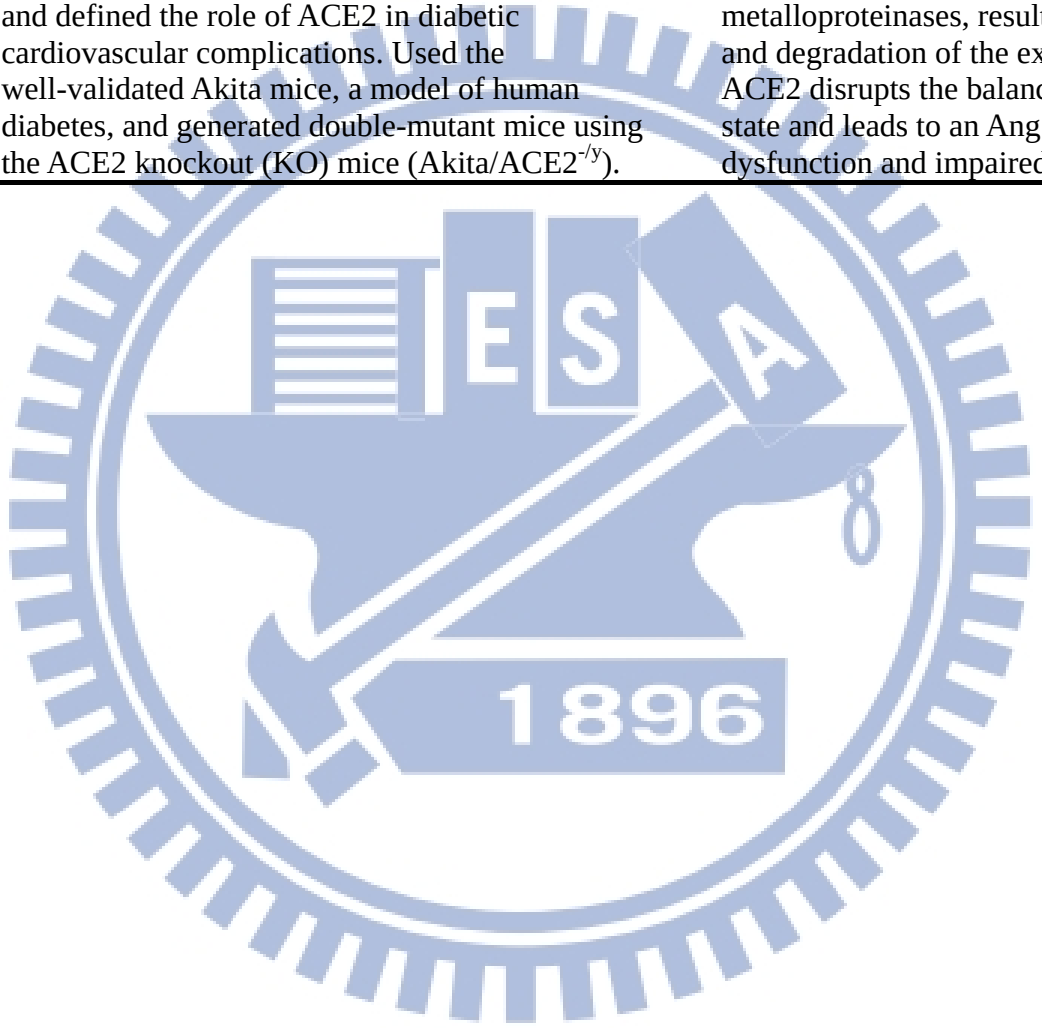
Patel et al.	2012	Investigated whether ACE2 polymorphisms are associated with hypertension, left ventricular (LV) mass, and cardiac function in type 2 diabetes. ACE2 (rs1978124, rs2074192, rs4240157, rs4646156, rs4646188) were examined in 503 Caucasian subjects with type 2 diabetes.	In men, hypertension was more prevalent with the ACE2 rs2074192 C allele, rs4240157 G allele and rs4646188 T allele; the rs1978124 A allele was associated with a significantly lower ejection fraction compared to the G allele. In women, the prevalence of hypertension was higher with the rs4240157 G allele, and the rs1978124 A allele was associated with significantly higher LV mass.
Murça et al.	2012	Evaluated whether the activation of endogenous ACE2 would improve the cardiovascular autonomic dysfunction of diabetic rats. The type 1 diabetes rats that streptozotocin-induced were treated with ACE2 activator, XNT, or saline. Autonomic cardiovascular parameters were evaluated in conscious animals, and an isolated heart preparation was used to analyze cardiac function.	Diabetes induced a significant decrease in the baroreflex bradycardia sensitivity was been improved by XNT treatment. XNT administration also enhanced the bradycardia induced by the chemoreflex activation in non-diabetic animals. Thus, XNT protects against the autonomic and cardiac dysfunction induced by diabetes.
Burchill et al.	2012	Assessed the effect of an ACEi and ARB, alone and in combination, on cardiac ACE2 in a rat MI model to evidence the benefits of ACEis and ARBs are mediated through increasing ACE2 after MI.	Ramipril and valsartan improved remodeling through ACE inhibited, and angiotensin receptor blocked, neither treatment alone nor in combination augmented cardiac ACE2 expression. Results suggest that the cardioprotective effects of ramipril and valsartan are not mediated through up-regulation of cardiac ACE2.

Alghamri et al.	2012	Conducted studies in ACE2 deficient mice to determine whether enzyme loss would exacerbate the cardiac and vascular pathological responses to chronic subcutaneous (sc) Ang II infusion. ACE2 knockout (KO) and wild type (WT) mice were infused with Ang II using mini-osmotic pumps, and the cardiac function, and vascular inflammation were examined.	Cardiac dysfunction was associated with hypertrophic cardiomyopathy shown by increased left-ventricular wall thickness, average cardiomyocyte cross-sectional area, and heart weight/body weight ratio. Oxidative stress and collagen staining reveal heart remodeling in the myocardium and aorta in Ang II infused ACE2 KO mice.
Tikellis et al.	2012	Examine the effect of ACE2 deficiency on the early cardiac and vascular changes associated with experimental diabetes. Streptozotocin diabetes was induced in male C57BL6 mice, Ace2-KO (knockout) mice, ApoE (apolipoprotein E)-KO mice and ApoE/Ace2-double-KO mice, and markers of RAS (renin-angiotensin system) activity, cardiac function and injury were assessed.	The induction of diabetes in wild-type mice led to reduced ACE2 expression and activity in the heart, elevated circulating AngII levels and reduced cardiac Ang 1-7. The major phenotypic differences between Ace2-deficient and Ace2-replete mice with respect to BP (blood pressure) and cardiac hypertrophy were eliminated following the induction of diabetes.
Patel et al.	2012	Defined the role of ACE2 in diabetic cardiovascular complications. Akita mice, a model of human diabetes, and generated double-mutant mice using the ACE2 knockout (KO) mice (Akita/ACE2$^{-/y}$) were using in this study. Loss of ACE2 in diabetic mice leads to increased plasma and tissue Ang II, resulting in systolic dysfunction on a background of impaired diastolic function.	Systolic dysfunction in Akita/ACE2KO mice was linked to enhanced activation of NADPH oxidase and metalloproteinases, resulting in greater oxidative stress and degradation of the extracellular matrix. Treatment with the AT1R blocker, irbesartan rescued the systolic dysfunction, normalized altered signaling pathways, flow-mediated dilation, and the increased oxidative stress in the cardiovascular system.

Appendix 8-2. ACE2 and gelatinase (MMP-2 and MMP-9)

Authors	Year	Experimental model and study design	Key findings
Kassiri et al.	2009	In response to MI (induced by left anterior descending artery ligation), there was a persistent increase in ACE2 protein in the infarct zone in wild-type mice, whereas loss of ACE2 enhanced the susceptibility to MI. In ACE2-deficient hearts, elevated myocardial levels of Ang II and decreased levels of Ang 1-7 in the infarct-related zone was associated with increased production of reactive oxygen species.	ACE2 deficiency leads to upregulate inflammatory cytokines, interferon-gamma, interleukin-6, and the chemokine, monocyte chemoattractant protein-1, as well as increased phosphorylation of ERK1/2 and JNK1/2 signaling pathways, and then increased MMP-2 and MMP-9 levels with MMP-2 activation in the infarct and peri-infarct regions, as well as increased gelatinase activity leading to a disrupted extracellular matrix structure after MI.
Bodiga et al.	2011	Using the aortic constriction model, we subjected wild-type ($Ace2^{+/y}$), ACE2 knockout (ACE2 KO, $Ace2^{-/y}$), $p47^{phox}$ knockout ($p47^{phox}$ KO), and ACE2/$p47^{phox}$ double KO mice to pressure overload. Examined changes in peptide levels, NADPH oxidase activity, gene expression, MMPs activity, pathological signalling, and heart function.	Activation of Ang II-stimulated signalling pathways in the ACE2-deficient myocardium was associated with increased expression and phosphorylation of $p47^{phox}$, NADPH oxidase activity, and superoxide generation, leading to enhanced MMP-mediated degradation of the extracellular matrix. Ang 1-7 supplementation suppressed the increased NADPH oxidase and rescued the early dilated cardiomyopathy in pressure-overloaded ACE2 KO mice.
Feng et al.	2011	The role of Ang II and ACE2 in the metastasis of non-small cell lung cancer (NSCLC) and the effects on MMPs are still unknown. The anti-invasive effect and mechanism of ACE2 were investigated *in vitro* and *in vivo*.	The overexpression of ACE2 reduces the invasive ability of A549 cells *in vitro*, and the inhibitory role of ACE2 was mediated through the down-regulation of MMP-2 and MMP-9.

Patel et al.	2012	Hypothesized that loss of ACE2 exacerbates cardiovascular complications induced by diabetes and defined the role of ACE2 in diabetic cardiovascular complications. Used the well-validated Akita mice, a model of human diabetes, and generated double-mutant mice using the ACE2 knockout (KO) mice (Akita/ACE2$^{-/y}$).	Systolic dysfunction in Akita/ACE2 KO mice was linked to enhanced activation of NADPH oxidase and metalloproteinases, resulting in greater oxidative stress and degradation of the extracellular matrix. Loss of ACE2 disrupts the balance of the RAS in a diabetic state and leads to an Ang II/AT1R-dependent systolic dysfunction and impaired vascular function.



Appendix 8-3. The study of ACE2 overexpression

Authors	Year	Experimental model and study design	Key findings
Huentelman et al.	2005	Evaluated overexpression of ACE2 protect the heart from Ang II-induced hypertrophy and fibrosis. WT mice and ACE2 overexpression mice (lenti-mACE2) were infused Ang II and then detected the body weight and myocardial fibrosis.	ACE2 overexpression were significant attenuation of the increased HW:BW and myocardial fibrosis induced by Ang II infusion. These results demonstrate that ACE2 overexpression results in protective effects on Ang II-induced cardiac hypertrophy and fibrosis.
Díez-Freire et al.	2006	SHR and normotensive WKY rats received a single intracardiac bolus injection of lentiviral vector containing either murine ACE2 or control enhanced green fluorescent protein (EGFP) genes. Systolic BP, cardiac functions, and perivascular fibrosis were evaluated 4 mo after ACE2 gene transduction.	ACE2 gene transfer resulted in a significant attenuation of high BP in the SHR. Lenti-ACE2-treated SHR showed an 18% reduction in left ventricular wall thickness, a 12% increase in left ventricular end diastolic and a 21% increase in end systolic diameters.
Grobe et al.	2007	Examined the effects of ACE2 gene delivery to cultured cardiac fibroblasts after acute hypoxic exposure. Cardiac fibroblasts from SD rat hearts transduced with a lentiviral vector containing murine ACE2 cDNA under transcriptional control by the EF1alpha (elongation factor 1alpha) promoter (lenti-ACE2).	Endogenous ACE2 activity is observed in cardiac myocytes, but not in cardiac fibroblasts. A significant attenuation of both basal and hypoxia/re-oxygenation-induced collagen and TGF-β production by the lenti-ACE2 transduced fibroblasts.

Yamazato et al.	2007	Compared ACE2 expression in the rostral ventrolateral medulla (RVLM) of Wistar-Kyoto rats and spontaneously hypertensive rats and to determine whether RVLM ACE2 is involved in blood pressure control by ACE2 immunoreactivity, western blot, mean arterial pressure and heart rate.	The western blots assay revealed a 40% decrease in ACE2 in the RVLM of spontaneously hypertensive rat compared with Wistar-Kyoto rats. Lentiviral-mediated overexpression of ACE2 (lenti-ACE2) was associated with a decrease in mean arterial pressure exclusively in the spontaneously hypertensive rat and heart rate; it also appeared that decrease in ACE2 in the RVLM is associated with hypertensive state.
Feng et al.	2008	Ad-hACE2-eGFP infection produced time-dependent expression and activity in the neuronal cells and mouse subfornical organ (SFO).	ACE2 over-expression was associated with down-regulation of the AT1R expression which suggests that ACE2 over-expression in the SFO impairs Ang II-mediated pressor and drinking responses at least by inhibiting the AT1R expression.
Guo et al.	2008	A truncated form of mouse ACE2 was cloned into adenovirus vector (Ad-ACE2) and transfected into human monocyte cell line (THP-1) macrophages. Examined expression of monocyte chemoattractant protein-1 (MCP-1) by administration of a selected Ang 1-7 antagonist (A779) to show the effect of ACE2 overexpression on MCP-1 level induced by Ang II.	Ang II-induced MCP-1 expression in THP-1, however, transduction of THP-1 with Ad-ACE2 with a significant attenuation of Ang II-induced MCP-1 production. ACE2 overexpression in the THP-1 attenuates Ang II-induced MCP-1 production and that this reduction is likely mediated by increased Ang 1-7 level.

Der et al.	2008	Used a gene overexpression approach to investigate the role of ACE2 in cardiac function and remodeling after MI. The cardiac functions, viability, and pathophysiology of coronary artery ligation (CAL) rat with/without ACE2 injection were assessed by MRI and by histological analysis.	Lenti-ACE2 rats demonstrated a complete rescue of cardiac output, a 41% rescue of ejection fraction, a 44% rescue in contractility, a 37% rescue in motion, and a 53% rescue in LV anterior (infracted) wall thinning compared with control CAL rats. Cardiac overexpression of ACE2 exerts protective influence on the heart during MI by preserving cardiac functions, LV wall motion and contractility, and by attenuating LV wall thinning.
Dong et al.	2008	Atherosclerotic plaques were induced and divided into 3 subgroups as a recombinant ACE2 expressing vector (Ad-ACE2), a control vector AdEGFP and Ad-ACE2+A779.	Local ACE2 overexpression resulted in stable plaque compositions, ie, fewer macrophages, less lipid deposition and more collagen contents, higher plaque stability scores, decreased Ang II levels, and increased Ang 1-7 levels in plaque tissues in the Ad-ACE2 subgroup compared with those in the Ad-EGFP subgroup.
Rentzsch et al.	2008	Generated transgenic rats on a spontaneously hypertensive stroke-prone rats (SHRSP) genetic background expressing the human ACE2 in vascular smooth muscle cells by the use of the SM22 promoter, called SHRSP-ACE2. Comfired the ACE2 expression of transgenic rats vascular smooth muscle by RNase protection, real-time RT-PCR, and ACE2 activity assays.	Transgene expression leads to significantly increased circulating levels of Ang 1-7 and the arterial blood pressure was reduced in SHRSP-ACE2 compared to SHRSP rats. Vascular ACE2 overexpression in SHRSP reduces hypertension probably by locally degrading Ang II and improving endothelial function.

Dong et al.	2009	Atherosclerosis (AS) plaques were induced in the abdominal aorta of rabbits by endothelial injury and atherogenic diet for 3 months. Injected of a recombinant adenoviru Ad-ACE2 or control vector Ad-EGFP to obtain Ad-ACE2 and Ad-EGFP rabbit (n = 19). One month later, all rabbits were sacrificed and plaques from aortic segments were analyzed.	Macrophage infiltration and MCP-1 expression were significantly reduced in Ad-ACE2 group compared to Ad-EGFP group. Overexpression of ACE2 inhibited atherosclerotic plaque inflammation response in hypercholesterolemic rabbits.
Masson et al.	2009	Used a recombinant adeno-associated virus 6 delivery system to provide 11-week overexpression of ACE2 in the myocardium of stroke-prone spontaneously hypertensive rats. Assessed by histological analysis with concomitant deficits in ejection fraction and fractional shortening measured by echocardiography to indicated cardiac fibrosis or not.	ACE2, as well as the ACE inhibitor enalapril, significantly reduced systolic blood pressure. However, sustained overexpression of ACE2 in the heart *in vivo* leads to the onset of severe fibrosis.
Zhao et al.	2010	The left anterior descending coronary artery was ligated to produce anterior MI in Wistar-Kyoto rats that were randomly divided into Ad-ACE2, Ad-ACE2+A779, Ad-EGFP, model, and sham groups. LV volume and systolic function, the extent of myocardial fibrosis, and levels of ACE2, Ang II, and collagen I protein expression were evaluated.	ACE2 overexpression favorably affected the pathological process of LV remodeling after MI by inhibiting ACE activity, reducing Ang II levels, and up-regulating Ang 1-7 expression, attenuated LV fibrosis and improved LV remodeling and systolic function.
Feng et al.	2010	The baseline hemodynamic parameters (telemetry), autonomic function, spontaneous baroreflex sensitivity (SBRS), AT2/AT1 receptor and Mas/AT1R ratios of Syn-hACE2 (SA) transgenic mice and nontransgenic littermates were assayed.	ACE2 overexpression attenuates the development of neurogenic hypertension partially by preventing the decrease in both SBRS and parasympathetic tone, this result might be mediated by enhanced NO release in the brain resulting from Mas and AT2R receptor upregulation

Bindom et al.	2010	Both db/db and nondiabetic lean control (db/m) mice were infected with an adenovirus expressing human ACE2 (Ad-hACE2-eGFP) or the control virus (Ad-eGFP) via injection into the pancreas. Glycemia and β-cell function were assessed 1 week later at the peak of viral expression.	Ad-hACE2-eGFP significantly improved fasting glycemia, enhanced intraperitoneal glucose tolerance, increased islet insulin content and β-cell proliferation, and reduced β-cell apoptosis compared with Ad-eGFP. ACE2 as a novel target for the prevention of β-cell dysfunction and apoptosis occurring in type 2 diabetes.
Shenoy et al.	2010	Lentiviral packaged Ang 1-7 fusion gene or ACE2 cDNA was intratracheally administered into the lungs of male Sprague Dawley rats to exam Ang 1-7 treatment would exert protective effects against PF and PH.	ACE2/Ang 1-7/Mas axis is a cardiopulmonary protective role in the treatment of lung disorders, prevented the pathophysiological conditions such as excessive collagen deposition, decreased expression of ACE and ACE2, increased mRNA levels for TGF-β and other proinflammatory cytokines, and increased protein levels of the AT1R.
Liu et al.	2011	Wistar rats were divided into normal group and diabetic model group, the diabetic rats were divided into no treatment group, Ad-ACE2 group, Ad-GFP group, ACE inhibition (ACEI) group receiving benazepril and Ad-ACE2 + ACEI group. The physical, biochemical, renal functional and morphological parameters were measured.	ACE2 transfection attenuated Ang II-induced glomerular mesangial cell proliferation, oxidative stress and collagen IV protein synthesis. ACE2 exerts a renoprotective effect similar to that of ACEI treatment, decreased renal Ang II, increased renal Ang 1-7 levels, and inhibited oxidative stress
Bu et al.	2011	Exam the ACE2 ameliorates the profibrotic effects of Ang II-mediated, Akt-dependent pathways in the ACE2 transfected mouse mesangial cell line, MES-13.	ACE2 overexpression appeared blocking phosphorylation of Akt in mesangial cells, regulated Ang II-mediated AT1R–TGFβRI–PI3K–Akt signaling and involved the synthesis of collagen.

Sriramula et al.	2011	Hypothesized that ACE2 overexpression in the PVN will have beneficial effects in counteracting Ang II-induced hypertension. Bilateral microinjection of an adenovirus encoding hACE2 (Ad-ACE2) into the PVN of SD rat was used to overexpress ACE2 within this region.	Bilateral PVN microinjection of Ad-ACE2 attenuated Ang II-induced hypertension, it also significantly decreased AT1R and ACE expression and increased AT2R and Mas expression in the PVN. Additionally, ACE2 overexpression attenuated the Ang II-induced increase in the expression of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in the PVN.
Xia et al.	2011	A relationship between ACE2 and oxidative stress was confirmed in a mouse neuroblastoma cell line (Neuro2A cells) treated with Ang II and infected with Ad-hACE2 to prove ACE2 might reduce AngII-mediated oxidative stress in the brain and prevent autonomic dysfunction. ACE2 knockout ($ACE2^{-/y}$) mice and WT mice were infused with Ang II and infected with Ad-hACE2 in the paraventricular nucleus (PVN).	ACE2 overexpression resulted in a reduction of reactive oxygen species (ROS) formation in Neuro2A cells. Cardiac sympathetic tone, brain NADPH oxidase, SOD activities and Ang II were significantly increased in $ACE2^{-/y}$ *in vivo*. ACE2 gene therapy to the PVN to normalize cardiac dysautonomia in $ACE2^{-/y}$ mice by reducing NADPH oxidase activity and ROS formation that AngII-induced.
Xiao et al.	2011	Investigated central overexpression of ACE2 decreases sympathetic outflow and enhances baroreflex function in chronic heart failure (CHF). Transgenic mice overexpressing human ACE2 selectively in the brain (SYN-hACE2 [SA]) and wild-type mice induced CHF by permanent coronary artery ligation.	Compared with WT mice with CHF, brain-selective ACE2 overexpression attenuated left ventricular end-diastolic pressure, decreased urinary norepinephrine excretion, baseline renal sympathetic nerve activity, enhanced baroreflex sensitivity and AT1R expression significantly attenuated.

Dong et al.	2012	Investigated ACE2 overexpression may inhibit myocardial collagen accumulation and improve left ventricular (LV) remodeling and function in diabetic cardiomyopathy. Diabetic rats (induced by a single intraperitoneal injection of streptozotocin) were further divided into adenovirus-ACE2, adenovirus-EGFP, losartan, and mock groups. LV volume; LV systolic and diastolic function; extent of myocardial fibrosis; ACE2 and MMP-2 activities were evaluated.	The adenovirus-ACE2 group showed increased ACE2, MMP-2 activities, and LV ejection fractions; decreased LV volumes, myocardial fibrosis, and ACE, Ang II, and collagen expression in comparison with the adenovirus-EGFP and control groups. ACE2 was superior to losartan in improving LV remodeling and function and reducing collagen expression may through transforming growth factor-beta inhibited and enhance MMP-2 to degrade collagen.
Gong et al.	2012	Vascular smooth muscle cells (VSMCs) were divided into lentiviral-GFP and lentiviral-ACE2 groups and treated with Ang II and irbesartan, respectively. The proliferation of VSMCs, AT1R mRNA and protein expressions were detected, the signaling pathway of signal transducer and activator of transcription 3 (STAT3) was also detected.	ACE2 gene transfer significantly inhibited the VSMCs proliferation and downregulated AT1R expression in the absence or presence of Ang II. Similar to AT1R expression, STAT3 phosphorylation was also significantly inhibited by ACE2 overexpression.

Appendix 8-4. The study of ACE2 knockout

Authors	Year	Experimental model and study design	Key findings
Gurley et al.	2006	Generated mice with targeted disruption of the *ace2* gene. The heart function and blood pressure were detected in the ACE2-deficient mice with and without Ang II infused.	ACE2-deficient mice were viable, fertile, lacked any gross structural abnormalities and no effect on baseline blood pressures. The absence of functional ACE2 causes enhanced susceptibility to Ang II-induced hypertension.
Yamamoto et al.	2006	Used $ACE2^{-/y}$ mice to analyze the role of ACE2 in the response to pressure overload. $ACE2^{-/y}$ mice and wild-type (WT) mice received transverse aortic constriction (TAC) or sham operation and estimated cardiac function and heart morphologically.	In response to TAC, cardiac Ang II concentration and activity of mitogen-activated protein (MAP) kinases of $ACE2^{-/y}$ mice were increased and developed cardiac hypertrophy and dilatation; their hearts displayed decreased cardiac contractility and increased fetal cardiac gene induction, compared with WT mice, furthermore, in $ACE2^{-/y}$ mice in response to TAC.
Oudit et al.	2007	$Ace2^{-/y}$ mutant mice develop a progressive age-dependent dilated cardiomyopathy with increased oxidative stress, neutrophilic infiltration, inflammatory cytokine and collagenase levels, mitogen-activated protein kinase (MAPK) activation and pathological hypertrophy. Confirming a critical role of Ang II- AT1R- G-proteincoupled receptor (GPCR)-activated phosphoinositide 3-kinase gamma (PI3Kγ) and its downstream pathways.	The age-dependent cardiomyopathy in ACE2 null mice is related to increase Ang II-mediated oxidative stress and neutrophilic infiltration via AT1 receptors. These results reveal a critical role of ACE2 in the suppression of Ang II-mediated heart failure.

Wong et al.	2007	Examined the effect of deletion of the ACE2 gene on diabetic kidney injury with four groups of mice: $Ace2^{+/y}Ins2^{WT/WT}$, $Ace2^{-/y}Ins2^{WT/WT}$, $Ace2^{+/y}Ins2^{WT/C96Y}$, and $Ace2^{-/y}Ins2^{WT/C96Y}$.	$Ace2^{-/y}Ins2^{WT/C96Y}$ mice exhibited a two-fold increase in the urinary albumin excretion rate compared with $Ace2^{+/y}Ins2^{WT/C96Y}$ mice despite similar blood glucose levels. It also exhibit increased mesangial matrix scores and glomerular basement membrane thicknesses compared with $Ace2^{+/y}Ins2^{WT/WT}$ mice, accompanied by increased fibronectin and α-smooth muscle actin immunostaining in the glomeruli of $Ace2^{-/y}$ $Ins2^{WT/C96Y}$ mice.
Tikellis et al.	2008	Diabetes was induced by streptozotocin in male c57bl6 mice and ACE2 knockout (KO) mice. After 5 weeks of study, animals were randomized to receive the ACE inhibitor perindopril. Wild-type mice were further randomized to receive the selective ACE2 inhibitor MLN-4760 and followed for an additional 5 weeks. Markers of renal function and injury were then assessed.	In diabetic mice receiving MLN-4760 and in ACE2 KO mice, diabetes-associated albuminuria was enhanced, associated with an increase in blood pressure. Diabetic wild-type mice, ACE2 KO mice and diabetic mice receiving MLN-4760 treated with an ACE inhibitor experienced a reduction in albuminuria and blood pressure. The expression of ACE2 is significantly modified by diabetes, and it is a complex and site-specific modulator of diabetic kidney disease.
Niu et al.	2008	To investigate the role of ACE2 in regulating glucose homeostasis, glucose tolerance test, insulin secretion test, and insulin tolerance test were performed in age-matched male ACE2 knockout (KO) and wild-type (WT) mice.	Male ACE2 KO mice displayed a selective decrease in first-phase insulin secretion in response to glucose and a progressive impairment of glucose tolerance compared with age- and sex-matched WT mice.

Osterreicher et al.	2009	ACE2 knockout (KO) mice and wild-type (wt) littermates underwent different models of acute and chronic liver injury, the liver pathology was analyzed by histology, immunohistochemistry, alpha smooth muscle actin (α-SMA) immunoblotting, and quantitative polymerase chain reaction (qPCR).	ACE2 is a key negative regulator of the RAS and functions to limit fibrosis through the degradation of Ang II and the formation of Ang1-7. Whereas loss of ACE2 activity worsens liver fibrosis in chronic liver injury models, administration of recombinant ACE2 shows therapeutic potential.
Kassiri et al.	2009	In response to MI (induced by left anterior descending artery ligation) in wild-type ($Ace2^{+/y}$) and ACE2-deficient mice ($Ace2^{-/y}$). The nicotinamide-adenine dinucleotide phosphate oxidase activity, infarct size, MMP activation, and myocardial inflammation were analyzed.	ACE2 deficiency leads to increased MMP-2 and MMP-9 levels, up-regulation of inflammatory cytokines, interferon-gamma, interleukin-6, and the chemokine, monocyte chemoattractant protein-1, as well as increased phosphorylation of ERK1/2 and JNK1/2 signaling pathways. Loss of ACE2 facilitates adverse post-MI ventricular remodeling by potentiation of Ang II effects by means of the AT1 receptors, and supplementing ACE2 can be a potential therapy for ischemic heart disease.
Zhong et al.	2010	Ang II infusion resulted in worsening cardiac fibrosis and pathological hypertrophy in ACE2 knockout ($Ace2^{-/y}$) mice compared with wild-type (WT) mice. Daily treatment of Ang II–infused wild-type mice with recombinant human ACE2. Observed the hypertrophic response and the expression of hypertrophy markers superoxide production in mice.	Elevated Ang II induced hypertension, myocardial hypertrophy, fibrosis, and diastolic dysfunction, which were exacerbated by ACE2 deficiency, whereas rhACE2 attenuated Ang II and pressure overload induced adverse myocardial remodeling.
Thomas et al.	2010	C57Bl6, ACE2 knockout (KO) were followed until 30 weeks of age. Bone marrow macrophages and endothelial cells isolated from WT and Ace2 KO mice, assessed the inflammatory responsiveness to LPS, Ang II and TNF-α.	Genetic ACE2 deficiency is associated with upregulation of putative mediators of atherogenesis and enhances responsiveness to proinflammatory stimuli.

Shiota et al.	2010	ACE2-KO and wild-type C57BL/6 mice were rendered diabetic by intraperitoneal injection of streptozotocin and observations of kidneys at early (4 weeks) and advanced (18 weeks) stages. The serum creatinine, urea nitrogen levels and glomerular/tubulointerstitial damage were estimated.	Treated with AT1R blocker olmesartan ameliorated the functional and morphological deterioration of diabetic nephropathy in ACE2-KO mice. These results suggest that ACE2 might continuously protect from both glomerular and tubulointerstitial injury during the development of diabetic nephropathy.
Thatcher et al.	2011	Male $ACE2^{-/y}$ mice in an low-density lipoprotein receptor-deficient background were fed a high-fat diet for 3 months and assessed the atherosclerotic area. Macrophages isolated from $ACE2^{-/y}$ mice and measured the expression of Ang II and inflammatory cytokines.	ACE2 deficiency in bone marrow-derived cells promotes atherosclerosis through regulation of Ang II/Ang 1-7 peptides.
Bodiga et al.	2011	Examined changes in peptide levels, NADPH oxidase activity, gene expression, MMPs activity, pathological signalling, and heart function in pressure overload ACE2-KO ($Ace2^{-/y}$) and wild-type ($Ace2^{+/y}$) mice.	Increased production of superoxide, activation of MMPs, and pathological signalling leads to severe adverse myocardial remodelling and dysfunction in ACE2-KO mice.
Zhong et al.	2011	Ang II infusion resulted in higher renal Ang II levels and increased nicotinamide adenine dinucleotide phosphate oxidase activity in ACE2 knockout ($Ace2^{-/y}$) mice compared to wild-type mice. Ang II-infused wild-type mice were then treated with recombinant human ACE2. The level of proinflammatory cytokines and collagen I were measured.	Loss of ACE2 enhances renal Ang II levels and Ang II-induced renal oxidative stress, resulting in greater renal injury, whereas recombinant human ACE2 prevents Ang II-induced hypertension, renal oxidative stress, and tubulointerstitial fibrosis.

Xia et al.	2011	ACE2 knockout ($ACE2^{-/y}$) mice and non-transgenic (NT) littermates were infused with Ang II (10 days) and infected with Ad-hACE2 in the paraventricular nucleus (PVN). The blood pressure (BP), AngII, brain ROS levels, NADPH oxidase and SOD activities were assay.	ACE2 overexpression resulted in a reduction of reactive oxygen species (ROS) formation. Cardiac sympathetic tone, brain NADPH oxidase and SOD activities were significantly increased in $ACE2^{-/y}$ mice. Post Ang II infusion, Ang II and ROS levels of plasma and brain were also significantly higher in $ACE2^{-/y}$ mice. ACE2 gene therapy to the PVN reduced Ang II-mediated increase in NADPH oxidase activity and normalized cardiac dysautonomia in $ACE2^{-/y}$ mice.
Bharadwaj et al.	2011	Determined the influence of ACE2 deficiency on circulating and tissue RAS components, fetal and maternal growth characteristics, and maternal hemodynamics (mean blood pressure and cardiac output) at day 18 of gestation.	Gestational body weight gain was lower in the ACE2 knockout (KO) versus C57BL/6 (wild-type) mice. Fetal weight and length were less in KO. ACE2 deficiency and associated elevated placenta Ang II levels impact pregnancy by impairing gestational weight gain and restricting fetal growth.
Bernardi et al.	2012	Forty-eight rats randomly allocated to three different dietary contents of salt were studied for 4 weeks after undergoing a left uninephrectomy. The kidney functional, structural and molecular changes were obseved, furthermore, kidney molecular changes in 20 weeks old male ACE2-knockout mice (Ace2KO), with and without ACE inhibition also studies.	A high salt content diet significantly increased the glomerular ACE/ACE2 ratio was associated with increased oxidative stress. The renal oxidative stress in ACE2KO could be prevented by ACE inhibition. High-salt diet leads to renal damage seems to be the modulation of the ACE/ACE2 ratio which in turn is critical for the cause of oxidative stress, through Ang II.
Jin et al.	2012	Loss of ACE2 would facilitate Ang II-mediated vascular inflammation and peroxynitrite production. Wild type (WT, $Ace2^{+/y}$) and ACE2 knockout (ACE2KO, $Ace2^{-/y}$) mice received with mini-osmotic pumps with Ang II or saline for 2 weeks.	Loss of ACE2 resulted in greater increases in Ang II-induced expressions of profilin-1, NADPH oxidase, superoxide, peroxynitrite and inflammatory cytokines MCP-1, IL-1β, and IL-6 without affecting TNF-α in aortas of ACE2KO mice.

Rey-Parra et al.	2012	Ang II contributes to lung injury and ACE2 may prevents Bleomycin (BLM)-induced lung injury. ACE2 knockout mice-male ($ACE2^{-/y}$) and female ($ACE2^{-/-}$)-and age-matched wild-type (WT) male mice received intratracheal BLM.	Compared with WT mice, $ACE2^{-/y}$ BLM injured mice exhibited poorer exercise capacity, worse lung function and exacerbated lung fibrosis and collagen deposition associated with α-SMA and TGF-ß1 expression. Treatment with intraperitoneal rhACE2 improved lung injury and BLM-induced fibrosis.
Patel et al.	2012	AT1R blockade and Ang 1-7 both resulted in marked recovery of systolic dysfunction in pressure-overloaded ACE2-null mice, compare the benefits of AT1R blockade versus enhancing Ang 1-7 action in pressure-overload-induced heart failure in ACE2 knockout mice.	AT1R blockade and Ang 1-7 reduced the biomechanical stress, MMP-2 and MMP-9 activities that pressure-overload-induced and attenuated the increase in NADPH oxidase activation by downregulating the expression of Nox2 and $p47^{phox}$ subunits and also by limiting the $p47^{phox}$ phosphorylation.
Alghamri et al.	2012	Conducted studies in ACE2 deficient mice to determine whether enzyme loss would exacerbate the cardiac and vascular pathological responses to chronic subcutaneous (sc) Ang II infusion. ACE2 knockout (KO) and wild type (WT) mice were infused with Ang II using mini-osmotic pumps, and the cardiac function, and vascular inflammation were examined.	Cardiac dysfunction was associated with hypertrophic cardiomyopathy shown by increased left-ventricular wall thickness, average cardiomyocyte cross-sectional area, and heart weight/body weight ratio. Oxidative stress and collagen staining reveal heart remodeling in the myocardium and aorta in Ang II infused ACE2 KO mice.
Takeda et al.	2012	Assessed the role of endogenous ACE2 in maintaining insulin sensitivity. Male ACE2 knockout (ACE2KO) and wild-type (WT) mice had normal insulin sensitivities. High-fat high-sucrose (HFHS) diet impaired glucose tolerance and insulin sensitivity in ACE2KO mice were more severely than WT mice.	The strain difference in glucose tolerance was not eliminated by an AT1R blocker but was eradicated by Ang 1-7 or an AT1R blocker combined with the Ang 1-7 inhibitor (A779). ACE2 protects against high-calorie diet-induced insulin resistance in mice may involve the transcriptional regulation of GLUT4 via an Ang 1-7-dependent pathway.

Patel et al.	2012	Defined the role of ACE2 in diabetic cardiovascular complications, the human diabetes model mice, Akita mice, and double-mutant mice (Akita/ACE2$^{-/y}$) were using in this study.	Compared to Akita mice, Akita/ACE2KO mice appeared more Ang II and linked to enhanced activation of NADPH oxidase and metalloproteinases. The systolic dysfunction that Ang II induced was rescued by AT1R blocker, irbesartan treatment. These results demonstrate that ACE2 serves as a protective mechanism against diabetes-induced cardiovascular complications.