# 行政院國家科學委員會專題研究計畫　成果報告

## 類胰島素生長因子-II 訊息傳遞誘發心肌細胞凋亡過程中雌激素接受體α及β　調控 Angiotensin-II 及 ZAK 基因之角色探討

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一：研究題目:

# 類胰島素生長因子-II 訊息傳遞誘發心肌細胞凋亡過程中雌激素接受體 α 及 β 調控 Angiotensin-II 及 ZAK 基因之角色探討(94-2320-B-040-017-)

## 二：摘要


已知在臨床心臟疾病試驗以及誘發心臟疾病的動物模式和細胞實驗中，均指出IGF-I具有改善心臟功能、減緩心衰竭發生以及增加心肌細胞存活的功能，且主要透過IGF-IR/PI3K/Akt訊息路徑。然而在發育過程中，過多的IGF-I會經由IGF-II receptor進行細胞攝粒作用而分解。於是我們提出一個問題：在心臟病變過程中，對於IGF-I receptor訊息路徑的抗性是否是因為過度活化IGF-II receptor所造成。首先我們使用H9c2 cardiofibroblast和neonatal cardiomyocytes，處理以AngII、ISO、Inomycin、LPS and TNF-α刺激物以及先天性高血壓的大白鼠(SHRs)和惡性肥胖的大白鼠(Zucker Rats)和心肌梗塞病患檢體之組織晶片，證實在心臟病變過程中和誘發心肌細胞凋亡時，IGF-II receptor會被大量開啟。然而IGF-II receptor基因的開啟主要透過JNK訊息路徑促進下游c-jun結合於在IGF-II receptor promoter上AP-1 site和改變Histione modification (acetyl and trimethyl Histione H3)所造成，但與promoter上CpG island的 DNA methylation無關。接著我們使用IGF-II analog (IGF-II Leu27)探討IGF-II receptor除了為生長清除者的角色之外，是否具有能開啟細胞內訊息的功能?我們證實IGF-II receptor經IGF-II活化後，能與Gαi結合和活化Calcineurin，不同於IGF-I receptor的存活路徑，為一促凋亡的角色。最後我們使用IGF-II receptor RNAi證實IGF-II receptor訊息路徑透過活化Calcineurin參與在AngII造成心肌細胞的機制中。這樣的發現將有助於在生物醫學上提供一個治療和減緩心衰竭發生的標地基因。

Previous study demonstrated that overexpression of ZAK induced cardiac hypertrophy and elevated ANF expression. ZAK also causes the apoptosis of hepatoma cell line. A critical role of apoptosis was suggested as a pathogenic mechanism of cardiac diseases. In this study, we investigate whether overexpression of ZAK could enhance cardiomyocytes death and alter

ECM components to cause cardiac fibrosis. The data revealed that expression of wild type, continuous active, but not dominative negative ZAK show apoptosis analyzed by TUNEL assay, and promoted caspase 3 activity by western blotting in H9C2 cells. Downregulation of phospho-Bad and phospho-PLB, and calcineurin-induced nuclear translocation of NF-ATc1 were also shown. ZAK-overpressed H9C2 cells treated with CsA, a calcineurin inhibitor, demonstrated the inhibition of ZAK-induced apoptosis. None of wild type, continuous active and dominative negative expression of ZAK in AngII-treated H9C2 affects apoptotic signaling activity. We indicate that aberrantly increasing ZAK in the sections of cardiac infraction (22/27) and myocardial scar (9/10) compare with in normal ventricular tissue (1/10) by tissue assay. ZAK enhanced MMP-2 activity, but not MMP-9, through JNK and p38 signal pathway and induced the protein level of TIMP-1/2that indicates the ZAK activated signaling cascade through regulating MMP-2 activity and TIMP1/2 gene expression to alter the ECM structure involved in ventricular remodeling. These results demonstrate ZAK induced cardiomyocyte apoptosis via calcineurin signaling pathway without AngII signaling involvement, and promoted cardiac fibrosis through evoking MMP-2 activity and TIMP1/2 gene expression.

# 三：研究動機、目的

心肌肥大(Myocardial hypertrophy)為心臟在面對各種刺激下的適應性反應，主

要發生在心衰竭(Heart failure)的早期，並會進一步造成個體的死亡。此種適應性反應是透過增加肌肉的重量(Muscle mass)，來維持正常心臟所需要的功能。而心肌細胞的肥大生長被認為開始於個體 Endocrine、Paracrine 和 Autocrine factors 的分泌，並結合於位在心肌細胞膜上多樣的接受體，並活化了 Multiple cytoplasmic signal transduction cascades，進而影響 Nuclear factors，調節基因的表現。而參與在心肌肥大的分子訊息傳遞的機制，重要的傳遞者(Transdacers)包括了：Specific G protein isoforms、Low-molecular-weight GTPase (Ras、RhoA and Rak)、Mitogen-activated protein kinase cascades(MAPK)、Protein kinase C、Calcineurin/NFAT、gp130-signal transducer and Activator of transcription、Insulin-like growth factor I receptor pathway、Fibroblast growth factor and transforming growth factor β receptor pathways。而這些有關於心肌肥大路徑中的傳遞者(Transducers)彼此有著相互連接的關係，顯示在心肌肥大的過程中，多樣的分子和多種不同的訊息傳遞路徑參與其中（1, 2）。

在本論文中主要探討在傳統功能上會透過細胞攝粒作用進而結抗 Insulin-like growth factor I receptor 細胞存活訊息路徑的 Insulin-like growth factor II receptor 以及參與在活化 Mitogen-activated protein kinase cascades(MAPK)訊息路徑中的 leucine-zipper and sterile-alpha motif kinase(ZAK),兩者在心肌細胞受外力刺激下所導致的肥大作用以及在心臟衰竭時心肌細胞的凋亡作用中,所扮演的角色及其分子機轉為何?

已知心肌細胞( Cardiac myocytes )在接受外來刺激下所產生的肥大現象,可分為三種型態：**A**.生理性肥大( Physiologic hypertrophy )：此種肥大現象為心肌細胞( Cardiac myocytes )厚度和長度呈現等比例的增加。所造成心肌細胞形態的改變唯一可逆的反應，當刺激消失後，心肌細胞肥大的型態會回復到正常狀態。**B**.增厚型肥大( Concentric hypertrophy )：為一增厚型的肥大，主要在於增加心肌細胞( Cardiac myocytes )的厚度，造成收縮的強度增強，在臨床上所造成心臟疾病稱為肥大型心肌症(Hypertrohic cardiomyopathy)，可經由兩心室中隔增厚，在心室壁增厚，左心室腔室變窄分辨出來。**C**.拉長型肥大( Eccentric hypertrophy )：為一拉長型的肥大，主要在於增加心肌細胞( Cardiac myocytes )的長度，造成收縮的速度增加，在臨床上所造成的心臟疾病稱為擴張型心肌症(Dilated cardiomyopathy)，主要特徵為：左心室腔室擴張，兩心室中隔和左心室壁變薄。增厚型肥大和拉長型肥大其所造成心肌細胞的肥大型態的變化為不可逆的反應，故此二型的肥大又被稱為病理性的肥大( Pathological cardiac hypertrophy )（2）。在病理性的肥大中，心肌細胞除了細胞大小有著明顯的變化，亦發現關於 Natriuretic petides 和 Fetal contractile protein 的胚胎基因( Embryonic gene )大量表達，例如：β-myosin heavy chain(β-MHC)，skeletal α-actin 和 atrial natriuretic factor(ANF) 都被認定為是心臟肥大的指標性蛋白(3)。若以進入心衰竭的時程上加以區分，肥大型心肌症被認為一代償性的肥大(Compensated hypertrophy)能維持受外力刺激下的心臟有著較長時間的正常心功能，減緩心衰竭的發生，而擴張型心肌症( Dilated cardiomyopathy )則為非代償性的肥大( Maladaptive decompensated hypertrophy)為心衰竭發生的前徵。心衰竭的發生亦被認為是，心臟由代償性的肥大走向非代償性肥大(4)，心肌細胞由肥大反應走向

凋亡反應所導致的（5,6,7,8,9,10）。在心血管系統中，也有許多疾病是與心肌凋亡有著相關性，如：Dilated cardiomyopathy、Ischemic cardiomypathy（5）、Arrhythmogenic right ventricular dysplasia（11）、Acute myocardial infarction（12）、Atherosclerosis（13）、Myocarditis syndromes（14）和 Congenital atrioventricular block（15）。然而心肌細胞由代償性的肥大轉變為心肌細胞的凋亡，將會加速心衰竭的發生，在目前已知的研究中尚無法解釋心肌肥大如何轉變為凋亡的分子機制?

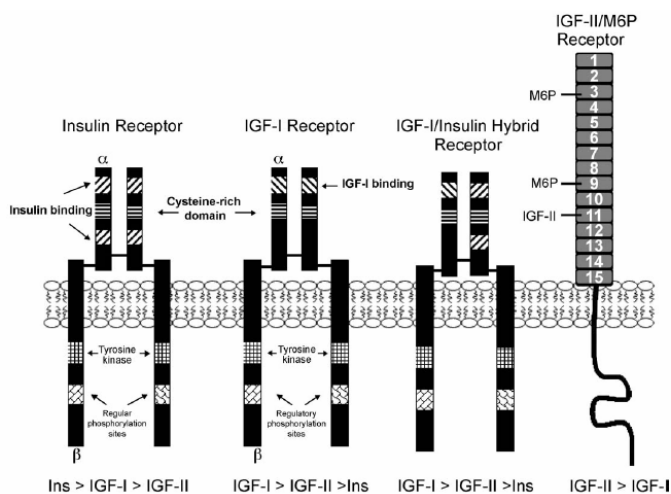
在臨床心臟疾病試驗、誘發心臟疾病的動物模式和細胞實驗中，均指出 IGF-I 具有改善心臟功能、減緩心衰竭發生以及增加心肌細胞存活的功能(16,17,18,19,20)，但在心衰竭末期以及持續刺激壓力下所誘發的心臟疾病，則無法顯示出 IGF-I 保護心臟的效果(21,22,23)，我們認為此時經由 Insulin-like growth factor I receptor pathway 的心肌細胞存活路徑受到了擷抗作用。

整個類胰島素生長因子的系統是由結合體( Ligand )、接受體( Receptor )、結合蛋白( Binding protein )所組成。結合體有二：類胰島素生長因子-I(IGF-I)和類胰島素生長因子-II(IGF-II)，主要在肝臟中產生可行內分泌並經由 IGF 結合蛋白運送到標地器官及在部份組織中也能自行分泌行外分泌和自体分泌作用，並透過位於細胞膜上的類胰島素生長因子-I 接受體(IGF-I R )對細胞造成影響。另一方面當體內的 IGFs 過多時，則會透過類胰島素生長因子-II 接受體(IGF-IIR)進行細胞攝粒作用(Endocytosis)，經溶酶體( Lysosome )分解(24)。

IGF-I 和 IGF-II 被證實能直接造成心肌細胞的肥大（25,26）。而 IGF-I造成心室肥大的訊息路徑，主要透過 IGF-IR/PI3K/Akt/p70S6k signaling pathway（27）。 IGF-I R 為一個 Tyrosine kinase receptor，下游路徑包括了：IRS-1、Shc、Ras、Raf 和 Mitogen-activated protein (MAP) kinase。在 IGF-I、IGF-II 和 IGF-I R 之基因刪除鼠中，顯示了嚴重的生長缺失(Severe growth deficiency)，顯示著 IGFs 透過 IGF-I R 的訊息傳遞路徑( IGF-IR signaling pathway )為調節發育和生長的重要機制（27）。另外，IGF-IR signaling pathway 也被證實具有抗凋亡(Anti-apoptosis)的能力，主要經由 PI3K-protein kinase B/ Akt pathway(28,29)及 Ras-extracellular signal regulated kinase (ERK) signaling pathway，透過影響 Caspases 的活性及 Bcl-2 family 的表現量（30,31），例如：增加抗凋亡蛋白 Bcl-xL、Bc1-2 的蛋白表現量或減少前凋亡蛋白 Bax、Bcl-xs 的蛋白表現量及抑制 Caspase1、3、7 的活性，被認為一重要的細胞存活路徑(Cell survial signaling(28)。因此在心臟中，Insulin-like growth factor I receptor pathway 訊息傳遞路徑所誘發之心肌肥大被認為是代償性生理性肥大作用。

而 IGF-II 造成心肌細胞肥大的機制中，可依其結合的接受體分為兩類：一為 IGF-IR-dependent pathway；二為 Lysosome-dependent pathway（32）。而 IGF-IR-dependent pathway 則跟 IGF-I 所誘發之心肌細胞代償性生理性肥大具有相同的作用；而 Lysosome-dependent pathway 則是透過結合於類胰島素生長因子-II 接受體(IGF-IIR)所誘發的，目前詳細的分子機轉則不清楚。IGF-Ⅱreceptor 為 Cation -independent mannose-6-phosphate receptor，為一高度保留的基因，在不同物種間有很高的相似度(24,33)。就蛋白結構而言，為一 Single-chain membrane-Spanning glycoprotein，由 2451

個 amino acid 所組成，結構中包括：一個較大部分的 Extracellular domain（2264 amino acid）、Transmembrane region（23 amino acid）和一個 Carboxyl-terminal intracytoplasmic domain（164 amino acid）(33)。在 Extracellular domain 有 15 個 Contiguoas segments (26)，每一個區域有 8 cysteines Repeating。Motif 13 為 43 個胺基酸組成的 Fibronectin type Ⅱ repeat，其能與 Collagen 結合；Repeating motif 1-3 稱為 M-6-P binding domain 1；Repeating motif 7-9 稱為 M-6-Pbinding domain 2，為 mannose-6-phosphate (M-6-P)結合的位置；而 Repeating motif 11 則為 IGF-Ⅱbinding region(33)。




長久以來 IGF-II receptor 被認為只是一個生長因子清除者的角色（12），透過將 IGF-I 和 IGF-II 行細胞攝粒作用(Endocytosis)於溶酶體( Lysosome )分解，具有腫瘤抑制基因(Tumor suppressor gene)的特性。但隨著愈來愈多的結合體( Ligand )被發現可以結合於 IGF-II receptor 上，使得 IGF-II receptor 成為一個多功能的接受體調節著多樣的細胞功能(詳細資料如下表)(33)，例如: IGF-IIR 被證實參與在殺手 T 細胞所誘發的凋亡反應當中，且為一個死亡接受體(Death receptor)的角色。

| Non-M6P-containing ligands | Consequences of IGF-II/M6P receptor binding |
|---|---|
| Insulin-like growth factor-II | Endocytosis and lysosomal degradation, possible signal transduction |
| Retinoic acid | Growth inhibition and/or apoptosis |
| Urokinase-type plasminogen activator receptor | Participation in TGF-β activation at the cell surface; endocytosis and lysosomal degradation |
| Plasminogen | Conversion to plasmin and participation of TGF-β activation |
| **M6P-containing ligands** | |
| Lysosomal enzymes | Endocytosis and/or trafficking to lysosomes |
| Transforming growth factor-β precursor | Cell surface proteolytic activation |
| Leukemia inhibitory factor | Endocytosis and lysosomal degradation |
| Proliferin | Induction of endothelial cell migration and angiogenesis |
| Thyroglobulin | Endocytosis and lysosomal activaiton and/or degradation |
| Renin precursor | Endocytosis and proteolytic activation and/or degradation |
| Granzyme A | Targeting to lytic granules and possible role in apoptosis |
| Granzyme B | Internalization and induction of apoptosis |
| DNAse I | Possible targeting to lysosomes |
| CD26 | Internalization and T cell activation |
| Epidermal growth factor | Endocytosis and lysosomal degradation |
| Herpes simplex viral glycoprotein D | Facilitation of viral entry into cells and transmission between cells |
| Varicella-zoster viral glycoprotein I | Facilitation of viral entry into cells |

這也使著對於 IGF-II receptor 基因的研究趨於複雜。另一方面，對於接受體行 endocytosis 形成 endosomes 的過程中，不再簡單地認為只是將結合體( Ligand )送至

溶酶體( Lysosome )分解，而是參與其中的 endocytic compartment 本身可能具有調節細胞內訊息傳遞的功能(34)。然而在 3T3 cells 中，更發現 IGF-II 透過 IGF-II R 經由活化 Gαi2，會增加細胞內鈣離子濃度上升(35)。一般而言，當細胞內局部鈣離子濃度增高時，鈣離子會與攜鈣蛋白(Calmoduin)結合，進而活化去磷酸激酶 Calcineurin，能將帶磷酸根之 Bad 蛋白給去磷酸化，造成 Cytochrom c 由粒線體內釋放到細胞質，而導致細胞的凋亡(36)。另一方面，Calcineurin 所活化的 NFAT-3 訊息路徑(Calcineurin-NFAT$_3$ signaling)亦被證實為導致心臟肥大的重要路徑之一(37)。而在心肌細胞中，對於 IGF-II R 訊息路徑之角色尚未有所定義。

於是我們提出一個假設：心肌細胞在面對外來刺激下，心肌細胞內的 IGFs system 中，IGF-I R signaling pathway 及 IGF-II R signaling pathway 之間強弱的協調性，將決定心肌細胞選擇代償性的肥大存活下來或走向非代償性的凋亡加速心衰竭的發生。

在本實驗室先前的研究中指出：隨著年齡的增加，人體內IGF-I的分泌會逐漸下降或者在糖尿病患者所誘發的insulin-like growth factor (IGF)-I resistance中，此時若伴隨著高血壓(高血管昇壓素; AngII)，則會加乘促進心肌細胞的凋亡，而此一促凋亡之分子機轉為開啟IGF-II R signaling pathway所造成(38;附件1)。而在另一篇由實驗室發表的文章中指出：使用腹動脈結紮促使腎臟分泌過高的血管昇壓素進而造成心臟疾病的動物模式和直接刺激以血管昇壓素的心肌細胞實驗中，證實血管昇壓素能分別透過p38和JNK訊息路徑開啟IGF-II和IGF-II R基因表現進而活化細胞內 IGF-II R signaling pathway，而經活化的IGF-II R signaling pathway則會透過 Calcineurin/Bad造成心肌細胞凋亡，而此一IGF-II R signaling pathway的活化為血管昇壓素造成心肌細胞凋亡所必須的(39;附件2)。但對於IGF-II R基因如何被調節以及在病態心臟疾病時，IGF-II R誘發心肌凋亡的直接詳細地分子機轉為何?則是接下來本論文急於回答的問題。

在本論文的第二部分，則將主題放在被認為是調節心肌功能重要路徑的 Mitogen-activated protein kinase (MAPK) cascades(1,2)中的新成員：leucine-**zi**pper and sterile-**a**lpha motif **k**inase(ZAK)(40)，在此要特別介紹ZAK基因是在西元2000年時，由楊肇基教授首次發現並命名的，在本實驗中所使用ZAK的穩定表現型心肌細胞株(Tet-on system H9c2 cell)都是由楊肇基教授所建構完成的。MAPK 主要可以區分為三個成員，分別為JNK、p38 以及ERK。而JNK 和p38 被歸類為stress activity protein kinases (SAPK)(41,42)，當心臟受到外在壓力下即可被活化誘導，其中包括了會對心肌造成凋亡作用的血管昇壓素(AngII)。ZAK屬於mixed lineage protein kinase(MLK)家族(43)，並被證實為一MAP3K可以透過MKK7 活化下游JNK 進而調節基因的表現。ZAK (蛋白全長為800 個氨激酸，分子量為91KDa) 在其蛋白結構上與MLK 家族有些不同，ZAK 除在N端有一個kinase catalytic domain(16-260 amino acids)外，還有一個名為SAM(sterile-alpha motif kinase) domain (336-410 amino acids)，在這兩個domain 間夾帶著一個leucine-zipper(40)。

在先前的報導中，指出在hepatoma cell line 大量表現ZAK(MAP3K)會造成細胞毒

性產生細胞凋亡的現象。並且能經MKK7(MAP kinase kinases)活化JNK/SAPK pathway 造成cell aresst，以及活化NF-kB 及促使actin stress fibers的重組進而改變了細胞的型態(44)。在心臟組織中亦偵測到ZAK 蛋白的表現。而大量表現ZAK 會造成心肌細胞的肥大，且被證實參與在Transforming growthfactor-beta(TGF-β)造成心肌細胞(H9c2)肥大及活化肥大標地基因ANF表現的機制中(45,46)。然而心臟在接受外力刺激後，即會產生心臟的重塑作用(cardiac remodeling)，此時心肌細胞容易因持續的壓力而由病理性肥大轉向細胞凋亡，而心纖維母細胞(cardiac fibroblasts) 則呈現過度增生， 改變細胞外物質(extracellular matrix ;ECM)的組成分，使得心臟產生纖維化的現象，加速心衰竭的發生。

已知ZAK 為Transforming growth factor-beta(TGF-β)造成心肌肥大以及活化ANF所必須的下游蛋白分子，並能活化MAPK cascade ：JNK/SAPK訊息路徑。然而在心臟中JNK 路徑的過度活化已被證實會促使心肌細胞走向凋亡(47)；此外TGF-β 亦被證實為組織纖維化過程中重要的調節生長因子，調節著影響ECM 組成的相關酵素，如：matrix metalloproteinases(MMP)，另外TGF-beta 訊息路徑的下游蛋白：MAPKKK7;TGF-beta activated kinase，亦被證實能促進心肌細胞的凋亡(48)。在本篇研究中，將探討ZAK 是否參與在cardiac remodeling 的過程中，ZAK 所扮演的角色為何?以及詳細的分子作用機轉為何?

# 四：研究架構

第一部分：類胰島素生長因子-II 接受體之基因調控與其訊息路徑誘發心肌凋亡之分子機轉探討 The molecular mechanism of regulating IGF-II receptor gene expression and the IGF-II receptor signaling pathway induce apoptosis in the cardiomyocytes.

1. 使用 H9c2 cardiofibroblasts 和 neonatal cardiomyocytes 處理以各種不同的刺激物，包含：AngII, ISO, Inomycin, LPS and TNF-α，測定 IGF-II receptor 基因的表現。
2. 使用先天性高血壓大白鼠(SHR)以及先天性肥胖鼠(Zucker Rat)與正常大白鼠相互比較其心臟檢體中，IGF-II receptor 基因表現之差異。
3. 使用人類心肌梗塞的組織晶片，測定 IGF-II receptor 蛋白表現之差異。
4. 分析 IGF-II receptor promoter 上可能之轉錄活化因子結合的區域。
5. 建構不同片段的 IGF-II receptor promoter 之報導基因，分析對於開啟 IGF-II receptor 基因表達具有功能之片段；透過具有功能片段之序列分析找出可能之轉錄活化因子以及使用 EMSA 及 ChIP 證實可能之轉錄活化因子與 IGF-II receptor promoter 之結合能力。
6. 分析 IGF-II receptor promoter 上的 CpG island 中，DNA methylation 的變化是否參與調節 IGF-II receptor 基因之表現；MeCP2 (DNA methylation binding protein)與 CpG island 之結合能力是否參與調節 IGF-II receptor 基因之表現。
7. 分析 IGF-II receptor promoter 上的 CpG island 中，Histione modification (acetyl and trimethyl Histione H3)是否參與調節 IGF-II receptor 基因表現。
8. 使用 IGF-I, IGF-II and IGF-II analog (IGF-II Leu27;對於 IGF-II receptor 有著極高的親合力)刺激心肌細胞，偵測其是否具不同之細胞訊息傳遞功能及其相互關西為何，方向有二個：
   a. IGF-I receptor signaling pathway: p-Akt, Akt, p-ERK and ERK.
   b. IGF-I receptor signaling pathway: Gαi and Calcineurin.
9. 刺激以 AngII 和 Insulin/IGF-I receptor inhibitor(AG1024;抑制 IGF-I receptor signaling pathway)，然後在分別加入 IGF-I, IGF-II and IGF-II analog，比較三者對於調節細胞凋亡以及細胞週期之不同。
10. 建構 IGF-II receptor RNAi 之載體，以期能抑制 IGF-II receptor 基因表現。刺激以 AngII 且同時轉殖 IGF-II receptor RNAi，觀察 AngII 造成之心肌細胞肥大與凋亡在抑制 IGF-II receptor 基因表現後是否受到影響。
11. 建構 IGF-I receptor RNAi 之載體，以期能抑制 IGF-I receptor 基因表現。在轉殖 IGF-I receptor RNAi 後，分別加入 IGF-I, IGF-II and IGF-II analog，觀察細胞凋亡蛋白：活化型 Caspase3 蛋白表現量之變化；建構 Calcineurin RNAi 之載體，以期能抑制 Calcineurin 基因表現，在上述處理所造成之活化型 Caspase3 蛋白表現量之變化是否因抑制 Calcineurin 基因表現而有所影響。

第二部分：ZAK 透過 JNK/p38 訊息路徑活化 MMP-2 以及活化 calcineurin 誘發心肌細胞凋亡參與在心肌病態的重塑作用

ZAK involves in pathologically cardiac remodeling through enhance MMP-2 by JNK/p38 and induce apoptosis by activation of calcineurin in H9c2 cardiomyoblast cells.

1. 使用穩定表現型心肌細胞株(Tet-on system H9c2 cell)誘發大量表現 ZAK 基因，觀察心肌細胞是否有肥大以及凋亡變化；偵測細胞內有關存活路徑的蛋白：p-Akt, Akt and p-Bad 以及凋亡蛋白：Bad, Bid and Caspase3 之蛋白變化量。
2. 使用 ZAK-wt(野生型), ZAK-E/E (S230E S234E;高激酶活性型), ZAK-dn (K45M;無激酶活性型)三株不同 KINASE 活性之穩定表現型心肌細胞株(Tet-on system H9c2 cell)，誘發不同 ZAK 活性表達，並處理以 AngII，觀察 ZAK 是否參與在 AngII 所造成之心肌凋亡路徑中。
3. 使用人類心肌梗塞的組織晶片，測定 ZAK 蛋白表現之差異。
4. 使用穩定表現型心肌細胞株(Tet-on system H9c2 cell)誘發大量表現 ZAK 基因，觀察 ZAK 是否影響 Calcineurin/NFAT3 之活化；以及使用不同路徑之抑制劑(SP-600125, SB-203580, U-0126, Ly-294002, FK-506 and CsA)探討 ZAK 透過哪一訊息路徑造成心肌凋亡。
5. 使用穩定表現型心肌細胞株(Tet-on system H9c2 cell)誘發大量表現 ZAK 基因，觀察參與在 cardiac remodeling 相關蛋白(MMP-2, MMP-9, TIMP-1, TIMP-2, TIMP-3, TIMP-4, uPA and PAI-1)之活性和表現是否受到影響；以及使用不同路徑之抑制劑(SP-600125, SB-203580, U-0126, Ly-294002, FK-506 and CsA)探討 ZAK 透過哪一訊息路徑活化 MMP-2。

# 五：研究方法

## Cell Culture

H9c2 cells were obtained from American Type Culture Collection (ATCC) and were cultured in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1 mM pyruvate in humidified air ($CO_2$ 5%) at 37 °C. H9c2 cells were cultured in serum-free medium for 12h, and then treated with or without Ang II ($10^{-8}$M) (Sigma Chemical Co., St. Louis, MO, USA), antisense IGF-I (14 μM), antisense IGF-II (14 μM), IGF-I receptor antibody (100 ng/ml) (Neo Markers, Fremont, CA, USA) or IGF-II antibody (100 ng/ml) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The incubation was continued for another 24 h, and then the cells were harvested and extracted for the analysis.

## DNA Fragmentation

H9c2 cells were lysed in 50 μl of lysis buffer (50mM Tris-HCl (pH 7.4), 20 mM EDTA, 1%IGEPAL-630) followed by incubation with 1%SDS and 5 μg/ μl RNase (Roche Molecular Biochemicals, Mannheim, Germany) for 2 h at 56 °C and 2.5g/ μl proteinase K (Roche) for 2 h at 37 °C, and only fragmented DNA was extracted. DNA was ethanol-precipitated and finally resuspended in distilled water. The fragmented DNA was electrophoretically fractionated on 1.5% agarose gel and stained with ethidium bromide.

## Protein Kinase Inhitors

H9c2 cells treated several protein kinase inhitors, including SB203580 (p38 MAP kinase inhibitor; Promega), U0126 (MEK1 and MEK2 inhibitor; Promega), SP600125 (JNK inhibitor; Promega) and LY294002 (PI 3-kinase inhibitor; Promega). The final concentration of SB203580 is 10 μM and the final concentration of U0126 is 30 μM and the final concentration of SP600125 is 20 μM and the final concentration of LY294002 is 10 μM.

## Total RNA extraction

Total RNA will be extracted using the Ultraspec RNA isolation System (Biotecx Laboratories, Inc.) according to directions supplied by the manufacturer. Respectively, Cells and Cardiac tissues were thoroughly homogenized (1 ml Ultraspec reagent per 100 mg tissue) with a homogenizer. The RNA precipitate was washed twice by gentle vortexing with 70% ethanol, collected by centrifugation at 12,000g, dried under vacuum for 5-10min., dissolved in 50-100 ul of diethylpyrocarbonate-treated water, and incubated for 10-15 min. at 55-60°C.

## Reverse transcription and PCR Amplification

cDNA was prepared in a buffer containing 50 mM Tris-HC1, pH 8.5,30 mM KCl, 8 mM MgCl,,1 mM dithiothreitol, 0.25 mM each dCTP, dGTP, dTTP, and dATP, 20 units of recombinant ribonuclease inhibitor, 1 pg of random hexamers, 5 pg of total RNA, 40 units of avian myeloblastosis virus reverse transcriptase, in a volume of 20 pl. This mixture was incubated for 10 min at room temperature followed by 1 h a t 42 "C to initiate cDNA synthesis. This mixture was then used for amplification of specific cDNAs by PCR. The buffer for PCR contained 50 m~ KCl, 10 mM Tris-HC1, pH 8.3 at 20 "C, 0.2 m~ each dCTP, dGTP, d'MP, and dATP, 0.5 p~ oligonucleotide PCR primers, 2.5 units of Taq polymerase, and various MgCl, concentrations in a final volume of 100 pl. Following the hot start (5 min at 95 "C, 80 "C hold), the samples were subjected to 35 cycles of 45 s at 95 "C, 2 min at 52 "C, and 45 s at 72 "C; for the IGF-II, IHG-II receptor and GAPDH primers the primer annealing temperature was 56 "C. This was followed by a final extension step at 72 "C for 10 min. All RNA samples used were demonstrated to have intact 18 S and 28 S RNA bands on ethidium bromide-strained formaldehyde-agarose gels.

### Protein Extraction and Western blot analysis

Cultured H9c2 cells were scraped and washed once with phosphate-buffered saline. Cell suspension was then spun down, and cell pellets were lysed for 30 min in lysis buffer (50 mM Tris (pH 7.5), 0.5M NaCl, 1.0 mM EDTA (pH 7.5), 10% glycerol, 1mM BME, 1% IGEPAL-630 and proteinase inhibitor cocktail table (Roche)) and spun down 12,000 rpm for 10 min. Then, apply the supernatants to new eppendorf tube for western blot. Cardiac tissue protein extracts from the aminals processed complete coarctation of the abdominal aorta were prepared by homogenizing the left ventricle samples in a PBS buffer (0.14M NaCl, 3mM KCL, 1.4mM KH2PO4, 14mM K2HPO4) at a concentration of 1 mg tissue/10 $\mu$l PBS for 5 min. The homogenates were placed on ice for 10 min and then centrifuged at 12,000 rpm for 30 min. The supernatant was collected and stored at -70℃ for further western esperiments. Proteins from H9C2 cell line or amimal heart extracts were then separated in 12% gradient SDS-PAGE and transferred to nitrocellulose membranes. Nonspecific protein binding was blocked in blocking buffer (5% milk, 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) and blotted with specific antibodies (Caspase 3, Akt-p, ZAK and $\alpha$-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA))as indicated for each experiment in the blocking buffer at 4 °C overnight. For repeated blotting, nitrocellulose membranes were stripped with Restore Western blot stripping

buffer (Pierce Biotechnology, Inc, Rockford, IL, USA) at room temperature for 30 min.

## Terminal Deoxynucleotide Transferase-mediated dUTP Nick End Labeling (TUNEL)

H9c2 cells plated on a cover glass were fixed with 4% paraformaldehyde solution for 30 min at room temperature. After a rinse with phosphate-buffered saline, the samples were first incubated with phalloidin-rhodamine for 1 h and with TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and fluorescein isothiocyanate-dUTP(Roche Applied Science). In tissues, the 3-µm thick paraffin sections were deparaffinized by immersing in xylene, rehydrated, and incubated in phosphate-buffered saline with 2% $H_2O_2$ to inactivate endogenous peroxidases. Next, the sections were incubated with proteinase K (20 µg/ml), washed in phosphate-buffered saline, and incubated with terminal deoxynucleotidyl transferase for 90 min and fluorescein isothiocyanate-dUTP for 30 min at 37 °C using an apoptosis detection kit (Roche Applied Science). Samples can be analyzed in a drop of PBS under a fluorescence microscope at this state. Use an excitation wavelength in the range of 450–500 nm and detection in the range of 515–565 nm (green). Or After Incubate slide with Converter-POD in a humidified chamber for 30 min at 37°C, the sections were stained with diaminobenzine for 10 min at room temperature, washed in phosphate-buffered saline, and mounted for light microscopic observations. The number of TUNEL-positive cardiac myocytes was determined by counting $3 \times 10^5$ cardiac myocytes. All morphometric measurements were performed by at least two independent individuals in a blinded manner.

## Measurement of $[Ca^{2+}]_i$ Transients

H9c2 were loaded with the fluorescent $Ca^{2+}$ indicator indo-1-AM (1.25 mM, Calbiochem) for 30 min at room temperature. Thereafter, the culture dish was mounted on the heated stage of an inverted microscope (Diaphot 300, Nikon), and H9c2 were field-stimulated at 1 Hz and excited at a wavelength of 350 nm. $[Ca^{2+}]_i$ transients were recorded at emission wavelengths of 405 nm and 490 nm with a photomultiplier (PTI 814, Photon Technology International, Lawrenceville, NJ).

# 六：結果（附數據圖）與說明　　七：討論

類胰島素生長因子-II 接受體之基因調控與其訊息路徑誘發心肌凋亡之分子機轉探討

The molecular mechanism of regulating IGF-II receptor gene expression and the IGF-II receptor signaling pathway induce apoptosis in the cardiomyocytes.

心臟的功能主要為透過血液循環供應氧氣和養分給予全身各個器官進行代謝利用，而當環境改變時，人體會透過調節賀爾蒙的分泌改變心臟的功能，其中心肌細胞則會以肥大的方式來增加收縮的強度或者速度以期能夠適應環境的變遷，但持續性賀爾蒙的刺激亦會造成心肌細胞凋亡，在心衰竭的病患中亦發現到有著過高的賀爾蒙，這些賀爾蒙包括：AngII 和 ISO…等(49)。另外，心肌細胞在刺激以誘發發炎反應之細胞激素：TNF-α和細胞毒素：LPS 亦被證實能造成凋亡的發生(50)。於是在實驗一開始，我們使用 H9c2 cardiofibroblast 和 neonatal cardiomyocytes 細胞培養的方式，處理以 AngII、ISO、Inomycin、LPS and TNF-α 刺激物，觀察 IGF-II receptor 基因是否受到開啟。由圖 1a-b 中得知，AngII、Inomycin、LPS and TNF-α 刺激下能夠促進 IGF-II receptor 基因表現增加，其中 AngII 和 Inomycin 在刺激後 18 小時能活化 IGF-II receptor 基因表現增加，LPS 和 TNF-α 則在 6 小時；另外，刺激以 ISO 則無法開啟 IGF-II receptor 基因表現。而在先天性高血壓的大白鼠(SHRs)中，與正常的大白鼠(Wistars)相較之下，心臟有著顯著的肥大現象並伴隨著較高 IGF-II receptor 基因表現(Fig.1c)。而在因基因缺陷造成惡性肥胖的大白鼠(Zucker Rats)中，觀察到與正常組(Lean Rats)比較下，左心室的心肌細胞有著顯著凋亡現象並伴隨著較高 IGF-II receptor 基因表現(Fig.1d)。接著我們使用包含正常人、急性心肌梗塞以及心癲痂病患檢體之組織晶片觀察 IGF-II receptor 蛋白表現之差異，結果顯示在十名正常人中只有一名偵測到 IGF-II receptor 蛋白表現，但在急性心肌梗塞和心癲痂病患檢體中，則高達百分之九十以上的比率偵測到 IGF-II receptor 蛋白表現(Fig.1e)。在上述的實驗結果，我們分別使用心肌細胞刺激以各種不同的刺激物的細胞模式以及先天性高血壓大白鼠和病態肥胖大白鼠的動物模式，最後使用人類心肌梗塞的臨床檢體，證實在各種誘發心臟病變的條件下，均會增加 IGF-II receptor 基因和蛋白的表現量。

然而在心肌細胞受到刺激下時，IGF-II receptor 基因又是如何被調節的?首先我們經由 EMBL-EBI(http://www.ebi.ac.uk/ensembl/)搜尋出 IGF-II receptor 基因在 exon1 前 2000bp 的 genomic DNA 序列，然後將此序列送到 TFSEARCH: Searching Transcription Factor Binding Sites(http://www.cbrc.jp/research/db/TFSEARCH.html)進行可能的轉錄活化因子的結合分析，結果顯示於 table1 中。接著我們建構含有不同片段之 IGF-II receptor promoter 的報導基因試圖去尋找可能具有功能的序列(Fig.2a; table2)，目前此一實驗仍在進行中。而由先前實驗室的結果得知：AngII 會透過 JNK 路徑開啟

IGF-II receptor 基因表現(Fig.2b)，而在 table1 轉錄活化因子的結合序列分析中，約在 promoter 上-550bp 的序列上顯示出有 AP-1 之結合位置，而 AP-1 蛋白為 JNK 路徑中的下游轉錄活化因子，於是我們合成 II R-p-AP-1 序列: 5' labeling BIOTEIN -TGTTAACTCTGAATCAACTTGCAA，在刺激以 AngII 和 Inomycin 後進行 EMSA assay 實驗，觀察 c-jun 蛋白結合於 II R-p-AP-1 上的能力是否增加。由結果得知，在處理以 AngII 和 Inomycin 後會增加 c-jun 蛋白結合於 II R-p-AP-1 上的能力，但令人感到意外的是同時加入 c-jun antibody 則未出現 super-shift 的現象，反而減弱了原先因刺激 AngII 和 Inomycin 後增加的結合能力，我們認為加入 c-jun antibody 反而競爭掉了原先 AP-1 與 II R-p-AP-1 的結合能力(Fig.2b)。

另一方面已知 IGF-II receptor 為一 imprint gene(51)。在胚胎發育時期，IGF-II receptor 基因會選擇性地開啟，主要受到 promoter 上 CpG island 區域的 DNA Methylation 所調控。於是我們提出一個問題：CpG island 上的 DNA Methylation 是否參與在心肌細胞受刺激下開啟 IGF-II receptor 基因的機制中?在這實驗中，我們分別使用 H9c2 cardiofibroblast 和 neonatal cardiomyocytes 細胞培養的方式，處理以 AngII 和 Inomycin 以及比較先天性高血壓的大白鼠(SHRs)和正常大白鼠(Wistars)，加上臨床因心臟疾病開刀取得的心臟檢體進行 methylation specific PCR (MSP)實驗，由結果得知在處理以 AngII 和 Inomycin 刺激下以及其他心臟病變的檢體中，IGF-II receptor 基因的開啟與其 CpG island 區域的 DNA Methylation 並相關(Fig.3a)。然而在 Histione modification 亦被報導參與在調節 IGF-II receptor 的開啟(52)，於是在接下來的實驗中，我們將偵測在 IGF-II receptor CpG island 區域裡，Histione H3 的乙醯化和甲基化(acetyl and trimethyl Histione H3)以及 MeCP2 (DNA methylation binding protein)的結合能力是否受到 AngII 和 Inomycin 刺激下而有所改變。由 ChIP 的實驗結果得知，在處理以 AngII 和 Inomycin 會增加 Histione H3 acetylation、減弱 Histione H3 trimethylation 和減低 MeCP2 的結合能力(Fig.3b)。

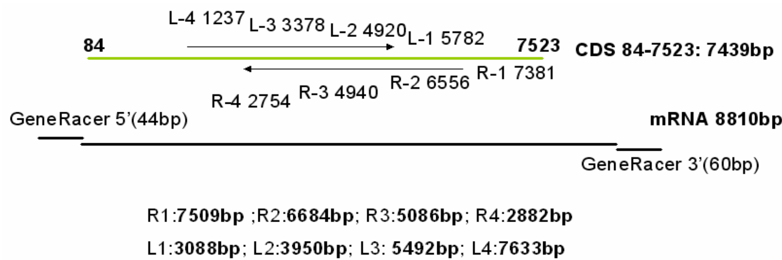
為了探討在心臟病變過程中，大量活化的IGF-II receptor基因的功能為何?除了扮演清除IGFs的角色外，是否具有其他活化細胞內訊息傳遞的功能。於是我們使用IGF-II analog (IGF-II Leu27)(53)，其氨機酸Leu 27經過修飾，對於IGF-II receptor有著極高的親合力，來探討經在IGFs結合於IGF-II receptor後，對心肌細胞的凋亡影響為何以及其可能存在的分子機轉為何?由實驗結果得知，IGF-II analog與IGF-I/IGF-II相較，並無法開啟由IGF-I receptor signaling所活化的ERK和Akt(Fig.4a)，也顯示出IGF-II receptor的角色不同於IGF-I receptor，但單獨處理以IGF-II analog亦無法造成心肌細胞凋亡(Fig.4a)。接著我們將細胞刺激以AngII和Insulin/IGF-I receptor inhibitor(AG1024;抑制IGF-I receptor signaling pathway)，然後在分別加入IGF-I, IGF-II and IGF-II analog，比較三者對於AngII和AG1024造成生長停止和細胞凋亡之影響為何?由實驗結果得知，在AngII和AG1024造成心肌細胞凋亡中，只有加入IGF-I具有抗凋亡的保護效果；加入IGF-II時抗凋亡的保護效果不佳；然而加入IGF-II analog則有加乘凋亡的現象(Fig.4b, d)。而AG1024處理下造成細胞週期G1的停頓增加，只有在加入IGF-I後細胞

周期中的S期和M期同時增加(Fig.4C)。上述的實驗結果指出IGF-II receptor訊息路徑不同於IGF-I receptor的存活路徑和活化細胞週期的能力，具有促凋亡的影響。先前的文獻指出：在3T3 cells中，IGF-II R經IGF-II活化後能與Gαi2結合，但是否存在心肌細胞中仍為未知。另外有報導指出在心肌細胞受到stretch時，會活化Gαi/PLC/$IP_3$/$Ca^{2+}$/Calcineurin此一路徑(54)。於是我們使用免疫沉澱的實驗方法，在分別刺激以IGF-II和IGF-II analog時，觀察在不同時間點下，Gαi與IGF-II R之結合能力。由實驗結果得知，在處理IGF-II後五分鐘可以觀察到Gαi與IGF-II R結合，但在三十分鐘時Gαi與IGF-II R的結合即消失；然而處理IGF-II analog一小時後，仍能觀察到Gαi與IGF-II R的結合(Fig.4e)以及增加Calcineurin的活化(Fig.4e)，另外若在加入IGF-I時，同時加入IGF-II analog，則IGF-I所活化之存活蛋白：磷酸化態的Bad和Akt，則受到抑制(Fig.4e)。

在先前的實驗中，我們證實在心臟病變過程中IGF-II receptor基因會被大量開啟以及IGF-II receptor signal不同於IGF-I receptor signal的存活路徑為一促凋亡路徑。但仍缺乏直接證據指出：在心肌細胞受凋亡刺激下，IGF-II receptor參與其中。於是我們建構IGF-II R RNAi，在處理以AngII造成心肌細胞肥大與凋亡時，轉殖IGF-II R RNAi抑制IGF-II R基因的表達，觀察在AngII造成心肌細胞肥大與凋亡過程中，IGF-II R是否參與其中。由實驗結果得知，在抑制IGF-II R基因的表達後，AngII造成心肌細胞的凋亡受到抑制，對於肥大則沒有影響(Fig.5a)。在先前Fig.4a的實驗中並無法觀察到IGF-II和IGF-II analog直接造成細胞凋亡的現象，為了排除IGF-II和IGF-II analog可能會結合於IGF-I receptor上，而減弱了到IGF-II receptor的凋亡路徑，於是我們建構IGF-I R RNAi，抑制細胞內的IGF-I R基因表現，在分別加入IGF-I, IGF-II and IGF-II analog，觀察凋亡蛋白Caspase3之變化。由實驗結果得知，單獨轉殖IGF-I R RNAi並不會增加Caspase3的活化，但在同時加入IGF-II and IGF-II analog時則增加Caspase3的活化，特別是在加入IGF-II analog(Fig.5b)。另外，我們使用Calcineurin RNAi和Calcineurin inhibitors(FK506;CsA)則抑制住了由同時轉殖IGF-I R RNAi和加入IGF-II analog所造成Caspase3的活化(Fig.5c)。由上述的實驗結果，顯示在心臟病變過程中，IGF-II receptor基因會被大量開啟，而活化的IGF-II receptor signal會進而促進心肌細胞凋亡，加速心衰竭的進程。這樣的發現將有助於在生物醫學上提供一個治療和減緩心衰竭發生的標地基因。

在接下來的計畫中，我們打算cloning IGF-II receptor基因全長(CDS)，並依照已知結合於IGF-II receptor的不同結合體( Ligand )，將IGF-II receptor的extracellular domain分成不同片段，首先將IGF-II receptor基因全長短暫轉直到心肌細胞內觀察其是否會造成心肌細胞凋亡，然後透過不同片段的IGF-II receptor extracellular domain基因轉殖，找出可能具有促凋亡的結合體( Ligand )為何?但在我們進行IGF-II receptor基因全長cloning時，意外地發現到IGF-II receptor的長度比預期的長度少了1000-2000bp(Fig.6a)。這樣的實驗結果，讓我們做了一個大膽的假設：在心臟中可能存在著一個新的IGF-II receptor isoform。為了印證這個假設，我們使用Rapid amplification of cDNA ends (RACE) end PCR，共設計了5'和3'各4組引子(如下圖)，試圖

找出新的isoform可能缺少的片段為何?在使用R-4 2785 primer進行5’ RACE end PCR




時,顯示出和Fig.6a一樣的結果,原先預期長度應為2882bp,如今卻短少為約1800bp左右(Fig.6b)。於是我們收集此一片段進行定序和進行分析，結果相當令人興奮，序列顯示出此一片段與原先IGF-II receptor基因序列相較少了exon1-9的片段，另外在exon10序列開始之前存在著部份intron9-10的片段(Fig.6c)。接著我們想要回答的這個新的IGF-II receptor isoform是否真的能被轉錄出來以及在各個器官之間的分布情形為何?於是我們購買Rat polyA+ RNA Northern Blot(包含12 major tissues: brain, heart, kidney, liver, lung, muscle, placenta, small intestine, spleen, stomach, testis, thymus)，接著利用In vitro transcription和Random primed DNA labeling實驗方法作出含有intron9-10片段的三種不同探針，然後將Rat polyA+ RNA Northern Blot膜與含有intron9-10片段的探針進行雜合，結果一致地顯示，此一含有部分intron9-10片段的IGF-II receptor isoform確實能夠被轉錄出來，並且廣泛的在各個組織中表現，但其卻存在著三個不同的分子量，分別為6000bp、5000bp和2000bp(Fig.7a- c)。然後，我們試著定義出此一IGF-II receptor isoform的全長為何?這次我們在intron9-10片段上設計5’ primer進行3’ RACE end PCR實驗，實驗結果顯示出在2500-3000bp中出現一條band(Fig.7d)，這樣的結果與Fig.7a- c所定義出的分子量有所衝突。實驗進行到這裡可以說：是遇到了瓶頸。目前而言，我們依舊試圖cloning IGF-II receptor基因全長和將Fig.7d中出現2500-3000bp的band定序出來或者調整PCR的條件以期能定義出含有部分intron9-10片段的IGF-II receptor isoform。

## ZAK 透過 JNK/p38 訊息路徑活化 MMP-2 以及活化 calcineurin 誘發心肌細胞凋亡參與在心肌病態的重塑作用

## ZAK involves in pathologically cardiac remodeling through enhance MMP-2 by JNK/p38 and induce apoptosis by activation of calcineurin in H9c2 cardiomyoblast cells.

**ZAK both induces hypertrophy and apoptosis of H9c2 cell line.**

In our previously study, we had created that inducible stable clone of expressing ZAK gene in H9c2. In this Tet-on system using the doxtcycline to induce signification of zak gene accompany with increased the hypertrophic marker gene ANF through RT-PCR (Fig. 8a). ZAK had been show caused cardiac hypertrophy earlier but whether ZAK generates the apoptotic

effect is not known. Therefore resulted in overexpression ZAK not only induced hypertrophic influence but also enhanced apoptosis that determined respectively by Actin stain and TUNEL assay (Fig.8b). Response extensively in AngII make non-compensatory hypertrophy accompany with apoptosis in cardiomyocytes is a critical process to cause heart failure. Consequently we investigate whether ZAK-inducing MAPK signaling pathway could involve in apoptotic effect of AngII-treated. Using the over expression of different JNK kinase activity of ZAK included ZAKwt, ZAKE/E (S230E S234E), ZAKdn (K45M) in treated AngII simultaneously indicate ZAK affect the apoptosis of H9c2 without AngII involved (Fig.9c). We suggested the ZAK stimulated both hypertrophy and apoptosis in H9c2 is a pathological hypertrophy as non-compensatory influence but AngII not participated in.

**ZAK aberrantly express in myocardial infraction and enhance activation of caspase3**

Cardiac infraction is acute heart disease caused by coronary thrombosis then make heart remodeling (55). In response to this injury included machinery stress, hypoxia and starvation, many intercellular signaling cascade were been activated such as stress-inducible protein kinase JNK and p38 pathway, which subsequently transmit the signals into cell nucleus to regulate gene expression. ZAK, up stream molecule of JNK signaling pathway, never were reported involved in cardiac remodeling from cardiac infraction of clinical specimen. We indicate that increasing ZAK aberrantly in the sections of cardiac infraction (22/27) and myocardial scar (9/10) compare with in normal ventricular tissue (1/10) (Fig.10). The resulting imply that during heart remodeling ZAK may have a critical role in regulated ECM re-organization and facilitated development of heart failure. Further as inducing ZAK expression to increase JNK1/2 activity (Fig.9c), which accompany reducing phosphorylation of cell survival factors, Akt and Bad (Fig.9a), with enhancing Caspase3 activation but not Bid (Fig.9b). These data evidenced ZAK was able to induce apoptosis of cardiomyocytes through attenuated phosphorylation of Akt and Bad to activated Caspase3 period the cardiac infraction.

**ZAK activate calcineurin/NFATc1 signaling pathway and affect calcium influx.**

Several growth factors such like IGF-I binding their receptor have a benefic effect in response to heart remodeling through intercellular signal pathway cascade, PI3K- Akt- Bad survival pathway, to prevent apoptosis of cardiomyocytes. But if aberrant stimulation in heart has been extended, the PI3K- Akt- Bad survival pathway could be repressed by the calcium-inducible protein phosphotase Calcineurin. We investigate whether ZAK activated the Calcineurin/ NFAT signaling pathway to induce apoptosis and to modulate calcium-handing contraction. In the over-expressed ZAK gene, the binding of Calcineurin with CaM1 be increased and the downstream effectors of Calcineurin NFAT3 transmitted from cytoplasm into nucleus exhibit that ZAK can regulate the activation of Calcineurin/ NFAT signaling pathway (Fig.11a-b). Using differently various protein kinase inhibitors contained SP, SB, U, Ly, FK and CsA with over-expression of ZAK to identify that ZAK induced activation of Caspase3 through Calcineurin signaling pathway (Fig.11d). Calcium recycling in the SR by RYR and SERCA was

crucial for contraction-relaxation mechanism in cardiomycytes (56). The results of calcium staining revealed that ZAK increased calcium influx (Fig.11c). And to investigate what the calcium modulators to be regulated, displayed ZAK diminished the expression of non phosphor-PLB (Fig.4d) which can inhibit SERCA recover calcium from cytoplasm into SR to let the calcium accumulation in cytoplasm(57,58). In conclusion we considered that ZAK affect activation of Calcineurin/NFAT signal pathway to modulate calcium recovery by de-phosphorylation of PLB to imply that ZAK may affect physiologically functional contraction of cardiomycytes.

**Over-expression of ZAK altered ECM by evoked MMP-2 activity via JNK pathway involved in ventricular remodeling.**

During heart remodeling in response to multi-stimulators make the adapted effects that not only regulating the size of cardiomyocytes but also altered the structure of composing ECM. Several enzymes such like MMP and TIMP involved in assembling ECM associated with irreversible morbidity effects to heart failure. ZAK has been indicated that aberrant expression in acute infraction and myocardium scar (Fig.10), whether its influence through activation or suppression of MMP/TIMP activity involved with cardiac remodeling. ZAK enhanced MMP-2 activity, but not MMP-9, through JNK and p38 signal pathway (Fig.12) and induced the protein level of TIMP-1/2 (Fig-5b) that indicates the ZAK activated signaling cascade through regulating MMP-2 activity and TIMP1/2 gene expression to alter the ECM structure involved in ventricular remodeling.

Fig.1a

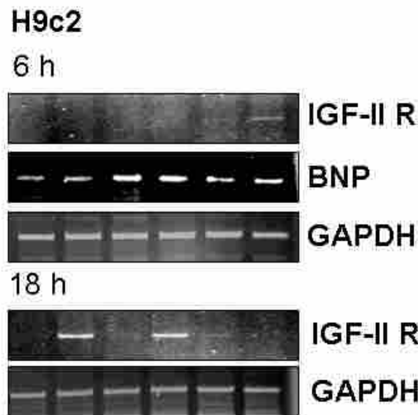



Fig.1b

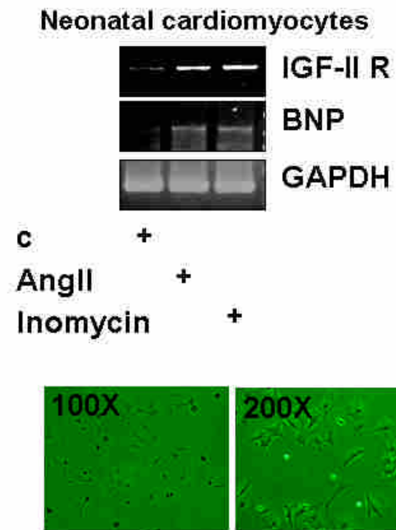



Fig.1c

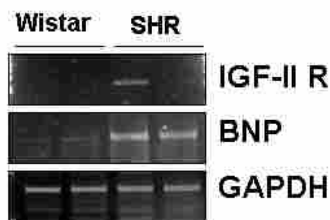



Fig.1d

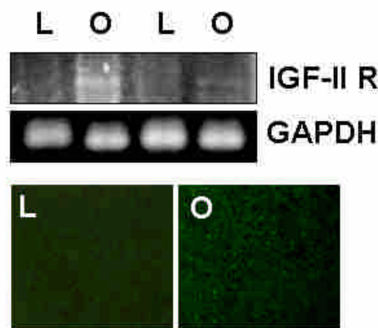
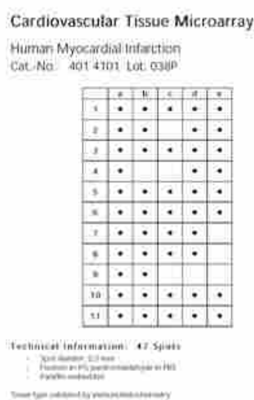



Fig.1e



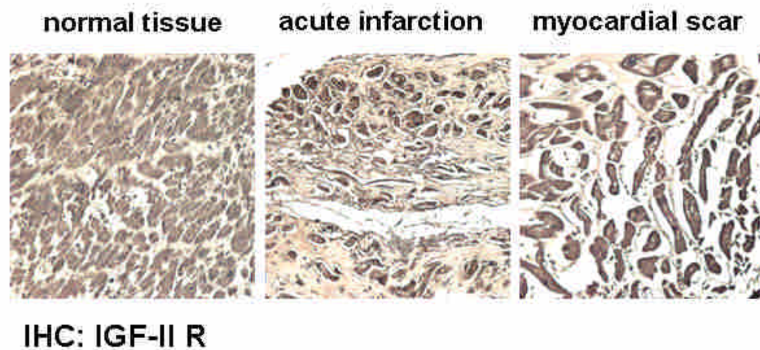
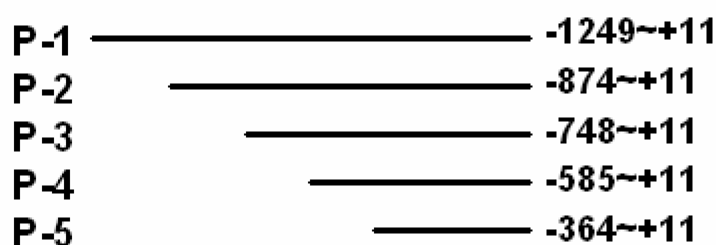




Figure1. Increasing IGF-II receptor expression in variously stimulated to pathological hypertrophy.

(a) Several stimuli included AngII, ISO, Inomycin, LPS and TNF-$\alpha$ treated H9c2 cardiofibroblast at 6 or 18 hours to identify IGF-II receptor expression by RT-PCR.
(b) In neonatal cardiomyocytes treated with AngII and Inomycin to indicate IGF-II receptor expression by RT-PCR.
(c) Compare the Wistar rats with SHR in the heart of IGF-II receptor gene expression using RT-PCR.
(d) To examine the expression level of IGF-II receptor between Lean and obese rats by RT-PCR.
(e) Using human tissue assay about acute infraction and myocardial scar section to observe the expression IGF-II receptor protein by IHC.

Fig.2a



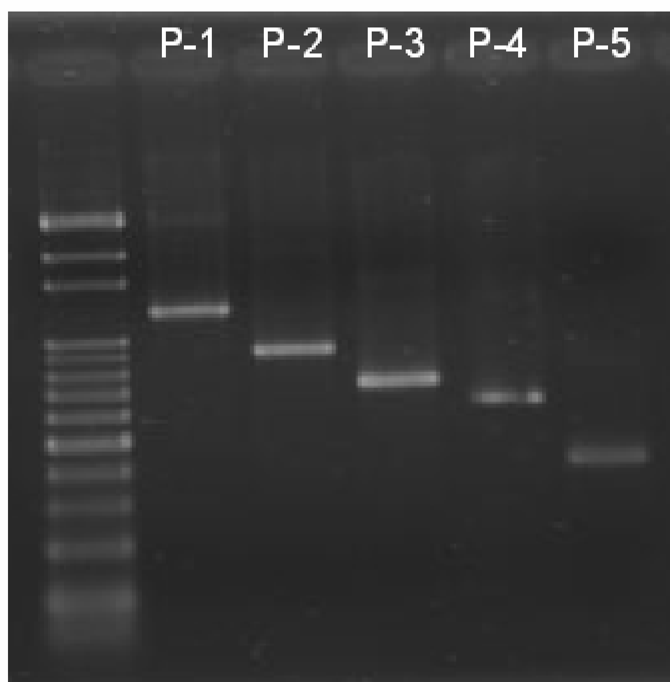




Table 2.IGF-II receptor promoter sequences **(**5' upstream sequence; 2000bp**)**

tcccagcttgctctccctag

cttctgcctagcttgctttcttatacagtccacaactccctgtctaggggatggtactgc

tcacagtgggctgtgcccttgtctaacaatttttgatcaagaaaatgttccaaagacatg

cccatggggcagtctccctctgacagctctgggctgtgtcacatcgagagctgaagctaa

ctaggagacaccatcagatcatgaatacatgaatggattacttcattgattaggtcatga

cttaatcatctctcagtagtgccaccacctggggacatagacttcatcaagggcttggtt

tacgggtgctccatttccaagtcatcaaaagaaaatgtattttgggggtgatactttgag

tagaggtaaacattctggttcatagttaactcttcatttctttcttcttcttttttt

ttttcaataactcttgcctgcagtagttctaacgtgaaggcaaacatgaactcccacttt

tatcagaagattactcctgtacccaggacccacaattggtccttcttccagcaggcagct

cctggagggacgcacgtggagtgaaggaatggaatatccgtctagctagctagatcattt

gatcaaccctggtgtcacagtcccggattgtcttcccgcccagtcacctcttcttgattt

cttgacaacagtagacatctagggagctctgggctattcagtatagcgaatcaatcaatt

tctctctctctctctctctctctctctctctctctctctctgaggtgtgtgtcagggt

P1-L

ctcatatgtag**caatgactagtcttcatgtaacagcct**tcgaactgttgggattaaaggt

acatgctaccacacgcagtagaattaccttattctttcctgtgtgtatgcttgcaaactc

ccttggaggccagaggttttgtttacttttccatctcttcgagacagtacccggacctgg

ctagattcagctagactggctggccagcaaacatcggagatccccctcatccctgcctcc

ccacagaggttccaggggtttgcagctacagactttattatatgggtgccgggacttgag

P2-L

ttcaggcctcatgcttctggagcaca**ttacagacggagccatttcc**taagccacatattt

attttactttcactaggtcaccgccttacataatatgggtgctgcatagatctttacaga

acaataaaactttgttaacagaggtggtggaacacgcttgtaaacccagcattcggaagg

P3-L

atccgaactccagtcctgtctgagctacatag**gcagcagtttcacaccaaaa**gttttgtg

tgtatgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgatttgcttgttacactttggtt

P4-L

ttctgtacactgcat**attagttcatcacggtcgcc**tgacttgctgcagaaagccctgctt

gggaaatcacacttccacagagctcctgtgaggccctcccggcaccagtcggaaactccc

acgcaggattctagaaaagctgacctgttaactctgaatcaacttgcaacactaagaatc

P5-L

aacctgggcctttccacctaactccatctcggccaccgtactggtctcggttgaaa**aaga**

**gagttaggaagcgcc**caagcgcagacgcaaccagggtgctggacggggaaactgaggtct

gggctctgaggcgtcacccctcgctgcgcgtgaccaaaccctggggttgccaggcttcga

gtaggtacctggcgctcgtgcccggcccacaacacttcctgtcccgcgcgcttgcgatgc

tcatgtgacccggggctgggcggagagcacctgaacgagaacgtcacgtgacaggaggcg

gggcaggggcgggccgactccggtcacgtgacgctttggggacggccacggagcgcctcc

P6-R

tcatctcactccccccctggctccggctctctctcctctttctccctccagctc**ccgttgc**

**AGCTTCGACTC**CGCTGTTGCGGCGCGATCGTGTCCCAGGCGCGGCTCGGAACGGCCAGCC

GCCGTGAGCCCCACGCCACACGCG

Primer design:

P1-L: **caatgactagtcttcatgtaacagcct**

P2-L: **ttacagacggagccatttcc**

P3-L: **gcagcagtttcacaccaaaa**

P4-L: **attagttcatcacggtcgcc**

P5-L: **aagagagttaggaagcgcc**

P6-R: **gagtcgaagctgcaacgg**

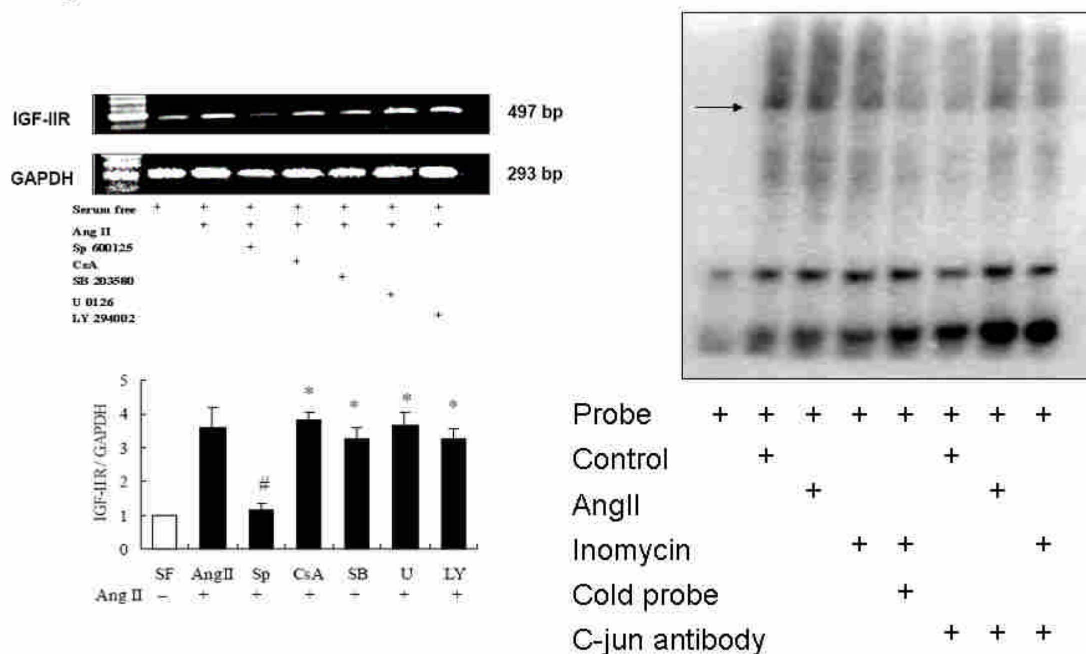


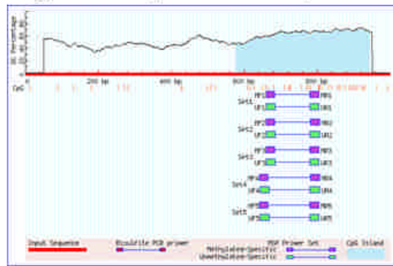

Figure2. AngII and Inomycin induced IGF-II receptor expression through JNK pathway and raised c-jun binding in AP-1 site in the IGF-II receptor promoter.

(a) Constructing the different length of IGF-II receptor promoter to the pGL3 Luciferase Reporter vector to explain the regulating mechanism of IGF-II receptor expression using Luciferase Assay System.

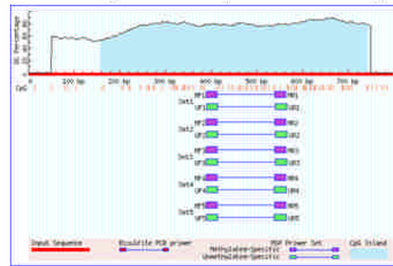
(b) To study the AP-I binding activity in the promoter of the IGF-II receptor through mixed the nucleus protein of H9C2 treated-AngII or Inomycine with the probe as the AP-1 site sequences of the IGF-II receptor promoter and antibody of c-jun by EMSA.

Fig.3a

Rat Igf-2r 5' upstream sequence



Human-Igf-2r 5' upstream sequence

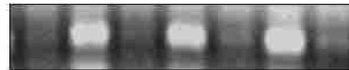


H9c2

M U M U M U

Wistar SHR

M U M U M U M U



Neonatal cardiomyocytes

M U M U M U

Human heart disease

M U M U M U M U

c + +

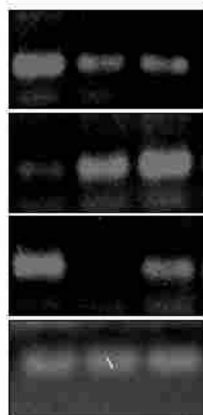
AngII + +

Inomycin + +

Fig.3b

ChIP assay

IGF-II receptor



Trimethyl Histone H3

Acetyl Histone H3

MeCP2

INPUT

c +

AngII +

Inomycin +

Chromatin Immunoprecipitation

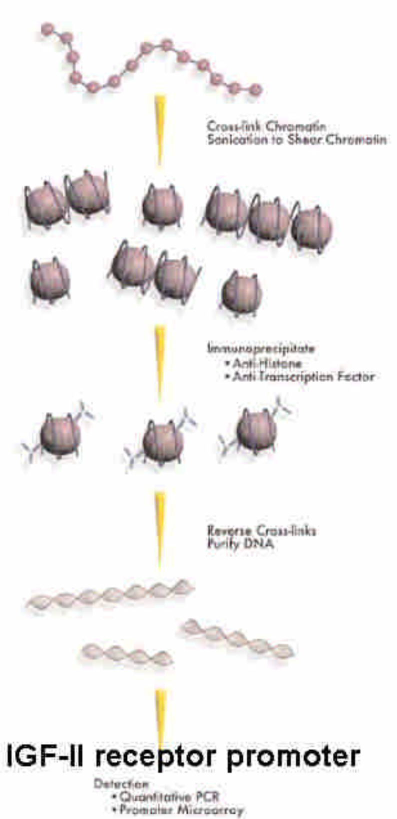



Figure3. Histone modification involved in regulating IGF-II receptor gene expression, but not DNA methylation of CpG island in IGF-II receptor promoter.

(a) H9c2 cardiofibroblasts and neonatal cardiomyocytes were treated with AngII or Inomycin to explore the DNA methylation in the CpG island of the IGF-II receptor promoter by MSP (left site) as well as the heart specimens of Wistar rats, SHR and human heart disease(right site).

(b) H9c2 cardiofibroblasts were treated with AngII or Inomycin to indicate the modification of acetyl and trimethyl Histione H3, and MeCP2 binding in the CpG island of the IGF-II receptor promoter by CHIP.

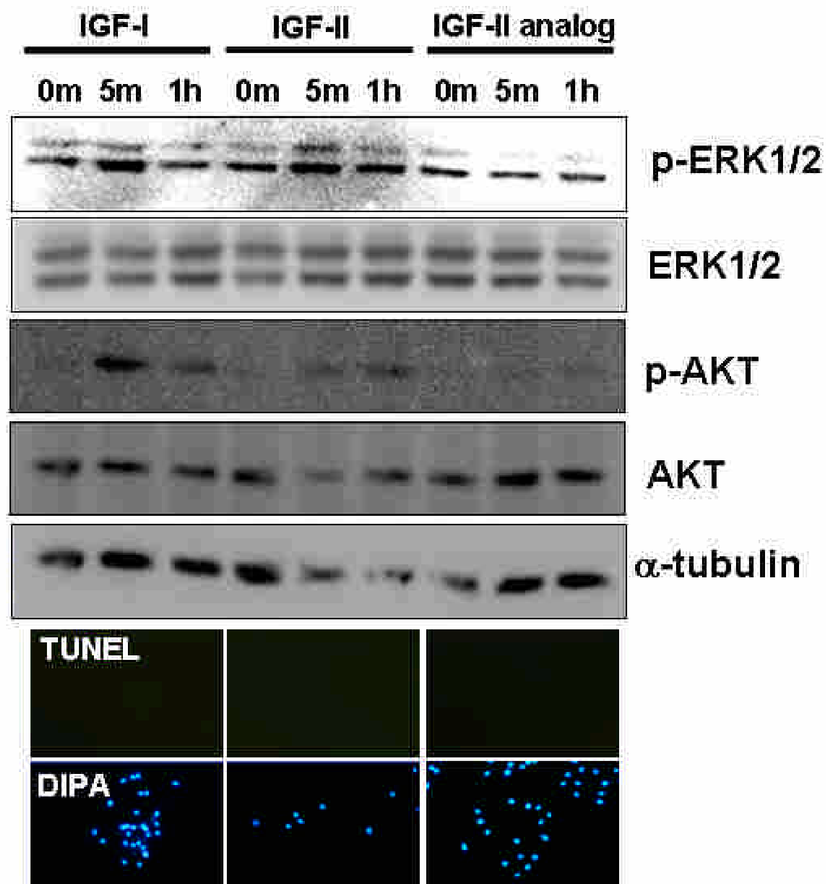
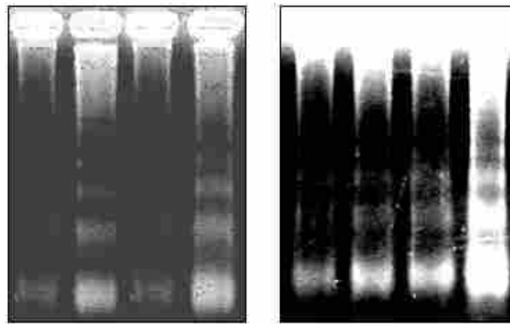


Fig.4b



| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Ang II** | + | + | + | | + | + | + |
| **IGF-I** | | + | | | | | |
| **IGF-II** | | | | | | + | |
| **IGF-II L27** | | | + | | | | + |

Fig.4c

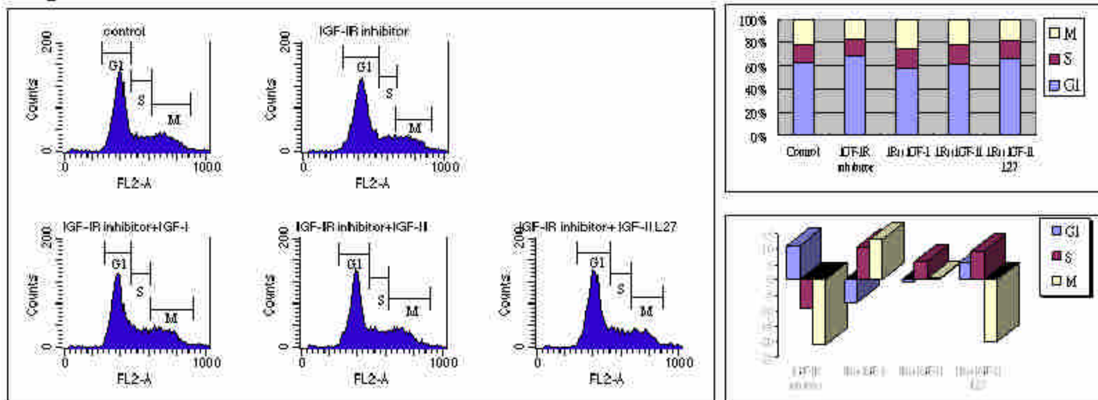
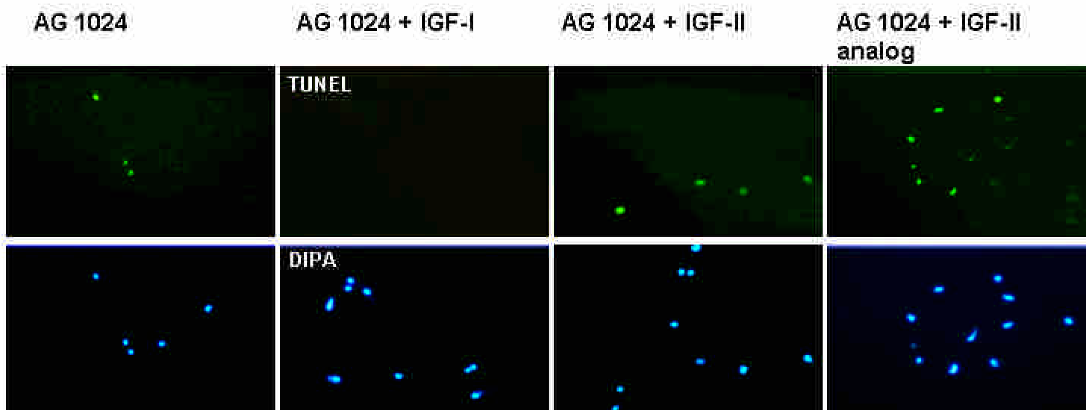



Fig.4d



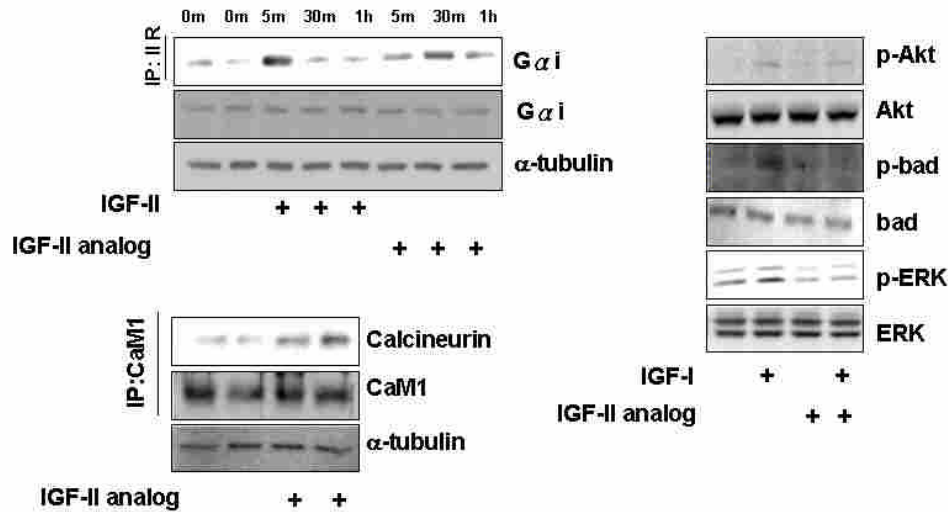



Figure4. The role of IGF-II receptor intracellular signaling pathway is apoptotic character, distinct from survival effect of IGF-I receptor.

(a) H9c2 cardiofibroblasts were treated with IGF-I, IGF-II and IGF-II analog, then recognized the protein level of p-ERK, ERK, p-Akt, α-tubulin and Akt by western blotting and detect the apoptotic effect by TUNEL assay. α-tubulin was a loading control.

(b) H9c2 cardiofibroblasts treated AngII and combined with or without IGF-I, IGF-II and IGF-II analog, then identify the distinct effect of the IGFs in AngII-induced apoptosis by DNA fragmentation and (c),(D) in AG 1024, IGF-I R and insulin R inhibitors, caused cell cycle influence by Flow Cytometry and apoptosis by TUNEL assay.

(e) IGF-II and IGF-II analog separable treated in H9c2 cardiofibroblasts at 5 min and 30min, then using the IGF-II R antibody to immuno-precipitating before detected the Gαi protein expression by western blotting (upper picture). And calcineurin activity have been reported through detected interacting between CaM1 and calcineurin by immuno–precipitation (lower picture). Add respectively IGF-I, IGF-II analog or together into the H9c2 cardiofibroblasts to investigate the protein expression of p-Akt, Akt, p-Bad, Bad, p-ERK, ERK by western blotting (left of picture).

Fig.5a

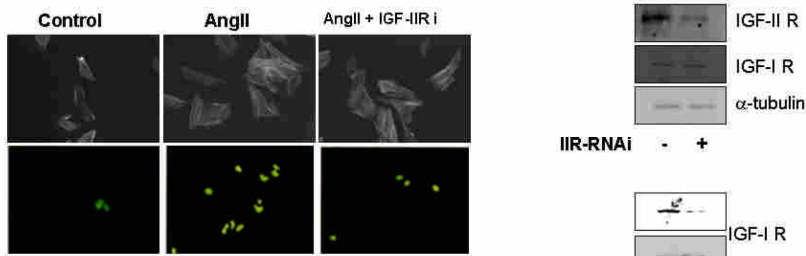



Fig.5b

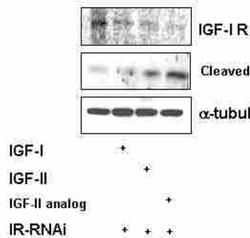



Fig.5c

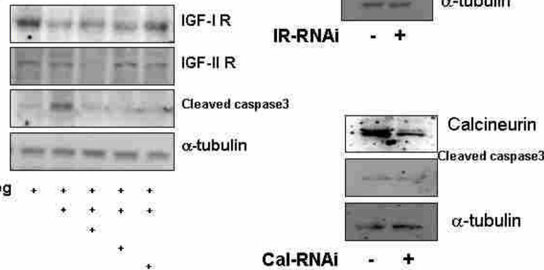



Figure5. Apoptotic effect of IGF-II receptor signaling involved in AngII-treated through activated Calcineurin.

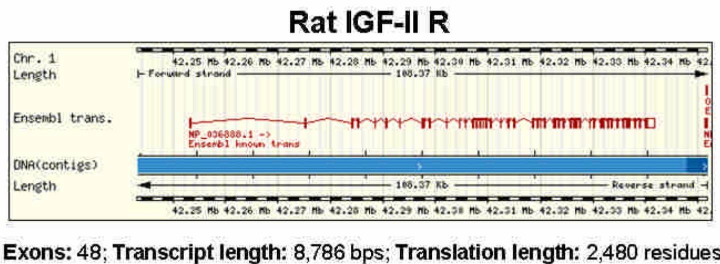
(a). H9c2 cardiofibroblasts treated AngII and transfected with or without IGF-II receptor RNAi then to recognize the hypertrophy and apoptosis through staining respectively the Actin protein and TUNEL assay.

(b). Treated with IGF-I, IGF-II and IGF-II analog individually in the transfect with IGF-I receptor RNAi situation, then demonstrate the protein expression of IGF-I receptor, caspase3 and $\alpha$-tubulin by western blotting.

(c). Condition of treated IGF-II analog with or without transfecting IGF-I receptor RNAi, then transfect with IGF-II receptor RNAi and plus CsA or FK506 respectively to observe the protein expression of IGF-I receptor, IGF-II receptor, caspase3 and $\alpha$-tubulin by western blotting.

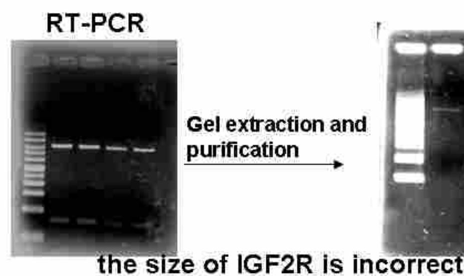
Fig.6a

Rat IGF-II R



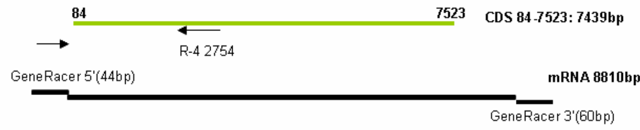

Exons: 48; Transcript length: 8,786 bps; Translation length: 2,480 residues

RT-PCR

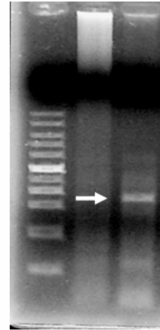



the size of IGF2R is incorrect

Fig.6b




5' RACE end PCR



the size is about 1800bp

Fig.6c

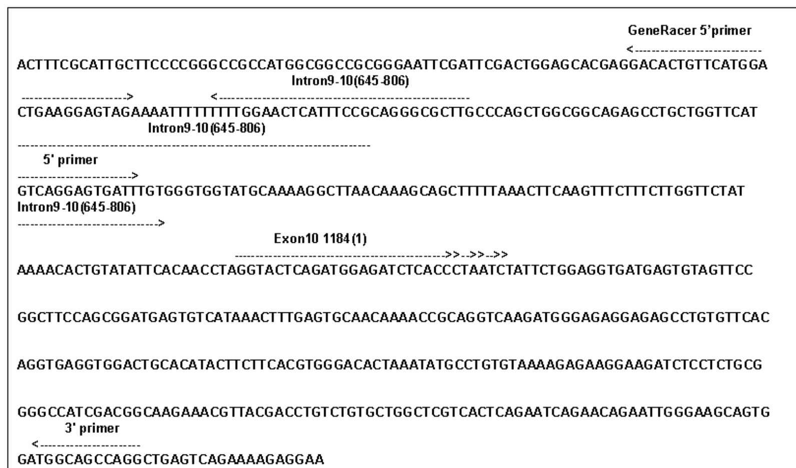
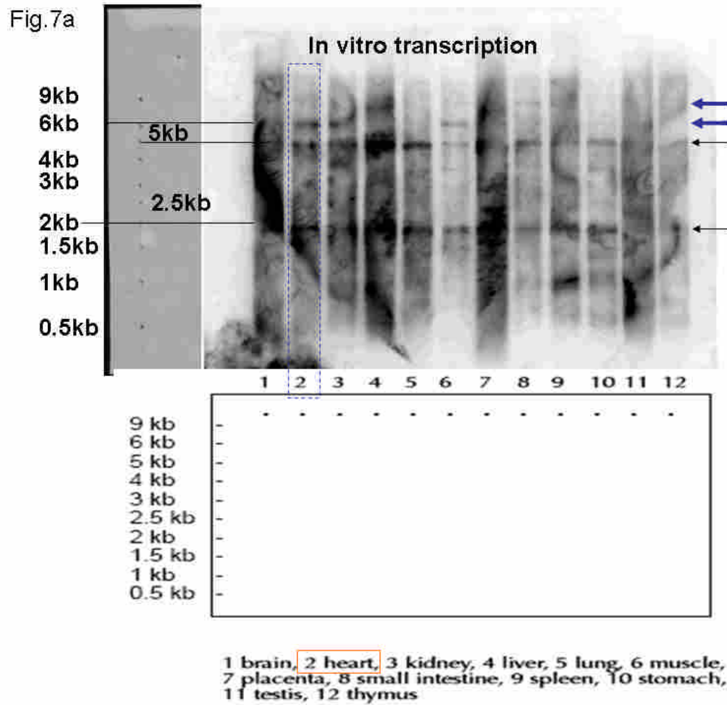
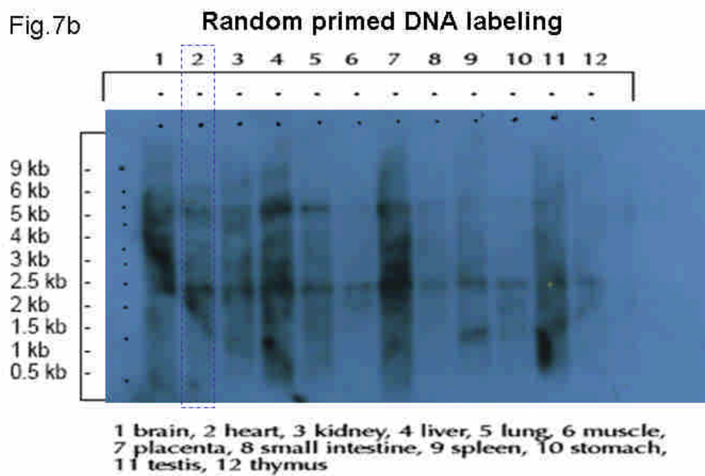
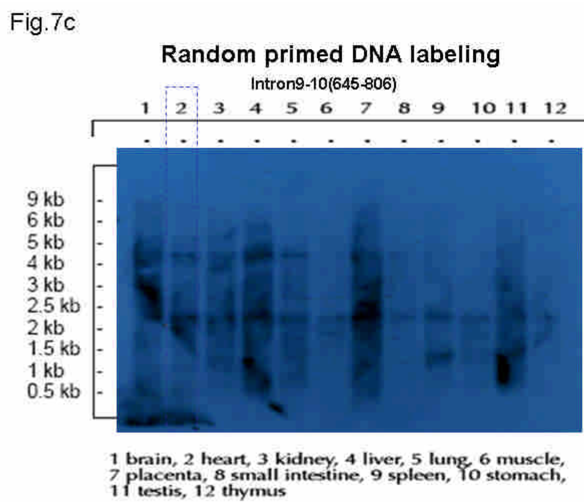



Figure6. Identification of novel IGF-II receptor isoform by 5' RACE end PCR.



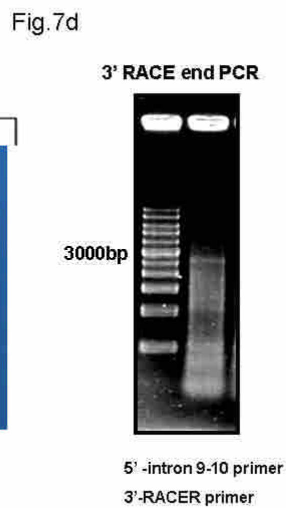




Figure7. Novel IGF-II receptor isoform widely distribute over many tissues.

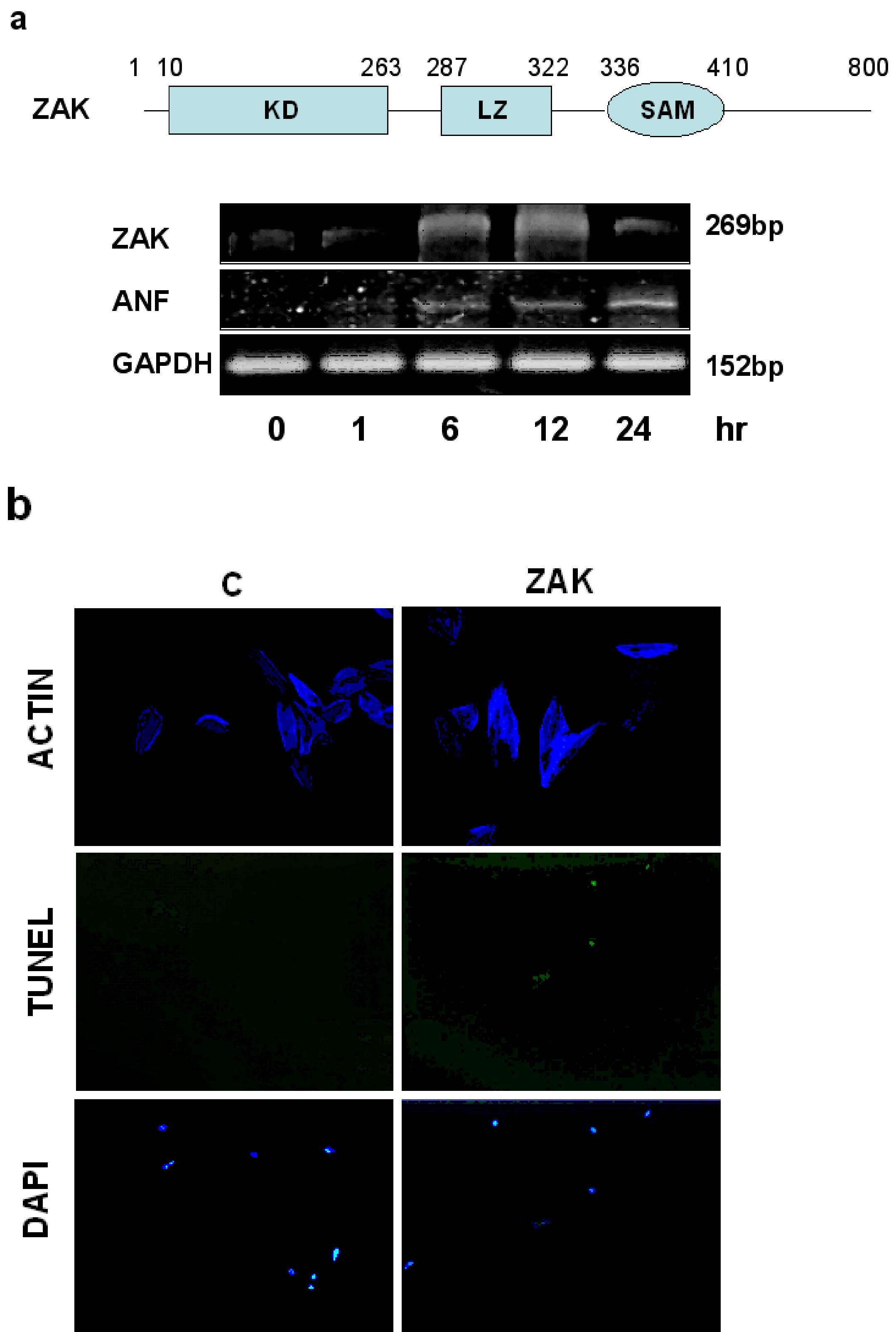
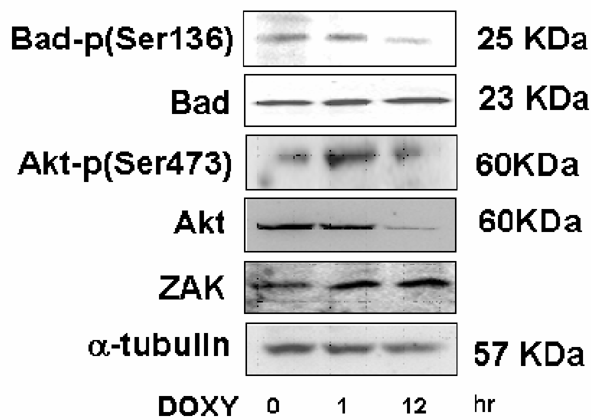
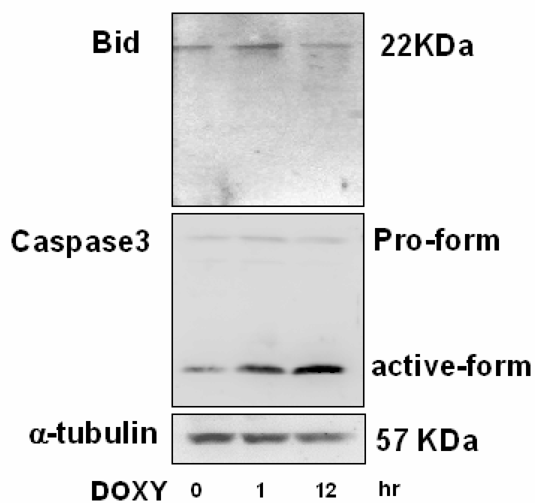



**Figure 8.ZAK both induces hypertrophy and apoptosis in H9c2.**

(a) Stable clone of ZAK gene constructed and selected in H9c2 cell, treated Doxycyclin can induce ZAK gene expression. Expression of ZAK and ANF gene are monitored by RT-PCR in 0, 1, 6, 12, 24 hour.
(b) Cell hypertrophy and apoptosis detected respectively by Actin staining and TUNEL assay to compare ZAK overexpression with control in *left* site; hypertrophy size and apoptosis percentage calculate by student *t* test statistics, and schematize in *right* site.

Fig.9



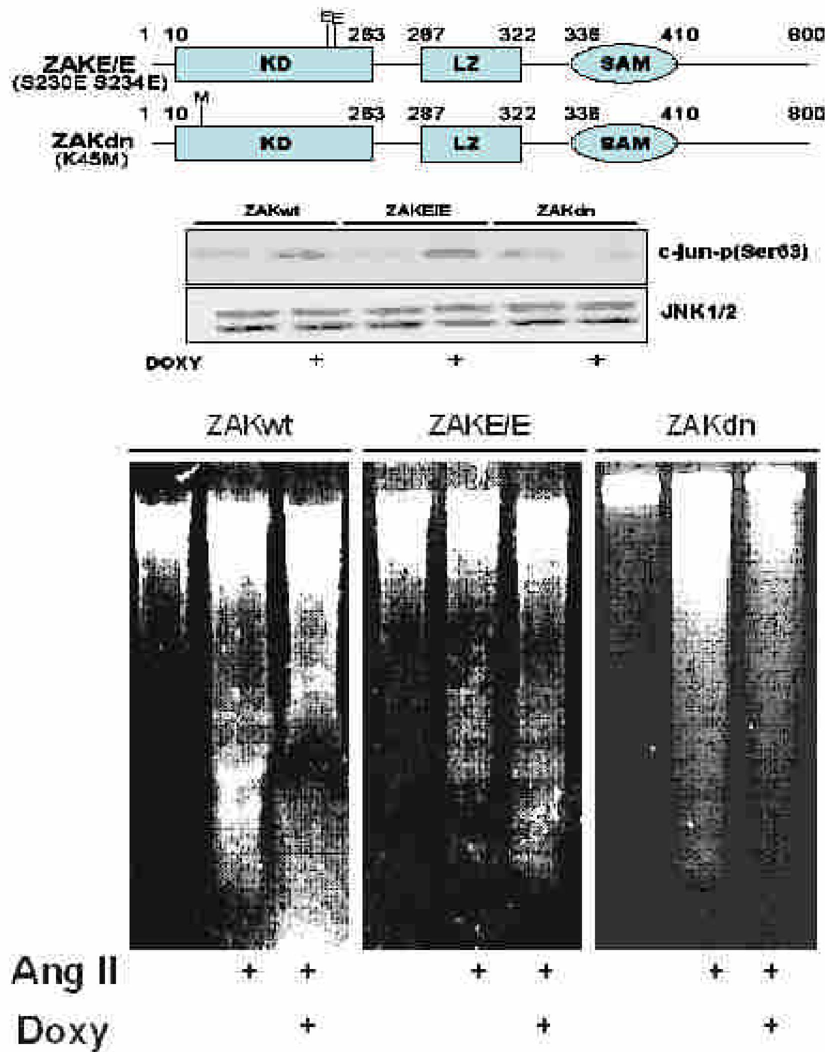



**Figure 9.ZAK enhance active-form of Caspase3, and weaken in phosphorylation of Bad and Akt without AngII signaling involvement.**

(a-b) In treated Doxycyclin to induced ZAK gene expression, we observe that protein expressional level of p-Bad, Bad, p-Akt, Akt, Bid and caspase3 by western blotting in 0, 1, 12 hour. The α-tubulin was designated to internal control.

(c) We invented three type of ZAK including ZAK-wt, ZAK-E/E (S230E S234E), ZAK-dn (K45M) those had been determined to have different JNK kinase activity. In the three stable clone of ZAK in H9c2 cell treated AngII with or without Doxycyclin to identify apoptosis by DNA fragmentation.

Fig.10

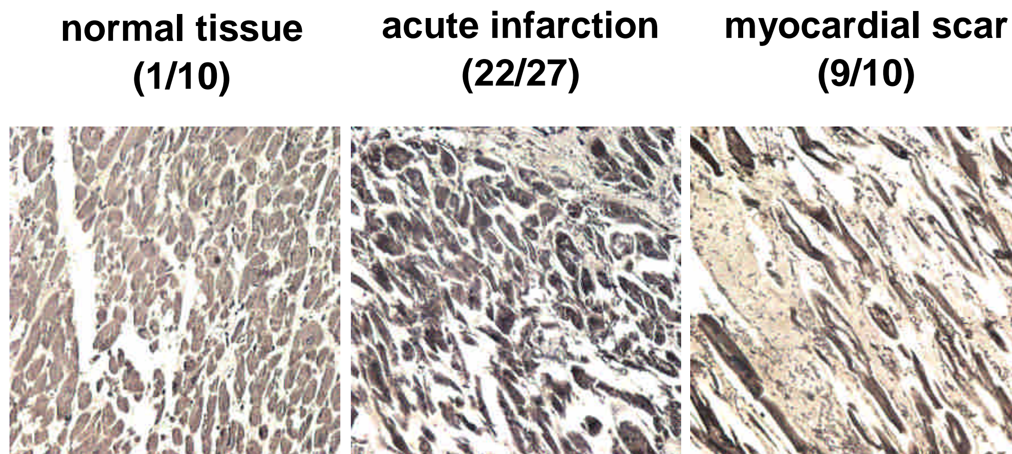
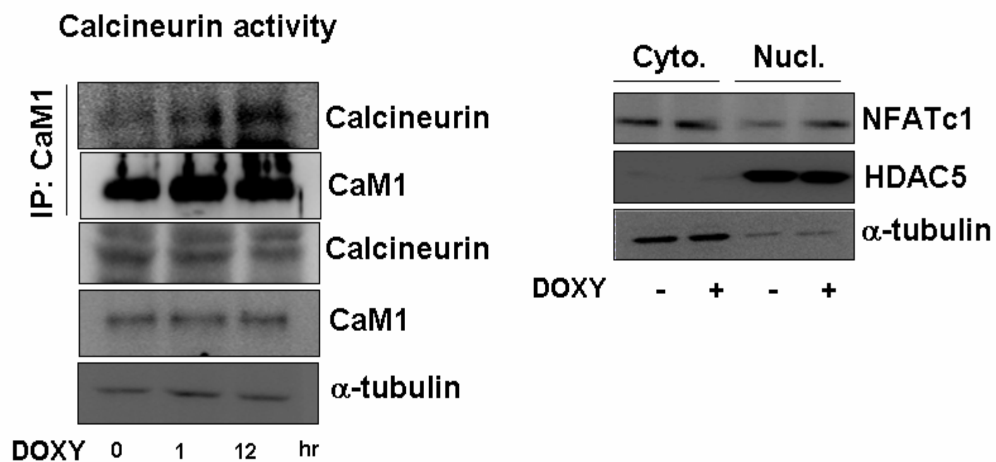



**Figure 10 Aberrantly enormous expression of ZAK in myocardial infraction, but not in normal ventricular tissue.**

We used cardiovascular disease tissue assay that have 47 tissue spot contain 27 acute infarction, 10 myocardium scar and 10 normal tissue to detect ZAK protein expression by immunohistochemistry.

Fig.11



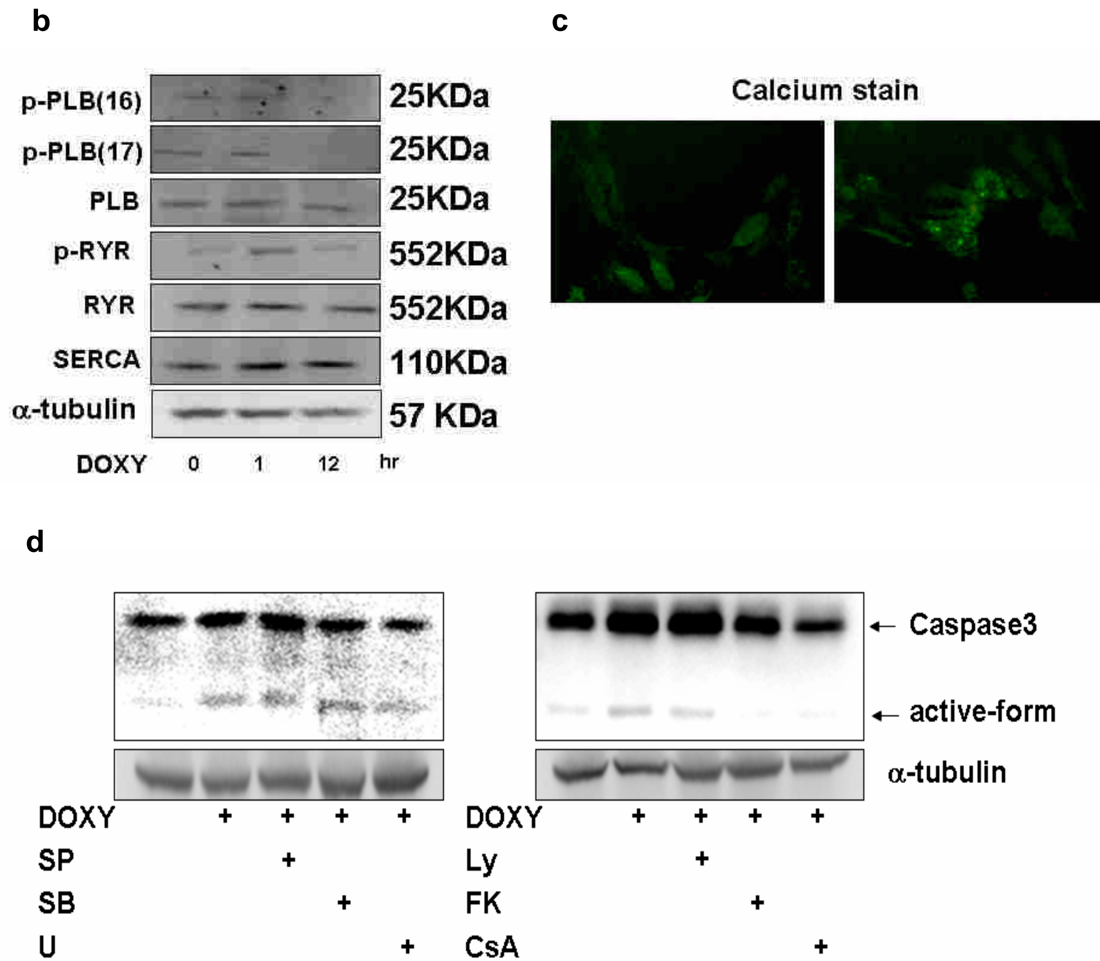



**Figure11 ZAK induces apoptosis through activating calcineurin/NFATc1 signaling pathway and affect calcium influx.**

(a) Calcineurin activity was recognized through CaM1 interaction with calcineurin by immnoprecipitation of CaM1, then immnoblotting of calcineurin in Doxycyclin -induced ZAK expression manner. Western blotting with CaM1 is loading control. We isolated cell partition of cytoplasm and nucleus to detect NFAT-c4 translocation from cytoplasm to nucleus by western blotting in Doxycyclin-induced ZAK expression manner. HDAC5 is nucleus marker.

(b) To evaluated that expression of contraction-relaxation related protein, PLB, p-PLB, SERCA, p-SERCA by western blotting.

(c) Calcium in the cytoplasm were stained by calcium indicator with or without treated Doxycyclin following that observed through with fluorescent microscopy.

(d) Using six differently protein kinase inhibitor contained SP-600125, SB-203580, U-0126, Ly-294002, FK-506 and CsA in ZAK overexpression of H9c2 cell line to expose signification of caspase3 in western blot.

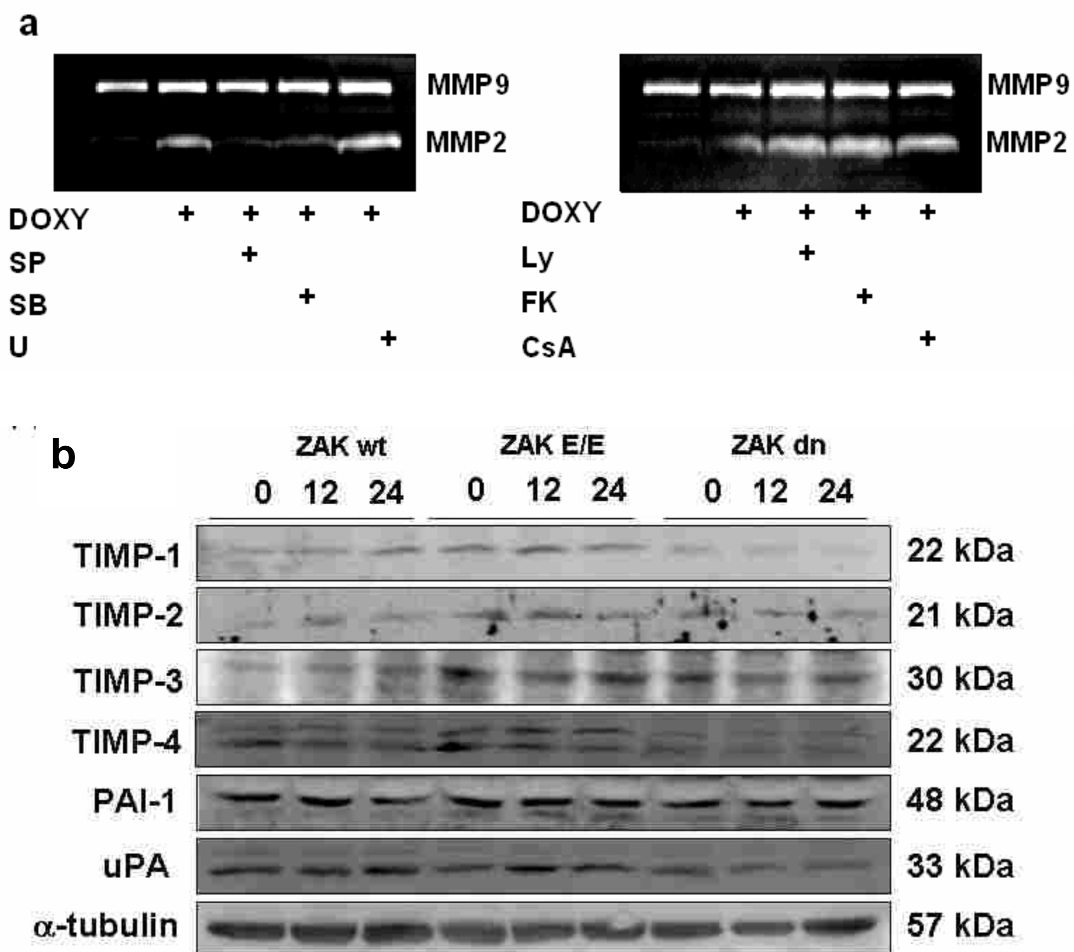



**Figure 12.Over-expression of ZAK not only evoked MMP-2 activity by JNK and p38 signaling pathway but also evoked TIMP-1 and TIMP-2 involve in ventricular remodeling.**

(a) Using six differently protein kinase inhibitor contained SP, SB, U, Ly, FK, CsA in ZAK overexpression of H9c2 cell line to measure the MMP2/MMP9 activity by Zymorgrophy.

(b) To observe that expression level of the related-cardiac remodeling proteins expression level contain TIMPs, PAI-1 and uPA among over-expression of three differently type ZAK in 0, 12, 24 hour time-point by western blotting.

# 八：未來繼續之研究

第一部分:

1. Cloning IGF-II receptor 基因全長(CDS)以進行轉暫轉殖，觀察是否具有刺激心肌細胞凋亡之作用，並透過將其 extracellular domain 分成不同片段，找出除了 IGF-II 外是否還有其他的 ligands 參與其中。
2. 探討在 Fig.1a 中，LPS 和 TNF-$\alpha$ 如何調節 IGF-II receptor 基因的表達，與 IGF-II receptor promoter 具有 NF-$\kappa$b binding site 是否相關。
3. 由 Fig.3b 的結果，探討 histone deacetylases (HDACs)是否參與在開啟 IGF-II receptor 基因的機制中(59,60,61)。使用 class I HDAC-selective inhibitor, SK-7041 或者 nonspecific HDAC inhibitors trichostatin A and valproic acid。
4. 找出 IGF-II receptor 如何與 small G protein 結合，以及確定 Gαi 與 Calcineurin 之上下游的關西。
5. 將 Fig.7d 中出現 2500-3000bp 的 band 定序出來或者調整 PCR 的條件以期能定義出含有部分 intron9-10 片段的 IGF-II receptor isoform；並探討此一新的 IGF-II receptor isoform 功能為何?與已知 IGF-II receptor 有何差異之處。

第二部分:

1. 在 Fig.11b 中顯示大量表現 ZAK 基因會使得磷酸化態之 Phospholamban(p-PLB)表現量下降，然而 PLB 在調節心臟收縮上扮演著重要的角色(58)，對於 ZAK 如何參與在心臟收縮和放鬆的循環中仍然未知。
2. 在本實驗室中，對於雌激素和雌激素接受體如何保護心肌細胞具有高度性興趣(62,63)，而在初步的實驗結果中，顯示出在 ISO 刺激下，雌激素和雌激素接受體會透過活化 Akt 和 ERK 保護心肌細胞免於 ISO 刺激下所造成之凋亡，故在未來會進一步探討 ZAK 是否參與在 ISO 刺激下的凋亡路徑中，以及 ZAK 基因的表達如何被調控，雌激素和雌激素接受體是否參與其中，其相關性為何?

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