**Title page**

Full title: Histone acetylation is essential for ANG-II-induced IGF-IIR gene expression in H9c2 cardiomyoblast cells and pathologically hypertensive rat heart.

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**Abstract**

The IGF-II/mannose 6-phosphate receptor (IGF-IIR/Man-6-P) up-regulation correlates with heart disease progression and its signaling cascades directly trigger pathological cardiac hypertrophy, fibrosis and cardiomyocytes apoptosis. IGF-IIR gene expression/ suppression is able to prevent myocardial remodeling. However, the regulating mechanisms for the IGF-IIR gene remain unclear. This study performed reverse transcriptase PCR (RT-PCR) and methylation-specific PCR (MS-PCR) to detect expression and DNA methylation of CpG islands within the IGF-IIR genomic DNA region. Our finding revealed that the IGF-IIR gene was up-regulated both in H9c2 cells treated with tumor necrosis factor-alpha (TNF-α), lipopolysaccharide (LPS), Angiotensin II (ANGII) and inomycin and aged dependently in spontaneously hypertensive rat (SHR) heart. For the DNA methylation study, although there were four CpG islands within IGF-IIR genomic regions, the DNA methylation distribution showed no change either in cells treated with ANGII or in the SHR heart. Using chemical inhibitors to individually block histone acetyltransferase (HAT) and histone deacetylase (HDAC) activity, we found that histone acetylation was essential for ANGII-induced IGF-IIR gene expression using RT-PCR and luciferase assay. The Chromatin immuno-precipitation assay indicated that acetyl-Histone H3 and acetyl-Histone H4 associated with the IGF-IIR promoter increased in the presence of ANGII, otherwise Methyl-CpG binding domain protein 2 (MeCP2) is disassociated with this. Taken together, this study demonstrates that histone acetylation plays a critical role in IGF-IIR up-regulation during pathological cardiac diseases and might provide a targeting gene in transcriptional therapies for the failing heart.

## INTRODUCTION

The IGF-IIR, a 300-kDa type I trans-membrane glycoprotein, triggers various cellular functions involved in lysosomal enzyme trafficking, fetal organogenesis, tumor suppression and T cell-mediated immunity by interaction with several distinct ligand classes, including IGF-II and the proteins containing Man-6-P on carbohydrate side chains (Braulke, 1999; Jones and Clemmons, 1995). The IGF-IIR gene is active in the early embryo and expressed widely, but its transcript and protein abundance drops dramatically during the early postnatal period (Baker et al., 1993). IGF-IIR-deficient mice uniformly have very large, malformed hearts, with marked abnormalities in the ventricular and intra-ventricular septum, indicating that IGF-IIR plays a vital role in normal cardiac morphogenesis and growth (Lau et al., 1994). The tissue specific knockout of the IGF-IIR gene in mouse heart exhibits that IGF-IIR is not necessary for adult stage normal physiological conditions (Wylie et al., 2003). However, up-regulation of IGF-IIR dose-dependently correlated with the progression of heart disease following complete abdominal aorta ligation (Lee et al., 2006), and IGF-IIR protein disruption mediated by ribozyme leads to cellular protection and against hypoxia- and TNF-induced apoptosis in cardiac myocytes (Chen et al., 2004). Overall, those investigations implicate IGF-IIR functions in normal heart embryogenesis and development, but also in the development of heart disease in adults.

Our previous studies showed a significant association of IGF-IIR over expression with myocardial infarction and myocardial scars (Chang et al., 2008; Chu et al., 2008). Results from specifically activated IGF-IIR signaling through either inhibition of the IGF-I receptor (IGF-IR) activity by IGF-IR siRNA and AG1024 (a chemical kinase inhibitor for IGF-IR) or using Leu27IGF-II analog, reveal that IGF-IIR activated by IGF-II binding acted like a G protein-coupled receptor to activate PKC-α/CaMKII and calcineurin by association with Gαq, leading to pathological hypertrophy and mitochondria-dependent cell apoptosis in cardiomyocytes (Chen et al., 2009; Chu et al., 2009a; Chu et al., 2009b). Additionally, IGF-IIR signaling activation causes the unbalance of matrix metallopeptidase 9 (MMP-9)/ tissue inhibitor of metalloproteinase 2 (TIMP-2) expression and increased plasminogen activator (PAs) expression (Chang et al., 2008), resulting in the development of myocardial remodeling. Based on those findings, suppression of the IGF-IIR gene expression and its signaling may contribute to the prevention of pathological hypertrophy, myocardial remodeling, cardiomyocyte apoptosis and heart failure progression retardation. However, the mechanisms in which transcriptional regulation of the IGF-IIR gene expression responsible for pathological cardiac stresses are still unclear.

IGF-IIR has been reported as a paternally imprinted gene (Barlow et al., 1991). Its transcriptional regulation is associated with the epigenomic marks, particular in the DNA methylation within two differential DNA methylation regions (DMRs) that referred as CpG island (Wutz and Barlow, 1998). The first DMR (DMR1) includes the promoter for the

IGF-IIR transcript and the second DMR (DMR2) contains the antisense *AIR* promoter within the intron 2 of the gene (Wutz and Barlow, 1998). In the mouse model, IGF-IIR gene expression depends upon the paternal expression of antisense *AIR* RNA, which inhibits the expression of the sense IGF-IIR on the paternal chromosome (Barlow et al., 1991; Wutz and Barlow, 1998). The evolutionarily divergent imprint status of IGF-IIR indicates that the presence of an imprinted antisense *AIR* is neither necessary nor sufficient for consistent IGF-IIR imprinting across species (Vu et al., 2006). More recently, some studies have revealed that histone modification, rather than the presence of DNA methylation and antisense transcripts, correctly respond to the imprinting statue of IGF-IIR in a tissue-specific and species-specific manner (Hu et al., 2000; Vu et al., 2006; Vu et al., 2004). Furthermore, the histone acetylation in the promoter in conjunction with the absence of H3-K9Me3, which may interact with DNA methylation to mark the promoter for transcription silencing, is required for transcriptional activation of the IGF-IIR gene (Hu et al., 2000), suggesting that the acetylating histone may play a critical role in the up-regulation of IGF-IIR gene expression. It is poorly understood whether epigenomic marks such as DNA methylation and histone acetylation were able to regulate IGF-IIR gene expression in adults with pathological heart disease. However, either in H9c2 cells treated with ANGII or in the SHR heart, the DNA methylation distribution within IGF-IIR's CpG islands showed no differences.

The aim of this study is to investigate whether genomic modifications such as DNA methylation and histone acetylation are involved in IGF-IIR gene expression induced by pathologically hypertrophic stresses. Our results observed that histone acetylation, but not DNA methylation, play an essential role in ANGII-induced IGF-IIR expression. This might provide a new approach for IGF-IIR gene expression inhibition and its signaling cascades to further prevent heart failure.

## MATERIALS AND METHODS

### H9c2 Cardiomyoblast Cell and Neonatal Rat Ventricular Myocyte (NRVM) Culture

H9c2 cardiomyoblast cells were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified essential medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 1 mM pyruvate in humidified air (5% $CO_2$) at 37°C. NRVM were prepared and cultured using a Neonatal Rat/Mouse Cardiomyocyte Isolation Kit (Cellutron Life Technology, Baltimore, MD) according to the manufacturer's instructions. Animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals under a protocol approved by the Animal Research Committee of China Medical University, Taichung, Taiwan. Hearts were dissected from 1- to 3-day-old Sprague-Dawley rats and transferred into a sterile beaker with a stir bar. The heart was digested with a digestion buffer and stirred in the beaker at 37℃ for 12 minutes. The supernatant was then transferred to a new sterile 15ml round bottom tube and spun at 1200 rpm for 1 minute. After removing as much supernatant as possible, the isolated cell pellets were resuspended in D3 buffer and preplated for 1 hour by seeding in an uncoated plate at 37℃ in $CO_2$ incubator in order to select out the cardiac fibroblasts. The unattached cells were then transferred onto precoated plates with NS medium. After overnight culture, the NS medium was replaced with a serum-free NW medium. The cardiomyocytes cultures were ready for experiments 48 hours after initial plating.

### Animals

SHR and WKY rats were purchased from the National Laboratory Animal Breeding and Research Center, Taipei, Taiwan. These animals, whose ages were 4 and 16 weeks, were kept in a temperature-controlled environment (25℃) illuminated for 12 hours daily (05.00–17.00 hours), and fed commercial pellets and water ad libitum. These animals were sacrificed and the hearts removed for further analysis. All protocols were approved by the Institutional Animal Care and Use Committee of China Medical University and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

### Reagents and antibodies

The following reagents were purchased from Sigma (St. Louis, MO): ANGII (A9525), ISO (I2706), TNF-α (T5944), Inomycin (I3903), LPS and the HDAC inhibitor Valproic acid (P4543). The HAT inhibitor Curcumin (ET-135) was purchased from BIOMOL (Plymouth Meeting, PA). The HDAC inhibitor Trichostatin A (1460) were purchased from TOCRIS bioscience (Ellisville, MI). Anti-Actyl-Histone H3 (#07-352) and Actyl-Histone H4 (#06-946) were purchased from Upstate (MILLIPORE). Anti-MeCP2 (Ab3725) was purchased from Abcam. Anti-IGF-IIR (sc-25462), anti-BNP (SC-18817), anti-α-tubulin (sc-5286) and all secondary antibodies (anti-rabbit-HRP, sc-2004; anti-mouse-HRP, sc-2005; anti-goat-HRP, sc-2020) and enhanced chemiluminescence were purchased from Santa Cruz Biotechnology

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(Santa Cruz, CA).

**Total RNA extraction**

Total RNA was extracted using the Ultraspec RNA isolation System (Biotecx Laboratories, Inc., Houston, TX) according to directions supplied by the manufacturer. Respectively, cells and cardiac tissues were thoroughly homogenized (1 ml Ultraspec reagent per 100 mg tissue or per 10 mm cell dish) 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 5-10 minutes, dissolved in 50-100 ul of diethylpyrocarbonate-treated water and incubated for 10-15 minutes at 55-60℃.

**Reverse transcription-polymerase chain reaction (RT-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 dNTP (MD. Bio.), 20 units of RNasin® Plus RNase Inhibitor (N2611; Promega), 1 pg of random hexamers (MD. Bio., Taipei, Taiwan), 5 pg of total RNA and 40 units of M-MLV Reverse Transcriptase (M1701；Promega, Wadison, WI), those concentrate into a volume of 20 μl. This mixture was incubated for 10 min at room temperature followed by 1 hour at 42℃ to initiate cDNA synthesis. This mixture was then used for specific cDNA amplification by PCR. The buffer for PCR contained 50 mM KCI, 10 mM Tris-HC1, pH 8.3 at 20℃, 0.2 mM dNTP, 0.5 pM oligonucleotide PCR primers, 2.5 units of Taq polymerase (R001A；*TaKaRa*, Shiga, Japan), and various MgCI, that related to a final concentration of 50 μl. Following the hot start (2 min at 95℃, 80℃ hold), the samples were subjected to 35 cycles of 45 seconds at 95℃, 2 minutes at distinct primer annealing temperature (IGF-IIR: 60℃；BNP：62℃；GAPDH：56℃) and 2 minutes at 72℃by used GeneAmp PCR system 2400 (Perkin Elmer, Waltham, MA). This was followed by a final extension step at 72℃ for 10 minutes. All RNA samples used were demonstrated to have intact 18 S and 28 S RNA bands on ethidium bromide-strained formaldehyde-agarose gels. Primers list in table 1.

**Protein Extraction and Western Blot Analysis**

H9c2 cells were scraped and washed once with phosphate-buffered saline (PBS). The cell suspension was then spun down and the cell pellets lysed for 30 minutes in lysis buffer [50 mM Tris, pH 7.5, 0.5 M NaCl, 1.0 mM EDTA, pH 7.5, 10% glycerol, 1 mM basal medium Eagle, 1% Igepal-630, and proteinase inhibitor cocktail tablet (Roche, Mannheim, Germany)] and spun down 12,000 rpm for 10 minutes. The supernatants were then poured into new eppendorfs for Western blot analysis, which was performed as described previously (Lee et al., 2006). Proteins were then separated in 6-12% gradient SDS-PAGE and transferred to nitrocellulose membranes (MILLIPORE, Billerica, MA). 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 as indicated for each experiment in blocking buffer at room temperature for 1 hour. Chemi-luminescent detection was accomplished with

Western Blotting Luminol Reagent. Densitometric analysis of immunoblots was performed using FUJIFILN LAS-1000 (Tokyo, Japan).

**Plasmid constructions and report assay**

The IGF-IIR upstream DNA fragment sequence was generated by PCR (standard reaction) using normal Rat genomic DNA as substrates. The primers were designed based on Rat genome sequence data from EMBL. The -1249~+11 upstream region of IGF-IIR promoter region was amplified using sense primer: 5′-CAATGACTAGTCTTCATGTAACAGCCT-3 and antisense primer: 5′-AAGCTTGAGTCGAAGCTGCAACGG-3′. PCR was performed with Platinum Taq DNA Polymerase High Fidelity (11593-019; Invitrogen, Carlsbad, CA). The PCR products were cloned into the pGEM-T vector (A1360; Promega, Wadison, WI), then sub-cloned into the luciferase vector pRL-null vector (E2273; Promega, Wadison, WI) used by the *Sph* I and *Sal* l restriction sites (Promega, Wadison, WI). Transient transfections of H9c2 cells were performed using TransFast$^{TM}$ Transfection Reagent (E2413; Promega Wadison, WI) as per the manufacturer's instructions. Briefly for the luciferase reporter experiments, cells at 70–80% confluence were co-transfected with *Renilla* luciferase reporter constructs (vacant vector or IGF-IIR-deriving promoter) and a *firefly* luciferase construct, pGL3-control (E1741, Promega, Wadison, WI), was used as an internal control. After transfection, cells were rested for 24 hours and then assayed for luciferase activity using a Dual-Glo$^{TM}$ luciferase assay system (E1919；Promega, Wadison, WI). Plates were read on a Reporter Microplate Luminometer (Turner Biosystems, Sunnyvale, CA). To normalize for potential variations in transfection or lysis efficiency, luciferase signals were normalized to control *Renilla* luciferase signal.

**Bisulfite Modification and Methylation specific PCR (MSP)**

The entire DNA was separated from H9c2 cells and animal hearts using the Genomic DNA Purification Kit (Gentra SYSTEM, Minneapolis, MN) and submitted for chemical modification using a CpGenome DNA Modification Kit (S7820; MILLIPORE, Billerica, MA**)** according to the manufacturer's instructions. Briefly, DNA was denatured with 2 M NaOH, followed by treatment with 10 mM hydroquinone and 3 M sodium bisulfite. After purification, the DNA was treated with 3 M NaOH and precipitated with three volumes of 100% ethanol, and a one-third volume of 10 M $NH_4OAc$ at room temperature over 30 minutes. The precipitated DNA was washed with 70% ethanol and dissolved in 20 μl of distilled water. PCR was conducted with primers specific for either the methylated or the unmethylated sequence of the five IGF-IIR CpG islands as indicated in figure 3.a. Methylated and unmethylated primers list in table 1. The total 25 μl volume of PCR reaction contained 2 μl of modified DNA, 1% DMSO, dNTP (200 μM), 1.5 mM $MgCl_2$, 0.4 μM PCR primers, and 0.25 units of Platinum Taq DNA Polymerase High Fidelity (11593-019；Invitrogen, Carlsbad, CA). DNA was amplified using an initial cycle at 95°C for 5 minutes, followed by 40 cycles of 94°C for 30 seconds, 66°C for 1 minute, and 72°C for 1 minute and ending with a 10-minutes

extension at 72°C for 1 cycle in a thermocycler (Applied Biosystems, Foster City, CA). PCR products were separated on 2.5% agarose gels and visualized after staining with ethidium bromide. The methylation index (%) in a sample was calculated using the following equation: Methylation index = M / M + U * 100% where M is the quantity of methylated IGF-IIR's CpG island sequences measured by the IGF2R-M primers MSP following bisulfite conversion and U is the quantity of unmethylated CpG island of IGF-IIR sequences measured using IGF-IIR-U primers MSP following bisulfite conversion.

**Chromatin Immuno-precipitation assay**

ChIP assay was performed from H9c2 cell lysates, treating the cells with ANGII using EZ ChIP Chromatin (MILLIPORE, Billerica, MA) according to the manufacturer's instructions. The cells were washed in PBS three times and incubated for 10 min with 1% formaldehyde. Subsequent to quenching, the reaction with glycine 0.1 M, the cross-linked material was sonicated into chromatin fragments with an average length of 500~800 bp. Chromatin was kept at –80°C. After the chromatin solution contain (100 µl chromatin sample and 900 µl Dilution Buffer) was pre-cleared by adding Protein G Agarose for 2 hours at 4 °C, immuno-precipitation was performed with Protein G Agarose and 1–10 µg of the indicated antibodies overnight at 4°C on a rotating wheel. Immuno-precipitated material was washed 5 times with cold washing buffer. Cross-links were reversed by incubating samples with 8µl of 5M NaCl for 5 h at 65°C and 10 µg of RNase A to eliminate RNA. Recovered material was treated with proteinase K, using Spin Columns, and precipitated. The pellets were re-suspended in 50 µl of $H_2O$ and analyzed using PCR with Platinum Taq DNA Polymerase High Fidelity (11593-019；Invitrogen, Carlsbad, CA). The PCR products were then analyzed using agarose gel electrophoresis and stained with EtBr for visualization. The primer set was designed to cover the DNA sequence from position -585 to +11 (IGF-IIR) in which the promoter-associated CpGs is located.

**Statistics**

The relative PCR intensities were analyzed using the Digital Sciences 1D program from Kodak Scientific Imaging Systems (New Haven). All results were expressed as the mean ± S.D. or the means and the coefficient of variation of at least three independent experiments, as indicated. Standard curves were plotted and the data obtained fell within the linear range of the curve. In addition, all values were normalized to their respective lane load controls. Densitometric RT-PCR analysis in bar charts (Figs. 4b and 4d) and the fold-activation of IGF-IIR-promoter luciferase activity (Figs. 5c-d) were analyzed using one way ANOVA with preplanned contrast comparisons against the serum free control group or against the treated group. Results in figures 1b, 1d, 1f, 2b, 2d, 5b and 5f were analyzed using unpaired *Student's* t test. In all cases, $P$ values < 0.05 were considered statistically significant.

## RESULTS

### IGF-IIR gene expression is up-regulated in pathological cardiac hypertrophy models

IGF-IIR up-regulation in patient heart has been previously linked with cardiac infarction and fibrosis (Chang et al., 2008; Chu et al., 2008). To determine whether pathologically hypertrophic stimulus may increase the IGF-IIR gene expression at the cellular level, we cultured the H9c2 cardiomyoblast cell line and neonatal cardiomyocytes and treated them with various pathological stimulators including TNF-α, LPS, ANGII, ISO and inomycin to detect the IGF-IIR transcript using RT-PCR. These stimulators were examined as a pathological inducer for hypertrophy and apoptosis that further contributes to heart failure. The RT-PCR results revealed that all of these stimulators significantly induced the hypertrophic marker, BNP ($p < 0.05$ vs. untreated control) gene expression (Fig.1A, B). After 6 hours treatment, IGF-IIR transcript elevated in the presence of TNF-α ($p < 0.05$ vs. untreated control; Fig.1A, B). A significant increase in IGF-IIR ($p < 0.05$ vs. untreated control) transcript was detected in the ANGII, inomycine and LPS treatment at 18 hours (Fig.1A, B). However, no significant change in ISO treatment occurred (Fig.1A, B). Consistent with up-regulating IGF-IIR at the gene expression level, western blots exhibited that treatment with ANGII both significantly increased BNP and IGF-IIR ($p < 0.05$ vs. untreated control) protein levels at 12 and 24 hours (Fig.1C, D). Similarly the IGF-IIR transcript ($p < 0.05$ vs. untreated control) elevation was found in the neonatal cardiomyocytes after treatment with ANGII (Fig.1E, F). In contrast to the *in vitro* cellular study, we compared the hearts of SHR (n=6) representing an animal model of human essential hypertension (Conrad et al., 1995) with WKY rat (n=6) hearts in the mRNA BNP and IGF-IIR expression levels. At 4 weeks age the BNP ($p < 0.05$) gene expression, but not IGF-IIR, increased in the SHR hearts compared with WKY (Fig.2A, B). However, both BNP and IGF-IIR ($p < 0.05$) were significantly up-regulated in the hearts of 16 week old SHR (Fig.2C, D). Consistent with our previous studies (8-12), all of these results suggest that IGF-IIR up-regulation might be the critical factor that promotes heart disease progression.

### DNA methylation marks are not associated with IGF-IIR gene expression induction under pathological hypertrophic stresses

IGF-IIR is a paternally imprinting gene (Barlow et al., 1991) and its gene expression is correlated with genomic modification such as DNA methylation and histone acetylation (Vu et al., 2006). We would like to find out how many CpG islands are located within the IGF-IIR genomic DNA in the rat species and whether DNA methylation plays a role in ANGII-induced IGF-IIR gene expression. Using the software at http://www.ebi.ac.uk/Tools/emboss/cpgplot/index.html, we found that there are four CpG islands within the IGF-IIR genomic DNA regions, including one IGF-IIR-promoter associated with the CpG island (DMR1, indicated as numbered (1); fragment size: -422~+163 bps), one IGF-IIR intron1 associated CpG island (as numbered (2); fragment size: 47~253 bps) and

three IGF-IIR intron2 associated CpG island (DMR2, as numbered (3-4); fragment size: 6910~7167 and 7173~7947 bps) (Fig. 3A). Methyl-specific PCR (MS-PCR) was performed to detect the variation in DNA methylation at those four CpG islands in the H9c2 cardiomyoblast cells treated with or without ANGII. These primers for MS-PCR were designed using the MethPrimer program (Li and Dahiya, 2002), as represented in figure 3A. As results shown in figure 3B and C, treatment with ANGII did not alter the DNA methylation status and methylation index (MI) in these four CpG islands, implying no correlation between IGF-IIR gene expression and its genomic DNA methylation status in ANGII-treated cardiomyoblast cells was found. Likewise, there was no difference in DNA methylation status and MI between the SHR and Wistar rat hearts on DMR1 (Fig. 3D, E). Taken together, our finding indicated that DNA methylation may not be involved in ANG-induced IGF-IIR expression.

**Histone acetylation plays a critical role in the ANG-II-induced IGF-IIR gene expression**

Several investigations reported histone modifications such as acetylation in Histone H3 and H4 associated with gene imprinting status and cooperating with DNA methylation in IGF-IIR gene expression regulation (Hu et al., 2000; Vu et al., 2004). To determine whether histone acetylation is essential to ANGII-induced IGF-IIR gene expression, the HAT inhibitor Curcumin and the HDAC inhibitors Valproic acid and Trichostatin A were individually used to specifically inhibit the enzyme activity. The ANGII-induced IGF-IIR expression was found to be significantly inhibited ($p < 0.05$ vs. only ANGII treated) in the Curcumin treated cells (Fig. 4A, B). Moreover, IGF-IIR gene induction by ANGII was synergistically enhanced ($p < 0.05$ vs. only ANGII treated) using the addition of either Valproic acid or Trichostatin A (Fig. 4C, D). These findings suggested that ANGII-induced IGF-IIR gene expression through histone acetylation.

**Promoter-restricted histone acetylation determines the increasing expression of IGF-IIR gene by ANGII**

To confirm the above RT-PCR results in IGF-IIR gene regulation, we constructed the IGF-IIR promoter-luciferase reporter, as illustrated in figure 5A. We performed the luciferase report assay to detect the IGF-IIR promoter activity in H9c2 cells treated with ANGII. The IGF-IIR promoter activity increased in a time-dependent manner after treatment with ANGII (Fig. 5B). IGF-IIR promoter activity had approximately 9 fold-increases ($p < 0.05$ vs. untreated control) 24 hours after ANGII treatment compared with the un-treated control (Fig. 5B). According to figure 3 the DNA methylation of CpG islands within IGF-IIR genomic DNA had no change with or without ANGII. We further used DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-aza-dC) to confirm whether DNA methylation was involved in IGF-IIR-promoter activity induction by ANGII. The luciferase assay results showed that treatment with 5-aza-dC had no statistical effect on ANGII-induced IGF-IIR-promoter activity (Fig. 5C). Interestingly, treatment alone with 5-aza-dC

significantly reduced ($p < 0.05$ vs. untreated control) the IGF-IIR promoter activity compared with the un-treated control (Fig. 5C). Similar to the RT-PCR results in figure 4, ANGII-induced IGF-IIR-promoter activity could be significantly rescued (55-60%) by HAT inhibitor, Curcumin. At the same time, the HDAC inhibitor, Trichostatin A enhanced (2-fold) IGF-IIR-promoter activity induction by ANGII (Fig. 5D). ChIP analysis was performed to further identify histone modification marks within the IGF-IIR-promoter region in H9c2 cells treated with ANGII. Densitometric analysis revealed # and %-fold increase in acetyl-Histone H3 and acetyl-Histone H4 at 24 hours ANGII treatment, indicated that acetyl-Histone H3 and H4 modification in IGF-IIR-promoter region were enhanced in the presence of ANGII. Otherwise, DNA methylation binding protein MeCP2 is disassociated with this promoter region after ANGII treatment (Fig 5E, F).

## DISCUSSION

We investigated the epigenotic modifications behind IGF-IIR gene expression induction under pathological hypertrophic stimulus in myocardial models. RT-PCR and luciferase report assays were performed to detect IGF-IIR gene expression and showed that IGF-IIR gene up-regulation occurred under various stimulus including ANGII, LPS, inomycin and TNF-α, in SHR hearts positively associated with age (Fig.1, 2 and 5B,C). The DNA methylation and histone acetylation within the IGF-IIR-promoter associated with CpG islands in these pathological statuses were detected using MS-PCR analysis and ChIP assay, respectively (Fig. 3 and 5D). Our findings suggest that modulating histone acetylation, not DNA methylation, correlated with the IGF-IIR gene up-regulation in the pathological cardiac hypertrophy model.

Classically, IGF-IIR stabilizes the local IGF-II concentration through internalization and lysosomal degradation, referred to as a "clearance receptor" (Braulke, 1999; Jones and Clemmons, 1995). According to its conventional role, IGF-IIR functions as a death receptor or a tumor suppression gene involved in the cytotoxic T cell-induced apoptosis and tumorigenesis (Leboulleux et al., 2001; Motyka et al., 2000). In the heart, the IGF-IIR disruption leads to cellular protection against cardiomyocyte apoptosis (Chen et al., 2004) and IGF-II and IGF-IIR up regulation in cardiomyocytes have been identified in a variety of heart disease models (Chang et al., 2008; Chu et al., 2008; Chu et al., 2009b; Lee et al., 2006). IGF-IIR could also function as a G protein-coupled receptor to trigger its downstream signaling modulators such as PKC-α/CaMKII, calcineurin and MMP/TIMP, contributing to heart failure progression (Chang et al., 2008; Chen et al., 2009; Chu et al., 2009a; Chu et al., 2008; Chu et al., 2009b). The up-regulation of IGF-IIR gene expression occurred in this study after treatment with ANGII, LPS, inomycin and TNF-α (Fig. 1), but not ISO (Fig. 1). These findings suggest that the IGF-IIR gene may particularly be regulated by individual cellular signaling pathways and involved in aspects of heart disease progression aspects (Olson, 2004; Vega et al., 2003). Although the H9c2 cell is an undifferentiated cell line, we also reconfirmed the gene expression (Fig. 1E,F and Fig. 2) and DNA methylation (Fig. 3C) results using neonatal cardiac myocytes and rat animals. From the SHR animal model study, we found the increase in IGF-IIR gene expression in rat hearts within 16 weeks old, but not 4 weeks old (Fig. 2), indicating that IGF-IIR up-regulation might correlate with the development of cardiovascular disease (Conrad et al., 1995). It is crucial to uncover the regulating mechanisms for IGF-IIR gene expression, which might not only improve the prevention strategy for cardiac diseases but also in cancer treatment (Martin-Kleiner and Gall Troselj, 2009).

In gene imprinting studies the IGF-IIR gene has been recognized as a great model and focuses on the human and mouse species (Wutz and Barlow, 1998). Numerous studies have implicated that DNA methylation within the CpG island is a major modulator in IGF-IIR gene

expression regulation (Barlow et al., 1991; Vu et al., 2006; Wutz and Barlow, 1998). We found that the IGF-IIR-promoter association with the CpG island was present in rats to be similar in humans and mice (Fig. 3A). However, there were two CpG islands within the IGF-IIR-intron 2 region and one CpG island in the IGF-IIR-intron 1 region (Fig. 3A). Our finding exhibited that under pathological stresses the DNA methylation distribution did not show any correlation with IGF-IIR expression in all five CpG islands (Fig. 3B-E). However, in the results in Fig. 5C, treatment with methyltransferase inhibitor 5-aza-dC alone significantly reduced IGF-IIR promoter activity compared with the untreated control. This result might raise some insights about the IGF-IIR-imprint mechanism in the rat species (Hu et al., 2000; Vu et al., 2006; Vu et al., 2004), suggesting the reduction in IGF-IIR gene expression by 5-aza-dC might due to the increasing expression of anti-sense *AIR*. However, antisense *AIR* expression in the rat species has not been recognized. Additionally, in spite of no change in DNA methylation, treatment with ANGII diminished the MeCP2-associated with IGF-IIR-promoter associated CpG island (Fig. 5E). Some investigations report that histone regulatory protein complex such as HAT, HDAC and DMBP can interact with each other in order to affect basal transcriptional complex to turn on or turn off gene expression (Margueron et al., 2005). It might be possible that under ANGII stimulation, increased histone acetylation could interact to form a complex regulatory network that dissociates MeCP2 from IGF-IIR promoter without affecting DNA methylation and modulates gene expression (Hite et al., 2009). Unlike well-documented human and mouse species, the imprint status of IGF-IIR in rat species during developmental and epigenetic factors responsible for patho-physiological function is another interesting issue for further study.

A number of studies demonstrated that histone modification, including methylation, phosphorylation, ubiquitination and acetylation on the conserve residue of histone protein H3, H4, H2A and H2B can contribute to repression or activation of gene expression (Bode and Dong, 2005; Jenuwein and Allis, 2001). The acetylation of histones by HAT stimulates gene expression through relaxing the chromatin structure, allowing DNA access to transcription factors. Otherwise, histone deacetylation by HDACs promotes chromatin condensation and transcriptional repression (Luo and Dean, 1999; Roth et al., 2001). Our finding showed the inhibition of histone acetylation by Curcumin, blocked IGF-IIR gene expression induced by ANGII (Fig. 4A,B and D) and increased in acety-histone H3 and H4 marks by ANGII treatment (Fig. 5E). Some reports also indicated that through inhibiting p300 HAT activity Curcumin prevents cardiac hypertrophy and heart failure in rats (Li et al., 2008; Morimoto et al., 2008). Therefore, we predicted that IGF-IIR may play the role of targeting gene for HAT inhibitors in transcriptional therapies for the failing heart (Olson et al., 2006).

Several protein complexes such as the GNAT super family, MYST family, nuclear receptor coactivators, TFIIIC and HDACs have been identified as involved in gene expression release through regulating histone acetylation (Roth et al., 2001). Additionally, the E-box-binding

protein such as transcription factors Sp1, ngf-1a, and related protein are required for modulating the IGF-IIR gene promoter activity contributes to early hepatic fobrogenesis (Weiner et al., 1998; Weiner et al., 2000). It is possible that by activating HAT or other transcriptional activators, the IGF-IIR gene may be up-regulated in response to pathological cardiac stresses. Moreover, diverse agonists act through G-protein-coupled receptors to activate the PKC-PKD axis, CaMK, Rho, and other effectors leading to class II HDAC phosphorylation, resulting in exportation of phospho-HDACs to the cytoplasm, contributing to gene activation (McKinsey and Olson, 2004; Olson et al., 2006). For the future, it would be interesting to further investigate which signaling cascades and modulators are involved in IGF-IIR gene expression regulation.

In conclusion, this study suggests that acetyl-histone marked on IGF-IIR promoter is required to increase IGF-IIR gene expression in response to pathological hypertrophic stresses. Our findings may provide a new insight into IGF-IIR gene expression inhibition and its signaling cascades to further prevent heart failure.

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**FIGURE LEGENDS**

**Figure 1. Pathological hypertrophic stimulators induce IGF-IIR gene expression.**

A: RT-PCR using primers specific for IGF-IIR, BNP and GAPDH (as a control) in the H9c2 cardiomyoblast cells were either untreated or were stimulated for 6 and 12 hours with the indicated agonists, as described in Materials and Methods. BNP indicated as the pathological hypertrophic marker.

B: Data were quantified using densitometry and presented as means ± SEM. Results are from three independent experiments run in triplicate on cultured cells. Bars indicate averages. P values represent comparison with untreated control, *$p < 0.05$; $^{\#}p < 0.01$. $n = 3$ independent experiments for each data point.

C: Western blot analysis of H9c2 cardiomyoblasts treated with ANGII ($10^{-8}$ M) at 12 and 24 hours using anti-IGF-IIR antibody, anti-BNP antibody and anti-α-tubulin antibody. IGF-IIR and BNP protein levels increased with ANGII treatment.

D: These bolts were quantified using densitometry. α-tubulin served as a loading control. Data are presented as means ± SD. Bars indicate averages, *$p < 0.05$. $n = 3$ independent experiments for each data point.

E: The neonatal rat ventricular myocytes (NRVM) were shown in the upper panel of figure 1f (magnification: 200X). Transcripts for IGF-IIR, BNP and GAPDH (as a control) detected by RT-PCR in primary neonatal rat cardiomyocytes treated with or without ANGII ($10^{-8}$ M) for 12 and 24 hours (lower panel).

F: Data were quantified using densitometry and expressed as fold change of untreated control. Data are shown as results ± SD of three independent experiments performed in triplicate. Statistical significance: *$p < 0.05$, ANGII versus untreated control.

**Figure 2. Highly expressed IGF-IIR mRNA level in heart of SHR**

A, C: Detection of transcripts for IGF-IIR, BNP and GAPDH (as a control) using RT-PCR in hearts from WKY rats (n = 6) and SHR (n = 6) at 4 week or 16 week age, respectively.

B, D: Data were quantified using densitometry and expressed as fold change of Wistar control rats. Data are shown as means ± SD. WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats. Statistical significance: #$p < 0.01$, SHR versus WKY.

**Figure 3. DNA methylation is not involved in up-regulation of IGF-IIR gene expression in ANGII treated H2c2 cells or in SHR hearts.**

A: Representative view showing the GC percent, CpG island, and CpG site within the IGF-IIR genomic DNA region: (1). one CpG island in Igf2r-Promoter region at -422~+163 bps (left panel); (2) one CpG island located in Igf2r-intron1 region at 47~253 bps (middle panel); (3-4). two CpG islands are distinctly positions in 6910~7167 and 7173~7947 bps of Igf2r-intron2 regions (right panel) were predicted using the soft program on the website

(http://www.ebi.ac.uk/Tools/emboss/cpgplot/index.html).

B: Methylation-specific PCR (MS PCR) analysis of the distinct IGF-IIR CpG islands region detected in the A, H9c2 cardiomyoblast cell were left untreated or stimulated with ANGII ($10^{-8}$ M) for 24 hours. M indicates hypermethylated IGF-IIR; U indicates unmethylated IGF-IIR. MethPrimer program (http://www.urogene.org/methprimer/index1.html) was performed to design primers of MS PCR. *Arrow* indicated as position of the MS-PCR product.

C: The methylation indices (MI) were based on results from direct quantitative fragments from three independent MS PCR assays spanning each of four CpGs in H9c2 cardiomyoblasts treated with or without ANGII ($10^{-8}$ M).

D: DNA methylation of IGF-IIR upstream regulatory region in hearts from 16 weeks age WKY rats and SHR, respectively, which were detected using methylation-specific PCR analysis.

E: The methylation indices (MI) were based on results of directly quantitative fragments from independently three MS PCR assays in WKY and SHR.

**Figure 4. Histone acetylation is required for ANGII-induced IGF-IIR gene expression.**

A: Detection of transcripts for IGF-IIR and GAPDH (as a control) by RT-PCR in the H9c2 cardiomyoblast cells stimulated with or without ANGII ($10^{-8}$ M) and HAT inhibitor, Curcumin ($10^{-6}$ M or $2*10^{-6}$ M) treatment.

B: Data were quantified using densitometry and presented as means ± SEM. Results are from three independent experiments run in triplicate on cultured cells. Bars indicate averages. Statistical significance: *$p < 0.05$ values represent comparison with untreated control; # $p < 0.05$ values represent comparison with only ANGII treated.

C: Detection of transcripts for IGF-IIR and GAPDH (as a control) by RT-PCR in the H9c2 cardiomyoblast stimulated with or without ANGII ($10^{-8}$ M) and HDAC inhibitors: Valproic acid or Trichostatin.

D: Data were quantified using densitometry and presented as means ± SEM. Results are from three independent experiments run in triplicate on cultured cells. Bars indicate averages. Statistical significance: *$p < 0.05$ values represent comparison with untreated control; #$p < 0.05$ values represent comparison with only ANGII treated.

**Figure. 5 Promoter-restricted histone acetylation marks the increasing expression of IGF-IIR gene by ANGII.**

A: Schematic diagram of IGF-IIR promoter-luciferase reporters (P1). Nucleotide acid identities within IGF-IIR genomic DNA are shown. Statistical significance: *$p < 0.05$ values represent comparison with untreated control.

B: H9c2 cardiomyoblast cells were transfected with a P1-IGF-IIR luciferase reporter for 24

hours; then followed with ANGII ($10^{-8}$ M) treated as indicated time. Values are expressed as the fold-increase in luciferase expression (± SD) compared to the absence of ANGII.

C: H9c2 cardiomyoblast cell transfected with a P1 construct encoding luciferase driven by the upstream regulatory region of IGF-IIR and then respectively treated with ANGII ($10^{-8}$ M) and/or methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-aza-dC) or combined with each other. Cells assayed for the luciferase activity were assayed. Values are expressed as the fold-increase (± SD) compared to the reporter alone. Statistical significance: *$p < 0.05$ represents comparison with untreated control.

D: H9c2 cardiomyoblast cell stimulated with or without ANGII ($10^{-8}$ M) and ANGII plus Curcumin or Trichostatin A, along with the IGFIIR-luciferase reporter (250 ng) treatment. Cells were assayed for luciferase activity, values are expressed as the fold-increase (± SD) compared to the reporter alone. Statistical significance: *$p < 0.05$, represents comparison with untreated control; #$p < 0.05$, represents comparison with only ANGII treated.

E,F: H9c2 cardiomyoblast cells were left untreated or stimulated with ANGII ($10^{-8}$ M) for 24 hours, ChIP analysis was performed with the indicated antibodies, and PCR was performed with primers flanking the IGF-IIR-promoter associated CpG island, as indicated in table 1. Input DNA serviced as a loading control. The results from 3 independent experiments were quantified by densitometry as shown in (F). Bars indicate averages. Statistical significance: *$p < 0.05$ values represent comparison with untreated control.

Table 1. List of primers used for PCR and Methylation specific PCR

| | Sequence forward | Sequence reverse |
|---|---|---|
| IGF-IIR | 5′-ATGCACCGTGCGGAATGGAAGCTCG-3′ | 5′-TCACCTGGCAGATGTTGGCACCGGA-3′ |
| BNP | 5′-GGGCGCTCCTGCTCCTGCTCTTC-3′ | 5′-ACACCTGTGGGACGGGGGCTCTC-3′ |
| GAPDH | 5′-TCCCTCAAGATTGTCAGCAA-3′ | 5′-AGATCCACAACGGATACA TT-3′ |
| IGF-IIR (ChIP assay) | 5′- ATTAGTTCATCACGGTCGCC -3′ | 5′- AAGCTTGAGTCGAAGCTGCAACGG -3′ |
| *Igf2r*-intron2-1-CpG island-M primer | 5′-TAT**C**GGAATTA**C**GTTAAAATTTTT**C**-3′ | 5′-CTATAAACCAAAATACC**G**C**G**CT-3′ |
| *Igf2r*-intron2-2-CpG island-M primer | 5′-T**C**G**C**GGAATTTTTAGAAATTT**C**-3′ | 5′-ATTCTACTCC**G**AACTACACCAC**G**-3′ |
| *Igf2r*-promoter-CpG island-U primer | 5′-TTAGGAAGTGTTTAAGTGTAGATGT-3′ | 5′-CAAAACAAAAAATATTATAAACCAAA-3′ |
| *Igf2r*-intron1-CpG island-U primer | 5′-AGGGTTTTGGGGATATGTAGATT-3′ | 5′-TACAACAAACACACTAAACATCAAA-3′ |
| *Igf2r*-intron2-1-CpG island-U primer | 5′-TTGGAATTATGTTAAAATTTTTTGA-3′ | 5′-ACACTATAAACCAAAATACCACACT-3′ |
| *Igf2r*-intron2-2-CpG island-U primer | 5′-GTTGTGGAATTTTTAGAAATTTTGT-3′ | 5′-ATTCTACTCCAAACTACACCACACT -3′ |

The bold indicated as specific cytosines analyzed in CpG islands.