ESC Congress 2014, Barcelona, Spain

30 August – 3 September 2014

EUROPEAN SOCIETY OF CARDIOLOGY®

European Heart Journal

www.eurheartj.org

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P1514 | BENCH

Mechanisms of increased oxygen radical formation in alcoholic cardiomyopathy in a mouse model of acetaldehyde overload and potential therapeutic approaches

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Background: Growing evidence suggests, that NADPH-oxidases (NOX) are involved in the pathogenesis of ethanol related organ damage. We sought to investigate the role of NADPH oxidase derived superoxide in the development of alcoholic cardiomyopathy (ACM) using ALDH-2–/– mice as an animal model of acetaldehyde overload and whether pharmacologic or genetic ablation of NOX2 could beneficially influence the disease model.
Methods: Male B6, ALDH2–/– and ALDH2–/–NOX2/gp91phox–/– were fed a 2% ethanol vs. liquid control diet. fter 5 weeks, cardiac function was assessed with echocardiograpgy, malondialdehyde levels in internal organs were measured by dot blot. NOX activity in cardiac membrane fractions was quantified by chemiluminscence, while the expression of NOX2 was assessed by WB. For in vitro studies, cardiomyocytes (CMs) were isolated from B6 mice via cannulation and retrograde perfusion of the aorta. Plated CMs were incubated in culture media containing increasing concentrations of ethanol (EtOH) or acetaldehyde (MeCHO) in the presence or absence of the NOX inhibitor apocynin. Subsequently, CM mitochondrial membrane potential and mitochondrial ROS production was assessed by automated cellular imaging using the fluorescent probes TMRM+ and MitoSOX.
Results: ALDH2–/– fed the 2% ethanol diet displayed a significantly decreased LV systolic function compared to ALDH2–/– fed the liquid control diet and to B6 mice. Furthermore, ethanol-fed ALDH2–/– mice exhibited significantly increased levels of malondialdehyde in internal organs (highest in the heart), increased heart/body and lung/body weight ratios, significantly increased cardiac NOX activity and significantly increased expression of the NOX2 subunit p67phox and the NOX2 activator Rac1 compared to control fed ALDH2–/– and B6 mice. These results were corroborated by in vitro studies: cultured CMs which incubated with media containing MeCHO showed significantly increased levels of mitochondrial ROS and significantly decreased mitochondrial membrane potential. Pharmacologic ablation of NOX in CMs exposed to MeCHO lowered mitochondrial ROS and partially preserved mitochondrial membrane potential. Finally, we subjected mice double negative for ALDH2 and NOX2/gp91phox to the ethanol diet for 5 weeks. Echo revealed that the additional lack of NOX2/gp91phox protects mice from the acetaldehyde-overload induced cardiomyopathy.
Conclusions: Our results in vitro and in vivo data show that NOX derived ROS contributes to the development of ACM. We further provide evidence that ablation of NOX-2 can beneficially influence the disease in in vitro and in vivo.

P1515

P1516 | BENCH

Nav1.5 N-terminus is responsible for the Nav1.5 and Kir2.x reciprocal modulation

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Purpose: It has been demonstrated that the N-terminal domain of Nav1.5 channels (Nter), a 132 aminoacids peptide, is able to increase the Nav1.5 current (INav1.5) density. Additionally, it is known that there is a reciprocal modulation of the expression of Nav1.5 and Kir2.1 and Kir2.2 channels. Thus, we tested whether Nter is able to increase the expression of Kir2.x channels.
Methods: Currents were recorded using the patch-clamp technique in Chinese ovary cells transiently transfected with wild-type or site directed mutated Nter, Kir2.1, Kir2.2, and Nav1.5 channels.
Results: Cotransfection of Nter with Kir2.1 and Kir2.2 channels significantly increased Kir2.1 current (IKir2.1) and IKir2.2 measured at -120 mV from -126±12 to -183±18 pA/pF (n=25, $P<0.05$) and from -72±9 to -114±13 pA/pF (n=22, $P<0.05$), respectively. Conversely, cotransfection of Nter with Kir2.3 channels, which did not bind to syntrophin with a high affinity as demonstrated in immunoprecipitation experiments, did not significantly modify IKir2.3. Nter did not increase the current density generated by Nav1.5 channels lacking their C-terminal PDZ domain (Nav1.5ΔPDZ). Noteworthy, Nav1.5ΔPDZ channels still coimmunoprecipitated with syntrophin. We identified in the Nter peptide a sequence which could act as a "PDZ-like" binding domain (18-RESLA) and we tested its involvement in the Nter "chaperon" effect by site directed mutagenesis. The results demonstrated that mutants of all residues, except p.S20A Nter, increased IKir2.x. To further confirm the role of Ser20, p.S20A Nav1.5 channels were cotransfected with either Kir2.1 and Kir2.2 channels. Cotransfection of Kir2.1 channels with p.S20A Nav1.5 channels did not increase IKir2.1 as cotransfection with Nav1.5 channels did (from -126±12 to -192±16 pA/pF, n=35, $p<0.05$). Finally, to test the Nter chaperon effect in a cardiac model, adult rat ventricular myocytes were enzimatically dissociated, cultured, and infected with either a control adenoviral construction (Ad-GFP) or an Nter codifying adenoviral construction (Ad-Nter). Results demonstrated that myocyte infection with Ad-Nter significantly increased both the inward sodium (INa) and inward rectifier (IK1) currents.
Conclusions: The N-terminal domain of Nav1.5 channels exerts "chaperon-like" effect increasing INav1.5 and IKir2.x densities. This effect depends on the residue Ser20, which probably determines the binding to syntrophin via an "internal" PDZ binding domain.

P1517 | BENCH

Characteristics of pre-clinical models for the study of atherosclerosis and restenosis

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Purpose: Animal models are necessary for the evaluation of new endovascular therapies. We compared relevant models of restenosis with and without atherosclerotic injury in pigs and rabbits. Our aim was to compare the characteristics and feasibility of these models.
Methods: We compared atherosclerotic lesions induced by balloon injury and atherogenic diet in pig coronary arteries (n=33) and rabbit aorta (n=12). We also studied the lesions of WHHL rabbits (n=6), which portray a familiar hypercholesterolemia type phenotype and have naturally high circulating cholesterol levels. In-stent restenosis (ISR) was also evaluated in different branches of the pig coronary tree by OCT imaging (n=12). Stents were implanted in the right (RCA), the left anterior descending (LAD) and left circumflex (LCX) coronary artery.
Results: Injured rabbit aortas developed lipid rich and uniform lesions. Porcine coronary injury produced lesions with varying characteristics and creation of lipid rich lesions was difficult even with high fat diet (picture). OCT follow up revealed very different characteristics in the different coronary branches in regards to the formation of in-stent restenosis. The RCA was found most resistant to ISR (RCA 17.9±3.5% vs LAD 33.7±3.8% vs LCX 39.9±9.2%) six weeks after stent implatation.

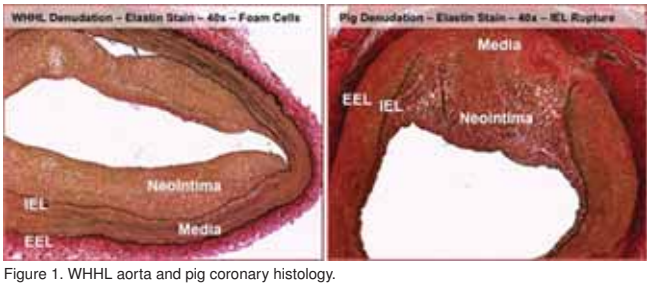

Figure 1. WHHL aorta and pig coronary histology.

Conclusions: The rabbit aorta with double injury of high cholesterol and mechanical de-endothelialization produced most atherosclerosis- like lesions. The

to express an inhibitory Gi-protein-coupled Drosophila allatostatin receptor (AlstR). Application of the AlstR ligand – the insect peptide allatostatin (5 μl) produced rapid and reversible silencing of targeted vagal preganglionic neurones. These were targeted with two stereotaxic microinjections of a viral suspension containing either PRSx8-AlstR-eGFP-LV or PRSx8-eGFP-LV control.
Results: Activation of neurones in the left caudal DVMN resulted in a significant load-independent decrease in LVdP/dt max by -692±193 mmHg/s (n=11, $p<0.0025$). This effect was abolished following intravenous administration of atropine methyl nitrate (2mg/kg, n=7). Acute inhibition of the DVMN neurones expressing AlstR following application of allatostatin resulted in a significant increase in LVdP/dt max by 922.3±193 mmHg/s (n=6, $p<0.001$).
Conclusion: The data obtained challenges the prevailing view which persists in both the scientific and educational literature that the vagal innervation of the ventricle is sparse and insignificant. We demonstrate direct load- and heart rate-independent tonic control of ventricular contractility by a distinct population of vagal preganglionic neurones.

P2527 | BENCH
MiR-208a/b decreases cardiac L-type Ca2+ current by targeting CACNA1C and CACNB2 mRNA

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Purpose: Recent evidence have demonstrated that microRNAs, small non-coding RNA that regulate expression of target mRNA by binding to their 3'-untranslated regions (3'-UTR), can be partially responsible of the atrial fibrillation (AF)-induced electrical remodelling. Among them, microRNA-208a (miR-208a) and miR-208b are cardiac-specific microRNAs. It was demonstrated that miR-208b expression increases in right atrial appendages of AF patients. Since reduction of L-type Ca2+ current (ICa,L) density is a hallmark of the electrical remodelling, the aim of the present study was to determine whether miR-208a and miR-208b modulate ICaL.
Methods: ICa,L was recorded at room temperature using Ba2+ as charge carrier (IBa) in HL-1 cells transfected or not with miR-208a and miR-208b mimics (at a final concentration of 30 nM) by whole-cell patch-clamp.
Results: Neither miR-208a nor miR-208b modified cell size as assessed by cell capacitance measurement (48.6±8.7 and 39.8±9.9 vs 34.7±2.0 pF; $P>0.05$). Under control conditions, maximum IBa density was reached at +20 mV (-5.9±1.0 pA/pF). miR208a and miR208b decreased peak IBa density at potentials positive to +10 (-2.2±0.4 pA/pF for miR208a and -1.8±0.3 pA/pF for miR208b at +20 mV; $P<0.01$ vs control). None of these microRNAs modified the voltage dependence of activation and inactivation. Importantly, a negative control miRNA failed to modify IBa, demonstrating the specificity of the observed effects. Bioinformatic algorithms such as miRanda and TargetMiner predicted that 3'UTR of both α1C (CACNA1C) and β2 (CACNB2), but not α2δ (CACNA2D) subunits can be targeted by miR-208a and miR-208b. These predictions were confirmed by luciferase reporter assays demonstrating that miR-208a and miR-208b produced a concentration-dependent decrease in the luciferase activity in CHO cells transfected with CACNA1C and CACNB2 3'UTR regions.
Conclusions: These results suggest that miR-208a and miR-208b may contribute to the development of AF-induced electrical remodelling, by means of a reduction of ICa,L.

P2528 | BEDSIDE
Association of genetic variants of the phosphatase and actin regulator 1 gene (PHACTR1) and the CDKN2B antisense RNA 1 (CDKN2BAS1) with coronary heart disease in Japanese individuals

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Purpose: Although various loci and genes that confer susceptibility to coronary heart disease (CHD) or myocardial infarction (MI) have been identified for Caucasian populations in genome-wide association studies (GWASs), genetic variants related to these conditions in Japanese individuals have not been identified definitively. The purpose of the present study was to examine a possible association of CHD or MI in Japanese with 15 polymorphisms identified as susceptibility loci for these conditions in the previously GWASs.
Methods: Study subjects comprised 4746 Japanese individuals (2462 subjects with CHD including 1822 with MI, 2284 controls) who visited the participating hospitals between 2002 and 2012. We selected 15 polymorphisms (each with a minor allele frequency of ≥ 0.05 in Japanese) reported by the previous GWASs for CHD or MI. Genotypes of polymorphisms were determined by the multiplex bead-based Luminex assay. To compensate for multiple comparisons, we adopted the criterion of a false discovery rate (FDR) of <0.05 for statistical significance of association.
Results: Comparisons of allele frequencies for 2462 subjects with CHD and 2284 controls by the chi-square test revealed that the rs9369640 of the phosphatase and actin regulator 1 gene (PHACTR1) (P=0.00001, FDR = 0.0002) and rs4977574 of the CDKN2B antisense RNA 1 (CDKN2BAS1) (P=0.0042, FDR = 0.0315) were significantly associated with CHD. Multivariable logistic regression analysis with adjustment for age, sex, body mass index, smoking status, and the prevalence of hypertension, diabetes mellitus, and dyslipidemia revealed that the rs9369640 (P=0.0006; odds ratio, 0.73; dominant model) and rs4977574 (P=0.0043; odds ratio, 1.26; recessive model) were significantly associated with CHD. The minor C allele of rs9369640 was protective against CHD, whereas the minor G allele of rs4977574 was a risk factor for this condition. Next, a relation of 15 polymorphisms to MI was examined among 1822 subjects with MI and 2284 controls. Comparisons of allele frequencies by the chi-square test revealed that the rs9369640 of PHACTR1 (P=0.00002, FDR = 0.0003) and rs4977574 of CDKN2BAS1 (P=0.0003, FDR = 0.0019) were significantly associated with MI. Multivariable logistic regression analysis with adjustment for covariates revealed that the rs9369640 (P=0.0005; odds ratio, 0.70; dominant model) and rs4977574 (P=0.0004; odds ratio, 1.37; recessive model) were significantly associated with MI.
Conclusions: The rs9369640 of PHACTR1 and rs4977574 of CDKN2BAS1 may be susceptibility loci for CHD and MI in Japanese individuals.

COMPUTED TOMOGRAPHY IN ASYMPTOMATIC HIGH RISK SUBJECTS

P2530 | BEDSIDE
Is metabolic syndrome predictive of coronary artery disease burden, and prognosis beyond its individual components?Results from the CONFIRM Registry

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Background: Metabolic Syndrome (MetS) is shown to increase the risk of cardiovascular disease and mortality. However, it is not yet known if MetS has any additive prognostic value beyond its individual risk factors (RF). The purpose of this study is to compare the prevalence, severity of coronary artery disease (CAD) and prognosis of patients with and without MetS.
Methods: The study cohort consisted of 27,125 consecutive individuals who underwent 64 detector row CCTA at 12 centers from 2003 to 2009. Patient with known CAD, unknown statin use or unavailable lipid profile were excluded. MetS was defined as per NCEP/ATP III criteria. We focused on 3900 patients who had measured RF for MetS and were not on statin therapy. Of these, 690 patients met the diagnostic criteria for MetS. Propensity matching was performed for age, sex, smoking status and family history of premature CAD with patients without MetS (no-MetS) with 0, 1, or 2 MetS RF. CAD was defined as none, non-obstructive (1–49% stenosis), or obstructive ($\geq$50% stenosis). Major adverse cardiac events (MACE) was defined as MI, acute coronary syndrome, all cause mortality and late revascularization. MACE was assessed by risk-adjusted Cox proportional hazards models.
Results: MetS group had higher rates of obstructive 1, 2 and 3-vessel/left main disease compared to no-MetS group with 0 or 1 RF (13.8 vs. 8.8%, 4.5% vs. 2.4% and 2.3% vs. 0.9%, respectively; $p<0.01$). However, presence of obstructive 1, 2 and 3-vessel/ left main disease was not significantly different between MetS and no-MetS group with 2 RF (13.8 vs 10.5%,4.5% vs 2.8% and 2.3% vs 1.3%, respectively; p= non significant (NS)). At 2.2 year follow up, MetS group had higher MACE compared to no-MetS subgroups with 0 or 1 RF (4.4% vs 1.6%; $P<0.01$). There was no statistically significant difference in MACE between MetS group and no-MetS subgroup with 2 RF (4.4% vs 3.2% P=NS).
Conclusion: The prevalence and severity of CAD as well as MACE rate are significantly higher in MetS group compared to no-MetS group. However, these differences are not significant when comparing MetS group with no-MetS subgroup with 2 RF. These findings challenge the prognostic value of MetS as a syndrome beyond the sum of its components.

P2531 | BEDSIDE
Assessing the pretest probability of obstructive coronary disease - can we afford to ignore the cardiovascular risk factors?

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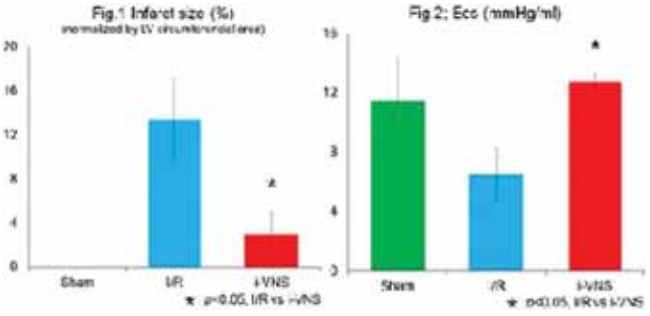
Background: The European Society of Cardiology (ESC) 2013 Guidelines on

VNS has been shown to exert powerful anti-infarct effects, the technical difficulty associated with VNS precludes its application under emergent settings of AMI. In this study, we developed a novel technique of intravenous VNS and evaluated how the VNS affects the infarct size and cardiac function 4 weeks after AMI with reperfusion.

Method: In 11 mongrel dogs, we ligated a left anterior descending coronary artery for 3 hours, then reperfused. For the intravenous VNS, we performed the field electrical stimulation between a pacing catheter in the superior vena cava and an electrode pad attached to the back. We delivered VNS from the beginning of ischemia to 1 hour after reperfusion. We titrated the strength of VNS to lower heart rate by 20–30%. We divided animals into 3 groups, sham operation/no stimulation (Sham, N=3), ischemia-reperfusion (I/R, N=4), and I/R+VNS (I/R-VNS, N=4). 4 weeks after ischemia, we evaluated hemodynamics and left ventricular function in terms of end-systolic elastance (Ees). We also histologically estimated the infarct size.

Results: During operation, mean heart rate were significantly lower in I/R-VNS than in I/R (107±20 vs. 134±16 bpm, $p<0.05$), while blood pressure didn't differ among 3 groups. I/R-VNS strikingly decreased the infarct size more than 80% ($p<0.05$, Fig. 1) and improved Ees ($p<0.05$, Fig. 2). I/R-VNS markedly decreased left ventricular end-diastolic pressure (5.0±1.6 vs. 23.8±2.5 mmHg, $p<0.05$) and serum NT-pro BNP (843±256 vs. 3667±1637 pmol/ml, $p<0.05$).



The effect of VNS for infarct size & Ees.

Conclusion: Intravenous VNS in AMI markedly reduces the infarct size and improves cardiac function in the chronic phase.

P5360 | BENCH

Funny current mediated pacemaker activity in the sinoatrial node of sodium-calcium exchanger knockout mice

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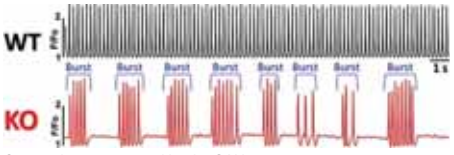
Purpose: The sodium-calcium exchanger (NCX) is the major Ca extruder of myocytes. In the sinoatrial node (SAN) both NCX and funny current (If) participate in the depolarization that initiates pacemaker activity. To clarify the relative contribution of NCX to SAN pacing, we created an atrial-specific NCX knockout (KO) mouse. This mouse does not express any NCX in the atrial region, including the SAN. Phenotypically, NCX KO mice lack P waves on their electrocardiograms and have quiescent isolated SAN cells. Recording the Ca dynamics in a novel preparation that includes the SAN and atria, we investigated wether atrial remodelling may have obscured residual pacemaker activity in NCX KO SAN tissue.

Methods and results: Using high speed 2D confocal microscopy we found that the KO SAN exhibited bursts of organized Ca transients alternating with pauses, characterized by abundant intracellular Ca waves. Although, the overall rate of Ca transients in NCX KO SAN (~2Hz; n=25) was reduced by the numerouse pauses, the frequency of Ca transients during the bursts was rapid. Their average frequency (~4Hz; n=24) was not significantly different from WT (~5Hz; n=24). When considering only the rate during the burst, we found that 6 out of 10 KO SANs responded significantly to β-adrenergic stimulation (isoproterenol 10μM; 52±16% rate increase). This response was smaller but still comparable to the increase in WT SANs (70±6%; n=5). The pacemaker activity of both genotypes responded to the If blocker ivabradine (IVA, 9μM). At higher doses of IVA (27μM) WT SANs decreased their rate by 43±4%, while KO SAN Ca transients were effectively eliminated.



Ca transients generated by the SAN.

Conclusions: These results indicate that If generates the burst pacemaker activity found in the NCX KO SAN, and allows the partial β-adrenergic responsiveness of the KO.

P5361 | BENCH

Pitx2 decreases L-type Ca2+ current and increases the slow delayed rectifier K+ current in cardiac cells

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Purpose: Genome wide scan analyses demonstrated that single nucleotide polymorphisms in the human chromosome 4q25, proposed to regulate the activity of the adjacent transcription factor Pitx2, were associated with an increased risk in atrial fibrillation (AF). Recent evidence has shown that Pitx2 could play a role in AF-induced electrical remodelling. However, its putative role in the control of expression/function of the ion channels responsible for atrial action potential is currently unknown. This work was undertaken to determine the effects of Pitx2 on voltage-gated cardiac Ca2+ and K+ channels.

Methods: Currents were recorded in HL-1 cells transfected or not with the cardiac Pitx2 isoform (Pitx2c) by using whole-cell patch-clamp. L-type calcium current (ICa,L) was recorded by using Ba2+ as charge carrier (IBa).

Results: Under control conditions, peak IBa density was reached at +20 mV (-4.6±0.6 pA/pF). Pitx2c significantly reduced peak IBa density (-2.8±0.3 pA/pF at +20 mV, $P<0.05$) without modifying activation, inactivation, and reactivation kinetics or voltage- dependent activation and inactivation. Regarding voltage-gated K+ channels, under control conditions 2 groups of cells were identified based on the predominant voltage-gated K+ current exhibited. In most of the cells (≈80%), a rapid delayed rectifier current (IKr) sensitive to dofetilide could be recorded, which reached a mean density of 1.9±0.2 pA/pF at 0 mV. In the rest of the cells (≈20%), IKr was absent and the predominant current was a fast activating and slow inactivating outward current sensitive to 4-aminopyridine (2 mM), with biophysical properties compatible with the ultrarapid delayed rectifier K+ current (IKur) recorded in human atrial myocytes. In the presence of Pitx2c, only a small subset of the cells exhibited IKur (≈10%) sensitive to 4-aminopyridine. Importantly, most of the cells (≈90%) exhibited a voltage-gated, dofetilide-resistant, K+ current with a very slow activation kinetics (τact at +60 mV= 1.8±0.3 s) that reached 8.9±3.0 pA/pF after 5-s pulses to +60 mV. The mean midpoint of the activation curve was 20.0±3.9 mV. This current was completely abolished by HMR-1556 (1 μM). All the biophysical and pharmacological properties of the Pitx2c-induced current resembled those of the human cardiac IKs.

Conclusions: The results demonstrated that Pitx2c decreased ICa,L and increased IKs and suggested that this transcription factor could contribute to the reduction of ICa,L and the increase of IKs that characterise the AF-induced electrical remodelling.

CARDIAC HYPERTROPHY AND HEART FAILURE

P5363 | BENCH

CTCF is prerequisite for the fetal gene expression in the process of hypertrophy of the cardiomyocyte

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Purpose: CCCTC-binding factor (CTCF) is a multifunctional chromatin modulator protein in the nucleus and regulates the phenotypic changes in differentiation or carcinogenesis in various types of cells. We hypothesized that CTCF is one of the master weaver of the cardiac genome and first investigated its roles in the fetal gene expression on the process of cardiac hypertrophy.

Methods: Neonatal rat cardiomyocytes (2-3 do) were cultured. CTCF mRNA was either knocked-down using siRNA or over-expressed by transfection with plasmid loading full-length CTCF cDNA. Hypertrophic stimulation were given by angiotensin II (AII: 0.1-0.4μM) or norepinephrine (NE: 1μM) for 24hrs. Gene expression was investigated by real-time PCR analysis using SYBR-Green method. Microarray profiling analysis of mRNAs was performed.

Results: Immunofluorescence and western blot showed CTCF localized in the nuclei of the cardiomyocytes. Knocking down of CTCF mRNA (-61±4%,n=4) suppressed the gene expression of both β-myosin heavy chain (β-MHC) (-45±7%, $p<0.01$) and α-skeletal actin (α-SA) (-25±3%, $p<0.01$). Pre-treatment with 5-Aza-2'-deoxycytidine (2μM) cancelled these suppressive effects by knocking down of CTCF (n=3), indicating DNA methylation is involved in the function of CTCF. After stimulation with AII or NE cell surface area was significantly increased, indicating hypertrophy occured. Under knocking down of CTCF (n=8), up-regulation of β-MHC mRNA to 190±17% following stimulation with AII (n=4) was suppressed to 123±21% ($p<0.01$), and up-regulation of α-SA to 191±22% was also suppressed to 108±9% ($p<0.01$). With NE (n=4) up-regulation of β-MHC to 155±24% was suppressed to 51±7% ($p<0.01$), and up-regulation of α-SA to 159±23% was also suppressed to 42±5% ($p<0.01$). On the other hand, only over-expression of CTCF (n=4) increased the gene expression of β-MHC to 204±18% andα-SA to 134±17% ($p<0.01$). But, unexpectedly it suppressed the up-regulation of β-MHC to 110±12% and α-SA to 106±11% following stimulation with AII ($p<0.01$,n=4). The extent of α-MHC mRNA was not significantly changed after gain or loss of CTCF. Microarray profiling analysis after knocking down of CTCF demonstrated significant down-regulation of 56 genes including WNT1 inducible signaling pathway protein 2, and significant up-regulation of 50 genes including G-protein coupled receptor 37.