Transcriptional Control of Pacemaker Channel Genes *HCN2* and *HCN4* by Sp1 and Implications in Re-expression of These Genes in Hypertrophied Myocytes

Huixian Lin^{1,3*}, Jiening Xiao^{1,3*}, Xiaobin Luo^{1,2}, Guorong Chen^{1,2}, Zhiguo Wang^{1,2}

¹Research Center, Montreal Heart Institute, Montreal, ²Department of Medicine, University of Montreal, ³School of Life Sciences, Guangzhou University, Guangzhou, *Authors with equal contribution to the work

Key Words

HCN2 • HCN4 • I_f • Sp1 • Transcription • Hypertrophy

Abstract

Cardiac hypertrophy is characterized by electrical remodeling with increased risk of arrhythmogenesis. Enhanced abnormal automaticity of ventricular cells may contribute to hypertrophic arrhythmias. The pacemaker current I_f , carried by the hyperpolarization-activated channels encoded mainly by the *HCN2* and *HCN4* genes in the heart, plays an important role in rhythmogenesis. Their expressions reportedly increase in hypertrophic and failing hearts, contributing to arrhythmogenesis under these conditions. However, how their expressions are controlled remained unclear. We performed a study to characterize the regulatory elements and transcriptional control of *HCN2* and *HCN4* genes. We identified the transcription start sites by 5'RACE and core promoter regions of these genes using luciferase reporter assay, and revealed the ubiquitous Sp1 protein as a common transactivator of *HCN2* and *HCN4* genes. We further unraveled robust increases in *HCN2/HCN4* transcripts and protein levels, using

real-time RT-PCR and Western blot analyses, in a rat model of left ventricular hypertrophy and in angiotensin II-induced neonatal ventricular hypertrophy. The upregulation of *HCN2* and *HCN4* transcription was accompanied by pronounced elevations of Sp1 and silencing of Sp1 by siRNA prevented overexpression of *HCN2/HCN4* in hypertrophic cardiomyocytes. Our data indicate that Sp1 drives *HCN2/HCN4* transcription and determines the functional level of *HCN2/HCN4* mRNAs, and upregulation of Sp1 underlie the abnormal re-expression of *HCN2/HCN4* genes in hypertrophied myocytes. This study also provides the first evidence for the role of Sp1 in the reactivation of 'fetal' cardiac genes, *HCN2* and *HCN4*, in ventricular myocytes, and thereby in the pathological electrical remodeling in hypertrophied myocytes.

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Introduction

Cardiac hypertrophy is characterized by electrical remodeling with increased risk of arrhythmogenicity. This electrical remodeling process is at least partially

determined by a reprogramming of cardiac gene expression and the reactivation of 'fetal' cardiac genes. Among these genes are the ones encoding the ion channel proteins that generate pacemaker current I_p , the hyperpolarization-activated cyclic nucleotide-gated channel (HCN) gene family [1-3]. The HCN family is composed of at least four members (HCN1-HCN4), of which HCN4 is believed to be the major molecular component for sinus I_f and HCN2 the dominant isoform for ventricular I_f . In the mammalian cardiac sinus node, I_f plays a crucial role in setting the heart rate and sensing its autonomic control. In addition to the automatic cells, I_f also exists in non-automatic (non-pacemaker) regions of the heart, such as atria and ventricles, where it contributes to abnormal automatic activities [4-6]. Abnormally enhanced automaticity of ventricular cells may contribute to arrhythmias in hypertrophied hearts. Consistent with this notion, robust enhancement of functional I_f and upregulation of HCN gene expression have been observed in a variety of animal models of cardiac hypertrophy and heart failure and in human failing hearts as well [1, 6-14]. Normally, I_f expresses in ventricular myocytes only during fetal and neonatal life and the abundance progressively decreases toward adulthood in terms of the number of cells expressing I_f and of the density of expressed I_f as well [4-6, 9, 12, 15, 16]. This results in a loss of the capacity of adult ventricular cells to generate spontaneous activity. In cardiac hypertrophy, I_f is at least doubled in left ventricular (LV) cells, depending upon severity of conditions [5, 13, 14].

However, mechanisms underlying the pathological re-expression of I_f and their encoding genes *HCN2/HCN4* in hypertrophic heart remained yet to be elucidated. To shed light on this issue, we performed detailed analyses of regulatory elements in the 5'-flanking regions of *HCN2* and *HCN4* genes. Our study identified the core promoter regions of *HCN2* and *HCN4* genes and the stimulating protein (Sp1) as a common transactivator for *HCN2/HCN4* transcription and established a significant role of Sp1 in hypertrophic re-expression of *HCN2* and *HCN4* genes.

Materials and Methods

Rapid amplification of cDNA Ends (5'RACE)

The transcription start sites (TSSs) of *HCN2* and *HCN4* were determined with Ambion's RNA-Ready cDNA Human Heart RNA ligase-mediated 5'RACE kit, as previously described (18-20). Human RNA sample was purchased from Clontech. The gene specific primers were designed based on the human

HCN2/HCN4 cDNAs (GenBank accession NM_001194 and NM_005477, respectively).

Construction of Promoter-Luciferase Fusion Plasmids

A series of fragments of varying length from position -1 for *HCN2* and position +157 for *HCN4* (TSS=+1, Fig. 1) were amplified using human genomic DNA (Promega: Cat G3041) and the Homo sapiens bacterial artificial chromosome (BAC) clone RP11-424J10 for *HCN2* and *HCN4*, respectively, as templates. PCR products were subcloned into luciferase-containing PGL3-Basic vector (Promega), as previously described [15-17].

Electrophoresis mobility shift assay (EMSA)

EMSA was performed with the DIG Gel Shift kit (Roche, Mannheim, Germany). Varying amounts of nuclear protein extracts from SKBr3 cells were incubated with digoxigenin (DIG)-labeled double-stranded oligonucleotides containing the putative Sp1 *cis*-elements in *HCN2* and *HCN4* promoter regions or the mutated fragments. The oligo fragments (Figure 1) were synthesized by Invitrogen, as shown below. For competition experiments, 100-fold excess of unlabeled double-stranded Sp1 consensus oligonucleotides were added to the reaction. The generated chemiluminescent signals were recorded on the X-ray film.

Sp1- containing fragment in *HCN2* promoter region (lower-case letters indicate mutations made within the core of the Sp1 binding motifs):

Wild-type: 5'-CGC GGC TCC GCC CCG CCC CGC CCC CGC-3'
GCG CCG AGG CGG GGC GGG GCG GGG GCG

Mutant: 5'-CGC ttc TCC GcT tCG Ctt CGC Ctt CGC-3'
GCG aaG AGG Cga aGC Gaa GCG Gaa GCG

Sp1- containing fragment in *HCN4* promoter region:

Wild-type: 5'-CCT CTC ACC CCG GCC CCG AA-3'
GGA GAG TGG GCC GGG GCT T

Mutant: 5'-CCT CTC Act tCG GCt tCG AA-3'
GGA GAG Tga aGC Cga aGC TT.

Mutagenesis

Deletion mutations and base-substitution mutations were created by direct oligomers synthesis or by PCR-based methods. Mutations were made to the 5'-flanking regions of *HCN2* and *HCN4* for promoter studies [15].

Small Interference RNA (siRNA) Treatment

The Stealth™ siRNAs targeting Sp1 and a scrambled control siRNA (Cat. 12935-300) were synthesized by Invitrogen. Rat LV myocytes in primary culture were transfected with the siRNAs, using lipofectamine 2000, according to the manufacturer's instructions (Invitrogen). Before siRNA treatment, cells were starved to synchronize growth by incubating in serum- and antibiotics-free medium for 12 h. Cell lysates were prepared 36 h post-transfection for real-time RT-PCR and Western blot analysis.

Cell Culture

The cell lines used in this study were all purchased from American Type Culture Collection (ATCC, Manassas, VA). H9c2

Fig. 1. The core promoter sequences of *HCN2* (A) and *HCN4* (B). The transcription start sites (TSS) are indicated by backward arrows and designated position +1. The consensus binding sequences for the Sp1 transcription factor are bold and italic and the core sequences of the *cis*-elements are underlined. For convenience, the positions of the Sp1 consensus sites are numbered in the brackets. The position of translation start codon (ATG) relative to the TSS is also indicated. The sequences within black boxes indicate the fragments used for EMSA.

(rat ventricular cell line) and HEK293 (human embryonic kidney cell line) were cultured in Dulbecco's Modified Eagle Medium (DMEM). H9c2 and HEK293 cells were used for our experiments involving luciferase assays because these cells do not have apparent endogenous pacemaker currents to contaminate the analysis and can serve as reasonable models for comparisons of HCN expression between muscle cells and non-muscle cells.

Hypertrophic Model

Left ventricular (LV) hypertrophy was induced by an aortic stenosis technique, similar to that described by Fernández-Velasco et al [5, 10]. Briefly, adult male Sprague-Dawley rats weighing 180-200 g were used. The animals were anesthetized via an intraperitoneal injection of sodium pentobarbital (50 mg/kg). A silver clip was placed around (0.3 mm aperture) the abdominal aorta above the left renal artery. The LV pressure was progressively developed as the animals grew and LV hypertrophy was established at eight weeks after surgery. Sham-operated and age-matched rats were used as control. Hypertrophied hearts were defined as the ratio of heart weight (wet)-to body weight >25% of control hearts. The use of animals was in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Harbin Medical University.

Myocyte Isolation and Primary Cell Culture

Neonatal rat ventricular cardiomyocytes were isolated and cultured as described previously [30]. These cells were used to investigate angiotensin-II (ANG-II)-induced cardiomyocyte hypertrophy because, unlike the proliferating H9c2 and HEK293 cells, neonatal rat ventricular myocytes are non-proliferating cells that would grow in size (not in number) upon ANG-II stimulation, better representing adult ventricular myocytes for studying a hypertrophic condition.

Angiotensin II-Induced Hypertrophy and Cell Surface Area Analysis of Cardiomyocytes

Isolated neonatal rat ventricular cells were cultured for 24 h in serum-containing medium after which they were serum-starved for 24 h before treatment. To induce hypertrophy, cells were stimulated by angiotensin II (ANGII, 1 μ M) for 36 h in serum-free culture medium [18, 19]. Cell surface area was analyzed using a Leica inverted microscope equipped with a Polaroid digital camera at 200 $\times$ magnification.

A. <i>HCN2</i> Core Promoter Region	
-751	CCCACCAGACCTAGAGGAGACCCCGCCGACGGCTCCCAGGGCACT
-701	GTTCGAGGGTGGGTGGGAACCCACACCTCGGGTGGCTTTCCAGTTT
-651	TCCTTGCTGGCTGGGCTTGGCTCAGTTTCTCAAATGCAGACAGGGA
-601	GAGGAGTGCCTGAGTCTCCAGAACGGCGCCCAAGCCGGCTGCCCTCC
	Sp1 (-566)
-551	CCACGCACTCAAGGGCTCTGTCCAGGGCCACAGGCGACCCAGCAAGGG
-501	CTCCTGTGACCTTTAGAAACACCTTTCGTGCTGGAAGTCTTCAAACCTC
-451	ACAGAAAGCAAGGAGGCGCTGGCGTGGCGGCTCTCCCGTCTCCAGGG
-401	CCCGCTCCACCTCCCGCGCGGCTGCCCTCCGACAGAGGTGGAAGCG
	Sp1 (-396)
-351	CGGCGCAGGAGAGGCGGCTCCGGCCGGGACGGGGCCCGGTGACCTTGG
-301	GCCTGCTCTACAGTCCGCGGCTCAGTTTCCCTCTCTGGGAGAGGCC
-251	CGCGCGGGCTCCGAGGCTCGGGCCGCGTACCTTGGGGGCCCCCGAC
-201	CCCGCGGCCGAGTTTCCCGCGCGGAGCGGAGCGGCGCGGGGAGGG
	Sp1 (-162)
-151	GGGCGAGCCGAGGGGGTCTCCCGCCCGCGCTCTCCCGCGCCCTCCCG
	Sp1 (-135) Sp1 (-118)
-101	CGCCGCGAGGCTCCCGCCCGCGGCTCCCGCGCGCGCGCGCGCTC
	Sp1 (-94) Sp1 (-69)
-51	CCCCCTCCTCGGGCTCCGCGCGCGCGCGCGCGCGCGCTCCGCTCCGC
+1	ACTGCCCGCGCGCGCTCCGCTATG
	↓ (TSS +1) ↓ (Translation start codon)
B. <i>HCN4</i> Core Promoter Region	
-270	GGGGAATTTCGCAACTGAAGCGGAGCTTCAGACTTGGGGCGCACTCCAG
-220	CTTAGCCAGGAAAGAGATTAAAGGCGCAGCAGTGTGGATACCTCTC
-170	CCGGGCGCGAGGCTGCTAGCAGGGTCTAACTTGGGCCCCCTTGGCAGGCC
	Sp1 (-175)
-120	CGCCCCCGCCCCCTTCAGCCCCCGGCCCGTGCGCGCTGCCCTTTAA
	Sp1 (-121)
-70	GAAAGCCAGGTAGGAGGCGCGGCTGCTGAGCGCTCCTATGGCAACCC
-20	CGAGCTGCGGGCTTCATGATATATTCGGGGCGCGGAGCCGAGCGC
	↓ (TSS +1)
+31	TGCCGAGGGCGCTTCGGGGAGGCGGCGCTGATGTAAGCCCGCGGGT
+81	CGCTGGCTCCGCTCGGTTGCGGGGAGCCCGGGACGGGCGGACGGG
	↓ (+157)
+131	CCGGGCGAGAGGAGGCGAGGCTCGCGGTGGCCAGCCACAAAGCCC
+181	GGGCGCGAGACAGACGGACAGCCAGCTCCCGGGAGCGACGCCGG
	Sp1 (+175)
+231	GACCCGCGGGGCGTGCCTCTGCACTCCGAGCGGTTCCCTGAGCGCC
+281	CGGGCGCAGAGCTCTCCGCGCGGCCATTGTTCCTCCGGGGGCGG
	Sp1 (+326)
+331	GGCGCTGGAGCCCGGGCGCGCGCCGCTGACGCCAGAGGGAGGG
	Sp1 (+345) Sp1 (+357)
	3' - 666
	AGGGAGGgAAGAAAGGAG-5' (Triplex DNA)
+381	AGGGAGGCAAGAAAGGAGCGCGGGTCCCGCGCCAGCGGGCCGGGA
+431	GGAGGTGTAGCGCGGAGCCCGGGGACTCGAGCGGGACTAGGATCTC
	↓ (+520)
+481	CCCGCGCGCGAGCTGCCAAGCATGGGCGCTGAGGCTGCCCCACG
+531	CCGGCGCAAGGAGCGGTCCCGCGGCGGCTGACCGGGGCGGAGCC
	Sp1 (+554) Sp1 (+570)
+581	TGGAGCCCGTCCGCGGCGCGCGCTCCCGCGGCGCGGCGGCTCCGACCC
	Sp1 (+607)
+631	GGCCCTGGCGGCTATG
	Sp1 (+632) ↓ (Translation start codon)

Transfection and Luciferase Assay

Luciferase activities were measured with a dual luciferase reporter assay kit (Promega) on a luminometer (Lumat LB9507), as described previously [15-17]. The plasmid vector carrying stimulating protein 1 (Sp1; CMVp-Sp1) was a generous gift from Dr. Robert Tjian (University of California, Berkeley, CA) [20, 21].

Drosophila Schneider cells (SL2)

SL2 cells were cultured at 25°C in Schneider's *Drosophila* medium (Gibco). Transfection was performed using Lipofectamine 2000. The DNA transfection mixture contained 250 ng reporter plasmid, varying amounts of pPac-Sp1 plasmid, and empty pPac0 vector (kind gifts from Dr. Robert Tjian, University of California) as control [20, 21].

Quantification of mRNA Levels

For quantification of HCN2, HCN4 and Sp1 transcripts, conventional TaqMan real-time RT-PCR was carried out with total RNA samples extracted from HEK293 cells or rat LV 36 h after various treatments, as previously described in detail [15-17].

Western Blot

The procedures for protein extraction from neonatal rat ventricular myocytes and immunoblotting analysis of HCN2/HCN4 and Sp1 proteins were the same as described in detail elsewhere [15-17, 22]. For HCN2/HCN4, membrane protein samples were extracted, and for Sp1, cytosolic protein samples were used. Affinity purified rabbit polyclonal anti-HCN2 and anti-HCN4 (Alomone Labs, Israel) antibodies (1:500 dilution), and polyclonal anti-Sp1 antibody (1:500 dilution; Santa Cruz Biotechnology Inc., CA) were used as the primary antibodies.

Data Analysis

Group data are expressed as mean $\pm$ S.E. Statistical comparisons (performed using ANOVA followed by Dunnett's method) were carried out using Microsoft Excel. A two-tailed $p < 0.05$ was taken to indicate a statistically significant difference.

Results

Transcriptional Activation HCN2 and HCN4 Expression by Sp1

5'RACE was performed to obtain the 5'ends of HCN2 and HCN4 transcripts using RNA samples from human heart. The experiments yielded a single discrete band (fragment) for each of the genes. Sequence analysis revealed that the fragments contain the transcription start sites (TSS, designated +1) for HCN2 and HCN4, respectively. The HCN2 TSS is located to 21 bp, and the HCN4 TSS located to 593 bp, upstream the translation start codon (ATG) of HCN2 (GenBank accession No.

Fig. 2. Analysis of HCN2 and HCN4 promoter activities in H9c2 rat ventricular cells and HEK293 human embryonic kidney cells. The 5' deletion constructs of the promoter regions are schematically shown on the left panels. Nucleotides of fusion plasmids are numbered with respect to the TSS (+1) identified by 5'RACE. Firefly luciferase expression levels were divided by co-expressed Renilla luciferase activity and expressed as relative activity divided by the promoter-less construct (PGL3-Basic). The results revealed -191/-1 fragment as HCN2 core promoter (A) and -267/+157 fragment as HCN4 core promoter (B). The data were averaged from 5 experiments in duplicate for each cell. * $p < 0.05$ vs. PGL3-Basic, $n = 6$. (C) EMSA for HCN2 and HCN4. Lane 1: free probe; lane 2: wild-type core promoter sequence with normal Sp1 binding sites; lane 3: core promoter sequence with mutated Sp1 binding sites; lane 4: unlabeled fragment as competitor. Arrows indicate the shifted bands.

DQ854814) and of HCN4 (GenBank accession No. DQ854815).

Computer analysis using the *MatInspector* program of 3-kb 5'-flanking regions of HCN2 and HCN4 revealed that it contains no TATA box proximal to their TSSs and other common promoter elements that are required for transcription initiation complex. There is no GATA4 binding

Fig. 3. Role of stimulating protein 1 (Sp1) as an essential transactivator for *HCN2* and *HCN4* genes. (A) Effects of Sp1 inhibitor mithramycin (MM, 100 nM) on the expression of *HCN2* and *HCN4* as reported by luciferase activities in H9c2 embryonic rat ventricular cells, and by real-time RT-PCR quantification of endogenous *HCN2/HCN4* transcripts and Western blot analysis of *HCN2/HCN4* proteins in cultured neonatal rat ventricular myocytes. The bands representing *HCN2* and *HCN4* were abolished if the antibodies had been pretreated with their respective antigenic peptides (AP), verifying the specificities of the antibodies. * $p < 0.05$ vs. control, $n = 6$ batches of cells for each group. (B) Effects of Sp1 overexpression (CMVp-Sp1) on the expression of *HCN2* and *HCN4* in HL-1 mouse atrial tumor cells. CMVp: co-transfection of Sp1-null CMVp vector and target DNA-PGL-3 vectors containing the core promoter fragments of *HCN2* (-191/-1) and *HCN4* (-267/+157). * $p < 0.05$ vs. CMVp, $n = 6$ batches of cells for each group. (C) Luciferase reporter activities of the *HCN2* or *HCN4* promoter in Sp1-null *Drosophila* Schneider cells (SL2). Cells were transfected with Sp1-free plasmid (pPac) or with Sp1-carrying plasmid (pPac-Sp1). * $p < 0.05$ pPac-Sp1 vs. pPac, $n = 4$.

site in either *HCN2* or *HCN4*, which is known to be the heart-specific element as the potent transactivator in cardiac cells. Of particular note is the high GC content around the TSSs common to both *HCN2* and *HCN4*. These regions contain multiple Sp1 consensus sequences at -69, -94, -118, -135, -162 for *HCN2* (Fig. 1A) and at -121 and -175 in the *HCN4* promoter (Fig. 1B).

Varying lengths of putative promoter fragment-luciferase constructs were generated and tested for their ability to drive expression of the reporter gene in H9c2 myoblasts line derived from embryonic rat ventricle, a permanent cell line which maintains some features of cardiac myocytes, and HEK293 human embryonic kidney cell line, to map the promoter activities in the *HCN2* and *HCN4* 5'-flanking regions, respectively. *HCN2* and *HCN4* promoter constructs had approximately 10-30 times the activity of the promoterless vector (pGL3-Basic) and demonstrated similar activities in the two different cell lines (Fig. 2). The core promoter of the *HCN2* gene was defined to the -191 region and that of *HCN4* defined to -267 region, as these fragments elicited the maximum luciferase activities.

Sp1 is a widely distributed member of a multigene family of zinc-finger transcription factors that binds DNA in mammalian cells primarily via interaction with GC-box elements. The binding of Sp1 to GC-boxes is often critical to achieving significant levels of transcription from TATA-less promoters and is intimately involved in the determination of the transcription start site(s) [20, 21, 23]. To investigate the role of Sp1 in the transcription of *HCN2* and *HCN4* genes, we first assessed the effects of Sp1 inhibitor mithramycin on the luciferase activities driven by the core promoters of these genes in H9c2 cells and endogenous expressions of these genes at mRNA and protein levels in cultured neonatal rat ventricular myocytes. As depicted in Figure 3, mithramycin produced considerable reduction of the promoter activities and of expression levels of native *HCN2* and *HCN4* transcripts and proteins. We then evaluated the effects of Sp1 overexpression on *HCN2* and *HCN4* expression by co-transfection of CMVp-Sp1 vectors with *HCN2*- or *HCN4*-carrying PGL-3 vectors and by transfection of CMVp-Sp1 to study endogenous mRNA levels of *HCN2*/ and *HCN4* genes in HL-1 cells that express much lower

level of endogenous Sp1. Sp1 overexpression strikingly enhanced the promoter activities and native transcripts of *HCN2* and *HCN4*, as compared with transfection of Sp1-null CMVp vectors (Fig. 3A and 3B). We further demonstrated that in SL2 cells, a Sp1-null environment, the luciferase reporter activity was undetectable with any one of the core promoter fragments without supplying exogenous Sp1 cDNA, whereas in the SL2 cells co-transfected with Sp1 cDNA, it was readily detectable (Fig. 3C). The data in Figure 2A also suggest that Sp1 *cis*-elements are required for *HCN2* transcription initiation because the -54/-1 fragment containing no Sp1 site show no reporter activity at all. And similarly, the -17/+157 fragment excluding the Sp1 consensus sites also failed to drive the *HCN4* promoter.

To verify that the above observations were indeed consequent to interactions between the *cis*-elements and proteins, we examined the ability of the putative *cis*-elements for Sp1 contained in the *HCN2* and *HCN4* promoters to bind purified Sp1 protein *in vitro* using EMSA. The results are illustrated in Figure 2C. Binding of the promoter fragment with Sp1 protein produced an up-shift of the probe band, which was not seen with mutated fragments. Further, the shifted bands were efficiently weakened if excessive unlabeled probes had been added to the reaction.

Roles of Sp1 in Overexpression of f-Channels in Hypertrophic Cardiomyocytes

Consistent with previous studies, we observed that the mRNA level of both *HCN2* was nearly doubled in hypertrophic LV relative to healthy LV and that of *HCN4* was increased by 55%. *HCN2* expression at the protein level was also markedly elevated (Fig. 4A). *HCN4* protein was not detectable under the same conditions; however, with increased loading of the membrane protein sample (from 30 μ g to 150 μ g), *HCN4* protein was detected and found 70% increase in hypertrophic LV.

To explore the potential role of Sp1 in hypertrophic overexpression of *HCN2/HCN4*, we first quantified the levels of Sp1 protein. An anti-Sp1 Western blot with human samples showed a Sp1 protein doublet at 110 kDa and 95 kDa positions (19). For quantification, the sum of the two bands was used to determine the total Sp1 level. Our results showed a remarkably greater Sp1 level, nearly tripled, in hypertrophic LV relative to healthy LV (Fig. 4B). These results were reproduced in neonatal rat ventricular myocytes with hypertrophy induced by angiotensin II. When comparing the Sp1 levels between healthy neonatal and adult hearts, we found around 2-

Fig. 4. Role of overexpression of Sp1 in re-expression of *HCN2/HCN4* in a rat model of left ventricular hypertrophy (adult, HLV) and in angiotensin II-induced hypertrophic neonatal rat ventricular cardiomyocytes (neonatal, HC). (A) Overexpression of *HCN2* and *HCN4* in HLV relative to healthy age-matched control hearts at both mRNA and protein levels. * $p < 0.05$ vs. Ctl; $n = 7$ for each group. (B) Overexpression of Sp1 in HC relative to non-treated control cells at both mRNA and protein levels. The data on Sp1 protein represent the sum of 110 kDa and 95 kDa. * $p < 0.05$ vs. Ctl; $n = 7$ for each group.

fold higher protein level in the former than in the latter (neonatal/adult: 1.96 ± 0.33 ; $p < 0.05$, unpaired t-test, $n = 7$ for each group).

We went on to evaluate the possibility of reversing the hypertrophic increases of *HCN2/HCN4* expression by correcting the Sp1 level in angiotensin II-induced hypertrophic neonatal rat ventricular cardiomyocytes (HC). Transfection of cells with Sp1-siRNA to knockdown Sp1 caused significant reduction of *HCN2* and *HCN4* at both mRNA (Fig. 5A) and protein (Fig. 5B) levels, whereas the negative control siRNA (scrambled siRNA) had no significant effects. Quantification of Sp1 protein levels confirmed the

Fig. 5. Suppression of HCN2/HCN4 overexpression by Sp1 knockdown in angiotensin II-induced hypertrophic neonatal rat ventricular cardiomyocytes (HC). (A) and (B) Suppressing effects of Sp1-siRNA on overexpression of HCN2 and HCN4 at both mRNA and protein levels. The HC was transfected with SRF-siRNA (30 nM) and 36 h after RNA and protein samples were extracted. siRNA: Sp1-specific siRNA; NC siRNA: scrambled negative control siRNA. * $p < 0.05$ vs. Ctrl, + $p < 0.05$ vs. HC; $n = 5$ for each group. (C) Verification of the ability of Sp1-siRNA to knockdown Sp1 at both mRNA and protein levels in HC. * $p < 0.05$ vs. Ctrl; $n = 4$ for each group.

Fig. 6. Cardiac hypertrophy induced by aortic stenosis and by incubation with angiotensin II (AngII) for 24 h. (A) Ratio of heart weight (mg) over body weight (g) (HW/BW) in control (Ctrl) and HLV rats. * $p < 0.05$ vs. Ctrl; $n = 7$ for each group. (B) Surface area of hypertrophic cardiomyocytes (HC), compared with the untreated control cells (Ctrl). Values are mean $\pm$ SEM ($n = 6$ for Ctrl and $n = 6$ for AngII). For cell surface area, the mean of 40 cells was used to provide one value. * $p < 0.05$ vs. Ctrl. (C) Increases in the expression of β -myosin heavy chain (β -MHC), myosin light chain-2 (MLC-2) and atrial natriuretic factor (ANF). The values were calculated by first normalizing to GAPDH for internal control and then by further normalizing to non-hypertrophic control myocytes to obtain the fold changes of mRNA levels. Data are presented as means $\pm$ SEM ($n = 8, 6$, and 6 for β -MHC, MLC-2 and ANF, respectively). * $p < 0.05$ vs. Ctrl.

downregulation of Sp1 by Sp1-siRNA (Fig. 5C). In order to see if in addition to Sp1, any other signaling pathways related to angiotensin II may also contribute to HCN transcription regulation, we applied 50 μ M losartan to the cells in the presence of Sp1-siRNA. This high concentration of losartan produced additional $11.3 \pm 2.1\%$ ($p > 0.05$, $n = 5$ batches of cells) reduction of HCN2 mRNA level and $14.7 \pm 2.6\%$ ($p < 0.05$, $n = 5$ batches of cells) reduction of HCN4 mRNA level.

To confirm that the myocytes treated with angiotensin II indeed developed hypertrophic conditions, we measured the changes of cell size and expression of several biomarkers for cardiac hypertrophy including β -myosin heavy chain (β -MHC), myosin light chain-2 (MLC-2) and atrial natriuretic factor (ANF) [24]. As depicted in Figure 6, cell surface area was significantly enlarged, and β -MHC, MLC-2 and ANF were all upregulated in their expression.

Discussion

One of the characteristic alterations of hypertrophic hearts is the re-expression or overexpression of pacemaker channel genes thereby I_f current, which may contribute significantly to the enhanced abnormality and the risk of arrhythmias under such pathological conditions [1-14]. However, the mechanisms remained poorly understood. In this study, we demonstrated overexpression of *HCN2* and *HCN4* genes in left ventricular (LV) hypertrophy induced by an aortic stenosis technique and in neonatal cardiomyocyte hypertrophy induced by angiotensin II. We revealed Sp1 transcription factor as a common and essential transactivator of *HCN2* and *HCN4* promoters. The hypertrophic upregulation of *HCN2* and *HCN4* expression was accompanied by robust elevation of the Sp1 protein level. Moreover, correction of Sp1 overexpression largely prevented re-expression of *HCN2/HCN4* in hypertrophied myocytes. We conclude that Sp1 drives *HCN2/HCN4* transcription and determines the functional level of *HCN2/HCN4* expression under physiological and pathological conditions.

Our finding that Sp1 plays an essential role in *HCN2/HCN4* transcription may have important implications in increased expression of *HCN2/HCN4* thereby I_f in hypertrophied myocytes. Sp1 is an oncoprotein that is critically involved in cell growth regulation and overexpresses during tumorigenesis. Recent studies also consistently demonstrated elevations of Sp1 levels in cardiac hypertrophy resulted from increased left-right shunting, volume loading of the left ventricle, pressure loading of the right ventricle, or angiotensin II stimulation [25-27]. In agreement with these previous studies, we also found a robust increase in Sp1 expression in left ventricular hypertrophy (Fig. 4). Most strikingly, abrogation of this increase reversed the increased expression of *HCN2* and *HCN4* in hypertrophied cardiomyocytes (Fig. 5), reconsolidating the significance of Sp1 in determining ionic remodeling of hypertrophied myocytes in culture.

The binding of Sp1 to GC-boxes is often critical to achieving significant levels of transcription from TATA-less promoters and is intimately involved in the determination of the transcription start site(s) [20, 21, 23]. Sp1 as a transactivator of *HCN2* and *HCN4* explains why there was no significant difference of the activities of the core promoters in between cardiac H9c2 and kidney HEK293 cells (Fig. 2), since Sp1 is a housekeeping gene ubiquitously expressed in a variety of tissues/cells. Intriguingly, Sp1 seems to be a common transactivator for many ion channel genes; we have previously found

that Sp1 drives transcription of *KCNH2* (encoding HERG K^+ channel) [15], *KCNQ1* (encoding KvLQT1 K^+ channel) [16, 17], and *KCNE1* (encoding a K^+ channel β -subunit) [16]. This study provides the first evidence for the role of Sp1 in the reactivation of 'fetal' cardiac genes, *HCN2* and *HCN4*, in ventricular myocytes, and thereby in the pathological electrical remodeling in hypertrophied cardiomyocytes.

It should be noted that our study does not explain the characteristic developmental changes of expression of *HCN* genes and the differential regional distribution of the transcripts of different *HCN* isoforms [4, 5, 8, 9, 12]. Nonetheless, Sp1 expression also undergoes similar developmental alterations and Sp1 knockout mice further revealed that Sp1 is essential for normal vertebrate embryogenesis [28-32].

In the course of our study, Kuratomi *et al* reported identification of the mouse *HCN4* promoter region and this region drove a high activity in embryonic myocytes, but not in neonatal myocytes treated with hypertrophic agents [33]. The authors further demonstrated the presence of a conserved NRSE motif in intron 1, which is known to bind the transcriptional factor neuron-restrictive silencing factor (NRSF). With wild-type NRSE, the promoter activity correlated well with the developmental and hypertrophic changes of *HCN4* expression, whereas mutant NRSE constructs failed. While the authors did not present any data regarding the regulatory elements in the promoter regions of the mouse *HCN4*, our analysis revealed a cluster of three putative Sp1 binding sites within the core promoter region (-446/+1). These data suggest that multiple regulatory elements are involved in the transcriptional regulation of *HCN4* expression though Sp1 is likely the major transactivator.

The present study aimed to understand the underlying mechanisms for the re-expression of *HCN2* and *HCN4* in hypertrophic conditions and our findings indicate the altered expression of Sp1 as one of the important factors. The results obtained from cultured cells in this study may not be directly applied to *in vivo* conditions of hypertrophic hearts. It should also be emphasized, however, that regulation of *HCN2/HCN4* expression must be a complex process and our study merely suggests the potential contributions of Sp1 to this process but does not at all exclude other regulatory factors. We have recently reported that downregulation of microRNAs miR-1 and miR-133 contributes to re-expression of *HCN2/HCN4* in hypertrophic hearts [34]. Taken together, it is likely that upregulation of Sp1 and downregulation of

miR-1/miR-133 can either or both act to enhance *HCN2/HCN4* expression.

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