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HOTAIR inhibited intracellular Ca^{2+} via regulation of $Ca_V1.2$ channel in human cardiomyocytes

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Abstract

HOTAIR, a long non-coding RNA (lncRNA), is reported to regulate chromatin organization and promote tumor progression. However, little is known about the roles of this gene in the modulation of calcium homeostasis in human cardiomyocytes. In the present study, we demonstrated that up-regulation of HOTAIR could suppress the expression of $Ca_V1.2$ in human cardiomyocytes. However, HOTAIR knockdown promoted $Ca_V1.2$ expression in human cardiomyocytes. In addition, we found that HOTAIR overexpression significantly reduced the intracellular Ca^{2+} contents; while knockdown of HOTAIR enhanced the Ca^{2+} contents in the cardiomyocytes. Moreover, enforced expression of $Ca_V1.2$ increased the calcium level in cardiomyocytes overexpressing HOTAIR. down-regulation of HOTAIR and up-regulation of $Ca_V1.2$ further enhanced the Ca^{2+} contents in the cardiomyocytes Taken together, these results for the first time demonstrate that HOTAIR inhibited the intracellular Ca^{2+} via regulation of $Ca_V1.2$ in human cardiomyocytes.

Key words: Cardiomyocyte, HOTAIR, $Ca_V1.2$, calcium.

Introduction

Calcium homeostasis plays a critical role in various types of tissues. The calcium entry through the L-type calcium channels leads to excitation/contraction coupling in the heart, neurotransmitter release in brain, endocrine secretion in gland tissues, and tension development in smooth muscle (1-3). Alterations in the density and function of L-type calcium channels have been involved in a variety of cardiovascular diseases such as cardiac hypertrophy, atrial fibrillation, ischaemic heart disease, and heart failure (4-6). Thus, it is imperative to understand the underlying mechanisms that regulate calcium channels.

Non-coding RNAs (ncRNAs) are functional RNAs that do not encode proteins. microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) are the major regulatory ncRNAs that regulate gene expression at epigenetic, transcriptional, and post-transcriptional processing levels (7). Over the last decade, much attention focused on the miRNAs. More recently, lncRNAs, defined as non-coding RNAs of more than 200nt in length, have risen to prominence with central roles in a diverse range of functions in cell biology, such as such as gene expression, RNA splicing, and ligand-receptor engagement (8). Increasing studies have demonstrated that HOTAIR, one of the few biologically well-documented lncRNAs, is overexpressed in a variety of human cancers and may represent negative prognostic markers in breast, liver, colon, and pancreatic cancer patient survival (9-12). However, the biological function of HOTAIR in regulation of calcium channels has never been investigated. Therefore, the present study aimed to reveal the relationship between the lncRNA HOTAIR and calcium channel gene using an *in vitro* human cardiomyocytes.

Materials and methods

Cell culture and transfection

Human cardiomyocytes (HCM) were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA) and cultured in polylysine-coated plates using complete cardiac myocyte medium according to the manufacturer's recommendation as previously described (13). The vector PCDH was purchased from Addgene, USA. HOTAIR overexpression plasmid was identified by digestion with Xhol and BamHI (Fig. 1). The HOTAIR siRNAs were synthesized by GenePharma (Shanghai, China). Transfection of cells was performed using Lipofectamine 2000 reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions.

Real time PCR

Total RNA was extracted by using TRIzol reagent (Invitrogen) and complementary DNA was synthesized using Reverse Transcription Kit (Toyobo, Osaka, Japan) according to the manufacturer's protocol. The sequences of primers used were as follows:
HOTAIR-F:5'-CCGCTCGAGCCTCCAGGCCC-TGCCTTCT-3';
HOTAIR-R:5'-CGGGATCCTTTATATTCACCACA-TGT-3';
GAPDH-F: 5'-CACCATCTTCCAGGAGCGAG- 3';
GAPDH-R: 5'-TCACGCCACAGTTTCCCGGA -3'.
PCR was performed according to the instruction by the manufacturer using the SYBR Green PCR Master Mix (Toyobo, Japan). Amplification protocols were fol-

lowed: 95°C for 3 min; 40 cycles of 95°C15 s, 60°C/15 s and 72°C/30 s. The transcript levels of interest genes were normalized to the GAPDH and were calculated with $2^{-\Delta\Delta Ct}$ method.

Western blotting

Total cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and transferred on a nitrocellulose filter membrane. The membrane was incubated with primary antibodies against $Ca_V1.2$ (1:1000 dilution) and GAPDH (1:5000 dilution). Horseradish peroxidase-conjugated secondary antibodies (1:10000) were applied on the membrane and the bound secondary antibody was detected with the enhanced chemiluminescence reagents (Pierce, Rockford, IL). All antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA.

Intracellular calcium measurements

The intracellular Ca^{2+} signals were recorded using Fluo-3 staining kit (Beyotime institute of biotechnology, Jiangsu Province, China) according to the manufacture's instruction. Cells were incubated with 5 μm Fluo-3-AM at room temperature for 20 min. The fluorescence signals were detected with an Olympus fluorescence microscope at 528 nm (excitation: 490-500 nm).

Statistical analysis

Data are expressed as mean ± SD. The difference between groups was analyzed by ANOVA and a Student's t-test using the SPSS 19.0 statistical software (SPSS, Inc., Chicago, IL, USA). A P value of <0.05 was considered statistically significant.

Results

Up-regulation of HOTAIR suppressed the protein level of $Ca_V1.2$ in human cardiomyocytes (hCMs)

Firstly, we transfected hCMs with PCDH-HOTAIR to up-regulate the levels of HOTAIR in hCMs. 72 h after transfection, real time PCR was perform to measure the expression of HOTAIR in hCMs. Data showed that HOTAIR levels in PCDH-HOTAIR-transfected hCMs were increased by nearly eight-folds compared with that in PCDH transfected hCMs (P<0.05) (Fig. 2A). The average Ct in non-transfectd cells of HOTAIR was 22.63

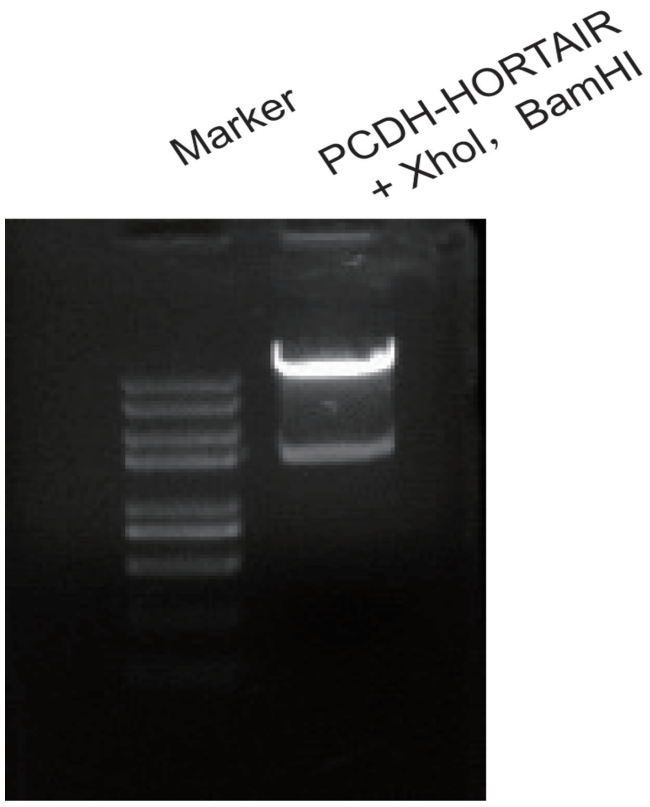

Figure 1. Identification of HOTAIR overexpression plasmid with XhoI and BamHI. The plasmid PCDH-HOTAIR was digested with XhoI and BamHI and then identified by gel electrophoresis.

with a standard error of 0.39; meanwhile, the Ct of U6 in this group cells was 14.41. These results suggested that HOTAIR was median level in such cells. It is well known that the carboxyl-terminal intracellular tail of the L-type Ca^{2+} channel $Ca_V1.2$ modulates various aspects of channel activity. In order to elucidate the effects of HOTAIR on calcium channel activity, we evaluated the expression of $Ca_V1.2$ in HCM overexpressing HOTAIR by western blot. Western blot analysis indicated that up-regulation of HOTAIR dramatically inhibited the protein expression of $Ca_V1.2$ in hCMs (P<0.05) (Fig. 2B and C). These data suggested that up-regulation of HOTAIR inhibited $Ca_V1.2$ expression in hCMs.

HOTAIR knockdown promoted the expression of $Ca_V1.2$ in HCM

Next we transfected hCMs with HOTAIR siRNA

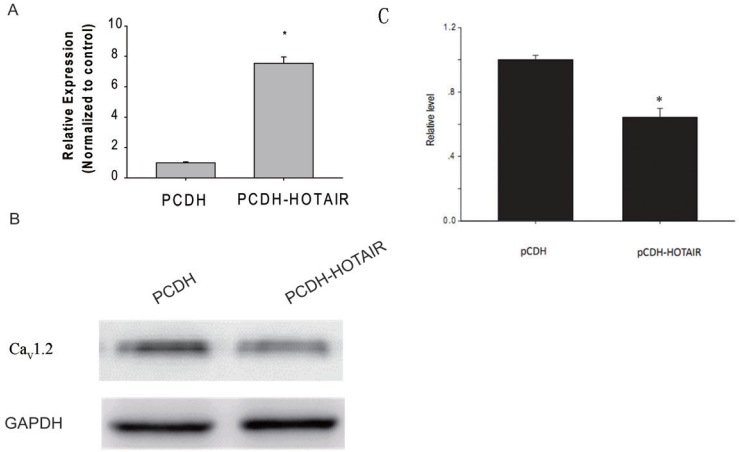

Figure 2. HOTAIR overexpression suppressed $Ca_V1.2$ in human cardiomyocytes. Human cardiomyocytes was transfected with plasmids encoding HOTAIR. 72 h after transfection, real time PCR was perform to measure the expression of HOTAIR in hCMs (A). Western blot was used to measure the expression of $Ca_V1.2$ in hCMs (B).Quantification analysis of protein expression (C). * P<0.05, compared with control.

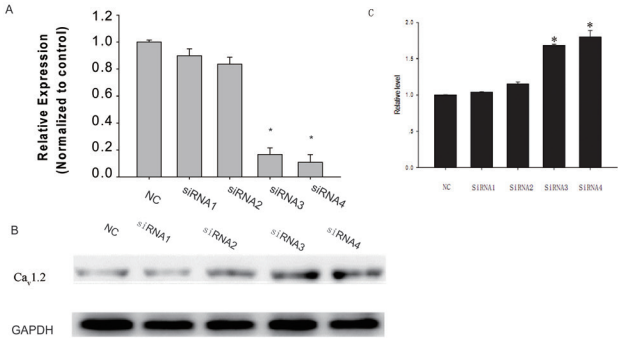

Figure 3. HOTAIR knockdown up-regulated $Ca_V1.2$ in human cardiomyocytes. Human cardiomyocytes was transfected with HOTAIR siRNA and negative control. 72 h after transfection, the expression of HOTAIR was examined by real time PCR (A). The expression of $Ca_V1.2$ was detected with western blot (B). Quantification analysis of protein expression (C). * P<0.05, compared with control.

and negative control siRNA to down-regulate HOTAIR expression in hCMs. Real time PCR analysis showed that the expression of HOTAIR in hCMs was efficiently down-regulated after transfection with HOTAIR siRNA3 and HOTAIR siRNA4 (P<0.05). While there were no significant changes in the HOTAIR contents in hCMs transfected with HOTAIR siRNA1 and HOTAIR siRNA2 (Fig.3A). In order to examine the effects of HOTAIR knockdown on $Ca_V1.2$ levels, western blot was used to determined the expression of $Ca_V1.2$ in hCMs. Our results demonstrated that down-regulation of HOTAIR led to a remarkable increase in $Ca_V1.2$ expression (P<0.05) (Fig. 3B and C). These results suggested that knockdown of HOTAIR in hCMs enhanced the expression of $Ca_V1.2$ in hCMs.

HOTAIR regulated intracellular Ca^{2+} contents via $Ca_V1.2$ in hCMs

The above results demonstrated that HOTAIR may function as a negative regulator for $Ca_V1.2$, we further explored the effects of HOTAIR on intracellular Ca^{2+} contents in hCMs. Compared with control group, we found that HOTAIR expression significantly reduced the intracellular Ca^{2+} contents; while knockdown of HOTAIR enhanced the Ca^{2+} contents in the cardiomyocytes. However, enforced expression of $Ca_V1.2$ increased the calcium level in cardiomyocytes overexpressing HOTAIR. Moreover, down-regulation of HOTAIR and up-regulation of $Ca_V1.2$ further enhanced the Ca^{2+} contents in the cardiomyocytes (P<0.05) (Fig. 4A and B). In order to ensure pH was not affected by the transfection with PCD-HOTAIR, we measure the pH level in supernant of cell cultures and cell lysis as shown in Table 1. Data suggested that PCD-HOTAIR transfection has little effects on pH levels (P>0.05). We also measured the Ca^{2+} level as shown in Table 2. Moreover, We detect the mRNA level of other calcium channels (Cav1.1, Cav1.3, and Cav1.4) after cells transfected with HOTAIR siRNA and PCDH-HOTAIR vectors, and results showed that PCD-HOTAIR transfection has little effects on other calcium channels (P>0.05) (Table 3). Taken together, these data suggested that HOTAIR inhibited the intracellular Ca^{2+} via $Ca_V1.2$ in human cardiomyocytes.

Discussion

LncRNAs are a novel class of ncRNAs that are larger than 200 nucleotides but do not encode proteins. Thou-

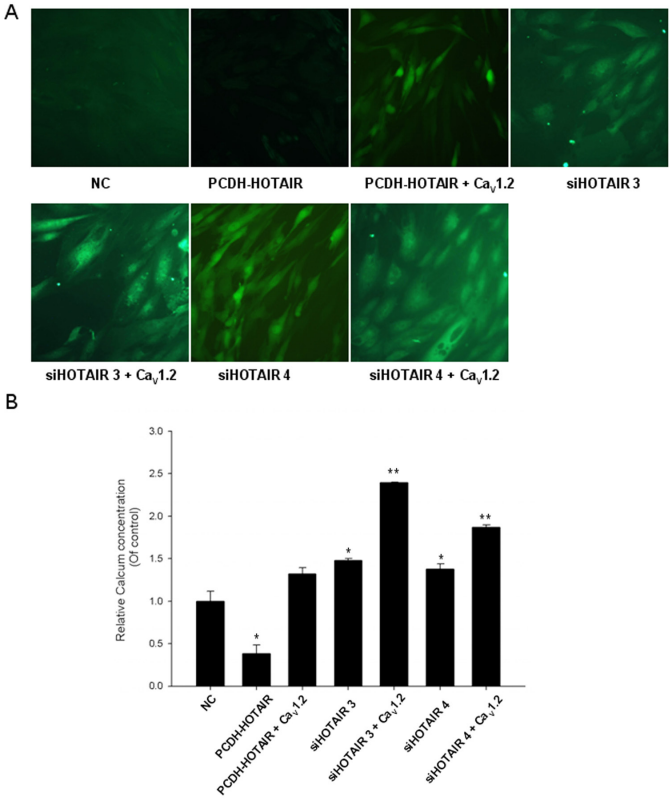

Figure 4. HOTAIR regulated intracellular Ca^{2+} contents via $Ca_V1.2$ in human cardiomyocytes. Human cardiomyocytes was transfected with PCDH-HOTAIR or siHOTAIR3/4 alone or combined with $Ca_V1.2$. Then the intracellular calcium contents were measured by fluorescence microscopy (A and B). * P<0.05, compared with control.

Table 1. Determination of pH levels in different groups.

	NC	siRNA1	siRNA2	siRNA3	siRNA4
pH in supernant	7.40	7.71	6.96	7.25	7.74
	7.30	6.66	6.24	7.46	7.86
	7.47	8.05	7.59	7.47	7.39
pH in cell lysis	7.24	6.05	5.93	7.04	7.06
	7.03	7.43	6.18	7.32	7.96
	7.04	7.00	6.47	7.73	7.36

Table 2. Calcium concentrations in different groups.

	NC	PCDH+HOTAIR	PCDH+HOTAIR + CaV1.2	siHOTAIR	siHOTAIR + CaV1.2
Ca^{2+}(mM)	0.39	0.19	1.21	0.61	1.27
	0.38	0.17	1.65	0.68	1.34
	0.25	0.08	1.07	0.58	1.42

Table 3. Calcium channels expression in different groups.

Genes	NC	HOTAIR siRNA3	PCDH	PCDH-HOTAIR
Cav1.1	27.40	26.21	26.09	26.20
	27.19	27.59	28.34	27.08
	27.20	27.16	26.63	28.59
Cav1.3	29.57	28.38	30.26	29.37
	29.36	29.76	29.51	29.25
	29.37	29.33	28.80	30.76
Cav1.4	25.57	24.38	24.26	27.37
	25.36	25.76	24.51	27.25
	25.37	25.33	24.80	26.76
GADPH	15.57	14.38	15.26	15.37
	15.36	15.76	15.51	15.25
	15.37	15.33	14.80	15.76

sands of lncRNAs have been identified in different species. Emerging evidence has suggested that lncRNAs have crucial roles in controlling gene expression and other cellular processes during both developmental and differentiation processes (14). lncRNAs regulate gene expression at the levels of epigenetic control, transcription, RNA processing, and translation (15). Many lncRNAs have recently been discovered and their function in a variety of biological processes is emerging. However, relatively little is known about the involvement of lncRNAs in the cardiovascular system.

Recently, the lncRNA Braveheart has been shown to be required for activation of the cardiovascular gene network and regulation of mesoderm posterior 1, a master regulator of a common multipotent cardiovascular progenitors (16). This first description of the critical regulatory role of a lncRNA in the cardiovascular system helps us better understand the epigenetic mechanisms and develop therapeutic strategies of cardiovascular diseases. In the present study, we for the first time demonstrated that one of the well-documented lncRNA, HOTAIR, participated in regulation of calcium content via $Ca_V1.2$ in human cardiomyocytes.

HOTAIR was firstly discovered as a lncRNA that recruits Polycomb Repressive Complex 2, a transcriptional co-repressor, to repress the expression of the homeobox gene D cluster (17). Up to now, much attention has been drawn to explore the role of HOTAIR in cancers with significant discoveries achieved. For instance, the up-regulation of HOTAIR was observed in several cancers, including breast cancer (9), hepatocellular carcinoma (10), pancreatic cancer (11) and colorectal cancer (12). In addition, HOTAIR exhibits diverse effects on tumor cells including cell proliferation, apoptosis, migration and invasion. In our study, we demonstrated that up-regulation of HOTAIR suppressed the expression of $Ca_V1.2$, whereas HOTAIR knockdown promoted $Ca_V1.2$ expression in hCMs.

High voltage-activated calcium channels have been classified as L-type and non-L-type channels. L-type channels are encoded by four genes including $Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$ and $Ca_v1.4$ (18). Substantial evidences suggest that increased activity of L-type calcium channels drives many of the markers of pathology in cardiovascular systems (19, 20). These voltage-gated Ca^{2+} channels play critical roles in the influx of activity-induced Ca^{2+} into neurons and regulation of multiple neuronal cell functions, including synaptic plasticity and excitability (21). Accordingly, aberrant activity of L-type channels and dysregulation of Ca^{2+} homeostasis has been shown to be associated with cardiovascular diseases, such as atrial fibrillation, heart failure, cardiac hypertrophy, and ischaemic heart disease (22). The α1C subunit of L-type calcium channel functions as a voltage sensor as well as a Ca^{2+}-selective pore (23). In the present study, we found that HOTAIR expression signi-

ficantly reduced the intracellular Ca^{2+} contents; while knockdown of HOTAIR enhanced the Ca^{2+} contents in the cardiomyocytes. However, enforced expression of $Ca_V1.2$ diminished the regulatory effects of HOTAIR on calcium contents, demonstrating that HOTAIR inhibited intracellular Ca^{2+} via regulation of $Ca_V1.2$. Moreover, HOTAIR has no obvious effects on other ion channels, including K^+ and Na^+, $Ca2^+$. In addition, treatment with L-type Ca2+ channel blockers has similar results with overexpression of HOTAIR in H9C2 cells.

In conclusion, the current study for the first time explored the role of HOTAIR in human cardiomyocytes. Our study demonstrate that HOTAIR inhibited the intracellular Ca^{2+} via regulation of $Ca_V1.2$ in human cardiomyocytes, highlighting the novel mechanism of lncRNA in calcium regulation.

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