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Delayed onset of beating and decreased expression of T-type Ca^{2+} channel in mouse ES cell-derived cardiocytes carrying human chromosome 21

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Abstract

The mouse ES cell line hcgp7/#21, which carries a human chromosome 21 (hChr.21), was used as an in vitro model to examine the effects of hChr.21 on cardiomyocyte differentiation. Cardiomyocytes derived from hcgp7/#21 showed a significant delay in the onset of spontaneous beating. The number of Nkx2.5/GFP(+) cardiac progenitor cells was comparable to that in control ES cells and they also expressed comparable mRNA levels for mesodermal markers, cardiac specific transcription factors, contractile proteins, and L-type Ca^{2+} channels. However, cells from hcgp7/#21 expressed significantly reduced levels of mRNA for Cav3.1 and Cav3.2, which was consistent with the decreased number of cells expressing T-type Ca^{2+} channels and decreased T-type Ca^{2+} channel currents. These findings suggest that the presence of human chromosome 21 suppresses expression of T-type Ca^{2+} channels in cardiomyocytes during differentiation, which may be responsible for delayed onset of spontaneous beating.

Keywords: Ca-channel; Congenital defects; Developmental biology; Stem cells

Down syndrome (DS), caused by the trisomy of human chromosome 21 (hChr.21), is characterized by facial dysplasia, mental retardation, immunodeficiency, and hypotonia [1], as well as congenital heart diseases such as endocardial cushion defects, atrioventricular valve malformation, and atrial and ventricular septal defects [2,3]. In addition, impaired functions of the cardiac conduction system, including asymptomatic progressive non-surgical atrioventricular block and bradycardia [4–6] are also common problems in patients with DS. The functions of both the Na^+ and K^+ channels [7,8] were reported to be altered in neuronal cells from DS patients as well as from DS mice carrying the trisomy of chromosome 16. Cardiac cells from these DS mice showed a reduced action potential duration, which suggested impaired Ca^{2+} influx through Ca^{2+} channels [9]. It was previously demonstrated that Ca^{2+} influx through Ca^{2+} channels induced transcription of the DS

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critical region (DSCR) through a calcineurin/NFAT-dependent pathway to contribute to electromechanical coupling and automaticity of the heart [10]. Cardiac Ca^{2+} channels are composed of L-type channels encoded by Cav1.2 and T-type channels, which are encoded by Cav3.1 and Cav3.2. However, it remains unknown whether DS itself has an effect on cardiac Ca^{2+} channels during fetal myocardial development.

Embryonic stem (ES) cells are useful for in vitro studies of cardiac development in DS. Inoue et al. recently demonstrated that the onset of beating in embryoid bodies (EBs) derived from ES cells carrying hChr.21 was significantly delayed as compared with that seen in control ES cells [11]. Chimeric mice derived from ES cells carrying hChr.21 showed congenital cardiac abnormalities, a ventricular septal defect or double outlet of the right ventricles [12]. However, the detailed molecular mechanism of altered cardiac function in DS remains unknown. Therefore, we established the Nkx2.5/GFP(+) ES cell line (hcgp7/#21) containing hChr.21 in order to clarify the molecular mechanisms of delayed onset of beating in cardiomyocytes derived from ES cells carrying hChr.21 and confirmed delayed beating in those cells. Further, the cells expressed unaltered levels of mesodermal markers, cardio-specific transcription factors, and contractile proteins as well as L-type Ca^{2+} channels, however, decreased expression and activities of T-type Ca^{2+} channels were also observed.

Materials and methods

Generation of hcgp7/#21 ES cells containing hChr.21. We used Nkx2.5/GFP(+) (hcgp7) cells [13], derived from ht7 ES cells, that carry a GFP reporter gene in one of the Nkx2.5 loci [14]. Mouse A9 cells containing hChr21 [15] tagged with pSTneo were used as a donor source for microcell-mediated chromosome transfer into hcgp7 cells. The hybrid cells were maintained in nonselective culture medium for 24 h and then selected in culture medium containing 300 μg/ml of G418. We isolated G418-resistant clones between 7 and 9 days after applying selection, and screened genomic DNA from the clones by PCR using hChr21 specific primers. The cells were grown under culture condition described elsewhere [14], then, differentiation of both hcgp7 and hcgp7/#21 cells was induced by formation of EBs.

Fluorescence in situ hybridization (FISH). Chromosome sampling and FISH analysis were carried out using standard methods [15]. Images were captured using a Nikon fluorescence microscope equipped with a photometric CCD camera and processed using the Cytovision Probe System (Applied Imaging). The probe used was digoxigenin (Boehringer)-labeled human COT-1 DNA (Invitrogen). Digoxigenin-labeled probes were detected with anti-digoxigenin–rhodamine (Roche). The chromosomes were counter stained with DAPI (Sigma).

Genomic DNA analysis. PCR analyses were carried out using standard techniques [15] with the following markers and primer pairs: D21S265, 5′-GGGTAAGAAGGTGCTTAATGCTC-3′ and 5′-TGAATATGGGTTCTGGATGTAGTG-3′; CBR1, 5′-GATCCTCCTGAATGCCTG-3′ and 5′-GTAAATGCCCTTTGGACC-3′; HLCS, 5′-TTCAGTACCTCCCCAGATGC-3′ and 5′-CTTAGTAGTGCAGACCTTTACCCC-3′; D21S266, 5′-GGCTTGGGGACATTGAGTCATCACAATGTAGATGT-3′ and 5′-GAAGAAAGGCAAATGAAGACCTGAACATGTAAGTT-3′; and PFKL, 5′-AGGGCTTCTGAGGCCAGC-3′ and 5′-AGGGCACTCTGTCCTCCTGC-3′. Amplifications were performed with an annealing temperature of 62°C for 30 cycles and the products were analyzed on a 2% agarose gel.

Flow cytometry. Cells were dissociated from EBs by trypsinization and gentle pipetting, then resuspended in Hanks' balanced salt solution containing 1% bovine serum albumin, and subjected to flow cytometry (EPICS XL, Beckman Coulter, USA) with WinMDI software. The sort gate for GFP(+) cells was established on the basis of forward-scattered, and side-scattered light, and GFP fluorescent intensities of control ES cells. Typically, $1–2 \times 10^5$ GFP(+) cells were obtained from 1×10^7 cells dissociated from day 8 EBs.

Real-time PCR. Total RNA was isolated from EBs on days 5, 6, 7, 8, and 10 using an RNeasy Mini Kit (Quiagen). RNA samples were treated with DNaseI (Promega) to eliminate genomic DNA and cDNA was synthesized using SuperScriptTM II reverse transcriptase (Gibco-BRL). Real-time PCR was performed as described elsewhere [16]. The primers used are shown in Table 1.

Electrophysiological recordings. T-type Ca^{2+} channel currents were recorded in Nkx2.5/GFP(+) cells from day 10 EBs using a whole cell patch-clamp technique as described elsewhere [16]. The T-type Ca^{2+} channel currents elicited every 3 s by 500 ms step pulses from a holding potential of −90 mV to test potential of −90 to −50 mV at 10 mV were defined and measured as nifedipine (3 μM)-insensitive Ca^{2+} currents. To obtain the current density, we divided the current amplitude by cell capacitance, which averaged 25.0 ± 2.6 (hcgp7) and 22.1 ± 1.1 (hcgp7/#21) pFs.

Immunofluorescence. Anti-α1G (Santa Cruz), anti-α1H (Santa Cruz), and tropomyosin (Sigma) staining of fixed cells was performed as described previously [17] using either Alexa fluor-conjugated anti-mouse or anti-gout IgG as a secondary antibody. The nuclei were stained with DAPI. Images were collected using a Bio-Rad MRC1024 confocal microscope.

Western blotting. Western blotting analyses were carried out as described elsewhere [17]. The membranes were tested with antibodies against dual-specificity tyrosine-phosphorylation regulated kinase 1 (Dyrk1A) (Invitrogen) and β-actin (Oncogene), which were developed using an ECL system.

Results

Characterization of hcgp7/#21 cells containing human chromosome 21

hChr21 in hcgp7/#21 cells was characterized by PCR, FISH, and Western blotting analyses. PCR analysis with 5 hChr21-specific primers revealed that hcgp7/#21 cells contained markers equivalent to A9/#21 donor cells (Fig. 1A). FISH analysis with the human COT-1 DNA probe showed that the transferred hChr21 was retained as an independent chromosome in hcgp7/#21 cells (Fig. 1B). The expression of Dyrk1A encoded in DSCR on hChr.21 was also detected in the hcgp7/#21 cells by immunoblotting (Fig. 1C), which suggested that the expression of the gene on hChr21 normally occurred in hcgp7/#21 cells, as previously reported [11]. Together, these findings indicated that the hcgp7/#21 cells contained intact and functionally active hChr21.

Delayed differentiation of hcgp7/#21 cells into beating cardiomyocytes

The onset of beating of wild-type (hcgp7) EBs occurred on day 5 and the proportion of beating EBs gradually increased to over 90% by day 10. In contrast, beating EBs from hcgp7/#21 cells were first detected on day 8 and then increased to over 90% by day 10

Table 1
Sequence of oligonucleotides used as real time RT-PCR primers

Target	Accession No.		Sequence (5′ → 3′)
β-Actin	X03672	Left primer	CAACCGTGAAAAGATGAC
		Right primer	CAGGATCTTCATGAGGTAGT
Brachyury	NM_009309	Left primer	CATTACACACCACTGACGCA
		Right primer	CATAGATGGGGGTGACACAG
Nodal	NM_013611	Left primer	GGGGCTCCTGGATCATCTAC
		Right primer	GCTCAGTGGCTTGGTCTTCA
Nkx2.5	NM_008700	Left primer	ACCGTCGCTACAAGTGCAA
		Right primer	CCATAGGCATTGAGACCCA
Mef2c	NM_025282	Left primer	CCCTTCGAGATACCCACAAC
		Right primer	TGCCCATCCTTCAGAGAGTC
GATA4	NM_008092	Left primer	CTGCGGCCTCTACATGAAGC
		Right primer	TCTTCACTGCTGCTGCTGCT
Mlc2a	NM_822879	Left primer	TGACCCAGGCAGACAAGTTC
		Right primer	CGTGGGTGATGATGTAGCAG
Mlc2v	NM_010861	Left primer	AAGGTGTTTGATCCCGAGGG
		Right primer	GGGAAAGGCTGCGAACATCT
Cav1.2	NM_009781	Left primer	TCTGCCTCTCTAGGTCGAA
		Right primer	GGGAATGTGGTAGGAGAATG
Cav3.1	NM_009783	Left primer	ACCCTCCCCAAAGAAAGAT
		Right primer	GCTTACATGGGACTTTTCAG
Cav3.2	NM_021415	Left primer	GCTGTTTGGGAGGCTAGAAT
		Right primer	CGAAGGTGACGAAGTAGACG

RT-PCR, reverse transcriptase-polymerase chain reaction.

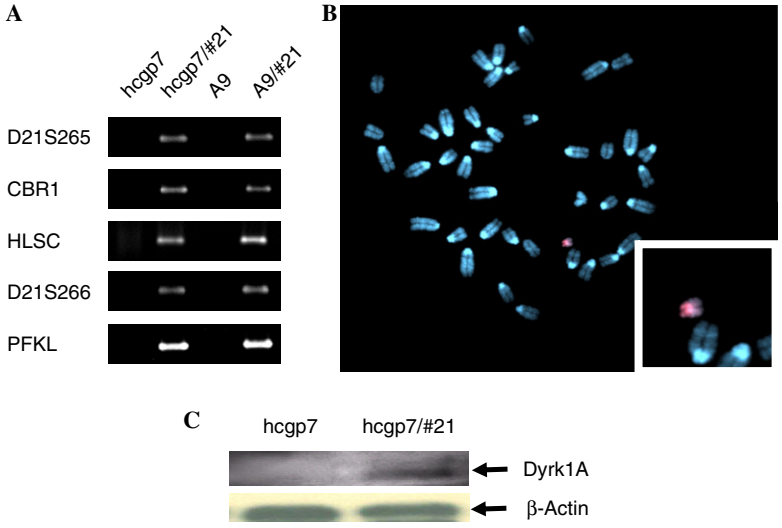

Fig. 1. Cytogenetic and molecular analyses of transferred hChr21 in hcgp7 cells. (A) PCR analyses of hcgp7 cells containing human Chr21 (hcgp7/#21) using primers for Chr21-specific STS markers and genes (see Materials and methods). hcgp7 cells were analyzed, with A9 cells with and without intact hChr21 (A9/#21) used as controls. (B) Detection of hChr21 by FISH with human COT-1 DNA to metaphase chromosomes of hcgp7/#21 cells. The transferred hChr21 (red color) was detected as an independent extra copy in the mouse background. (C) Immunoblotting results of expressed Dyrk1A in DSCR in hcgp7/#21 cells. Cell lysates were subjected to Western blotting with the indicated antibodies. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

(Fig. 2A). These results suggested that transferred hChr.21 had an effect on the process of cardiomyocyte differentiation or general mesodermal differentiation. mRNA levels of the early mesodermal marker Brachyury and mesoderm-inducing factor Nodal in the hcgp7/#21 cells were comparable to those in the hcgp7 cells (Fig. 2B), which indicated that mesodermal differentiation by hcgp7/#21 cells was not inhibited by the acquisition of hChr21.

Similar mRNA levels of Nkx2.5, Mef2C, and GATA4 were detected in EBs derived from hcgp7 and hcgp7/#21 cells (Fig. 2B). Further, the same number of Nkx2.5/GFP(+) cardiac progenitor cells was thought to be present in the two groups during the differentiation process (Fig. 2C), as the

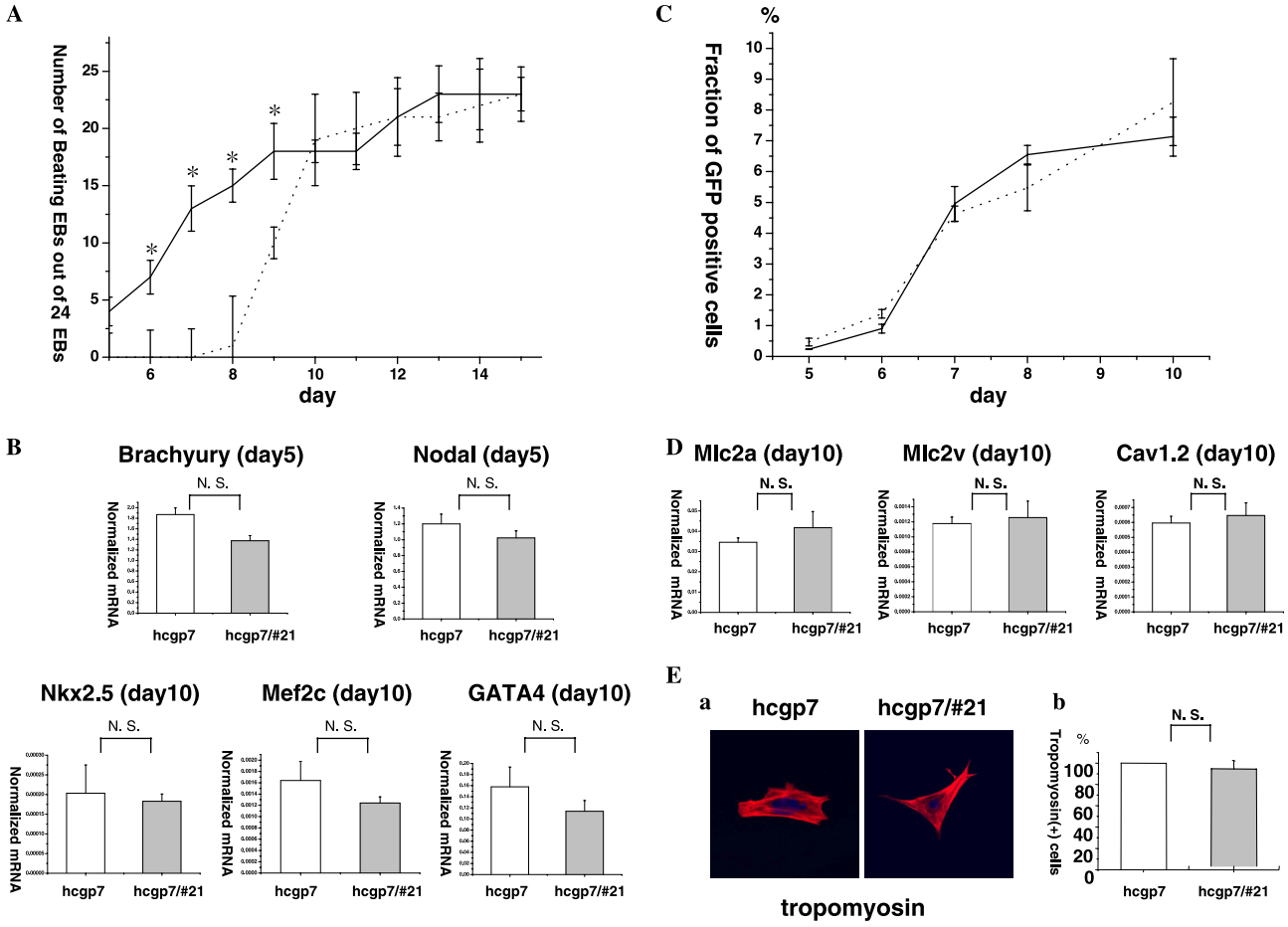

Fig. 2. Effects of hChr.21 on cardiac differentiation. (A) Quantification of contracting EBs. Solid and dotted lines show the numbers of beating EBs derived from hcgp7 and hcgp7/#21 cells, respectively. The number of contracting EBs out of a total of 24 EBs was counted daily. Values are shown as means ± SE of six independent experiments $*p < 0.05$. (B) Expression of mesodermal markers, genes, and cardiac specific transcriptional factors during differentiation of EBs derived from hcgp7 (open column) and hcgp7/#21 (gray column) cells at the indicated time points. Values are shown as means ± SE of six independent experiments. (C) Percentage of Nkx2.5/GFP(+) cells. The solid and dotted lines show the percentages of Nkx2.5/GFP(+) cells in EBs derived from hcgp7 and hcgp7/#21 cells, respectively, at the indicated time points. Values are shown as means ± SE of six independent experiments. (D) Expression of cardiac contractile protein genes and L-type Ca^{2+} channel gene during differentiation of EBs derived from hcgp7 (open column) and hcgp7/#21 (gray column) cells on day 10. Values are shown as means ± SE of six independent experiments. (E) Expression of tropomyosin in Nkx2.5/GFP(+) cardiac progenitor cells from hcgp7 and hcgp7/#21 cells. (a) Cells were sorted by FACS, then fixed and stained with anti-tropomyosin antibody. Nuclei were stained using DAPI. Representative images obtained with a confocal microscope are shown. (b) Percentages of tropomyosin(+) cells of total Nkx2.5/GFP(+) cells are shown. hcgp7 cells: open column, hcgp7/#21 cells: gray column. Values are shown as means ± SE of six individual experiments. NS, not significant.

hcgp7/#21 cells showed the same proportion of beating EBs after 10 days as hcgp7 cells. Thus, hChr.21 did not impair cardiogenic differentiation of the ES cells. In addition, the same levels of transcription of Mlc2a and Mlc2v were seen in both types of cells (Fig. 2D), and the same number of cells expressing tropomyosin was seen in the two groups (Fig. 2E), which indicated that hChr.21 did not hamper the capacity of ES cells to differentiate into a mesodermal lineage and subsequently to cardiomyocytes. Since the onset and proportion of beating EBs was not only determined by the capacity for cardiac differentiation, but also by pacemaker activity to regulate the beating rate of the cardiac precursor cells, we considered that hChr21 may interfere with the development of pacemaker activities, which resulted in a delayed onset of beating.

Impaired expression of T-type Ca^{2+} channels in hcgp7/#21-derived cardiomyocytes

It has been reported that the pacemaker activities regulating the beating rate of sino-atrial nodal cells [18] and ES-derived cardiac progenitor cells [19] are dependent on the activities of both T-type and L-type Ca^{2+} channels. The transcriptional levels of Cav1.2 were similar between EBs from hcgp7 and hcgp7/#21 (Fig. 2D) cells. The mRNA levels of Cav3.1 in EBs from hcgp7 cells showed a steady increase during the course of differentiation, whereas those of Cav3.2 were highest on day 8 and then decreased. Further, the levels of both Cav3.1 (a in Fig. 3A) and Cav3.2 (b in Fig. 3A) in EBs from hcgp7/#21 cells were significantly lower than those in the controls. The immunoreactivity of

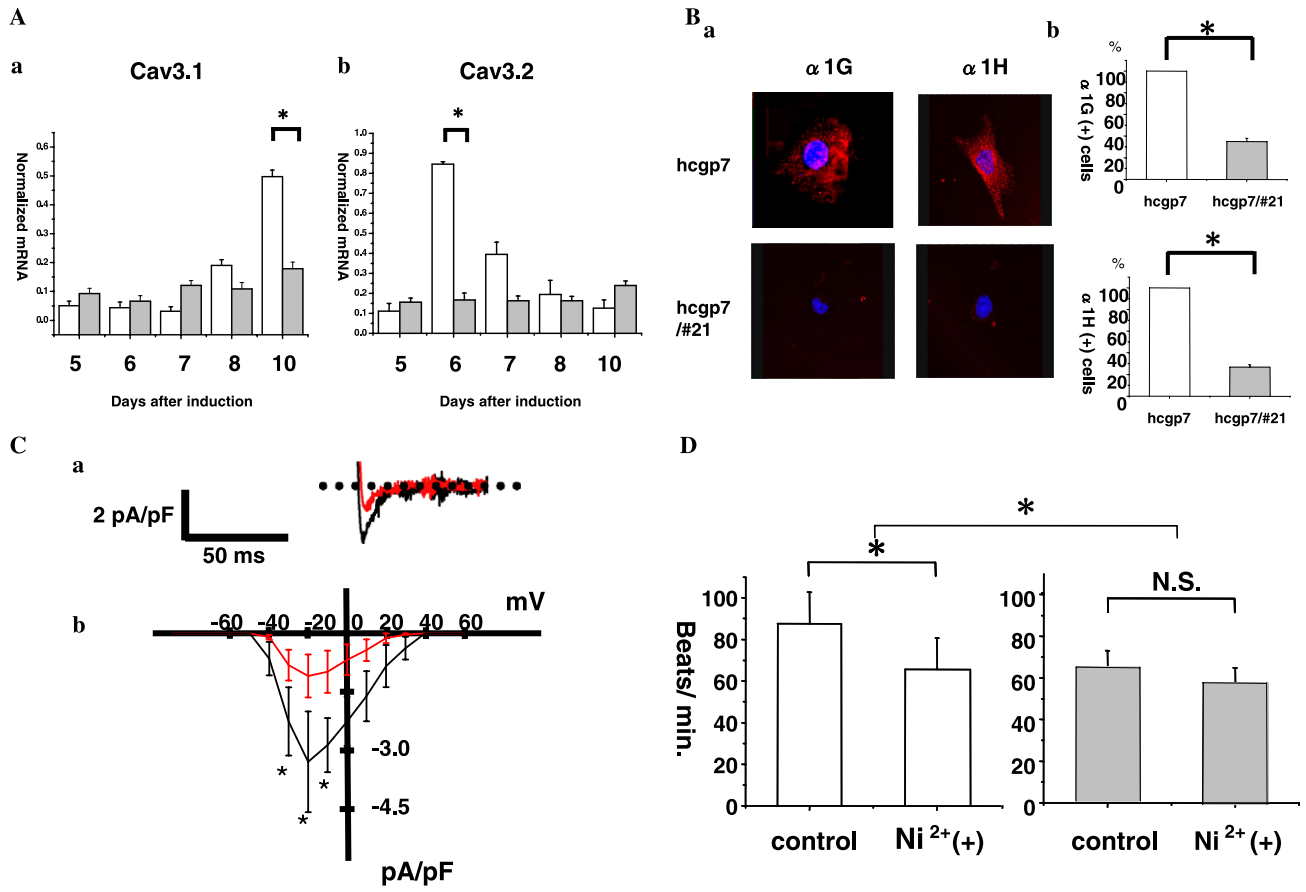

Fig. 3. Impaired expression of T-type Ca^{2+} channels during cardiac differentiation of hcgp7/#21 cells. (A) Developmental changes in the expression of mRNA encoding T-type Ca^{2+} channels in EBs from hcgp7 (open column) and hcgp7/#21 (gray column) cells at the indicated time points. (a) Cav3.1, (b) Cav3.2. Abscissa indicates the number of days after induction of differentiation. Values are shown as means ± SE of six independent experiments. (B) Expression of α1G and α1H in Nkx2.5/GFP(+) cells differentiated from hcgp7 and hcgp7/#21 cells. (a) Cells were sorted by FACS, then fixed and stained with anti-α1G and anti-α1H antibodies. Nuclei were stained using DAPI. Representative images obtained with a confocal microscope are shown. (b) Percentages of α1G and α1H positive cells of total Nkx2.5/GFP(+) cells are shown. hcgp7 cells: open column, hcgp7/#21 cells: gray column. Values are shown as the means ± SE of six independent experiments $*p < 0.05$. (C) Decreased T-type Ca^{2+} channel currents of Nkx2.5/GFP(+) cells differentiated from hcgp7/#21 cells. (a) Representative T-type Ca^{2+} channel currents elicited by 500 ms step pulses from a holding potential of −90 mV to a test potential of −20 mV in Nkx2.5/GFP(+) cells differentiated from hcgp7 (black line) and hcgp7/#21 (red line) cells. (b) Current–voltage relationships obtained from Nkx2.5/GFP(+) cells differentiated from hcgp7 (black line) and hcgp7/#21 (red line) cells. Values are shown as means ± SE of six independent experiments $*p < 0.05$. (D) Beating rates of EBs from hcgp7 and hcgp7/#21 cells on day 8 in the presence and absence of Ni^{2+}. hcgp7 cells: open column, hcgp7/#21 cells: gray column. Control: before administration of Ni^{2+}, Ni^{2+}(+): during administration of 40 μM $NiCl_2$. Values are shown as means ± SE of six independent experiments $*p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

both α1G, encoded by Cav3.1, and α1H, encoded by Cav3.2, was detected in the cardiac progenitor cells from hcgp7 cells on day 8. However, the number of cells expressing either α1G or α1H was significantly lower in cardiomyocytes from hcgp7/#21 cells on day 8 than in those from hcgp7 cells (b in Fig. 3B). These results suggested that the reduction of activities in the T-type Ca^{2+} channel, but not the L-type Ca^{2+} channel, may have been related to the lower pacemaker activity considered responsible for the delayed onset of beating in hcgp7/#21 cells.

Whole cell patch clamp studies on day 10 revealed that the amplitude of the T-type Ca^{2+} channel currents elicited by the step pulse was smaller in cardiac progenitor cells from hcgp7/#21 cells (a in Fig. 3C) than in those from hcgp7 cells. Also, the peak current–voltage curves showed that the density of the T-type Ca^{2+} channel currents in cardiac progenitor cells from hcgp7/#21 cells was significantly smaller than in those from hcgp7 cells (b in Fig. 3C). On day 8, the beating rate of EBs from hcgp7/#21 cells was significantly lower than that of EBs from hcgp7 cells and Ni^{2+} (40 μM), a selective T-type Ca^{2+} channel blocker [18,19], did not have an effect on the beating rate of EBs from hcgp7/#21 cells (Fig. 3D), while beating in EBs from hcgp7 was significantly suppressed by Ni^{2+} at the same concentration. Further, the beating rate of hcgp7 cells was reduced to the level of hcgp7/#21 cells after inhibition by Ni^{2+}. Taken together, these results indicated that impaired transcription of T-type Ca^{2+} channels caused a reduction of pacemaker activity, leading to the reduced beating rate of EBs from hcgp7/#21 cells.

Discussion

In the present study, hcgp7/#21 cells showed delayed onset of beating of EBs, which was consistent with the results of a previous report that utilized another ES cell line [11]. In that study, a short fragment of hChr.21 did not delay beating onset, which suggests that this phenomenon is specific to the effects of intact hChr.21. Since the expression levels of early mesodermal markers, cardiac specific transcription factors, cardiac contractile proteins, and L-type Ca^{2+} channels, as well as number of cardiac progenitor cells in hcgp7/#21 cells, were comparable to those in hcgp7cells, in the present study, we concluded that hChr.21 did not interfere with mesodermal induction of cardiac progenitor cells.

The most prominent finding in the present study is that acquisition of hChr.21 impaired the expression of Cav3.1 and Cav3.2, leading to reduced levels of T-type Ca^{2+} channel proteins. These findings suggest that hChr.21 has an influence on those transcriptional levels in the early stage of cardiac differentiation. Impairment of Kir3.2 and Kir4.2 encoded in DSCR has been reported in DS models [20,21]. However, Cav3.1 and Cav3.2 are on chromosome 17q22 and 16p13.3 in humans, respectively, which suggests that hChr.21 has a transacting effect to lower the expression of genes encoded elsewhere in the genome. Orozco-Buenrostro et al. [9] reported that the action potential duration was significantly shorter in cardiomyocytes from DS mice, though they concluded that it was secondary to an increase in voltage-sensitive K^+ conductance. The reduction of T-type Ca^{2+} channels demonstrated in our study provides an alternative explanation for their findings of reduced action potential duration.

Cardiac differentiation has also been reported to require automaticity of cardiac precursor cells. It was recently demonstrated that depolarization through activation of Ca^{2+} channels induces transcription of the DSCR1 protein via a calcineurin/nuclear factor that controls a wide variety of physiological processes [22,23]. Thus, impaired Ca^{2+} influx through T-type Ca^{2+} channels may explain the impaired differentiation of hearts from chimeric mice containing hChr.21 [12] as well as in patients with trisomy of chromosome 21 [2,3].

It is well known that T-type Ca^{2+} channels contribute to the pacemaker activity responsible for the beating rate of the heart [18,24–26]. Inhibition of cardiac T-type Ca^{2+} channels by a specific antibody has been reported to cause congenital heart block [4]. Further, it has been indicated that sinus bradycardia and conduction block are frequently associated in patients with Down syndrome [4–6]. In the present study, EBs from hcgp7/#21 cells had a slower rate of beating that was resistant to Ni^{2+}. Since Ni^{2+} at a low concentration has been shown to selectively block T-type Ca^{2+} channel current [18,19], the decrease in beating rate of cardiac progenitor cells containing hChr.21 might have been due to impaired T-type Ca^{2+} channels. Thus, impairment of T-type Ca^{2+} channel activities of cardiac progenitor cells carrying hChr.21 might explain heart conduction block or bradycardia in patients with Down syndrome.

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