LEOPARD-type SHP2 mutant Gln510Glu attenuates cardiomyocyte differentiation and promotes cardiac hypertrophy via dysregulation of Akt/GSK-3β/β-catenin signaling

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LEOPARD SYNDROME (LS; OMIM 151100) is an autosomal dominant inherited multisystemic disorder characterized by multiple lentigines, electrocardiographic abnormalities, ocular hypertelorism, pulmonary stenosis, abnormalities of genitalia, retardation of growth, and deafness. LS shares many clinical phenotypes with Noonan syndrome (NS; OMIM 163950). In both syndromes, germline missense mutations in the PTPN11 gene, which encodes the protein tyrosine phosphatase Src homology 2-containing protein phosphatase 2 (SHP2), are the most common pathogenic alteration.

We (26) previously reported a novel SHP2 mutation, Gln510Glu (Q510E), in a patient with NS-like clinical features that showed the most severe type of HCM in LS, in cardiomyocyte differentiation, and in morphological changes. We generated mutant P19CL6 cell lines, the most convenient cardiomyocyte differentiation model, which continuously expressed SHP2-Q510E, SHP2-D61N (Noonan-type mutant), wild-type SHP2, and green fluorescent protein (native SHP2 expression only). SHP2-Q510E mutant P19CL6 cells showed significant attenuation of myofibrillogenesis, with increased proliferative activity. Mature cardiomyocytes from the SHP2-Q510E mutant were significantly larger than those of controls and the other mutants. However, expression of cardiac-specific transcriptional factors (Gata4, Tbx5, and Nkx2.5) did not differ significantly between the LS-type SHP2-Q510E mutants and the other mutants and controls. Our results indicate that SHP2-Q510E mutants can differentiate into cardiac progenitors but are inhibited from undergoing terminal differentiation into mature cardiomyocytes. In contrast, Akt and glycogen synthase kinase (GSK)-3β phosphorylation were upregulated, and nuclear β-catenin at the late stage of differentiation was highly accumulated in SHP2-Q510E mutant P19CL6 cells. Supplementation with the phosphoinositide 3-kinase/Akt inhibitor LY-294002 during the late stage of differentiation was found to partially restore myofibrillogenesis while suppressing the increase in size of individual mature cardiomyocytes derived from the SHP2-Q510E mutants. Our findings suggest that dysregulation of the Akt/GSK-3β/β-catenin pathway can contribute to the pathogenesis of HCM in LS patients, not only through hypertrophic changes in individual cardiac cells but also via the expansion of cardiac progenitors.

hypertrophic cardiomyopathy; LEOPARD syndrome; Src homology 2-containing protein phosphatase 2; cardiomyocyte differentiation; glycogen synthase kinase-3β

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LS patients. Together, these clinical and basic studies suggest that the pathogenesis of HCM in LS may involve cell-autonomous mechanisms and may progress during cardiomyogenesis. Other previous studies (9, 14, 17, 23, 31) have demonstrated that SHP2 deletion leads to inhibition of cardiomyocyte differentiation from embryonal stem cells in vitro and gastrointestinal defects in vivo. These findings indicate that SHP2 plays a regulatory role in the development of organs (including the heart) and that LS-type SHP2 mutants can affect cardiomyogenesis. Therefore, we hypothesized that LS-type SHP2 mutants could affect the regulation of cardiomyocyte differentiation and morphogenesis.

P19CL6 cells are clonal derivatives of murine P19 embryonal carcinoma cells, which are a line of pluripotent stem cells maintained in culture in an undifferentiated state without requiring to be supplemented with leukemia inhibitory factor. P19CL6 cells can differentiate efficiently into cardiomyocytes after treatment with DMSO, in the absence of prior embryoid body formation (11). P19CL6 cells thus provide a very useful model for studying cardiomyocyte differentiation in vitro.

Here, we generated mutant P19CL6 cell lines expressing the LS-type SHP2-Q510E mutant and the NS-type SHP2-D61N mutant. We demonstrated that the SHP2-Q510E mutant showed increased Akt and GSK-3β phosphorylation, which subsequently induced aberrant β-catenin signaling during the late stage of cardiac differentiation. This resulted in the attenuation of terminal differentiation toward mature cardiomyocytes, with increased proliferative activity. The mature cardiomyocytes derived from P19CL6 cells with the SHP2-Q510E mutation were significantly larger than those derived from the controls via the Akt/GSK-3β signaling pathway.

**MATERIALS AND METHODS**

**Cell culture and differentiation.** P19CL6 mouse embryonic carcinoma cells were kindly provided by Prof. I. Komuro (Osaka University Graduate School of Medicine, Osaka, Japan). Cells were cultured in minimal essential medium (α-MEM; Wako) supplemented with 10% FBS (Invitrogen), penicillin, and streptomycin. To induce differentiation, cells were seeded at 1 × 10^5 cells/well on a six-well plate with differentiation medium (α-MEM with 10% FBS, penicillin, streptomycin, and 1% DMSO). To inhibit aberrant PI3K/Akt signaling, we added LY-294002 (20 μM) from 6 days after the initiation of cardiac differentiation by supplementation with 1% DMSO supplement.

**Establishment of P19CL6 cell lines continuously expressing SHP2 mutants.** The lentivirus vector cFUGW-2 was used to produce recombinant lentiviruses encoding LS-type mutant SHP2-Q510E, NS-type mutant SHP2-D61N, wild-type (WT) SHP2, and green fluorescent protein (GFP) (native SHP2 expression only). The FLAG tag was added to each SHP2 protein. We used these lentiviruses to infect P19CL6 cells and obtained three stable clonal lines by the limiting dilution method.

**Proliferation and apoptosis assay.** To measure proliferative activity, each mutant P19CL6 cell was differentiated as described above. Cells were incubated with 10 μM 5-ethyl-2′-deoxuryridine (EdU) for 30 min every 3 days until day 15 and then harvested. Incorporated EdU was detected by anti-EdU antibody conjugated with Alexa fluor 488 using an EdU detection kit (Invitrogen). EdU-positive cells were counted by flow cytometry (Becton Dickinson).

A TUNEL assay was conducted using a Takara apoptosis detection kit. P19CL6 cells were seeded on glass cover slips and cultured with differentiation medium (α-MEM with 10% FBS, penicillin, streptomycin, and 1% DMSO) for 18 days. Cells were then incubated with 5% BSA-PBS for 30 min to block the antigen. To detect mature cardiomyocytes, cells were incubated for 16 h at 4°C with primary antibody (anti-α-actinin, 1:200, Sigma-Aldrich). Cells were then incubated for 60 min with secondary antibody (Alexa fluor 594, Invitrogen), and nuclei were counterstained with 4′,6-diamidino-2-phenylindole. α-Actinin-positive mature cardiomyocytes were observed by LSM510 confocal microscopy (Carl Zeiss). The α-actinin-positive area was measured by ImageJ software (http://rsb.info.nih.gov/ij/).

**Morphological analysis of mature cardiomyocytes.** To assess the hypertrophic alterations of each SHP2 mutant cardiomyocyte derived from P19CL6 cells, P19CL6 cells were cultured with differentiation medium for 15 days, dissociated with trypsin-EDTA for 5 min for single cell suspension, and seeded on glass coverslips coated with laminin. The following day, cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Tween 20 for 5 min. Cells were then incubated for 30 min with 5% BSA-PBS to block the antigens. To delineate mature cardiomyocytes, cells were incubated for 16 h at 4°C with primary antibody (anti-α-actinin, 1:200), because differentiated P19CL6 cells contain various types of cells, including fibroblast-like cells, endothelial cells, and cardiomyocytes. Cells were then incubated for 60 min at room temperature with secondary antibody (Alexa fluor 594), and nuclei were counterstained with 4′,6-diamidino-2-phenylindole. α-Actinin-positive mature cardiomyocytes were observed by LSM510 confocal microscopy. Cell surface areas were measured by ImageJ software. In each case, four independent experiments were performed, and median values were calculated. These values were then used to calculate mean cell surface areas.

In addition to this cell surface area analysis, the same cell suspensions of mature cardiomyocytes from P19CL6 cells were provided for fluorescence-activated cell sorting (FACS) analysis. Individual cells were fixed and permeabilized using a DAKO Intrastain kit and immunostained with anti-α-actinin (1:100) and phycocerythrin-conjugated secondary antibody (1:50 Becton Dickinson). The forward scatter of α-actinin-positive cells of each mutant was measured using a FACSscan (Becton Dickinson). Median values of forward scatter of each mutant were normalized to that of the control (GFP-expressing P19CL6 cells). Mean values of three independent experiments were calculated.

**Western blot analysis.** All mutant P19CL6 cells were cultured with differentiation medium. Every 3 days until day 15, cells were scraped off in modified RIPA buffer containing a protease inhibitor mixture (Roche Diagnostics). Cells were homogenized by a freeze and thaw method and centrifuged at 16,000 g for 15 min. Supernatants were then collected. Nucleic proteins were collected using a NE-PER kit (Thermo Scientific) in accordance with the manufacturer’s instructions. Protein lysates were diluted in Laemmli’s sample buffer, boiled, and then subjected to electrophoresis using 10% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, washed with Tris-buffered saline containing 0.05% Tween 20, and incubated with BlockingOne solution (Nakalai Tesque) for 30 min. The monoclonal antibodies used were anti-α-actinin and anti-α-myosin heavy chain (α-MHC) (both from Abcam); anti-SHP2, anti-phospho-Erk1/2, anti-Akt, and anti-β-catenin (all from Cell Signaling); and anti-FLAG, anti-β-actin, and anti-PCNA (all from Sigma-Aldrich). The polyclonal antibodies used were anti-Erk1/2, anti-phospho-Akt (Ser^473 and Thr^308), anti-phospho-GSK-3β, and anti-GSK-3β (all from Cell Signaling). Horseradish peroxidase-conjugated secondary antibodies were from Promega. Blots were visualized using SuperSignal West Dura (Thermo Scientific).
Akt, GSK-3β, and Erk1/2 phosphorylation. P19CL6 cells with each mutant were differentiated by supplementation with 1% DMSO for 6 days to ensure commitment to the cardiac lineage. Cells were then serum starved for 16 h and stimulated by mouse recombinant EGF (150 ng/ml, R&D) for 0, 10, and 30 min or by mouse recombinant leukemia inhibitory factor (500 IU/ml, R&D) for 0, 15, and 45 min. After this, cells were scraped and used for Western blot analyses.

Quantitative real-time PCR analysis. Total RNA was extracted using an RNeasy minikit (Qiagen). An equal amount of total RNA (2 μg) was used for reverse transcription using a High-Capacity RNA-to-cDNA kit (Applied Biosystems). Quantitative PCR was conducted in a total volume of 20 μl using a LightCycler ST300 kit and a LightCycler FastStart DNA MasterPLUS SYBR-Green I kit (both from Roche Diagnostics). PCR cycles were 95°C for 15 s, 60°C for 10 s, and 72°C for 20 s.

Fig. 1. The Src homology 2-containing protein phosphatase 2 (SHP2)-Q510E mutation attenuates myofibrillogenesis of P19CL6 cells. A: α-actinin-positive mature cardiomyocytes were gradually expanded in medium supplemented with 1% DMSO. P19CL6 cells with the SHP2-Q510E mutation showed a significant delay in myofibrillogenesis compared with P19CL6 cells expressing SHP2-D61N, SHP2-wild type (WT), and green fluorescent protein (GFP). Scale bars = 50 μm. B: quantification of α-actinin-positive areas. Three different clonal lines for each SHP2-overexpressing P19CL6 cell and one clonal line for GFP-expressing P19CL6 cells were differentiated. Three independent experiments were performed, and at least ten different fields for each experiment were measured by confocal microscopy and ImageJ software. *P < 0.05 compared with P19CL6 cells expressing GFP, SHP2-WT, and SHP2-D61N. C: Western blot analysis for α-actinin expression confirmed significant attenuation of myofibrillogenesis in the SHP2-Q510E mutant. *P < 0.05 compared with P19CL6 cells expressing GFP, SHP2-WT, and SHP2-D61N. D: Western blot analysis for α-myosin heavy chain (α-MHC) expression. *P < 0.05 compared with P19CL6 cells expressing GFP, SHP2-WT, and SHP2-D61N. E: quantitative real-time PCR analysis of α-MHC mRNA expression. *P < 0.05 compared with P19CL6 cells expressing GFP, SHP2-WT, and SHP2-D61N.
Sequences of primers are provided in the Supplemental Material. The copy numbers for each transcript were expressed relative to that of GAPDH, which was used as a constitutive internal control.

**Statistical analysis.** Results are expressed as means ± SE. Statistical analyses were performed using a Turkey-Kramer multiple-comparison test. Statistical significance was defined as $P < 0.05$.

**RESULTS**

**LS-type SHP2-Q510E mutant attenuated mature cardiomyocyte differentiation in P19CL6 cells.** To explore the possible effects of SHP2 mutants on the differentiation of P19CL6 cells into cardiomyocytes, we assessed the transitions of -actinin-positive areas of differentiating P19CL6 cells every 3 days, from the initiation of 1% DMSO supplementation until day 18. At first, we confirmed the expression levels of mutant or native SHP2 in each P19CL6 clonal line by Western blot analysis. There were no significant differences among the mutants (Supplemental Fig. S1). Expression levels of mutant SHP2 or native SHP2 did not change significantly during the differentiation (data not shown). LS-type SHP2-Q510E mutants demonstrated significant attenuation of myofibrillogenesis compared with P19CL6 cells expressing SHP2-WT, SHP2-D61N, and GFP (Fig. 1, A–C). Spontaneous beating foci were observed from day 9 in P19CL6 cells with SHP2-WT, SHP2-D61N, and GFP expression, whereas no beating foci were observed in the SHP2-Q510E mutant until day 18. To confirm this attenuation of myofibrillogenesis, we conducted Western blot analysis and quantitative real-time PCR analysis for -MHC expression, which is another marker of mature cardiomyocytes. We found that -MHC expression was significantly suppressed in P19CL6 cells with the SHP2-Q510E mutation at the protein and mRNA levels (Fig. 1, D and E). After day 18, SHP2-Q510E mutant P19CL6 cells gradually expanded -actinin-positive areas, reaching ~40% on day 27. However, cells did not survive after 1 mo (data not shown).

Next, we investigated the proliferative ability and apoptosis of each mutant SHP2 and control. LS-type SHP2-Q510E mutants showed higher EdU uptake compared with P19CL6 cells expressing SHP2-WT, SHP2-D61N, and GFP on days 6, 9, and 12 (Fig. 2A). This suggests that P19CL6 cells with the SHP2-Q510E mutation maintained higher numbers of immature proliferative cardiac cells after the initiation of differentiation. In contrast, the TUNEL assay showed no significant differences between mutants and controls (Fig. 2B), suggesting that apoptosis was not implicated in the suppression of cardiomyocyte differentiation.

Next, we explored mRNA expression of the cardiac-specific transcriptional factors Gata4, Nkx2.5, and Tbx5. Surprisingly, the expression of these transcriptional factors did not differ significantly between the LS-type SHP2-Q510E mutant and the other mutants and controls, despite the suppression of mature cardiomyocyte differentiation (Fig. 2C). Taken together, these results indicate that P19CL6 cells with the SHP2-Q510E mutation can differentiate into cardiac progenitors expressing Gata4, Nkx2.5, and Tbx5 but not into mature cardiomyocytes expressing -actinin and -MHC.

Fig. 2. Proliferation, apoptosis, and transcriptional factor expression analyses. All data were obtained from three independent experiments. A: for each mutant, differentiating P19CL6 cells were treated with 5-ethyl-2'-deoxyuridine (EdU) for 30 min every 3 days until day 15, and EdU-positive cells were analyzed by flow cytometry. P19CL6 cells with the SHP2-Q510E mutation remained highly proliferative on days 6, 9, and 12 after treatment with 1% DMSO. *$P < 0.05$ compared with P19CL6 cells expressing GFP, SHP2-WT, and SHP2-D61N. B: TUNEL-positive apoptotic cells were counted by flow cytometry. Apoptotic cells did not differ significantly between the SHP2-Q510E mutant, SHP2-D61N mutant, and controls. C: quantitative real-time PCR analyses for cardiac-specific transcriptional factors (Gata4, Nkx2.5, and Tbx5). The factors were gradually expressed according to commitment to the cardiac lineage and differentiation into mature cardiomyocytes. There were no significant differences between the SHP2-Q510E mutant, SHP2-D61N mutant, and controls.
Mature cardiomyocytes from P19CL6 cells with the SHP2-Q510E mutation showed hypertrophic changes. To explore the morphological changes occurring in mature cardiomyocytes derived from P19CL6 cells with the SHP2 mutation, each mutant P19CL6 cell was differentiated for 15 days and replated on laminin-coated glass coverslips. Immunostaining of α-actinin clearly delineated the mature cardiomyocytes from each P19CL6 cell (Fig. 3A). Computational surface area analysis revealed that mature cardiomyocytes from SHP2-Q510E mutants were significantly larger than those from the other mutants and controls (Fig. 3B). FACs analysis of the α-actinin-positive mature cardiomyocytes further demonstrated the larger size of cardiomyocytes from the SHP2-Q510E mutant (Fig. 3, C and D). The original proliferating P19CL6 cells were smaller than the mature cardiomyocytes, and there were no differences between any of the mutants and controls (Supplemental Fig. S2, A and B). These results are consistent with those of a previous study by Carvajal-Vergara et al. (1) using iPSCs from LS patients with the T468M mutation.

P19CL6 cells with the SHP2-Q510E mutation showed Akt and GSK-3β hyperphosphorylation. Recently, it has been reported that Akt and GSK-3β phosphorylation are elevated in skin fibroblasts from LS patients. This signaling axis has been implicated in the hypertrophic mechanism of cardiomyocytes (7). Thus, we investigated the state of phosphorylation of Akt from SHP2-Q510E mutants and controls. In contrast, during the late phase of differentiation (on day 12 after supplementation with 1% DMSO), β-catenin expression in the nucleus was higher than day 0 (before supplementation with DMSO) but did not differ between mutants and controls. In contrast, during the late phase of differentiation (on day 12 after supplementation with 1% DMSO), β-catenin expression remained higher in SHP2-Q510E mutant than in the other mutants and controls (Fig. 4C). mRNA expression

The baseline phosphorylation of Akt Ser473 and GSK-3β was also significantly increased in the SHP-Q510E mutant (Supplemental Fig. S3, A and B). In contrast, Erk1/2 phosphorylation was significantly decreased in the SHP2-Q510E mutant (Fig. 4D), as previously reported (15).

In the original proliferating P19CL6 cells, Akt Ser473 and GSK-3β phosphorylation were also significantly more robust in the SHP2-Q510E mutant (Supplemental Fig. S4, A and B). Taken together, these results indicate that the LS-type SHP2-Q510E mutation has a positive effect on Akt activation and a negative effect on GSK-3β in original proliferating as well as differentiating P19CL6 cells.

P19CL6 cells with the SHP2-Q510E mutation showed aberrant nuclear β-catenin accumulation at the late stage of differentiation. Canonical Wnt signaling has been shown to play a biphasic role in cardiomyogenesis. It has a positive effect during the early phase of differentiation and a negative effect during the late phase of differentiation. Therefore, we hypothesized that dysregulation of Wnt/β-catenin signaling in P19CL6 cells with the SHP2-Q510E mutation causes the inhibition of terminal differentiation from cardiac progenitors into mature cardiomyocytes. To test this hypothesis, we determined whether mutants exhibited a change in β-catenin accumulation at the nucleus during P19CL6 cell differentiation. During the early phase of differentiation (on day 6 after supplementation with 1% DMSO), β-catenin expression in the nucleus was higher than day 0 (before supplementation with DMSO) but did not differ between mutants and controls. In contrast, during the late phase of differentiation (on day 12 after supplementation with 1% DMSO), β-catenin expression remained higher in SHP2-Q510E mutant than in the other mutants and controls (Fig. 5A).
of Axin2, a downstream transcriptional factor of β-catenin, was also higher in the SHP2-Q510E mutant during the late stage of differentiation (Fig. 5B). These results suggest that in P19CL6 cells with the SHP2-Q510E mutation, aberrant (higher) β-catenin signaling during the late phase of differentiation can attenuate terminal differentiation into mature cardiomyocytes.

PI3K inhibitor LY-294002 enhanced the attenuation of myofibrillogenesis and also suppressed hypertrophic changes in P19CL6 cells with the SHP2-Q510E mutation. The PI3K/Akt pathway plays an important role in cardiomyocyte differentiation by regulation of canonical Wnt signaling. Inhibition of the PI3K/Akt pathway, and thus phosphorylation of GSK-3β, has been shown to decrease the expression levels of nuclear β-catenin in DMSO-treated P19CL6 cells (20). Therefore, we hypothesized that hyperphosphorylation of Akt/GSK-3β in P19CL6 cells with the SHP2-Q510E mutation induces higher β-catenin signaling at the late stage of differentiation, thereby attenuating terminal differentiation into mature cardiomyocytes. To explore this hypothesis, we added the PI3K inhibitor LY-294002 to the differentiation medium from 6 days after supplementation with 1% DMSO. SHP2-Q510E mutant P19CL6 cells supplemented with LY-294002 showed significantly lower phosphorylation of Erk1/2. *P < 0.05 compared with P19CL6 cells expressing GFP, SHP2-WT, and SHP2-D61N.

To confirm the effect of the PI3K/Akt pathway on morphological alterations, we evaluated the sizes of mature cardiomyo-
cytes from mutant P19CL6 cells after PI3K inhibition by LY-294002 during the late stage of differentiation (after day 6). Cells were harvested on day 15, and the cell surface area of mature cardiomyocytes was analyzed by the method shown in Fig. 3. The increase in size of mature cardiomyocytes differentiated from SHP2-Q510E mutant P19CL6 cells was remarkably suppressed in the presence of LY-294002 (Fig. 6E). These results indicate that PI3K/Akt inhibition after myofibrillogenesis can reduce the size of terminally differentiated cardiomyocytes with the LS-type SHP2-Q510E mutation.

**DISCUSSION**

In LS patients, HCM is the most common and life-threatening cardiac abnormality. HCM associated with LS differs significantly from other types of HCM (usually caused by sarcomeric protein mutations), in that it occurs even from the fetal period (19). A recent study (1) using iPSCs from LS patients suggested that the pathogenesis of HCM in LS is associated with cell-autonomous mechanisms. However, the precise molecular mechanisms were not clearly elucidated.

Here, we studied the differential regulation of cardiomyocytes in LS-type SHP2 mutants and demonstrated that the SHP2-Q510E mutation attenuated terminal differentiation of cardiomyocytes via dysregulation of Akt/GSK-3β/β-catenin signaling. Numerous previous studies (3, 22) have revealed that Wnt/β-catenin signaling plays pivotal roles in cardiac lineage specification, differentiation, and proliferation. Interestingly, Wnt/β-catenin signaling is considered to have different effects depending on the time of action. During the early stage of differentiation, Wnt/β-catenin signaling induces cardiac cell specification and amplification. In contrast, at the late stage of differentiation, Wnt/β-catenin signaling inhibits differentiation toward mature cardiomyocytes (10, 20, 21, 30). In the present study, we demonstrated that the LS-type SHP2-Q510E mutation attenuated myofibrillogenesis in P19CL6 cells and elevated nuclear β-catenin accumulation at the late stage of differentiation. Our results indicate that the SHP2-Q510E mutation suppresses terminal differentiation into mature cardiomyocytes via aberrant β-catenin signaling at the late stage of differentiation. We consider that this aberrant (high) β-catenin accumulation is derived from Akt and GSK-3β hyperphosphorylation. Edouard et al. (7) showed that EGF-induced Akt and GSK-3β phosphorylation were elevated in skin fibroblasts from LS patients. LS-type SHP2 mutants showed decreased phosphatase activity and were unable to efficiently dephosphorylate GAB1 PI3K-binding sites, resulting in upregulation of the PI3K/Akt pathway (12). Similarly, in the present study, EGF-stimulated Akt and GSK-3β phosphorylation were elevated in P19CL6 cells with the SHP2-Q510E mutation compared with P19CL6 cells with the SHP2-D61N mutation and controls. Moreover, the PI3K inhibitor LY-294002 reversed the aberrant (high) accumulation of nuclear β-catenin at the late stage of differentiation. Naito et al. (20) demonstrated that DMSO-treated P19CL6 cells, which were transfected with constitutively active Akt, showed significantly enhanced Wnt/β-catenin activity and that the PI3K inhibitor LY-294002 decreased nuclear β-catenin expression. It was further reported that the Akt/GSK-3β pathway, and not Wnt activation, induces stabilization of β-catenin in isolated cardiomyocytes and in vivo (13). Taken together, these studies demonstrate the possible interaction between the PI3K/Akt pathway and β-catenin signaling. The LS-type SHP2 mutant shows impairment in the dephosphorylation of GAB1 PI3K-binding sites, which results in PI3K/Akt hyperactivation (12) and GSK-3β degradation (7). Our present findings suggest that this aberrant Akt/GSK-3β signaling axis might lead to impairment of normal downregulation of β-catenin signaling at the late stage of cardiac differentiation, thereby resulting in the attenuation of terminal differentiation into mature cardiomyocytes. We further speculate that this high β-catenin signaling may promote the proliferation and expansion of cardiac progenitors, thereby increasing the number of cardiac cells in the hearts of LS patients. Recent studies have demonstrated that stabilized β-catenin overexpressed under the specific Nkx2.5 enhancer induced the expansion of cardiac progenitors. Additionally, the GSK-3β inhibitor elevated β-catenin activity and led to the proliferation of cardiomyocytes in vitro (16, 29). It has also been shown that β-catenin promoted the expansion of Islet-1-positive, second-heart field cardiac progenitors (2). Thus, the activation of GSK-3β/β-catenin signaling is considered to be important for cardiomyocyte growth and proliferation (13). In the present study,
we demonstrated that the proliferative activity of P19CL6 cells with the SHP2-Q510E mutation was significantly increased during cardiomyogenesis. Further in vivo studies using animal models are required to elucidate the possible involvement of increased numbers of cardiac cells in the pathogenesis of HCM associated with LS during cardiac development.

The morphological alterations of terminally differentiated cardiomyocytes in iPSCs from skin fibroblasts of LS patients clearly suggest that the LS-type SHP2 mutation affects the hypertrophic mechanisms of cardiomyocytes during cardiomyogenesis by cell-autonomous mechanisms (1). In the present study, we demonstrated that terminally differentiated mature cardiomyocytes derived from P19CL6 cells with the LS-type SHP2-Q510E mutation were larger than those derived from the other mutant cells and controls. However, individual cardiomyocyte hypertrophy (i.e., increased sarcomeric protein and fetal gene expression) cannot be reliably assessed in iPSCs and P19CL6 cells because various types of cells are obtained using these cardiac differentiation procedures. In a recent study of iPSCs, Carvajal-Vergara et al. (1) suggested the possible involvement of calcinurin-NFAT signaling in the morphological changes of cardiomyocytes in LS. However, the mechanism by which the LS-type SHP2 mutation could affect the calcinurin-NFAT system has not been elucidated. On the other hand, Edouard et al. (7) suggested that elevated Akt signaling could induce cardiac hypertrophy in LS patients. In the present study, we showed that the hypertrophic changes of mature cardiomyocytes from SHP2-Q510E mutant P19CL6 cells could be associated with Akt and GSK-3β hyperphosphorylation. Moreover, the PI3K/Akt inhibitor LY-294002 suppressed these hypertrophic changes. Thus, it appears that the Akt/GSK-3β pathway plays a pivotal role in the pathogenesis of HCM in LS patients. It may appear contradictory that the SHP2-Q510E mutants attenuate myofibrillogenesis (i.e., decrease sarcomeric protein expression) and promote hypertrophy (i.e., increase sarcomeric protein expression). However, we evaluated the morphological alterations in terminally differentiated cardiomyocytes after myofibrillogenesis. The two steps may be differently affected by PI3K/Akt and β-catenin signaling.

There are several limitations to our study. Most importantly, P19CL6 cells comprise a unique in vitro cardiomyocyte differentiation model. Therefore, it is not clear to what extent P19CL6 cells mimic in vivo cardiomyocyte differentiation and human pathogenesis. Although P19CL6 cells are recognized as a well-established in vitro cardiomyocyte differentiation system, further experiments using more specific cells, such as...
primary human cardiomyocytes or iPSCs, are needed to elucidate the precise mechanism of HCM in LS patients.

In conclusion, two mechanisms can contribute to the pathogenesis of severe progressive HCM in LS patients with the SHP2-Q510E mutation: 1) aberrant proliferation and expansion of cardiac cells, with attenuation of mature cardiomyocyte differentiation, and 2) hypertrophic changes in individual cardiomyocytes. The LS-type SHP2 mutation causes dysregulation of Akt/GSK-3β/β-catenin signaling, which may play a pivotal role in these mechanisms. Therefore, inhibition of the PI3K/Akt or β-catenin pathways represents a possible treatment strategy for HCM in LS patients.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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