

Myocardin Functions as an Effective Inducer of Growth Arrest and Differentiation in Human Uterine Leiomyosarcoma Cells

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Abstract

Myocardin is an important transcriptional regulator in smooth and cardiac muscle development. We noticed that the expression of myocardin was markedly downregulated in human uterine leiomyosarcoma cells. Restoration of myocardin expression induced the reexpression of smooth muscle marker proteins and the formation of well-developed actin fibers. A concomitant increase in the expression of a cyclin-dependent kinase inhibitor, p21, led to significantly reduced cell proliferation, via p21's inhibition of the G₁-S transition. A p21 promoter-reporter assay showed that myocardin markedly increased p21's promoter activity. Furthermore, a serum response factor (SRF)-binding *cis*-element CArG box in the p21 promoter region was required for this myocardin effect. Chromatin immunoprecipitation and DNA-protein binding assays showed that myocardin indirectly bound to the CArG box in the p21 promoter through the interaction with SRF. Furthermore, immunohistochemistry revealed that the levels of myocardin and p21 were both lower in leiomyosarcoma samples than in normal smooth muscle tissue. Taken together, our results indicate that the downregulation of myocardin expression facilitates cell cycle progression via the reduction of p21 expression in human leiomyosarcomas and suggest that myocardin could be a useful therapeutic target for this disease. *Cancer Res*; 70(2); 501-11. ©2010 AACR.

Introduction

Leiomyosarcoma is a relatively rare smooth muscle tumor, accounting for ~10% of all sarcomas. It is an aggressive disease with a propensity to spread systemically and is associated with high recurrence rates and a poor prognosis (1-3). It most frequently occurs in the gynecologic tract, particularly the uterus, and in soft tissues. Uterine leiomyosarcomas have an estimated annual incidence of 0.64 per 100,000 women. They account for one third of uterine sarcomas and 1.3% of all uterine malignancies; the 5-year survival rate is only 15% to 25% (4).

Although in many tissues differentiation is irreversible and accompanied by cell cycle exit, the differentiation of smooth muscle cells (SMC) is readily reversible. Malignancies of the SMCs switch from the differentiated to the undifferentiated phenotype in response to changes in the local environment (5-10), and normal myofibroblasts exhibit an intermediate

stage in the phenotypic spectrum between fibroblasts and SMCs (11). Thus, the phenotypic modulation is tightly correlated with the progression of leiomyosarcoma.

Recently, myocardin was reported to be a key regulator of cardiac and smooth muscle differentiation. Its expression is restricted to smooth and cardiac muscle lineages, and it regulates the differentiation of these muscle types by inducing the expression of the appropriate genes (12, 13). Myocardin drives transcription through its interaction with a MADS box-containing transcription factor, serum response factor (SRF), which binds to a consensus DNA sequence, CC[A/T]_nGG (the CArG box), found in the promoter regions of many smooth muscle and myofibroblast genes (14, 15). Although the importance of myocardin in smooth muscle development is firmly established, its role in the progression of leiomyosarcoma is not well understood.

In the present study, we found that myocardin was significantly reduced in human uterine leiomyosarcoma cell lines. Restoration of myocardin expression in the uterine leiomyosarcoma cells induced phenotypic modulation to the differentiated phenotype and significantly suppressed cell proliferation, accompanied by an increase in the level of a cyclin-dependent kinase inhibitor (CDKI), p21. In agreement with these findings, the expression levels of myocardin and p21 were both lower in the clinical tissue samples of leiomyosarcoma patients than in normal uterine smooth muscle tissue. This is the first report to describe the molecular mechanism underlying the myocardin-mediated conversion of malignancy of leiomyosarcoma cells. Our present results indicate that myocardin may prove to be an important therapeutic target for the treatment of human leiomyosarcomas.

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi: 10.1158/0008-5472.CAN-09-1469

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Materials and Methods

Materials. DMEM was purchased from Sigma. Fetal bovine serum (FBS) was obtained from Invitrogen. Anti-myocardin antibody was generated in rabbits using recombinant human myocardin proteins (amino acids 752–903) as an antigen. IgGs were affinity-purified from the antisera raised against myocardin, using an antigen-conjugated affinity column. Anti-caldesmon antibody was prepared and purified as described previously (16). The following primary antibodies were purchased from Sigma: mouse anti- α -tubulin, mouse anti-tropomyosin, mouse anti- α -smooth muscle actin (α SMA), mouse anti-calponin, and rabbit anti-FLAG. The rabbit anti-SRF and mouse anti-p21 were from Santa Cruz. The other antibodies and their sources were as follows: mouse anti-green fluorescent protein (GFP; Invitrogen), mouse anti-SM22 (Novocastra), mouse anti-smooth muscle 2 myosin heavy chain (SM2-MHC; Yamasa), rat anti-HA (Roche), mouse anti-p21 (Calbiochem), and mouse anti-5-bromodeoxyuridine (BrdUrd; Dako).

Cell culture. The human uterine leiomyosarcoma cell lines SKN (Health Science Research Resources Bank), SK-UT-1, SK-LMS-1 (American Type Culture Collection), human uterine SMCs (Hu.USMC; Cambrex BioScience Walkersville, Inc.), and human hepatocellular carcinoma cell line HepG2 (American Type Culture Collection) were cultured in DMEM with 10% FBS and 1% antibiotic solution (Life Technologies).

Immunofluorescence analysis. Cells grown on coverslips were fixed with 4% formaldehyde and 4% sucrose in PBS for 30 min at room temperature and then permeabilized in blocking solution (0.2% Triton X-100, 0.2% bovine serum albumin, 10% normal goat serum in PBS). The cells were incubated with primary antibody diluted in blocking solution, followed by the secondary antibody. To visualize actin filaments, Alexa 568-conjugated phalloidin (Invitrogen) was added to the secondary antibody solution. For nuclear staining, Hoechst 33342 (Invitrogen) was used. Samples were observed under a Biorvo BZ-9000 fluorescence microscope (Keyence).

BrdUrd labeling and cell counting. Hu.USMCs and the human uterine leiomyosarcoma cell lines (SK-UT-1, SKN, and SK-LMS-1) were cultured on coverslips to 70% confluence and then incubated with 10 μ mol/L BrdUrd for 8 h. The cells were fixed with 4% formaldehyde and 4% sucrose solution for 1 h at room temperature and then treated with 2 N HCl for 5 min at 37°C for BrdUrd staining. BrdUrd-labeled cells were illuminated by epifluorescence and counted. The percentages of BrdUrd-positive cells were determined by counting four fields at a 100 \times magnification. Results were obtained from three independent experiments.

Adenoviral gene transfection. Adenoviral vectors encoding enhanced green fluorescent protein (EGFP) and FLAG-tagged mouse myocardin were produced using the BD Adeno-X Universal Adenoviral Expression System (Clontech) and stored in aliquots at -80°C . When the cells became 60% confluent, they were incubated with adenoviral vectors for 1 h at a multiplicity of infection of 100. Under these conditions, an almost 100% infection efficiency was obtained. The cells were used for immunostaining and Western blotting analysis for 72 h after the infection.

Cell cycle analysis. Human uterine leiomyosarcoma cell lines were infected by adenoviral vectors encoding FLAG-myocardin and control EGFP and then cultured to 70% confluence for 72 h. The cells were trypsinized, fixed in 100% ethanol for 15 min at -20°C , and suspended in a DNA staining solution (10 $\mu\text{g}/\text{mL}$ DNase-free RNase and 50 $\mu\text{g}/\text{mL}$ propidium iodide in 0.85% NaCl). Fluorescence-activated cell sorting (FACS) analysis (ModFit LT2, Topsham, ME) was performed to determine the cell cycle distribution.

Quantitative real-time reverse transcription-PCR. Total RNAs were extracted from 70% to 80% confluent cultured cells using the TRIzol reagent (Invitrogen) and reverse-transcribed with MultiScribe Reverse Transcriptase (Applied Biosystems). The cDNA was amplified with gene-specific primer pairs using the SYBR GreenER qPCR SuperMix Universal reagent (Invitrogen). The primer sequences used in this paper are listed in the supplementary material (Supplementary Table S1). The quantities measured by real-time PCR were normalized to the glyceraldehyde-3-phosphate dehydrogenase expression level in each sample.

RNA interference. Small interference RNAs (siRNA) targeting human myocardin, Myocd siRNA1 (CTCGCCGATGATCTCAATGAA, Qiagen) and Myocd siRNA2 (CAGCTAAGGAACCAAATGAA, Qiagen), and a control siRNA (Santa Cruz) were purchased. The cells were transfected with the siRNAs using Lipofectamine RNAiMAX transfection reagent (Invitrogen) and cultured for 1 wk; the medium was replaced with fresh siRNA-containing medium every 2 d.

Luciferase reporter assay. A fragment of the promoter region of the human p21 gene (-830 to $+10$) was amplified by PCR and cloned into pGL3-basic (Promega; pGL3-p21-Luc CarG WT). A mutation in the CarG box motif of the human p21 promoter was introduced by PCR mutagenesis (from CCTTTTTTGG to CCTTTTTTTT; pGL3-p21-Luc CarG mut). The cDNA of mouse myocardin was amplified by reverse transcription-PCR (RT-PCR) and inserted into the mammalian expression plasmid pCS2+ with a FLAG-tag sequence (pCS2+-FLAG-myocardin). HepG2 cells were cotransfected with reporter plasmid (pGL3-p21-Luc CarG WT or pGL3-p21-Luc CarG mut), pCS2+-FLAG-myocardin, and with pGL3- β -gal for normalization of the transfection efficiency. Twenty-four hours after the transfection, the cells were lysed with passive lysis buffer (Promega), and the luciferase and β -galactosidase activities were measured using the luciferase assay system (Promega) and Luminescent β -galactosidase detection kit II (Clontech), respectively.

Chromatin immunoprecipitation assay. The chromatin immunoprecipitation (ChIP) assay was performed using a ChIP kit (Upstate Biotechnology), according to the manufacturer's instructions. In Hu.USMCs, immunoprecipitation was performed using anti-SRF antibodies. In SK-UT-1 cells infected with adenoviral vectors encoding FLAG-tagged myocardin or EGFP, immunoprecipitation was performed using anti-FLAG antibodies. The sequence surrounding the CarG box (-788 to -632) in the human p21 promoter was amplified from the immunoprecipitants using the primer pair AAGCTGCTGCAACCACAG and GGCCTTCGTACTGTAGT.

DNA affinity precipitation assay. The coding regions for human SRF were amplified by PCR and cloned into the pCS2+ expression vectors (pCS2+–HA-SRF) with an HA-tag sequence. FLAG-myocardin and HA-SRF proteins were synthesized using the TnT high yield *in vitro* transcription/translation system (Promega). Two pairs of complementary single-strand oligonucleotides, Biotin-ATCAGGTTGCCCTTTTTTGG-TAGTCTCTCC/ggagagactacaaaaagggaacctgat (wild-type CarG probe) and Biotin-atcaggttgcccttttttTtagtctctcc/ggagagactaAaaaaagggaacctgat (mutated CarG probe), were synthesized, annealed, and used as DNA affinity probes. The synthesized proteins were incubated with 1 µg of the biotinylated DNA probes in DNA-binding buffer [20 mmol/L HEPES-KOH (pH 7.9), 80 mmol/L KCl, 1 mmol/L MgCl₂, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, 10% glycerol, 0.1% Triton X-100, 50 µg/mL herring sperm DNA, 1% protease inhibitor cocktail (Nacalai Tesque)] at 4°C for 1.5 h. Dynal Dynabeads M280 streptavidin (Invitrogen) was then added to the reaction mixtures and incubated at room temperature for 1 h. After three washes with the DNA-binding buffer, SDS sample buffer was added to the Dynabeads to elute the DNA-protein complex.

Immunohistochemistry. A microarray tissue section slide (SO2081, US Biomax) that included samples of human leiomyosarcomas and normal smooth muscle tissue was heated at 58°C for 2 h to soften the covering layer of paraffin. The slide was dewaxed with xylene for 10 min twice, then with 100% ethanol for 3 min thrice, and with 90% ethanol, 80% ethanol, and 70% ethanol (each for 3 min). The slide was rehydrated in PBS for 20 min and then boiled for 20 min in an antigen retrieval buffer (Trilogy, Cell Marque). After cooling for 20 min at room temperature, the slide was washed twice more in PBS for 10 min each. The slide was incubated in 0.4% Triton-X-100-containing blocking solution for 1 h, washed in PBS for 15 min, and incubated with anti-p21 and anti-myocardin antibodies in Can Get Signal immunoreaction enhancer solution B (Can Get Signal, Toyobo) overnight, followed by the appropriate secondary antibodies with Hoechst 33342. The fluorescence intensities of myocardin and p21 staining were quantified using a Biorevo BZ-9000 fluorescence microscope (Keyence). Staining intensity was assessed as the mean integrated absorbance per unit area.

Statistical analyses. Statistical analyses were carried out using Stat Flex ver. 4.2 for Windows (Artec). The nonparametric Mann-Whitney *U* test was used to evaluate the significance of differences between normal smooth muscle tissue samples and leiomyosarcoma samples in immunohistochemistry analyses. The association between the fluorescence intensity of anti-p21 staining and that of anti-myocardin staining was assessed by the Spearman rank correlation test. Other statistical analyses were performed using Student's *t* test. In all these analyses, *P* < 0.05 was regarded as significant.

Results

Human uterine leiomyosarcoma cell lines have a dedifferentiated smooth muscle phenotype. We first observed the organization of the actin cytoskeleton in Hu.USMC and human uterine leiomyosarcoma (SK-UT-1, SKN, and SK-LMS-1)

cell lines by phalloidin staining. Hu.USMCs showed well-developed actin stress fibers (Fig. 1A). In contrast, the human uterine leiomyosarcoma cells showed thin actin filaments (Fig. 1A). The contractile apparatus of SMCs is characterized by high levels of αSMA, calponin, SM22, caldesmon, and SM-MHC, and therefore these proteins are informative indicators of the differentiation status of the cells (17, 18). Hu.USMCs expressed αSMA, calponin, SM22, SM2-MHC, and caldesmon. Caldesmon has two main splicing isoforms, high-molecular-weight caldesmon (h-caldesmon) and low-molecular-weight caldesmon (l-caldesmon), and h-caldesmon is specific for SMCs (19, 20). In the Hu.USMCs, both h-caldesmon and l-caldesmon were expressed. On the other hand, in the three leiomyosarcoma cell lines examined, h-caldesmon expression was not detected and the levels of αSMA, calponin, SM22, SM2-MHC, and l-caldesmon were lower than in the Hu.USMCs (Fig. 1B). Tropomyosin, an actin-binding protein involved in the stabilization of actin filaments and smooth muscle contraction (21), was also downregulated in the leiomyosarcoma cells.

Because myocardin is an important regulator for the transcription of SMC marker genes, we compared the expression levels of myocardin in Hu.USMCs and the leiomyosarcoma cell lines. We found that myocardin was markedly downregulated at both the mRNA and protein levels in all three leiomyosarcoma cell lines compared with their levels in Hu.USMCs (Fig. 1B and C).

The malignant transformation process is also tightly associated with defects in cell cycle regulation. We investigated whether the cell proliferation rate differed between Hu.USMCs and the leiomyosarcoma cell lines using BrdUrd. We found a significant increase in BrdUrd-positive cells among the leiomyosarcoma cells compared with the Hu.USMCs (potential doubling time was 41.8 ± 12.9 h for Hu.USMCs, 12.8 ± 1.0 h for SK-UT-1, 16.6 ± 0.4 h for SKN, and 13.1 ± 0.7 h for SK-LMS-1; Fig. 1D). Taken together, our immunostaining and proliferation analyses indicated that leiomyosarcoma cells have a less differentiated phenotype than SMCs, and their cell cycling is dysregulated.

Myocardin overexpression induces phenotypic modulation in human uterine leiomyosarcoma cells. To evaluate the functional significance of myocardin for the maintenance of smooth muscle phenotype in leiomyosarcoma cells, we expressed exogenous myocardin in human uterine leiomyosarcoma cells, using an adenoviral expression system. Cells infected with control EGFP-encoding adenovirus did not show morphologic changes (Fig. 2A). In contrast, the expression of exogenous myocardin resulted in the reorganization of the actin cytoskeleton and the formation of well-developed stress fibers with a stretched cell shape (Fig. 2A). Consistent with these changes, the transfected cells highly expressed αSMA, SM22, SM2-MHC, l-caldesmon, tropomyosin, and calponin (Fig. 2B). In particular, h-caldesmon expression was substantially rescued in myocardin-expressing SKN cells. Taken together, these results indicate that the expression of myocardin induces phenotypic modulation to the differentiated SMC phenotype in human uterine leiomyosarcoma cells.

Myocardin induces a cell cycle delay at the G₁-S phase transition in leiomyosarcoma cells. Recent reports showed that the inactivation of myocardin is associated with malignant tumor growth (22) and that myocardin inhibits cell proliferation by interfering with NF- κ B-dependent cell cycle regulation (23). Here we found that proliferation of myocardin-expressing leiomyosarcoma cells was significantly slower than that in GFP-expressing control cells (Fig. 3A). Using DNA flow cytometry, we investigated the phase distribution of the cell cycle in the myocardin- and GFP-expressing cells. All the myocardin-expressing leiomyosarcoma cells displayed a significantly lower population of cells in S phase and a significantly higher population of cells in G₀-G₁ phase compared with the GFP-expressing control cells (Fig. 3B), suggesting

that myocardin expression induced a delayed G₁-S phase transition in the leiomyosarcoma cells.

Myocardin-induced cell cycle delay is accompanied by upregulation of CDKI p21. CDKIs are critical cell cycle regulators. To elucidate the transcriptional targets of myocardin that were linked to the cell cycle delay, we examined whether the expression levels of CDKIs were affected by myocardin expression in the leiomyosarcoma cell lines. Although a number of CDKI genes showed slight differences in expression level in the myocardin-expressing cells, depending on the cell line, only the p21 mRNA was dramatically upregulated in all three cell lines (Fig. 4A). We then confirmed that the expression of p21 protein was also upregulated in these cells (Fig. 4B).

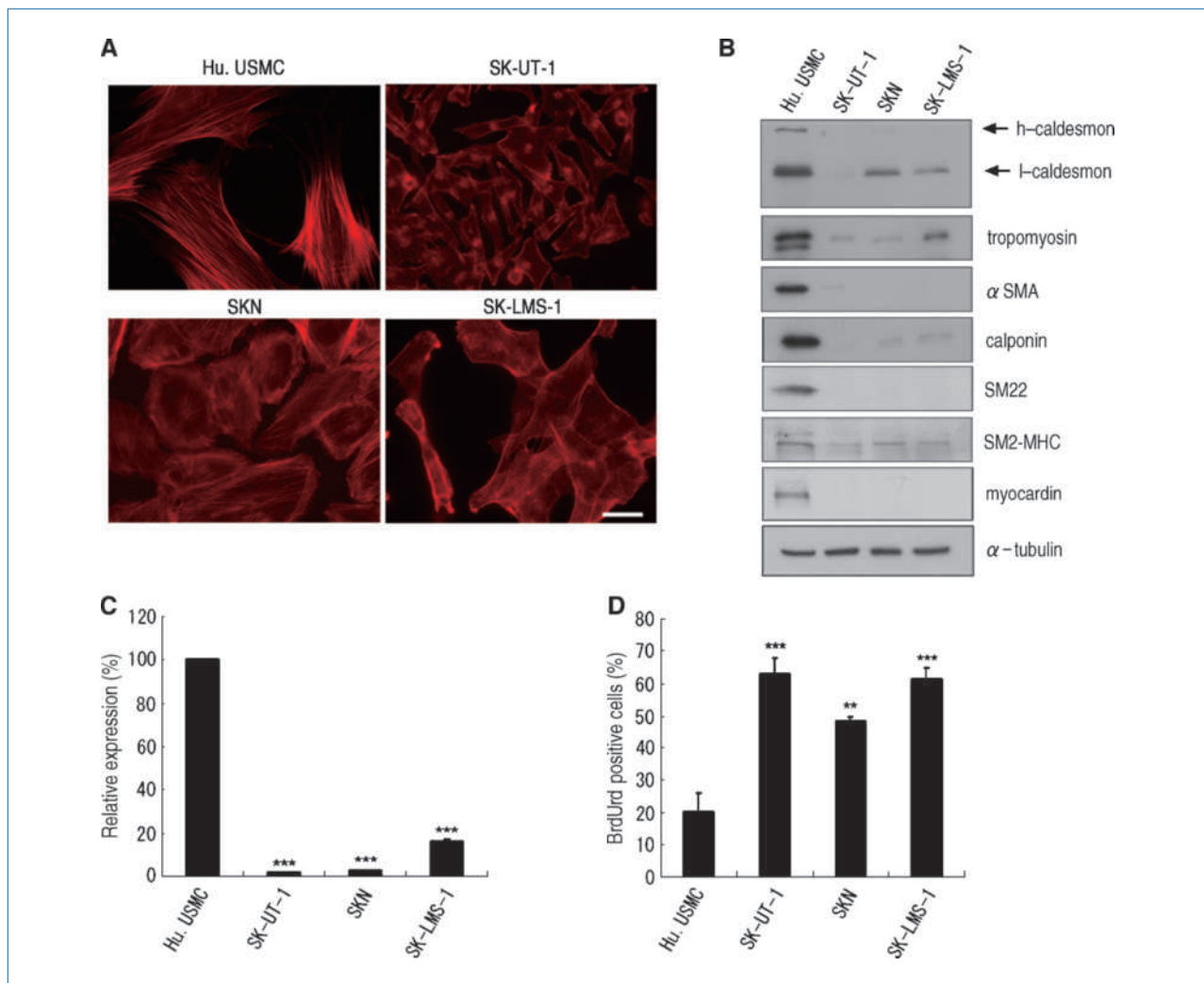
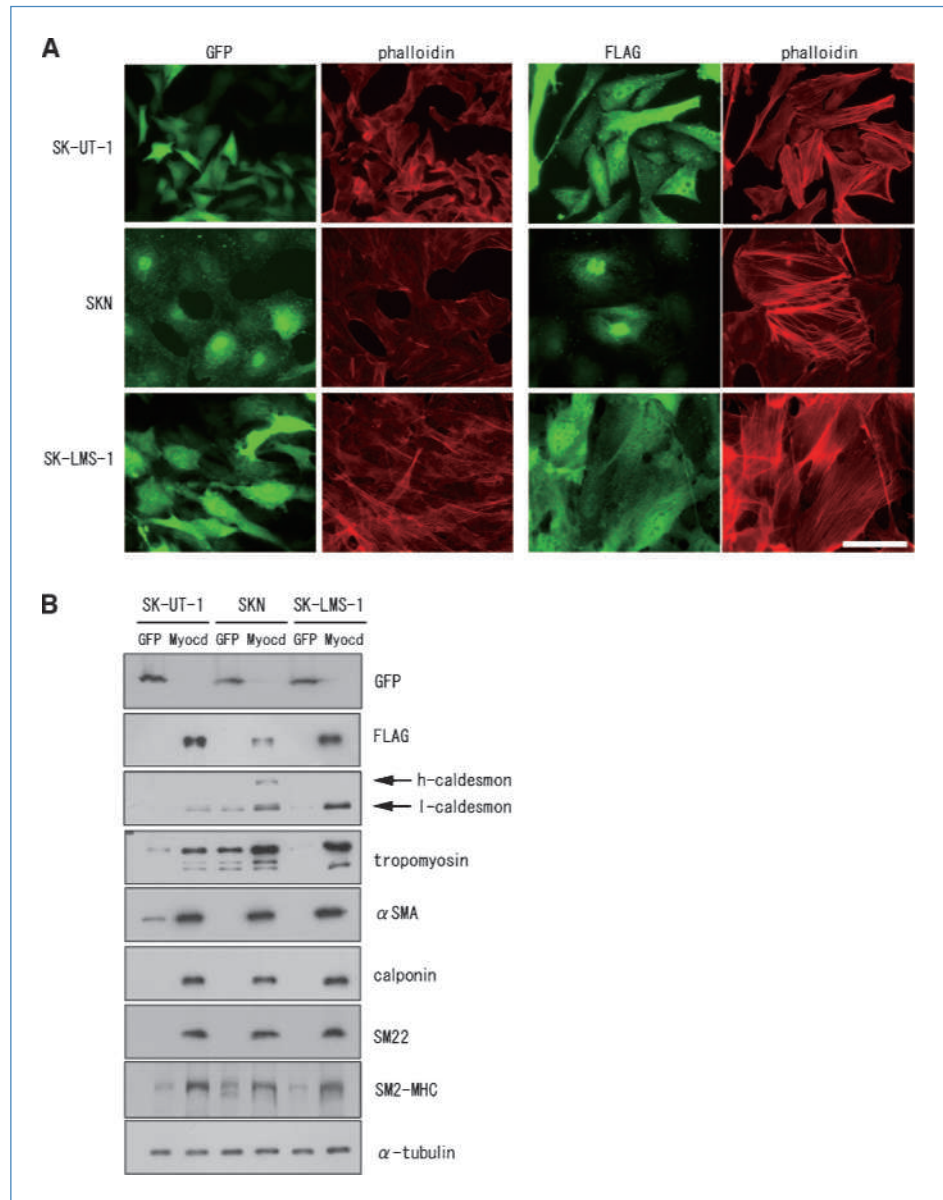


Figure 1. Human uterine leiomyosarcoma cell lines (SK-UT-1, SKN, and SK-LMS-1) were fixed and stained with Alexa 568-conjugated phalloidin. Scale bar, 10 μ m. B, total proteins were extracted from the Hu.USMC and the leiomyosarcoma cell lines and analyzed by immunoblotting analysis with the indicated antibodies. C, myocardin mRNA levels in Hu.USMCs and the leiomyosarcoma cell lines determined by quantitative real-time RT-PCR. Statistical analysis was carried out for three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by Student's t test. D, Hu.USMCs and the leiomyosarcoma cell lines stained with an anti-BrdUrd antibody after an 8-h incubation with BrdUrd. Percentages of BrdUrd-positive cells were determined by counting four 100 \times magnification fields. Statistical analysis was carried out for three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by Student's t test.

Figure 2. Characterization of myocardin-overexpressing cells. A, FLAG-tagged myocardin or control EGFP was introduced into the leiomyosarcoma cell lines using an adenovirus expression system. After 48 h, the cells were fixed and stained with anti-GFP or anti-FLAG antibody (green) and phalloidin (red). Scale bar, 10 μ m. B, immunoblotting analysis of the leiomyosarcoma cell lines expressing control GFP or FLAG-myocardin with the indicated antibodies.



To examine the effect of myocardin knockdown on p21 expression, two siRNAs against the human myocardin gene were designed (Myocd siRNA1 and siRNA2). The depletion of myocardin expression led to reduced p21 expression (Fig. 4D). Furthermore, the endogenous p21 expression was significantly lower in the leiomyosarcoma cell lines than in the Hu.USMCs (Fig. 4C). Collectively, these results indicate that myocardin is an upstream regulator of p21 expression and that decreased myocardin levels might cause reduced p21 expression in the leiomyosarcoma cell lines.

Myocardin transactivates the p21 promoter via the CARG box. To determine the molecular mechanism underlying the transcriptional regulation of the p21 gene by myocardin, we performed a luciferase reporter assay using a

fragment of the human p21 gene promoter. Sequence analysis showed a consensus SRF-binding sequence CARG box -728 bp upstream of the first exon (Fig. 5A). In the reporter assay, myocardin markedly enhanced the p21 promoter activity, but this response was completely eliminated by mutation of the CARG box sequence (Fig. 5B). Thus, the CARG box is required for the activation of the human p21 promoter by myocardin.

We next performed a ChIP assay to determine whether myocardin recognizes the human p21 promoter sequence *in vivo*. In Hu.USMCs, endogenous SRF was bound to the human p21 promoter region at or in the vicinity of the CARG box *in vivo* (Fig. 5C). Because the level of endogenous myocardin was too low to immunoprecipitate it using an anti-myocardin antibody, we performed the ChIP assay using

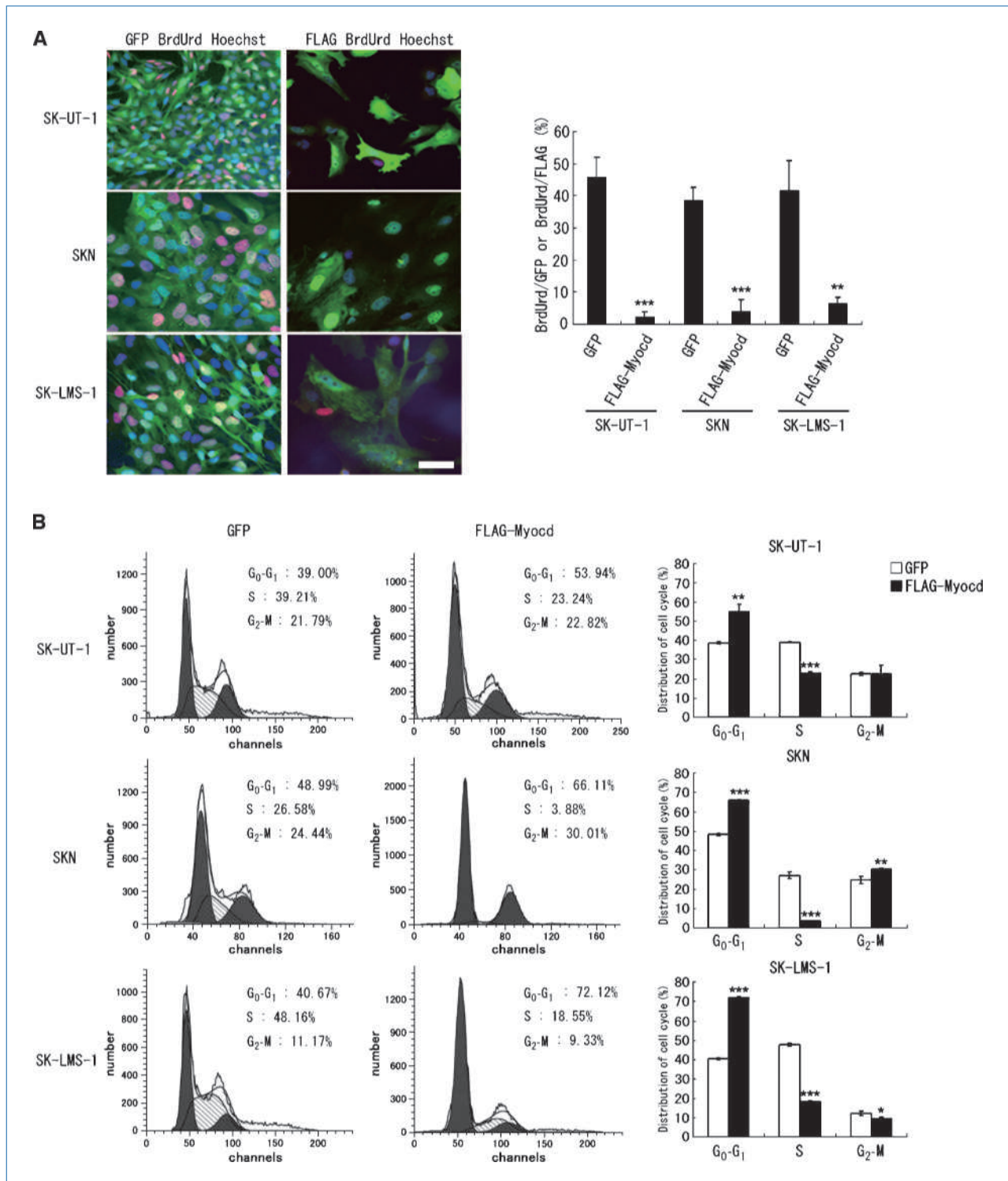


Figure 3. Myocardin induces cell cycle delay in leiomyosarcoma cells at the G₁-S phase transition. **A**, the leiomyosarcoma cell lines expressing FLAG-tagged myocardin or control EGFP were incubated with 10 μmol/L BrdUrd for 12 h and stained with anti-BrdUrd antibody (red) and anti-GFP or anti-FLAG antibody (green). Nuclei were counterstained with Hoechst 33342 (blue). Scale bar, 10 μm. Percentages of BrdUrd/FLAG-positive and BrdUrd/GFP-positive cells were determined by counting eight 100× magnification fields. Statistical analysis was carried out for three independent experiments. **B**, FACS analysis of cell cycle phase distributions in the leiomyosarcoma cell lines expressing FLAG-myocardin or control EGFP. Statistical analysis was carried out for three independent experiments. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001 by Student's *t* test.

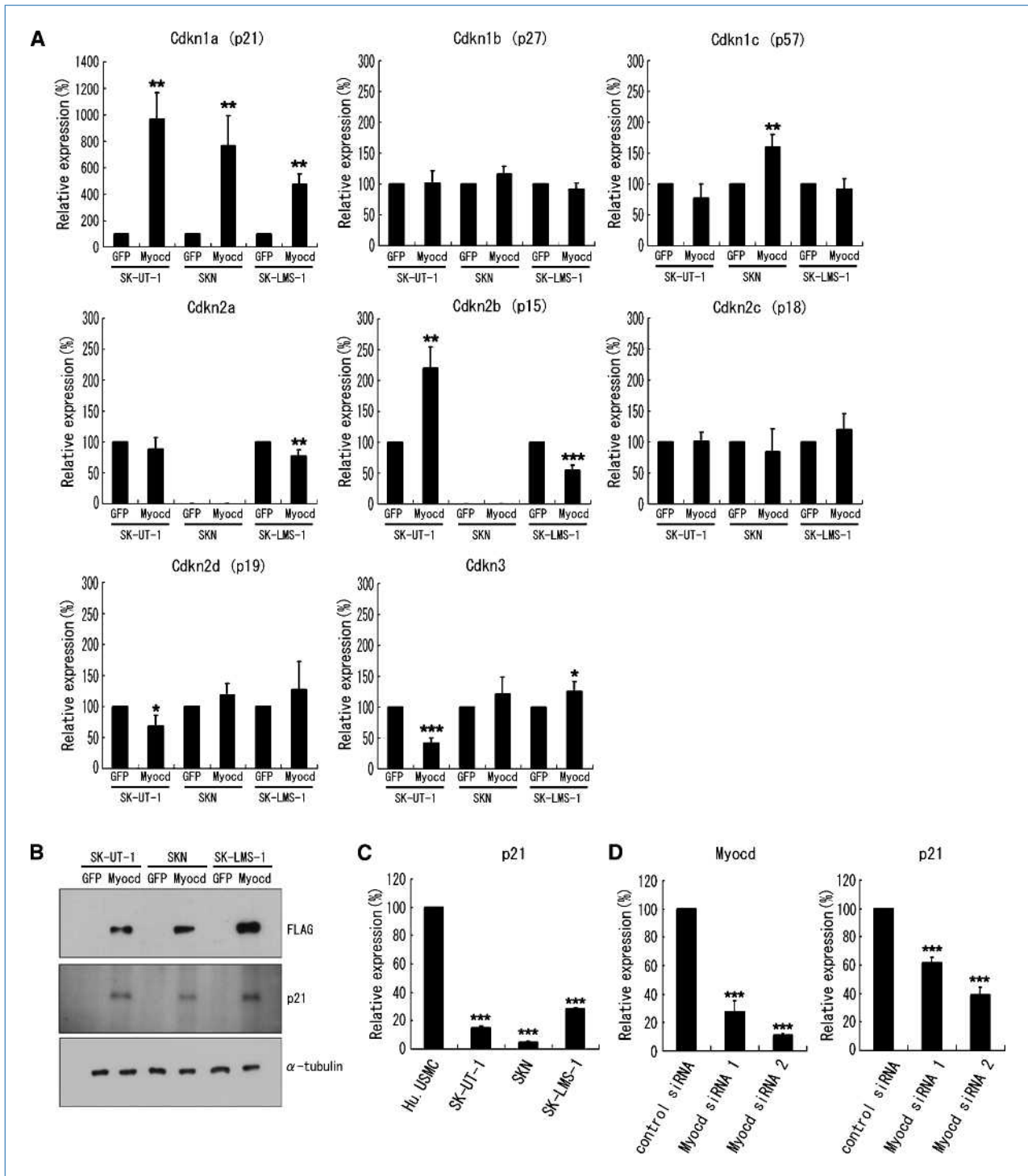


Figure 4. Cell cycle delay by myocardin is accompanied by upregulation of p21. **A**, mRNA levels of CDKs in the leiomyosarcoma cell lines expressing FLAG-tagged myocardin or control EGFP were determined by quantitative real-time RT-PCR. Statistical analysis was carried out for three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by Student's *t* test. **B**, p21 protein level evaluated by Western blotting of the leiomyosarcoma cell lines expressing FLAG-tagged myocardin or control EGFP. **C**, mRNA levels of p21 in Hu.USMCs and the leiomyosarcoma cell lines determined by quantitative real-time RT-PCR. Statistical analysis was carried out for three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by Student's *t* test. **D**, expression levels of myocardin and p21 mRNA in Hu.USMCs expressing a control siRNA or two myocardin siRNAs (Myocd siRNA1 and siRNA2) determined by quantitative real-time RT-PCR. Statistical analysis was carried out for three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by Student's *t* test.

SK-UT-1 cells infected with adenovirus vectors encoding FLAG-myocardin or EGFP. The result showed that myocardin was also bound to the human p21 promoter region *in vivo* (Fig. 5C).

Previous studies showed that myocardin does not bind directly to DNA; rather, it is recruited to target promoters by forming a stable complex with SRF that binds to a CARg box (12, 24, 25). We therefore performed a DNA-protein binding assay to determine whether myocardin binds to the p21 promoter directly or indirectly. Myocardin did not directly bind to a DNA probe containing the p21 promoter fragment with the CARg box (Fig. 5D). In the presence of SRF, however, myocardin did bind the DNA probe by forming a myocardin-SRF-DNA probe ternary complex (Fig. 5D). On the other hand, SRF alone also bound to the DNA probe (Fig. 5D).

When the CARg box sequence was mutated, neither SRF nor myocardin bound to it (Fig. 5D). These results showed that myocardin accesses the p21 promoter region indirectly via its interaction with SRF.

Correlation between p21 and myocardin expression levels in human leiomyosarcoma and normal smooth muscle tissue samples. Because the downregulation of myocardin expression was responsible for the reduced expression of p21 in leiomyosarcoma cell lines, we compared the expression levels of myocardin and p21 in normal smooth muscle tissues and tumor tissues from leiomyosarcoma patients. The expression levels were observed using a muscle disease spectrum array (SO2081, US Biomax), containing many samples on a single slide, by immunohistochemical analysis (Fig. 6A). Myocardin and p21 were

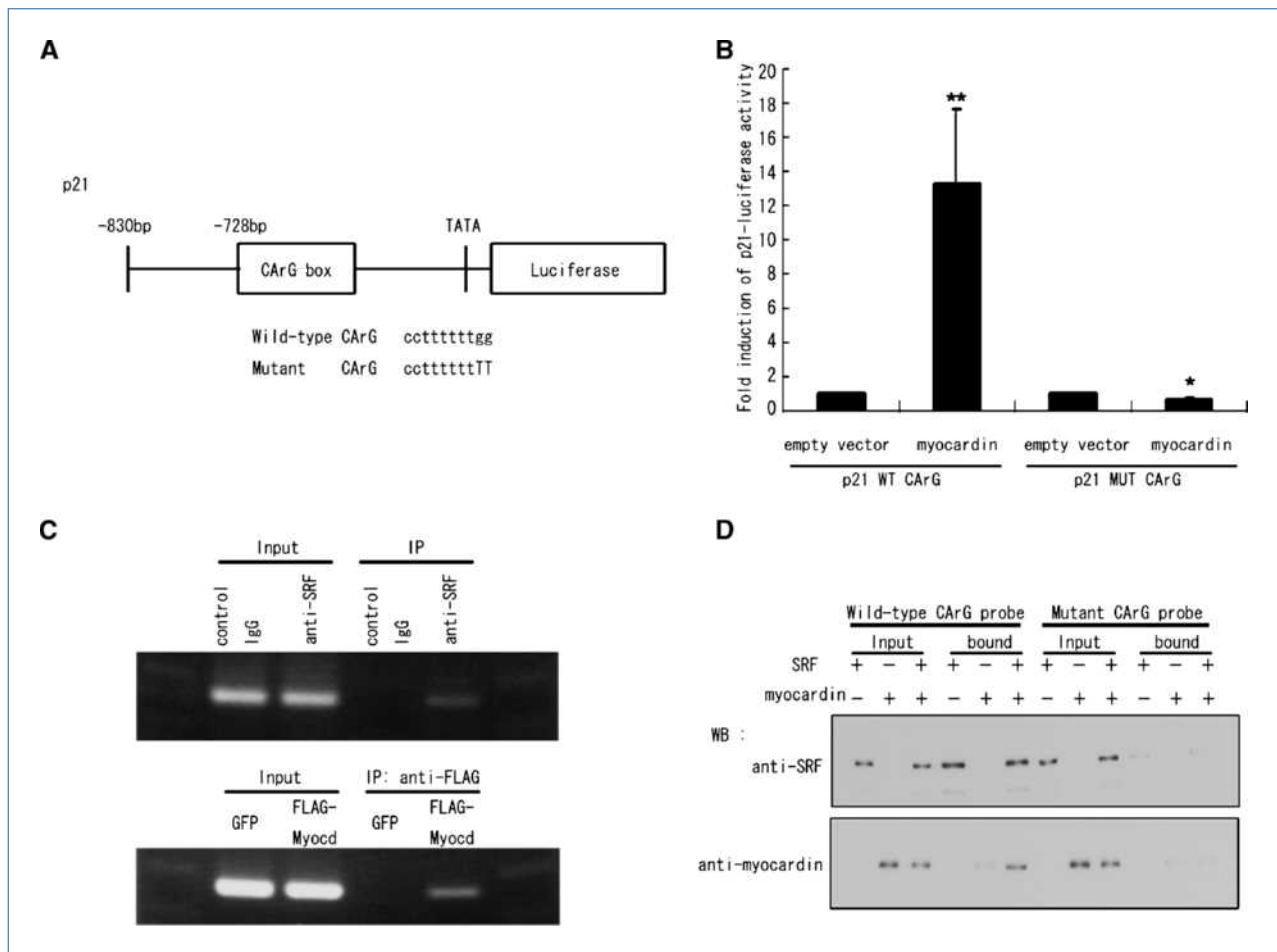


Figure 5. Analysis of myocardin-mediated transactivation of the p21 promoter. **A**, schematic representation of the human p21 promoter construct. The CARg box mutation was introduced by changing the consensus sequence from CCTTTTGG to CCTTTT. **B**, luciferase activities were measured in HepG2 cells transfected with the reporter constructs and FLAG-tagged myocardin. Statistical analysis was carried out for three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by Student's *t* test. **C**, ChIP assay in Hu.USMCs using anti-SRF antibody and in SK-UT-1 cells expressing FLAG-myocardin or control EGFP using anti-FLAG antibody. The PCR primers specifically amplified a fragment of human p21 promoter sequence that included the CARg box. **D**, DNA-protein binding assay. Biotinylated DNA probes containing wild-type or mutant p21 promoter CARg box sequence were combined with *in vitro* synthesized FLAG-tagged myocardin and HA-tagged SRF. The proteins that bound to the DNA probe were detected by immunoblotting analysis with the indicated antibodies.

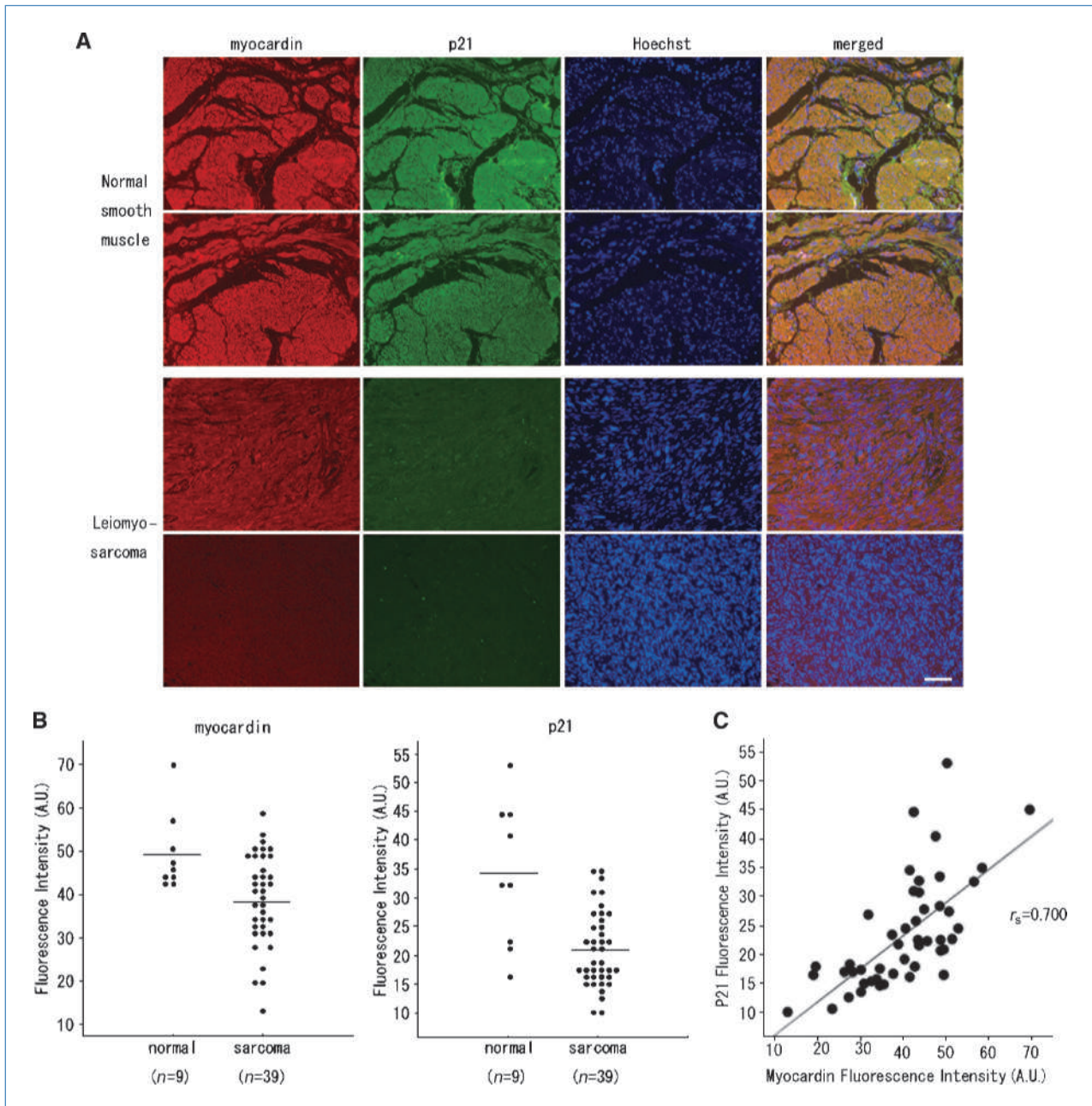


Figure 6. Correlation between myocardin and p21 expression in human leiomyosarcoma and normal smooth muscle tissue samples. **A**, representative immunohistochemical stainings of myocardin (red) and p21 (green) in human normal smooth muscle tissue (top two panels) and leiomyosarcomas (bottom two panels) are shown. Nuclei were counterstained with Hoechst 33342 (blue). Scale bar, 10 μ m. **B**, myocardin and p21 levels quantified by their immunofluorescence intensities in normal smooth muscle tissue and leiomyosarcoma samples. A.U., arbitrary units. *P* values were determined by the nonparametric Mann-Whitney *U* test. Bar, median value. **C**, scatter plot between the fluorescence intensities of myocardin and p21 staining. Statistical relationships between the immunofluorescence intensities were determined using the Spearman rank correlation analysis [coefficient (r_s) = 0.700, $P < 0.001$].

abundant in normal smooth muscle tissues, but their expression levels were significantly lower in leiomyosarcomas (Fig. 6B). We quantified the difference by measuring the samples' immunofluorescence intensity and found that the median immunofluorescence intensities of myocardin and p21 in the leiomyosarcoma samples were, respectively, 85% and

58% of normal smooth muscle tissues (Fig. 6B). Importantly, the expression level of p21 was positively and significantly correlated with that of myocardin (correlation coefficient $r_s = 0.700$, $P < 0.001$; Fig. 6C). These data were in accord with our result from the cultured cell lines that showed p21 expression to be tightly regulated by myocardin in SMCs.

Discussion

Myocardin is a key regulator of cardiac and smooth muscle differentiation, and its expression is restricted to cardiac and smooth muscles. It regulates the differentiation of these muscle types via transactivation of the appropriate differentiation marker's expression (12, 13). Although the importance of myocardin in smooth muscle development is firmly established, its role in leiomyosarcoma progression has barely been addressed. In the present study, we showed that leiomyosarcoma cell lines exhibited less-differentiated smooth muscle phenotypes with diminished myocardin expression compared with normal SMCs. Also in clinical samples from leiomyosarcoma patients, the expression level of myocardin was downregulated. Recently, Milyavsky and colleagues also reported that myocardin is frequently repressed during human malignant transformation (22). Thus, the reduction in myocardin level is tightly correlated with leiomyosarcoma progression. The exogenously myocardin-expressing leiomyosarcoma cells showed phenotypic switching to a differentiated phenotype, as assessed by the increased expression of caldesmon, α SMA, calponin, SM2-MHC, and SM22. These proteins are informative indicators of the differentiation status of SMCs (17, 18). These results supported our idea that myocardin loss during malignant transformation could account for the differentiation defects of human leiomyosarcoma cells.

Inducing myocardin expression in human uterine leiomyosarcoma cells also inhibited cell proliferation, consistent with a recent report that ectopic expression of myocardin leads to the growth inhibition of fibrosarcoma cells (22). We elucidated by flow cytometry, real-time RT-PCR, and immunoblotting analysis that myocardin induced p21 expression and suppressed cell cycle progression at the G₁-S phase in human uterine leiomyosarcoma cells. Given that p21 is a key regulator of cell cycle progression and plays a critical role in the G₁-S transition, the p21 protein seemed a likely candidate for mediating the myocardin-induced cell cycle delay in the leiomyosarcoma cells. We additionally examined the mechanism of the myocardin-induced p21 expression in leiomyosarcoma cells by p21 promoter analysis, ChIP assay, and DNA-protein binding assay. All these results support our model, in which myocardin controls the G₁-S phase transition by the direct regulation of p21 expression. In Chinese hamster ovary (CHO) cells however, myocardin is reported to induce a G₂-M phase arrest by repressing NF- κ B-mediated c-myc and CDK2 expression, but not by affecting p21 and p27 levels (23). We investigated the effect of myocardin on p21 expression in CHO and other cell lines (human uterine SMCs, rat thoracic smooth muscle A7r5 cells, and mouse embryo fibroblasts 10T1/2) and observed that the p21 protein was markedly upregulated in all the myocardin-expressing cells (Supplementary Fig. S1). We think that this discrepancy might be caused by a difference in the methods used to overexpress myocardin.

Myocardin, a potent coactivator of SRF, induces the expression of smooth muscle-specific genes in an SRF-dependent manner (12, 13, 24, 25). We previously reported

that MRTF-A and MRTF-B, which are other myocardin family members, act as pivotal mediators of stress fiber and focal adhesion formation via the transcriptional regulation of a subset of cytoskeletal/focal adhesion genes in an SRF-dependent manner (26). Thus, SRF binding is very important for the functions of myocardin family proteins. However, there is also the possibility that myocardin family proteins indirectly regulate the organization of the actin cytoskeleton through interacting with other proteins. In this paper, we identified a consensus SRF-binding sequence, CArG box, within -830 bp upstream of the first exon of the human p21 gene. Myocardin markedly transactivated the p21 promoter in a CArG box-dependent manner and upregulated the expression level of endogenous p21. Furthermore, the results of ChIP assay and DNA-protein binding assay indicate that myocardin accesses the p21 promoter region indirectly via its interaction with SRF. Thus, SRF is a critical mediator for myocardin function in the expressional regulation of p21 gene.

Although the myocardin-induced upregulation of p21 expression was observed in cell lines from human, rat, and mouse, the CArG box sequence present in the human p21 promoter was not conserved in the p21 promoter of other mammals. However, a CArG-like sequence with one or two nucleotide differences from the consensus CC(A/T)₆GG sequence was present in the rat and mouse p21 promoter regions. Some transcription factors tolerate differences of more than one nucleotide from the consensus binding sequence, and in many SRF-driven promoters, the CArG-like sequence functions as an SRF-binding *cis*-element (27–31). Therefore, the CArG-like sequence in other species may serve the same function as the CArG box does in the human p21 promoter.

In summary, here we showed that myocardin overexpression induced cell phenotypic switching in human uterine leiomyosarcoma from a dedifferentiated to a differentiated phenotype and that it delayed the cell cycle progression by increasing p21 levels, thereby inhibiting the G₁-S phase transition. This is the first report to describe myocardin's regulation of human p21 gene transcription. Furthermore, we showed correlative downregulation between myocardin and p21 in clinical leiomyosarcoma samples. Our findings suggest that myocardin can exert an antiproliferative function in human uterine leiomyosarcoma and may therefore be a useful therapeutic target for the treatment of human uterine leiomyosarcoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

Grant-in-aid for Scientific Research 20240038 from Japan Society for the Promotion of Science (K. Sobue).

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Received 4/21/09; revised 9/27/09; accepted 10/27/09; published OnlineFirst 1/12/10.

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