Enhancer of Polycomb1, a Novel Homeodomain Only Protein-binding Partner, Induces Skeletal Muscle Differentiation*

Received for publication, December 6, 2006 Published, JBC Papers in Press, December 27, 2006, DOI 10.1074/jbc.M611198200

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Homeodomain only protein, Hop, is an unusual small protein that modulates target gene transcription without direct binding to DNA. Here we show that Hop interacts with Enhancer of Polycomb1 (Epc1), a homolog of a Drosophila polycomb group gene product that regulates transcription, to induce the skeletal muscle differentiation. Yeast two-hybrid assay with the human adult heart cDNA library revealed that Hop can associate with Epc1. The amino-terminal domain of Epc1 as well as full Epc1 physically interacted with Hop in mammalian cells and in yeast. Epc1 is highly expressed in the embryonic heart and adult skeletal muscles. Serum deprivation induced differentiation of H9c2, a myoblast cell line, into skeletal myocytes, and Epc1 was up-regulated. Differentiation of H9c2 was induced by Epc1 overexpression, although it was severely impaired in Epc1-knockdown cells. Co-transfection of Hop potentiated Epc1-induced transactivation of myogenin and myotube formation. Hop knock-out mice elicited a decrease in myosin heavy chain and myogenin expressions in skeletal muscle and showed delay in hamstring muscle healing after injury. Differentiation was impaired in skeletal myoblasts from Hop knock-out mice. These results suggest that Epc1 plays a role in the initiation of skeletal muscle differentiation, and its interaction with Hop is required for the full activity.

In cardiac muscle differentiation, the fine regulation of temporal and spatial expression of heart-specific genes in response to developmental events may require precise interactions among various transactivators and repressors, giving rise to cardiomyocytes from primordial cardiomyoblasts. Hop (homeodomain only protein) is an example of fine regulation of cardiomyocyte-specific gene transcription (1, 2). Likewise, muscle-specific gene regulations are dependent on the precise orchestration between series of specific transcription factors and other ubiquitous factors.

Hop encodes a 73-amino acid protein that includes a 60-amino acid motif homologous to the homeodomain of Hox transcription factors. Unlike other Hox homeodomains that regulate embryonic patterning, cell fate specification, and organ formation, however, Hop is unable to bind DNA. Nevertheless, Hop can function to modulate transcription by interfering serum-response factor (SRF)\textsuperscript{4}-dependent transcription of cardiac-specific genes. Thus, half of homozygous Hop mutant mouse embryos die of failure of cardiac muscle compaction (1), and the hearts of Hop mutant neonates elicited hypercellularity (2).

Enhancer of Polycomb1 (Epc1) is an unusual member of the polycomb group (PcG) gene family. Although homozygotic mutations of E(Pc) in Drosophila are lethal in the embryo, heterozygous mutations do not by themselves result in a zygotic homeotic phenotype. Rather, mutations in E(Pc) enhance the phenotypes in mutations of other PcG genes (3, 4). Although Epc1 itself does not have enzymatic activity, the complex, including Epc1, is reported to possess both activating and repressive activities as a transcription regulator. For example, human EPC1 was also shown to interact with the transcriptional repressor RET finger protein, RFP (similar to RING1) (5). Likewise, transcriptional repressor complex E2F6 containing Epc1 has a function to regulate the cell cycle.

As an effort to understand the role of Hop in muscle-specific gene regulation, we sought a Hop-binding candidate by the yeast two-hybrid screening technique and found that Epc1, a novel Hop-interacting partner, initiates skeletal muscle differentiation and that its interaction with Hop is required for the full activity.

\textsuperscript{1}This work was supported in part by Korea Research Foundation Grant KRF-2004-015-E00089 and Korea Health 21 R&D Project Grant A060380 from the Ministry of Health and Welfare, Republic of Korea. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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\textsuperscript{4}The abbreviations used are: SRF, serum-response factor; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; RT, reverse transcription; MHC, myosin heavy chain; VDCC, voltage-dependent calcium channel; MCK, muscle creatine kinase; PcG, polycomb group; E, embryonic day.
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Experimental Procedures

Animals and Plasmid Constructs—Pregnant CD1 mice were purchased from Daehan Biolink (Daejeon, Korea). Hop knock-out mice were kindly provided by Prof. Jonathan A. Epstein (University of Pennsylvania, Philadelphia). The experimental protocols were approved by the Chonnam National University Medical School Research Institutional Animal Care and Use Committee.

pcDNA3.1-mouse Hop-myc was described previously (6). pGBK7-human HOP and pcDNA3.1-human HOP-V5/His were prepared by subcloning after PCR amplification of coding region of Hop from pCDNA3.1-HOP-myc or from human adult heart cDNA library (BD Biosciences). All constructs were confirmed by sequencing. pCMV-myc-mouse Epc1 was kindly provided by Dr. Kristian Helin (Biotech Research and Innovation Centre, Copenhagen, Denmark). Structures of human EPC1 and its truncated mutants were described previously (5).

Four Epc1 truncated mutants of the EPC domain-containing region (EPC A, amino acids 2–285), EPCB domain-containing region (EPC B, amino acids 280–496), EPCc domain- and glutamine-rich-containing region (EPC CQ, amino acids 493–620), or CQ and carboxyl-terminal region (EPC CQCT, amino acids 493–836) are shown in Fig. 2A. For myogenin minimal promoter (7), −185 to +45 bp from the transcription start sequence was amplified from mouse genomic DNA and subcloned into pGL3 basic vector (Promega, Madison, WI). For antisense strategy, complementary sequences of the 1–516-nucleotide region of rat Epc1 was amplified and subcloned into pcDNA6/myc-HisA (Invitrogen) containing the blasticidin resistance gene.

Yeast Two-hybrid Screening for Binding Candidates—All assays were carried out according to the protocols of MATCH-MAKER GAL4 two-hybrid system 3 (BD Biosciences). pGBK7-human HOP plasmid was co-transfected with human adult heart cDNA library into the AH109 strain of Saccharomyces cerevisiae. Approximately 1250 independent clones were found to grow on a minimal medium lacking leucine, tryptophan, and histidine with 2.5 mm 3-amino-1,2,4-triazole. To confirm the positive reactions, a yeast one-to-one hybrid was performed in the AH109 yeast strains to detect the initiation of the lacZ reporter gene transcription qualitatively. Colony-lift filter assay was used to check the activity of β-galactosidase. Plasmid DNA from positive yeast clones were further characterized by sequencing and analyzed for gene homology by the BLAST data base.

Epc1 domain mapping study was done by yeast one-to-one match experiments. The pGBK7-human HOP plasmid was co-transfected with pACT2-EPC A, pACT2-EPC B, pACT2-EPC CQ, or pACT2-EPC CQCT into the AH109 strain of S. cerevisiae, and the histidine “jump-start” method was utilized.

Antibodies, Cell Cultures, and Transfection Study—Epc1 antibody was generated from Peptron (Daejeon, South Korea), with the epitope region of human Epc1 195–209 amino acids. The antibodies able to recognize myogenin (Sc-576, Santa Cruz Biotechnology), FLAG (M2, Sigma), V5 (Invitrogen) embryonic myosin heavy chain (MHCemb, MF20, Developmental Studies Hybridoma Bank), and desmin (Chemicon, Temecula, CA) were utilized.

H9c2, HeLa, COS7, and 293T cells were obtained from Seoul Korean Cell Line Bank (Seoul, Korea) and maintained with Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). Differentiation of H9c2 cells was induced by depriving the serum with 1% FBS as described previously (8).

To establish antisense Epc1 cell lines, pcDNA6/myc-HisA-antisense Epc1 and vector was transfected to H9c2 cells. The cells were treated with 5 μg/ml blasticidin (Invitrogen), and positive colonies were selected 2 weeks later. Epc1-overexpressing cell lines were generated by co-transfecting pCMV-myc-Epc1 and pSV2-hygro (Invitrogen). Positive cells were selected by adding 50 μg/ml hygromycin (Invitrogen) for 2 weeks.

For transient transfection of Hop and Epc1, pcDNA3.1-HOP-V5/His and/or pCMV-myc-Epc1 was introduced to H9c2 cells by Lipofectamine Plus reagent (Invitrogen). The differentiation rates were determined morphologically by analyzing multinucleated myotube formation and nuclei clustering using phalloidin staining. The nuclear fusion indices were measured as described (9). The rate of myoblast fusion was expressed as the percentage of nuclei in fused cells of the total number of nuclei in randomly chosen fields as viewed under a microscope. Cells containing more than three nuclei were regarded as fused cells.

The effect of Hop and Epc1 on cell proliferation/survival was measured by direct cell counting. Transfection of pcDNA3.1-HOP-V5/His and/or pCMV-myc-Epc1 to H9c2 cells was repeated every other day; the cells were lifted at the 5th days, and the numbers were counted as described previously (10).

Primary myoblasts from either wild type or Hop knock-out mice were prepared as described (11) with a slight modification. Muscle tissues from hindlimbs were dissociated by mincing with microdissecting scissors followed by treatment with collagenase (2 mg/ml; Worthington) for 60 min and trypsin/EDTA (0.25% Invitrogen) for 20 min. Then the enzymatic digestion was stopped by the addition of DMEM containing 15% FBS. The cell suspension was filtered through a 100-μm sieve (Cell Strainer Nylon, Falcon) and collected. The cells were resuspended, and the contaminating fibroblasts were removed by preplating the cell suspension for 1 h at 37 °C, 5% CO2. The myoblasts were plated on culture dishes coated with 0.2% collagen and grown in 15% FBS in DMEM supplemented with 5 nx basic fibroblast growth factor (BIOSOURCE). Forty eight hours later, the cells were preplated once more to remove the extra fibroblast. To induce differentiation, the growth medium was changed to 2% horse serum in DMEM.

Promoter analysis was described previously (1). H9c2 cells were plated in 24-well plates, and transfections were carried out. Total DNA used in each transfection was adjusted by adding the pcDNA3.1 vector. Cells were harvested 48 h after transfection, and luciferase activity was measured (Promega). Transfection rate was normalized by β-galactosidase activity.

Immunoprecipitation and Western Blot—Protocols for immunoprecipitation and Western blot were described previ-
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For immunoprecipitation, 293T cells were transfected with pCMV-FLAG-Epc1 full or truncated mutants together with pcDNA3.1-HOP-V5/His by FuGENE 6 (Roche Diagnostics). The cells were harvested by lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, with protease inhibitor mixture (Roche Diagnostics), and 0.5% v/v Igepal CA-630. Whole cell lysates were incubated with specific antibody or normal mouse IgG. Immunocomplexes was pulled down by incubating the samples with protein A/G-agarose (Santa Cruz Biotechnology). After washing the agarose beads three times, the immunoprecipitates were separated by electrophoresis on 10% SDS-PAGE and subjected to immunoblot. The antibodies for Western blot analysis were anti-myogenin (1:500), Epc1 (1:200), actin (1:2000), and tubulin (1:1000).

Immunohistochemistry and Fluorescent Immunocytochemistry—To examine the tissue distribution of Epc1 in the mouse embryo, immunohistochemistry was performed. Mouse embryos were obtained from pregnant mice at 13.5, 15.5, and 17.5 days post-coitum. Sagittal sections were obtained and utilized for immunohistochemistry as described (12). To test the specificity of the α-Epc1 antibody, an excess amount of blocking peptide that had been used to raise the antibody was simultaneously incubated with α-Epc1 antibody. Protocol for fluorescent immunocytochemistry was described previously (6).

Northern Blot Analysis and RT-PCR—Northern blot analysis was performed using a specific probe spanning 432 bp in EPC A region (13). Total RNA from embryonic mouse heart and differentiated H9c2 cells was extracted using TRizol reagent (Invitrogen) and subjected to reverse transcription reaction followed by semi-quantitative PCR amplification. The amplified DNA fragment was confirmed by sequencing after cloning into pCR2.1 TOPO TA vector (Invitrogen). The primer sequences for RT-PCRs can be provided upon request.

In Vivo Wound Healing Assay—Seven- or 8-week-old wild type or Hop knock-out mice were utilized for in vivo wound healing assay as described previously (14). Under anesthesia, right hamstring muscles were cut transversely using a 15 scalpel and then sutured with a modified Kessler stitch using nylon 7-0 wires. After 1 or 3 weeks, the mice were sacrificed, and the muscles were isolated. The transverse sections at suture sites were obtained and utilized for hematoxylin and eosin staining or desmin staining or desmin immunohistochemistry.

RESULTS

Identification of Hop-interacting Partners—We postulated that Hop might be involved in muscle differentiation by interacting with other transcription regulators. By utilizing a yeast two-hybrid technique, we screened adult human heart cDNA library with a bait of full-length human HOP. From this screen we identified 300 clones that were His- and β-galactosidase-positive. Among them ~100 clones were sequenced and blasted to the public data base. Six positive clones were proven to encode sequences of the amino-terminal part of Epc1. Enhancer of polycomb is highly conserved and is known to have several subtypes by gene duplication or alternative splice in mice as follows: Epc1L, Epc1S, Epc2L, and Epc2S (5, 15). In our yeast two-hybrid screening experiments, Epc1L was detected.

To confirm the interaction further, in vivo interaction assay in yeast was performed with pGBK7-human HOP and pACT2-Epc1. HOP did show a strong association with Epc1, although it had no apparent interaction with an empty pACT2 vector (Fig. 1A). To test if the interaction takes place in mammalian cells, we performed immunoprecipitation and found that Hop successfully recruited Epc1 (Fig. 1B).

We next tried to see the subcellular localization of Epc1 in relation to that of Hop by immunofluorescent staining. After transient transfection of both pCMV-myc-Epc1 and pcDNA3.1-HOP-V5/His, COS7 cells were stained with anti-Epc1 polyclonal and anti-V5 monoclonal antibodies. As shown in Fig. 1C, the majority of Epc1 (Fig. 1C, panel a) and Hop (Fig. 1C, panel b) was co-localized in the nucleus of COS7 cells, as demarcated by 4,6-diamidino-2-phenylindole nucleus staining (Fig. 1C, panel c). The merged image is shown in Fig. 1C, panel d.

Identification of Hop-interacting Domain of Epc1 by Deletion Mapping—Epc1 has four distinct structures named EpcA, EpcB, EpcC, and a Qx domain; all of them are conserved in many species, including Drosophila, Caenorhabditis elegans, yeast, mouse, and human (15). To identify the interacting domains, pACT2 vector versions of the truncated human Epc1 (Fig. 2A) were used for yeast one-to-one match experiments. Strong interaction was detected in the EPC A region. In contrast, the EPC B, EPC CQ, and EPC CQCT regions showed no activity or very weak interacting activity (Fig. 2B). Each experiment was done by using four independent colonies, and the results are summarized in Fig. 2C.
Hop-interacting domain of Epc1 was further confirmed by immunoprecipitation in mammalian cells. FLAG-tagged full or truncated human EPC1 plasmids were co-transfected with pcDNA3.1-human HOP-V5/His to 293T cells. The upper panel of Fig. 2D shows the expression of each truncated mutant of Epc1 with diverse molecular weights, and the middle panel shows the constant expression of V5-tagged HOP in each experimental condition. After immunoprecipitation with anti-FLAG antibody, HOP was detected with anti-V5 antibody. HOP was pulled down by EPC full or EPC A, confirming that EPC A is responsible for the interaction with HOP (Fig. 2D).

We next investigated whether the lack of interactions of truncated mutants was caused by loss of nuclear localization. Epc1 fragments as well as full clone were transfected into HeLa cells and stained with α-FLAG antibody (Fig. 2E). EPC A (Fig. 2E, panel b) and EPC B (panel c) were still localized in the nucleus as was EPC full (panel a), but EPCs, which have carboxy-terminal regions only, were seen in the cytoplasm (panels d and e), suggesting that alteration in the structure and thereby failure of nuclear localization might be one of the causes of losing the interaction with Hop in the mammalian cells.

**Expression Patterns of Epc1**—The tissue distribution of Epc1 was examined. Northern blot analysis for Epc1 showed expression of the 4-kb transcript. Although it was expressed ubiquitously, Epc1 was abundant in heart and skeletal muscles (data not shown). In Western analysis with mouse adult tissue blot, 90-kDa bands were detected (Fig. 3A, upper panel), which was confirmed by the presence of the same size band in pCMV-myc-Epc1-transfected 293T cells (rightmost lane). Those bands completely disappeared when the antibody was premixed with blocking peptide at excess amounts. Muscle tissues such as skeletal or ventricular muscles were strongly positive for Epc1 expression.

We further investigated the time course of Epc1 expression at the ages ranging from embryonic day 11.5 to postnatal week 8. Northern blot analysis showed that Epc1 expression in the heart is gradually decreased with aging (Fig. 3B). The changes in the Epc1 expression as well as cardiac specific genes were further examined by semi-quantitative RT-PCR, and the typical gel pictures are shown in Fig. 3C. As expected, α-myosin heavy chain (α-MHC), one of the cytoskeletal proteins in the differentiated cardiomyocytes, was turned on in late embryonic stage and after birth, whereas β-MHC, a fetal cardiac protein, was expressed only in the embryonic period and in postnatal day 1, as suggested in previous reports (16). As shown in Northern blot, Epc1 tran-
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FIGURE 3. Expression patterns of Epc1 in mouse. A, tissue distribution of Epc1 in 8-week-old mice. Upper panel, typical Western blot analysis for the tissue distribution. α-EPC1 polyclonal antibody was utilized for the Western blot. Epc1 is highly expressed in skeletal muscles and heart ventricle. Rightmost lane shows the Epc1 detected with α-EPC1 antibody at expected size in pCMV-mouse Epc1-myc-transfected 293T cells. Lower panel, expression of actin. Sk, skeletal. B, Northern blot analysis for Epc1 mRNA expression in the embryonic heart. The expression of Epc1 mRNA is gradually decreased with aging from embryonic day (d) 11.5 to postnatal week (w) 8. C, RT-PCR for Epc1 and Hop as well as cardiac markers, α-MHC and β-MHC. D, Epc1 expression in embryonic mouse. Epc1 expression in the heart in the embryonic 13.5-day period (panels a and b). Panel b, antibody was preincubated with blocking peptide. E, whole mount sagittal section of embryonic 13.5-day (panel c), 15.5-day (panel d), and 17.5-day (panel e) mouse embryo probed with α-Epc1 antibody. Arrow in panel c indicates staining in the heart, whereas arrow in panel e shows Epc1 immunoreactivity in the tongue. Epc1 expression is gradually decreased in the heart, and it is increased in the skeletal muscles such as tongue by aging (panels c–e).

FIGURE 4. Epc1 expression is up-regulated during H9c2 differentiation. A, Epc1-knockdown and vector-transfected H9c2 cell lines were established, and those cell lines underwent serum deprivation. Note that H9c2 cells were elongated to form myotube in vector cell lines (upper panels) and that Epc1-knockdown cells failed to differentiate (lower panels). d, days. B, RT-PCR analysis to show changes of expression of Epc1 or Hop in H9c2 differentiation. Epc1 was increased in the early phase of differentiation, whereas Hop was not changed. C, Western blot analysis to check the Epc1 expression. Epc1 protein level was dramatically increased by serum deprivation and then it was down-regulated in the late phase of differentiation. Myogenin, a key transcription factor for muscle differentiation, transiently increased in the early phase.

The expression patterns of Epc1 in embryonic tissue were further investigated by immunohistochemistry with E13.5 to E17.5 embryo sections. Strong positive immunoreactivity was detected in E13.5 heart (Fig. 3D, panel a), which disappeared completely by premixing the excess blocking peptides (Fig. 3D, panel b). However, Epc1 expression in the heart (arrow in Fig. 3D, panel c, at E13.5) was gradually decreased by aging (Fig. 3D, panel d, E15.5, and Fig. 3D, panel e, E17.5 day). Interestingly, skeletal muscles such as tongue were gradually increased by aging (arrow in Fig. 3D, panel e), suggesting the differences in the expression patterns in both types of sarcomeric muscles. Epc1 was also well expressed in the endothelium, spinal ganglia, and cartilage. Although choroidal plexus and meninges were positive, brain parenchymal structures did not show any significant immunoreactivities.

Changes of Epc1 Expression in Differentiating H9c2, a Myoblast Cell Line—Changes in expression patterns of Epc1 in skeletal and cardiac muscle led us to speculate that Epc1 is involved in the process of muscle differentiation. H9c2, a cardiomyoblast cell line originated from rats, is widely employed for the in vitro model of muscle differentiation (17). Differentiating H9c2 cells could have either cardiac or skeletal phenotypes, depending on the presence of retinoic acid in differentiation media (18). In our experimental model, serum starvation with 1% FBS induced elongation of H9c2 cells and multinucleation. Typical serial changes in morphology are shown in Fig. 4A, which is accompanied by induction of myogenin (Fig. 4C).

In our model, H9c2 seemed to undergo phenotype switch from cardiac to skeletal muscles during differentiation; the expression of cardiac type of voltage-dependent calcium channel (VDCC)α1 was decreased by differentiation, whereas those of muscle creatine kinase (MCK) and skeletal type of VDCCα1 were gradually up-regulated by differentiation (Fig. 5B). Interestingly, Epc1 was transiently up-regulated in the early phase of differentiation, whereas Hop was not changed (Fig. 4, B and C), suggesting that Epc1 plays an important role in the initiation of H9c2 differentiation.

To delineate the role of Epc1 in the differentiation, we generated the Epc1-knockdown cell lines with antisense strategy as well as the stable cell lines overexpressing Epc1 (Fig. 5A) and
induced the differentiation by serum starvation. As evidenced in Fig. 4A in which H9c2 differentiation is markedly impeded in knockdown cell lines, the increases in differentiation markers such as MCK and skeletal VDCCa1 were significantly reduced (Fig. 5C). In Epc1-overexpressing cell lines, however, induction of those markers was greatly enhanced (Fig. 5D).

Skeletal muscle fate determination/differentiation processes are governed by several distinct transcription factors such as MyoD, Myf5, myogenin, and Myf6/Mrf4 (19). Therefore, to investigate whether H9c2 cells elicit the skeletal muscle phenotype during its differentiation process, we tried to examine the expression of those four myogenic transcription factors. Myf5 and Myf6/Mrf4 were increased in the late phase of H9c2 differentiation, whereas MyoD was transiently up-regulated in the early phase of differentiation (Fig. 5B). Myogenin, a key step regulator of differentiation of skeletal muscle (20), was abruptly increased in the beginning of the serum starvation. Strikingly, all of those changes in expression levels were significantly blunted or deranged in Epc1-knockdown cells (Fig. 5C), although they were enhanced in Epc1-overexpressing cells (Fig. 5D).

**Forced Expression of Hop and Epc1 Induces H9c2 Differentiation**—The roles of Epc1 and Hop in the differentiation of H9c2 cells were further investigated by transient transfection, followed by the evaluation of cell morphology (Fig. 6A) or myogenin expression (Fig. 6B). Hop itself did not induce significant changes in the cell morphology (Fig. 6A, panel b), compared with mock-transfected H9c2 cells (Fig. 6A, panel a). Epc1 transfection, however, caused the cells to be thinned or elongated and often fused to form multinucleated syncytia appearing like myotubes (Fig. 6A, panel c). The changes of the cell shapes became more prominent by co-transfection of Epc1 and Hop (Fig. 6A, panel d). The number of myogenin-positive nuclei as well as the expression level were increased in Epc1-transfected H9c2 cells (Fig. 6B, panel g) compared with mock- (Fig. 6B, panel e) or Hop-transfected cells (Fig. 6B, panel f). The myogenin staining was much stronger in Hop/Epc1-co-transfected H9c2 cells (Fig. 6, panel h). The expression of myogenin was quantified by Western blot analysis after transfection of Epc1 or Hop. Myogenin expression was increased when Epc1 or Epc1 + Hop was transfected to H9c2 cells (Fig. 6C).

The transcript level of myogenin was also increased by Epc1 or Hop + Epc1 transfection (Fig. 6D). Interestingly, skeletal VDCCa1 was significantly increased by the co-transfection of Hop and Epc1 (Fig. 6D). On the contrary, the absence of Epc1 reduced myogenin and skeletal VDCCa1, and Hop did not affect the expression (Fig. 6, C and D).

We next investigated if Epc1 can activate myogenin promoter. Because the myogenin promoter, which spans from −185 to +48 bp of its transcription initiation site, has been reported to have essential regulatory functions of the gene (7), we subcloned the minimal promoter region to pGL3 basic vector to drive luciferase activity. By testing the basal promoter activity, 20 ng of myogenin minimal promoter (−185 to +45)-luciferase construct was selected for optimal reporter constructs (data not shown). Epc1 dramatically activated the myogenin minimal promoter activity in a dose-dependent fashion (Fig. 6E), although Hop did not increase the activity in the range from 25- to 100-ng doses (data not shown). To check if Hop potentiates the transactivation of Epc1 on the myogenin minimal promoter, we selected the doses of 100 and 50 ng for Epc1 and Hop, respectively. One hundred nanograms of Epc1 doubled the promoter activity, but the transactivation was further increased by co-transfection of 50 ng of Hop (Fig. 6F).

We further analyzed the myotube formation by counting the multinucleated cells after the transfection. As shown in Fig. 6G, Epc1 transfection increased the number of multinucleated cells, and co-transfection of Hop further increased the multinucleation. In serum deprivation, Epc1-knockdown cells failed to form multinucleation, whereas Epc1-overexpressing cells elicited potentiation of myotube formation (Fig. 6H). Cellular differentiation into specialized cells usually accompanies blunting of cellular proliferation. Fig. 6I showed the reduced cell number when Epc1 and Hop were co-transfected into H9c2 cells, suggesting those transfected cells underwent differentiation.

**Delayed Wound Healing in Hop Knock-out Mice**—The observations that Epc1 initiates myocyte differentiation and that Hop is required for the full activity led us to speculate if myocyte
maturation is impaired in Hop knock-out mice. To confirm this, the expressions of myogenin and MHCemb were investigated in postnatal day 1 mice with either wild type or Hop knock-out. As expected, Hop knock-out mice showed reduction of the expression of cardiac or skeletal myogenin as well as MHCemb (Fig. 7A), which was quantified by immunoblotting the myogenin in the skeletal muscle at postnatal day 1 (Fig. 7C).

Hop was expressed in skeletal muscle, although the amount was smaller than in heart (Fig. 7B).

We further investigated if the differentiation capacity of myoblast is attenuated in Hop knock-out mice. Muscle-specific genes as well as myogenic regulatory factors were significantly reduced in Hop-lacking skeletal myoblasts (Fig. 7E). Next, skeletal myoblasts from either adult wild type or knock-out mice underwent serum deprivation, and multinucleation at the 3rd day of differentiation (Fig. 7D) and myogenin expression during differentiation (Fig. 7F) were significantly reduced, suggesting the impairment of skeletal muscle differentiation.

Although we and others (1) could not observe any clear skeletal deformities in Hop knock-out mice, we assumed that muscle regeneration to injury to reactivate the myoblasts might be altered. Thus, we evaluated the capacity of myoblast reactivation by an in vivo wound-healing assay. At the 1st week after injury, the cutting margins of hamstring muscle of Hop knock-out were filled with inflammatory cells (Fig. 7G, panel h), and desmin-positive muscle bundles were barely seen (Fig. 7G, panel j), compared with wild type control (Fig. 7G, panels g and i). In Hop knock-out mice, at the 3rd week after injury, inflammatory cells were reduced (Fig. 7H, panel i); however, the muscle fibers were smaller and scantier (Fig. 7H, panel n) than wild type control (Fig. 7H, panels k and m), showing that muscle regeneration was impaired in those knock-out mice.

**DISCUSSION**

To delineate the functional role of Hop in the muscle differentiation by seeking its interacting protein, we utilized yeast two-hybrid assay with the human adult heart cDNA library and found Epc1 as a novel Hop-interacting partner. In addition, our results highlight Epc1 as a regulator in skeletal muscle differentiation, in association with Hop, by inducing the expression of key transcription factors in myoblast differentiation.

The previous observations that some of the Hop null mice die as embryos because of defects in cardiac muscle maturation implicate its roles in cardiac differentiation. Because Hop was...
first reported to repress SRF-dependent gene transcription (1), the alterations in SRF-dependent signal cascade would be considered first as the cause of death in Hop null mice. Indeed, SRF and its cognate DNA-binding CArG element also control expression of genes restricted to the muscles as well as a number of growth-related genes (21). Not only is it involved in cardiac muscle formation, it is also important in differentiation of smooth muscle (22) or skeletal muscle (23).

In this study, in addition to SRF, we found that Epc1, a novel muscle differentiation regulator, together with Hop, could be an alternative answer for the skeletal muscle maturation; we identified Epc1 as an interacting protein of Hop and demonstrated that it induces H9c2 myoblast differentiation. Epc1 and Hop were detected in the nucleus and largely co-localized, suggesting the functional importance of their interaction. The EPC A domain, the amino-terminal part, is highly conserved (15) and has also been known to interact with other transcription factors such as RET finger protein (5) or E2F6 (24). Indeed, by computer-based prediction methods, Perry (25) recently reported that the amino-terminal part of the EPC protein (96–199 amino acids), which spans a substantial part of the EPC A region (2–285 amino acids) that we used in this study, mediates protein-protein interactions necessary for chromatin structure formation and/or recognition. We also demonstrated that the EPC A region determines the nuclear localization of the protein and is responsible for the interaction with Hop, suggesting its possible chromosome regulatory role in association with other transcription factors.

Although Epc1 was abundant in embryonic heart, its expression was gradually down-regulated with age, whereas it is up-regulated in skeletal muscle, suggesting that it may function to determine the direction of cell fates during myoblast differentiation. Indeed, in the H9c2 differentiation model, Epc1 was transiently up-regulated in the differentiation process. When cultured in low serum-containing medium, H9c2 myoblasts, derived from embryonic rat heart (17), exit the cell cycle and differentiate into myocytes to form myotubes. Thus, H9c2 has been used as an in vitro differentiation model of both skeletal and cardiac muscle, because these cells can show electrophysiological and biochemical properties of both tissues, expressing acetylcholine or skeletal or cardiac isoforms of the L-type voltage-dependent Ca\(^{2+}\) channels (26). Most interestingly, in our experimental model we observed that cardiac phenotypes such as cardiac VDCC\(a1\) (Fig. 5B) dominate in the early phase of differentiation. However, persistent serum deprivation induces skeletal phenotype determined by MCK or skeletal VDCC\(a1\) (Fig. 5B) in the late phase. Strikingly, these phenotypic shifts were completely abolished in Epc1-knockdown H9c2 cells (Fig. 5C), although they were potentiated in Epc1-overexpressing cell lines (Fig. 5D), implicating the critical role of Epc1 in the initiation of sarcomeric muscle differentiation.

Next we tried to prove the assumption that interaction of Epc1 with Hop may also play a role in the myoblast differentiation process. To H9c2 cells, we transfected Epc1 and Hop and examined cell survival, morphology, and expression of myogenin, a key transcription factor in H9c2 myoblast differentiation (27, 28). Our results clearly indicate that Epc1 induces myogenin expression as well as elongation of the cells to make multinucleated syncytia (myotubes), followed by reduction in cell survival. In contrast, knocking down Epc1 significantly
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reduces the myotube formation. Interestingly, those differentiation phenotypes were further potentiated by co-transfection of Hop (Fig. 6).

Requirement of Hop in muscle differentiation is further confirmed with Hop knock-out mice. In the absence of Hop, myogenin and MHCemb expressions in heart and skeletal muscles were significantly reduced. Moreover, isolated skeletal myoblasts from Hop knock-out mice showed defects in differentiation induced by serum deprivation. In addition, these defects in muscle differentiation seem to cause the delay in wound healing after hamstring injury in Hop knock-out mice (Fig. 7).

The PcG gene family, to which Epc1 belongs, has many component proteins that primarily induce gene silencing and thereby establish memory system in dividing cells by modifying chromatin structures (29). One of PcG protein prototypes, Enhancer of Zeste Homolog 2 (EZH2), which has been implicated as a cell cycle regulator in various cancers (30–32), is also directly associated with muscle differentiation (33). In addition, Rae28, an alternate PcG protein member regulates cardiac morphogenesis (34). Likewise, one of Epc1-binding partners, E2F6 (24), is recently emphasized in the regulation of the exit and re-entry of myocytes from the cell cycles (35). Thus, mammalian PcG genes are believed to play a key role in organogenesis, including muscles, by modifying the expression of a target gene or by regulating cell cycle.

Still the precise functional role of Epc1 in the skeletal muscle differentiation remains unclear. Although we have observed that some muscle-specific transcription regulators are increased by Epc1, it is questionable if Epc1 switches on to make myocytes by turning on the transcription factors or it may simply make the cells exit from the cell cycle. Indeed, the previous report that E2F6, together with Epc1, regulates transcription in cell proliferation-dependent manner (24) raises the possibility that Epc1 has a cell cycle regulatory role rather than a differentiation-initiating function mediated by transactivation. Also, rather than by Epc1 itself, Epc1-mediated initiation of muscle differentiation might be caused indirectly by the interaction with SRF. Although we have observed that Epc1 interferes with the interaction of Hop with SRF (data not shown), the significance of the Hop-Epc1 interaction in association with SRF in muscle differentiation or in cell cycle regulation remains to be elucidated. Rather, those proteins could be simple components of a large complex regulating chromatin structure/function.

In this study, we clearly demonstrate the novel phenotype of Hop knock-out mice, i.e. a defect in skeletal myoblast differentiation. The delay in muscle formation seems to be subtle, because we and others (1) could not observed any significant deformities in musculature. Of course, we cannot conclude that the defect in muscle formation is solely caused by loss of the Hop-Epc1 interaction, because other binding partners of Hop should also be considered. However, at least in part, the interaction might play a critical role in the initiation of muscle differentiation. The phenotype evaluation of Epc1 knock-out mice that lose Epc1 expression in specialized tissues is expected to answer the questions.

Acknowledgments—We are grateful to Prof. Jonathan A. Epstein, University of Pennsylvania, for the helpful suggestions and to Professor Emeritus Young Joong Kook, Chonnam National University Medical School, for reviewing the manuscript.

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