Basic Helix-Loop-Helix Transcription Factor Epicardin/capsulin/Pod-1

Suppresses Differentiation by Negative Regulation of Transcription*

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Running Title: Epicardin/capsulin /Pod-1 is an Inhibitor of Differentiation
Summary

Epicardin/capsulin/Pod-1, expressed in skeletal myoblasts within branchial arches and in the condensing mesenchyme, is a member of the basic helix-loop-helix (bHLH) transcription factor family that is involved in various cell differentiation processes. In this study, we examined the functional properties of epicardin/capsulin/Pod-1 in differentiation. The yeast and mammalian two-hybrid systems showed physical associations between epicardin/capsulin/Pod-1 and E2A, both of which were present in the nuclei. The bHLH domains mediated this association. Ectopic expression of epicardin/capsulin/Pod-1 inhibited E2A-dependent activation of the exogenous and endogenous expression of the cyclin-dependent kinase inhibitor, \textit{p21 (WAF1/Cip1)} gene, and the \textit{muscle creatine kinase} gene that encodes the predominant creatine kinase isoform expressed in mammalian skeletal muscle. Transfection with epicardin/capsulin/Pod-1 siRNA abolished the epicardin/capsulin/Pod-1-mediated suppression of E12-dependent activation of the p21 promoter. Chromatin immunoprecipitation assay showed that epicardin/capsulin/Pod-1 was physically associated with the muscle creatine kinase promoter \textit{in vivo}. Moreover, terminal differentiation of C2C12 myoblasts was inhibited by exogenous introduction of epicardin/capsulin/Pod-1. These inhibitory functions of epicardin/capsulin/Pod-1 closely resemble those of the bHLH inhibitor Twist protein. These results indicate that epicardin/capsulin/Pod-1 functions as a negative regulator of differentiation of myoblasts through transcription in at least two distinct steps, cell growth arrest and lineage specific differentiation.
Introduction

Basic helix-loop-helix (bHLH) transcription factors have been demonstrated to play critical roles in cell fate determination including differentiation in a variety of tissues of both vertebrates and invertebrates (1, 2). The bHLH proteins form homodimers or heterodimers through the helix-loop-helix (HLH) domains and enable the basic regions to form a bipartite DNA-binding motif that recognizes the so-called E-box sequences, CANNTG (3). Typically, tissue-specific bHLH factors, such as MyoD and BETA2/NeuroD, dimerize with ubiquitously expressed bHLH factors, such as E2A gene products, and promote cell fate determination to differentiate into specific lineages (3, 4). The E2A gene encodes two alternatively spliced products, E12 and E47, which differ in their bHLH domains and hence their DNA-binding properties (5, 6).

Epicardin (7)/capsulin (8, 9)/Pod-1 (10) is a bHLH transcription factor expressed in branchial muscle precursors and mesenchymal cells at sites of epithelial-mesenchymal interactions in the kidney, lung, intestine, pancreas, spleen, developing respiratory, gastrointestinal, urogenital, and cardiovascular systems (7, 8, 10). The phenotypic analysis of homozygous epicardin/capsulin/Pod-1 mouse mutants reveals a critical role for epicardin/capsulin/Pod-1 in the formation of spleen (11), lung and kidney (12). Epicardin/capsulin/Pod-1 binds the E-box consensus sequence as a heterodimer with the ubiquitous bHLH protein E12 (8, 9). While epicardin/capsulin/Pod-1 seems to be involved in organogenesis in vivo, little is known concerning the functions of epicardin/capsulin/Pod-1 in controlling tissue-specific gene expression and differentiation.
bHLH factors are involved in at least two distinct steps, cell cycle arrest and tissue-specific gene expression. The E2A proteins, another bHLH factor Twist and the dominant negative-type HLH proteins Ids regulate expression of the gene for an inhibitor of cyclin-dependent kinases (Cdk) p21 (WAF1/Cip1) (13, 14), which is induced early during the differentiation program in myogenesis (15, 16). Previous studies using cultured cells have shown that MyoD promotes cell cycle arrest through induction of p21 in differentiating myoblasts (17, 18). In addition, the combination of p21 and a related molecule p57 has been shown to be essential for muscle differentiation during embryonic development in mice (19). bHLH factors also regulate expression of fibroblast growth factor receptor 3 (FGFR3) and muscle creatine kinase (MCK) genes (14, 20), which are responsible for differentiation of osteoblasts and myoblasts, respectively (21, 22).

To investigate the implication of epicardin/capsulin/Pod-1 in differentiation, we examined how epicardin/capsulin/Pod-1 is involved in the transcriptional regulation of cell cycle arrest and lineage specific gene expression. Ectopic expression of epicardin/capsulin/Pod-1 inhibited E12- and MyoD-induced transactivation of p21. This is also the case in the regulation of MCK expression, demonstrating that common transcriptional regulation controls cell cycle arrest and differentiation of myoblasts. Furthermore, introduction of epicardin/capsulin/Pod-1 hampered terminal differentiation of C2C12 myoblasts. These functional characteristics indicate that epicardin/capsulin/Pod-1 is a negative transcriptional regulator of differentiation-related genes similar to Twist.
EXPERIMENTAL PROCEDURES

_Yeast two-hybrid screening_—All plasmids and strains for two-hybrid analysis were obtained from the Matchmaker System-3 kit (Clontech). The bHLH domain of E12 (amino acids 510-654) was cloned in-frame with the GAL4-DNA-binding domain (DBD) in pGBK7 (Clontech), yielding pGBK7-E12 (bHLH). An MG63 cDNA library in the pGADT7 prey plasmid was co-transfected with the pGBK7-E12 (bHLH) bait plasmid into AH109 yeast cells. Yeast two-hybrid screening was performed as described by the manufacturers protocol (Clontech Matchmaker Two-Hybrid Protocol). For conditional growth assays, yeast cells transfected with the expression plasmids for pGAL4DBD-fusion constructs and pGAL4AD-epicardin were grown on the appropriate selective medium at 30°C for 5 days.

_Mammalian two-hybrid assay_—All plasmids and strains for mammalian two-hybrid assays were obtained from Promega. Subconfluent cultures of C3H10T1/2 cells ($2\times10^5$ cells/dish of a 60-mm diameter) were transfected with 2 µg of pG5luc reporter, pBIND chimeras, and pACT chimeras together with pCMV-β-Gal by the calcium-phosphate method. After incubation overnight with the DNA precipitate, cells were then cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 2% horse serum for a further 48 h for the luciferase assay. Luciferase and β-galactosidase assays were done.

_Cell culture_—The human osteosarcoma osteoblast-like cell line MG63, which is negative for p53 (23), C3H10T1/2 fibroblasts (Riken Cell Bank) and myoblast cell line C2C12 (Riken Cell Bank) were cultured in DMEM with 10% fetal bovine serum (FBS) at 37°C under a humidified atmosphere of 5% CO2.
Plasmid construction—E12 and E47 cDNAs were kindly given by C. Murre (University of California at San Diego). Expression vectors, pCMV-E12, pCMV-E47, pCMV-Twist (14), and pCMV-Id1 (24) were used. Reporter plasmids p21-luc (25) and pMCK-luc were generous gifts of X. F. Wang (Duke University), and S. D. Hauschka and J. Buskin (University of Washington), respectively. The expression vectors, pCMV-MyoD (26) and pRSV-CBP were generous gifts of S. J. Tapscott (Fred Hutchinson Cancer Research Center) and T. Nakajima (St. Marianna University), respectively.

The epicardin/capsulin/Pod-1 cDNA fragment was recovered from the pGADT7 prey plasmids by digestion with EcoRI and subcloned into the EcoRI site of pcDNA3 (Invitrogen), generating pCMV-epicardin. Epicardin/capsulin/Pod-1 cDNA was fused to pEGFP-C2 (Clontech) for expression of the green fluorescent protein (GFP), generating the vector pGFP::epicardin. Epicardin/capsulin/Pod-1 cDNA was fused to pCMV-Myc (Clontech) or pCMV-HA (Clontech) for expression of the Myc- or HA-tagged epicardin/capsulin/Pod-1, generating the vector pMyc-epicardin or pHA-epicardin, respectively.

Transient transfection and luciferase assay—Subconfluent cultures of MG63 cells, C3H10T1/2 cells or C2C12 myoblasts (2×10^5 cells/dish of a 60-mm diameter) were transfected with a total of 10 µg of expression and reporter plasmids by the calcium-phosphate method together with the β-galactosidase expression vector, pCMV-β-Gal, which was used as an internal control to monitor the transfection efficiency. After incubation overnight with the DNA precipitate, cells were then cultured in DMEM containing 2% or 10% FBS or 2% horse serum for a further 48 h for the luciferase assay and 10% FBS for a further 24 h or 72 h for immunostaining. Luciferase activity was assayed using the luciferase assay system (Promega).
and normalized to β-galactosidase activity, which was determined by the method of Rose and Botstein (27). All assays were performed at least three times in duplicate and representative data are presented. The results are the mean of different experiments ± standard error.

**Immunofluorescence stains**—Cells were fixed in phosphate-buffered saline (PBS) with 3.7% formaldehyde, permeabilized with Triton X-100 (0.1%) in PBS, and then treated with goat or horse serum to block nonspecific binding sites. Polyclonal rabbit antibodies against E2A (sc-349, Santa Cruz) and a mouse monoclonal antibody to human p21 (sc-817, Santa Cruz) were used. After permeabilization, cells were incubated for 1 h at room temperature with the above-mentioned primary antibodies at the respective dilutions of 1:100 to 1:250. Immune complexes containing E2A and p21 were detected with rhodamine-conjugated anti-rabbit immunoglobulin G (IgG) (Santa Cruz) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Chemicon), respectively. DNA in the nuclei was detected with 4’, 6’-diamidino-2-phenylindole (DAPI). In the quantitative analysis, a minimum of 150-positive cells was examined in each transfection under an Olympus fluorescence microscope (BX-FLA) or a confocal laser-scanning microscope (LSM510; Carl Zeiss).

**Differentiation of C2C12 myoblasts**—The myoblast differentiation assays were performed as described previously (28). In brief, C2C12 myoblasts were transiently transfected in DMEM containing 10% FBS (growth medium) with 2.5 µg of pCMV-β-Gal together with or without 2.5 µg of pCMV-epicardin per a 30-mm well. The total amount of DNA added to C2C12 cells was adjusted to 2.5 µg by addition of empty pCMV vector. After culture in DMEM containing 2% horse serum (differentiation medium) for 96 h, cells were fixed, permeabilized and stained with anti-troponin T (TnT) antibody (Sigma). Differentiation was evaluated by counting the number
of TnT-positive cells relative to that of β-galactosidase-positive cells.

Small interfering RNA (siRNA) —The coding strand sequence of the siRNA for epicardin/capsulin/Pod-1 was TTAAGGCCTTCTCCAGACTCAAG. siRNA duplexes were prepared by annealing two pairs of 21-ribonucleotide oligonucleotides synthesized by Qiagen. As a control, non-silencing siRNA was purchased from Dharmacon. Twenty-four h before transfection, mammalian cells were trypsinized and transferred to 6-well plates (5x10^5 cells/well). Reporter plasmids and expression plasmids were transfected by the calcium-phosphate method. Cells were incubated for 5 h and then transfected with 0.5 µg siRNA duplexes using TransMessenger Transfection Reagent (Qiagen) as described by the manufacturer for adherent cell lines. Forty-eight h later, cell lysates were prepared and subjected to Western blotting and luciferase assay, respectively.

Immunoblot analysis —Total cell lysates were fractionated by SDS-PAGE in a 12% gel according to standard protocols, and proteins were transferred to nitrocellulose filters. Polyclonal antibodies against β-galactosidase (Zymed), and mouse monoclonal antibodies to Myc epitope (Clontech), p21 (sc-817; Santa Cruz Biotechnology), MHC (MY-32; Sigma) and α-tubulin (DM1A; Sigma) were used at the respective dilutions of 1:400 to 1:1000 in blocking buffer (1% BSA and 0.1% Tween-20 in PBS) at room temperature for 2 h and immune complexes were detected by enhanced the chemiluminescence (ECL) method as described by the manufacturer (Amersham).

Chromatin immunoprecipitation (ChIP) assays—ChIP assays were done as described previously by Shang et al. (29). Approximately 1x 10^7 C2C12 cells were transfected with 5 µg of pMCK-luc and 15 µg of pHA-epicardin by the calcium-phosphate method and cultured in
DMEM containing 2% horse serum for 48 h. After washing twice with PBS, cells were cross-linked with 1% formaldehyde at room temperature for 10 min, rinsed with ice-cold PBS twice, and collected into ice-cold PBS. Cells were then resuspended in 0.3 ml of lysis buffer (1% SDS, 5 mM EDTA, 50 mM Tris-HCl, pH 8.1) with protease inhibitor cocktail (Roche) and sonicated three times for 15 s each (Tomy Ultrasonic disruptor, Model UD-201) followed by centrifugation for 10 min. Supernatants were collected and diluted in a dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1) with protease inhibitor cocktail followed by immunoclearing with 2 µg sheared salmon sperm DNA and protein G-Sepharose (45 µl of 50% slurry in 10 mM Tris-HCl, pH 8.1, 1 mM EDTA) for 2 h at 4°C. Immunoprecipitation was performed overnight at 4°C with anti-HA antibody (3F10; Roche) or anti-Myc antibody (Clontech). After immunoprecipitation, 45 µl protein G-Sepharose and 2 µg of salmon sperm DNA were added and incubation was continued for another 1 h. Precipitates were washed sequentially for 5 min each in TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), and buffer III (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), further washed three times with TE buffer and extracted with 1% SDS, 0.1 M NaHCO₃. Elutes were heated at 65°C for 6 h to reverse the formaldehyde cross-linking. DNA fragments were purified with a QIAquick Spin Kit (Qiagen, CA). A portion (4%) of purified DNA extraction was subjected to 30 cycles of PCR with primers specific for either the MCK enhancer ([+], 5'-GACACCGAGATGCCTGGTT-3'; [-], 5'-GATCCACCAGGGACAGGGTT-3'). As a control for DNA content, PCR reactions were performed with samples without immunoprecipitation. A portion (20%) of PCR product of each
reaction mixture was resolved through a 5% native acrylamide gel.
RESULTS

*Association of epicardin/capsulin/Pod-1 with bHLH of E12.* —We employed the yeast two-hybrid system to search for proteins that bind to E12, using the E12-bHLH region fused to the GAL4 DNA-binding domain as bait. In a screen of $2\times10^6$ human MG63 cell library clones, 35 clones specifically interacted with E12-bHLH. DNA sequence analysis of these clones revealed that half of them encoded epicardin/capsulin/Pod-1 fused in-frame to the transcriptional activation domain in the prey plasmid. We confirmed interaction of intact epicardin/capsulin/Pod-1 with either full length E12 or the bHLH domain of E12 (E12-bHLH) in a yeast two-hybrid assay (Fig. 1A and 1B). The result is consistent with the observation that epicardin/capsulin/Pod-1 heterodimerizes with the ubiquitous bHLH protein E12 (8). Epicardin/capsulin/Pod-1 did not bind to the Twist bHLH domain (Fig. 1B), indicating that the association of epicardin/capsulin/Pod-1 with E12 was specific.

Similar observations were made in mammalian cells. The pG5luc vector contains five tandem copies of the GAL4-binding site upstream of a minimal TATA box linked to the firefly luciferase gene (Fig. 1C). Cotransfection of this reporter plasmid with the expression vectors for the fusion proteins of GAL4DBD-epicardin bHLH and VP16-E12 effectively induced profound activation of the promoter, while introduction of each of GAL4DBD-epicardin bHLH and VP16-E12 showed slight and little, if any, activation, respectively (Fig. 1D). These results clearly indicate that E12 physically interacts with the bHLH domain of epicardin/capsulin/Pod-1.

This conclusion was further supported by colocalization of epicardin/capsulin/Pod-1 and E12 in the nuclei. GFP alone was in both the cytoplasm and nuclei in MG63 cells (Fig. 2b), while
the epicardin/capsulin/Pod-1-GFP fusion protein from pGFP::epicardin were found in the nuclei (Fig. 2e).

<<Figure 1>> <<Figure 2>>

**Transcriptional inhibition of the p21 gene by epicardin/capsulin/Pod-1**—Expression of p21 has been shown to parallel the irreversible withdrawal from the cell cycle, following the induction of MyoD in myogenic differentiation (17, 18, 30). We have previously shown that there is a close link between p21 induction and cell cycle arrest in MG63 cells (14). The MyoD/E12 heterodimer has been shown to bind to specific E-box DNA sequences and activate muscle specific genes (5, 20, 31). To gain insight into the biological significance of the interaction of epicardin/capsulin/Pod-1 with E12, we tested the role of epicardin/capsulin/Pod-1 in E2A-dependent activation of p21 transcription with MG63 cells, in which we have previously shown the inhibitory effects of Twist on E2A-dependent p21 promoter activation (14). MG63 cells were cotransfected with p21-luc, an E2A expression plasmid (pCMV-E12 or pCMV-E47) or/and pCMV-epicardin and luciferase activities were determined after 48 h culture. Concentrations of the epicardin/capsulin/Pod-1 plasmid were equivalent to those for Twist, at which muscle differentiation is inhibited (20, 32). When p21-luc was transfected along with pCMV-epicardin, a major reduction in luciferase activities was observed in either E12 or E47 expressing cells over a range of pCMV-epicardin concentrations used, similar to Twist (Fig. 3B). The magnitude of inhibition by epicardin/capsulin/Pod-1 was equivalent to those induced by Twist and Id1 except for the case of Id1 in E47-driven transcription (Fig. 3A). The inhibition of the E2A-dependent p21 promoter activation by epicardin/capsulin/Pod-1 was further enhanced by the addition of Twist or Id1 (Fig. 3A), suggesting that epicardin/capsulin/Pod-1, Twist and Id1 inhibit p21
expression in a cooperative manner. These results demonstrate that the epicardin/capsulin/Pod-1 functions as a negative transcriptional regulator in the E2A-dependent p21 promoter activation in MG63 cells, like Twist and Id1.

We further tested if epicardin/capsulin/Pod-1 protein could inhibit the expression of the endogenous p21 gene. MG63 cells were transfected with expression plasmids for E12 with or without epicardin/capsulin/Pod-1 and then examined for p21 expression by immunostaining. Upon introduction of E12, endogenous p21 expression was induced in cells expressing high levels of E12 even when cultured in high serum (Fig. 4A) as reported previously (14). Overexpression of epicardin/capsulin/Pod-1 abolished the endogenous p21 expression induced by E12 (Fig. 4B and Table 1), confirming the antagonistic function of epicardin/capsulin/Pod-1 in E12-induced gene expression.

The coactivator p300/CBP (cyclic AMP-responsive element binding factor CREB-binding protein) harboring histone acetyltransferase (HAT) activity has been shown to interact with E12 (33). HAT activity is important for decondensing the chromatin (34), and changing the accessibility of the transcription machinery (35). To analyze the functional implications of the interactions between E12-CBP complex and epicardin/capsulin/Pod-1, we studied the effects of epicardin/capsulin/Pod-1 on E12-CBP-dependent transcriptional activation of the p21 gene. Epicardin/capsulin/Pod-1-induced inhibition of p21 promoter activation in response to E12 was partly overcome by the addition of CBP in a dose-dependent manner (Fig. 3C). Taken together, these findings support the view that epicardin/capsulin/Pod-1 suppresses transcriptional activity conferred by a combination of E12 and CBP.

To confirm involvement of epicardin/capsulin/Pod-1 in regulation of p21 gene expression,
the effect of loss of function of epicardin/capsulin/Pod-1 was examined using siRNA specific to epicardin/capsulin/Pod-1. Transfection of MG63 cells with epicardin/capsulin/Pod-1 siRNA along with the epicardin/capsulin/Pod-1 expression vector relieved epicardin/capsulin/Pod-1-mediated suppression of E12-dependent activation of the p21 promoter (Fig. 3F), in concord with reduction of the expression of epicardin/capsulin/Pod-1 at the protein level (Fig. 3E). Introduction of epicardin/capsulin/Pod-1 siRNA alone induced little, if any, change in p21 expression (Fig. 3F).

**Figure 3** **Figure 4** **Table 1**

*Transcriptional inhibition of the MCK gene by epicardin/capsulin/Pod-1—*Expression of epicardin/capsulin/Pod-1 and MyoR has been shown to overlap in head muscle precursors and the lack of an appreciable change in phenotype of the head musculature in epicardin/capsulin/Pod-1 mutant mice (36) raises the possibility of functional redundancy between epicardin/capsulin/Pod-1 and MyoR. We thus investigated the transcriptional regulatory mechanism that involves epicardin/capsulin/Pod-1 in myofibroblast differentiation beyond the cell cycle arrest. Terminal differentiation of skeletal myoblasts is accompanied by induction of a series of tissue-specific genes including the *MCK* gene. MyoD and E12 have been shown to efficiently transactivate the *MCK* gene in a cooperative manner (20, 37). The effects of epicardin/capsulin/Pod-1 on expression of the *MCK* gene were examined. 10T1/2 cells were cotransfected with a luciferase reporter construct pMCK-luc containing the 1.3-kb promoter sequence of the *MCK* gene, along with pCMV-E12, pCMV-MyoD and pCMV-epicardin. The epicardin/capsulin/Pod-1 expression plasmid was added at concentrations over a range equivalent to those for Id1 and Id2 which showed inhibition of muscle differentiation through suppression of production of molecules, such
as MCK (20, 38, 39) and p204 (40). Epicardin/capsulin/Pod-1 reduced luciferase activity driven by the MCK promoter in response to E12 and MyoD in a dose-dependent manner (Fig. 5A and B). Similar results were obtained in the regulation of p21 and MCK expression in C2C12 myoblasts (Fig. 3D and 5C). These results demonstrate that the MCK gene is negatively regulated by epicardin/capsulin/Pod-1, similar to the p21 gene. Regulation of the MCK promoter was also examined with epicardin/capsulin/Pod-1 siRNA. The addition of siRNA partly prevented epicardin/capsulin/Pod-1-mediated suppression of the MCK promoter (Fig. 5D), similar to the effect of siRNA on the p21 promoter (Fig. 3F).

**Binding of epicardin/capsulin/Pod-1 to the MCK promoter in vivo**— Since epicardin/capsulin/Pod-1 was initially found as a molecule physically interacting with E12 (Fig. 1B), integration of epicardin/capsulin/Pod-1 into a complex with the MCK promoter would be expected. To address this view, we further investigated the recruitment of epicardin/capsulin/Pod-1 to the MyoD-dependent MCK enhancer by the ChIP assay in vivo.

Soluble chromatin from C2C12 transfected with pHA-epicardin and pMCK-luc was immunoprecipitated with an antibody specific to the HA epitope, and examined for the presence of the MyoD-binding site by PCR. PCR produced only one band with a molecular size of 162 bp (Fig. 5E) and DNA sequencing confirmed that the product contained the MyoD binding sequence in the MCK promoter. The soluble chromatin treated with an antibody specific to the Myc epitope did not produce any PCR products (Fig. 5E). These results demonstrate that epicardin/capsulin/Pod-1 binds to the MCK promoter in C2C12 cells.

<<Figure 5>>

**Inhibition of muscle gene expression by epicardin/capsulin/Pod-1**— The results in this
study convinced us that induced the expression of epicardin/capsulin/Pod-1 could interfere with
the activation of endogenous muscle-specific genes. To examine this possibility, C2C12
myoblasts capable of differentiation to myotubes were transfected with pCMV-epicardin along
with pCMV-β-Gal and then cultured in the differentiation medium. Induction of
epicardin/capsulin/Pod-1 greatly inhibited expression of TnT, a marker of differentiated muscle
cells. Immunostaining analysis exhibited that epicardin/capsulin/Pod-1 expression decreased
percentage of TnT positive cells to 26.6% among cell expressing β-galactosidase, while nearly
all cells expressing β-galactosidase were positive for TnT in a mock empty vector transfection
experiment (Fig. 6). In all experiments, β-galactosidase-negative cells differentiated to muscle
with expression of TnT to a similar extent. Thus, the results indicate that the terminal
differentiation to myotubes of C2C12 cells is greatly suppressed by epicardin/capsulin/Pod-1
presumably via its inhibitory function on the expression of genes specific to muscle cells.

<<Figure 6>>
Discussion

In this study we found that epicardin/capsulin/Pod-1 acts as a transcriptional suppressor of E2A-induced gene expression. The inhibitory effect was seen on the expression of p21 and MCK, which are activated by E2A proteins and by the combination of E12 and MyoD, respectively. Physical interaction of epicardin/capsulin/Pod-1 with the bHLH domain of E12 was shown by the yeast and mammalian two-hybrid methods in addition to the demonstration of colocalization of epicardin/capsulin/Pod-1 and E12 in the nuclei. Moreover, ectopic expression of epicardin/capsulin/Pod-1 blocked the terminal differentiation of C2C12 myoblasts. Collectively, epicardin/capsulin/Pod-1 is involved in the inhibition of differentiation through the regulation of the expression of genes related to cell cycle arrest and of tissue-specific differentiation.

Little is known about the transcriptional suppression which regulates early processes of organogenesis. Our results provide a hypothesis that epicardin/capsulin/Pod-1 normally suppresses differentiation through inhibition of cell cycle arrest by regulating p21 expression, presumably resulting in precursor cells remaining in an undifferentiated state. This in turn, leads to the expansion of precursor cells for the generation of the organ. Epicardin/capsulin/Pod-1 is expressed in branchial muscle precursors and mesenchymal cells at sites of epithelial-mesenchymal interactions in various organs (8, 10, 36). In this study, we showed that myoblast differentiation was inhibited by epicardin/capsulin/Pod-1-mediated suppression of p21 expression. Regulation of expression of the \( p21 \) gene is presumably involved in the differentiation of other cell lineages. Indeed, we have previously reported that bHLH proteins regulate \( p21 \) expression and osteoblast differentiation (14). Since E12 has been shown to activate
p16 and p15 (41) besides p21, epicardin/capsulin/Pod-1 may participate in the regulation of other Cdk inhibitors, and such a regulation may be essential for differentiation. We observed that, in 10T1/2 cells, introduction of p21 alone could not rescue epicardin/capsulin/Pod-1-mediated inhibition of MCK promoter activity induced by E12 and MyoD (data not shown). Our observation is compatible with a previous report that ectopic expression of either p21 or p16 partially reversed the inhibitory effect of the growth medium on the MCK promoter, and that coexpression of p21 and p16 resulted in the reverse of the suppressed MCK promoter activity (42). Previous studies demonstrated that the Cdk inhibitors are implicated in myogenesis; p21 and p16 promote differentiation (42), p21, p27, p57 and p18 are all upregulated coincident with terminal growth arrest (17-19, 30) and the expression of p21 and p57 is essential for muscle differentiation during mice embryonic development (19). Simultaneously, tissue-specific gene expression is inhibited by epicardin/capsulin/Pod-1, resulting in suppression of the organ development. A set of lineage differentiation-specific genes obviously differs from tissue to tissue and seems to be commonly regulated by transcriptional signals from tissue-specific master molecules, such as MCK for myoblasts and FGFR3 for osteoblasts. In addition, epicardin/capsulin/Pod-1 has been reported to suppress expression of Ad4BP/SF-1, a zinc finger transcription factor, which plays important roles in gonadogenesis (43).

Epicardin/capsulin/Pod-1-mediated suppression of transcription may be selective within genes that are regulated by E2A. The expression of epicardin/capsulin/Pod-1 seems to be more restricted than that of E2A in terms of location and timing during differentiation (7, 8). This may be a mechanism underlying the selective suppression by epicardin/capsulin/Pod-1. Related molecules have been shown to function selectively depending on promoters. OUT, structurally
related to epicardin/capsulin/Pod-1, inhibited the induction of E-box-dependent MCK promoter activation by MyoD-E12 heterodimers, whereas similar bHLH factors, neural bHLH TAL2 and placental bHLH Mash2, failed to inhibit the MCK expression (44). It may also be possible that the specificity of action of epicardin/capsulin/Pod-1 is dependent on the context of DNA elements in a promoter; interaction of epicardin/capsulin/Pod-1 with another factors that bind different DNA elements in the same promoter may be important. Alternatively, there may be an unidentified mechanism by which epicardin/capsulin/Pod-1 selects an E-box in E2A-targeted genes, as shown in the case of MyoD, which selectively binds some of the E-boxes (45).

It is also possible that epicardin/capsulin/Pod-1 may inhibit apoptosis. Some bHLH factors are implicated in inhibition of apoptosis by negative regulation of transcription. Twist and Dermo-1, which suppresses transcriptional activity of myogenic bHLH proteins (46), have been shown to inhibit oncogene-dependent and p53-dependent cell death (47). This notion may be supported by the observation that apoptosis was readily detected in the presumptive splenic-forming region of epicardin/capsulin/Pod-1 mutant embryos, whereas only random and occasional apoptosis was observed in the internal organs of wild-type embryos (11).

The importance of negative regulation by bHLH factors has been emphasized in cell differentiation of Drosophila and mice. Inactivation of twist results in defective dorso-ventral patterning due to disturbed gastrulation in Drosophila (48) as well as defects in cranial neural tube closure and mesodermal derivatives in mice (49). Saethre-Chotzen syndrome (acrocephalosyndactyly type III; OMIM 101400), characteristic of skull deformity due to craniosynostosis, is caused by mutations in the gene encoding Twist (50, 51), which is a transcriptional suppressor for the p21 and FGFR3 genes in osteoblasts (14).
The asplenic phenotype of *epicardin/capsulin/Pod-1* mutant mice resembles those of mice lacking *Bapx1* (52, 53) and *Hox11* (54, 55), as well as the Wilm’s tumor suppressor gene, *WT-1* (56). They are both coexpressed with epicardin/capsulin/Pod-1 in the spleen during development and function as essential regulators of spleen organogenesis. These genes may constitute a genetical cascade that acts in concert to regulate a common early event in spleen organogenesis. In the developing lung, epicardin/capsulin/Pod-1 in the mesenchyme has been shown to be required to activate expression of bone morphogenetic protein-4 (BMP-4) in the adjacent epithelium; in the absence of BMP-4 expression, the airway epithelium fails to differentiate (12). This result together with our findings suggest that epicardin/capsulin/Pod-1 acts as a transcriptional suppressor and raises the possibility that epicardin/capsulin/Pod-1 does not directly regulate expression of BMP-4. Rather that it is implicated in regulation upstream of an unknown transcription factor that suppresses transcription of BMP-4.

Among bHLH members, epicardin/capsulin/Pod-1 shows a high degree of homology to a group of bHLH factors that are expressed in tissues of mesodermal origin. In particular, epicardin/capsulin/Pod-1 is closely related to *MyoR* (57) with 94.5% identity in the bHLH region at the amino acid level, presumably generating a subfamily within the bHLH factors. *MyoR* acts in undifferentiated skeletal myoblasts as a potent transcriptional suppressor that can block myoblast differentiation by interfering with the activity of MyoD (36, 57). The expression of *epicardin/capsulin/Pod-1* and *MyoR* overlaps in head muscle precursors (7, 57), suggesting functional redundancy between *epicardin/capsulin/Pod-1* and *MyoR*. This notion may be supported by the phenotypic observation made in *epicardin/capsulin/Pod-1* mutant mice, which have no defect in head musculature (11). Our preliminary experiments showed that
epicardin/capsulin/Pod-1 siRNA did not affect p21 in MG63 cells and TnT and myosin heavy-chain in C2C12 cells, markers of myoblast differentiation. This may result from functional redundancy between epicardin/capsulin/Pod-1 and TWIST, Id1, or MyoR.

In a broader context, our study demonstrates that epicardin/capsulin/Pod-1 inhibits cell cycle arrest and tissue-specific gene induction, resulting in regulation of cell differentiation. Identification of other molecules that bind to epicardin/capsulin/Pod-1 and elucidation of the functional significance of the interaction of those molecules represent important issues for the future.

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FOOTNOTES

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1The abbreviations used are: bHLH, basic helix-loop-helix; HLH, helix-loop-helix; Cdk, cyclin-dependent kinase; FGFR3, fibroblast growth factor receptor 3; MCK, muscle creatine kinase; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; IgG, immunoglobulin G; kb, kilobase pair; bp, base pair; TnT, troponin T; GAL4DBD, GAL4 DNA-binding domain; AD, activation domain; CBP, cyclic AMP-responsive element binding factor CREB-binding protein; HAT, histone acetyltransferase; siRNA, small interfering RNA; ChIP, chromatin immunoprecipitation.
Legends to Figures

Fig. 1. Association of epicardin/capsulin/Pod-1 with E12. (A) Schematic representation of wild-type and mutant proteins of E12 and Twist. (B) Interaction of epicardin/capsulin/Pod-1 with the wild-type E12 protein in yeast. A plasmid encoding full-length epicardin/capsulin/Pod-1 fused to the GAL4 activating domain (AD) was cotransfected into yeast AH109 cells with plasmids encoding the indicated forms of E12 or Twist fused to the GAL4 DNA-binding domain (DBD). Transformants were streaked onto a control plate lacking leucine and tryptophan (SD-Leu-Trp) and an indicator plate lacking leucine, tryptophan, histidine and adenine (SD-Leu-Trp-His-Ade). (C) Diagrammatic representation of the mammalian two-hybrid assay. Constructs for chimeric proteins of GAL4-DBD and epicardin/capsulin/Pod-1 bHLH and of VP16 and E12 were generated and named pGAL-epi (bHLH) and pVP16-E12, respectively. (D) Interaction of epicardin/capsulin/Pod-1 with E12. MG63 cells were transfected with combinations of expression plasmids and control plasmids along with the reporter plasmid pG5-luc, and luciferase activity was determined. Fold activation is expressed as a ratio of luciferase activity relative to that obtained with the GAL4 DBD and VP16 cassettes, which is arbitrarily set at 1.

Fig. 2. Co-localization of epicardin/capsulin/Pod-1 with E12 in the nucleus. MG63 cells were transfected with pGFP (a-c) or pGFP::epicardin (d-f) together with expression vector for E12 (pCMV-E12). The GFP signal (green; b and e) was subsequently visualized by confocal microscopy. Immunofluorescence associated with E12 expression was detected by immunostaining with the antibody specific to E12 (rhodamine; a and d). Panels c and f show
images merged with a and b, d and e, respectively. Scale bars, 20µm.

Fig. 3. Effects of by epicardin/capsulin/Pod-1 on E2A-dependent transactivation of p21. (A) Inhibition of E12- and E47-dependent transactivation of p21 by epicardin/capsulin/Pod-1. MG63 cells were cotransfected with 2.5 µg of p21-luc in combination with pCMV-E12, pCMV-E47, pCMV-epicardin, pCMV-Twist and pCMV-Id1. The cells were harvested 48 h after transfection and then assayed for reporter gene expression. Empty vector pcDNA3 was included to adjust DNA amounts. Activation mediated by pcDNA3 is arbitrarily set at 1. (B) Inhibition of E2A-dependent transactivation of p21 by epicardin/capsulin/Pod-1 in a dose-dependent manner. MG63 cells were cotransfected with 2.5 µg of p21-luc in combination with pCMV-E12 and pCMV-E47 and with indicated amounts of pCMV-epicardin or pCMV-Twist. Empty vector pcDNA3 was included to adjust DNA amounts. (C) Inhibition of E12- and CBP-dependent p21 transcription by epicardin/capsulin/Pod-1. MG63 cells were cotransfected with 1.0 µg of p21-luc and 0.75 µg of either pcDNA3 or pCMV-E12. The cells were cultured in DMEM containing 2% FBS for 48 h and collected for the luciferase assay. (D) Inhibition of E12- and MyoD-dependent transactivation of p21 by epicardin/capsulin/Pod-1. C2C12 myoblasts were cotransfected with 2.5 µg of p21-luc in combinations with pCMV-E12, pCMV-MyoD and pCMV-epicardin. The cells were cultured in DMEM containing 10% FBS for 48 h and collected for luciferase assay. Activation mediated by pcDNA3 is arbitrarily set at 1. (E) Reduced epicardin/capsulin/Pod-1 in MG63 cells transfected with epicardin/capsulin/Pod-1 siRNA. Five h prior to siRNA transfection, cells were cotransfected with pCMV-Myc-epicardin and the β-galactosidase expression vector, pCMV-β-Gal, which was used as an internal control to monitor
the transfection efficiency. Cells were harvested 48 h following siRNA transfection and cell lysates were generated. Samples were immunoblotted with antibodies to the Myc epitope, β-galactosidase and α-tubulin. (F) Abolishment of epicardin/capsulin/Pod-1-mediated E12 inhibition by epicardin/capsulin/Pod-1 siRNA. Five h prior to siRNA transfection, MG63 cells were cotransfected with 1.0 µg of p21-luc in combination with 1.0 µg of pCMV-E12 and pMyc-epicardin. The cells were harvested 48 h later and luciferase activities were determined. Empty vector pCMV-Myc was included to adjust DNA amounts.

Fig. 4. Inhibition of E12-dependent endogenous p21 induction by epicardin/capsulin/Pod-1. MG63 cells were transfected with pCMV-E12 and either pcDNA3 (an empty vector) (A) or pCMV-epicardin (B). The cells were maintained in DMEM containing 10% FBS for 72 h. The cells were fixed and stained for E12 (rhodamine, a), p21 (fluorescein, b) and DNA (DAPI, c). Arrows indicate cells expressing E12. Scale bar, 20 µm.

Fig. 5. Inhibition of MCK transcription by epicardin/capsulin/Pod-1. (A) Inhibition of E12- and MyoD-dependent transactivation of MCK by epicardin/capsulin/Pod-1. 10T1/2 cells were cotransfected with 2.5 µg of pMCK-luc in combination with pCMV-E12, pCMV-MyoD, pCMV-epicardin, pCMV-Twist and pCMV-Id1. The cells were harvested 48 h after transfection and then assayed for reporter gene expression. (B) Inhibition of MyoD- and E12-dependent transactivation of MCK by epicardin/capsulin/Pod-1 in a dose-dependent manner.
10T1/2 cells were cotransfected with pMCK-luc, pCMV-E12 and pCMV-MyoD in combination with indicated amounts of either pCMV-epicardin, pCMV-Id1 or pCMV-Id2. The cells were harvested 48 h after transfection and then assayed for reporter gene expression. Empty vector pcDNA3 was included to adjust DNA amounts. (C) Inhibition of MyoD- and E12-dependent transactivation of MCK by epicardin/capsulin/Pod-1 in C2C12 myoblasts. C2C12 myoblasts were cotransfected with 2.5 μg of pMCK-luc in combinations with pCMV-E12, pCMV-MyoD and pCMV-epicardin. The cells were cultured in DMEM containing 10% FBS for 48 h and collected for the luciferase assay. Activation mediated by pcDNA3 is arbitrarily set at 1. (D) Abolishment of epicardin/capsulin/Pod-1-mediated E12 inhibition by epicardin/capsulin/Pod-1 siRNA. Five h prior to siRNA transfection, C3H10T1/2 cells were cotransfected with 1.0 μg of pMCK-luc in combination with 1.0 μg of pCMV-E12 and pCMV-Myc-epicardin. Empty vector pCMV-Myc was included to adjust DNA amounts. (E) Physical association of epicardin/capsulin/Pod-1 to the MCK promoter. Chromatin immunoprecipitated from C2C12 cells transfected with pHA-epicardin and pMCK-luc was subjected to PCR with primers specific to the MCK promoter. A portion (10%) of total amounts of chromatin prior to immunoprecipitation was also subjected to PCR (Input). Anti-Myc antibody was used as an irrelevant control antibody (Mock).

Fig. 6. Effect of epicardin overexpression on muscle-specific gene expression. C2C12 cells were transfected in the growth medium with pCMV empty vector or pCMV-epicardin together with the β-galactosidase expression vector and then induced to differentiate in the differentiation medium for 96 hr. After fixation in paraformaldehyde, productively transfected cells were
visualized by the expression of cotransfected β-galactosidase (green). Myogenic differentiation was scored by determining expression of TnT (red) in β-galactosidase-positive cells. Scale bars, 20µm. Vector: pcDNA3-transfected cells; Epicardin: pCMV-epicardin-transfected cells.
TABLE 1. Effects of epicardin/capsulin/Pod-1 on E12-dependent p21 expression.

| Transfection       | E12+ Cells | E12− Cells |
|--------------------|------------|------------|
|                    | p21+ (%)   | p21− (%)   | p21+ (%) | p21− (%) |
| E12                | 88.3       | 11.7       | 0.1      | 99.9     |
| E12+epicardin      | 43.6       | 56.4       | 0.4      | 95.9     |

MG63 cells were transiently cotransfected with pCMV-E12 and pCMV-epicardin/capsulin/Pod-1. The cells were maintained in DMEM containing 10% FBS. The expression of p21 was monitored by immunostaining of E12 and p21 (Fig. 4). At least 150 cells were counted in each category, and results are representative of multiple experiments performed.
A  GAL4 DBD-Fusion Constructs

E12

TAD1  TAD2  bHLH

E12(bHLH)

Twist

NLS NLS  bHLH

100 206

Twist(bHLH)  bHLH

510 654

B  GAL4 AD-epicardin

E12

E12 (bHLH)

Twist

Twist (bHLH)

GAL4 DBD

-Gal4 AD-epicardin

-Trp/-Leu  -Trp/-Leu/-His/-Ade

C

GAL4 DBD-epicardin (bHLH)

VP16  E12

GAL4 sites  TATA

Luc

D

Fold Activation

pG5Luc

pVP16  + + - -
pVP16-E12  - - + +
pGAL  + - + -
pGAL-epi(bHLH)  - + - +
**A** MCK-luc

- C3H10T1/2

**B** MCK-luc

- C3H10T1/2

**C** MCK-luc

- C2C12

**D** MCK-luc

- C3H10T1/2

**E** *ChIP assay*

*Input αHA Mock* MCK (162 bp)
Basic helix-loop-helix transcription factor epicardin/capsulin/Pod-1 suppresses differentiation by negative regulation of transcription
Noriko Funato, Kimie Ohyama, Takayuki Kuroda and Masataka Nakamura

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