Sox4-mediated caldesmon expression facilitates differentiation of skeletal myoblasts

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Accepted 2 September 2013
Journal of Cell Science 126, 5178–5188

doi: 10.1242/jcs.131581

Summary

Caldesmon (CaD), which was originally identified as an actin-regulatory protein, is involved in the regulation of diverse actin-related signaling processes, including cell migration and proliferation, in various cells. The cellular function of CaD has been studied primarily in the smooth muscle system; nothing is known about its function in skeletal muscle differentiation. In this study, we found that the expression of CaD gradually increased as differentiation of C2C12 myoblasts progressed. Silencing of CaD inhibited cell spreading and migration, resulting in a decrease in myoblast differentiation. Promoter analysis of the caldesmon gene (*Cald1*) and gel mobility shift assays identified Sox4 as a major trans-acting factor for the regulation of *Cald1* expression during myoblast differentiation. Silencing of *Sox4* decreased not only CaD protein synthesis but also myoblast fusion in C2C12 cells and myofibril formation in mouse embryonic muscle. Overexpression of CaD in *Sox4*-silenced C2C12 cells rescued the differentiation process. These results clearly demonstrate that CaD, regulated by *Sox4* transcriptional activity, contributes to skeletal muscle differentiation.

Key words: Skeletal muscle differentiation, Caldesmon, Transcription factor Sox4

Introduction

Myoblast differentiation is a highly ordered process that requires a particular combination of gene expression and signaling (Clatworthy and Subramanian, 2001; Handwerger and Aronow, 2003; Marshall, 2003). Myogenic cells withdraw from the cell cycle and undergo dramatic morphological changes. Mononucleated myoblasts align and their membranes fuse to form multinucleated myotubes that then mature into muscle fibers. Concomitantly, transcriptional cascades regulated by multiple groups of muscle-specific transcription factors initiate the *de novo* synthesis of various muscle-specific proteins (Braun and Gautel, 2011). Several lines of evidence indicate that the migration of myoblast cells is required for membrane fusion (Biressi et al., 2007; Braun and Gautel, 2011). This dynamic cellular behavior is accompanied by massive changes in cytoskeletal proteins, including the dramatic rearrangement of actin filaments and related signaling proteins such as integrin and focal adhesion kinase (Burattini et al., 2004; Formigli et al., 2007).

Caldesmon (CaD) is an actin-binding protein found in almost all vertebrate cells. There are two types of CaD protein: the heavy form (h-CaD) is specifically detected in smooth muscle cells (SMCs) (Lehman et al., 1992; Sobue et al., 1981; Wang, 2001), whereas the light form (l-CaD) is ubiquitously expressed (Bretscher and Lynch, 1985; Matsumura and Yamashiro, 1993). The known functions of the CaD proteins suggest that they have crucial roles during muscle development. The expression of h-CaD in differentiated SMCs plays an important role in the maintenance of the actomyosin contractile structure (Bretscher and Lynch, 1985; Fürst et al., 1986). Moreover, the insufficient development of organs composed of SMCs and cardiac muscle in the CaD-knockout mouse suggests that CaD is required for myogenesis (Zheng et al., 2009a; Zheng et al., 2009b). Nevertheless, the function of CaD in skeletal muscle development is not clear.

The CaD isoforms originate from alternative splicing and/or promoter usage (Mayanagi and Sobue, 2011). Two independent *CALD1* promoters have been identified in both chickens and humans: gizzard-type and brain-type in chickens and fibro-type and HeLa-type in humans (Hayashi et al., 1992; Yano et al., 1994). The activities of gizzard-type and fibro-type promoters are higher than the activities of brain-type and HeLa-type promoters (Fukumoto et al., 2009; Yano et al., 1995). Therefore, most studies of *CALD1* promoter activity and associated transcription factors have focused on the gizzard-type or fibro-type promoter. Nonetheless, it is not clear why l-CaD expression is higher than h-CaD expression in mouse skeletal muscle (Yano et al., 1995).

Sox4, a transcription factor in the SRY (sex-determining region Y) family, contains a highly conserved high-mobility group (HMG) DNA-binding domain (DBD) that plays important roles in many developmental processes, including nervous system development (Cheung et al., 2000) and endocrine islet formation (Wilson et al., 2005). Cardiac development in $Sox4^{-/-}$ mice showed defective outflow tract formation and valve development, thus indicating a role for Sox4 in myogenesis (Schilham et al., 1996). Moreover, *Sox4* transcripts have been detected in skeletal myoblasts and in myocardium (Ling et al., 2009; Tomczak et al., 2004). These reports strongly suggest that Sox4 participates in myoblast differentiation. However, the

molecular mechanisms underlying Sox4-mediated myogenesis have not been elucidated.

In this study, we investigated the molecular mechanism of CaD expression and its role in myoblast differentiation by using C2C12 murine skeletal myoblasts as a model system for skeletal muscle differentiation. CaD expression was induced before the onset of membrane fusion and gradually increased as myoblast differentiation proceeded. Silencing of CaD expression by siRNA significantly reduced membrane fusion and myotube formation and inhibited muscle-specific protein expression. Furthermore, the *Cald1* gene in C2C12 myoblasts contained a HeLa-type promoter, and its activity was controlled by the direct interaction of the Sox4 transcription factor with cis-acting elements on the promoter. In conclusion, we show that CaD expression, regulated by Sox4, plays an important role in myofibril formation during muscle development in the mouse embryo.

Results

CaD expression is induced during C2C12 myoblast differentiation

First, we examined the expression of CaD during C2C12 skeletal myoblast differentiation. To induce differentiation, confluent C2C12 myoblasts were switched from growth medium (GM) to differentiation medium (DM). The level of CaD protein gradually increased along with the expression of skeletal muscle markers, including myogenin and myosin heavy chain (MHC), as C2C12 myoblast differentiation progressed (Fig. 1A,B). RT-PCR results indicated that the changes in CaD protein level during myoblast differentiation were controlled by transcriptional regulation (supplementary material Fig. S1). To examine the cellular localization of CaD in myoblasts, we performed immunocytochemistry with cells incubated in DM. Consistent with the immunoblot results, CaD fluorescence was low under proliferation conditions, and CaD became enriched in MHC-positive myotubes as differentiation proceeded (Fig. 1C).

CaD is required for C2C12 cell spreading and migration

To examine the effect of CaD on skeletal muscle differentiation, we constructed four small interfering RNA (siRNA) constructs specific for *Cald1* mRNA and transfected control siRNA or CaD siRNA (#1 and #2) into C2C12 cells before inducing differentiation. Fig. 2A shows a representative western blot performed using cell lysates prepared from cells transfected with siRNA #1 or #2 and induced to differentiate for 2 days. The level of CaD in cells transfected with CaD-siRNA #2 was reduced by 80%. The level of β-actin was unaffected.

CaD is involved in actin-dependent cell morphology and motility in various cells (Castellino et al., 1995; Eppinga et al., 2006; Li et al., 2004; Mayanagi et al., 2008; Mirzapoiazova et al., 2005). Therefore, we examined the effect of depletion of CaD on the spreading of C2C12 cells. Microscopy observation of siRNA-transfected C2C12 myoblasts showed that *Cald1* silencing decreased cell spreading by ~50% (Fig. 2B). We confirmed the correlation between the effectiveness of *Cald1* silencing and the myoblast phenotype with two additional CaD siRNA constructs (supplementary material Fig. S2A,B). Wild-type C2C12 cells were elongated, and CaD colocalized with actin-rich lamellar structures at the edges of the cells. By contrast, *Cald1* silencing markedly inhibited cell polarity, decreased the cell area and disrupted lamellar formations (Fig. 2B and supplementary material Fig. S3). These results suggest that CaD is necessary for actin-mediated cell spreading and lamellipodia formation in C2C12 myoblast differentiation.

Because cell motility regulated by actin rearrangement is crucial for myoblast alignment and differentiation (Burattini et al., 2004; Formigli et al., 2007), we examined the effect of *Cald1* silencing on cell migration in a scratch-wound healing assay. As

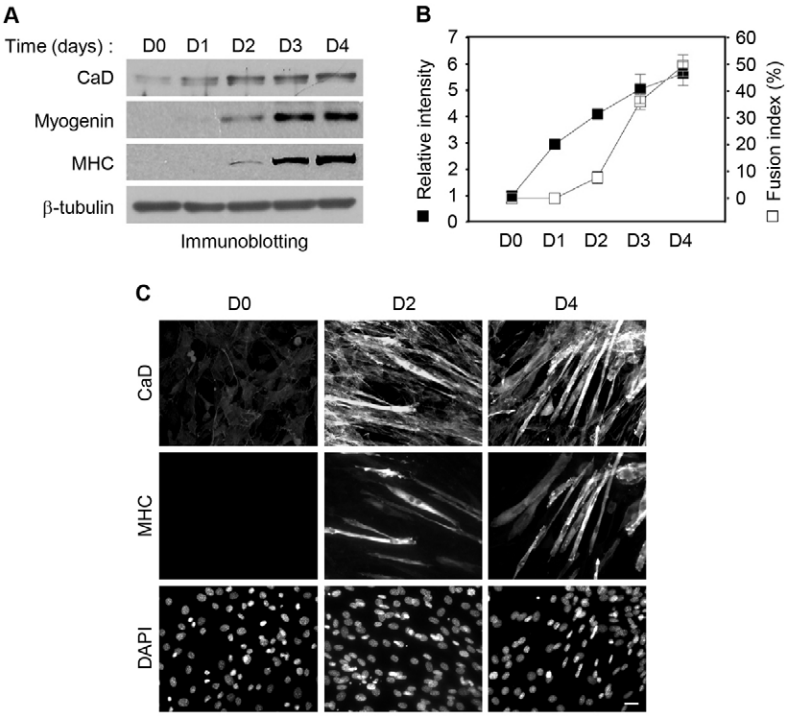

Fig. 1. Expression of CaD is induced during C2C12 myoblast differentiation. (A) C2C12 myoblast lysates prepared at the indicated times (D0–D4, day 0 to day 4) after the induction of differentiation were examined by immunoblot analysis with specific antibodies against CaD and the muscle-specific markers myogenin and MHC. β-tubulin was assessed as a loading control. These experiments were conducted at least three times, and similar results were obtained. (B) The amount of CaD was normalized to the amount of β-tubulin and quantified by image analysis of a blot similar to that shown in A. The intensity of normalized CaD (■) in proliferating C2C12 myoblasts in growth medium (GM; D0) was arbitrarily set at 1. The total fusion index (□) represents the number of nuclei in multinucleated myotubes (at least three nuclei) divided by the total number of nuclei in the field. Data are the means ± s.e.m. from three independent experiments. (C) C2C12 myoblast cells induced to differentiate in differentiation medium (DM) for the indicated times were examined by immunofluorescence analysis with anti-MHC and anti-CaD antibodies. The nuclei were stained with DAPI. Scale bar: 50 μm.

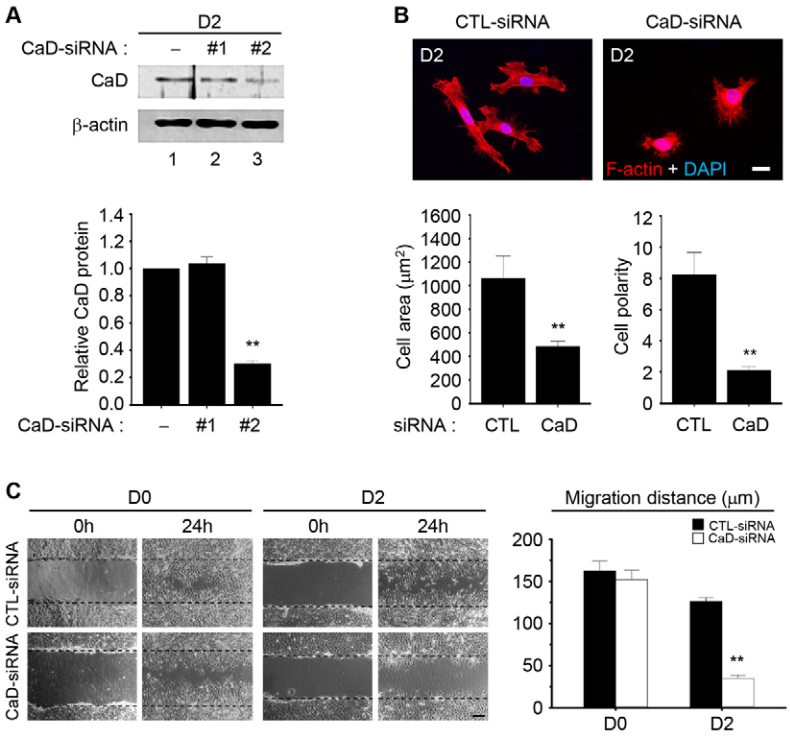

Fig. 2. CaD is required for cell spreading and migration during C2C12 myoblast differentiation. (A) C2C12 myoblasts were transfected with control-siRNA or one of two siRNAs specific for CaD (CaD-siRNA #1 and CaD-siRNA #2). Cells were induced to differentiate by incubation for 2 days in DM. Lysates from the myoblasts were subjected to immunoblot analysis with CaD-specific antibodies. β-actin was used as a loading control (top panel). The expression of CaD, quantified by scanning the blots shown in the top panel, was normalized to the β-actin expression level (bottom panel). Data are presented as the means ± s.e.m. from three independent experiments. $^{**}P<0.01$. (B) C2C12 myoblasts were transfected with the indicated siRNA for 2 days and incubated in differentiation medium for 2 days. Merged images of Rhodamine-Phalloidin and DAPI are shown (top panel). Scale bar: 20 μm. The mean area and the polarity of myoblasts were calculated from 10 random microscopic fields ($n=50$). Data are the means ± s.e.m. from three independent experiments. $^{**}P<0.01$. (C) C2C12 myoblast monolayers transfected with control siRNA or CaD siRNA were wounded and allowed to migrate in proliferation (D0) or differentiation (D2) medium for the indicated times. The migration distance, quantified from at least three photographed fields obtained from three independent experiments, is represented as the mean ± s.e.m. Scale bar: 100 μm. $^{**}P<0.01$.

shown in Fig. 1A, under proliferation conditions in which CaD expression was extremely low (D0), cell migration was not affected by CaD siRNA transfection, indicating that the siRNA used in the experiment had no side effects on cell migration. However, under differentiation conditions (D2) in which CaD expression increased transiently, silencing of *Cald1* substantially reduced cell migration into the wound region (Fig. 2C and supplementary material Fig. S2C). These results suggest that the activity of CaD in C2C12 myoblasts is essential for the regulation of cell motility, which is required to initiate myoblast alignment in response to the differentiation signal.

CaD is required for C2C12 myoblast differentiation

Next, we investigated the role of CaD in C2C12 myoblast differentiation. In control C2C12 cells, myoblast fusion began on day 2 after the application of differentiation medium and gradually increased to form myotubes (Fig. 3A,B). By day 4, the frequency of MHC-positive myotubes increased to ~50%. Membrane fusion was significantly lower in CaD-silenced C2C12 cells, thus reducing the frequency of MHC-positive myotubes to ~10%. Measurement of myotube diameter, as an indicator of myotube development (Burattini et al., 2004; Doyle et al., 2011), suggested that CaD plays a major role in myoblast

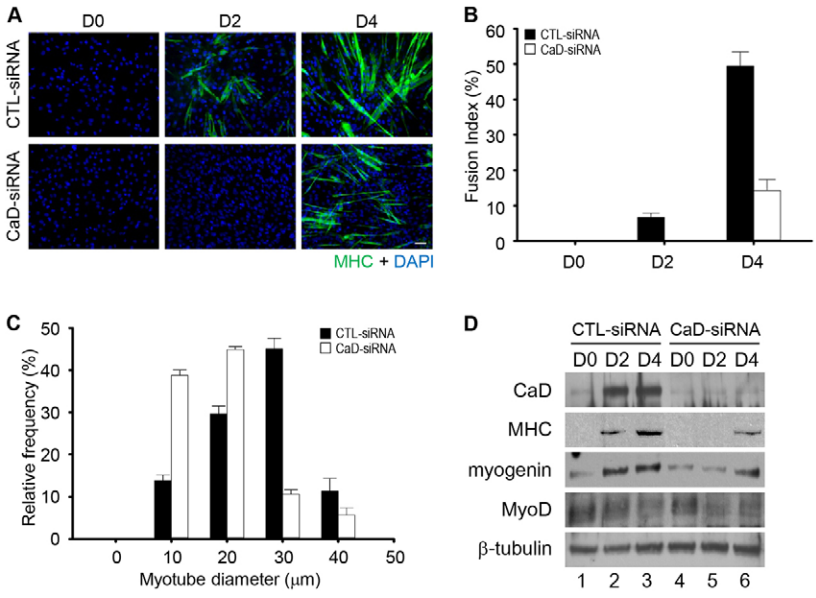

Fig. 3. CaD is required for the differentiation of C2C12 myoblasts. (A) C2C12 myoblast cells were transfected with control siRNA or CaD siRNA and were induced to differentiate by incubation for the indicated times in DM. The myoblast cells were then subjected to immunofluorescence analysis for MHC (green) and DAPI (blue). Scale bar: 50 μm. (B) The total fusion index represents the number of nuclei in multinucleated myotubes (at least three nuclei) divided by the total number of nuclei in the field. Data represent the means ± s.e.m. from three independent experiments. (C) The myotube diameter, in myotubes with at least three nuclei after 4 days in DM, was quantified from at least three photographed fields obtained from three independent experiments. The myotube diameter distribution is presented as a frequency curve. Data are the means ± s.e.m. from a representative experiment. (D) Lysates of myoblast cells transfected as described in A and incubated in DM for the indicated times were subjected to western blot analysis with antibodies against the indicated proteins. β-tubulin was used as a loading control.

fusion by increasing myotube size during differentiation (Fig. 3C and supplementary material Fig. S2D).

Myoblast differentiation is orchestrated by the expression of muscle-specific proteins that initiate and guide the differentiation process. We, therefore, investigated the effect of *Cald1* silencing on the expression of specific myogenic marker proteins. The expression of myogenin and MHC was markedly reduced in CaD-siRNA-transfected C2C12 myoblasts (Fig. 3D), whereas MyoD expression was similar in CaD-siRNA-transfected cells and control cells in the proliferation stage. These results, together with those in Fig. 2, clearly indicate that CaD functions in the early stage of myoblast differentiation to regulate initial cell spreading and motility.

Cald1 promoter activity is regulated by Sox4 cis-acting elements during C2C12 myoblast differentiation

The results described above demonstrated that the expression of CaD in response to the differentiation signal is an important event in C2C12 myoblast differentiation. To understand the molecular mechanism underlying CaD expression during C2C12 differentiation, we characterized the promoter of the gene encoding mouse CaD (*Cald1*). In previous studies, characterization of the promoters of avian and human *CALD1* showed that a CArG box (Yano et al., 1995) and glucocorticoid-response element (GRE)-like sequences (Mayanagi et al., 2008), respectively, were cis-acting elements important for the regulation of promoter activity in each species. However, we found that these cis-acting elements are located −110 kbp upstream of the transcription initiation site of mouse *Cald1*. Moreover, the nucleotide sequence of the putative promoter region in mouse *Cald1* is ~70% identical to that of the HeLa-type promoter in human *CALD1*.

Because the HeLa-type promoter has not been extensively characterized, we first searched the mouse *Cald1* promoter for putative cis-acting elements and their binding partners using TFSEARCH software (Searching Transcription Factor Binding Sites, Version 1.3; http://www.cbrc.jp/research/db/TFSEARCH.html). Sequence analysis identified several putative cis-acting elements in the mouse *Cald1* promoter between −759 bp and +41 bp for the transcription factors GATA, Sox4, AP1, Nkx2.5, SP1 and CP2. The sequences are almost conserved in the human HeLa-type promoter (Fig. 4A). To determine which cis-acting elements are responsible for *Cald1* promoter activity, we constructed reporter plasmids containing 800 bp of the *Cald1* promoter (WT-Luc) or truncated fragments (mut-1-Luc–mut-4-Luc) (Fig. 4B). Each plasmid was introduced into C2C12 myoblasts and promoter activity was measured after culturing cells for 2 days in proliferation medium (D0) or differentiation medium (D2). With the WT-Luc plasmid, basal levels of promoter activity were observed in proliferating myoblasts (D0); promoter activity increased by ~fourfold in differentiating C2C12 myoblasts (D2), indicating that *Cald1* promoter activity is associated with C2C12 myoblast differentiation. However, the promoter activity in differentiating myoblasts was reduced considerably when cells were transfected with mut-3-Luc or mut-4-Luc, lacking 459 bp and 656 bp, respectively, at the 5′ end of the promoter, indicating that the region between −532 bp and −300 bp is crucial for mouse *Cald1* promoter activity (Fig. 4C).

Sox4 is responsible for mouse *Cald1* promoter activity

Because promoter analysis indicated that the nucleotide sequence between −421 bp and −415 bp contained a Sox4-binding site (Fig. 4A), we examined whether Sox4 directly binds this region

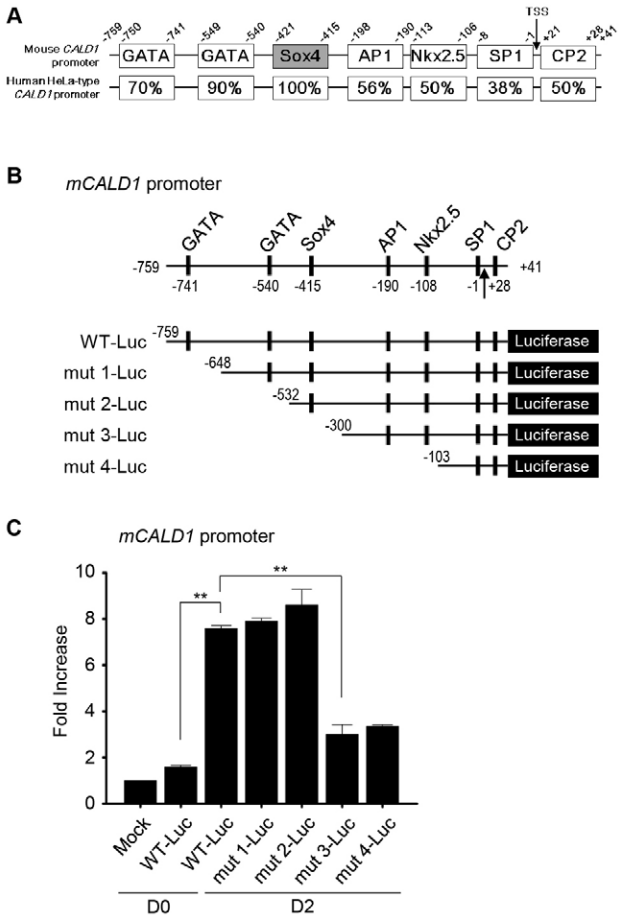

Fig. 4. The putative Sox4 cis-acting elements enhance *Cald1* promoter activity during C2C12 myoblast differentiation. (A) The mouse *Cald1* promoter (−759 to +41) was analyzed with a motif search program to identify putative transcription factor binding sites. These binding motifs were aligned with the human HeLa-type *CALD1* promoter. The percentage of conserved nucleotides is indicated. Possible binding factors are shown with their distance from the transcription start site (TSS; arrow, +1). (B) Schematic of four truncated promoter constructs. (C) C2C12 myoblast cells were co-transfected with each of the constructs shown in B and with pCMV-β-galactosidase. Transfected cells were maintained in proliferation (D0) or differentiation medium (D2). The luciferase activity of cells transfected with *Cald1*-WT-luciferase (WT-Luc) in differentiation medium was normalized to β-galactosidase and expressed as the fold increase over the mean value derived from cells co-transfected with *Cald1* WT-Luc in proliferation medium. The luciferase activity of cells transfected with truncated *Cald1*-luciferase constructs (mut-1-Luc–mut-4-Luc) in differentiation medium was expressed as the fold increase over the mean value derived from cells co-transfected with *Cald1* WT-Luc in differentiation medium. Data represent the means ± s.e.m. from three independent experiments. $^{**}P<0.01$.

of the *Cald1* promoter in a electrophoretic mobility shift assay (EMSA) using a recombinant Sox4 DNA-binding domain (DBD) fused to glutathione S-transferase (GST). As shown in Fig. 5A, incubation of a radiolabeled *Cald1* probe with the Sox4 DBD slowed the migration of the probe in a concentration-dependent manner (lanes 4, 5), indicating the formation of DNA–protein complexes. Incubation with GST alone did not produce DNA–protein complexes (lanes 2, 3). The DNA-protein complex was further shifted in the presence of anti-GST antibody (lane 6),

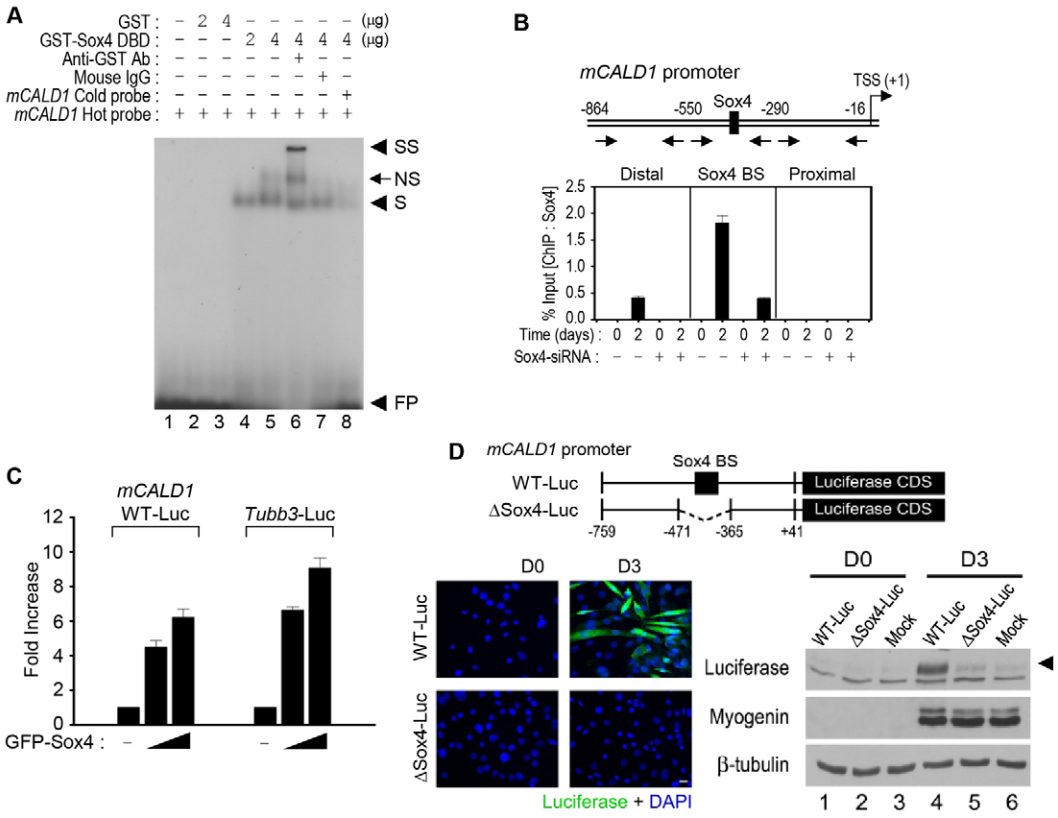

Fig. 5. Transcription factor Sox4 directly binds the *Cald1* promoter and enhances its activity during C2C12 myoblast differentiation. (A) EMSA showing the binding of Sox4 to Sox4-binding sites in the *Cald1* promoter. The lanes are indicated below the autoradiograph. Two or four micrograms of purified Sox4 DNA-binding domain protein (Sox4 DBD) was used for the assay. For the competition experiment, lane 8 included a 10-fold molar excess of unlabeled oligonucleotide containing the Sox4-binding site sequence. Lane 6 contained 0.1 μg of antibody against GST, and lane 7 contained the same quantity of mouse IgG as a negative control for the GST antibody. The arrowheads mark specific shifted bands (SS, supershift; S, shift; FP, free probe). The arrow marks non-specific bands (NS). These experiments were performed at least three times, and similar results were obtained. (B) Schematic diagram of primer pairs in the distal region, middle region containing the Sox4-binding site, and proximal region of the *Cald1* promoter used in the ChIP assay (top panel). C2C12 myoblast cells were transfected with control siRNA or Sox4 siRNA and maintained in proliferation or differentiation medium as indicated in the figure (bottom panel). ChIP analysis was conducted using anti-Sox4 antibodies, and the recruitment of Sox4 to the *Cald1* promoter region was normalized to the input. Data represent the means ± s.e.m. from three independent experiments. (C) HEK293 cells were co-transfected with *Cald1* WT-Luc, pCMV-β-galactosidase, and increasing amounts of plasmid encoding Sox4 cDNA. Two days after transfection, luciferase activity was measured and normalized to β-galactosidase. Data are expressed as the fold increase over relative luciferase units, normalized to the control. The *Tubb3* promoter was used as a positive control. Data represent the means ± s.e.m. from three independent experiments. (D) Schematic showing a promoter construct with deletion of the putative Sox4-binding sequence (Sox4 BS; top panel). *Cald1* WT-Luc or ΔSox4-Luc constructs were transfected into C2C12 myoblast cells. The cells were maintained in proliferation or differentiation medium as indicated in the figure (bottom panel). The cells were examined by immunofluorescence analysis with anti-luciferase (green) or DAPI nuclear stain (blue; left panel). Scale bar: 20 μm. Cell lysates were subjected to immunoblot analysis with anti-luciferase, myogenin, and β-tubulin antibodies (right panel). Myogenin was used as a differentiation marker and β-tubulin was used as the loading control. The arrowhead indicates the band for synthesized luciferase protein.

whereas IgG had no effect on the mobility shift of the DNA–protein complex (lane 7). Moreover, the DNA-protein complex disappeared when unlabeled *Cald1* probe was added (lane 8). The results indicate that Sox4 can bind directly to the *Cald1* promoter region between −421 bp and −415 bp *in vitro*.

To examine whether Sox4 binds to the *Cald1* promoter *in vivo*, we performed a ChIP analysis with C2C12 myoblast cells transfected with control or Sox4 siRNA and cultured in proliferation medium (D0) or differentiation medium (D2). As shown in Fig. 5B, neither the proximal nor distal region of the *Cald1* promoter was specifically precipitated by the Sox4 antibody. The precipitation of the distal region of the *Cald1* promoter was probably nonspecific because the region does not contain a Sox4-binding sequence. By contrast, anti-Sox4 antibody precipitated DNA corresponding to the Sox4-binding sequence in the *Cald1* promoter. The amount of DNA precipitated was reduced considerably when the cells were transfected with Sox4 siRNA (Fig. 5B, middle panel). As a negative control, we performed ChIP analysis using IgG instead of Sox4 antibody; no significant signal was detected (supplementary material Fig. S4). In addition, acetylated histones H3 and H4, which are markers for active transcription, were associated with the *Cald1* gene promoter in differentiating, but not proliferating, C2C12 cells, and their recruitment to the *Cald1* promoter was reduced in *Sox4*-silenced C2C12 cells (supplementary material Fig. S4). Taken together, these results indicate that Sox4 can bind directly to the *Cald1* promoter in the early stage of C2C12 myoblast differentiation.

To confirm that Sox4 induces CaD expression, HEK293 cells were co-transfected with the *Cald1* WT-Luc construct and Sox4-GFP constructs. The activity of *Cald1* WT-Luc increased up to ~sixfold with the expression of GFP-Sox4 protein (Fig. 5C). A reporter assay with the 622 bp non-coding region of the mouse *Tubb3* gene was used as a positive control for Sox4-mediated gene expression (Bergsland et al., 2006).

To determine whether Sox4-dependent *Cald1* promoter activity is associated with C2C12 myoblast differentiation, constructs containing the full-length sequence of the *Cald1* promoter (WT-Luc) or the *Cald1* promoter with deletion of the Sox4-binding sequence (ΔSox4-Luc) were transfected into C2C12 myoblast cells. Newly synthesized luciferase protein was analyzed by immunofluorescence and western blot. At 3 days after differentiation, cells transfected with the WT-Luc construct showed strong expression of luciferase in myotubes, whereas luciferase expression was nearly absent in cells transfected with the ΔSox4-Luc construct (Fig. 5D, left panel). These results were confirmed by western blot analysis (Fig. 5D, right panel). As expected, none of the cells expressed luciferase when cultured in proliferation medium (D0). Meanwhile, cells transfected with WT-Luc, but not ΔSox4-Luc, synthesized luciferase when cultured in differentiation medium (D3). Myoblast differentiation was confirmed by the expression of myogenin. These results suggest that Sox4 is the core transcription factor regulating the promoter activity of *Cald1* in myoblast differentiation.

Sox4-mediated CaD expression is required for skeletal muscle differentiation

Having shown that Sox4 regulates the expression of CaD in differentiated myoblasts, we next examined whether *Sox4* silencing inhibits myoblast differentiation. As shown in Fig. 6A, Sox4 was expressed in proliferating C2C12 myoblasts, and the protein level increased modestly as differentiation progressed (Fig. 6A,B). To examine the effect of *Sox4* silencing on myoblast differentiation, we transfected C2C12 myoblasts with Sox4 siRNA or with control siRNA and analyzed the lysates by western blot analysis 2 days later. In cells transfected with Sox4 siRNA (#1 and #2), but not in cells transfected with control siRNA, the level of Sox4 decreased by almost 90% (Fig. 6C). The expression of CaD and myogenic marker proteins was also reduced in the *Sox4*-silenced cells. The level of tubulin was unaffected. Consistent with this finding, the percentage of fused myoblasts also decreased in *Sox4*-silenced C2C12 myoblasts, which expressed MHC weakly (Fig. 6D,E). We then examined whether CaD overexpression could rescue the inhibitory effect of Sox4 siRNA on myoblast fusion.

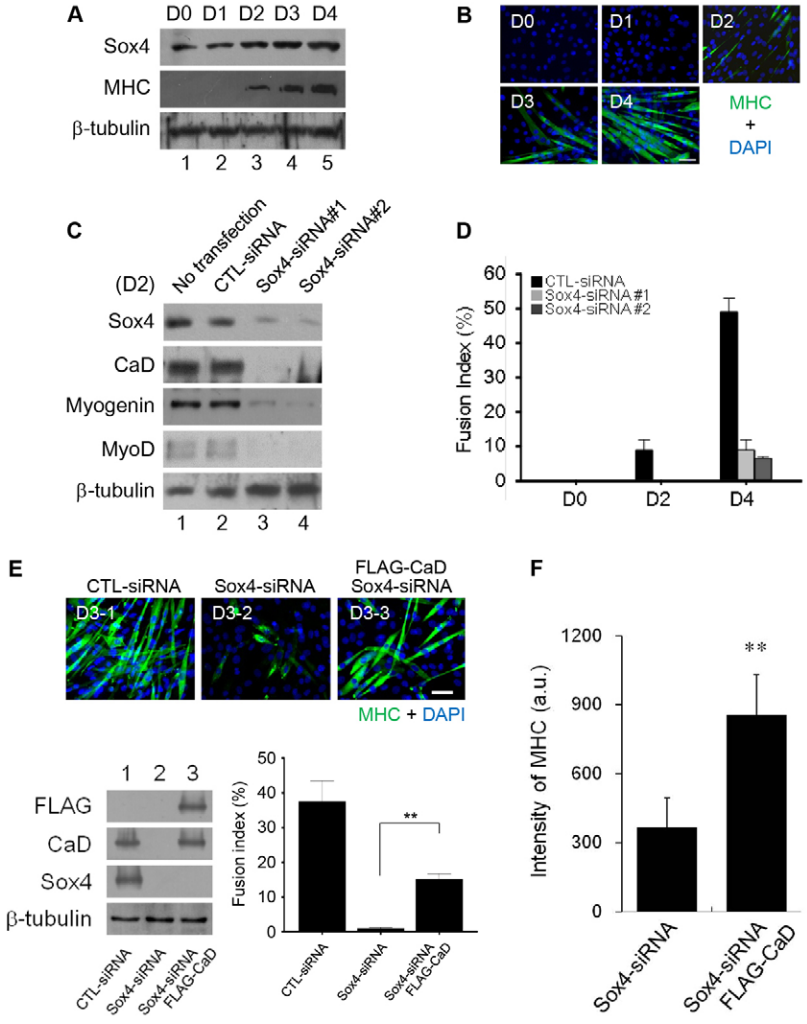

Fig. 6. Sox4-mediated CaD expression is required for skeletal muscle differentiation. (A) C2C12 myoblast lysates prepared at the indicated times after the induction of differentiation were analyzed by western blot with specific antibodies against the indicated proteins, including Sox4 and a muscle-specific marker (MHC). (B) C2C12 myoblasts at the indicated times after differentiation were subjected to immunofluorescence analysis for MHC (green) and DAPI (blue). Scale bar: 20 μm. (C) C2C12 myoblast cells transfected with control siRNA or Sox4 siRNA were cultured for 2 days in DM to induce differentiation. Cell lysates were analyzed by immunoblotting with antibodies against Sox4, CaD, myogenin, MyoD and β-tubulin. (D) The total fusion index represents the number of nuclei in multinucleated myotubes (at least three nuclei) divided by total number of nuclei in the field. Data represent the means ± s.e.m. from three independent experiments. (E,F) C2C12 myoblast cells were transfected with Sox4-siRNA with or without a FLAG-CaD expression plasmid and maintained in differentiation medium for 3 days. The cells were examined by immunofluorescence analysis with anti-MHC (E, top panel), and the fusion rates were quantified (E, right panel). Scale bar: 50 μm. Lysates from the remaining transfectants were subjected to immunoblot analysis with anti-FLAG, Sox4, CaD, and β-tubulin antibodies (E, left panel). From 10 randomly selected fields, the intensity of MHC expression was quantified using NIS Element Image analysis software and expressed in arbitrary units (F). $^{**}P<0.01$.

Sox4-silenced C2C12 cells were transfected with a FLAG-CaD construct and incubated for 3 days in differentiation medium. To verify the transfection efficiency, we transfected C2C12 myoblasts with an equal amount (3.0 μg) of GFP cDNA and examined GFP expression with florescence microscopy. GFP fluorescence was observed in almost 90% of the cells and this signal was detectable for more than 3 days. Thus, the transfection efficiency was high enough to perform the experiment with transient expression (supplementary material Fig. S5). Although overexpression of CaD in *Sox4*-silenced cells did not fully restore myoblast fusion to the level observed in mock-siRNA-transfected cells, both myotube formation and MHC expression were moderately rescued by CaD overexpression (Fig. 6E,F). To ensure that myoblast fusion and MHC expression in *Sox4*-silenced C2C12 cells was rescued only in cells that overexpressed FLAG-CaD, we co-transfected FLAG-CaD and GFP constructs at a 5:1 molar ratio. Immunofluorescence analysis confirmed that myoblast differentiation and MHC expression were restored only in cells expressing CaD (supplementary material Fig. S6).

Sox4-mediated CaD expression is required for myofibrillogenesis during embryonic skeletal muscle differentiation

To understand the role of Sox4-mediated CaD protein expression in skeletal muscle differentiation *in vivo*, we introduced control or Sox4 siRNA, with or without a GFP expression vector, directly into the hindlimb muscle of mouse embryos at embryonic day (E) 14 by electroporation (Cardoso et al., 2004). To verify the transfection efficiency, we electroporated various amounts of GFP cDNA into tissue samples and quantitatively analyzed the extent of transfection. GFP fluorescence was detected in more than 90% of myofibers after transfection of 3 μg of GFP cDNA (supplementary material Fig. S7). Consistent with the results from C2C12 myoblasts, silencing of *Sox4* inhibited CaD and MHC expression in the hindlimb muscle of mouse embryos (Fig. 7A).

Because decreased MHC expression during skeletal muscle differentiation results in skeletal muscle hypotrophy (smaller myofibers) or hypoplasia (fewer myofibers) (O'Rourke et al., 2007), we analyzed the morphological characteristics of myofibers in the *Sox4*-silenced mouse embryonic hindlimb muscle by staining for MHC and actin (Fig. 7B). As shown in Fig. 7C,D, the number of myofibers did not significantly differ between the samples, but the projected area of the myofibers decreased slightly upon silencing of *Sox4*. Of interest, MHC in control-siRNA-transfected muscle appeared as spot-like structures that completely colocalized with actin in the myofibers. However, in muscle transfected with Sox4 siRNA, distinct MHC structures were rarely detected, whereas some fibers still exhibited spot-like actin structures (Fig. 7B,E). Because actin and MHC assembly is crucial for myofibril formation in functional muscle development (Sanger et al., 2005), our results suggest that Sox4-mediated CaD expression is important for myofibril assembly during embryonic skeletal muscle differentiation.

Discussion

During skeletal muscle differentiation, the regulation of cytoskeletal structure in myoblasts is essential for the initiation of membrane fusion at an early stage of differentiation and for the formation of a contractile unit in the myotube (Richardson et al., 2008). The establishment of actin cytoskeletal interactions with the extracellular matrix and between cells is important for myoblast differentiation (Kim et al., 2007). In addition, numerous studies have shown that actin-regulating proteins are involved in muscle differentiation and development (Bongiovanni et al., 2012; Nowak et al., 2009; Richardson et al., 2008). Suppression of Rho family small-GTPase activity by pharmacological inhibitors interferes with actin remodeling and the expression of myogenic factors such as myogenin and MRF4 (Bryan et al., 2005). However, little is known about the transcriptional regulation of actin-regulating proteins and its role in myoblast differentiation. In this study, we found that CaD regulates cell spreading and migration in the early stage of C2C12 myoblast differentiation. The expression of CaD in the early stage of differentiation is controlled at the transcriptional level by Sox4 transcription factor.

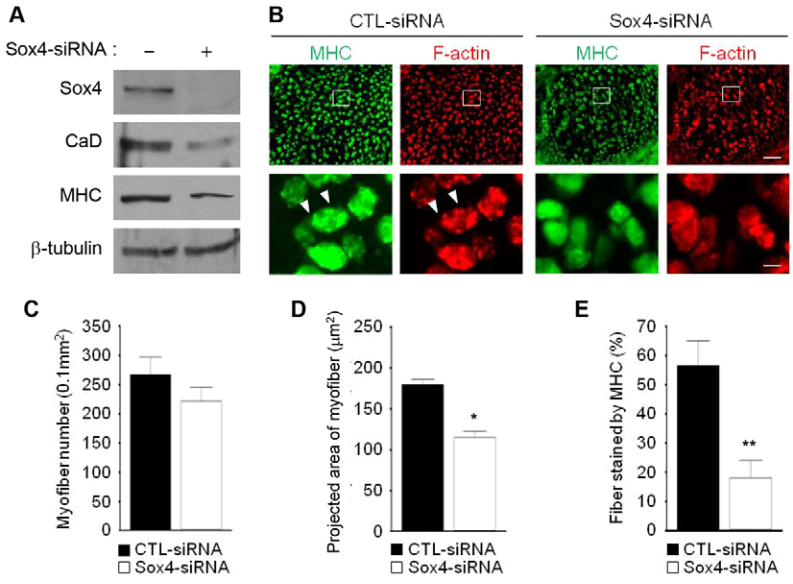

Fig. 7. Sox4-mediated CaD expression is required for myofibril formation during embryonic skeletal muscle differentiation. (A,B) Control siRNA or Sox4 siRNA constructs were introduced directly into the hindlimb muscle of E14 mouse embryos by *in vivo* electroporation. Injected tissues were incubated using an *in vitro* tissue culture method. Lysates of muscle tissues were subjected to immunoblot analysis with antibodies to the indicated proteins (A). Cross sections of muscle tissues at 3 days post injection were immunostained with anti-MHC (green) and with Rhodamine-Phalloidin (red) to visualize F-actin. Higher magnification images of the boxed regions in the upper panel are shown in the bottom panel. Scale bars: 50 μm (top panel) and 7 μm (bottom panel). Structures enriched for MHC and F-actin are indicated by the white arrowheads (B). (C) The number of myofibers in an area of 0.1 mm^2, quantified from at least three photographed fields from three independent experiments, is presented as the means ± s.e.m. (D) A cross sectional area of differentiating myofibers was quantified and expressed as the means ± s.e.m. (n=100). (E) The number of myofibers that strongly expressed MHC in myofibrils was quantified in three independent experiments and expressed as the percentage of myofibers stained by MHC (n=100). Data are the means ± s.e.m. from a representative experiment. *P<0.05, **P<0.01.

The cellular functions of CaD are controversial. There is evidence that h-CaD expression enhances stress fiber stability and reduces cell motility in non-muscle cells (Castellino et al., 1992; Castellino et al., 1995; Eppinga et al., 2006; Li et al., 2004; Mayanagi et al., 2008; Mirzapoiazova et al., 2005). Moreover, CaD expression induced by p53 suppresses Src-kinase-dependent podosome formation, which inhibits fibroblast and rat aortic SMC migration (Mukhopadhyay et al., 2009). By contrast, HeLa–l-CaD expression in endothelial cells and endothelial progenitor cells in the vasculature of various human tumors promotes cell migration for vasculogenesis and angiogenesis during tumor development (Zheng et al., 2004; Zheng et al., 2007). Of note, we observed that endogenous CaD in C2C12 myoblasts localized to the membrane ruffles, suggesting that CaD is involved in cell migration in C2C12 myoblasts. Consistent with this idea, silencing of CaD in C2C12 cells strongly inhibited cell spreading and migration (Fig. 2 and supplementary material Fig. S2). Disassembly of vinculin- and talin-containing focal adhesions was observed in HeLa–l-CaD-positive endothelial cells (Zheng et al., 2007). Overexpression of l-CaD promotes cell movement, but inhibits cell contractility with a decrease in focal adhesions (Helfman et al., 1999). Meanwhile, the increase in cell contractility with vinculin- and talin-mediated strong adhesions prevents the cell spreading of fibroblasts (Park et al., 2002). Thus, it is plausible that CaD in C2C12 myoblasts controls the dynamics of focal adhesion assembly, followed by the induction of cell migration in response to differentiation signaling.

CaD is expressed as two different isoforms, which are mainly generated by alternative splicing (Mayanagi and Sobue, 2011). Selective splicing of exon 1a or 1b produces the fibro-type or HeLa-type N-terminal sequence of CaD, respectively. Amino acid sequence comparison of mouse CaD and human fibro l-CaD-I or l-CaD-II showed that mouse CaD has no significant homology with human fibro l-CaD proteins in the N-terminal region, which corresponds to the first encoding exon. Instead, mouse CaD shares 78% identity with the N-terminus of HeLa l-CaD proteins (supplementary material Fig. S8A). Moreover, additional amino acid alignments of mouse CaD and human HeLa l-CaD-I or l-CaD-II confirmed that the CaD protein in C2C12 myoblasts is identical to HeLa l-CaD-II in humans (supplementary material Fig. S8B,C).

Furthermore, analysis of the genomic structure of *Cald1* indicates that the transcripts encoding the CaD isoforms in chickens and humans are generated by independent promoters (Hayashi et al., 1992; Yano et al., 1994). In the human *CALD1* gene, the promoter responsible for the fibro-type CaD protein is located in the proximal region of exon 1a-1, which generates h-CaD and l-CaD proteins; the HeLa-type promoter located upstream of exon 1b generates only l-CaD proteins (HeLa l-CaD-I and l-CaD-II) (Hayashi et al., 1992). Thus, it is reasonable to expect that the expression of mouse CaD is also regulated by the HeLa-type promoter. We also identified a fibro-type-like promoter for the mouse *Cald1* gene within the coding region of an uncharacterized protein (LOC100861595; gene ID: 100861595). The promoter sequence is positioned approximately −110 kbp upstream from the transcription start site of the mouse *Cald1* gene. Thus, it does not appear that the fibro-type-like promoter sequence is involved in mouse *Cald1* gene expression. This was not a major concern for the analysis of HeLa-type promoter activity related to CaD expression because HeLa-type promoter activity is weaker than fibro-type promoter activity in various tissues (Fukumoto et al., 2009; Yano et al., 1995).

To date, several cis-acting elements such as the CArG box and GRE sequence, which are primarily responsible for the transcription of *CALD1*, have been identified (Fukumoto et al., 2009; Mayanagi et al., 2008; Momiyama et al., 1998; Yano et al., 1995). These conserved cis-acting elements are located in the fibro-type promoter. However, little is known about the cis-acting elements in the HeLa-type promoter. Sequence analysis of the mouse *Cald1* promoter indicates that the cis-acting elements in the HeLa-type promoter are completely different from those in the fibro-type promoter. The promoter of mouse *Cald1* is nearly identical to the HeLa-type promoter in humans. In particular, the Sox4-binding site is completely conserved in both promoters (Fig. 4A). Thus, the Sox4-binding site might serve as a crucial cis-acting element for HeLa-type promoter activity.

The promoter of the mouse *Cald1* gene has several other conserved sequences that are capable of binding to transcription factors, including GATA2, GATA3, AP1, SP1 and Nkx2.5 (Fig. 4). However, these transcription factors repress myoblast differentiation, and their expression decreases during differentiation of C2C12 myoblasts (Itoh et al., 2008; Lehtinen et al., 1996; Riazi et al., 2005). Therefore, these trans-acting factors, except for Sox4, appear not to be involved in CaD expression during myoblast differentiation. Some Sox transcription factors might require binding partners to selectively recognize their target promoters (Wilson and Koopman, 2002). For example, Sox2, Sox8 and Sox10 interact with POU (Pit-1, Oct and Unc-86) transcription factors to recognize their target genes (Bernard and Harley, 2010). However, no POU transcription factors have yet been reported to bind Sox4 to regulate its transcriptional activity. Interestingly, we found that the mouse *Cald1* promoter contains binding sequences for the Oct-1 transcription factor, a member of the POU family, near the Sox4-binding sequence, with lower score threshold (supplementary material Fig. S9). Oct-1 interacts with other Sox family proteins through their HMG domain, and the HMG domain in Sox4 is highly conserved with other Sox proteins. Thus, further study of the regulation of Sox4 activity by Oct-1 might provide new insight into Sox4-mediated differentiation of myoblasts.

Although the exact functions of Sox4 were not elucidated until a few years ago, Sox4 has been considered an important factor for the development of various organs and tissues, including the heart (Schilham et al., 1996), thymocytes (Schilham et al., 1997), nervous system (Cheung et al., 2000) and osteoblasts (Nissen-Meyer et al., 2007). Recently, functional studies of Sox4 have focused on its undefined roles in developmental processes. For instance, Sox4 is required for neural and glial cell development because it regulates pan-neuronal gene expression, including the transcription of the class-III β-tubulin gene *Tubb3*, which is required for neuronal maturation (Bergsland et al., 2006). Members of the SoxC group, including Sox11 and Sox12, in addition to Sox4, might have essential function in early mouse embryonic organogenesis; they directly target the *Tead2* gene, a crucial transcription factor in the Hippo signaling pathway (Bhattaram et al., 2010). Recent reports have also shown that Sox4 and Sox11 are necessary for the control of corticospinal system development; Sox4 and Sox11 directly regulate *Fezf2*, which encodes a key factor for the specification of identity and the connectivity of corticospinal neurons (Shim et al., 2012). In

C2C12 myoblasts, our quantitative RT-PCR results showed that the levels of *Sox11* and *Sox12* transcripts were extremely low compared with the level of Sox4; their expression pattern did not change during myoblast differentiation. Thus, of the Sox family members, Sox4 is probably the primary transcription factor for C2C12 myoblast differentiation (supplementary material Fig. S10).

The expression of myogenic regulatory factors (MRFs), including MyoD and myogenin, is believed to orchestrate the gene regulatory network that controls muscle differentiation. Proliferating myoblasts express the MyoD by which the cell can be committed to the muscle lineage. Myogenin expression increases dramatically at the onset of differentiation to regulate the expression of various downstream genes, including myosin (Braun and Gautel, 2011). Sox4 is expressed in proliferating cells, and its expression increases moderately upon serum deprivation. In this study, silencing of *Sox4* by siRNA inhibited myoblast differentiation and reduced MyoD expression, indicating that Sox4 functions as a myogenic factor. Promoter sequence analysis showed that the promoter of the *Myod* gene contains Sox4-binding sites at various positions, although the score threshold was low. Thus, it is likely that Sox4 can control the transcription of *Myod*. Alternatively, Sox4 might have a similar function as a muscle-determining factor upstream of MyoD. The identification of target genes regulated by Sox4 activity will help define the molecular mechanism of skeletal muscle differentiation.

Consistent with our *in vitro* results, Sox4 siRNA decreased the levels of CaD and MHC when electroporated into the hindlimb of mouse embryos (Fig. 7). Interestingly, immunofluorescence staining with anti-MHC antibody and phalloidin demonstrated that the projected area of myofibers decreased in *Sox4*-silenced embryonic muscle tissue. Furthermore, MHC organization in individual myofibrils appeared defective, but the overall number of myofibers was similar to the number in control-siRNA-transfected muscle. CaD can interact with F-actin, myosin, calmodulin (CaM) and tropomyosin (TM) (Graceffa, 1987; Smith et al., 1987; Sobue et al., 1981; Sobue and Sellers, 1991; Wang et al., 1997). Moreover, CaD enhances the binding of F-actin to TM and competes with gelsolin, an actin-severing protein that binds to F-actin (Ishikawa et al., 1989; Warren et al., 1994). Consequently, because it promotes stable actin-myosin structures in smooth muscle, CaD is an important regulatory protein for thin filament organization (Sobue and Sellers, 1991). In developing skeletal muscle, the actin-myosin system is also required for normal muscle functions such as contraction or relaxation. Taken together, our results suggest that Sox4-mediated CaD expression is involved in actomyosin organization in myofibrils during skeletal muscle development.

Materials and Methods

Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells and C2C12 cells, a myogenic cell line derived from mouse, were obtained from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (50 units/ml; Invitrogen) in a humidified atmosphere of 5% CO_2 at 37℃. For all experiments, C2C12 cells were seeded in proliferation medium (GM) at a density of 2×10^4 cells/cm^2. Differentiation was induced 24 hours after seeding by exchanging GM for differentiation medium (DM; DMEM supplemented with 2% horse serum). Cells were transiently transfected with different plasmid DNA using a Neon electroporation system (Invitrogen) according to the manufacturer's instructions.

Plasmid constructs

The full-length coding region of *SOX4* was amplified from HEK293 cell cDNA generated by reverse transcriptase (Intron, Korea). Amplified *SOX4* cDNA was introduced into a pEGFPC2 vector (6083; Clontech, Mountain View, CA). The full-length coding region of mouse *Cald1* was amplified from C2C12 cell cDNA, generated by reverse transcriptase, and introduced into a pFLAG-CMV2 vector (E7033; Sigma-Aldrich). *Tubb3* and mouse *Cald1* promoter fragments were generated by PCR using genomic DNA extracted from C2C12 cells. Truncated mutants and Sox4-binding site deletion mutants were amplified from the WT *Cald1* promoter using sequential PCR and were introduced into a pGL.4.12 basic vector (E6671; Promega, Madison, WI). For siRNA constructs, oligonucleotides for CaD siRNA and Sox4 siRNA were introduced into the pBabe-dual vector. All clones were verified by DNA sequencing. The PCR primers used in this study and their sequences are listed in supplementary material Table S1.

Luciferase assay

HEK293 or C2C12 cells were transfected with vectors containing the firefly luciferase reporter gene (0.1 μg) and pCMV-β-galactosidase (0.1 μg) together with GFP-Sox4 or siRNA Sox4 using an electroporation method. Thereafter, transfected cells were partially selected under puromycin or neomycin for 3–4 days. Transfected cells were lysed in reporter lysis buffer (Promega), and cell extracts were analyzed with a luciferase reporter assay system using a GloMax luminometer (Promega). Luciferase activities were normalized to the β-galactosidase activity of the co-transfected vector.

Sox4 polyclonal antibody production

Polyclonal antibodies against Sox4 were produced in a rabbit using a Sox4-specific peptide (TNNAENTEALLAGESSDSGA) (Peptron, Korea) as an antigen. Pre-immune serum from the same rabbit was obtained before immunization. After boosting four times at 2 week intervals, the serum was tested by western blot analysis using purified GST-Sox4 DBD protein. The specificity of the purified antibodies was further analyzed by western blotting using serial dilution.

Electrophoretic mobility-shift assay

Oligonucleotide labeling and EMSA were performed as described by Hellman and Fried (Hellman and Fried, 2007). The oligonucleotides for the *Cald1* promoter and their sequences are listed in supplementary material Table S1. The DNA–protein binding reaction was conducted in a mixture containing 10× binding buffer (100 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1 M KCl, 1 mM DTT, 50% v/v glycerol, 0.1 mg/ml BSA), 4000 cpm of ^{32}P-labeled oligonucleotide and affinity-purified GST-Sox4 DBD protein for 30 minutes at 30℃, and resolved on a 6% acrylamide gel that had been pre-run at 150 V for 1 hour with 400 mM TAE (Tris, acetic acid and EDTA) buffer. The loaded gel was run at 200 V for 40 minutes, dried and placed on Kodak X-ray film (Eastman Kodak, Rochester, NY) for autoradiography. After 16 hours of exposure at −20℃, the film was developed.

Chromatin immunoprecipitation

The ChIP assay was performed following a protocol provided by Millipore (Temecula, CA). C2C12 cells were sonicated to shear chromatin to 500–1000 bp fragments. For immunoprecipitation, 2 mg of anti-Sox4 antibodies were incubated with the cell lysates overnight at 4℃ with rotation. Lysates were immunoprecipitated with IgG as a control. Precipitated DNA was analyzed by quantitative real-time PCR using *Cald1* promoter-specific primers, as indicated in supplementary material Table S1.

Immunoblotting and immunofluorescence microscopy

Anti-CaD (sc-15374; Santa Cruz Biotechnology), myogenin (sc-12732; Santa Cruz Biotechnology), MHC (MF20; Developmental Studies Hybridoma Bank) and luciferase (ab21176; Abcam) antibodies were used for immunoblotting. Horseradish-peroxidase-conjugated secondary antibodies were used for detection and immune complexes were visualized with ECL-chemiluminescence (sc-2048; Santa Cruz Biotechnology). Immunocytochemistry and immunohistochemistry were performed following a protocol provided by Abcam. Primary antibodies were used at 1:200 for CaD, 1:200 for MHC, and 1:500 for luciferase. Cy3-conjugated donkey anti-mouse IgG (1:200; Jackson ImmunoResearch Laboratories) and FITC-conjugated donkey anti-rabbit IgG (1:500; Abcam) were used as secondary antibodies. DAPI (Sigma) was used for nuclear staining. Rhodamine-Phalloidin was used for polymerized actin staining (PHDR1; Cytoskeleton). Samples were incubated with 300 nM DAPI in PBS for 2 minutes at room temperature and visualized using a Nikon Eclipse 80i microscope (Melville, New York).

Electroporation and *in vitro* skeletal muscle culture

The DNA constructs indicated in Fig. 7 were prepared using a Qiagen maxiprep kit (Qiagen, Valencia, CA) and concentrated using an ethanol-precipitation method to a final concentration of 3.0 μg/μl DNA. A solution of DNA (1 μl) was injected into the hindlimb muscles using a pulled glass capillary connected to an IM6 microinjector. The injected muscle tissue was then placed between the electrodes

of the electroporation chamber and covered with ice-cold PBS. Square-wave pulses (35 V; pulse length, 150 milliseconds; three pulses at 100 millisecond intervals) were delivered by the electroporator (BTX ECM 830; Harvard Apparatus, Holliston, MA). Electroporated hindlimb muscles of mouse embryos were incubated using *in vitro* muscle culture systems as described by Cardoso and co-workers (Cardoso et al., 2004).

Fusion index

The extent of myoblast fusion was expressed as the percentage of the number of nuclei in fused cells relative to the total number of nuclei in randomly chosen fields under a microscope. Cells were considered fused only when there was clear cytoplasmic continuity and at least three nuclei were present in each myotube.

Acknowledgements

We are grateful to Dr Kun Ho Lee (Chosun University, Korea) for helpful comments and suggestions.

Author contributions

S.-M.J., J.-W.K., K.-H.C. and S.R. conceived and designed experiments; S.-M.J. performed experiments; S.-M.J., J.-W.K., D.K., C.-H.K., J.-H.A., K.-H.C., and S.R. analyzed the data; S.-M.J. and D.K. prepared figures; S.-M.J. and S.R. wrote the paper.

Funding

This research was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) [grant number 2012008662].

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.131581/-/DC1

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