# Role of H1-Calponin in Pancreatic AR42J Cell Differentiation Into Insulin-Producing Cells

**Tomoaki Morioka,[1] Hidenori Koyama,[1] Hisako Yamamura,[2] Shinji Tanaka,[1] Shinya Fukumoto,[1] Masanori Emoto,[1] Hiroyuki Mizuguchi,[3] Takao Hayakawa,[3] Itaru Kojima,[4] Katsuhito Takahashi,[2] and Yoshiki Nishizawa[1]**

**Basic or h1-calponin is a smooth muscle–specific, actin-binding protein that is involved in the regulation of smooth muscle contractile activity. We found in this study the expression of mRNA and protein for h1-calponin in AR42J-B13 cells, which is a useful model for investigating islet β-cell differentiation from pancreatic common precursor cells. Following treatment of AR42J cells with activin A and hepatocyte growth factor, the protein levels of h1-calponin decreased in a time-dependent manner during the course of the cell differentiation. When h1-calponin was continuously overexpressed by utilizing recombinant adenovirus-mediated gene transfer, the percentage of cell differentiation in h1-calponin overexpressing cells was markedly suppressed as compared with that in the cells without overexpression (6.7 ± 2.5 vs. 28.6 ± 3.2%, $P < 0.001$, Student's $t$ test). Finally, overexpression of h1-calponin (65.6 ± 3.4), or that lacking actin-binding domain (55.9 ± 3.4%), significantly ($P < 0.001$) suppressed the activin A–stimulated transcriptional activity of activin responsive element (ARE), whereas calponin homology-domain disruption mutant did not (100.6 ± 1.9%). These results suggest that regulation of h1-calponin is involved in the regulation of differentiation of AR42J cells into insulin-producing cells at least partly through modulating ARE transcriptional activity. *Diabetes* 52: 760–766, 2003**

From the [1]Department of Metabolism, Endocrinology, and Molecular Medicine, Osaka City University Graduate School of Medicine, Osaka, Japan; the [2]Department of Molecular Medicine and Pathophysiology, Osaka Medical Center for Cancer and Cardiovascular Diseases, the Graduate School of Pharmaceutical Science, Osaka University, SORST, JST, Osaka, Japan; the [3]Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, Tokyo, Japan; and the [4]Department of Cell Biology, Institute for Molecular and Cellular Regulation, Gunma University, Maebashi, Japan.

Address correspondence and reprint requests to Hidenori Koyama, MD, PhD, Department of Metabolism, Endocrinology and Molecular Medicine, (Second Department of Internal Medicine), Osaka City University Graduate School of Medicine, 1-4-3, Asahi-machi, Abeno-ku, Osaka 545-8585, Japan. E-mail: hidekoyama@med.osaka-cu.ac.jp.

Received for publication 29 May 2002 and accepted in revised form 9 December 2002.

ABD, actin-binding domain; ARE, activin responsive element; β-gal, β-galactosidase; CHD *mt*, calponin homology-domain disruption mutant; ERK, extracellular regulated kinase; FITC, fluorescein isothiocyanate; FSH, follicle-stimulating hormone; GABA, γ-aminobutyric acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HEK, human embryonic kidney; HGF, hepatocyte growth factor; MOI, multiplicity of infection; pGL2-ARE, pGL2-basic containing activin responsive element; SMC, smooth muscle cell; UTR, untranslated region.

H1-calponin is a 34-kDa troponin-like molecule that is present in most vertebrate smooth muscles that binds to actin, tropomyosin, and calmodulin and is involved in the regulation of smooth muscle contractile activity (1–6). Three isoforms of calponin, basic (or h1), neutral (or h2), and acidic calponin have been identified according to their isoelectric points and molecular size (5,7,8). Basic or h1 isoform of calponin, originally isolated from smooth muscle, is recently shown to be expressed in other mesenchymal cells including osteoblasts and mesangial cells (9,10). H2 and acidic calponin is ubiquitously expressed in both smooth muscle and non–smooth muscle tissues, such as adrenal gland, platelets, fibroblasts, brain tissues, cerebellar cells, endothelial cells, keratinocytes, and mesangial cells (5,10–12).

Activin A, a member of the transforming growth factor β family, was initially isolated from gonads as a stimulator of follicle-stimulating hormone (FSH) secretion. The peptide is a dimer of the β-subunit of inhibin, which in turn inhibits FSH secretion (see recent review in 13). Inhibins consist of either a βA or βB subunit linked by disulfide bonds. Activin A is a homodimer of the βA-subunit (βA-βA) with potent activities in diverse biological systems including pancreatic hormone release (14). Activin signaling occurs via binding to a heterotrimeric receptor complex with transmembrane serine/threonine kinase activity (13). The receptor encompasses two subgroups, type I (ActR I and ActR IB) and type II (ActR II and Act IIB) receptors (13). Transgenic mice expressing dominant negative activin receptor mutants show hypoplasia of pancreatic islets (15,16). Activin gene expression has been shown in rat (17) and human fetal pancreas (18). Moreover, immunoreactive activin A has been found in rat (19) and human pancreatic endocrine cells (20,21).

AR42J cell is derived from a chemically induced rat pancreatic acinar cell tumor and possesses both exocrine and neuroendocrine properties characterized by the secretion of digestive enzymes, the expression of voltage-sensitive ionic currents, and the release of small neurotransmitters such as glycine, glutamate, γ-aminobutyric acid (GABA), and synaptophysin (22). When treated with activin A, amylase-secreting AR42J cells are converted into the cells that possess neuron-like properties and express ATP-sensitive potassium channel and mRNAs for glucose transporter-2 and pancreatic polypeptide (23,24).

Thus, activin A converts AR42J cells into endocrine cells, although the activin-treated cells die due to apoptosis. In the presence of either betacellulin (24) or hepatocyte growth factor (HGF) (25), activin-treated AR42J cells survive and differentiate into insulin-producing cells. In pancreatic AR42J cells, mRNA for Smad2, a signaling molecule activated by activin receptor, is abundantly expressed. Activation of Smad2 pathway induces apoptosis and morphological changes of the cells, and is shown to be involved in activin A–induced differentiation of the pancreatic AR42J cell into insulin-producing cells (26). These observations indicate AR42J cells can provide a useful in vitro model system to investigate the differentiation of islet β-cell from pancreatic common precursor cells.

Recently, a transient expression of h1-calponin has been observed in dorsal pancreatic bud in E10-E11 of fetal mice, and also in cytokeratin-positive duct cells of three-dimensional organ culture of pancreas rudiment (K.T. and H.Y., unpublished observation). In this study, we show that h1-calponin is expressed in AR42J cells, and expression levels of h1-calponin are downregulated during AR42J cell differentiation induced by activin A. Overexpression of h1-calponin via adenoviral vector suppressed differentiation of AR42J cells into insulin-producing cells. Finally, we show that transcriptional activity stimulated by activin A is significantly suppressed by overexpression of h1-calponin in AR42J cells.

## RESEARCH DESIGN AND METHODS

**Materials.** Recombinant human activin A was a kind gift from Dr. Y. Eto (Central Research Laboratory, Ajinomoto, Kawasaki, Japan). Recombinant human HGF was obtained from Genzyme/Techne (Minneapolis, MN). Guinea-pig anti-porcine insulin antibody was kindly provided by the Institute for Molecular and Cellular Regulation, Gumma University, Maebashi, Japan (antibody code HAC-PC94-03-GPP94). Monoclonal mouse anti-human calponin (h1) antibody (clone hCP) was from Sigma (St. Louis, MO). This antibody was shown to recognize only h1-calponin protein without cross-reactivity with h2-calponin (11). Polyclonal rabbit anti-p70 S6 kinase antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti–β-galactosidase (β-gal) monoclonal antibody was from Chemicon International (Temecula, CA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-guinea pig IgG antibody and FITC-conjugated anti-mouse IgG antibody were purchased from ICN Pharmaceutical (Aurora, OH). Texas red–conjugated horse anti-mouse IgG antibody was from Vector Laboratories (Burlingame, CA). Purified mouse IgG1 was from Ancell Corporation (Bayport, MN). Affinity purified peroxidase-labeled horse anti-mouse IgG (H+L) antibody and goat anti-rabbit antibody were from Vector Laboratories.

**Cell culture.** AR42J-B13 cells, a subclone of AR42J cells that convert into insulin-producing cells in response to activin A and HGF, were cultured as previously described (25–27). Cells were routinely plated at a density of $1.2 \times 10^5/cm^2$ in dishes or on glass coverslips.

**Immunofluorescent analysis.** AR42J-B13 cells were cultured on glass coverslips and treated with or without activin A and HGF according to the experimental protocols. Immunocytochemistry and confocal microscopy were performed as previously described (28). Cells were incubated with anti-insulin antibody (1:5,000 dilution) for 60 min at 37°C or with anti-calponin antibody (1:1,000) for overnight at 4°C, and consecutively incubated with FITC-conjugated anti-guinea pig (for insulin) or anti-mouse IgG (calponin). Nuclei were stained by 0.04 μg/ml propidium iodide (Sigma) for 5 min. For staining actin fiber, cells were treated with 0.2 μmol/l TRITC-labeled phalloidin (Sigma) for 20 min. The immunofluorescence was analyzed by confocal microscopy with the help of the Fluoview software (Olympus Optical, Tokyo, Japan). In some experiments, double immunofluorescent analysis for insulin and calponin was performed. Cells cultured on glass coverslips were infected with recombinant adenovirus encoding β-gal (LacZ) or h1-calponin, and treated with activin A and HGF according to the experimental protocols. After fixing, permeabilization, and blocking of the cells, insulin was first stained with anti-insulin antibody and FITC-conjugated anti-guinea pig IgG antibody. Then the cells were refixed, blocked, and incubated with the anti-calponin antibody followed by Texas red–conjugated anti-mouse IgG antibody as the second color.

**Western blot analysis.** Preparation of whole cell protein extracts and Western blot analysis was performed as previously described (28). Anti–h1-calponin or anti-p70S6 kinase antibody was used at 1:10,000 or 1:400 dilution, respectively. Blots were developed by enhanced chemiluminescence using the ECL method (Amersham, Buckinghamshire, UK). Autoradiographs were developed using Kodak X-OMAT 300RA.

**RT-PCR analysis.** mRNA was extracted from AR42J-B13 cells using Fast-Track 2.0 Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. mRNA isolated from rat A7r5 cells, clonal rat smooth muscle cells (SMCs), was used as a positive control template. Reverse transcription of 2 μg of mRNA was performed using reaction mixture of Ready-To-Go You-Prime First-Strand Beads (Amersham, Piscataway, NJ) with 1 μg of oligo dT primer (Life technologies). PCR was conducted using Advantage cDNA PCR Kit (Clontech, Palo Alto, CA) in a DNA Thermal Cycler (Perkin Elmer, Norwalk, CT) under the condition of 30 cycles of denaturation (94°C, 40 s), annealing (60°C except for h1 calponin at 61°C, 30 s), and extension (72°C, 90 s). Forward and reverse oligonucleotide primers used, and predicted size of PCR products were as follows: h1 calponin 5′GGACGAGCCACCCAC AATCAC3′ (forward), 5′GGGCCCCCTTCTTCCTACTGC3′ (reverse), 207 bp; h2-calponin 5′GTTCATGCCGTAGCTGACCATG3′ (forward), 5′GAGCTCC ACGCAGTTCAACAAG3′ (reverse), 300 bp; acidic calponin 5′GAACAGC CATGACCCACTTCAAC3′ (forward), 5′TCGGGGTATTCTGCCTGGTAATC3′ (reverse), 935 bp; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) 5′CCCATCACCATCTTCCAGGA3′ (forward), 5′TTGTCATACCAGGAAAT GAGC3′ (reverse), 731 bp. The products were detected by separation on 1% Agarose gel electrophoresis. PCR products were cloned into pCR2.1 vectors using TA-cloning system (Invitrogen) and their sequences were analyzed by Big Dye Terminator Cycle Sequencing using ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

**Northern blot analysis.** Isolation of total cellular RNA and Northern blot analysis were performed as described previously (28). cDNAs for rat h1-, h2-, and acidic calponin and GAPDH were cloned by RT-PCR as described above and used as probes for hybridization.

**Construction and generation of recombinant adenovirus vectors.** Recombinant adenovirus vector expressing β-gal (Lac Z) containing Arg-Gly-Asp (RGD) sequence in the HI loop of the fiber knob was generated as previously described (29). The fiber-mutant adenovirus containing RGD-containing peptide in the fiber knob can be efficiently infected to mammalian cells through integrins on the cell surface (29,30). Human h1-calponin–expressing pAdHM15-RGD-calponin (Ad-Calponin) was constructed using in vitro ligation method as described below (31,32). In brief, a full-length human h1-calponin cDNA was first inserted into the shuttle plasmid, pHMCMV5, and was subcloned into the pAdHM15-RGD, which contains RGD sequence in the h1 loop of the fiber knob (30), using unique *I-Ceu*I and *PI-Sce*I sites in the E1 deletion region. Ad-Calponin vector was transfected to human embryonic kidney (HEK) 293 cells using Superfect transfection reagent (Qiagen) according to the manufacturer's protocol.

**Construction of deletion mutant of h1-calponin plasmid and activin A–stimulated transcriptional activity.** Deletion mutant cDNA of human h1-calponin lacking actin-binding domain [ABD(-)] (33) was constructed by deleting *Msc*I-*Stu*I region of full-length h1-calponin cDNA and linking *Nhe*I linkers as an universal codon terminator to its 3′-untranslated region (UTR) after blunting. Calponin homology-domain disruption mutant (CHD *mt*) (34), which is full-length h1-calponin lacking 62 amino acids including $NH_2$-terminal region of CH-domain, was constructed by deleting the 5′-coding region from *Eco*RI to *Xho*I sites of full-length h1-calponin cDNA subcloned in the pBluescript SK(+) vector (Stratagene, La Jolla, CA) and connecting its adenine thymine guanine sequence to 5′-UTR. Then they were subcloned into pCAGGS vector. Activin A-dependent transcriptional activity was determined by using pcDNA3-FAST-1 and pGL2-basic containing activin responsive element (pGL2-ARE) (kindly provided by Dr. Takeshi Imamura, the Cancer Institute of Japanese Foundation for Cancer Research, Tokyo, Japan, and Dr. Kohei Miyazono, University of Tokyo, Tokyo, Japan) as originally described (35). AR42J-B13 cells cultured in DMEM containing 10% fetal bovine serum (FBS) were plated in 6-well plates ($1.2 \times 10^5/cm^2$) and incubated for 24 h. The cells were transfected with 0.45 μg of reporter plasmids, 0.45 μg of pcDNA3-FAST-1, 0.375 μg of effector plasmids, and 0.1 μg of pRL-TK R*luc* reference plasmid using Lipofectamine PLUS reagent (Life Technologies) according to the manufacturer's protocol. As reporter plasmids, we used pGL2-ARE or pGL2-basic alone. pCAGGS plasmid containing human h1-calponin (pCAGGS/hCN), pCAGGS/hCN-CHD*mt*, pCAGGS/hCN-ABD(-), pCAGGS/β-gal, or pCAGGS vector alone was used as effector plasmids. An equal amount of DMEM containing 20% FBS was added to the media 3.5 h later. The cells were treated with 2 nmol/l activin A at 12 h after transfection. Cell lysates were prepared using passive lysis buffer 24 h later, and then firefly luciferase and *Renilla* luciferase activities, expressed by pGL2 reporter and pRL-TK R*luc*

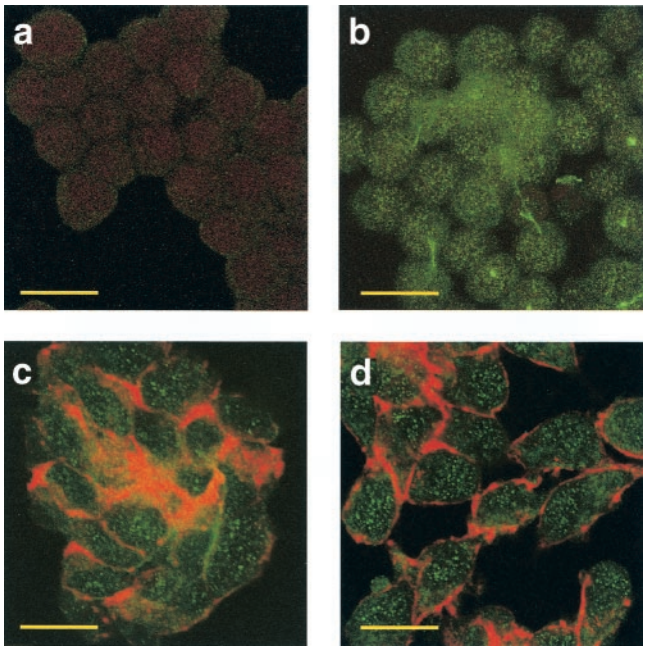


**FIG. 1. Immunofluorescent analysis for h1-calponin in AR42J-B13 cells. AR42J-B13 cells cultured in DMEM containing 10% FBS were fixed and immunostained for calponin (FITC), then nuclei were stained by propidium iodide (*a* and *b*). *a*: Immunofluorescence with mouse IgG for negative control. *b*: Immunofluorescence with anti–h1-calponin antibody. AR42J cells treated with (*d*) or without (*c*) activin A and HGF for 72 h and double-stained first with anti–h1-calponin antibody and then with TRITC-phalloidin. Each bar represents 20 μm.**

reference plasmid, respectively, were sequentially assayed using Dual-Luciferase Reporter Assay System (Promega, Madison, WI) with a ARVOsx 1420 multilabel counter (Wallac, Turku, Finland) according to the manufacturer's protocol. Firefly luciferase activities were normalized by *Renilla* luciferase activities. Data are expressed as means ± SD relative to the value of pCAGGS vector without activin A treatment.

**Statistical analysis.** All data are represented as mean ± SD. Differences were analyzed by Student's *t* test. $P < 0.05$ was considered significant difference.

## RESULTS

**Presence of h1-calponin in AR42J-B13 cells.** We initially examined if h1-calponin was expressed in AR42J-B13 cells by using immunofluorescent microscopy, Western blot analysis, and RT-PCR. h1-calponin was mainly stained diffusely in the cytosol of untreated AR42J-B13 cells in immunofluorescent analysis (Fig. 1*a* and *b*). Unlike other non-SMCs such as platelets, fibroblasts, and mesangial cells in which h1-calponin was colocalized with actin cytoskeleton (10,36), h1-calponin was not colocalized with actin filament which was strongly stained along the margin of the AR42J-B13 cells (Fig. 1*c*). In the cells treated with 2 nmol/l activin A and 500 pmol/l HGF for 72 h, intracellular distribution of h1-calponin and actin fiber was not dramatically altered (Fig. 1*d*). In Western blot analysis, h1-calponin was detected as a single band, the size of which was ~34 kDa (Fig. 2*A*). RT-PCR analyses also confirmed that h1-calponin mRNA was expressed in AR42J cells. Ubiquitously expressed isoforms of h2- and acidic calponin were also detected in AR42J-B13 cells (Fig. 2*B*). These PCR products were identified as respective calponin isoforms by cloning and sequencing.

**Downregulation of h1-calponin during activin A– and HGF-induced differentiation of pancreatic AR42J-B13 cells to insulin-producing cells.** To examine the role of h1-calponin in pancreatic AR42J cells, we examined its regulation during differentiation of the cells to insulin-producing cells. For this purpose, we treated AR42J cells with activin A and HGF and analyzed cell differentiation by immunostaining for insulin (25). Untreated AR42J-B13 cells were round-shaped, formed clusters, and none of the cells made morphological change or expressed insulin after incubation for 72 h (Fig. 3*A*). After treatment of cells with 2 nmol/l activin A and 500 pmol/l HGF for 72 h, AR42J-B13 cells grew singly and extended long processes, and 31.1 ± 8.2% (18.6–44.4% in five independent experiments) of the cells expressed insulin in immunofluorescent analysis (Fig. 3*B*). Insulin-producing cells were detected as early as 2 days after the treatment as previously described (data not shown).

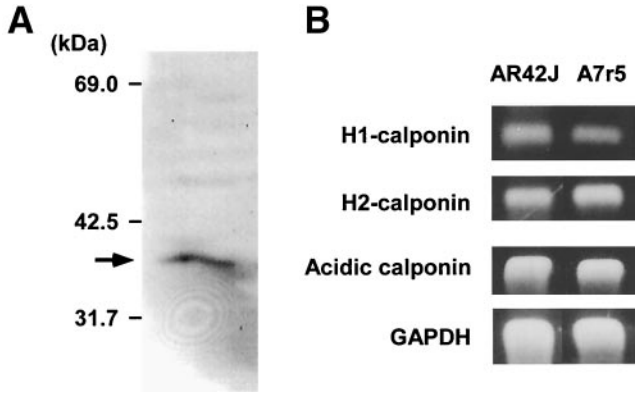


**FIG. 2. *A*: Western blot analysis for h1-calponin in AR42J-B13 cells. Whole cell lysates were separated by SDS-PAGE and immunoblotted with anti–h1-calponin antibody. *B*: RT-PCR analyses for isoforms of calponin. Poly A mRNA was extracted from AR42J-B13 cells and mRNAs for h1, h2, acidic calponin, and GAPDH were determined by RT-PCR. A7r5 rat smooth muscle cells were used for positive control.**

Using this system, we examined the expression of h1-calponin protein by Western blot analysis during differentiation of AR42J-B13 cells (Fig. 4*A*). Immunoblot for p70 S6 kinase protein, the expression of which was not significantly affected by the cell differentiation, was also performed to adjust the loaded samples. Compared with the untreated cells (Fig. 4*A*, "-" lanes), the levels of the 34-kDa of h1-calponin protein adjusted with the levels of p70 S6 kinase protein were markedly decreased in the cells treated with activin A and HGF (Fig. 4*A*, "+" lanes). Treatment of cells with activin A and HGF suppressed

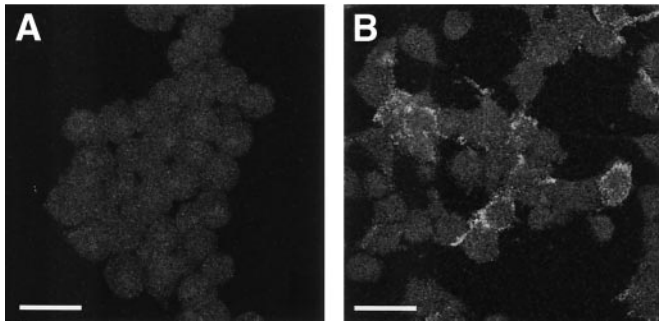


**FIG. 3. AR42J cell differentiation into insulin-producing cells by activin A and HGF. AR42J-B13 cells were cultured in DMEM containing 10% FBS without (*A*) or with (*B*) 2 nmol/l activin A and 500 pmol/l HGF for 72 h, and immunostained for insulin (FITC). Each bar represents 20 μm.**

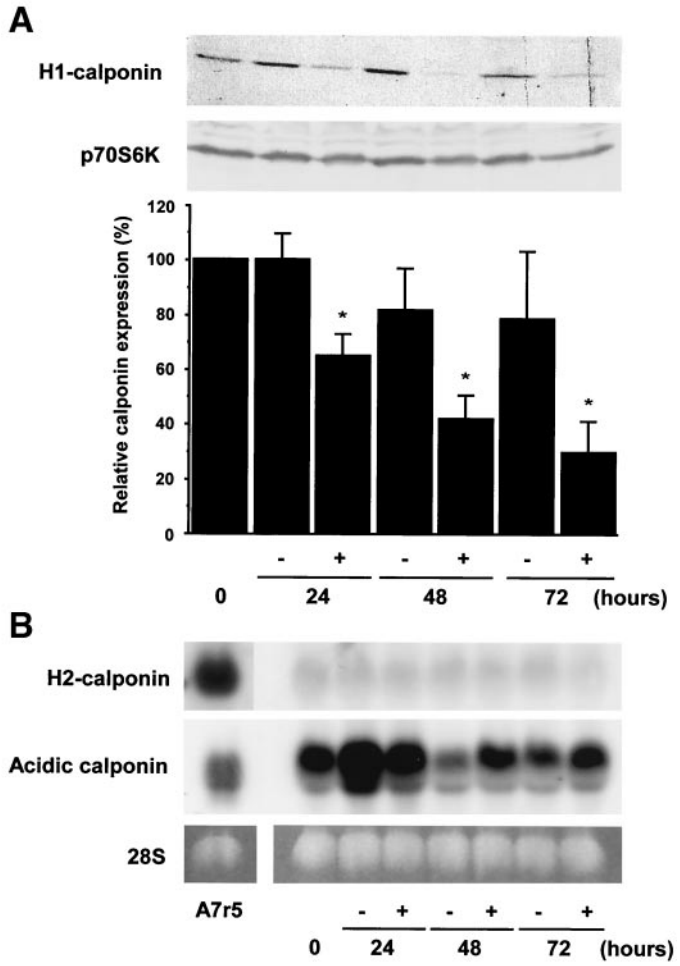


**FIG. 4. Protein levels of h1-calponin decrease during the course of AR42J-B13 cell differentiation. *A*: AR42J-B13 cells were treated with ("+") or without ("-") activin A and HGF for the times indicated. Cell lysates were obtained and Western blot analysis for h1-calponin or p70 S6 kinase as a control protein was performed. Lower panel shows summary of four independent experiments. Expression levels of h1-calponin were corrected by that of p70 S6 kinase and relative levels of calponin protein at 0 h are expressed as 100%. *$P < 0.05$ vs. without activin A and HGF at each hour. *B:* Total cellular RNA was extracted from the AR42J cells in the same condition as in *A* and from A7r5 rat smooth muscle cells for positive control, then subjected to Northern blot analysis for h2- or acidic calponin. Reference 28S rRNA lane s were also shown.**

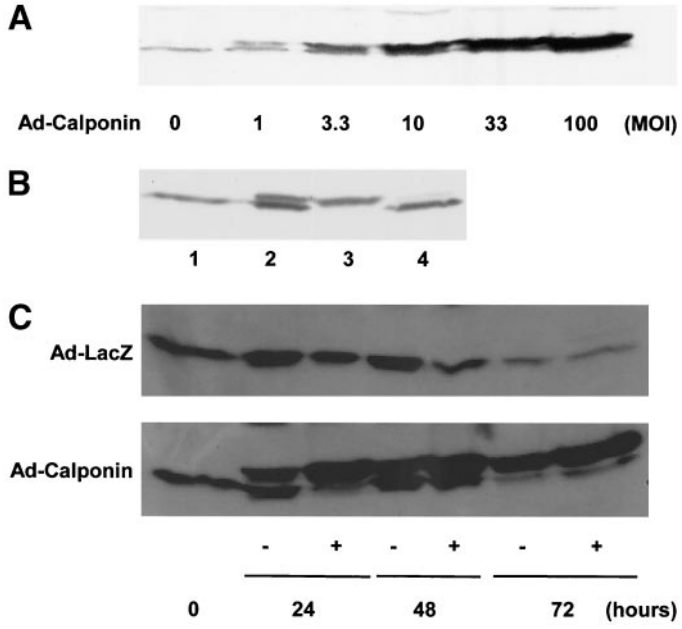


**FIG. 5. Adenovirus-mediated dose-dependent overexpression of h1-calponin protein in AR42J-B13 cells. *A*: Cells were infected with Ad-calponin for 3 h at multiplicity of infection (MOI) indicated and then incubated for 48 h. Total cell lysates were extracted and analyzed for h1-calponin by Western blot analysis. *B*: Western blot analysis for h1-calponin in total cell lysates from AR42J cells (*lane* 1), AR42J cells infected with Ad-calponin for 24 h (*lane 2*), human umbilical smooth muscle cells (*lane 3*), and A7r5 rat smooth muscle cells (*lane 4*). *C*: Cells were infected with Ad-LacZ (upper panel) or Ad-calponin (lower panel) at MOI of 30 and then incubated with ("+") or without ("-") activin A and HGF for the times indicated. Expression of h1-calponin protein was analyzed by Western blot analysis.**

h1-calponin expression levels as early as 24 h, thus preceding cell differentiation. In this system, Northern blot analyses showed that h2-calponin mRNA was hardly detected, and acidic calponin mRNA was abundantly expressed (Fig. 4*B*). In contrast to the regulation of h1-calponin during the cell differentiation, mRNA expression levels of acidic calponin were higher in the differentiated cells than in untreated cells.

**Effect of overexpression of h1-calponin on AR42J-B13 cell differentiation.** To investigate the role of the decrease in h1-calponin expression during the AR42J-B13 cell differentiation, h1-calponin was overexpressed in AR42J-B13 cells using adenoviral system. Cell lysates were obtained and analyzed for the expression of h1-calponin with Western blot analysis 48 h after the infection of the cells with Ad-calponin. Dose-dependent overexpression of h1-calponin was observed in the cells infected with Ad-h1-calponin at multiplicity of infection (MOI) from 1 to 100 (Fig. 5*A*). As shown in Fig. 5*B*, the human h1-calponin migrated slower than the rat one, and the overexpressed human h1-calponin was observed as the upper band of doublet (Fig. 5*B*). Figure 5*C* shows Western blot analysis for h1-calponin in AR42J-B13 cells treated with ("+") or without ("-") activin A and HGF following Ad-LacZ or Ad-Calponin infection at a MOI of 30. Continuous overexpression of h1-calponin was observed at least until 72 h in the Ad-Calponin infected cells both with and without activin A and HGF treatment. In contrast, Ad-LacZ infection of the cells had no effect on endogenous h1-calponin. As in noninfected cells (Fig. 4*A*), treatment of cells with activin A and HGF decreased h1-calponin levels in Ad-LacZ–infected cells.

We next examined the effect of Ad-mediated overexpression of h1-calponin on AR42J-B13 cell differentiation into insulin-producing cell. Immediately after Ad-Calponin infection, AR42J-B13 cells were treated with activin A and HGF for 72 h, and double immunofluorescent analysis for insulin and h1-calponin was performed (Fig. 6*A-a*). Overexpression of h1-calponin was observed in 13.1% of Ad-Calponin–infected cells, whereas none of the cells infected with Ad-LacZ overexpressed h1-calponin. Among calponin-overexpressing cells, the percentages of insulin-producing cells were 5.7, 9.5, and 4.9% at MOI of 30, 100, and 300 of Ad-calponin, respectively, which were markedly suppressed as compared with that in the cells without calponin overexpression (26.2, 27.5, and 32.2%, respectively). As a whole, 28.6 ± 3.2% ($n = 3$) of the cells without calponin overexpression differentiated to insulin-produc-

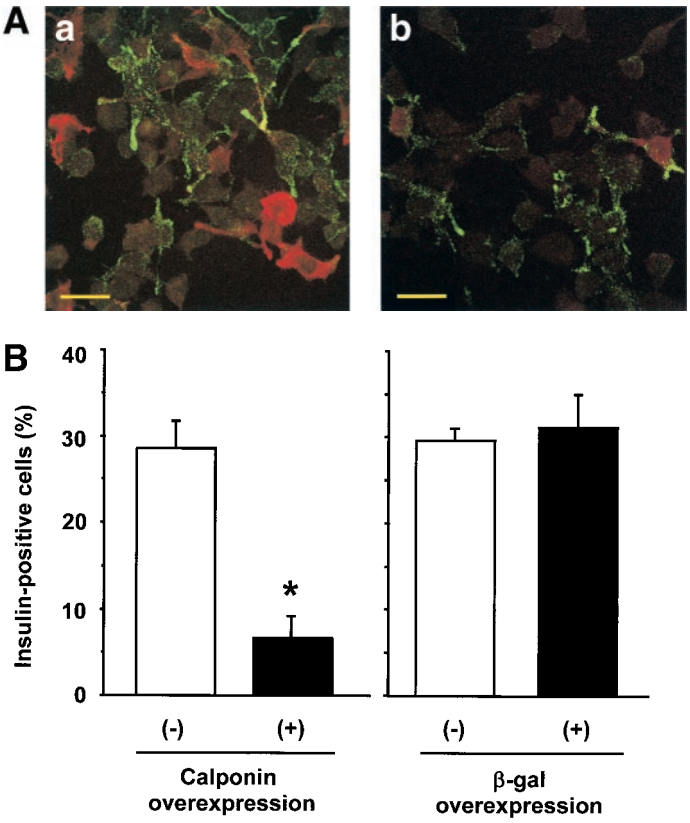


**FIG. 6.** *A*: **Effect of adenovirus-mediated overexpression of calponin or β-gal on AR42J-B13 cell differentiation. AR42J-B13 cells were infected with Ad-calponin (*a*) or Ad-LacZ (*b*) and incubated with 2 nmol/l activin A and 500 pmol/l HGF for 72 h. Cells were double-immunostained for insulin (FITC) and calponin or β-gal (Texas-red). Each bar represents 20 μm. *B*: Summary of the triplicate experiments. Values are expressed as mean ± SD. **P* < 0.05 vs. cells without overexpression of calponin.**

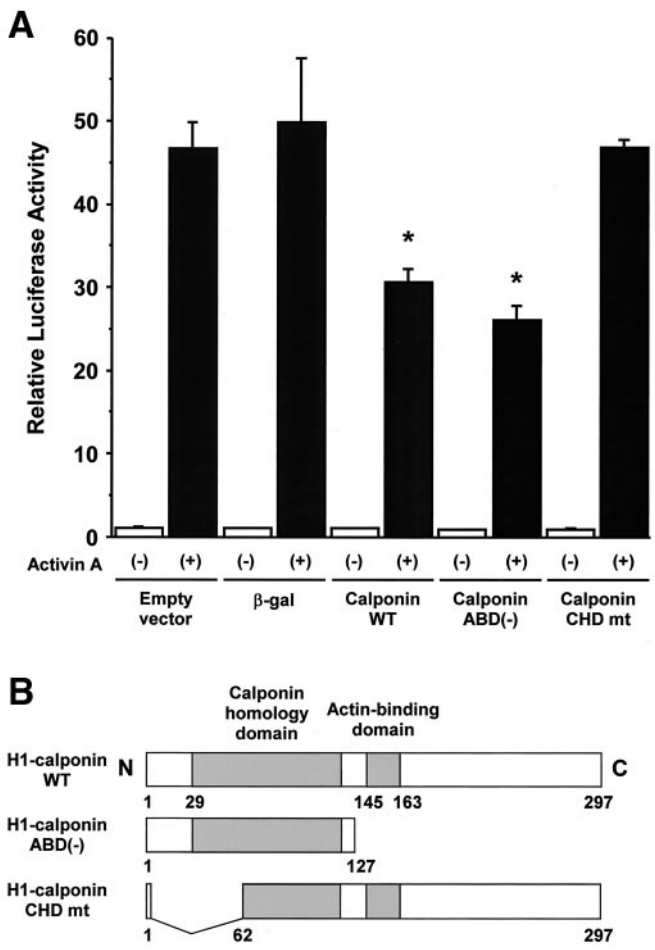


**FIG. 7. Effect of overexpression of h1-calponin on transcriptional activity of ARE. *A*: ARE-regulated pGL2-luciferase vector (pGL2-ARE-Luc) and pcDNA-FAST-1 vector were cotransfected with pCAGGS-empty, −β-gal, −h1-calponin (WT), or −h1-calponin with CHD *mt* or ABD(-) vector to AR42J-B13 cells. Cells were treated with vehicle or 2 nmol/l activin A 12 h after transfection and incubated for 24 h. The luciferase activity was measured as described in RESEARCH DESIGN AND METHODS. Values are expressed as mean ± SD ($n$ = 6). **P* < 0.001 vs. pCAGGS-empty vector with activin A. *B*: Schematic diagram of molecular structures of wild type human h1-calponin and h1-calponin ABD(-) or that with CHD *mt*.**

ing cells, while only 6.7 ± 2.5% of the cells with calponin overexpression did (Fig. 6*B*). This difference was statistically significant ($P < 0.001$).

To negate the artificial effect of Ad-mediated protein overexpression on AR42J-B13 cell differentiation, the effect of Ad-LacZ was examined (Fig. 6*A*-*b*). The percentages of insulin-producing cells with or without overexpression of β-gal was 31.2 ± 3.7 or 29.6 ± 1.5%, respectively ($n$ = 3; Fig. 6*B*). Thus, the cells with and without overexpression of β-gal differentiated equally into insulin-producing cells.

**Effect of h1-calponin on activin A–stimulated transcriptional activity.** To understand the role of h1-calponin in activin A–induced AR42J-B13 cell differentiation, we investigated the effect of h1-calponin overexpression on activin A–stimulated transcriptional activity using an ARE-regulated, luciferase-expressing reporter plasmid with a plasmid expressing FAST-1, a cofactor interacting ARE with Smad2 and Smad4. In this system, activin A treatment elicited ARE activity 26- to 47-fold higher than that in the cells without it. Overexpression of the full-length h1-calponin (WT) significantly repressed the ARE activity of AR42J-B13 cells treated with activin A by 65.6 ± 3.4% ($n$ = 6). Importantly, h1-calponin ABD(-) also suppressed activin A–stimulated ARE activity by 55.9 ± 3.7% ($n$ = 6). In contrast, *CHD mt* had no significant effect on ARE activity (100.6 ± 3.7%, $n$ = 6) (Fig. 7*A*). Overexpression of β-gal did not alter activin A–induced transcriptional activity, suggesting that the effect of h1-calponin is not the result of nonspecific overexpression of the protein driven by CMV promoter. Difference in effector plasmids did not significantly affect the *Renilla* luciferase activities by pRL-TK-R*luc* reference plasmids in the cells treated with activin A (data not shown).

## DISCUSSION

We showed in this study that h1-calponin, which is predominantly expressed in differentiated SMCs, is also expressed in rat pancreatic AR42J-B13 cells, and its expression is downregulated during differentiation of the cells into insulin-producing β-cells. Moreover, overexpression of h1-calponin in this system markedly impairs cell differentiation, indicating that h1-calponin downregulation in AR42J cell may be prerequisite for cell differentiation.

**Calponin and pancreatic β-cell differentiation.** Although recent reports have shown that h1-calponin is also expressed in non-SMCs such as less differentiated osteo-

blasts (9,37) and cultured human mesangial cells (10), its expression in pancreatic endocrine or exocrine cells has not been documented. Several lines of evidence suggest that calponin is involved in the regulation of differentiation of the cells. Increased expression of calponin gene has been observed in SMCs of the late embryo and in the adult (38), and in the developing and healing bone tissues and undifferentiated osteoblasts (37). Furthermore, Xenopus calponin variant in collaboration with OTX1/OTX2 is demonstrated to be involved in the formation of anterior/posterior axis (39). Recent observation that h1-calponin is transiently expressed in pancreatic duct cells preceding islet development in fetal mice (K.T. and H. Y. unpublished observation) have driven us to explore the role of calponin in the β-cell differentiation.

We showed that the expression of h1-calponin in rat pancreatic AR42J cells at both protein and mRNA levels. Ubiquitously expressed h2 and acidic isoforms of calponin are also detected in AR42J cells. Importantly, the level of h1-calponin protein was downregulated during the course of AR42J cell differentiation into insulin-producing cells following treatment with activin A and HGF. Since decrease in h1-calponin protein levels is observed within 24 h, at the time of which insulin-producing cells are hardly detected, both presence of h1-calponin and subsequent decrease in its expression could be involved in AR42J cell differentiation. These findings are in good agreement with the observations that h1-calponin is transiently expressed in the pancreatic duct cells of fetal mice, and quickly disappeared before islet development (K.T. and H.Y. unpublished observations). To address this hypothesis, we examined the effect of h1-calponin overexpression on differentiation of AR42J cells using recombinant adenovirus-mediated gene transfer, and found that continuous overexpression of h1-calponin in AR42J cells significantly suppressed the cell differentiation into insulin-producing cells. Thus, the presence and downregulation of h1-calponin appears to play a critical role in AR42J cell differentiation into β-cells. In this experimental system, when MOI of adenovirus was reduced, the percentage of infected cells were decreased, so we found it difficult to examine the dose-dependent effect of exogenously expressed calponin at the level of physiological stoichiometry. We could at least negate the nonspecific effect of protein overexpression, since adenovirus-mediated overexpression of nonspecific proteins (β-gal in this case) did not affect AR42J cell differentiation.

In the present system of AR42J cell differentiation, Northern blot analyses revealed that h2-calponin was hardly detected, while acidic calponin mRNA were abundantly expressed and regulated inversely to h1-calponin. Thus, our study does not exclude the possibility that acidic calponin also plays a role in β-cell differentiation, and the functional significance of acidic calponin remains to be determined.

**Modulation of activin A–stimulated transcriptional activity by h1-calponin.** TGF-β signaling is a major regulator of pancreatic endocrine and exocrine development (40), and in vitro exposure of embryonic mouse pancreas to TGF-β promotes development of endocrine cells, particularly β-cells and PP-cells (41). Recent observations show that subunits and receptors of activin are expressed in the pancreatic duct and are upregulated after streptozotocin injection or partial pancreatectomy where β-cell neogenesis is initiated (42). Activin A, a TGF-β family member, induces differentiation of amylase-secreting pancreatic AR42J cells into endocrine-like cells of islets through activating Smad2 pathway (23,26). Smad2, together with Smad4 and FAST-1, interacts directly with ARE in an activin-dependent manner and affects transcription of specific genes (43). Furthermore, activation of the Smad2 pathway is required for activin A–induced AR42J cell differentiation into insulin-secreting cells in the presence of HGF (26).

We showed here that ectopic overexpression of h1-calponin suppressed activin A–induced ARE activity in AR42J cells. We showed that mutant h1-calponin with CH-domain disruption failed to suppress activin-stimulated ARE activity, suggesting the essential role of CH-domain in regulating ARE activity. Multiple copies of a CH-domain are present in several actin-binding proteins, and single CH-domains are found in signal-transducing proteins like the proto-oncogene product Vav and Ras-GAP–like protein [reviewed in 6]. CH-domain also interacts directly with extracellular regulated kinase (ERK) (44), implicating the role of this domain on calponin-mediated signaling (44). In our system, actin-binding property of h1-calponin appears not essential to modulate activin A–mediated signaling. The actin-binding site of calponin is considered to be located primarily in more central region of the molecule named actin-binding domain (A145-I163) (33), secondarily in the COOH-terminal tandem repeats (45). Actin-binding activity of the single CH-domain is still controversial. It has been reported that the single CH-domain is neither sufficient nor necessary for F-actin binding of calponin (34), whereas a novel actin-binding site was recently identified in the $NH_2$-terminal region of the CH-domain (46). We showed in this study by immunofluorescent analyses that h1-calponin was not colocalized with actin fiber in both untreated and differentiated AR42J cells (Fig. 1*c* and *d*). Moreover, exogenously overexpressed h1-calponin also failed to associate with actin fiber in cells infected with Ad-calponin (data not shown). These observations are in good agreement with the findings that h1-calponin lacking actin-binding domain suppresses activin A–stimulated transcriptional activity to the similar level as full-length h1-calponin. Thus, h1-calponin appears to modulate activin A signaling through the property of the $NH_2$-terminal region of CH-domain as a signaling molecule rather than primary actin-binding property. Further studies are necessary to understand in detail the mechanism underlying h1-calponin modulation of activin A–mediated signaling and transcription.

In conclusion, expression and regulation of h1-calponin in AR42J cells is involved in cell differentiation into insulin-producing β-cells, at least partly through modulation of activin A–induced transcriptional activity.

**ACKNOWLEDGMENTS**

This study is supported in part by a Grant-in Aid for Scientific Research (13671197 to H.K., 12557128 to K.T.) from the Ministry of Education, Science and Culture, Japan, and by a Research Grant from Suzuken Memorial Foundation to K.T. (Nagoya).

The authors thank Dr. Takeshi Imamura, the Cancer Institute of Japanese Foundation for Cancer Research, Tokyo, Japan, and Dr. Kohei Miyazono, University of Tokyo, Tokyo, Japan, for providing reagents and helpful discussion, and Dr. Toshiyuki Takeuchi, Institute for Molecular and cellular Regulation, Gunma University, Maebashi, Japan, and Dr. Y. Eto, Ajinomoto, Kawasaki, Japan for providing us with reagents.

## REFERENCES


1. Takahashi K, Hiwada K, Kokubu T: Vascular smooth muscle calponin: a novel troponin T-like protein. *Hypertension* 11:620–626, 1988
2. Abe M, Takahashi K, Hiwada K: Effect of calponin on actin-activated myosin ATPase activity. *J Biochem (Tokyo)* 108:835–838, 1990
3. Winder SJ, Walsh MP: Smooth muscle calponin: inhibition of actomyosin MgATPase and regulation by phosphorylation. *J Biol Chem* 265:10148–10155, 1990
4. Lehman W: Calponin and the composition of smooth muscle thin filaments. *J Muscle Res Cell Motil* 12:221–224, 1991
5. Takahashi K, Nadal-Ginard B: Molecular cloning and sequence analysis of smooth muscle calponin [published erratum appears in *J Biol Chem* 267:26198, 1992]. *J Biol Chem* 266:13284–13288, 1991
6. Winder SJ, Allen BG, Clement-Chomienne O, Walsh MP: Regulation of smooth muscle actin-myosin interaction and force by calponin. *Acta Physiol Scand* 164:415–426, 1998
7. Strasser P, Gimona M, Moessler H, Herzog M, Small JV: Mammalian calponin: identification and expression of genetic variants. *FEBS Lett* 330:13–18, 1993
8. Applegate D, Feng W, Green RS, Taubman MB: Cloning and expression of a novel acidic calponin isoform from rat aortic vascular smooth muscle. *J Biol Chem* 269:10683–10690, 1994
9. Yamamura H, Yoshikawa H, Tatsuta M, Akedo H, Takahashi K: Expression of the smooth muscle calponin gene in human osteosarcoma and its possible association with prognosis. *Int J Cancer* 79:245–250, 1998
10. Sugenoya Y, Yoshimura A, Yamamura H, Inui K, Morita H, Yamabe H, Ueki N, Ideura T, Takahashi K: Smooth-muscle calponin in mesangial cells: regulation of expression and a role in suppressing glomerulonephritis. *J Am Soc Nephrol* 13:322–331, 2002
11. Masuda H, Tanaka K, Takagi M, Ohgami K, Sakamaki T, Shibata N, Takahashi K: Molecular cloning and characterization of human non-smooth muscle calponin. *J Biochem (Tokyo)* 120:415–424, 1996
12. Fukui Y, Masuda H, Takagi M, Takahashi K, Kiyokane K: The presence of h2-calponin in human keratinocyte. *J Dermatol Sci* 14:29–36, 1997
13. Massague J, Chen YG: Controlling TGF-beta signaling. *Genes Dev* 14:627–644, 2000
14. Totsuka Y, Tabuchi M, Kojima I, Shibai H, Ogata E: A novel action of activin A: stimulation of insulin secretion in rat pancreatic islets. *Biochem Biophys Res Commun* 156:335–339, 1988
15. Yamaoka T, Idehara C, Yano M, Matsushita T, Yamada T, Ii S, Moritani M, Hata J, Sugino H, Noji S, Itakura M: Hypoplasia of pancreatic islets in transgenic mice expressing activin receptor mutants. *J Clin Invest* 102: 294–301, 1998
16. Shiozaki S, Tajima T, Zhang YQ, Furukawa M, Nakazato Y, Kojima I: Impaired differentiation of endocrine and exocrine cells of the pancreas in transgenic mouse expressing the truncated type II activin receptor. *Biochim Biophys Acta* 1450:1–11, 1999
17. Roberts V, Barth S: Expression of messenger ribonucleic acids encoding the inhibin/activin system during mid- and late-gestation rat embryogenesis. *Endocrinology* 134:914–923, 1994
18. Voutilainen R, Eramaa M, Ritvos O: Hormonally regulated inhibin gene expression in human fetal and adult adrenals. *J Clin Endocrinol Metab* 73:1026–1030, 1991
19. Ogawa K, Ono K, Kurohmaru M, Hayashi Y: Effect of streptozotocin injection on expression of immunoreactive follistatin and beta A and beta B subunits of inhibin/activin in rat pancreatic islets. *Eur J Endocrinol* 132:363–369, 1995
20. Yasuda H, Inoue K, Shibata H, Takeuchi T, Eto Y, Hasegawa Y, Sekine N, Totsuka Y, Mine T, Ogata E: Existence of activin-A in A- and D-cells of rat pancreatic islet. *Endocrinology* 133:624–630, 1993
21. Wada M, Shintani Y, Kosaka M, Sano T, Hizawa K, Saito S: Immunohistochemical localization of activin A and follistatin in human tissues. *Endocr J* 43:375–385, 1996
22. Christophe J: Pancreatic tumoral cell line AR42J: an amphicrine model. *Am J Physiol* 266:G963–G971, 1994
23. Ohnishi H, Ohgushi N, Tanaka S, Mogami H, Nobusawa R, Mashima H, Furukawa M, Mine T, Shimada O, Ishikawa H, et al: Conversion of amylase-secreting rat pancreatic AR42J cells to neuronlike cells by activin A. *J Clin Invest* 95:2304–2314, 1995
24. Mashima H, Ohnishi H, Wakabayashi K, Mine T, Miyagawa J, Hanafusa T, Seno M, Yamada H, Kojima I: Betacellulin and activin A coordinately convert amylase-secreting pancreatic AR42J cells into insulin-secreting cells. *J Clin Invest* 97:1647–1654, 1996
25. Mashima H, Shibata H, Mine T, Kojima I: Formation of insulin-producing cells from pancreatic acinar AR42J cells by hepatocyte growth factor. *Endocrinology* 137:3969–3976, 1996
26. Zhang YQ, Kanzaki M, Furukawa M, Shibata H, Ozeki M, Kojima I: Involvement of Smad proteins in the differentiation of pancreatic AR42J cells induced by activin A. *Diabetologia* 42:719–727, 1999
27. Furukawa M, Zhang YQ, Nie L, Shibata H, Kojima I: Role of mitogen-activated protein kinase and phosphoinositide 3-kinase in the differentiation of rat pancreatic AR42J cells induced by hepatocyte growth factor. *Diabetologia* 42:450–456, 1999
28. Ichii T, Koyama H, Tanaka S, Kim S, Shioi A, Okuno Y, Raines EW, Iwao H, Otani S, Nishizawa Y: Fibrillar collagen specifically regulates human vascular smooth muscle cell genes involved in cellular responses and the pericellular matrix environment. *Circ Res* 88:460–467, 2001
29. Okada N, Tsukada Y, Nakagawa S, Mizuguchi H, Mori K, Saito T, Fujita T, Yamamoto A, Hayakawa T, Mayumi T: Efficient gene delivery into dendritic cells by fiber-mutant adenovirus vectors. *Biochem Biophys Res Commun* 282:173–179, 2001
30. Mizuguchi H, Koizumi N, Hosono T, Utoguchi N, Watanabe Y, Kay MA, Hayakawa T: A simplified system for constructing recombinant adenoviral vectors containing heterologous peptides in the HI loop of their fiber knob. *Gene Ther* 8:730–735, 2001
31. Mizuguchi H, Kay MA: Efficient construction of a recombinant adenovirus vector by an improved in vitro ligation method. *Hum Gene Ther* 9:2577–2583, 1998
32. Mizuguchi H, Kay MA: A simple method for constructing E1- and E1/E4-deleted recombinant adenoviral vectors. *Hum Gene Ther* 10:2013–2017, 1999
33. Mezgueldi M, Mendre C, Calas B, Kassab R, Fattoum A: Characterization of the regulatory domain of gizzard calponin. *J Biol Chem* 270:8876–8876, 1995
34. Gimona M, Djinovic-Carugo K, Kranewitter WJ, Winder SJ: Functional plasticity of CH domains. *FEBS Lett* 513:98–106, 2002
35. Hayashi H, Abdollah S, Qiu Y, Cai J, Xu YY, Grinnell BW, Richardson MA, Topper JN, Gimbrone MA Jr, Wrana JL, Falb D: The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signaling. *Cell* 89:1165–1173, 1997
36. Takeuchi K, Takahashi K, Abe M, Nishida W, Hiwada K, Nabeya T, Maruyama K: Co-localization of immunoreactive forms of calponin with actin cytoskeleton in platelets, fibroblasts, and vascular smooth muscle. *J Biochem (Tokyo)* 109:311–316, 1991
37. Yoshikawa H, Taniguchi SI, Yamamura H, Mori S, Sugimoto M, Miyado K, Nakamura K, Nakao K, Katsuki M, Shibata N, Takahashi K: Mice lacking smooth muscle calponin display increased bone formation that is associated with enhancement of bone morphogenetic protein responses. *Genes Cells* 3:685–695, 1998
38. Duband J, Gimona M, Scatena M, Sartore S, Small J: Calponin and SM 22 as differentiation markers of smooth muscle: spatiotemporal distribution during avian embryonic development. *Differentiation* 55:1–11, 1993
39. Morgan R, Hooiveld M, Pannese M, Dati G, Broders F, Delarue M, Thiery J, Boncinelli E, Durston A: Calponin modulates the exclusion of Otx-expressing cells from convergence extension movements. *Nat Cell Biol* 1:404–408, 1999
40. Kim SK, Hebrok M: Intercellular signals regulating pancreas development and function. *Genes Dev* 15:111–127, 2001
41. Sanvito F, Herrera PL, Huarte J, Nichols A, Montesano R, Orci L, Vassalli JD: TGF-beta 1 influences the relative development of the exocrine and endocrine pancreas in vitro. *Development* 120:3451–3462, 1994
42. Zhang Y, Zhang H, Maeshima A, Kurihara H, Miyagawa J, Takeuchi T, Kojima I: Up-regulation of the expression of activins in the pancreatic duct by reduction of the beta-cell mass. *Endocrinology* 143:3540–3547, 2002
43. Heldin CH, Miyazono K, ten Dijke P: TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature* 390:465–471, 1997
44. Morgan K, Gangopadhyay S: Invited review: cross-bridge regulation by thin filament-associated proteins. *J Appl Physiol* 91:953–962, 2001
45. Mino T, Yuasa U, Nakamura F, Naka M, Tanaka T: Two distinct actin-binding sites of smooth muscle calponin. *Eur J Biochem* 251:262–268, 1998
46. Fujii T, Nakamura K, Ootani H, Koizumi Y, Takahashi K: A novel actin-binding site in the single calponin homology (CH) domain of basic calponin. *J Biochem Mol Biophys* 5:399–405, 2001