Phenotype-dependent expression of cadherin 6B in vascular and visceral smooth muscle cells

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Received 17 January 2000

Edited by Masayuki Miyasaka

Abstract We used mRNA subtraction of differentiated and dedifferentiated smooth muscle cells (SMCs) to reveal the molecular mechanisms underlying the phenotypic modulation of SMCs. With this approach, we found that a 10 kb mRNA encoding a homotypic cell adhesion molecule, cadherin 6B, was strongly expressed in differentiated vascular and visceral SMCs, but not in the dedifferentiated SMCs derived from them. In vivo, cadherin 6B was expressed in vascular and visceral SMCs, in addition to brain, spinal cord, retina and kidney, at a late stage of chicken embryonic development. These results suggest that cadherin 6B is a novel molecular marker for vascular and visceral SMC phenotypes and is involved in the late differentiation of SMCs.

Key words: mRNA subtraction; Smooth muscle; Phenotypic modulation; Cadherin 6B

1. Introduction

In serious diseases such as atherosclerosis, hypertension, and leiomyogenic tumorigenicity, smooth muscle cells (SMCs) display phenotypic modulation from a differentiated state to a dedifferentiated one [1]. Differentiated SMCs have a spindle-like shape and specific intracellular structures including well-developed dense membranes, dense bodies and myofibrils and show ligand-induced contractility. In dedifferentiated SMCs, these unique morphological and functional properties are lost and the expression of SMC-specific molecular markers also disappears [2,3]. Several cytoskeletal and contractile proteins can be used as SMC-specific molecular markers. Among them, caldesmon [4,5], calponin [6,7], α-tropomyosin [5] and α1 integrin [8,9] are upregulated in differentiated SMCs, but downregulated in dedifferentiated SMCs. Alternative splicing events that result in the expression of alternative isoforms of caldesmon [4,5], α-tropomyosin [5], vinculin/metavinculin [10] and smooth muscle myosin heavy chain [11,12] have been shown to be correlated with changes in SMC phenotype. Until recently, the molecular mechanisms underlying phenotypic modulation have not been well characterized because of a lack of primary culture systems or cell lines in which SMCs maintain a differentiated phenotype. We have recently established primary culture systems of vascular and visceral SMCs that maintain a fully differentiated phenotype under insulin-like growth factor-I (IGF-I)-stimulated conditions [13,14]. Using these culture systems, we have clarified that changes in the balance between the phosphatidylinositol 3-kinase (PI3-K)/protein kinase B (PKB(Akt)) pathway and the extracellular signal-regulated kinase (ERK) and p38 mitogen activated protein kinase (p38MAPK) pathways determine the phenotypes of vascular and visceral SMCs [14]. The IGF-I signaling, which is mediated through the PI3-K/PKB(Akt) pathway, is critically involved in maintaining the differentiated SMC phenotype, whereas the co-activation of the ERK and p38MAPK pathways directly induces the dedifferentiation of SMCs. In this study, we attempted to isolate critical elements involved in the phenotypic modulation of SMCs. For this purpose, we used mRNA subtraction of differentiated and dedifferentiated SMCs and isolated cadherin 6B, whose expression is regulated in a phenotype-dependent manner in vascular and visceral SMCs.

2. Materials and methods

2.1. Cell culture

SMCs were prepared for primary culture from 15-day-old chicken embryo gizzards [13] and cultured on laminin-coated dishes under the following conditions: IGF-I stimulated, IGF-I depleted and serum stimulated. We used a monoclonal antibody against IGF-I (Upstate Biotechnology) to deplete IGF-I from the SMC culture medium. This antibody was able to neutralize the activity of the IGF-I secreted from the SMCs and to induce dedifferentiation of the SMCs [13]. Vascular SMCs from 15-day-old chicken aortae were prepared for primary culture using the explant method, as described elsewhere [9].

2.2. RNA subtraction

Total RNA was isolated from cultured gizzard SMCs under the three different conditions described above. mRNAs purified from each source were reverse-transcribed. Single-stranded cDNAs thus obtained were amplified by PCR using eight different arbitrary primers, according to the manufacturer's recommended protocols (RNAimage Kit 1, GenHunter) and separated by polyacrylamide gel electrophoresis. cDNA fragments specifically expressed in differentiated SMCs under IGF-I-stimulated conditions were selected and cloned. To confirm that these cDNA fragments were present in the differentiated but not the dedifferentiated cultured SMCs, we performed virtual Northern blotting using a CapFinder PCR cDNA synthesis kit (CLONTECH).

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PII: S0014-5793(00)01220-5

2.3. Northern blotting

Total RNAs from all three sources were separated on 1.0% agarose-formaldehyde denaturing gels and transferred to nylon membranes (GeneScreenPlus, NEN Life Science Products). The blots were hybridized with the ^{32}P-labeled antisense strands of the DD4 clone (one of the clones obtained in the RNA subtraction), *h*-caldesmon-specific cDNA and with calponin cDNA, as described elsewhere [9]. To visualize 28S rRNAs, membranes were stained with 0.02% methylene blue.

2.4. cDNA library screening

A cDNA library of gizzard SMCs from 15-day-old chicken embryos was constructed in λZAPII and was screened by plaque hybridization with a ^{32}P-labeled cDNA fragment obtained by the mRNA subtraction, as described above. Three overlapping cDNA clones were obtained and sequenced. To sequence the 5′-region, rapid amplification of cDNA end (RACE) was performed.

2.5. In situ hybridization of chicken embryos

Chicken embryos were frozen on dry ice and sectioned 6–8 μm thick on a cryostat (Bright, UK). [^{35}S]UTP-labeled riboprobes were hybridized to the sections and analyzed by light microscopy [9]. Hydrolyzed riboprobes labeled with digoxigenin were prepared and hybridized as described elsewhere [15]. After a high stringency wash, amplification was performed using the TSA Plus (AP) System (NEN Life Science Products, MA, USA) and fluorescent signals were detected with HNPP/Fast Red (Boehringer Mannheim, Germany). Images were obtained with a ZEISS Axiophot microscope.

2.6. Immunostaining and immunoblotting

Cryosections of aortae and gizzards from 15-day-old chicken embryos were prepared and stained with a monoclonal antibody against cadherin 6B, CCD6B-1 [16], which was kindly provided by Dr. M. Takeichi (Graduate School of Science, Kyoto University, Japan). The secondary antibody was conjugated with Alexa 488 (Molecular Probes, USA). For immunoblotting, whole lysates of aortae and gizzards from 15-day-old chicken embryos were fractionated on a 7.5% SDS–polyacrylamide gel and transferred onto nitrocellulose membranes. The blots were incubated with anti-cadherin 6B, anti-caldesmon [17] or anti-calponin [18] antibodies and processed for chemiluminescence with the ECL detection kit (Amersham Pharmacia Biotech).

3. Results

To better understand the molecular mechanisms underlying the phenotypic modulation of SMCs, we carried out mRNA subtraction of differentiated and dedifferentiated gizzard SMCs. In our first screening, we obtained 170 clones, which were predominantly expressed in differentiated SMCs. To select candidate genes from among these clones, we used virtual Northern blotting to distinguish the cDNAs expressed in differentiated and dedifferentiated SMCs and selected 48 positive clones. We determined their sequences and chose 5 unique clones. The 5′-RACE analyses revealed that two clones encoded partial sequences of adipoQ [19] and smooth muscle type myosin heavy chain cDNAs [20], respectively. Among the remaining three positive clones, we selected one that we named DD4 (318 bp). DD4 is not homologous with any sequences registered at the BLASTN Search (NIH), the UniGene database, or the human EST database, and we believed it to be a part of the 3′-noncoding region of some cDNA. Northern blotting analysis probed with DD4 revealed that a 10 kb mRNA is specifically expressed in differentiated gizzard SMCs, but not in the serum-induced or the IGF-I depleted dedifferentiated SMCs (Fig. 1, lanes 1 to 4). During serum-stimulated dedifferentiation of SMCs, this mRNA rapidly decreased and completely disappeared after 3 days of culture.

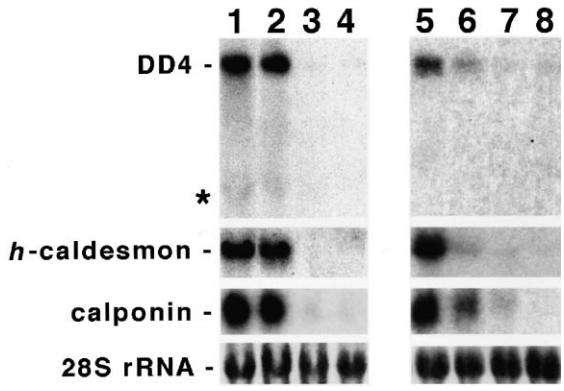

Fig. 1. Expression of the DD4 mRNA in different phenotypes of gizzard SMCs. The expressions of DD4 mRNA (first panel; 10 kb) in 15-day-old chicken embryo gizzards (lane 1) and cultured gizzard SMCs (lanes 2 to 8) were compared with that of SMC-specific molecular markers, *h*-caldesmon (second panel; 4.8 kb) and calponin (third panel; 1.8 kb). Gizzard SMCs were cultured on laminin-coated dishes under IGF-I-stimulated conditions (2 ng/ml IGF-I for 9 days; lane 2), IGF-I-deleted conditions (5 μg/ml anti-IGF-I antibody for 9 days; lane 3) and serum-stimulated conditions (10% FCS for 3 days; lane 4), respectively. Expression change of the DD4 mRNA in cultured during serum-stimulated dedifferentiation is shown in lanes 5 to 8: 1-day culture (lane 5), 2-day culture (lane 6), 3-day culture (lane 7), and 5-day culture (lane 8). The bottom panel shows the 28S rRNA stained by methylene blue. An asterisk indicates the positions of 28S rRNA in the top panel.

This decrease coincided with the downregulation of *h*-caldesmon and calponin (Fig. 1, lanes 5 to 8).

To isolate the full-length cDNA encoding DD4, we screened a cDNA library of gizzard SMCs by plaque hybridization. We obtained three overlapping cDNA clones (Fig. 2), and found that these clones encoded partial sequences of the chicken cadherin 6B cDNA [16]. However, none of them contained the amino-terminal sequence. To obtain the full coding sequence, we used the 5′-RACE method and were able to acquire an additional 1200 bp (Fig. 2), which encoded the amino-terminal sequence of cadherin 6B. The coding region of this cDNA was identical with that of cadherin 6B. Based on these results, we concluded that DD4 was actually derived from the cadherin 6B mRNA. The length of the total cDNA was 10 204 bp (from which the polyadenylation signal was absent), which is in good agreement with the results from our Northern blots and with the previously reported results of Nakagawa and Takeichi [16].

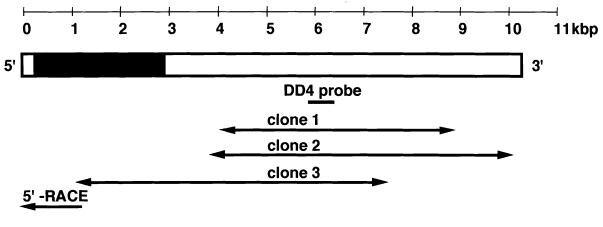

Fig. 2. Cloning of full-length DD4 cDNA. Alignment maps of DD4 cDNA and overlapping cDNA clones are schematically shown. Original cDNA fragment obtained by mRNA subtraction is indicated by a DD4 probe. The protein-coding region of DD4 cDNA, which is completely identical with cadherin 6B, is indicated with a closed box.

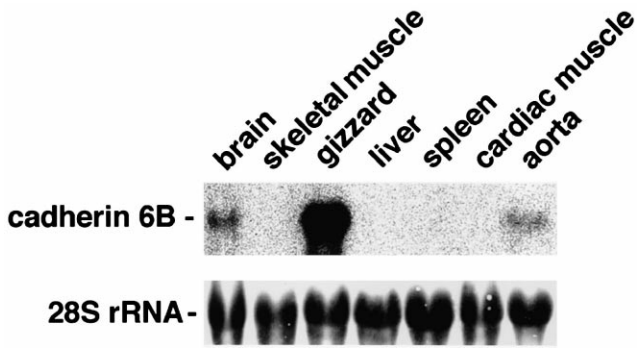

Fig. 3. Tissue-specific expression of the cadherin 6B mRNA by Northern blotting. Total RNAs were prepared from indicated tissues and the cadherin 6B mRNA was analyzed (top panel). The bottom panel shows the 28S rRNAs stained by methylene blue.

It has been well documented that cadherin 6B is detected in the invaginating neural plate and most strongly at the neural crest during the emergence of neural crest cells from the neural tube in the chicken embryo [16]. However, the expression of cadherin 6B in smooth muscle tissues has not been investigated. The results of our Northern blot analysis showed that cadherin 6B mRNA was strongly expressed in the gizzard muscle and significantly expressed in brain and aorta (Fig. 3). We further analyzed its mRNA expression in 15-day-old chicken embryos by in situ hybridization using radiolabeled riboprobes corresponding to the coding sequence of cadherin 6B. Fig. 4 shows that the cadherin 6B mRNA was co-expressed with *h*-caldesmon mRNA in smooth muscle tissues such as aorta, gizzard and the digestive tract. In addition, the cadherin 6B mRNA was definitely detected in brain, spinal cord, retina, and kidney. This result agrees with the results of our Northern blotting analysis. We further analyzed the detailed location of the cadherin 6B expression in the aorta and gizzard by in situ hybridization using digoxigenin-labeled riboprobes. The expression of the cadherin 6B transcript was restricted to the medial SMC layer of the aorta and to the SMC layer of the gizzard (Fig. 5). The cadherin 6B immunostaining was also localized to the SMC layer of the aorta and gizzard (Fig. 6).

As shown in Fig. 1, the expression of cadherin 6B mRNA in gizzard SMCs was regulated in a phenotype-dependent manner. To address whether the cadherin 6B expression is dependent on the vascular SMC phenotype, we compared whole aortae, which are largely composed of differentiated vascular SMCs, with serum-induced dedifferentiated vascular SMCs. The expression of cadherin 6B at the mRNA and protein levels was coordinately downregulated with that of *h*-caldesmon and calponin in dedifferentiated vascular SMCs (Fig. 7A,B). These results indicate that the cadherin 6B expression is regulated in a phenotype-dependent manner in vascular and visceral SMCs.

4. Discussion

The cadherin 6B cDNA was originally cloned from 2/3-day-old whole chicken embryos by RT-PCR and the expression of

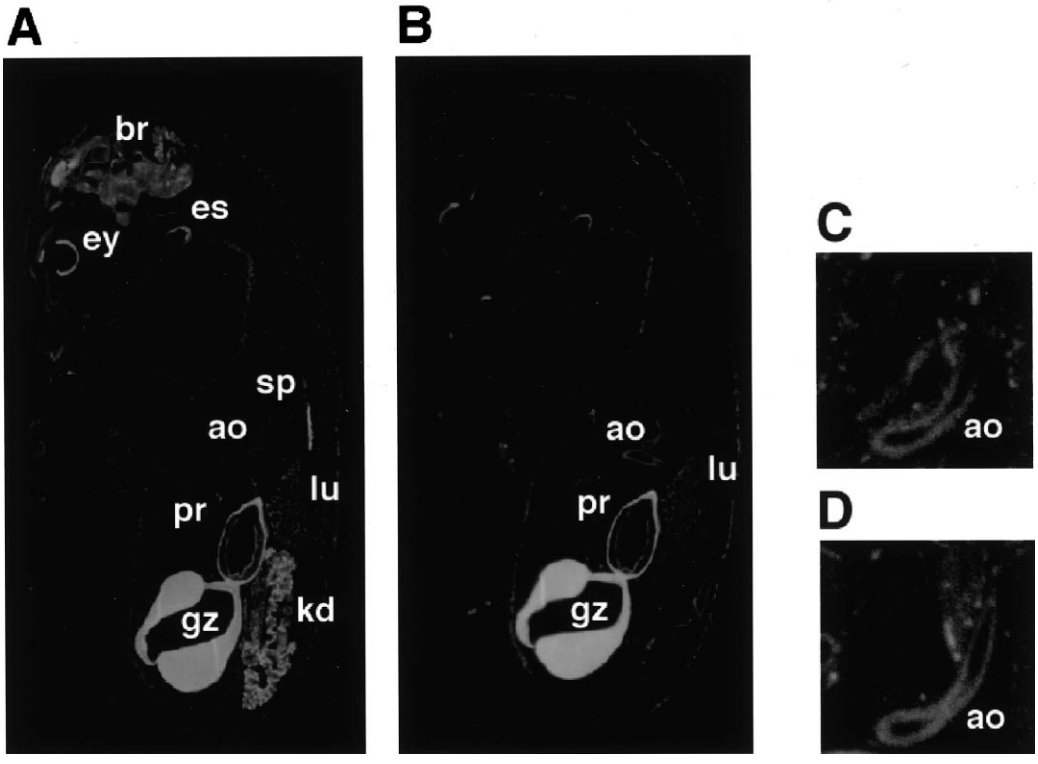

Fig. 4. Distribution of the cadherin 6B mRNA in 15-day-old chicken embryos. In situ hybridization was performed using [^{35}S]UTP-labeled riboprobes of cadherin 6B (panels A and C) and *h*-caldesmon (panels B and D). Sagittal sections of 15-day-old chicken embryos are presented. Panels C and D are the magnified regions of aorta in panels A and B. ao, aorta; br, brain; es, esophagus; ey, eye; gz, gizzard; kd, kidney; lu, lung; pr, proventiculus and sp, spinal cord.

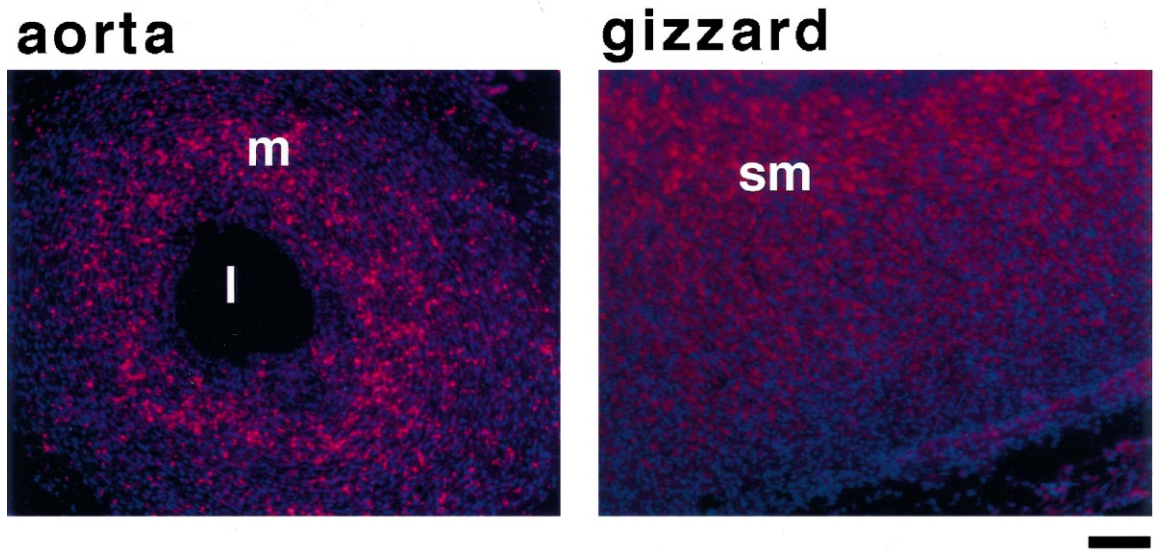

Fig. 5. Localization of the cadherin 6B mRNA in aorta and gizzard was analyzed by in situ hybridization using a digoxigenin-labeled probe. The SMC layers in aortic media and gizzard were hybridized with antisense cadherin 6B probe (red). Nuclei in cells in both tissues were stained with Hochest 33342. l, lumen; m, medial SMC layer of aorta; sm, SMC layer of gizzard. Bar, 100 μm.

cadherin 6B was well studied during neural crest development [16,21]. In chicken embryos, cadherin 6B appears during early neural tube formation. Its expression continues until neural tube closure, but fades away when the neural crest cells migrate from the neural tube. These studies clearly documented the regulation of neural crest cell–cell adhesion by the cadherin protein family, but cadherin 6B expression in other tissues or other developmental stages was not characterized. In this study, we first demonstrated that cadherin 6B was expressed in smooth muscle tissues such as aorta, gizzard and the digestive tract, in addition to the brain, spinal cord, retina and kidney at a late stage of chicken embryonic development. In aorta and gizzard, cadherin 6B was detectable late in SMC differentiation (data not shown). Rat K-cadherin [22] and human cadherin 6 [23] are highly homologous with chicken cadherin 6B with 88 and 87% identity, respectively. It seems, therefore, likely that chicken cadherin 6B is a family of these proteins. However, K-cadherin is preferentially expressed in fetal kidney but is not present in adult tissues [22], whereas human cadherin 6 is intensely expressed in adult brain and kidney, but not in smooth muscle tissues [23].

This paper provides the first demonstration that the vascular and visceral SMCs express cadherin 6B and that its expression is regulated in a SMC phenotype-dependent manner. Since the IGF-I stimulation affected the cadherin 6B expression, the signaling pathway mediated through PI3-K/PKB(Akt) is likely to be responsible for upregulating the transcription of its mRNA, as with other SMC molecular markers such as caldesmon and calponin [13,14]. These findings suggest that cadherin 6B is a novel molecular marker for the SMC phenotype. Although we do not directly address the function of cadherin 6B in SMCs, this protein might be in-

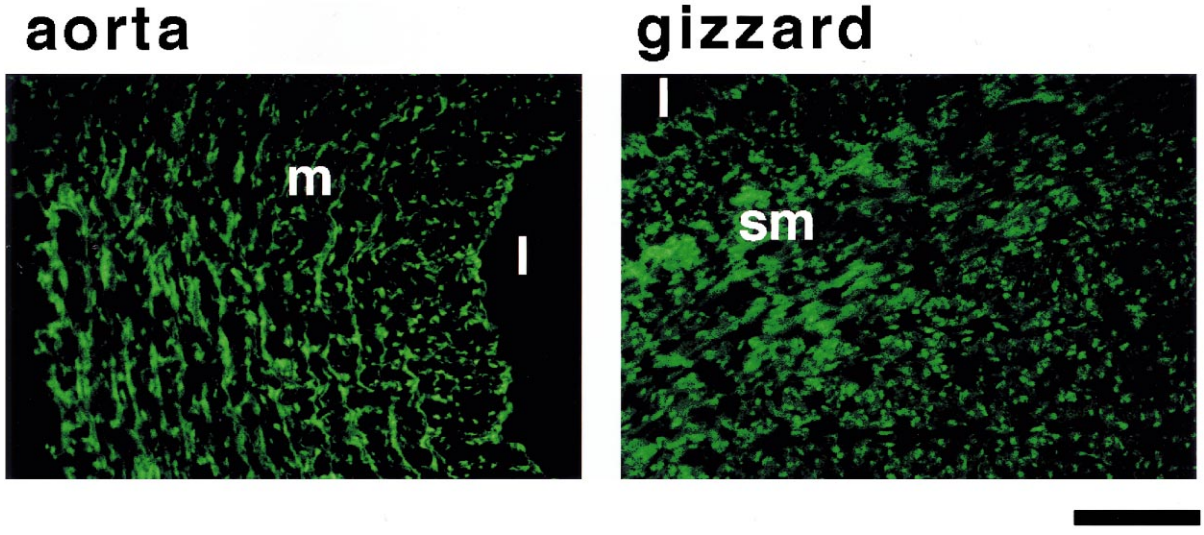

Fig. 6. Expression of the cadherin 6B protein in aorta and gizzard. The SMC layers in aorta and gizzard were stained with a monoclonal antibody against cadherin 6B (green). l, lumen; m, medial SMC layer of aorta; sm, SMC layer of gizzard. Bar, 100 μm.

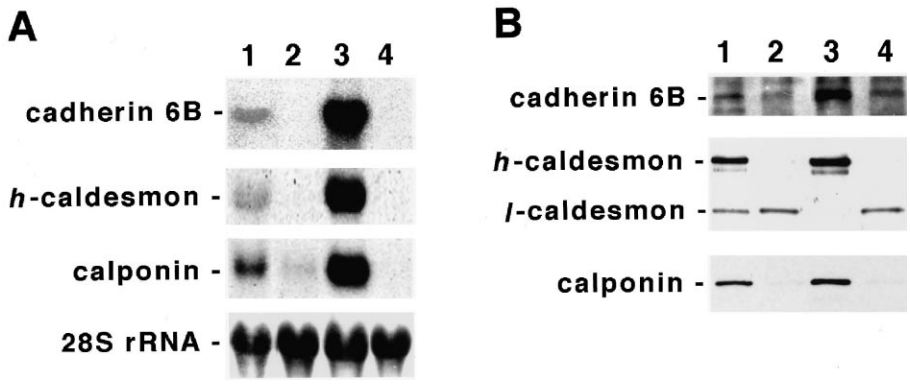

Fig. 7. Phenotype-dependent expression of cadherin 6B in vascular and gizzard SMCs. The expressions of cadherin 6B, *h*-caldesmon, and calponin mRNAs were analyzed by Northern blotting (A). The expression of cadherin 6B, caldesmon and calponin at a protein level was analyzed by immunoblotting using a monoclonal antibody against cadherin 6B, anti-caldesmon antibodies and anti-calponin antibodies (B). Total RNAs (A) or whole cell lysates (B) were isolated from aortae in 15-day-old chicken embryos (lane 1), dedifferentiated vascular SMCs under serum-stimulated conditions (lane 2), differentiated gizzard SMCs under IGF-I-stimulated conditions (lane 3) and dedifferentiated gizzard SMCs under serum-stimulated conditions (lane 4). Anti-caldesmon antibodies recognized both *h*- and *l*-caldesmons, respectively.

volved in the cell–cell interactions of differentiated SMCs or in the late differentiation processes of SMCs. Further analyses are required to understand the biological significance of the expression of cadherin 6B in SMCs.

Acknowledgements: We thank Dr. M. Takeichi (Graduate School of Science, Kyoto University, Japan) for providing a monoclonal antibody against cadherin 6B. This work was supported by a Grant-in-aid for COE Research (to K.S.) from the Ministry of Education, Science, Sports and Culture of Japan.

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