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DEVELOPMENTAL DYNAMICS 235:1648–1658, 2006

Muscularizing Tissues in the Endocardial Cushions of the Avian Heart Are Characterized by the Expression of h1-Calponin

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Muscularization of mesenchymal tissues in the developing heart is an important event in the morphogenesis of the valvuloseptal complex in four-chambered hearts. Perturbation of muscularization has been implicated in the pathogenesis of cardiac malformations in several animal models for congenital heart disease, including the Trisomy 16 mouse and the TGFβ2 knockout mouse. Studies to unravel the mechanism of muscularization, as well as studies to determine the extent of the process in frequently used animal-model systems for cardiac development, have, thus far, been hampered by the lack of useful differentiation markers for muscularizing tissues, albeit that it had been demonstrated that, in the mouse, muscularizing cells are characterized by an elevated level of smooth muscle actin expression. In this study, we investigated whether muscularization of endocardial cushions in the avian heart is also accompanied by the expression of smooth muscle cell markers. The results presented in this study demonstrate that, in quail and chick, a specific population of muscularizing cells is recognized by the expression of smooth muscle h1-calponin. Interestingly, other genes typically found in smooth muscle cells (e.g., smooth muscle actin and caldesmon) are not expressed in muscularizing tissues. We conclude that muscularization of cushion-derived mesenchymal tissues is associated with a discrete genetic program reflected by the expression of h1-calponin and predict that h1-calponin will prove an invaluable tool in elucidating the regulation of muscularization and other aspects related to this event. *Developmental Dynamics 235:1648–1658, 2006.*

Key words: heart development; muscularization; myocardialization; h1 calponin; differentiation

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INTRODUCTION

Muscularization of mesenchymal tissues is an essential process in cardiovascular remodeling and valvuloseptal morphogenesis. Previously, we have demonstrated that the formation of the muscular outlet septum and infundibulum is the result of muscularization of the fused endocardial cushions in the proximal outflow tract (Lamers et al., 1995; van den Hoff et al., 1999). It was also demonstrated that parts of the mesenchymal tissues that are derived from the atrioventricular cushions become muscularized (Kim et al., 2001; Kruithof et al., 2003a). Furthermore, we have shown

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that the extent of the process of muscularization is not the same in all species investigated, i.e., muscularization is more extensive in the avian heart than in the developing mammalian heart (Lamers et al., 1995; van den Hoff et al., 1999, 2001, 2004; Kim et al., 2001; Kruithof et al., 2003b).

The mechanism by which muscularization of the mesenchymal tissues occurs is still contentious. Whereas it was initially hypothesized that muscularization is resulting from active ingrowth of existing myocardium into flanking mesenchymal tissues, a process that we dubbed "myocardialization" (van den Hoff et al., 1999), more recent observations indicate that muscularization can also be established by a mesenchymal-to-myocardial differentiation (van den Hoff et al., 2001, 2004; Kruithof et al., 2003b). We define mesenchymal-to-myocardial differentiation, or MMD, as the process during which cushion mesenchymal cells obtain a myocardial phenotype, as indicated by the expression of characteristic myocardial differentiation markers. The relative contributions of myocardialization and MMD to muscularization of the different mesenchymal components in the respective regions of the developing heart, as well as the molecular mechanisms that govern the regulation of both events still need to be established.

Perturbation of muscularization has been implicated in the pathogenesis of congenital malformations in mice such as the Trisomy 16 mouse (Waller et al., 2000), the NF1 knockout mouse (Lakkis and Epstein, 1998), and the TGFβ2 knockout mouse (Sanford et al., 1997; Bartram et al., 2001). In addition, muscularization is also reportedly abnormal in avian models with experimentally induced heart abnormalities (Sedmera et al., 1999; Gittenberger-de Groot et al., 2000).

Central questions on which we have focused since the recognition of the importance of muscularization to the developing heart include (1) how is muscularization regulated, and (2) what is the actual contribution of muscularizing tissue to the overall composition of the heart in health and disease. Studies to address these questions have been hampered, because until recently, specific marker(s) to study the differentiation process were not identified in the avian heart albeit that previous studies in the mouse had shown that muscularizing cells were characterized by an elevated level of smooth muscle α-actin (SMA) expression (Kruithof et al., 2003a). However, given that, in the mouse, the level of SMA expression in the working myocardium is relatively high at stages during which muscularization occurs (see the Discussion section), it is difficult to use SMA expression as a differentiation marker for muscularization. Given the general similarities in regulatory mechanisms that govern cardiac morphogenesis, we hypothesized that muscularizing cells in the avian heart would also go through a stage with up-regulation of expression of smooth muscle cell markers.

In this study, we present data that demonstrate that, in the avian heart, a specific population of muscularizing cells is recognized by the expression of h1-calponin, a gene usually associated with smooth muscle cell differentiation, but that, in contrast to the finding in the mouse, SMA is not expressed in the avian muscularizing tissues. Our observations demonstrate that, during cardiac morphogenesis in the avian heart, muscularization is accompanied by a discrete genetic program.

RESULTS

CP93, an Antibody Raised Against Turkey h1-Calponin, Recognizes Muscularizing Tissues of the Developing Quail and Chick Embryo

The aim of this study was to investigate the expression of smooth muscle cell markers in the developing avian heart in the context of earlier reports on the relatively high expression of SMA in muscularizing tissue in the murine heart (Kruithof et al., 2003a). In the first series of experiments (Fig. 1), serial sections of quail embryos were immunohistochemically labeled with a panel of antibodies recognizing SMA, caldesmon (CALD), and h1-calponin (initially using CP93, a commercial antibody raised against turkey calponin). In addition, we used a set of antibodies to recognize specific subpopulations of cells including MF20 (recognizing myosin heavy chain [MHC] typically expressed in the myocardium) and an antibody recognizing vimentin (VIM) primarily expressed in epithelial and mesenchymal cells. Whereas we did not observe any staining of muscularizing tissues with either anti-SMA (see Fig. 1E,H) or anti-CALD (not shown), we found remarkably strong immunoreactivity of CP93 in the populations of cells that we have described previously as myocardializing/muscularizing tissues (van den Hoff et al., 1999, 2001). At late stages of muscularization, the strongly labeled cells were located in the proximal outflow tract (OFT; i.e., the forming muscular outlet septum), in atrioventricular (AV) cushion-derived mesenchyme (in particular in the cushion-derived tissues associated with the interventricular septum), and in the muscularizing tissues of the flap-valve in the right AV junction (Fig. 1B,D,G). Interestingly, immunoreactivity of CP93 was not observed in other cardiac regions of muscularization that are commonly associated with the craniocaudal expansion of the developing heart (i.e., distal OFT and inflow tract; see van den Hoff et al., 2004). Within the rest of the quail embryo, strong CP93 staining was seen in the smooth muscle cells of the esophagus/stomach, whereas smooth muscle cell populations in other embryonic tissues were hardly stained (intensity of staining depending on pretreatment of the sections, Fig. 2B), this is in contrast to SMA, which, at this stage, is expressed in all smooth muscle cell populations (Fig. 2C). Careful inspection of the serially incubated consecutive (or sister) sections treated with CP93 and MF20 strongly suggested that the antigens recognized by these antibodies were coexpressed in the same cells (Fig. 3A,B). This coexpression was confirmed by immunofluorescent colabeling that demonstrated that CP93 was coexpressed with myocardial markers such as myosin light chain (MLC; Fig. 3C–E). Importantly, our immunolabeling experiments showed that cells in the "leading edge" of the muscularizing field expressed the highest level of CP93 and the lowest level of MLC and MHC (Fig. 3A,B). Moreover, the immunofluorescent colabeling studies suggested that some mesenchymal

cells in the periphery of the field of muscularizing cells express CP93 before the onset of the myocardial differentiation program (Fig. 3C–E).

Having established that the observed immunoreactivity of muscularizing tissue with CP93 at late stages of valvuloseptal development indeed reflects the expression of h1-calponin (see next section), we set out to determine some additional features of the h1-calponin expression profile in the developing quail heart. Staining of serial sections of hearts before the onset of muscularization, i.e., before Hamburger and Hamilton stage (HH) 28 (van den Hoff et al., 1999), showed that the overall expression of h1-calponin in the developing myocardium in quail hearts at HH18–HH26 is low (Fig. 4C,F,I). There is a slightly elevated level of expression in the myocardium at the base (black arrows in Fig. 4C,F,I), and the more distal part of the OFT (black arrow in Fig. 4I), but these areas of the developing heart are also characterized by intense staining with MF20 (white arrows in Fig. 4,A,D,G). When hearts in the early stages of muscularization were investigated, h1-calponin expression was typically observed in the loosely arranged cells in the leading edge of muscularization. Like in the older stages, these muscularizing cells typically express relatively low levels of MF20.

Antibodies CP93, CP1, and CP/4291 Recognize h1-Calponin in the Developing Avian Heart

The cardiac expression pattern observed with CP93 strongly suggested that muscularizing tissue in the quail heart was characterized by the expression of h1-calponin, a significant observation in the context of ongoing studies on the role and regulation of this process in normal and perturbed heart development. However, the absence of detectable expression of other genes associated with smooth muscle differentiation (i.e., smooth muscle actin and caldesmon) in these cells, raised the question whether CP93 was indeed recognizing h1-calponin or whether this commercial antibody was cross-reacting with another antigen. To address this question, a series of experiments was conducted to prove unambiguously that h1-calponin is expressed in the developing avian heart and that h1-calponin is a bona fide marker for muscularizing tissue in the endocardial cushion derived tissues.

Application of CP1, a monoclonal antibody that was raised against

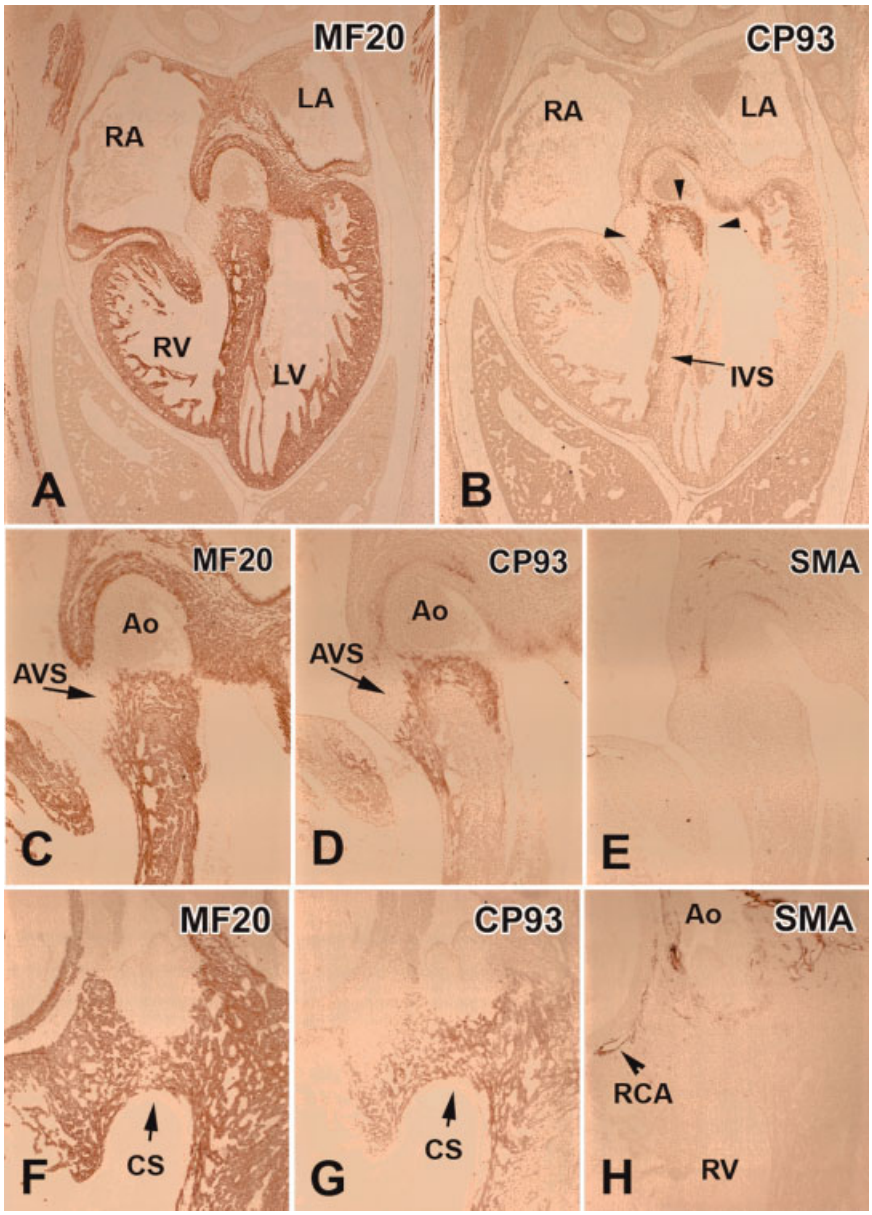

Fig. 1. h1-Calponin expression in muscularizing tissues of the embryonic quail heart. **A–H:** Immunolabeling of serial sections of an embryonic quail heart at Hamburger and Hamilton stage (HH) 34 with MF20 (recognizing myosin heavy chain; A,C,F), CP93 (raised against avian h1-calponin; B,D,G), and anti-smooth muscle actin (SMA; E,H). The respective panels clearly show that muscularizing cells in the endocardial cushion-derived mesenchymal tissues are characterized by the expression of h1-calponin, but not SMA. The overviews in A and B (enlarged in C and D, respectively) show the massive muscularization of the atrioventricular septal structures at the rim of the interventricular septum. The panels also show the ongoing muscularization in the flap valve of the right atrioventricular (AV) junction. F and G show the ongoing muscularization of the conal septum. As demonstrated in G, the muscularizing tissue in the conal septum of the developing outflow tract is also characterized by expression of h1-calponin. H: However, like in the AV septum, no expression of SMA is found in these muscularizing cells. Note that, in contrast, at this stage of development, the coronary smooth muscle cells are expressing SMA, however, they do not yet express h1-calponin. AO, aorta; AVS, atrioventricular septum; CS, conal septum; IVS, interventricular septum; LA, left atrium; LV, left ventricle; RA, right atrium; RCA, right coronary artery.

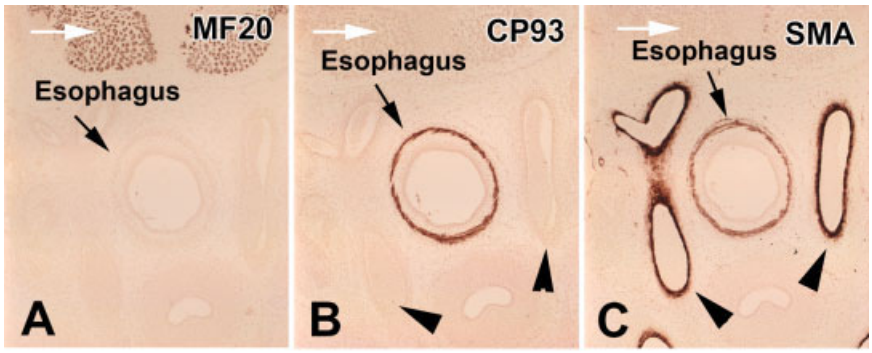

Fig. 2. h1-Calponin and smooth muscle actin (SMA) expression in smooth muscle cell population of the developing quail embryo. **B,C:** Immunolabeling of a Hamburger and Hamilton stage (HH) 34 embryo with CP93 (B) and anti-SMA antibodies (C) demonstrate that the smooth muscle cell markers h1-calponin and SMA are expressed in the smooth muscle cells of the developing esophagus. However, unlike SMA, h1-calponin is not expressed (or if so at very low levels) in the smooth muscle cells of the aortic arches (arrowheads in B and C). **A:** The myosin heavy chain isoforms that are recognized by MF20 and found in atrial and ventricular myocytes are not expressed in the smooth muscle cells of the esophagus. In addition, this panel shows that, at this stage, the smooth muscle markers h1-calponin and SMA are not expressed in the developing skeletal muscle cells (white arrows in B,C), characteristically expressing myosin heavy chain isoforms recognized by MF20 (white arrows in A). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

chicken gizzard h1-calponin (the generation and characterization of which has been described in detail elsewhere; Jin et al., 1996, 2003) to serial sections of the developing quail, as well as chick, heart resulted in a staining pattern identical to that found with CP93 (Fig. 5A–D). We also generated a polyclonal antibody, CP/4291, in rabbit using a synthetic multiple antigenic peptide (MAP) for an avian-specific region in the C-terminus of the h1-calponin (amino acid sequence: DAPGLLGEDGLNHSFYNSQ). Again, immunofluorescent labeling of serial sections obtained from embryonic quail and chick hearts with this antibody resulted in a staining pattern identical to the pattern seen with CP93 and CP1 (Fig. 5E,F). These immunohistochemical observations confirmed that muscularizing tissues in the chick and quail heart are characterized by the expression of elevated levels of h1-calponin.

α-h1 Calponin Is the Prevalent h1-Calponin Splice Variant Expressed in the Heart

Both in avian and mammalian tissues, two isoforms of h1-calponin have been described, designated α-h1 and β-h1 calponin. The mRNA for the β-h1 isoform (Fig. 6A′) is a splice variant of the α-h1 isoform mRNA (Fig. 6A) and is 120 bp shorter. As a result, the β-h1 calponin protein is 40 amino acids shorter at the C-terminus than the α-h1 calponin isoform. Alpha-h1 calponin has an approximate molecular weight of 34 kDa, while β-h1 calponin has an approximate weight of 29 kDa (Takahashi and Nadal-Ginard, 1991). To assess whether both h1 calponin isoforms were expressed in the developing heart, we performed Western blot (Fig. 6B) and reverse transcriptase-polymerase chain reaction (RT-PCR; Fig. 6C,D) analyses.

Using protein extracts from embryonic quail hearts, Western blot analyses were performed using the monoclonal antibodies CP1 and CP93. These experiments revealed immunoreactive bands at approximately 34–36 kDa, corresponding with the anticipated molecular weight for α-h1 calponin (Fig. 6B), suggesting that α-h1 calponin is the prominent isoform expressed in the developing quail heart. A (very) faint second band was occasionally observed, possibly indicating the presence of relatively small amounts of β h1 calponin.

The expression of the respective h1 calponin mRNAs in the developing quail was investigated by RT-PCR on total mRNA extracted from isolated quail heart and quail body (i.e., after removal of heart) at various stages of development. As the sequence of quail h1 calponin is not available in Genbank, primers were designed based on the chicken sequence available in Genbank (accession no. 63559). The first set of calponin-specific primers was designed to amplify, based on published chicken sequence, an ~380-bp fragment located in a common region of both the α-h1 and β-h1 splice-variants of calponin. This analysis with the "generic" h-1 calponin primer set showed the presence of h1-calponin mRNA in the developing heart and body (Fig. 6C), confirming the immunohistochemical and Western blot data. To determine whether there was differential expression of both calponin h1 variants, a "α/β" h-1 calponin primer set was designed that spanned the alternatively spliced area. The anticipated size of the amplified fragment derived from the mRNA encoding the h1 calponin isoforms using this primer set is 310 bp for α-h1 calponin mRNA and 190 bp for β-h1 calponin (see cartoon in Fig. 6A,A′). As predicted, the RT-PCR analysis revealed the expected DNA fragments confirming the expression of both isoforms in the heart and body (Fig. 6D). Subsequent sequence analysis revealed that the amplified 310-bp fragment from the quail tissue was 99% identical to the published corresponding chicken h1-calponin sequence, indicating that the amplified fragments indeed represent parts of quail h1-calponin. These observations were further substantiated by RT-PCR analysis using an "α-specific" h1 calponin primer set that was designed to specifically recognize the α-h1 calponin and by sequencing the purified PCR fragments directly. Using additional primer sets, the entire coding regions of both quail h1 calponin isoforms were amplified and sequenced. Analysis of the obtained sequence data demonstrated that the quail h1 calponin cDNA and published chick cDNA sequences (GenBank accession no. M63559) have a high degree of homology (97%, see Fig. 7). Moreover, translation of the cDNA sequence of quail α-h1 calponin into a derived amino acid sequence for α-h1 protein showed that the quail protein sequence is 100% identical (see Fig. 7) to the published chicken h1-calponin sequence (GenBank accession no. P26932). Thus, the results of these

analyses demonstrate the presence of α-h1 (predominant isoform) and β-h1 mRNA in both the developing quail heart and body, confirming the immunohistochemical and Western blot results.

DISCUSSION

Relevance of Expression of h1 Calponin in the Developing Heart

Although muscularization of the mesenchymal tissues of the heart was first described by Okamoto and colleagues (1978), the process did not receive widespread attention until the immunohistochemical studies on muscularization in the human heart by Lamers and colleagues (1995). In subsequent efforts to increase our understanding of the significance of the process during valvuloseptal morphogenesis, we explored a variety of aspects of muscularization in the developing avian and murine heart (van den Hoff et al., 1999, 2001, 2005; Kruithof et al., 2003a,b). These studies have provided new insights into the spatiotemporal morphological aspects of the process. Although muscularization of cushion-derived mesenchymal tissues is an important process (van den Hoff et al., 1999, 2001; Phillips et al., 2005), the mechanisms by which muscularization occur have yet to be fully elucidated.

Determining the cascade of events involved in the respective steps in muscularization and the assessment of the actual contribution of muscularized tissue to the overall myocardial architecture of the heart during normal and perturbed heart formation, however, thus far has been hampered by the lack of a specific marker (or markers) for muscularizing cells. Identification of "muscularization-specific" markers would allow delineation of the myocardium derived from muscularizing tissue and myocardium directly derived from the myocardial precursors in the precardiac mesoderm (i.e., the primary myocardium). Furthermore, "muscularization-specific" gene expression would allow the development of new in vitro assays to study regulation of muscularization. Here, we report that smooth muscle calponin (or h1-calponin) is expressed at significantly higher levels in muscularizing tissues than in flanking myocardium and demonstrate that h1-calponin can serve as an invaluable tool in studying the respective aspects of muscularization.

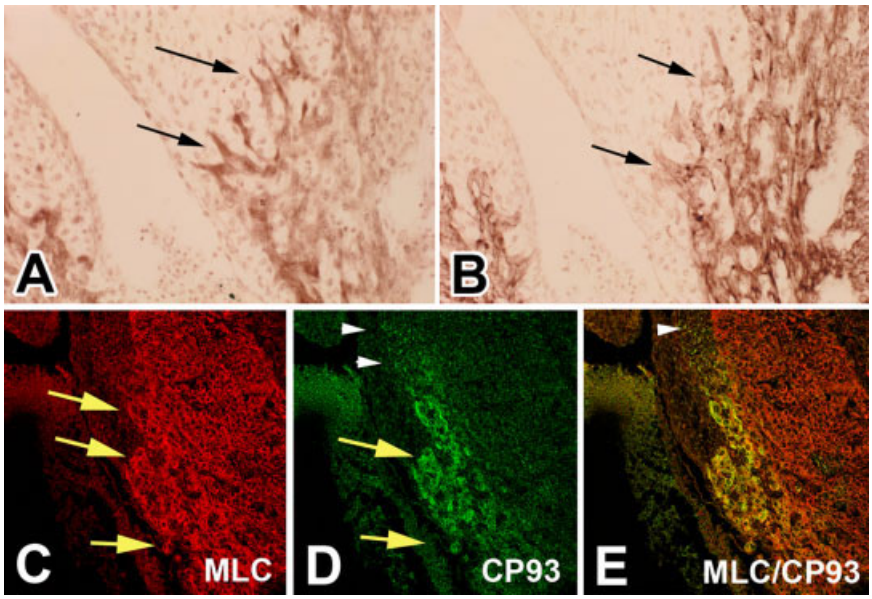

Fig. 3. h1-Calponin is expressed most strongly at the leading edge of the muscularizing tissue. **A,B:** Details of Figure 1C and 1D, respectively, stained for h1-calponin (A) and myosin heavy chain (MHC; B). The panels clearly demonstrate that cells at the leading edge of a field of muscularizing cells in endocardial cushion-derived tissues intensely express h1-calponin (arrows in A), whereas the same cells express relatively little MHC (arrows in B). **C–E:** Results of an immunofluorescent colabeling of a quail heart at Hamburger and Hamilton stage (HH) 34 stained for myosin light chain (red in C and E) and for h1-calponin (green in D and E). C and E are merged in E. D,E: The white arrowheads point to mesenchymal cells that appear to express h1-calponin but do not express myosin light chain (MLC).

What Does h1-Calponin Expression Tell Us Regarding the Mechanisms of Muscularization: "Ingrowth" Vs. "MMD"?

The very strong expression of h1-calponin in the leading edge of patches of muscularizing cells, combined with the low level of myosin heavy chain expression in the same cells (see Fig. 2), strongly suggests that h1-calponin expression precedes the robust expression of myocardial markers in the cells at the perimeter of muscularization. Moreover, the observation that some isolated mesenchymal cells, adjacent to muscularizing tissue, appear to express h1-calponin in the absence of MHC expression, indicates that upregulation of h1-calponin in mesenchyme is an early step in MMD, preceding the induction of expression of characteristic myocardial genes. In this context, it is important to note that preliminary in vitro experiments, using endocardial cushion mesenchyme of stage HH25 OFT explants, have demonstrated that h1-calponin is expressed in a subset of cushion mesenchymal cells in a filamentous pattern, characteristic for intracellular calponin (Gimona et al., 2003). We currently are optimizing a protocol for a new muscularization/differentiation assay based on this observation and plan to apply it in future experiments to specifically address the differentiation events of cushion mesenchyme during MMD.

Whereas we believe that the observations on the expression of h1-calponin in muscularizing tissues strongly supports the hypothesis that MMD is involved in the process of muscularization in the avian heart, it is noteworthy that, thus far, lineage tracing experiments have failed to provide conclusive evidence to support the MMD hypothesis. Specifically, studies in the mouse using endocardial-specific Cre-recombinase transgenic mice (Tie2-cre) and (cardiac) neural crest

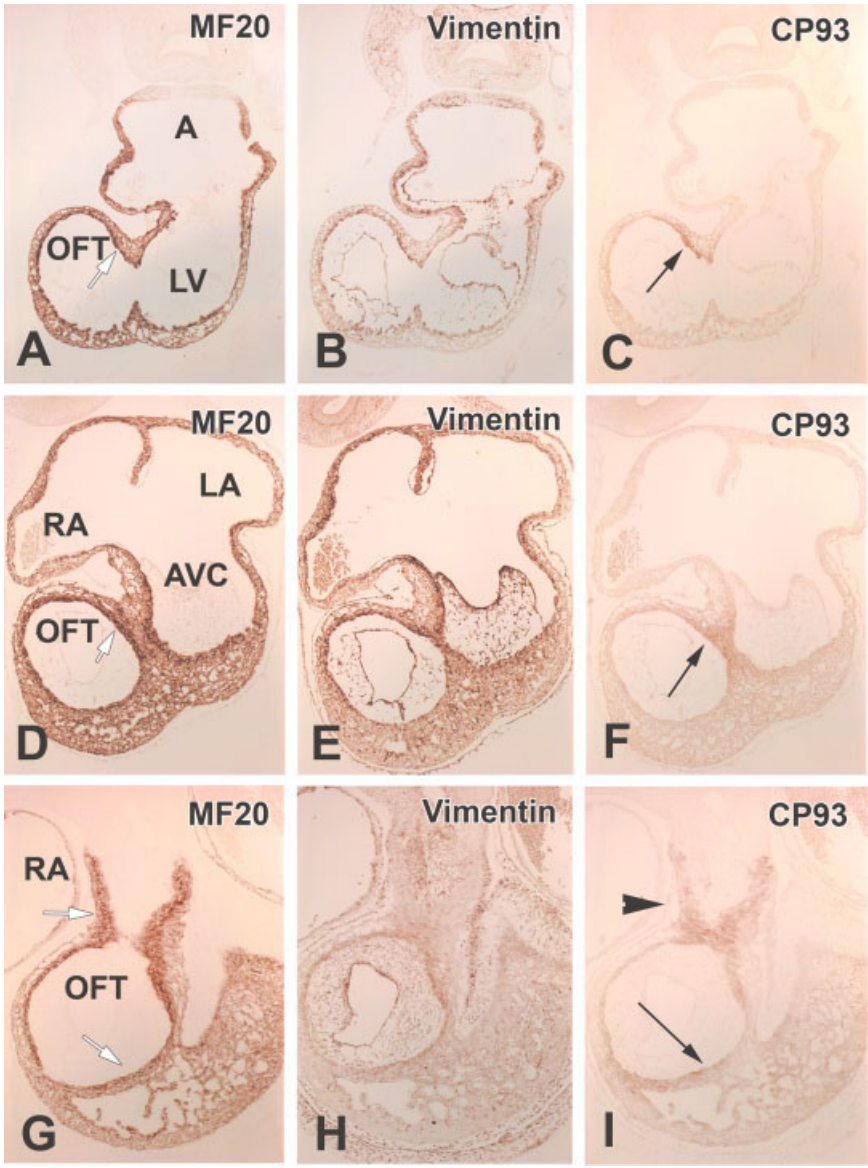

Fig. 4. h1-Calponin is expressed at low levels in the developing myocardium before onset of muscularization. **A–I:** Serial sections of quail hearts at Hamburger and Hamilton stage (HH) 18 (A–C), HH22 (D–F), and HH26 (G–I) were incubated with anti-myosin heavy chain (MF20; A,D,G), anti-vimentin (B,E,H), and anti–h1-calponin (CP93; C,F,I). C,F,I: The h1-calponin–immunolabeled sections show that some expression of h1-calponin is observed in myocardium lining the developing cushion tissues in the proximal (arrows in C,F,I) and more distal part of the outflow tract (arrowhead in I). A,D,G: Note that, unlike what is observed in the muscularizing tissue within the cushions, the areas with slightly elevated levels of h1-calponin are also characterized by intense staining for myosin heavy chain (white arrows). A, (common) atrium; AVC, atrioventricular canal; LA, left atrium; LV, left ventricle; OFT, outflow tract; RA, right atrium. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

specific mice (Wnt1-cre) have not revealed a mesenchymal origin for muscularizing tissue (de Lange et al., 2004). None of the cell-fate studies published to date, however, have specifically focused on muscularization. Therefore, it is conceivable that this specific aspect of heart development may have been overlooked. Alternatively, as the origin of cushion mesenchyme continues to be a contentious topic, it cannot be ruled out that other cell populations play an important role in this event. The most likely candidate in this context is the epicardially derived cell (EPDC). Several studies have demonstrated the abundance of EPDCs in the developing cushions of the embryonic heart (Dettman et al., 1998; Gittenberger-de Groot et al., 1998; Manner, 1999; Perez-Pomares et al., 2002), and we have convincing evidence that EPDCs, at least under in vitro conditions, can undergo a myocardial differentiation (Wessels and Perez-Pomares, 2004). It is important to note that an epicardial-cre mouse, that would allow tracing the fate of EPDCs in the mouse, is not yet available.

Functional Significance of h1-Calponin Expression in Muscularizing Cells

The observation that smooth muscle h1-calponin is expressed at high levels in muscularizing tissue was surprising, given that other markers for smooth muscle cell differentiation (e.g., SMA and CALD) were not found to be present at detectable levels in the muscularizing cells of the endocardial cushions in the avian heart. This finding was even more remarkable when observations on smooth muscle gene expression in the mammalian heart were taken into consideration. Thus, in previous studies, it was demonstrated that muscularizing cells in the mouse show (slightly) elevated levels of smooth muscle actin expression (Kruithof et al., 2003a). This finding indicates that, even though it appears that expression of genes considered characteristic for SMCs is a common feature of muscularizing tissue in fowl and mammals, there are significant differences in the profile of smooth muscle protein expression related to muscularization between the respective species.

One of the known functions of h1-calponin is as a negative regulator of bone formation by affecting bone morphogenetic protein (BMP) responsiveness of mesenchymal cells (Yoshikawa et al., 1998). Thus, in mice lacking h1-calponin, increased bone formation is observed that is associated with the enhancement of BMP-mediated signaling response. Of interest, the muscularizing cells that express h1-calponin in the avian heart are also characterized by the expression of BMPs, in particular BMP2 and BMP4 (Somi et al., 2004). This relationship between h1-calponin and BMPs is of particular significance in the light of

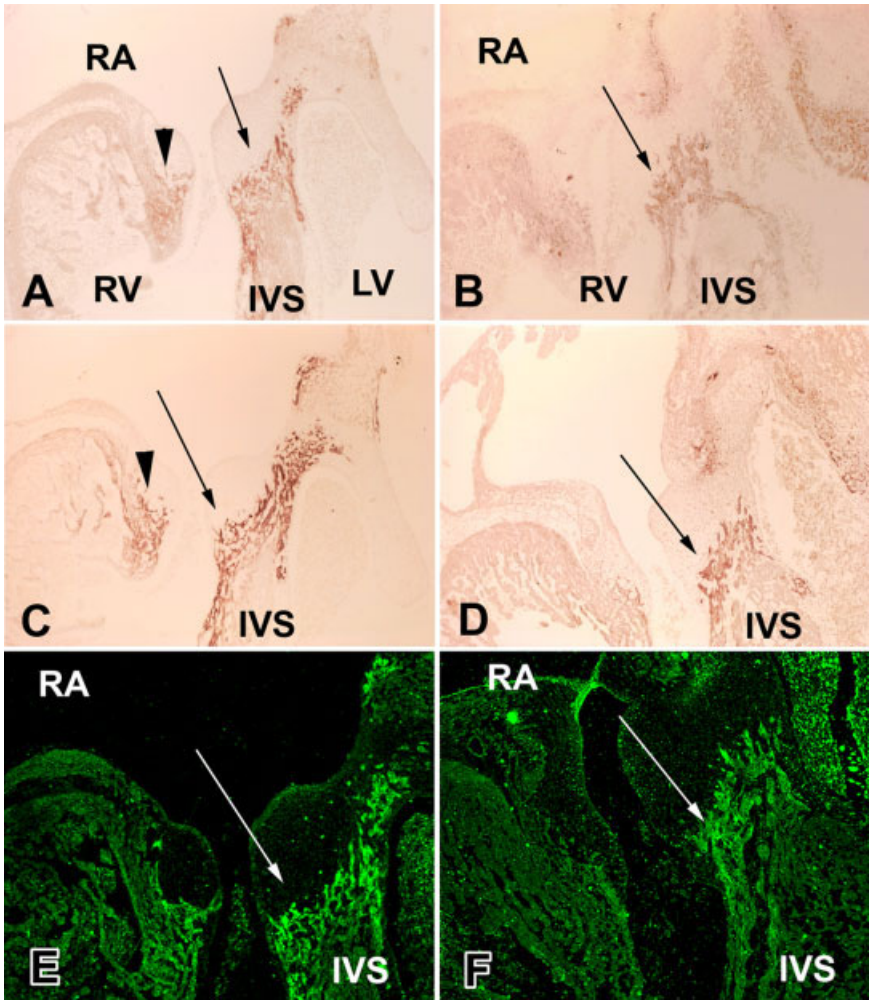

Fig. 5.

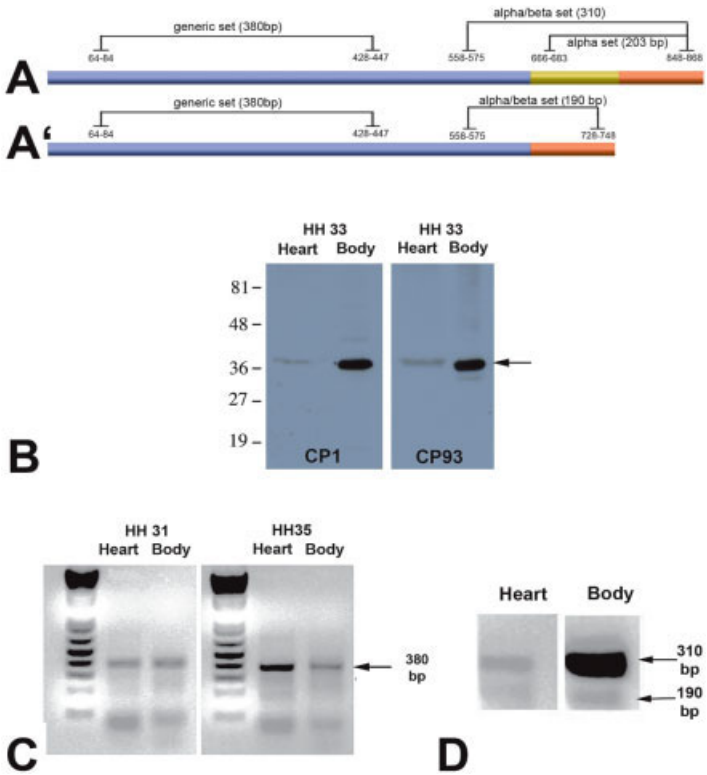

Fig. 6.

observations regarding the role of growth factors in muscularization. An increasing body of evidence points to the involvement of members of the TGFβ family of growth factors in the regulation of muscularization. Not only are these growth factors expressed within the region of muscularization (Abdelwahid et al., 2001; Molin et al., 2003), in vitro results obtained using our three-dimensional myocardialization assays also point to their specific role in this event (van den Hoff et al., 2005). A strong indication that TGFβ2 is involved in the reg-

Fig. 5. CP93, CP1, and CP/4291 recognize muscularizing cells in developing quail and chick heart. **A–F:** Serial sections of quail (A,C,E) and chick (B,D, F) were incubated with the monoclonal h1-calponin antibodies CP93 (A,B), and CP1 (C,D), as well as with the polyclonal antibody CP/4291 (E,F). Antibody binding for CP93 and CP1 was visualized immunohistochemically using peroxidase-conjugated secondary antibody, followed by diaminobenzidine (DAB) treatment. For visualization of CP/4291, a fluorescently labeled secondary antibody was used. The arrows in the respective panels point to the area of muscularization in the atrioventricular cushion-derived mesenchymal tissues characterized by the expression of h1-calponin. Note also the extensive muscularization of the right flap valve in the right atrioventricular junction (arrowheads in A and C). IVS, interventricular septum; LV, left ventricle; RA, right ventricle; RV, right ventricle.

Fig. 6. Alpha and beta h1-calponin and detection of their expression in the developing quail heart by Western blot and polymerase chain reaction (PCR) analysis. **A,A′:** Schematic representations of the mRNAs encoding for α h1-calponin (A) and β h1-calponin (A′). Indicated in these schematics are the primer sets (and the anticipated length of PCR products) used to determine expression of α and β h1-calponin as described in the text. **B:** The results of Western blot analysis using extracts of Hamburger and Hamilton stage (HH) 33 quail heart and body and the indicated primary antibodies (CP1 or CP93) as described in the Experimental Procedures section. The migration of standard proteins is indicated by their molecular weight divided by 1,000. The migration of α h1-calponin is indicated by an arrow. **C,D:** The results of PCR experiments that demonstrate expression of h1-calponin in the developing quail. C: The "generic" primer set (recognizing α as well as β h1-calponin; see A,A′) amplifies a fragment of ~380 bp, indicating expression of h1-calponin in the heart and body of HH31 and HH35 quail embryos. PCR analysis with the "alpha/beta" primers set, designed to amplify a ~310-bp fragment for α h1-calponin and ~190 bp for β h1-calponin (see A,A′) leads to the conclusion that α h1-calponin is the predominant isoform expressed in heart and body.

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Quail 1    ATGTCGAACG CGAACTTCAA CCGCGGGCCG GCCTACGGGC TGTCGGCAGA GGTGAAGAAC
Chick 1    ATGTCGAACG CGAACTTCAA CCGCGGGCCG GCCTACGGGC TGTCGGCCGA GGTGAAGAAC

Quail 61   AAGCTGGCAC AGAAATACGA CCCACAGACA GAGCGTCAGC TGCGTGTCTG GATCGAAGGA
Chick 61   AAGCTGGCAC AGAAATACGA CCCACAGACG GAACGTCAGC TGCGTGTGTG GATCGAGGGA

Quail 121  GCCACCGGGC GCCGCATCGG GGACAACTTC ATGGACGGCC TCAAGGACGG CGTCATCCTC
Chick 121  GCCACCGGGC GCCGCATCGG GGACAACTTC ATGGATGGCC TCAAGGATGG AGTCATCCTC

Quail 181  TGCGAGCTCA TCAATAAGCT GCAGCCTGGC TCGGTGCAGA AGGTGAACGA CCCCGTCCAG
Chick 181  TGCGAGCTCA TCAACACGCT GCAGCCTGGC TCAGTGCAAA AGGTGAACGA TCCCGTCCAG

Quail 241  AACTGGCACA AGCTGGAGAA CATTGGGAAC TTCCTGCGGG CCATCAAACA CTACGGAGTG
Chick 241  AACTGGCATA AGCTGGAGAA CATTGGGAAC TTCCTGCGGG CCATCAAACA CTACGGAGTG

Quail 301  AAGCCCCACG ACATCTTCGA GGCCAACGAC CTCTTTGAGA ACACCAACCA CACACAGGTG
Chick 301  AAACCCCACG ACATCTTTGA GGCCAACGAC CTCTTCGAGA ACACCAACCA CACACAGGTG

Quail 361  CAGTCCACCC TCATCGCCCT CGCCAGCCAG GCCAAGACGA AGGGGAACAA TGTGGGGCTG
Chick 361  CAGTCCACCC TCATCGCCCT CGCCAGCCAG GCCAAGACGA AGGGCAACAA CGTGGGTTTG

Quail 421  GGGGTGAAGT ACGCGGAGAA GCAGCAGCGT CGCTTCCAGC CTGAGAAGCT GCGCGAGGGC
Chick 421  GGGGTGAAGT ACGCAGAGAA GCAGCAGCGT CGCTTCCAGC CTGAGAAGCT GCGCGAGGGC

Quail 481  AGGAACATCA TCGGCCTCCA GATGGGCACC AACAAGTTCG CGAGCCAGCA GGGCATGACG
Chick 481  AGGAACATCA TCGGGCTCCA GATGGGCACC AACAAGTTTG CGAGCCAGCA GGGCATGACA

Quail 541  GCGTATGGCA CGAGGCGGCA CCTCTACGAC CCCAAGCTGG GCACAGACCA GCCCTTGGAC
Chick 541  GCGTACGGCA CGAGGCGGCA CCTCTACGAC CCCAAGCTGG GGACAGACCA GCCCTTGGAC

Quail 601  CAGGCCACCA TCAGCCTCCA GATGGGCACC AACAAGGGGG CCAGCCAGGC GGGGATGACG
Chick 601  CAGGCCACCA TCAGCCTCCA GATGGGCACC AACAAGGGGG CCAGCCAGGC GGGGATGACG

Quail 661  GCGCCGGGGA CCAAGAGGCA AATCTTCGAG CCGTCCCTGG GCATGGAGCG CTGCGACACA
Chick 661  GCGCCGGGGA CCAAGAGGCA GATCTTCGAG CCCTCGCTGG GCATGGAGCG CTGCGACACA

Quail 721  AACATCATCG GGCTGCAGAT GGGCAGCAAC AAGGGCGCCT CACAGCAGGG CATGACGGTG
Chick 721  AACATCATCG GGCTGCAGAT GGGCAGCAAC AAGGGCGCCT CACAGCAGGG CATGACGGTG

Quail 781  TATGGGCTGC CGAGGCAGGT CTACGACCCC AAGTACTGCG ACGCGCCCGG GCTGCTGGGC
Chick 781  TACGGGCTGC CGAGGCAGGT CTACGACCCC AAGTACTGCG ACGCGCCTGG GCTGCTGGGC

Quail 841  GAGGACGGCC TCAACCACAG CTTTTACAAC TCACAATAA
Chick 841  GAGGACGGCC TCAACCACAG CTTTTACAAC TCACAATAA
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Quail MSNANFNRGPAYGLSAEVKNKLAQKYDPQTERQLRVWIEGATGRRIGDNFMDGLKDGVILCELINKLQPGSVQ
Chick MSNANFNRGPAYGLSAEVKNKLAQKYDPQTERQLRVWIEGATGRRIGDNFMDGLKDGVILCELINKLQPGSVQ

Quail KVNDPVQNWHKLENIGNFLRAIKHYGVKPHDIFEANDLFENTNHTQVQSTLIALASQAKTKGNNVGLGVKYAE
Chick KVNDPVQNWHKLENIGNFLRAIKHYGVKPHDIFEANDLFENTNHTQVQSTLIALASQAKTKGNNVGLGVKYAE

Quail KQQRRFQPEKLREGRNIIGLQMGTNKFASQQGMTAYGTRRHLYDPKLGTDQPLDQATISLQMGTNKGASQAGM
Chick KQQRRFQPEKLREGRNIIGLQMGTNKFASQQGMTAYGTRRHLYDPKLGTDQPLDQATISLQMGTNKGASQAGM

Quail TAPGTKRQIFEPSLGMERCDTNIIGLQMGSNKGASQQGMTVYGLPRQVYDPKYCDAPGLLGEDGLNHSFYNSQ
Chick TAPGTKRQIFEPSLGMERCDTNIIGLQMGSNKGASQQGMTVYGLPRQVYDPKYCDAPGLLGEDGLNHSFYNSQ
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Fig. 7. Comparison of cDNA and protein sequence of quail and chick h1-calponin isoforms. The upper part of this figure shows the comparison of the sequence data for quail α h1-calponin cDNA and the published chick cDNA sequence information (GenBank accession no. M63559). The sequences show a high degree of homology (97%). Nucleotides that differ between the quail and chick cDNA sequence are underlined. The stretch of 120 bp not found in the β h1-calponin isoform (bp 649-768) is highlighted in light gray. GenBank has assigned the accession no. DQ370107 to quail α h1-calponin cDNA and accession no. DQ370108 to quail β h1-calponin cDNA. The lower part of this figure shows the (identical) amino acid sequence for quail and chick α h1-calponin protein. The stretch of 40 amino acids missing from β h1-calponin protein is highlighted in dark gray.

ulation of muscularization initially came from studies analyzing the phenotype of the TGFβ2 knockout mouse. TGFβ2$^{-/-}$ embryos were found to have a nonmuscularized outlet septum within a spectrum of other heart malformations (Sanford et al., 1997; Bartram et al., 2001). Subsequent in vitro studies confirmed that TGFβ2 plays a role in regulation of muscularization. The notion that BMPs may play an important regulatory role in muscularization comes from the observations that (1) the known BMP inhibitors noggin and follistatin interfere with muscularization in vitro (Somi et al., 2004); (2) misexpression of noggin, by injecting recombinant noggin-expressing retrovirus into chick embryos at stage 10–12 HH, leads to malformations of the outflow tract including double outlet right ventricle and apparent failure of muscularization (Allen et al., 2001); (3) in situ hybridization data in the mouse that indicate expression of BMP4 in the area of muscularization in the 14ED mouse heart (Abdelwahid et al., 2001); and (4) in situ hybridization studies on the expression of BMP isoform mRNAs in the developing chick heart that show colocalization with muscularizing tissue (Somi et al., 2004). These observations lead to the central working hypothesis that BMP is "initiating/conditioning" cardiac mesenchyme in the initial phase of muscularization and that TGFβ is involved in subsequent steps of this process. Future studies in our laboratory will focus on testing this hypothesis.

EXPERIMENTAL PROCEDURES

Tissue Preparation

After incubation of fertilized quail (*Coturnix coturnix japonica*) and chicken (*Gallus gallus*) eggs at 39°C, embryos were isolated in phosphate buffered saline (PBS) and staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951), fixed overnight at room temperature in Amsterdam's fixative (35% methanol: 35% acetone: 25% water: 5% acetic acid), dehydrated through a graded ethanol series, and embedded in Paraplast Plus. Serial 5-μm sections were prepared and mounted onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Once the sections were deparaffinized in xylene and rehydrated, immunohistochemistry was performed according to established protocols (Waller and Wessels, 2000).

Immunohistochemistry

Immunohistochemical labeling was performed using the following calponin antibodies: CP93 (C-6047; Sigma, St. Louis, MO), CP1 (Jin et al., 1996, 2003), and CP/4291 (a polyclonal antibody generated during this study; see below). Anti-SMA (A-2547) and anti-CALD (C-6542) were purchased from Sigma. The monoclonal antibodies MF-20 (anti-MHC, developed by D.A. Fischman) and AMF-17b (anti-VIM, developed by B. Caterson) were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa (Department of Biological Sciences, Iowa City, IA). Antibody binding was either detected using peroxidase-conjugated rabbit anti-mouse serum (Sigma), the color reaction being performed with the Pierce Metal Enhanced Diaminobenzidine (DAB) Substrate Kit (Pierce, Rockford, IL; product number 34065), or by immunofluorescence with Alexa 568–conjugated antibodies.

Preparation of Polyclonal Anti–h1-Calponin Antibodies

Polyclonal antibodies recognizing avian h1-calponin were made using a synthetic MAP for an avian-specific region in the C-terminus of the h1-calponin sequence (amino acid sequence: DAPGLLGEDGLNHSFYNSQ). The polyclonal sera obtained after immunization of two rabbits were designated CP/4291 and CP/4292. Total IgG was isolated from the immune sera and immune-IgG was affinity purified from the total IgG using an affinity matrix made by coupling the immunogenic peptide (synthesized with an additional N-terminal cysteine residue) to SulfoLink Coupling Gel (Pierce, Rockford, IL).

Immunofluorescent Colabeling

Colabeling was performed on quail embryos fixed in Amsterdam's fixative (see above) using the monoclonal antibody CP93 and a rabbit polyclonal myosin light chain (AB-2480; Novus Biologicals, Littleton, CO). Sections were incubated overnight at room temperature in a mixture of CP93 and AB-2480. After washes in PBS, binding of primary antibodies was detected with a mixture of DTAF-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) and AlexaFluor 568 goat anti-rabbit IgG (Molecular Probes, Eugene, OR) overnight at room temperature. After washing, slides were cover-slipped with Slowfade (Molecular Probes) and analyzed by confocal microscopy.

Western Blot Analysis

HH stage 33 quail hearts or whole bodies were homogenized in 10 volumes of 25 mM Tris (pH 8.0) containing a cocktail of protease inhibitors (N-ethylmaleimide [10 mM], benzamidine [5 mM], leupeptin [6.7 μg/ml], pepstatin A [5 μg/ml], phenylmethyl sulfoxide [2 mM]). Sodium dodecyl sulfate (SDS) from a 20% stock solution was added to a final 2% and the samples were denatured by heating for 15 min at 60°C. Samples were sonicated to degrade DNA (thereby decreasing their viscosity) and clarified by centrifugation at full speed in a Microfuge for 3 min. The protein content of the supernatants was determined using the Micro BCA Protein Assay Kit (Pierce). For Western blots, 30 μg of protein from each sample was resolved by SDS-polyacrylamide gel electrophoresis on a 12% gel and transferred to nitrocellulose using a Trans Blot SD Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA). For blots with monoclonal antibody CP1, blocking buffer was 0.1% bovine serum albumin in TTBS (0.05% Tween 20/50 mM Tris [pH 7.6]/0.5 M NaCl), antibody buffer was TTBS alone. For monoclonal antibody CP93, blocking buffer was 5% Blotting Grade Blocker Non-Fat Dry Milk (Bio-Rad) in TTBS, antibody buffer was 2.5% Non-Fat Dry Milk in TTBS. Bound primary antibodies were detected with appropri-

ate secondary antibodies and enhanced chemiluminescence.

RT-PCR

Total RNA was isolated from quail heart or body using Qiagen RNeasy Midi Kit (catalog no. 74104) and quantified by ultraviolet spectrophotometry (Hitachi U-2000,AN0-0431). First-strand cDNA was synthesized with ProSTAR First-Strand RT-PCR Kit (Stratagene, 200420) using a calponin-specific reverse primer (ctgctgcttctctgcgtact). Using various isoforms and nonisoform specific primer combinations, RT-PCR reactions were conducted using a Biometra T-gradient Thermocycler PCR machine (050-801).

A "generic" h-1 calponin PCR primer set (forward primer, ctggcacagaaatacgaccc; reverse primer, ctgctgcttctctgcgtact) was used to amplify a ~380-bp fragment located in a region that α-h1 and β-h1 splice-variants of calponin have in common. An "α/β" PCR primer set (forward primer, gcacctctacgaccccaa; reverse primer, tgtaaaagctgtggttgaggc), spanning the alternatively spliced area, was used to identify presence of the respective isoforms, with anticipated size of amplified fragment of 310 bp for α-h1 calponin and 190 bp for β-h1 calponin. Furthermore, an "α-specific" primer set (forward primer, ggggaccaagaggcagat; reverse primer, tgtaaaagctgtggttgaggc) was designed to specifically recognize α-h1 calponin. Finally, three additional overlapping primer sets were used in the process to sequence the entire α-h1 mRNA. To sequence the first part of the mRNA a "5′-alpha" set (forward primer, acttaaccgatgtcgaacgcgaacttcaa; reverse primer, ctgctgcttctctgcgtact) was used, for the middle section a "middle-alpha" primer set (forward, tgcgggccatcaaacacta; reverse, agcttggggtcgtagaggt) and for the end a "3′-alpha" set (forward, gcacctctacgaccccaa; reverse, ttattgtgagttgtaaaagct).

Reaction products were separated on agarose gels, DNA was visualized with 0.01% ethidium bromide, and approximate base pair length of PCR products was determined by comparing migration distance with 1-kb Plus DNA ladder (GibcoBRL). Amplified fragments were isolated from the gel, purified using the Qiagen QIAquick Gel Extraction Kit (catalog no. 28704), and sequenced.

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