Cellular Distribution of Smooth Muscle Actins during Mammalian Embryogenesis: Expression of the α-Vascular but not the γ-Enteric Isoform in Differentiating Striated Myocytes

N. M. Sawtell and J. L. Lessard
Childrens Hospital Research Foundation, Basic Science Research, Cincinnati, Ohio 45229

Abstract. The cellular distribution of the α-vascular and γ-enteric smooth muscle actin isoforms was analyzed in rat embryos from gestational day (gd) 8 through the first neonatal week by in situ antigen localization using isoactin specific monoclonal antibodies. The α-vascular actin isoform was first detected on gd 10 in discrete cells lining the embryonic vasculature. By gd 14, this isoform was also present in the inner layers of mesenchymal cells condensing around the developing airways and gut. The γ-enteric actin, however, was not detected until gd 15 when cells surrounding the developing aorta, airways, and gut labeled with the γ-enteric-specific probe. There was continued expression of these two actin isoforms in regions of developing smooth muscle through the remainder of gestation and first neonatal week at which time their distribution coincided with that found in the adult. In addition to developing smooth muscle, the α-vascular actin isoform was expressed in differentiating striated muscle cells. On gd 10, there was intense labeling with the α-vascular specific probe in developing myocardocytes and, within 24 h, in somitic myotomal cells. Although significant levels of this smooth muscle actin were present in striated myocytes through gd 17, by the end of the first postnatal week, α-vascular actin was no longer detectable in either cardiac or skeletal muscle. Thus, the normal developmental sequence of striated muscle cells includes the transient expression of the α-vascular smooth muscle actin isoform. In contrast, the γ-enteric smooth muscle actin was not detected at any time in embryonic striated muscle. The differential timing of appearance and distribution of these two smooth muscle isoforms indicates that their expression is independently regulated during development.

Actin is a protein fundamental to contractility and as such is a major component of the organized contractile apparatus of muscle cells (20). In mammals, muscle actin is comprised of a family of four distinct protein isoforms. Two of these isoforms, the α-cardiac and α-skeletal, are restricted to striated muscle, while the remaining two, the α-vascular and γ-enteric, are expressed in smooth muscle cells (26, 27). Although each of these isoforms has been identified as the predominant steady state actin in the corresponding mature muscle type (28), coexpression of the α-skeletal and α-cardiac actin mRNAs occurs in the normal developmental program of differentiating cardiac and skeletal myocytes in both mammals and aves (2, 9, 10, 14, 16, 17, 18). Expression of the smooth muscle actin isoforms during development, however, is not well defined because specific probes for these proteins and mRNAs have only recently become available (5, 12, 15, 24). Ruzicka and Schwartz (21) have examined early chick embryos for the expression of α-actin gene transcripts and have reported that α-vascular smooth muscle actin mRNA is the first muscle α-actin transcript detected in developing avian heart. However, evidence for translation of this mRNA in these developing cardiomyocytes was not provided. Woodcock-Mitchell, et al. (31) have shown that the α-vascular smooth muscle isoform is present in striated myocytes of late gestational (gestational day [gd] 18) rat embryos. However, earlier time points were not examined. Moreover, no information is available concerning γ-enteric actin gene expression in striated muscle.

To address these questions and to extend the observations of Ruzicka and Schwartz (21) and Woodcock-Mitchell, et al. (31), we have examined rat embryos from gd 8 through the first neonatal week for the appearance and distribution of both the α-vascular and γ-enteric smooth muscle actin isoforms. Using monoclonal antibody probes selective for these two isoforms, we have determined that the normal developmental sequence of striated muscle includes the transient synthesis of the α-vascular actin. This protein appears early in differentiating cardiac and skeletal myocytes and is present for a significant portion of in utero development. In contrast, although evident in developing smooth muscle cells, the γ-enteric muscle actin isoform was not detected in either striated muscle type at any time during development.

1. Abbreviation used in this paper: gd, gestational day.
This report delineates the expression of both smooth muscle actin isoforms during development. In addition, we document the specific incorporation of the α-vascular smooth muscle actin isoform into the sarcomeres of developing striated muscle cells in vivo.

Materials and Methods

Antibody Specificity

The actin isoform specific antibodies used in this study have been characterized extensively by ELISA assay, Western blot analysis, and immunohistochemical procedures as previously described (12, 22, 24) and their specificities are summarized in Fig. 1. mAb HUC 1-1 recognizes an epitope present on all the mammalian muscle actins that is located between residues 360-369 (J. L. Lessard, unpublished results). SM-1 recognizes an epitope contained within the first 10 amino terminal residues of the α-vascular smooth muscle isoform (24). The precise epitope for mAb B4 remains to be defined; however, removal of the first 13 amino acids of gizzard actin eliminates the binding of this antibody indicating that the amino terminal region of the actin molecule is important to the B4 epitope (J. L. Lessard, unpublished results). The affinity of mAb B4 is more than 50-fold greater for gizzard actin (γ-enteric smooth muscle isoform) than for the α-cardiac, α-skeletal, or α-vascular muscle actins and this mAb shows no binding to the cytoplasmic isoforms (12). Because of the large difference in the binding affinity of this antibody to the γ-enteric relative to the α-muscle actin isoforms, mAb B4, when used at the appropriate dilution, is selective for the γ-enteric smooth muscle isoform (22).

Immunohistochemistry

Timed pregnant rats (Sprague-Dawley, Harlan, Inc., Indianapolis, IN) were anesthetized with chloroform, decapitated, and the uterine horns were surgically removed and placed in PBS on ice. On gd 8-10, regions of implantation were identified, and the embryos in utero were fixed for 4 h in a solution of 6% mercuric chloride and 0.5 M sodium acetate containing 4% paraformaldehyde that was added just before use. At later time points, embryos were removed from the uterus before fixation. After fixation, embryos were dehydrated in a graded series of alcohols, embedded in paraplast, and serially sectioned at 4 μm.

Selected sections were deparaffinized in xylene, cleared in alcohol, rehydrated in PBS and placed for 5 min in Gram's iodine solution (0.01 M iodine, 0.036 M potassium iodide). After washing in distilled water, sections were placed in 0.01 M sodium thiosulfate for 5 min, washed in distilled water, and then in PBS. Endogenous peroxidase activity was eliminated by placing sections in a methanol bath containing 0.05% hydrogen peroxide for 10 min. After rinsing in PBS, nonspecific protein interactions were blocked by incubating sections for 30 min in PBS containing 5% nonfat dry milk, 0.5% NP-40, 0.03% sodium azide. Sections were rinsed thoroughly in PBS, and proteins of interest were detected using the specific probes in a three-step avidin-biotin system as described (11). Peroxidase was used as the enzyme reporter molecule.

The optimum dilution of mAb SM-1 was previously determined to be 1:10,000 of an ascites fluid stock. mAb HUC 1-1 was used at a concentration of 4 μg/ml (diluted from a purified IgG stock) and mAb B4 at 10 μg/ml as was P3X63, a nonimmune myeloma IgG that was used as a control antibody. The probes were layered onto serial sections of embryos, and incubated at room temperature for 30 min, rinsed in PBS and incubated for 30 min with a biotinylated horse anti-mouse IgG (Vector Laboratories, Inc., Burlingame, CA) that had previously been absorbed with normal rat serum. Sections were again rinsed, and then incubated for 30 min with the avidin-peroxidase conjugate that was prepared according to the method of Wilson, et al. (30). Sites of avidin-peroxidase deposition were detected by placing slides in a solution of 0.05% diaminobenzidine and 0.002% hydrogen peroxide in Tris, pH 7.6, for 10 min. The color reaction was enhanced by incubation in a solution of 2% copper sulfate for 5 min. After rinsing in distilled water, sections were counterstained with methyl green, dehydrated in xylene, and mounted using permount. Stained sections were viewed and photographed (model HB 42 camera with PM-10AK photomicrographic system; Olympus Corporation of America, New Hyde Park, NY; T-MAX 100 ASA film; Eastman Kodak Co., Rochester, NY).

Results

Smooth Muscle

In gd 10 embryos, intense labeling with HUC 1-1 (not shown) and SM-1, but not B4, was present in cells lining the developing vasculature (Fig. 2, a and b). Muscle actins were not detected in regions of developing smooth muscle other than the vasculature until gd 14, when the inner layer of mesenchyme condensing around the developing lung and gut stained with both HUC 1-1 and SM-1. Again, at this time point there was no detectable staining with mAb B4; i.e., slides incubated with this mAb were indistinguishable from negative control slides. On gd 15, there was an increase in the number of cells labeling with mAbs HUC 1-1 (not shown) and SM-1 in the thickening layer of differentiating smooth muscle around the aorta, airways, and gut (Figs. 2–4). Staining with mAb B4, now detectable in some of these cells, exhibited a proximal to distal gradient with strongest labeling in the airways and proximal intestinal loops (Figs. 2–4). Regions of developing smooth muscle continued to show increased staining throughout gestation with all three mAbs with the exception of the smaller vasculature that remained negative with mAb B4 (Figs. 2–4). Corresponding negative controls consisting of serial sections incubated with P3X63 immunoglobulin as the primary antibody showed no staining in these embryos as well as all other embryos examined in this study (not shown).

Cardiac Development

Cardiac development in the rat occurs rapidly between gd 9 and 11, progressing from the primordial cardiac mesenchyme to a four chambered beating “heart” (3). No staining with the muscle actin-specific mAbs was detected in embryos before gd 10. However, in gd 10 embryos, mAbs HUC 1-1 and SM-1 intensely stained the layer of cells comprising the myocardium of the developing heart while mAb B4 did not stain these cells. In late gd 10 embryos, the atrium, ventricle, and sinus venosus can be identified (Fig. 5), and intense labeling with both mAbs HUC 1-1 and SM-1 was present in all the cells comprising the myocardium of the atrial and ventricular walls (Fig. 5, a and b). In contrast, although most cells forming the sinus venosus stained with HUC 1-1,
Figure 2. Immunoperoxidase staining with mAbs SM-1 (a-c) and B4 (d-f) showing embryonic vasculature on gd 10 (a and d), gd 15 (b and e), and gd 17 (c and f). Arrowheads indicate smaller vessels branching from the aorta (c and f) that label intensely with SM-1 but not B4. Bar, 100 μm.

only a subpopulation of these showed detectable SM-1 staining. Although the α-vascular smooth muscle isoform was present in significant amounts in the developing myocardium as indicated by the strong immunoreactivity with mAb SM-1, no staining was detected in these cells with the γ-enteric smooth muscle specific mAb, B4 (Fig. 5 c).

Staining with mAbs HUC I-1 and SM-1 continued to be present in developing atrial and ventricular myocardia as shown in Fig. 6 (a and b) of gd 13 heart. However, sections through different regions of the heart at this gestational time point demonstrated that the intensity of SM-1 staining among cardiomyocytes was no longer homogeneous. Although very intense uniform staining was present in the walls of the bulbus arteriosus (which gives rise to the aortic and pulmonary trunk)(l), not all cardiomyocytes within the thickening ventricular wall contained equal SM-1 labeling (data not shown). As observed at earlier time points, there was no detectable staining in the heart with mAb B4 (Fig. 6 c).

By gd 17, there was a marked decrease in SM-1 staining in the inner layers of the ventricular myocardium (Fig. 7, b and e), in contrast to the uniform intensity of HUC I-1 staining in this region (Fig. 7, a and d). The atrial myocardium, however, continued to show strong labeling with both SM-1 and HUC I-1. Again, there was no detectable labeling with mAb B4, although at this time developing smooth muscle cells in the lung, aorta, and gut showed strong staining with this mAb (Fig. 7, c, f, and g).

Skeletal Muscle Development

Skeletal muscle is derived either from the myotomal region of the paired somites (neck, body wall, limb) or from mesenchyme of the branchial arches (head and, in part, neck)(l). Between gds 10 and 11, myotomal cells elongate giving rise to myoblasts (4). The appearance and distribution of staining with mAbs HUC I-1, SM-1, and B4 in developing skeletal muscle cells was similar to that seen in cardiomyocytes. The relative timing of the appearance of HUC I-1 and SM-1 staining, however, was different. Immunoperoxidase labeling with mAb HUC I-1 was first detected in the myotomal region of the developing somites on gd 10, at which time there was no detectable staining with either mAb SM-1 or B4 (Fig. 8, S.
Figure 5. Immunoperoxidase staining with mAbs HUC 1-1 (a), SM-1 (b), and B4 (c) of rat embryo at gd 10 cut in cross section through the heart. Ventricular myocardium is indicated by arrows. Note that in contrast to mAb HUC1-1 (a), SM-1 stains only a subset of cells forming the sinus venosus (b) (arrowheads). NT, neural tube; DA, dorsal aorta; ACV, anterior cardinal vein; FG, foregut; SV, sinus venosus; RV, right ventricle; LV, left ventricle; PC, pericardial cavity; S, somite. Bar, 75 μm.

It was not until early gd 11 that labeling of myotomal cells with mAb SM-1 first became evident, while staining with B4 remained negative (Fig. 8, d and f).

Developing skeletal muscle in somites in gd 13 embryos continued to show staining with HUC 1-1 and SM-1 but not B4 (Fig. 9, a–c). By gd 17, extensive migration of somitic myotomal cells has occurred in the embryo, and muscles of the head and neck derived from branchial arch mesenchyme

Figure 6. Immunoperoxidase staining of gd 13 embryonic rat heart (para-sagittal section) with mAbs HUC 1-1 (a), SM-1 (b), and B4 (c). V, ventricle; A, atrium. Bar, 80 μm.
are forming. All of these groups of developing skeletal muscle showed strong labeling with mAbs SM-1 and HUC 1-1 while staining with mAb B4 remained negative (Fig. 10).

In developing cardiac and skeletal muscle, a striated pattern of staining was apparent with both SM-1 (Fig. 11) and HUC 1-1 (not shown). Already by gd 10, many cardiomyocytes contained α-vascular smooth muscle actin in regularly aligned units as evidenced by the cross-striated pattern of SM-1 staining (Fig. 11 a). Striations in somitic myotomal cells, however, were not apparent until around gd 12 (not shown). The time of appearance of striations was also variable among the skeletal muscle groups forming in the embryo. Those arising proximal to the somites, such as the body wall muscles, exhibited well-developed cross-striations by gd 15, while those arising distant from somites, such as the digital muscles, did not show a striated staining pattern with either HUC 1-1 or SM-1 until after gd 17 (Fig. 11, b and c).

By the end of the first neonatal week, labeling of striated myofibers with mAb SM-1 was no longer detectable in either cardiac or skeletal muscle. At this time, the tissue distribution of staining with all three mAbs was the same as that found in the adult animal as exemplified in sections of normal adult rat myocardium (Fig. 12). mAb HUC 1-1 stains all the striated myocytes as well as the smooth muscle cells within the walls of the vasculature (Fig. 12 a). In contrast, the two smooth muscle actin specific mAbs, SM-1 and B4, label the smooth muscle cells surrounding the larger vessels but do not stain the striated muscle fibers (Fig. 12, b and c). In addition to the larger vessels, mAb SM-1 stains many smaller diameter vessels that show no immunoreactivity with mAb B4, reflecting variations in the distribution of the α-vascular and γ-enteric smooth muscle isoforms within the vascular tree. The labeling specificities demonstrated by these mAbs in adult rat tissues and shown here in myocardium are consistent with the previously reported specificities of these mAbs demonstrated by Western blot analysis of tissue homogenates (22).

Discussion

Our understanding of the adult tissue distribution of the isoactins has come primarily from analytical biochemical studies (peptide mapping and amino acid sequencing) of actins extracted from the various tissues (26, 27, 28, 29). Such an approach is largely impractical, if not impossible, in the early embryo. Furthermore, to obtain detailed information about cellular expression during early embryogenesis, in situ detection methods are necessary. Because the amino acid sequences of the four muscle actins vary at most in 8 out of 375 residues (27), obtaining isoform specific antibodies has been difficult and until recently, none have been available. Differences in the 3' untranslated regions of the mRNAs en-

Figure 7. Immunoperoxidase staining of gd 17 embryonic rat heart (para-sagittal section) with mAbs HUC 1-1 (a), SM-1 (b), and B4 (c). Higher magnification of ventricular wall stained with HUC 1-1 (d) and SM-1 (e). Arrowheads indicate cardiac trabeculae; asterisks indicate inner ventricular myocardocytes; arrows indicate the smooth muscle developing around pulmonary airways. A, atria; V, ventricle. Bar, (a–c) 200 μm; (d–e), 60 μm.
Figure 8. Immunoperoxidase staining with mAbs HUC 1-1 (a and b), SM-1 (c and d), and B4 (e and f) of gd 10 (a, c, and e) and gd 11 (b, d, and f) embryonic rat somites. Arrows indicate myotomal region of somites. Bar, 5 μm.

Coding these actins, however, have proven to be useful in delineating the transcriptional activation of the muscle actin genes during development. Using this approach, it has been demonstrated by several investigators that the coexpression of α-cardiac and α-skeletal transcripts occurs during the differentiation of striated muscle cells (9, 10, 14, 16, 17). Ruzicka and Schwartz (21) have recently shown using in situ hybridization that there is a sequential activation of the α-actin genes during early avian cardiogenesis. Surprisingly, in the heart, α-vascular transcripts precede those for the α-cardiac and α-skeletal actins. This information is clearly important to our understanding of the transcriptional regulation of the actin genes, however, the question remained regarding the translation of these smooth muscle actin mRNAs. Although the presence of the α-vascular smooth muscle isoform in isolated gd 18 rat heart and leg muscle has recently been reported (31), a comprehensive examination of the appearance and distribution of this isoform during early and late gestation was lacking. Furthermore, neither study addressed the issue of γ-enteric smooth muscle actin gene expression.

In this report, we have demonstrated that unlike striated muscle cells in the adult mammal, embryonic cardiac and skeletal myocytes synthesize significant amounts of α-vascular actin during in utero differentiation. In contrast, the γ-enteric smooth muscle isoform, although expressed in developing smooth muscle cells, is not detected in developing striated myocytes. These results are summarized in Fig. 13.

Transcripts encoding α-vascular actin were first detected in developing cardiac tissue in Hamburger-Hamilton stage 9 (7 somites) chick embryos (21). This is the approximate number of somites in a rat embryo early on gd 10 (3, 23), which is the time that we first detected the α-vascular smooth muscle actin in developing cardiomyocytes. Similarly, there is a good correlation in the somite number of chick and rat embryos at the time of the appearance in myotomal cells of actin transcripts in the chick and protein in the rat. The α-vascular actin mRNA was first detected in HH stage 14 (22 somites) chick embryos (21) and the α-vascular actin in gd 11 rat embryos (20–27 somites). Although we first observed α-vascular actin in the rat embryo early on gd 10, additional time
points spanning gds 8 and 9 must be examined to determine the precise stage of cardiogenesis at which the $\alpha$-vascular actin is first detectable.

By Hamburger-Hamilton stage 12 (2–2.5 gds) (8), mRNA for the smooth muscle actin was no longer detected in developing chick ventricular myocardial cells but was restricted to the conus (region of aortic and pulmonary trunk)(21). Thus, $\alpha$-vascular transcripts were detectable in the myocardium for only 16 h early in chick heart development. In contrast, although regional variations in the intensity of labeling were evident, the protein was detectable in developing rat cardiac and skeletal muscle cells until shortly after birth. This suggested that either developing chick and rat striated muscle differed markedly with respect to the duration of $\alpha$-vascular actin expression, or that detectable levels of the protein were present considerably longer than the corresponding mRNA. To address this, we examined cardiac and skeletal muscle in gd 8 chick embryos and found that the $\alpha$-vascular actin isoform was still present in these striated muscle cells (not shown). Thus, in the chick, it appears that a brief window of transcriptional activity of the $\alpha$-vascular actin gene results in the presence of mRNA for 16 h and the corresponding protein in these cells for more than 6 d, consistent with the reported 6–8 d half-life of actin (32). Further studies will be required to determine if a similar burst in transcriptional activity of the $\alpha$-vascular actin gene occurs early in the developmental program of striated muscle cells in the rat. Woodcock-Mitchell, et al. (31) have reported low levels of $\alpha$-vascular actin mRNAs in northern blots of total cellular mRNA extracts from gd 18 rat heart. In the absence of in situ examination, however, it is not clear from these data whether this mRNA is actually present in myocardiocytes or confined to the smooth muscle cells within the cardiac vasculature.

In contrast to chick heart, Ruzicka and Schwartz (21) detected transcripts in somites for $\alpha$-cardiac actin before those for $\alpha$-vascular actin. Consistent with this, HUC 1-1 immunoreactivity was detectable in rat somites before that of SM-1, indicating that expression of the $\alpha$-vascular isoform was preceded by that of another HUC 1-1 immunoreactive actin isoform. Since B4 immunoreactivity was also absent, HUC 1-1 staining does not reflect the presence of the $\gamma$-enteric
muscle actin, and thus suggests the presence of one or both of the striated isoforms (α-cardiac and α-skeletal). mAb HUC 1-1, however, also recognizes unique actins in the brush border of intestinal epithelial cells (22), and, therefore, it is possible that early HUC 1-1 immunoreactivity in the somites represents expression of these actins. The development of additional actin isoform specific probes will be necessary to determine which of these actins are reflected by early HUC 1-1 immunoreactivity in the somites.

In gd 10 embryos, we consistently observed a subset of cells within the sinus venosus which strongly labeled with HUC 1-1 but not SM-1 (Fig. 2). Since a detailed temporal examination of these cells was not performed, we do not know whether or not these same cells go on to express the α-vascular smooth muscle isoform at a later time. However, it would appear that they do since we did not observe SM-1 negative cells in this area of the heart at more advanced developmental stages. This regionally restricted heterogeneity of α-vascular actin expression on gd 10 suggests that the developmental program of actin isoform expression is not the same among all cardiomyocytes. The fact that cells from the sinus venosus eventually contribute to the sino-atrial node (cardiac pacemaker) (1, 6), raises the interesting possibility that these cells represent a population of specialized cardiomyocytes.

The biological significance of the expression of this smooth muscle actin during the development of striated muscle is not clear. The fact that the expression of this isoform is coincident with early myofibril formation and, further, that it is incorporated into forming sarcomeres throughout in utero development suggests that it may be important in establishing or modulating the degree of sarcomere organization and contractility. It has been demonstrated that the sarcomeric structure of embryonic striated muscle cells is significantly less organized than in mature muscle (7). There are also distinct physiological differences in the contractile properties of embryonic striated muscle myocytes that are, at the least, temporally linked to the differences in sarcomere organization (19). Although there is no evidence that the structure of the thin (actin) filaments per se varies in embryonic and adult striated myocytes, differences in the arrangement of these filaments in the sarcomeres of developing skeletal muscle cells have been reported (25). What differences in sarcomere organization or function, if any, result from the presence of the α-vascular actin have yet to be determined. It is of interest that the period in which cardiomyocytes contain the α-vascular smooth muscle actin (early cardiogenesis through 1 wk postnatal) is also the time in which these cells are characterized by relatively disorganized sarcomeres of low force generating potential (7, 19). In addition, the apparent gradual decrease in the levels of smooth muscle actin coincides with the gradual increase in organizational rigidity of the sarcomeres (7).

The α-vascular and γ-enteric actins are generally coexpressed in the smooth muscle of adult mammals although the relative amount of each isoactin varies with the type of smooth muscle (28). In the rat embryo, we found that many developing smooth muscle cells also coexpressed the two smooth muscle actins. The timing of expression, however, was sequential: the appearance of the α-vascular isoform preceding that of the γ-enteric. In contrast, the transient expression of smooth muscle actin in developing striated muscle cells was exclusively the α-vascular smooth muscle isoform, the γ-enteric actin isoform was not detected at any developmental time point studied. As with any negative result, we cannot exclude the possibility that the γ-enteric isoform is present at levels below the detection limit of our assay. In addition, although unlikely, it is possible that the B4 epitope is selectively masked in developing striated muscle cells. However, the timing of the appearance of the protein...
demonstrated by our in situ analysis corresponds to the appearance of the specific mRNA detected by Northern blot analyses of trisected rat embryo homogenates. In these studies, γ-enteric actin mRNA was not detected on gd 15 (15). On gd 16 very low amounts were detected which increased to modest levels on gd 17 (McHugh and Lessard, unpublished results). However, time points before gd 15 have not been examined, and thus, the possibility of early transient transcriptional activity of the γ-enteric actin gene cannot be eliminated. Examination of the developing striated muscle cells for γ-enteric smooth muscle actin transcripts throughout gestation will be necessary to determine whether the absence of detectable levels of protein results from the lack of transcriptional activation of the γ-enteric actin gene or a regulation of translational activity.

These studies have demonstrated that the normal developmental program of striated muscle cells includes the expression of the α-vascular smooth muscle actin. This smooth muscle isoform is present in both cardiac and skeletal myocytes in significant amounts during most of gestational development, and it is incorporated into sarcomeres. In contrast, the γ-enteric smooth muscle actin is not detectable in these cells, indicating that the two smooth muscle actins are regulated differently in developing striated muscle.

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