UvA-DARE (Digital Academic Repository)

Expression of the smooth-muscle proteins alpha-smooth-muscle actin and calponin, and of the intermediate filament protein desmin are parameters of cardiomyocyte maturation in the prenatal rat heart

Ya, J.; Markman, M.W.M.; Wagenaar, G.T.M.; Blommaart, P.J.E.

DOI

[10.1002/\(SICI\)1097-0185\(199712\)249:4<495::AID-AR9>3.0.CO;2-Q](https://doi.org/10.1002/(SICI)1097-0185(199712)249:4<495::AID-AR9>3.0.CO;2-Q)

Publication date

1997

Published in

The Anatomical Record

[Link to publication](#)

Citation for published version (APA):

Ya, J., Markman, M. W. M., Wagenaar, G. T. M., & Blommaart, P. J. E. (1997). Expression of the smooth-muscle proteins alpha-smooth-muscle actin and calponin, and of the intermediate filament protein desmin are parameters of cardiomyocyte maturation in the prenatal rat heart. *The Anatomical Record*, 249, 495-505. [https://doi.org/10.1002/\(SICI\)1097-0185\(199712\)249:4<495::AID-AR9>3.0.CO;2-Q](https://doi.org/10.1002/(SICI)1097-0185(199712)249:4<495::AID-AR9>3.0.CO;2-Q)

General rights

It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations

If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: <https://uba.uva.nl/en/contact>, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

UvA-DARE is a service provided by the library of the University of Amsterdam (<https://dare.uva.nl>)

Expression of the Smooth-Muscle Proteins α -Smooth-Muscle Actin and Calponin, and of the Intermediate Filament Protein Desmin are Parameters of Cardiomyocyte Maturation in the Prenatal Rat Heart

JING YA, MARRY W.M. MARKMAN, GERRY T.M. WAGENAAR,
PIET-JAN B. BLOMMAART, ANTOON F.M. MOORMAN, AND WOUTER H. LAMERS*

*Department of Anatomy and Embryology, University of Amsterdam,
Academic Medical Center, Amsterdam, The Netherlands*

ABSTRACT *Background:* Coexpression of α - and β -myosin heavy chain (MHC) is a characteristic of the primary myocardial tube. To establish if the smooth-muscle proteins α -smooth-muscle actin (α -SMA) and calponin, and the intermediate filament protein, desmin, contribute to the specific functional properties of these early cardiomyocytes, we studied their spatiotemporal expression pattern.

Methods: Sections of prenatal and neonatal Wistar rats were stained with antibodies against α - and β -MHC, α -SMA, calponin, and desmin.

Results: The expression of α -SMA and calponin in embryonic cardiomyocytes increases to reach its highest level at ED14. Subsequently, these proteins gradually disappear, beginning in the interventricular septum (IVS) and followed successively by the compact myocardium of the left ventricle, the right ventricle, and the central atrium. Expression of α -SMA persists longer in the ventricular conduction system, making it a convenient marker for the ventricular conduction system of the fetal rat. Desmin becomes expressed one day later than α -SMA, but also reaches its peak at ED14, suggesting that a relatively high concentration is required to form mature sarcomeres.

Conclusions: The results indicate that α -SMA, calponin, and desmin are involved in the myofibrillar development in rat heart. The presence of spatiotemporal differences in the expression of these proteins reveals regional differences in the developmental timing of cardiomyocyte maturation. The maturation process extends from the compact myocardium in the IVS to the left and right ventricular free walls, whereas the atrioventricular junction, the ventricular trabeculae, and developing ventricular conduction system show a relatively slow maturation. Smooth-muscle proteins may contribute to the slow shortening speed that is characteristic of the embryonic myocardium. *Anat. Rec.* 249:495–505, 1997.

© 1997 Wiley-Liss, Inc.

Key words: heart development; α -smooth-muscle-action; calponin; desmin

During embryonic development, myocardial function changes continuously, concomitant with a complex remodelling of the heart (see Moorman and Lamers, 1994, for a recent review). Heart development starts with the formation of a slowly conducting (Kamino, 1991) and peristaltoid-contracting (Patten and Kramer, 1933) single myocardial tube. Subsequently, fast-conducting, rapidly contracting atrial and ventricular chambers develop within the confines of the still slowly conducting primary myocardial heart tube (de Jong et al., 1992). As a result, five segments can be distinguished in such hearts, the slowly conducting inflow tract, the fast-conducting atrium, the slowly conducting atrioventricular canal, the fast-conducting ventricle, and the

slowly conducting outflow tract (Moorman and Lamers, 1994). Differences in conduction velocity in the consecutive segments of the embryonic heart tube explain to a large extent how the embryonic heart can function without valves and without a conduction system (de Jong et al., 1992).

This schematic summary of the developmental formation of functional segments within the embryonic heart

*Correspondence to: Wouter H. Lamers, Department of Anatomy and Embryology, University of Amsterdam, Academic Medical Center, Meibergdreef 15, 1105 AZ, Amsterdam, The Netherlands.

Received 30 September 1996; Accepted 19 March 1997

is based on the heterogeneous distribution of proteins involved in energy metabolism, contraction, and conduction (Moorman et al., 1995; Hasselbaink et al., 1990; Lamers et al., 1989; van Kempen et al., 1991; de Jong et al., 1987, 1990, 1992; de Groot et al., 1989; Wessels et al., 1991; Lyons, 1994). However, it is well known from electron microscopic studies that the structural maturation of the myocytes of the atrium and ventricle continues long after these segments have become identifiable. Nevertheless, regional differences within these segments relating to the level of maturation have attracted less attention. The temporary expression of the thin-filament protein α -smooth-muscle actin (α -SMA) in the cardiomyocytes of the embryonic heart of rat and chicken (Sugi and Lough, 1992; Ruzicka and Schwartz, 1988; Woodcock-Mitchell et al., 1988; Sawtell and Lessard, 1989), therefore, prompted us to investigate whether the coexpression of α - and β -myosin heavy chain (MHC) that is typical of the earliest phases of cardiomyocyte development is correlated with the expression of smooth-muscle proteins, and, if so, what this combination of phenotypic markers reveals about cardiomyocyte development. In this study, we investigated the spatiotemporal pattern of expression of α -SMA and the thin filament-binding proteins calponin and caldesmon (Shirinsky et al., 1992; Haeberle and Hemric, 1994; Gerthoffer and Pohl, 1994), and observed, indeed, a correlation in the expression of α -SMA and calponin, the troponin I/T-like molecule of smooth muscle, with the coexpression of both MHCs. Based on the electron microscopic data available in the literature, we argue that α -SMA expression is a very useful light microscope marker to follow the transition from the random arrangement of myofibrils in the embryonic heart to the highly ordered organization in the fetal heart; that is to say, to follow the myofibrillar maturation in the prenatal rat heart.

MATERIALS AND METHODS

Animals

Wistar rats were obtained from the HSD animal farm, Zeist, The Netherlands. The animals were kept in a controlled dark-light cycle (light, 7 a.m. to 7 p.m.), and had access to a standard chow diet (RMH 1410, Hope Farms, Woerden, The Netherlands) and water ad libitum. Animal welfare was in accordance with institutional guidelines of the University of Amsterdam. Females in estrus were selected as described (Lamers et al., 1987) and mated. The time of mating was regarded as the beginning (day 0) of gestation. Pregnant rats were decapitated after CO_2/O_2 anaesthesia at 9–20 days postcoitum and a total of 40 embryos were studied: four on the 9th day of pregnancy (ED9), two on ED10, five on ED11, four on ED12, five on ED13, five on ED14, three on ED15, five on ED16, two on ED18, three on ED19, and two on ED20. The embryos of ED9 and ED10 were left within the uterus. The hearts of the 7-day neonates ($n = 3$) were dissected. The specimens were fixed in an ice-cold mixture of methanol:acetone:water (2:2:1 v/v) for 4 hr and left in the same fixative at room temperature overnight. Hereafter, they were dehydrated in a graded series of ethanol and embedded in Paraplast plus (Oxford Labwarf, St. Louis, MO). Serial

sections of 7 μm thickness were cut and mounted on poly-L-lysine-coated slides and stored at 4°C until use.

Immunohistochemistry

After deparaffination, the sections were pretreated sequentially with 3% (v/v) H_2O_2 in phosphate-buffered saline (PBS, pH 7.4) for 30 min and TENG-T (10 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.25% gelatin, 0.05% Tween-20, pH 8.0) for 15 min to reduce endogenous peroxidase activity and non-specific antibody binding, respectively. The pretreated serial sections were incubated overnight with alternately monoclonal antibodies against α -myosin heavy chain (α -MHC, 1:10 [de Groot et al., 1989]), β -myosin heavy chain (β -MHC, 1:10 [Sant'Ana Pereira et al., 1995]), α -smooth-muscle actin (α -SMA, 1:1,000; Sigma IMM-2), desmin (1:50; Monosan, Mon 3001, Clone 33), calponin (1:500 and 1:1,000; Sigma C2687), and caldesmon (1:150 and 1:300; Sigma C4562), followed by consecutive incubations with rabbit-antimouse IgG (1:7,500; non-commercial), goat-antirabbit IgG (1:250; non-commercial), and rabbit peroxidase-antiperoxidase complex (1:750; Nordic, The Netherlands). Antibodies were diluted in PBS. All incubations were followed by three washes in PBS for 5 min each. Antibody binding was demonstrated by staining with 3,3'-diaminobenzidine tetrahydrochloride (Sigma) and 3 mM H_2O_2 . After staining, the sections were taken rapidly through graded ethanols, cleared in xylene, and mounted in Entellan (Merck, Darmstadt, Germany).

Western Blotting

Protein extracts were prepared in 4% sodium dodecylsulphate (SDS) from 21, 13, and 6 hearts of ED11, ED14, and ED20 embryos, respectively, as well as from adult rat aorta. Aliquots of the extracts equivalent to 5 μg of protein were electrophoresed for α -SMA and aliquots equivalent to 10 μg of protein for desmin, calponin, and caldesmon on denaturing 10% polyacrylamide gels. The proteins were blotted onto PVDF membranes (Immobilon-P, Millipore), using the BioRad Minitransblot. A prestained SDS molecular weight standard mixture (SDS-7B, Sigma) was electrophoresed and transferred simultaneously to enable identification of protein bands. After washing with the TBST buffer (10 mM Tris-HCl, 0.15 M NaCl, 0.5% Tween-20, pH 8) and treatment with the blocking buffer (5% nonfat dried milk powder in TBST buffer), the membranes were incubated with the same dilution of the antibodies against α -SMA, desmin, calponin, and caldesmon as used for immunohistochemistry. Primary antibody binding was detected using rabbit-antimouse-peroxidase-antiperoxidase (1:1,000) complex. The immuno-complex formed was demonstrated by incubation of the membranes for 5–10 min with 0.5 mg/ml 3,3'-diaminobenzidine and 9 mM H_2O_2 in PBS. The reaction was stopped by rinsing the membranes with distilled water.

RESULTS

Expression of α - and β -Myosin Heavy Chains

The expression patterns of α - and β -myosin heavy chains (MHC) in the developing rat heart are well established (Lompre et al., 1981; de Groot et al., 1989). However, to facilitate the delineation of the myocar-

TABLE 1. Spatio-temporal pattern of expression of α -SMA

Age	OFT	VT(R)	VC(R)	IVS	Atrium	SAN	PM	SCV
ED10	±	++	++	NP	+	±	NP	NP
ED11	++	++	++	NP	++	++	NP	+
ED12	++	++	++	++	++	++	NP	++
ED13	+++	+++	+++	+	+++	+++	++	++
ED14	+++	+++	+++	+	+++	+++	++	++
ED16	++	++	+	±	++	++	++	++
ED19	+	+	±	—	++	++	+++	++
ED20	±	+	±	—	+	±	++	+

Abbreviations: ED: embryonic day; OFT: outflow tract; VT(R/L): trabeculae of (right/left) ventricle; VC(R/L): compact myocardium of (right/left) ventricle; IVS: interventricular septum; SAN: sinoatrial node; PM: pulmonary myocardium; SCV: superior caval vein. NP: not present. Increasing intensity of staining is reflected in the number of plus signs.

dium, the expression patterns of α - and β -MHC were also studied in the present investigation (not shown).

Expression of α -Smooth-Muscle Actin (Table 1)

α -SMA is expressed in the myocytes of the ED9 myocardium (Fig. 1A). As in the case of α -MHC, the staining of the myocytes in this early stage is most intense in their periphery (Fig. 1, panels A–D). From ED9 to ED14, α -SMA gradually accumulates in all segments of the heart. Immunostaining of α -SMA can be detected throughout the tubular heart of ED10, but the intensity of staining is higher in the ventricle (Fig. 1B–D) than in the developing outflow tract (Fig. 1B). As was observed for both MHCs, α -SMA expression in the outflow tract does not extend all the way to the bifurcation into the branchial arteries at ED10 (Fig. 1B), but does so at ED11 (Fig. 1E). In the inflow tract, its expression is concentrated near the cells that also express α -MHC (Fig. 1F). Expression of α -SMA in the wall of the arteries becomes visible from ED11 onward (starting at the bifurcation of the aortic sac into the branchial arteries; Fig. 1E,G) and in the skeletal muscle from ED13 onward (Fig. 1H,I).

The expression of α -SMA in cardiomyocytes is transient, starting to decline in the interventricular septum from ED12 onward. In other parts of the ventricles, such a decline is seen from ED16, i.e., 3–4 days later (Fig. 1H–J). At ED16, staining is still strong in the appendages of both embryonic atria, in the atrioventricular canal and the adjacent part of the atrial wall, and in the ventricular trabeculae (Fig. 1J). Between ED16 and 7 days after birth, the staining intensity in these areas gradually declines so that by 7 days after birth, strong expression is restricted to the ventricular rim of the right atrium (Figs. 1K, 5A). Interestingly, the ventricular conduction system, including the His bundle and bundle branches, also retains a strong expression of α -SMA during this period (Figs. 1K, 5A–B), making α -SMA a very convenient marker for this structure. Concomitant with the cellular decline of α -SMA in most parts of the myocardium, the staining of α -SMA in the walls of the great arteries becomes very intense (Figs. 1J, 5B).

Expression of Calponin and Caldesmon (Table 2)

The staining intensity of calponin is substantially weaker than that of α -MHC, β -MHC, or α -SMA. Its

staining becomes detectable at ED11 and is most intense at ED14. Thereafter, its cellular level declines, becoming undetectable after ED16. In ED11 and ED12 hearts, a relatively high staining intensity is found in the outflow tract, with other parts of the heart showing a weaker staining (Fig. 2A–C). The staining intensity of calponin in ED13 and ED14 hearts increases further when compared with that in ED12, being more pronounced in the outflow tract and the ventricular trabeculae than in the atrioventricular canal, atria, or sinus venosus (Fig. 2D). The level of expression of caldesmon is so low that specific staining could not be established.

Expression of Desmin (Table 3)

The expression of desmin cannot be detected in the ED9 myocardium (Fig. 4A). In the ventricular and atrial myocytes of the ED10 heart, desmin is found mainly in the periphery of the cells, leaving most of the cytoplasm desmin-negative (Fig. 4B–D). Furthermore, the most intense staining of desmin in the myocytes of the outflow tract is initially confined to the cell surface facing the cardiac jelly (Fig. 4B). In contrast to the expression of both MHCs and α -SMA, however, the desmin-expressing cells in the arterial pole of the heart are found to extend all the way up to the bifurcation of the aortic sac into the branchial arteries. Between ED10 and ED13, the expression of desmin increases greatly in all parts of the heart (Fig. 4E–G). At ED11, desmin expression in the downstream arterial pole of the tubular heart has spread over the entire outflow tract into the adjacent pericardium (Fig. 4E), whereas in the upstream venous pole of the heart, desmin expression is seen in the mesenchyme surrounding the sinus venosus and the superior caval veins (Fig. 3F).

Starting at ED14, i.e., two days later than α -SMA, desmin staining begins to decline in the interventricular septum (Fig. 4I; cf. Fig. 1G). From ED15 onwards, the staining intensity of desmin in the myocytes of the compact myocardium also starts to decline, but the cellular content of desmin remains relatively high in the atrium and ventricular trabeculae (Fig. 4J,K). In the 7-day neonate, the slightly stronger staining of desmin in the His bundle and its branches allows one to distinguish the conducting cells from the working myocytes (Fig. 5C,D), although α -SMA is clearly a superior marker.

Expression of α -MHC, Desmin, and α -SMA in the Pulmonary Myocardium

The development of the pulmonary vein is described as a separate paragraph because the progressive formation of myocardium along its wall (the “pulmonary myocardium”) provides an exceptionally nice illustration of the temporal sequence of expression of muscle proteins during cardiomyocyte differentiation. Myocardium around the developing pulmonary vein can be detected in the dorsal wall of the left atrium from ED13 onward. In the ED14 embryo, expression of α -SMA and desmin is seen to reach for a short distance beyond the first bifurcation of the pulmonary vein, but its staining intensity tapers off (Fig. 4A,C), whereas α -MHC expression has only just reached this bifurcation (Fig. 4B). During subsequent days, expression of α -SMA in the wall of the pulmonary vein of the embryos is found to

Fig. 1. Expression pattern of α -SMA in transverse sections of embryonic rat hearts at ED9 (A), ED10 (B–D), ED11 (E, F), ED12 (G), ED13 (H), ED14 (I), ED16 (J) and ED20 (K). Note that myocytes enveloping the heart tube express α -SMA at ED9 (A), but that expression does not reach the arterial pole of the heart before ED11 (cf. B, E). Furthermore, the staining intensity in the ventricle of the ED10 heart (C) is higher than that in either the outflow tract (B) or the inflow tract (D). Whereas the overall staining intensity increases up to ED13 (H), it begins to decline in the interventricular septum from ED12 onward (G) and has virtually disappeared there at ED14 (I). Only then, α -SMA begins to disappear from the compact myocardium

of the left ventricular free wall (J), the right ventricular free wall, and the trabeculae (K). Expression is maintained longer in the atria, in particular in the appendages and atrioventricular canal, but also in the ventricular conduction system (J, K, arrows). Expression of α -SMA in the arterial wall is first seen at ED11 and becomes gradually stronger toward ED16 (J, ap: appendage of atrium). oft: outflow tract; v: ventricle; rv: right ventricle; lv: left ventricle; ivs: interventricular septum; a: atrium; ra: right atrium; la: left atrium; sv: sinus venosus; ccv: common cardinal vein; cs: coronary sinus; pv: pulmonary vein; ts: transverse septum. In A, B, C, D, E, and F, bars = 100 μ m. In G, H, and I, bars = 200 μ m. In J and K, scale bars = 400 μ m.

TABLE 2. Spatio-temporal pattern of expression of calponin

Age	OFT	VT	VC	IVS	Atrium	SAN
ED10	±	±	±	NP	±	±
ED11	++	+	+	NP	+	+
ED12	++	++	++	++	++	++
ED13	+++	+++	++	++	++	++
ED14	+++	+++	++	++	++	++
ED16	+	+	+	+	+	+
ED19	+	±	±	—	±	±
ED20	±	±	±	—	±	±

Abbreviations: ED: embryonic day; OFT: outflow tract; VT(R/L): trabeculae of (right/left) ventricle; VC(R/L): compact myocardium of (right/left) ventricle; IVS: interventricular septum; SAN: sinoatrial node; PM: pulmonary myocardium; SCV: superior caval vein.

NP: not present. Increasing intensity of staining is reflected in number of plus signs.

extend at least one bifurcation further into the lung than that of either α -MHC or desmin (Fig. 4D–F). This situation persists until birth and shows that α -SMA becomes expressed in the developing cardiomyocytes around the pulmonary vein before α -MHC and desmin.

Western Blotting

To substantiate our interpretation that the expression of α -SMA, desmin, and calponin in the developing heart is transient, with the highest cellular content being present at approximately ED14, we have analyzed extracts of embryonic hearts by Western blotting. The bands that were visualized are of the expected size: 42 Kd for α -SMA, 55 Kd for desmin, and 44 Kd for calponin. The results of this analysis demonstrate that the content of α -SMA, desmin, and calponin in total protein extracts of ED14 hearts is higher than that in extracts of ED11 or ED20 hearts (Fig. 6A–C). Even though we could demonstrate the presence of caldesmon as a specific band of 120 Kd in an extract of the aortic wall of an adult rat (not shown), we could not visualize such a band in extracts of embryonic hearts.

DISCUSSION

The structural organization of cardiomyocytes is considered to be (still) immature at birth, in particular in murine species (Manasek, 1970; Hirakow et al., 1980; Challice and Virágh, 1973; Virágh and Challice, 1973). The development toward a more mature myocardial organization coincides with a decline in the mitotic growth of the myocardium (Rumyantsev, 1977). This coincidence is probably not accidental, because mitotic divisions of cardiomyocytes are accompanied by a temporary dissolution of myofibrillar structure (Rumyantsev, 1977). It is, therefore, of interest that the transient expression of typical smooth-muscle proteins and the peak in the expression of the intermediate filament protein desmin in the embryonic heart coincide with the developmental period during which poorly organized sarcomeres of low force-generating potential are present (Manasek, 1970; Challice and Virágh, 1973), the rate of proliferation of the myocardium is highest (Rumyantsev, 1977; Cluzeaut and Maurer-Schultze, 1986; Thompson et al., 1990), and a major remodelling takes place of the architecture of the heart that leads to its septation into a right and left side. Although it is

evident that the proper development of cardiac muscle requires a carefully programmed appearance of muscle-specific proteins, the implications of the early expression of the intermediate filament-protein desmin and the typical smooth-muscle proteins α -SMA and calponin for myocardial structure and function are not yet clear. Nevertheless, the spatiotemporal pattern of expression of the contractile proteins sheds light on three aspects of myocardial development: the myocardialization of the arterial and venous poles of the heart, the transition of the embryonic into the definitive myocardium, and regional differences in the occurrence of this phenomenon.

The Myocardialization of the Arterial and the Venous Poles of the Heart

The first myocardium to develop forms the future ventricle, from where myocardialization progresses toward both the outflow and the inflow tract (De la Cruz et al., 1989; de Jong et al., 1987). In the rat, this process is completed on ED11 in the outflow tract (panel E in Figs. 1 and 4; panel A in Fig. 2) and on ED12 in the sinus venosus (Laan et al., 1989). Myocardial expansion along the outflow tract and sinus venosus can be followed temporally by the typical coexpression of α - and β -MHCs (de Jong et al., 1990, 1987; de Groot et al., 1989; Sweeney et al., 1987). We observed that the appearance of α -SMA at both poles of the heart coincides with that of the MHCs. The available data (see also Miano and Olson, 1996) show that the expression of calponin follows that of α -SMA, but its cellular concentration in relation to the avidity of the available antibody does not allow a very sensitive detection. On the other hand, it was observed that the appearance of α -SMA consistently preceded the expression of α -MHC in the pulmonary myocardium during its progressive prenatal expansion from the left atrium into the lungs (Fig. 4A–F [Kramer and Marks, 1965]). Findings in chicken embryos, in which expression of α -SMA is confined to the earliest stages of cardiogenesis only, further supports the observed relation between myocardialization and the expression of α -SMA, as expression of α -SMA in this species persists longest in the outflow and inflow tract (Sugi and Lough, 1992; Ruzicka and Schwartz, 1988), that is, in the areas with ongoing formation of myocardium (De la Cruz et al., 1989; de Jong et al., 1987). Cardiac muscle shares the property of temporally expressing α -SMA with skeletal muscle (Fig. 1H–I [Sawtell and Lessard, 1989]). Taken together, these data suggest that the expression of smooth-muscle proteins accompanies the early phases of muscle formation.

In contrast to α -SMA and MHCs, desmin expression is not observed in the initial stages of heart development (Theiler's stage 12, Fig. 4A), even though the surrounding myometrium of the uterus shows strong staining of desmin. However, the expression of desmin is strongly induced during the subsequent day, not only within the myocardium but also well beyond the cranial and caudal boundary (Fig. 2). This finding corresponds with that of other groups (Schaart et al., 1989; Baldwin et al., 1991; van der Loop et al., 1992) and indicates that desmin expression is not a prerequisite for myocardial differentiation. This conclusion is corroborated by the finding that the commitment and early differentiation

Fig. 2. Expression pattern of calponin in embryonic rat hearts of ED11–ED14. Panels A–C are adjacent serial sections of panels E, G, and I in Figures 1 and 4, respectively. In ED11 (A) and ED12 hearts

(B), the expression is stronger in the outflow tract than in other parts of the heart. At ED14 (C), the expression of calponin is stronger in the outflow tract and the ventricular trabeculae. Scale bar = 200 μ m

TABLE 3. Spatio-temporal pattern of expression of desmin

Age	OFT	VT(R)	VC(R)	IVS	VT(L)	VC(L)	AVC	Atrium	SAN	PM	SCV
ED10	+	+	+	NP	+	++	+	$\pm$	$\pm$	NP	NP
ED11	++	++	++	NP	++	++	+	+	+	NP	+
ED12	+++	+++	+++	++	+++	+++	++	++	++	NP	++
ED13	+++	+++	+++	++	+++	+++	+++	+++	+++	++	++
ED14	+++	+++	+++	++	+++	+++	+++	+++	+++	++	++
ED16	+++	+++	+++	+	+++	++	+++	+++	+++	++	++
ED19	+++	+++	++	+	+++	++	+++	+++	+++	+++	+++
ED20	++	+++	++	+	+++	++	+++	++	+++	+++	+++

Abbreviations: ED: embryonic day; OFT: outflow tract; VT(R/L): trabeculae of (right/left) ventricle; VC(R/L): compact myocardium of (right/left) ventricle; IVS: interventricular septum; SAN: sinoatrial node; PM: pulmonary myocardium; SCV: superior caval vein.

NP: not present. Increasing intensity of staining is reflected in number of plus signs.

of the cardiomyocytes is not affected after targeted disruption of both desmin alleles by homologous recombination (Weitzer et al., 1995; Li et al., 1996). However, as soon as the cardiomyocyte has differentiated, a quick increase in desmin expression appears to be a prerequisite for further maturation of the contractile apparatus of the cardiomyocytes (Fuseler and Shay, 1982; Osinska and Lemanski, 1989; Li et al., 1996; Weitzer et al., 1995).

The Transition of the Embryonic Into the Definitive Myocardium

Electron microscopic studies (Chacko, 1976; Virágh and Challice, 1973; Challice and Virágh, 1973; Hirakow et al., 1980) have shown that the accumulation of sarcomeres proceeds only gradually in the prenatal rodent heart. The temporal expression of smooth-muscle proteins and desmin, which peak at ED14 (Fig. 6), suggests that these proteins play a role in myofibril formation. The formation of the sarcomeres starts with the appearance of thin filaments, followed by the formation of Z-bands (Manasek, 1968; Myklebust et al., 1978). These structures appear to be the templates for the assembly of thick filaments and, hence, sarcomeres

(Legato, 1970, 1973; Myklebust et al., 1978; Chacko, 1976). In mature myocytes, desmin is found in close association with the Z lines of the sarcomeres and extends between the Z lines of adjacent myofibrils. It functions as a three-dimensional matrix by interconnecting individual myofibrils to one another and to the plasma membrane at the level of their Z lines (Osinska and Lemanski, 1989; Fuseler and Shay, 1982). Desmin in the initially diffusely organized Z-band material is therefore thought to play an important role in the lateral organization and registration of the randomly distributed myofibrils of embryonic cardiac and skeletal myocytes into the contractile myofibrillar apparatus (Osinska and Lemanski, 1989; Fuseler and Shay, 1982). In accordance with this model, both desmin and developing sarcomeres are initially found in the periphery of the cardiomyocytes (Figs. 1, 4, panels A–D) (Rash et al., 1970; Legato, 1970; Markwald, 1973). Reduced desmin expression interferes with the maintenance and performance of newly formed cardiomyocytes and results in an instability of the contractile apparatus (Weitzer et al., 1995). The increase in myocardial desmin content between ED11 and ED14 indicates that

Fig. 3. Expression patterns of desmin in embryonic rat hearts between ED9 and ED20. The respective panels are adjacent serial sections of those shown in Figure 1. Note that the myocytes of the ED9 heart do not yet show expression of desmin. In the ED10 heart, desmin expression in the arterial pole extends up to the bifurcation of the aortic sac into the branchial arteries, i.e., further than that of α -MHCs and α -SMA (B). Furthermore, its expression is still relatively weak,

with its distribution confined to the cell surface facing the cardiac jelly. Between ED11 (E, F) and ED13 (H), its expression greatly increases in all parts of the heart. Subsequently, its expression declines, but to a lesser extent in the atria than in the ventricles (I-K). As for α -SMA, the decline starts in the interventricular septum, extends first toward the left ventricular free wall and then to the right ventricular free wall, and only then to the trabeculae.

a relatively high concentration of desmin is required to form the mature Z-bands and sarcomeres.

Coexpression of both MHC isoforms in the myocytes of early embryonic heart and the incorporation of α -SMA into the thin filaments of the sarcomere (Ordahl, 1986) implies that these proteins may actually determine the contractile properties, including the

shortening speed, of the embryonic myocardium. The presence of smooth-muscle-like properties in the sarcomeres of the embryonic heart is further supported by the presence of calponin, a troponin I/T-like molecule of smooth muscle that binds the thin filament of the sarcomere and that regulates the sliding of these filaments along the thick filament in a Ca^{2+} -dependent

Fig. 4. Expression of desmin (A, D), α -MHC (B, E) and α -SMA (C, F) in the developing pulmonary myocardium at ED14 (A-C) and ED16 (D-F). Panels A-C and D-F, respectively, are serial sections. Note that the peripheral expansion of the expression of α -SMA and desmin precedes that of α -MHC at ED14 at the first bifurcation of the lung

vein (A-C), whereas at ED16, α -SMA expression extends further than that of either α -MHC or desmin at the third bifurcation (D-F). la: left atrium; pv and arrows: pulmonary vein. b: bronchus. Panels A-C, scale bar = 100 μ m; panels D-F, scale bar = 200 μ m, have the same magnification.

manner (Shirinsky et al., 1992; Haeberle and Hemric, 1994). After ED14, the coexpression of α - and β -MHC disappears and the expression of calponin and α -SMA begins to decline (Figs. 1I-K, 2). In mature cardiomyocytes, the regulation of cardiomyocyte contraction is carried out by the troponin-tropomyosin complex (Kendrick-Jones and Scholey, 1981). Western blot analysis has shown that the adult isoforms of troponin I and T, the functional equivalents of caldesmon and calponin, do not appear until ED16 in the embryonic rat heart (Sabry and Dhoot, 1989a,b). Although it is well established that α -cardiac and, to a lesser extent, α -skeletal actins are also expressed in the embryonic heart (Lyons, 1994), these data clearly suggest that typical smooth-muscle proteins play an important role as regulators of embryonic heart contraction. This inference is underscored by the previous conclusion that myocardium exhibiting coexpression of α - and β -MHC is generally characterized by a peristaltoid contraction pattern with a slow shortening speed of the myocytes (de Jong et al., 1992). In fact, the expression of the smooth-muscle proteins probably contributes to this functional property.

Regional Differences in the Timing of the Transition of the Embryonic Into the Definitive Myocardium

Detailed observations on the development of well-structured myofibrils in individual cardiomyocytes may easily obscure regional differences in the timing of this process. We have previously shown that the disappearance of β -MHC from the atrium and α -MHC from the ventricle are parameters that delineate the myocar-

dium of these respective compartments from the less well-differentiated remaining primary myocardium in the flanking segments, which continue to coexpress α - and β -MHC (de Jong et al., 1987, 1990; de Groot et al., 1989; Wessels et al., 1991; Sweeney et al., 1987). The transient expression of smooth-muscle proteins, in particular the intensely expressed α -SMA, in all cardiomyocytes makes it a very suitable single marker protein to survey the maturation of the myocardium. The interventricular septum is by far the first structure to discontinue expressing α -SMA and, hence, to develop more definitive structural characteristics, followed after 3-4 days (ED16) by the developing compact myocardium of the left and right ventricles and the central part of the atria, while the atrioventricular junctional myocardium and the ventricular trabeculae, including the developing ventricular conduction system, the appendages of the atria, and the developing pulmonary myocardium are slowest. A similar developmental pattern of α -SMA expression in developing rat heart can be observed in the study of Jackson et al. (1995), in which this marker was used to delineate the myocardium. The spatiotemporal pattern of the disappearance of α -SMA from the myocardium mirrors that of the disappearance of coexpression of α - and β -MHC, except that β -MHC disappears slightly faster from the atrium than α -MHC from the ventricle and that the pulmonary myocardium never shows expression of β -MHC. In aggregate, these data strongly suggest the presence of major regional differences in the timing of cardiomyocyte maturation. In particular, the relatively early maturation of the compact myocardium in the muscular ventricular sep-

Fig. 5. Expression of α -SMA (A, B) and desmin (C, D) in the atrial and ventricular junction of the heart of a 7-day old neonatal rat. Panels A and C and panels B and D, respectively, are serial sections. At one week after birth, α -SMA is still expressed abundantly in the myocardium of the right atrioventricular junction and in the ventricu-

lar conduction system (arrow). Desmin is expressed in all cardiomyocytes, with a slightly stronger expression in the ventricular conduction system (arrow) than in the working myocytes. ra: right atrium; ao: aorta. Panels A-D have the same magnification; scale bar = 400 μ m.

Fig. 6. Western blot analysis of the cardiac content of α -SMA (A; Mw: 42Kd), desmin (B; Mw: 55Kd), and calponin (C; Mw: 44Kd) in total protein extracts of ED11 (lane 1), ED14 (lane 2), and ED20 (lane 3) embryonic hearts. Note that the cardiac content of all three proteins reaches a maximum at ED14.

tum, followed by that of the left free wall and only thereafter that of the right free wall, are striking features. In addition, the relatively slow maturation of the right atrioventricular junction and the ventricular trabeculae, including the developing ventricular conduction system, is a notable feature. Furthermore, the right atrioventricular junction and the ventricular conduction system share their lineage from the interven-tricular myocardium that, in the human embryo, not only expresses the neural marker GLN2 (HNK) (Lamers et al., 1992; Wessels et al., 1992), but also co-expresses α - and β -MHC (Wessels et al., 1991). These observations fit very well with the frequent suggestion that the myofibrillar organization of the ventricular conduction system retains "primitive" characteristics (Canale et al., 1986; Virágh et al., 1987; Virágh and Challice, 1973, and references therein). These findings make α -SMA expression an excellent marker for the ventricular conduction system of the rat heart in the fetal and neonatal period.

LITERATURE CITED

- Baldwin, H.S., K.L. Jensen, and M. Solursh 1991 Myogenic differentiation of the precardiac mesoderm in the rat. *Differentiation*, 47:163–172.
- Canale, E.D., G.R. Campbell, J.J. Smolich, and J.H. Campbell 1986 *Cardiac Muscle*. Springer Verlag, Berlin.
- Chacko, K.J. 1976 Observations on the ultrastructure of developing myocardium of rat embryos. *J. Morphol.*, 150:681–710.
- Challice, C.E., and S. Virágh 1973 The architectural development of the early mammalian heart. *Tissue Cell*, 6:447–462.
- Cluzeaut, F., and B. Maurer-Schultze 1986 Proliferation of cardiomyocytes and interstitial cells in the cardiac muscle of the mouse during pre- and postnatal development. *Cell Tissue Kinet.*, 19:267–274.
- de Groot, I.J.M., W.H. Lamers, and A.F.M. Moorman 1989 Isomyosin expression pattern during rat heart morphogenesis: An immunohistochemical study. *Anat. Rec.*, 224:365–373.
- de Jong, F., W.J.C. Geerts, W.H. Lamers, J.A. Los, and A.F.M. Moorman 1987 Isomyosin expression patterns in tubular stages of chicken heart development: A 3-D immunohistochemical analysis. *Anat. Embryol.*, 177:81–90.
- de Jong, F., W.J.C. Geerts, W.H. Lamers, J.A. Los, and A.F.M. Moorman 1990 Isomyosin expression pattern during formation of the tubular chicken heart: A 3D immunohistochemical analysis. *Anat. Rec.*, 226:213–227.
- de Jong, F., T. Opthof, A.A.M. Wilde, M.J. Janse, R. Charles, W.H. Lamers, and A.F.M. Moorman 1992 Persisting zones of slow impulse conduction in developing chicken hearts. *Circ. Res.*, 71:240–250.
- De la Cruz, M.V., C. Sánchez-Gómez, and M. Palomino 1989 The primitive cardiac regions in the straight tube heart (stage 9) and their anatomical expression in the mature heart: An experimental study in the chick embryo. *J. Anat.*, 165:121–131.
- Fuseler, J.W., and J.W. Shay 1982 The association of desmin with the developing myofibrils of cultured embryonic rat heart myocytes. *Dev. Biol.*, 91:448–457.
- Gerthoffer, W.T., and J. Pohl 1994 Caldesmon and calponin phosphorylation in regulation of smooth muscle contraction. *Can. J. Physiol. Pharmacol.*, 72:1410–1414.
- Haerberle, J.R., and M.E. Hemric 1994 A model for the coregulation of smooth muscle actomyosin by caldesmon, calponin, tropomyosin, and the myosin regulatory light chain. *Can. J. Physiol. Pharmacol.*, 72:1400–1409.
- Hasselbaink, H.D.J., W.T. Labruyère, A.F.M. Moorman, and W.H. Lamers 1990 Creatine kinase isozyme expression in prenatal rat heart. *Anat. Embryol.*, 182:195–203.
- Hirakow, R., T. Gotoh, and T. Watanabe 1980 Quantitative studies on the ultrastructural differentiation and growth of mammalian cardiac muscle cells. I. The atria and ventricles of the rat. *Acta Anat.*, 108:144–152.
- Jackson, M., M.G. Connell, A. Smith, and R.H. Anderson 1995 Immunohistochemical evaluation of the developing outflow tract in the rat: Achieving aortic to mitral fibrous continuity. *Cardiovasc. Res.*, 30:262–269.
- Kamino, K. 1991 Optical approaches to ontogeny of electrical activity and related functional organization during early heart development. *Physiol. Rev.*, 71:53–91.
- Kendrick-Jones, J., and J.M. Scholey 1981 Myosin-linked regulatory systems. *J. Muscle Res. Cell Motil.*, 2:347–372.
- Kramer, A.W. Jr., and L.S. Marks 1965 The occurrence of cardiac muscle in the pulmonary veins of rodentia. *J. Morphol.*, 117:135–150.
- Laan, A.C., W.H. Lamers, D.P. Huijsmans, A. te Kortschot, J. Smith, J. Strackee, and J.A. Los 1989 Deformation-corrected computer-aided 3D-reconstruction of immunohistochemically stained organs: Application to the rat heart during organogenesis. *Anat. Rec.*, 224:443–457.
- Lamers, W.H., A. te Kortschot, A.F.M. Moorman, and J.A. Los 1987 Acetylcholinesterase in prenatal rat heart: A marker for the early development of the cardiac conductive tissue? *Anat. Rec.*, 217:361–370.
- Lamers, W.H., W.J.C. Geerts, A.F.M. Moorman, and R.P. Dottin 1989 Creatine kinase isozyme expression in embryonic chicken heart. *Anat. Embryol.*, 179:387–393.
- Lamers, W.H., A. Wessels, F.J. Verbeek, A.F.M. Moorman, S. Virágh, A.C.G. Wenink, A.C. Gittenberger-de Groot, and R.H. Anderson 1992 New findings concerning ventricular septation in the human heart — their implications for maldevelopment. *Circulation*, 86:1194–1205.
- Legato, M.J. 1970 Sarcomerogenesis in human myocardium. *J. Mol. Cell. Cardiol.*, 1:425–437.
- Legato, M.J. 1973 The role of Z-substance in sarcomerogenesis. *J. Mol. Cell. Cardiol.*, 205–206.
- Li, Z., E. Colucci-Guyon, M. Pinçon-Raymond, M. Mericskay, S. Pournin, D. Paulin, and C. Babinet 1996 Cardiovascular lesions and skeletal myopathy in mice lacking desmin. *Dev. Biol.*, 175:362–366.
- Lompre, A.M., J.J. Mercadier, C. Wisnewsky, D. Bouveret, C. Pantaloni, A. D'Albis, and K. Schwartz 1981 Species- and age-dependent changes in the relative amounts of cardiac myosin isoenzymes in mammals. *Dev. Biol.*, 84:286–290.
- Lyons, G.E. 1994 In situ analysis of the cardiac muscle gene program during embryogenesis. *Trends Cardiovasc. Med.*, 4:70–77.
- Manasek, F.J. 1968 Embryonic development of the heart. I. A light and electron microscopic study of myocardial development in the early chick embryo. *J. Morphol.*, 125:329–366.
- Manasek, F.J. 1970 Histogenesis of the embryonic myocardium. *Am. J. Cardiol.*, 25:149–168.
- Markwald, R.R. 1973 Distribution and relationship of precursor Z material to organizing myofibrillar bundles in embryonic rat and hamster ventricular myocytes. *J. Mol. Cell. Cardiol.*, 5:341–350.
- Miano, J.M., E.N. Olson 1996 Expression of the smooth muscle cell calponin gene marks the early cardiac and smooth muscle cell lineages during mouse embryogenesis. *J. Biol. Chem.*, 271:7095–7103.
- Moorman, A.F.M., J.L.M. Vermeulen, M.U. Koban, K. Schwartz, W.H. Lamers, and K.R. Boheler 1995 Patterns of expression of sarcoplasmic reticulum Ca^{2+} -ATPase and phospholamban mRNAs during rat heart development. *Circ. Res.*, 76:616–625.
- Moorman, A.F.M., and W.H. Lamers 1994 Molecular anatomy of the developing heart. *Trends Cardiovasc. Med.*, 4:257–264.
- Myklebust, R., T.S. Soetersdal, H. Engedal, M. Ulstein, and S. Odegård 1978 Ultrastructural studies on the formation of myofilaments and myofibrils in the human embryonic and adult hypertrophied heart. *Anat. Embryol.*, 152:127–140.
- Ordahl, C.P. 1986 The skeletal and cardiac α -actin genes are coexpressed in early embryonic striated muscle. *Dev. Biol.*, 117:488–492.
- Osinska, H.E., and L.F. Lemanski 1989 Immunofluorescent localization of desmin and vimentin in developing cardiac muscle of syrian hamster. *Anat. Rec.*, 223:406–413.
- Patten, B.M., and T.C. Kramer 1933 The initiation of contraction in the embryonic chicken heart. *Am. J. Anat.*, 53:349–375.
- Rash, J.E., J.J. Bieseke, and G.O. Gey 1970 Three classes of filaments in cardiac differentiation. *J. Ultrastruct. Res.*, 33:408–435.
- Rumyantsev, P.P. 1977 Interrelations of the proliferation and differentiation processes during cardiac myogenesis and regeneration. *Int. Rev. Cytol.*, 51:187–273.
- Ruzicka, D.L., and R.J. Schwartz 1988 Sequential activation during avian cardiogenesis: Vascular smooth muscle α -actin gene transcripts mark the onset of cardiac differentiation. *J. Cell Biol.*, 107:2575–2585.
- Sabry, M.A., and G.K. Dhoot 1989a Identification of and changes in the expression of troponin T isoforms in developing avian and mammalian heart. *J. Mol. Cell. Cardiol.*, 21:85–91.

- Sabry, M.A., and G.K. Dhoot 1989b Identification and pattern of expression of a developmental isoform of troponin I in chicken and rat cardiac muscle. *J. Muscle Res. Cell Motil.*, 10:85–91.
- Sant'Ana Pereira, J.A.A., A. Wessels, L. Nytmans, A.F.M. Moorman, and A.J. Sargeant 1995 New method for the accurate characterization of single human skeletal muscle fibres demonstrates a relation between mATPase and MyHC expression in pure and hybrid fiber types. *J. Muscle Res. Cell Motil.*, 16:21–34.
- Sawtell, N.M., and J.L. Lessard 1989 Cellular distribution of smooth muscle actins during mammalian embryogenesis: Expression of the α -vascular but not the gamma-enteric isoform in differentiating striated myocytes. *J. Cell Biol.*, 109:2929–2937.
- Schaart, G., C. Viebahn, W. Langmann, and F.C.S. Ramaekers 1989 Desmin and titin expression in early postimplantation mouse embryos. *Development*, 107:585–596.
- Shirinsky, V.P., K.G. Biryukov, J.M. Hettasch, and J.R. Sellers 1992 Inhibition of the relative movement of actin and myosin by caldesmon and calponin. *J. Biol. Chem.*, 267:15886–15892.
- Sugi, Y., and Lough 1992 Onset of expression and regional deposition of alpha-smooth and sarcomeric actin during avian heart development. *Dev. Dyn.*, 193:116–124.
- Sweeney, L.J., R. Zak, and F.J. Manasek 1987 Transitions in cardiac isomyosin expression during differentiation of the embryonic chick heart. *Circ. Res.*, 61:287–295.
- Thompson, R.P., J.R. Lindroth, A.J. Alles, and A.R. Fazel 1990 Cell differentiation birthdates in the embryonic rat heart. *Ann. N. Y. Acad. Sci.*, 588:446–448.
- van der Loop, F.T.L., G. Schaart, W. Langmann, F.C.S. Ramaekers, and C. Viebahn 1992 Expression and organization of muscle specific proteins during the early developmental stages of the rabbit heart. *Anat. Embryol.*, 185:439–450.
- van Kempen, M.J.A., C. Fromaget, D. Gros, A.F.M. Moorman, and W.H. Lamers 1991 Spatial distribution of connexin-43, the major cardiac gap junction protein, in the developing and adult rat heart. *Circ. Res.*, 68:1638–1651.
- Virágh, S., and C.E. Challice 1973 The impulse generation and conduction system of the heart. In: *Ultrastructure of the Mammalian Heart*. C.E. Challice, S. Virágh, eds. Academic Press, New York, London, pp. 43–89.
- Virágh, S., M.E. Stoeckel, and A. Porte 1987 Light and electron microscopic structure of the cardiac Purkinje fibers — review. *Physiol. Boh.*, 36:233–242.
- Weitzer, G., D.J. Milner, J.U. Kim, A. Bradley, and Y. Capetanaki 1995 Cytoskeletal control of myogenesis: A desmin null mutation blocks the myogenic pathway during embryonic stem cell differentiation. *Dev. Biol.*, 172:422–439.
- Wessels, A., J.L.M. Vermeulen, S. Virágh, F. Kálmán, W.H. Lamers, and A.F.M. Moorman 1991 Spatial distribution of "tissue specific" antigens in the developing human heart and skeletal muscle. II. An immunohistochemical analysis of myosin heavy chain isoform expression patterns in the embryonic heart. *Anat. Rec.*, 229:355–368.
- Wessels, A., J.L.M. Vermeulen, F.J. Verbeek, S. Virágh, F. Kálmán, W.H. Lamers, and A.F.M. Moorman 1992 Spatial distribution of "tissue-specific" antigens in the developing human heart and skeletal muscle. III. An immunohistochemical analysis of the distribution of the neural tissue antigen G1N2 in the embryonic heart; implications for the development of the atrioventricular conduction system. *Anat. Rec.*, 232:97–111.
- Woodcock-Mitchell, J., J.J. Mitchell, R.B. Low, M. Kieny, P. Sengel, L. Rubbia, O. Skalli, B. Jackson, and G. Gabbiani 1988 α -smooth muscle actin is transiently expressed in embryonic rat cardiac and skeletal muscles. *Differentiation*, 39:161–166.