Myocardium formation after the initial development of the linear heart tube
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Cardiac muscle cell formation after development of the linear heart tube

Boudewijn P.T. Kruithof, Maurice J.B. van den Hoff, Andy Wessels, and Antoon F.M. Moorman

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ABSTRACT

After the development of the linear heart tube, additional myocardium is formed leading to the muscular mantle around the caval and pulmonary veins and the muscular septa in the embryonic heart. Here we report the results of our in vivo and in vitro studies of this late myocardium-generating process in the mouse. Using an immunohistochemical approach we determined that myocardium formation starts around embryonic day 12 in the dorsal mesocardium. In subsequent stages of development, the process extends downstream into the intra-cardiac mesenchymal tissues of the atrioventricular canal and outflow tract, and upstream into the extra-cardiac mediastinal mesenchyme embedding the pulmonary and caval veins. Given the spatiotemporal pattern of myocardium formation we applied a 3D in vitro explant culture assay to investigate the myocardium-generating potential of the different cardiac compartments. We determined that this potential is stage- and mesenchyme dependent. This latter finding suggests an important role for mesenchyme in myocardium formation after the development of the linear heart tube.
INTRODUCTION

The tubular heart differentiates from splanchnic mesoderm (Rosenquist and de Haan, 1966; Stalsberg and de Haan, 1969). As development proceeds, myocardium is added to the posterior side of the tube leading to the formation of the inflow region (Virág and Challice, 1973; Stalsberg and De Haan, 1969; De la Cruz and Markwald et al., 1998) and to the anterior side, contributing to the outflow tract in chicken (De la Cruz and Markwald et al., 1998; Mjaatvedt et al., 2001; Waldo et al., 2001) and to the outflow tract and possibly the embryonic right ventricle in mouse (Virág and Challice, 1973; Kelly et al., 2001). This muscularization process is characterized by a continuous differentiation of the mesothelial lining of the pericardial cavity into cardiomyocytes (Virág and Challice, 1973). At the venous pole, myocardium formation proceeds in the extra-cardiac mesenchyme, which lines the caval and pulmonary veins. This results in the formation of caval and pulmonary myocardium (Ya et al., 1997; Van den Hoff et al., 2001). In addition, myocardium formation takes place in the intra-cardiac mesenchyme that forms the outlet and atrioventricular septa (De Jong et al., 1997; Ya et al., 1998; Mjaatvedt et al., 1999; Van den Hoff et al., 1999, 2001; Moorman et al., 2000).

Thus, after formation of the linear heart tube, myocardium formation takes place in the intra-cardiac and extra-cardiac mesenchyme. Two mechanisms, i.e. myocardialization and recruitment might underlie this myocardium formation. Myocardialization refers to growth of existing cardiomyocytes into the mesenchyme. Recruitment refers to differentiation of non-cardiac muscle cells into cardiac muscle cells. This late type of myocardium formation shows interspecies differences, being more extensive in avian than in mammalian hearts (Lammers et al., 1995; De Jong et al., 1997; Van den Hoff et al., 1999, 2001; Moorman et al., 2000; Kim et al., 2001).

Recently, an in vitro explant culture assay was developed to analyze this late type of myocardium formation in the chicken (Van den Hoff et al., 1999, 2001). We reported that explants from the venous pole, atrioventricular canal, and outflow tract display spontaneous myocardium formation in vitro, provided the proper stages are cultured. However, working myocardium of the atrial appendages and ventricles does not show spontaneous myocardium formation. We also showed that early outflow tract or atrioventricular canal explants that by themselves do not spontaneously form myocardial networks, can be induced to do so by culturing them in conditioned medium derived from older explant cultures that do show spontaneous in vitro myocardial network formation. Conditioned medium of ventricular explants that do not spontaneously form myocardial networks cannot induce these early explants. This analysis suggested that a common signaling pathway is operational throughout the entire heart tube (Van den Hoff et al., 1999, 2001).
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Aberrant muscularization of the outlet septum has been observed in several mouse models of cardiac dysmorphogenesis, including transforming growth factor β2 deficient mice (Sanford et al., 1997; Bartram et al., 2001), trisomy 16 mice (Waller III et al., 2000), and neurofibromin-1 deficient mice (Braman et al., 1994). Analyzing the in vivo myocardium formation in these transgenic mice might facilitate the elucidation of the underlying molecular mechanisms. As a basis for future studies on these mechanisms we have performed a comprehensive in vivo and in vitro study in the developing mouse from E11 onwards. Using immunohistochemistry, we showed that after the development of the heart tube additional myocardium is formed leading to the smooth walled atrial myocardium, the myocardial atrioventricular septum, the myocardial outlet septum, and the pulmonary and caval myocardium. The in vivo explant culture assay revealed stage- and mesenchyme-dependent myocardial network formation potencies of the different compartments, suggesting an important role for mesenchyme in myocardium formation after development of the linear heart tube.

MATERIAL AND METHODS

Mouse embryos

Young female FVB mice (Brockman) that had not yet developed an oestrous cycle were superovulated by injection of 5 units of folligonan (Intervet) followed after 48 hours by injection of 5 units of chorulon (Intervet) and mated with male FVB mice. The next day the mice were inspected for vaginal plugs. This day was scored as day 1 of gestation.

Chicken embryos

Fertilized chicken eggs were obtained from a local hatchery (Drost BV, Nieuw Loosdrecht, the Netherlands), incubated at 39°C in a moist atmosphere, and automatically turned every hour. After the appropriate incubation times, embryos were isolated and staged according to Hamburger and Hamilton (1951).

Immunohistochemistry

Embryos were fixed in ice-cold Modified Amsterdam’s Fixative (40% methanol; 40% acetic; 20% water) for 4 hours, dehydrated in a graded alcohol series and embedded in paraplast. Serial 7 μm sections were prepared and mounted onto polylysine-coated slides. After deparaffination and hydration in a graded alcohol series, endogenous peroxidase activity was blocked using 3% H2O2 in phosphate buffered saline (PBS: 150 mmol/L.
Myocardium formation in mouse

NaCl and 10 mmol/L sodium phosphate, pH 7.4). Following a pretreatment for 30 minutes in TNG-T (10 mmol/L Tris, 5 mmol/L EDTA, 150 mmol/L NaCl, 0.25% (w/v) gelatin and 0.05% (v/v) Tween-20, pH 8.0) to reduce non-specific binding, the sections were incubated overnight with a polyclonal antibody against SIRCA2a (kindly provided by F. Wuytack, Leuven, Belgium; Figgeumont et al., 1990), a monoclonal antibody against Myosin Light Chain 2a (MLC2a; kindly provided by K. Chien, San Diego, USA; Kubalak et al., 1994), MLC2v (kindly provided by W. Franz, Lübeck, Germany; Katus et al., 1982) α- and β Myosin Heavy Chain (α- and βMHC; Wessels et al., 1991). Antibody binding was visualized using the indirect unconjugated peroxidase-antiperoxidase technique as described previously (Wessels et al., 1990).

In vitro explant culture assay: preparation of collagen gels

Collagen gels were prepared essentially according to procedures previously described (Van den Hoff et al., 1999). In short, the day prior to the use of the collagen gels, rat-tail collagen type I (Collaborative Research Inc.) was diluted to a final concentration of 1.5 mg/ml in M199 (Life Technologies) culture medium and distilled water on ice. Polymerization of the collagen was initiated by adding NaOH to a final concentration of 15 mmol/L. Directly after addition of NaOH, aliquots of approximately 500 μl of collagen solution were pipetted into the wells of 4-well or 24-well NUNC plates. Subsequently, the gels were placed in a 37°C tissue-culture incubator at 5% CO2 and allowed to polymerize. After 30 minutes, 500 μl M199 culture medium containing penicillin/streptomycin (Life Technologies), 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml selenium (ITS, Collaborative Research Inc.) and 2 mM glutamine (Life Technologies) was added to the gels. The medium was changed once after another 30 minutes. After an overnight incubation of the gels in a 5% CO2 incubator at 37°C, the medium was changed again.

In vitro explant culture assay: isolation, culturing and staining of explants

Embryonic hearts were isolated under sterile conditions in filter-sterilized Earl’s Balanced Salt Solution (EBBS, Life Technologies). The cardiac regions of interest were isolated, cut open longitudinally and positioned on top of the drained collagen gel with the endocardial surface facing the gel. Prior to the addition of complete medium M199 with 1% heat-inactivated mouse serum for the mouse explants or 1% chicken serum for the chicken explants with or without heparin (Sigma), the explants were allowed to attach to the gel for at least 4 hours. After a culture period of one week (37°C, 5% CO2), the gels containing the explants were rinsed with PBS and fixed by incubation for at least 60 minutes in 70% methanol. Next, the gels were hydrated in a graded methanol series,
permeabilized with 1% Triton-X100 (v/v), blocked with PBS, 5mmol/L EDTA, 150 mmol/L NaCl, 0.25% (w/v) gelatin) and incubated overnight with MF20, a mouse monoclonal antibody specific to myosin heavy chain. MF20 was used for this analysis because these analyses require large amount of antibodies, which are provided by the MF20 hybridoma-cell line (Hybridomabank, Iowa City, IA). After extensive washing in PBS, the gels were incubated in FITC labeled rabbit anti mouse serum (Nordic) for at least 3 hours. After extensive washing, the gels were stained with propidium iodide (Molecular probes) to identify all nuclei. After a rinse in PBS, the gels were analyzed by confocal laser scanning microscopy (Biorad MRC1024).

**In vitro explant culture assay: scoring system**

The extent of muscularization was scored on an arbitrary scale, with scores ranging from 0 to 2, using the following criteria. A score of “0” was assigned to explants in which no or very few small myocardial projections could be observed. Explants that were washed away during the immune procedure, but which prior to fixation appeared to be alive by displaying endocardial cell outgrowth and/or spontaneous beating, were considered to have no protrusions and were given a score “0”. When a few myocardial protrusions were observed, the explants received the score of “1”. A score of “2” was assigned to explants in which the myocardial protrusions showed locally or around the entire explant extensive myocardial networks. The score 0 is considered no muscularization; score 1 a little muscularization and score 2 extensive muscularization.

**RESULTS**

**In vivo myocardium formation**

Recently, we have described myocardium formation in the intra- and extra-cardiac mesenchyme in the chicken embryo (Van den Hoff et al., 1999, 2001). In this study we focus on this myocardium formation during mouse development from E11 onwards. For the **in vivo** description, a comprehensive immunohistochemical study was performed on sections of staged mouse embryos. Serial sections of each embryo were sequentially stained for sarcoplasmatic reticulum Ca$^{2+}$-ATPase (SERCA2a), myosin light chain (MLC) 2v, MLC2a, α-Myosin Heavy Chain (MHC) and βMHC delineating myocardial from non-myocardial cells. As each of the respective antibodies can be used as a suitable marker to delineate myocardium throughout the entire heart or in a compartment, we show in the analysis presented below the section that most clearly illustrates the phenomenon described.
Myocardium formation at the level of the atrioventricular canal and dorsal mesocardium (Figure 1)

Soon after its formation, the tubular heart is populated by mesenchyme, resulting in the formation of the outflow tract and atrioventricular cushions, the dorsal mesocardium with its protrusion into the atrial cavity, referred to as spina vestibuli or dorsal mesocardial protrusion, and the cap of the primary atrial septum. This cap is contiguous dorsally, via the mesenchyme of the spina vestibuli and the dorsal mesocardium, with the extra-cardiac mediastinal mesenchyme, and ventrally with the superior atrioventricular cushion (Fig. 1h). During subsequent development, this cap also becomes contiguous with the inferior atrioventricular cushion via the spina vestibuli (Fig. 1h). Upon caudal expansion of the primary atrial septum, its mesenchymal cap, the spina vestibuli and the atrioventricular cushions fuse (Wessels et al., 2000).

At E11, the myocardium of the dorsal mesocardium and the atrioventricular canal is in an epithelial context and no myocardial protrusions are observed in the flanking mesenchyme (not shown). At E12 (Fig. 1a), small myocardial protrusions are observed at the interface of the inferior atrioventricular cushion and the muscular ventricular septum. In the mesenchyme of the dorsal mesocardium and spina vestibuli cardiomyocytes are present that show a low level of SERCA2a expression (Fig. 1c and 3b). Cardiomyocytes are not found in the superior atrioventricular cushion or in the mesenchymal cap of the primary atrial septum (Fig. 1b). At E14, when the mesenchymal components have completely fused, myocardial protrusions, contiguous with the primary atrial septum, are observed into this mesenchyme (Fig. 1e). At this stage, the mesenchyme of the dorsal mesocardium and the base of the spina vestibuli have almost completely become myocardial (Fig. 1d). Myocardial protrusions that are contiguous with the ventricular septum, spina vestibuli, and right venous valve are observed in the mesenchyme of the inferior atrioventricular cushion (Fig. 1d). At E15, myocardial protrusions contiguous with the ventricular septum are hardly observed in this mesenchyme anymore (Fig. 1g). Myocardial protrusions contiguous with the primary atrial septum (Fig. 1g) and the dorsal wall of the atrioventricular canal (arrow in fig. 1i), however, are observed in the cushion mesenchyme. Since the mesenchyme of the spina vestibuli, and of the atrioventricular cushions are continuous, it is not possible to determine from these sections whether myocardial protrusions are present only in the mesenchyme of the spina vestibuli or also in the mesenchyme of the inferior atrioventricular cushion. In the spina vestibuli, a non-myocardial core remains that will become the tendon of Todaro (indicated with a star in fig. 1g and j; Webb et al., 1998). Ventrally, the initially mesenchymal cap of the primary atrial septum has become myocardial, forming a myocardial cap (Fig. 1f). Caudally to this myocardial cap, myocardium is present which is contiguous with the ventral wall of the atrioventricular myocardium (arrow in fig. 1f and circle in fig. 1i).
Figure 1
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The myocardium formation at the level of the atrioventricular canal is complete at E16 (Fig. 1k). The remaining mesenchyme persists as the central fibrous body (Fig. 1k), the tendon of Todaro, and the tricuspid and mitral valves. Interestingly, small myocardial protrusions are observed at E15 at the base of the tricuspid leaflets (Fig. II), but extensive myocardial networks are never observed during subsequent development.

Myocardium formation at the level of the outflow tract (Figure 2)

At E11, the cardiomyocytes lining the outflow tract cushions form a smooth interface between cushions and myocardium, and are in an epithelial context. Mesenchymal cells are populating the outflow tract cushions (arrow in fig. 2a). At E12, the cardiomyocytes that face the cushions start to lose their epithelial context (arrow in fig. 2b). At E13, myocardial spikes are visible into the mesenchyme of the cushions (arrow in fig. 2c). At E14, the mesenchymal outlet septum has formed by fusion of the outflow tract cushions.
and extensive muscularization is observed (Fig. 2d). Myocardial protrusions are not only found to be contiguous with the flanking lateral outflow tract myocardium, but also with the ventricular septum (arrowheads in fig. 2e). At E15, the myocardial protrusions have reached each other at the sides flanking the lumens of the ventricle and arterial pole leaving an enclosed mesenchymal area. During subsequent development this mesenchymal area becomes gradually muscular (not shown). At E17, the muscularization of the outlet septum is completed (Fig. 2f).

**Myocardium formation around the pulmonary vein (Figure 3)**

At E11, the pulmonary pit is surrounded by myocardium (Fig. 3a). When the spina vestibuli has formed, the origin of the pulmonary vein is directed to the left side (Fig. 3b). Although cardiomyocytes are present in the mesenchyme of the dorsal mesocardium, the wall of the pulmonary vein is not myocardial yet (Fig. 3c). At E14, the first extensive muscularization of the pulmonary vein is observed. This begins at the origin of the pulmonary vein in the atrium and proceeds into the direction of the lungs (Fig. 3d). At E15, the myocardium of the pulmonary vein has reached the third bifurcation in the lungs (Fig. 3e). This muscularization process is observed to continue till at least E17. At this stage, the walls of the pulmonary veins are myocardial up to the fifth bifurcation (Fig. 3f).

**Myocardium formation around the caval veins (Figure 4)**

Before E14, caval myocardium is not observed beyond the pericardial border in the mediastinal mesenchyme (arrow in fig. 4a). At E14, loosely arranged myocardial cells are located beyond the pericardial border near the left caval vein (arrow in fig. 4b). At E15, cardiomyocytes are present in the wall of the left superior caval vein (Fig. 4c). At E17, the part of the right superior caval vein flanking the pericardial cavity is myocardial. In the right inferior caval vein, the myocardium does not extend beyond the venous valves (arrows in fig. 4d).

**In vitro myocardium formation**

To address the question of how myocardium formation in the mouse is regulated, we developed an *in vitro* explant culture assay in which the process of myocardium formation after development of the linear heart tube can be mimicked and manipulated. This culture system is based on the explant assay that was used for chicken explants (Van den Hoff et al., 1999, 2001). To successfully culture mouse explants and visualize the cardiomyocytes, several modifications had to be made in the culture conditions and the immunohistochemical staining procedure (see Materials and Methods).
Myocardium formation in mouse

In vivo muscularization of the various cardiac compartments is most pronounced at E14. Therefore, the venous pole, atrioventricular canal, outflow tract, and the apex of the left ventricle of this stage were cultured for one week and inspected daily using Varel-modulation microscopy. The venous pole refers to the part of the developing heart that is upstream of the atrioventricular canal, which comprises the atrial appendages, primary atrial septum, dorsal mesocardium, spin vestibuli and the proximal parts of the caval and pulmonary veins. After overnight incubation of the explants an epithelial monolayer starts to form on top of the collagen matrix, which expands during the subsequent culture period. In the cultures of the venous pole, atrioventricular canal and outflow tract, upstream of the atrioventricular canal, which comprises the atrial appendages, primary

Figure 6. Developmental window of in vivo myocardium formation of the venous pole, atrioventricular canal, outflow tract and apex of the left ventricle of the mouse. The extent of myocardial network formation in 1.5 mg/l collagen gels by these tissues between E11 and E19 (x-axis) was assessed after a week in culture as described in Material and Methods. On the y-axis, the extent of the formed myocardial networks is expressed in arbitrary units. Each point in the graph is based on the analysis of a separate in vitro experiment.

Figure 5. Characteristic examples of the extent of cellular outgrowth of the different cardiac compartments. Micrographs of myocardial explants derived of the venous pole of E13 (Panels a and d), atrioventricular canal of E12 (Panels b and e) and E14 (Panels c and f), outflow tract of E15 (Panels g and h), and of left ventricle of E15 ( Panels i, k-o) after a week of culture are shown. Panels a-c, g and h show Varel modulation micrographs. Panels d-f, i-o show brightest point projections of CLSM stacks of the explants after immunofluorescent staining of the myocytes using the monoclonal antibody MF20. Scale bar: 200 μm. For panels m-o scale bar is 400 μm. The boxed areas in panels i are magnified in panels l and m. Panels b and e show an example of an explant, which does not show myocardial protrusions. To illustrate that not all cells in the collagen matrix stain with MF20, the explants were not only stained with MF20 to identify the myocytes (Panels i and m), but also with propidium iodide to identify all nuclei (Panels n). Panel o shows the merged images of panels m and n, illustrating that nuclei of non-MF20 stained cells are present around the myocytes.
atrial septum, dorsal mesocardium, spina vestibuli and the proximal parts of the caval and pulmonary veins. After overnight incubation of the explants an epithelial monolayer starts to form on top of the collagen matrix, which expands during the subsequent culture period. In the cultures of the venous pole, atrioventricular canal and outflow tract, mesenchymal cells are first observed in the collagen matrix underneath the explant and epithelial monolayer after 2 days of culture. In cultures of the apex of the left ventricle mesenchymal cells are first observed after 3 days of culture. During subsequent culture period, the amount of mesenchyme increases. Although Varel-modulation microscopy (Fig. 5a-c, g and h) suggests that cardiomyocytes have populated the collagen matrix, the explants had to be fixed and immunostained for myosin heavy chain (MF20) to unambiguously distinguish cardiomyocytes from non-cardiomyocytes. The extent of myocardium formation in the collagen matrix was analyzed by confocal laser scanning microscopy (CLSM), and scored on an arbitrary scale as specified in Materials and Methods. Figure 5 shows typical examples of cellular outgrowth of the different cardiac compartments. CLSM analysis showed that myocardial protrusions are present that extend over the surface and into the collagen gel forming three-dimensional networks. In explant cultures of the outflow tract (Fig. 5j) and ventricle (Fig. 5i,k) myocardial protrusions are observed around the entire explant. In explant cultures of the venous pole and, albeit to a lesser extent, of the atrioventricular canal, regions of myocardial protrusions alternate with regions that have a smooth myocardial border (arrows in fig. 5d and f). In general, myocardial protrusions are contiguous with the original explant (Fig. 5m). Myocardial cells that do not seem to have contact with other myocardial cells (i.e. isolated myocardial cells) are rarely observed in the gel (arrows in fig. 5 l).

To further characterize the spontaneous formation of myocardial networks of the venous pole, atrioventricular canal, outflow tract and ventricular explants, we analyzed explants ranging from E11 to E19 (Fig. 6). Venous pole explants show extensive myocardial networks (score 2) from stage E13 to E15. Atrioventricular canal explants show the first extensive myocardial network formation at E13, whereas outflow tract explants do so one day later in development, i.e. at E14. Both atrioventricular canal and outflow tract explants do not form extensive myocardial networks at E17 anymore. Although the developmental window for myocardial network formation of ventricular explants resembles that of outflow tract explants, ventricular explants show hardly, if any, myocardial protrusions before E14.

**Involvement of mesenchyme in myocardium formation**

In cultures of ventricular explants younger than E14, mesenchymal cells and myocardial networks are not formed. Epithelial cells that have formed on the collagen gel do not survive until the end of the culture period (data not shown). From E14 up to E16,
Myocardium formation in mouse

however, ventricular explants display extensive outgrowth of epithelial and mesenchymal cells (Fig. 7a and b), which survive till the end of the culture period. This coincides with the developmental stages that show extensive myocardial network formation (Fig. 6 and 7c). From E17 onward, outgrowth of epithelial cells is observed, whereas the amount of both mesenchyme (Fig. 7d) and myocardial networks (Fig. 7h) are reduced. In venous pole, atrioventricular canal, and outflow tract explant cultures, mesenchymal cells are observed at the end of the culture period at all stages examined. However, the amount of mesenchyme at the later stages (E17-E19), when myocardial network formation has ceased (Fig. 6), seems to be reduced (not shown). Taken together, these observations suggest a correlation between mesenchyme formation and myocardial network formation.

To explore the correlation between mesenchyme formation and myocardial network formation we aimed at inhibiting the mesenchyme formation in ventricular explants. Ventricular explants were selected based on the following two observations. First, in the apex of the left ventricle of E14 no endocardial cushion mesenchyme is present, whereas in venous pole, atrioventricular canal and outflow tract mesenchyme is present. Secondly, when explants of venous pole, atrioventricular canal and outflow tract are used, mesenchymal cells form in the collagen gel after two days of culture, whereas mesenchymal cells form after three days of culture in ventricular explant cultures. These observations suggest that mesenchymal cells are formed \textit{de novo} in ventricular explant cultures. This unique feature of ventricular explants offers the opportunity to inhibit the formation of mesenchyme in ventricular explant cultures by heparin. Heparin interferes with FGF signaling (Powers et al., 2000) that is implicated in the formation of mesenchyme in the heart (Choy et al., 1996; De la Cruz and Markwald, 1998; Dettman et al., 1998; Morabito et al., 2001). Addition of heparin to ventricular explants of E15, which normally show extensive mesenchyme (Fig. 7h) and myocardial network formation (Fig. 7c), strongly reduced both the amount of mesenchymal cells (Fig. 7e and f) and the extent of myocardial network formation (Fig. 7g).

To determine whether the correlation between mesenchyme and myocardial network formation in cardiac explants also holds true for chicken explants, we extended our experiments to chicken ventricular explants. In a previous study, chicken left ventricular explants were generally not found to form myocardial networks at the stages examined (Van den Hoff et al., 1999), although a small percentage of ventricular explants between the stages HH21-26 showed myocardial network formation. Re-evaluation of the capacity to form myocardial networks using different culture conditions due to batch-to-batch variation revealed that most left ventricular explants taken from HH21-24 (Fig. 7k) and to lesser extent those taken from HH25-27 are capable of forming myocardial networks, comparable to mouse explants. The ventricular explants from these stages only, show a large amount of mesenchyme (Fig. 7i and j). In ventricular explants of HH28 hardly any mesenchyme (Fig. 7l) and no myocardial networks (Fig. 7m) are formed. Upon addition of heparin, the formation of both mesenchymal cells (Fig. 7n) and myocardial
networks (Fig. 7o) was blocked in chicken ventricular explant cultures of H124, resembling the observations in mouse explant cultures. These findings indicate that also in chicken explants a correlation exists between mesenchyme and myocardial network formation. In addition, whenever mesenchyme formation was not completely blocked, myocardial networks (Fig. 7r) are only formed at sites of mesenchyme formation (arrow and box in fig. 7p and q). Furthermore, close examinations of Vare1-modulation and confocal images show that mesenchyme-like cells (Fig. 7s and v) are myosin positive (Fig. 7t and w). Taken together, these observations suggest that the presence of mesenchymal cells is a prerequisite for myocardial network formation in both mouse and chicken explant cultures.

**DISCUSSION**

In this paper we report an *in vivo* and *in vitro* study of myocardium formation in intra- and extra-cardiac mesenchyme after development of the linear heart tube in mouse.

**In vivo myocardium formation**

Using an immunohistochemical approach we determined that myocardium formation starts around E12 in the dorsal mesocardium. In subsequent stages of development, the process extends downstream into the intra-cardiac mesenchymal tissues of the atrioventricular canal and outflow tract, and upstream into the extra-cardiac mediastinal mesenchyme embedding the pulmonary and caval veins. At E17, myocardium formation in the intra- and extra-cardiac mesenchyme has contributed the smooth walled atrial myocardium, the myocardial atrioventricular septum, the myocardial outlet septum, and the caval and pulmonary myocardium. The contribution of myocardium to the

**Figure 7.** Correlation of myocardial network formation with the appearance of mesenchymal cells in the collagen gel in ventricular explant cultures. Panels a, b, d-f, i, j, k, n, p, q, r, and s show Vare1 modulation micrographs. Panels e, g, h, k, m, o, r and u show brightest point projections of CLSM stacks of the respective explants after immunohistochemical staining of the myocytes using the monoclonal antibody ME20. Panel t shows one CLSM stack of an explant depicted in panels s and u. Panels a-c show mouse ventricular explant of E15. Panels d and h show mouse ventricular explant of E19. Panels e-g show mouse ventricular explant of E15 treated with 500 ng/ml of heparin. Panels i-k show chicken ventricular explant of HH124. Panels l and m show chicken ventricular explant of HH24. Panels n and o show chicken ventricular explant of HH24 treated with 50 mg/ml of heparin. Note the high amount of both mesenchymal cells and myocardial networks in ventricular explants of E15 mouse and HH124 chicken, and the little amount of both mesenchymal cells and myocardial networks in ventricular explants of E19 mouse and HH128 chicken, and ventricular explants of E15 mouse and HH24 chicken treated with heparin. Panels p-r show that myocardial networks are restricted to the site where mesenchymal cells are present (boxed area and arrow). Panels s-w show that myosin positive cells have mesenchymal-like shape. Scale bar: 200 μm.
atrioventricular septum is limited, because mesenchyme remains as the central fibrous body, the tendon of Todaro and the valvular leaflets. Although the tricuspid valve does not become muscular, myocardial protrusions are observed at the base of the developing tricuspid valve. This observation suggests that muscularization is initiated, but not sustained, or even inhibited. A similar phenomenon is observed in chicken. Whereas the mitral valve remains mesenchymal, myocardial protrusions are present at the base of the developing mitral valve. The atrioventricular septum and tricuspid valve, however, do become completely myocardial in chicken (Van den Hoff et al., 2001).

In addition to a difference in the extent of myocardium formation at the level of the atrioventricular canal, the following differences are seen between mouse and chicken. 1) In mouse, muscularization of the intra-cardiac mesenchyme takes 5 days (E:12-1°), whereas in chicken it lasts 9 days (HH19-HH38, E3-E12). 2) In mouse, muscularization is not observed in the right inferior caval vein, whereas the myocardial cells reach to the margin of the liver in chicken. 3) Muscle formation of the pulmonary vein is more extensive in mouse than in chicken. The myocardial cells reach to at least the fifth bifurcation of the pulmonary vein in the lungs of the mouse, whereas in chicken the myocardial cells do not extend beyond the first bifurcation.

In vitro analysis of the myocardium formation

As a first step towards the unravelling of the molecular mechanisms underlying this relative late process of myocardium formation, an in vitro assay to culture and analyze mouse cardiac explants was established. Using this in vitro explant assay, different cardiac compartments of various developmental stages were analyzed for their capacity to form myocardial networks. Explants of the venous pole, atrioventricular canal, and outflow tract are able to form myocardial networks provided the proper developmental stages are cultured. The in vitro developmental window of myocardium formation shows that myocardium formation in the intra-cardiac mesenchyme is initiated and completed in downstream direction, reflecting the in vivo observations. This indicates that myocardium formation in the in vitro explant assay mimics myocardium formation in vivo.

Mesenchyme is required for myocardial network formation

The following observations, which were made in mouse and chicken explant cultures, suggest a crucial role for mesenchymal cells in myocardium formation. 1) Myocardial networks were only found in in vitro explant cultures containing mesenchymal cells. 2) When mesenchyme formation was blocked, myocardial networks were not observed. 3) When mesenchyme was formed from a restricted part of the explant, myocardial networks were only found at these sites of mesenchyme formation.
Myocardium formation in mouse

Myocardial networks are first observed in atrioventricular canal explants at E13 and in outflow tract explants at E14, whereas mesenchymal cells are observed from the first stage examined (E11). *In vivo*, myocardium formation is observed about 2 days after the initial appearance of cushion mesenchyme in both the atrioventricular canal and outflow tract (Table 1). Endocardium-derived mesenchymal cells that invade the cushions are initially subjected to proliferation signals. As they migrate towards the interface with the myocardium they become subjected to differentiation signals. Together, these observations suggest that a sufficient amount of mesenchymal cells has to acquire a state of differentiation prior to the muscularization of cushion mesenchyme (Markwald et al. 1998; Mjaatvedt et al., 1999). Taking this into account, the observation that both *in vitro* and *in vivo* myocardial networks appear in the outflow tract one day later in development than in the atrioventricular canal is consistent with the observation that the first mesenchyme is observed one day later in the outflow tract than in the atrioventricular canal.

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<td>Atrioventricular canal</td>
<td>E10&lt;sup&gt;1&lt;/sup&gt;</td>
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<td>Outflow tract</td>
<td>E11&lt;sup&gt;1&lt;/sup&gt;</td>
<td>E13</td>
</tr>
<tr>
<td><strong>Chicken</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atrioventricular canal</td>
<td>E2.5&lt;sup&gt;2&lt;/sup&gt; (HH16)</td>
<td>E4.5&lt;sup&gt;2&lt;/sup&gt; (HH26)</td>
</tr>
<tr>
<td>Outflow tract</td>
<td>E3.5&lt;sup&gt;2&lt;/sup&gt; (HH21)</td>
<td>E5.5&lt;sup&gt;2&lt;/sup&gt; (HH28)</td>
</tr>
</tbody>
</table>

Table 1. Overview of the stages in which mesenchyme or myocardium formation is first observed in the atrioventricular canal and outflow tract of mouse and chicken. Ref: 1: Camensch et al., 2002; 2: Moreno-Rodríguez et al., 1997; 3: Van den Hoff et al., 2001; 4: Van den Hoff et al., 1999.

Mouse and chicken ventricular explants spontaneously form myocardial networks within a limited developmental period (E14-E16 and HH21-27, respectively), whereas no myocardial networks are observed *in vivo*. Because mesenchymal cells were found to be crucial for myocardial network formation *in vitro*, these apparent contradictory observations are likely to be due to the fact that *in vivo* mesenchymal cells are not formed in-between the ventricular myocardium and endocardium, whereas mesenchymal cells are formed *in vitro*.

**Potential mechanisms underlying myocardium formation**

Two mechanisms can be envisioned to underlie myocardium formation: (i) growth of existing cardiomyocytes into mesenchyme, defined as myocardialization, and (ii) differentiation (recruitment) of non-muscle cells into cardiac muscle cells. *In vitro* most of the myocardial networks are contiguous with the explant and were initially thought to be derived from already existing myocardial cells that had grown out of the explant.
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However, the presence of isolated myocardial cells suggests differentiation of mesenchymal or epithelial cells into cardiac muscle cells. The notion that mesenchymal cells are able to differentiate into cardiomyocytes is supported by the observations that (i) isolated cushion mesenchyme is able to differentiate into cardiac muscle cells (Van den Hoff et al., 2001), (ii) in vitro myocardial network formation is only observed in the presence of mesenchyme, (iii) myosin-positive mesenchyme-like cells are observed in explants cultures, and (iv) α-smooth muscle actin expression precedes the expression of myocardial markers during muscularization of intra- and extra-cardiac mesenchyme (Kruithof et al., submitted). Taken together, our observations suggest that differentiation of mesenchymal cells contributes to myocardium formation in the intra-cardiac mesenchyme and possibly also the extra-cardiac mesenchyme.

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