IN SITU HYBRIDIZATION AND IMMUNOCYTOCHEMICAL ANALYSES OF THE EXPRESSION OF CARDIAC-SPECIFIC GENES IN AN EXPERIMENTAL MODEL, WHICH PREVENTS THE FUSION AND SUBSEQUENT FORMATION OF THE SINGLE TUBULAR HEART IN AVIAN EMBRYOS

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In the chick embryo, precardiac cells migrate through the primitive streak and into bilateral sites within the lateral plate mesoderm (Garcia-Martinez and Schoenwolf, 1993) in close proximity to anterior endoderm, which has been implicated in heart differentiation (Sugi and Lough, 1994). Several other factors have been implicated in the process of heart development, including retinoids (Dickman and Smith, 1996) and activin-A, FGFs and TGFs (Sugi and Lough, 1995). However, the intrinsic mechanisms involved in the induction of precardiac cells resulting in the formation of paired primitive endocardial tubes and their subsequent posterior fusion are still largely unknown. In this work we analyze the response of a short caudal segment of a primitive endocardial tube, which under normal circumstances would form a segment of the sinus venosus, to differentiate into a contractile heart tube. This tube shows a structural arrangement similar to the normal heart (endocardium, myocardium and cardiac jelly), and a similar pattern of segmentation (atrium, ventricle and, likely, sinus venosus and trunco-conal region).

Material and Methods

Experimental procedure

Fertile White Leghorn chicken and Japanese quail eggs were incubated to stage 8 (Hamburger and Hamilton, 1951). Embryos were cultured ventral-side up on their vitelline membranes according to New (1955). Operations were performed at the stages of formation of paired primitive endocardial tubes; i.e. stages 8- to 9- HH, (3-6 somites). A tiny, rigid cactus needle was used to cut the caudal pole of one (left or right) or both primitive endocardial tubes (Figure 1) in a total of 110 embryos. Then embryos were placed in humidified chamber and cultured at 38°C for 14-36 h. One embryo from each session was videotaped at 12.5 second intervals during the culture period (Figure 2).

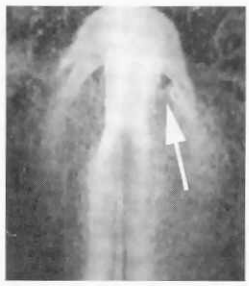

Figure 1. Ventral view of an embryo at stage 8b (time 0) to show the location (white arrow) of the section at the level of the caudal pole of the primitive endocardial tube.

Scanning electron microscopy

Several control and experimental embryos were fixed in 3% gluteraldehyde in 0.1M cacodylate buffer. After 24 h the embryos were subsequently cut at informative planes with a fine scalpel blade, processed for scanning electron microscopy and examined with a Jeol T-100 scanning electron microscope.

Region-, tissue- and cell-specific markers

A subset of embryos was examined with region-, tissue- or cell-specific markers; that is, riboprobes and antibodies specific for a particular region, tissue or cell type within the cardiac tube at the stages during which the results of the experiment were evaluated. The markers used were: the cardiac muscle marker cNkx-2.5 (tinman), a chick homeobox-containing gene that shares extensive sequence similarity with the *Drosophila* gene tinman (Schultheiss et al., 1995); the atrial-specific myosin heavy chain (AMHC1) and ventricular myosin heavy chain (VMHC1) probes (Yutzey et al., 1994; Bisaha and Bader, 1991), which mark their respective regions in the cardiac tube and are expressed at the onset of cardiomyocyte differentiation; and antibodies against sarcomeric myosin heavy chain (MF20), tropomyosin (CH1), and titin (9D10; all obtained from the Developmental Studies Hybridoma Bank, University of Iowa), and alpha-smooth muscle actin (Sigma). QH-1 antibody, which is specific for blood-vessel endothelial cells in quail, was used to analyze the endocardial cell layer. In whole mount embryos, the antibodies were visualized using peroxidase immunocytochemistry, whereas the remaining markers were visualized using *in situ* hybridization.

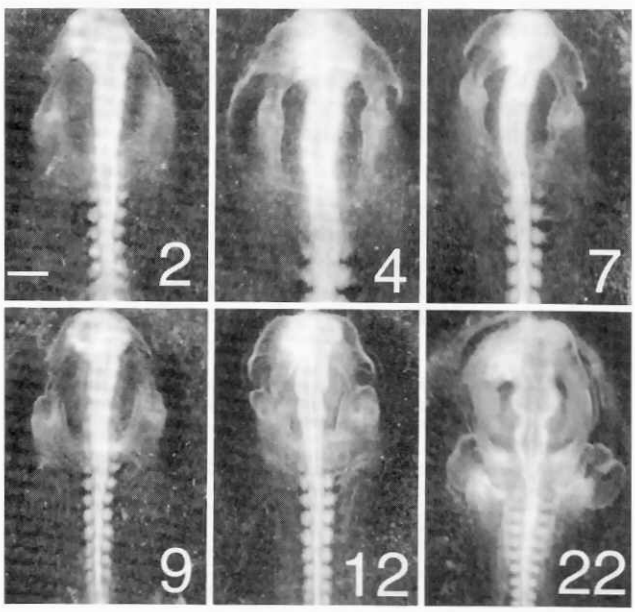

Figure 2. Sequential prints corresponding to an experimental embryo at 2, 4, 7, 9, 12 and 22 h after cutting the caudal pole of both endocardial tubes. Bar = 200 μm

Results and Conclusions

In our experimental model, the severance (on one side) of the caudal pole of the primitive endocardial tube at stage 8+ interrupts the normal fusion of the tubes. As a result (Figure 2), two cardiac tubes form, one solely from the caudal pole of the severed primitive endocardial tube. Both tubes develop the same structural and segmental characteristics as a normal heart tube. That is, each has both structural layers, the endocardium and myocardium, and the cardiac jelly in between (determined by scanning electron microscopy; not shown). The use of QH-1 in quail experimental embryos allowed the identification of a differentiated endocardial layer in each heart tube. Furthermore, the use of MF20, CH1, 9D10, and anti-alpha-smooth muscle actin, showed the identification of a process of myocardial layer differentiation in both heart tubes, with a similar pattern, in time and spatial distribution, to a normal heart tube (Figure 3). Moreover, tinman was expressed at the level of the two tubular structures originated after this microsurgical procedure (Figure 4). These data suggest that the process of fusion in early tubular heart formation is not required for the differentiation and establishment of the structural layers of the rudimentary heart.

In addition, our results show that, even the tubular cardiac structure originating from the caudal pole of the primitive endocardial tube sectioned, has the ability to differentiate and partition itself into the different segments which characterize the normal primitive heart tube during looping: atrium, ventricle and, presumably, sinus venosus and trunco-conal segment. As can be seen in Figure 5, both tubular structures express VMHC1 (Figure 5a) and AMHC1 (Figure 5b), which are specific for the primitive ventricle and the primitive atrium, respectively.

In this study we demonstrate the ability of a single primitive endocardial tube, or a segment of itself, to form contractile tubular structures during the initial stages of heart morphogenesis. We demonstrate the structural properties of the primitive tubular heart (endocardium, cardiac jelly and myocardium), and the typical pattern of segmentation in atrium, ventricle and, presumably, sinus venosus and trunco-conal region.

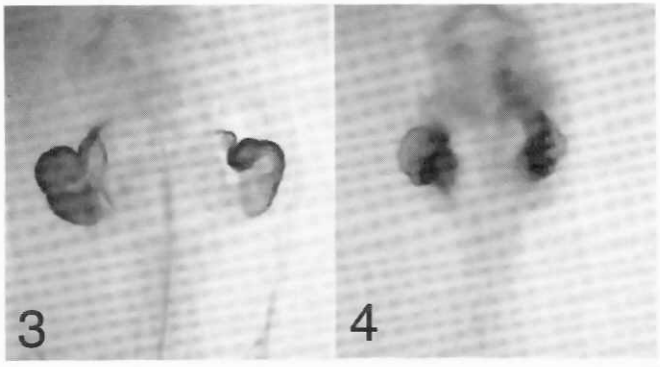

Figure 3. Whole mount immunocytochemistry with MF20 antibody.

Figure 4. Whole mount *in situ* hybridization using the cardiac marker cNkx-2.5, showing the expression of tinman in both tubes.

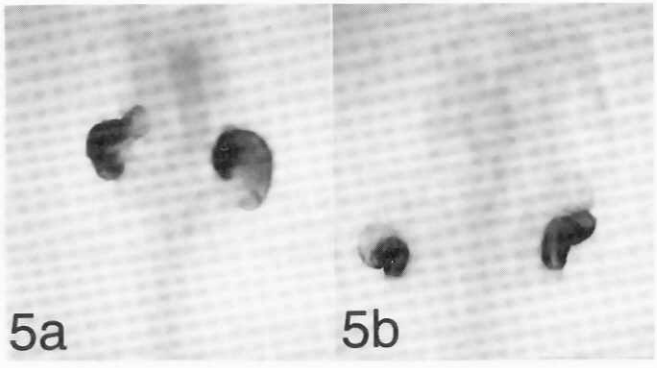

Figure 5. Whole mount *in situ* hybridization using VMHC1 (a) and AMHC1 (b) probes. Note the positive expression at the level of the respective segments in cardiac tubes.

Acknowledgments

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