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Short Report

The contribution of the proepicardium to avian cardiovascular development

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ABSTRACT The embryonic epicardial layer of the heart, which originates from the proepicardium, gives rise to a set of invasive pluripotent epicardially-derived cells (EPDCs) that contribute to the development of 1) subepicardial mesenchyme, 2) coronary vessels, 3) intermyocardial fibrous tissue, and 4) mesenchyme of the atrioventricular cardiac valve primordia. Here we report that chimeric tracing of proepicardial cells as well as a new experimental procedure reveal a non-proepicardial origin for the epicardial-like cells of the more distal part of the outflow tract of the heart (OFT). This observation is supported by the fact that the expression pattern of mesothelial antigens in the epicardial-like cells of the distal outflow tract is different from the expression pattern in the proepicardially-derived epicardium. The delay of epicardium and EPDC development using diverse microsurgical procedures leads to abnormal cardiac phenotypes in all cardiac areas colonized by proepicardially-derived epicardium and EPDCs. These results suggest the existence of a signaling role for epicardium and EPDCs in cardiac development and support the hypothesis that ontogenetical diversity of cardiac epicardium is involved in heart chamber morphogenesis and remodeling.

Objectives

The epicardium is the outer layer of the heart. Its development is a relatively late event compared to the formation of the myocardial and endocardial tissues, i.e. the only two cardiac cell lineages that directly derive from embryonic heart fields. The epicardial progenitor is the proepicardium, a mass of coelomic cells located between the posterior sinus venosus and the liver primordium. Proepicardial cells migrate over the surface of the heart around stages H/H17-18 and undergo an epithelial-to-mesenchymal transformation (EMT) which, in turn, gives rise to a population of pluripotent mesenchymal epicardially-derived cells (EPDCs) (Gittenberger de-Groot *et al.*, 1998; Pérez-Pomares *et al.*, 1998). This EPDC population shows an invasive behavior and differentiates into a variety of cell types including coronary endothelium, coronary smooth muscle, intermyocardial fibroblasts and mesenchymal cells found in the primordia of the atrioventricular (AV) valves (i.e. endocardial cushions; reviewed in Männer *et al.*, 2001). Here, we present a study of the impact of epicardium and epicardial-derived tissues in cardiac morphogenesis.

Material and Methods

We have traced proepicardial derivatives (epicardium and EPDCs) by a quail-to-chick chimera approach (Männer, 1999). QCPN, a monoclonal antibody that specifically recognizes a quail nuclear antigen, was used to establish the differential distribution of the donor-derived cells in the developing heart. QCPN was colocalized with other epicardial markers including the retinoic acid converting enzyme RALDH2 (Xavier-Neto *et al.*, 2000) and the Wilms' tumour transcription factor WT1 (Moore *et al.*, 1999) in a laser scanning confocal microscope. Microsurgical procedures allowed us to: 1) analyze the effects of a delay in epicardial and EPDC development and study the extent of proepicardial contribution to the epicardium of the different heart segments (eggshell membrane block of proepicardial attachment to the surface of the heart or direct proepicardial ablation) and 2) map other possible sources for epicardial-like tissue (inner curvature of the heart-aortic sac insertion of a piece of eggshell membrane). Results were analyzed by histological and immunohistochemical methods.

Results

Quail-to-chick proepicardial chimeras demonstrated the presence of a segment-specific patterning for epicardium and epicardially-derived cells. At stages H/H26-27 the sinus venosus, atria, ventricles and the most proximal part (conus) of the OFT were completely covered by donor-derived epicardium (quail origin). The epicardium and EPDCs showed a strong immunoreactivity against the RALDH2 antigen. However, the distal portion of the OFT epicardium (truncal epicardium) was formed by host tissue (chick) and its RALDH2 immunoreactivity was heterogeneous and weaker than that of the rest of the epicardium. Control non-chimeric embryos of the same age displayed a similar immunohistochemical pattern. Generation of EPDCs starts soon after the arrival of the proepicardial tissue to the surface of the heart but the cells remain in the subepicardial space until stages H/H26 when they start to invade the ventricular myocardium; only after the stages H/H29 the atrioventricular (AV) myocardium and AV endocardial cushion tissues are invaded by EPDCs. Some of these cells, like the epicardial epithelium, still express the WT1 antigen. Only the proximal part of the OFT epicardium generates EPDCs, but these cells do not invade the myocardial or cushion tissue of the segment. Alterations in epicardial and EPDC development directly affect AV values and ventricular morphogenesis. The resulting experimental phenotypes include dysmorphogenesis of the AV valves, hypoplastic ventricular myocardium, irregularities in the formation of the interventricular septum and changes in normal coronary patterning. Immunohistochemical characterization of the experimental embryos (proepicardial block or ablation) shows ab-

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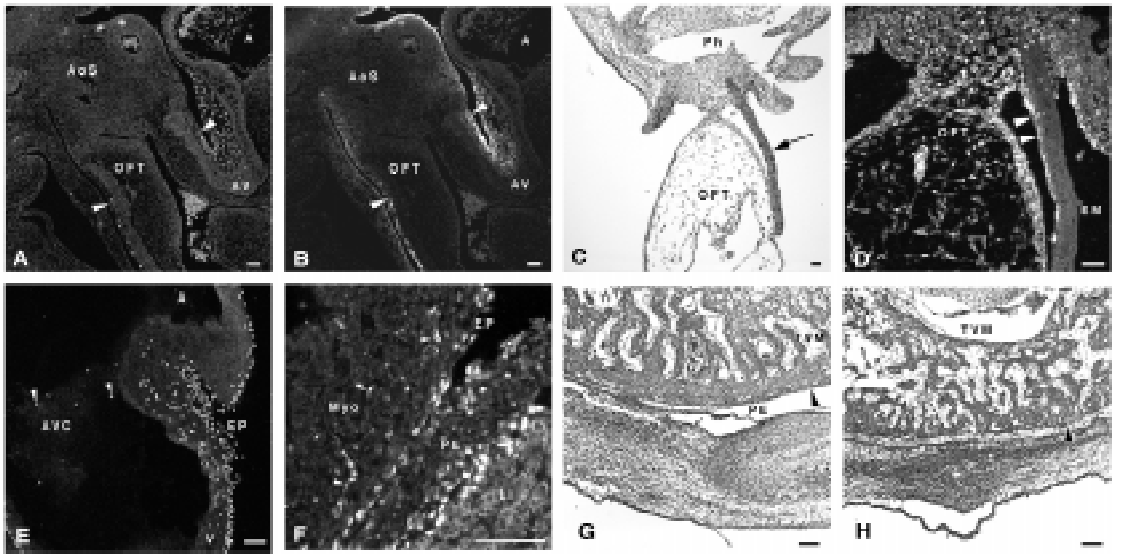

Fig. 1. (A,B) *OFT of a stage H/H29 quail-to-chick proepicardial chimera. The expression of QCPN and RALDH2 in the same confocal section are shown separately.* **(A)** *Quail nuclei in the chimeric epicardium are stained with the QCPN antibody while in* **(B)** *RALDH2 expression in the epicardium is shown. White arrowheads indicate the end of the donor-derived epicardium (note that in A the more distal/cranial epicardium is not of quail origin). Scale bars, 45 microns.* **(C)** *Insertion of a piece of eggshell membrane (arrow) in the distal OFT region of a stage H/H20 chick embryo. Scale bar, 30 microns.* **(D)** *In a section consecutive to C ,a magnification of the insertion is shown. The tissue is stained with an anti-cytokeratin antibody. An epicardial-like monolayer is indicated growing over the eggshell membrane (arrowheads). Scale bar, 30 microns.* **(E)** *The AV sulcus of a stage H/H28 quail-to-chick proepicardial chimera is showed. QCPN-positive EPDCs immigrate to the myocardial layers of the ventricle and the AV region where they reach the endocardial cushions (white arrowheads). No EPDCs invade the atrial myocardium. Scale bar, 130 microns.* **(F)** *A ventricular section of a stage H/H32 chick embryo is shown. Epicardium, pericardium and liver mesothelium express the WT1 protein. EPDCs, also WT1-positive, can be tracked into the myocardium (white arrowheads). Scale bar, 50 microns.* **(G)** *Ventricle of a control stage H/H35 chick embryo (hematoxylin-eosin stained). Note the thickness of the compact myocardial layer as indicated by the black arrowhead. Scale bar, 80 microns.* **(H)** *A stage H/H35 proepicardially-blocked chick embryo is shown (H&E stained). The ventricular region can be compared to the one in G. The compact myocardial layer is hypoplastic (its thickness is about 1/5 of that of the normal compact myocardium, black arrowhead) and the trabeculae look abnormal. Scale bar, 80 microns. A, atrium; AoS, aortic sac; AV, atrioventricular sulcus; AVC, atrioventricular cushion; EM, eggshell membrane; EP, epicardium; Li, liver; Myo, myocardium; OFT, outflow tract; PE, pericardium; Ph, Pharynx; TVM, trabeculated ventricular myocardium; V, ventricle.*

normalities in the amount of epicardium and EPDCs in the cardiac segments that were shown to be affected by the epicardial delays. Finally, insertion of small pieces of eggshell membrane between the inner curvature of the heart and the aortic sac indicated that this tissue is able to form an epicardial-like monolayer over the surface of the membrane and is likely to normally differentiate into distal OFT epicardium.

Conclusions

Our results support the hypothesis that two populations of epicardial cells (proepicardially-derived and aortic sac-derived) can differentially contribute to normal heart development. The RALDH2 immunohistochemical expression patterns in proepicardially-derived epicardium and aortic sac-originated epicardium may indicate differences regarding retinoic acid (RA) metabolism between those tissues; RA is known to play an essential role in cardiac development (Kubalak and Sucov, 1999). Thus, combination of disparity in EPDC immigration into the heart chambers and a diversity in epicardial inductive abilities -related with a different embryological origin- can be involved in differential segment-specific cardiac morphogenesis.

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