The Role of the Epicardium in Heart Development

Ismail Eralp

Impressum

Cover Image: “Le Sac à Malice” by René Magritte is one the less familiar works by this Belgian surrealist. It was painted in 1946 and represents a surrealistic image of a heart in which obviously something went terribly wrong with epicardial development. A naked myocardium is seen as well as aberrantly originating coronary arteries. Situated in front of a window, this is perhaps the scene of gazing into future research of cardiac development, and maybe even having opened the curtain on the right by the results presented in this thesis.

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Papa, ik lijk steeds meer op jou

Stef Bos

Dit proefschrift draag ik op aan mijn moeder

ter nagedachtenis aan mijn vader

Contents

Chapter 1

Introduction

Introduction

In early embryonic life, the primitive heart tube consists of two layers, the myocardium and the endocardium. Eventually the heart tube loops to form a four-chambered structure providing for a circulation of blood by rhythmic contractions of the myocardium. With the increase in heart size and myocardial work load, a separate vasculature for the free myocardial wall is required. The formation of this coronary system is preceeded by the outgrowth of the epicardium, covering the naked heart tube[1]. Our studies were performed on quail and chicken embryos, because of the high resemblance with human cardiac development. Avian embryos develop in approximately three weeks and were staged according to the Hamburger&Hamilton (HH) stages[2]. In avian embryos the epicardial development starts at stage HH15 which is at three days of embryonic development, and is completed at stage HH25, which is after seven days of incubation. Epicardial cells are derived from the proepicardial organ (PEO), that protrudes as a cauliflower-like structure from the mesothelial lining of the body cavity near the sinus venosus and primitive liver, towards the inner curvature of the heart[1]. The villi of the PEO reach the heart at the posterior atrial side where the sinus venosus myocardium is incorporated and the epicardial cells start to spread over the naked heart tube, until they eventually cover the myocardium completely[1, 3, 4].

Between the epicardium and the myocardium a subepicardial layer develops. Epicardium-derived cells (EPDCs[5]) cells, that detach from the epicardium by epithelial to mesenchymal transformation (EMT) make up this subepicardial mesenchyme. Both perihepatic endothelial cells from the dorsal mesocardium as well as EPDCs migrate into the subepicardial layer and produce extracellular matrix components[6]. The subepicardial mesenchyme is most prominent at the atrio-ventricular (AV) and interventricular grooves. In contrast, the epicardial mesothelium remains in closer contact with the free myocardium of the atria and ventricles[6].

The coronary vessel network develops within the subepicardial mesenchyme[6, 7]. The initial endothelial network is in open contact with the sinus venosus[8] and starts growing over the heart encircling the AV groove towards the ventral side. The coronary endothelial plexus reaches the ventriculo-arterial junction and forms a peritruncal ring. From here vessels grow into the aorta[9, 10] and into the right atrium[8]. This ultimately results in two coronary arteries that connect to the aorta[10].

Part of the EPDCs migrate into the underlying layers to differentiate into several cell types. Smooth muscle cells of coronary arteries, myocardial and subendocardial interstitial fibroblasts and a subpopulation of the mesenchymal cells of the endocardial cushions are derived from EPDCs[11 12-15]. Several mouse models (VCAM-1$^{-/-}$, α_4 integrin$^{-/-}$ and FOG2 null mutants[16-18]) as well as our avian models[19-21] have shown that coronary vessel formation is severely disturbed when the epicardium fails to develop properly.
EPDCs which have migrated into the myocardial layer and differentiated into interstitial fibroblasts have been hypothesized to provide for a signalling function in myocardial development[13, 20, 22, 23].

The peripheral Purkinje conduction cells are derived from cardiomyocytes that differentiate into Purkinje cells[24]. A close spatial relationship was observed between EPDCs and Purkinje cells differentiating in both subendocardial and periarterial locations in chicken embryos[13, 25]. It was suggested that signals from coronary vascular cells induce cardiomyocytes to differentiate into Purkinje cells[26-28].

A morphogenic role for EPDCs in cardiac fibrous tissue and valve development has also been postulated[5, 29]. With respect to valve development periostin was recently identified as an important factor[30]. The spatiotemporal expression of periostin closely resembles the migratory patterns of EPDCs[5;30], so one of our studies focusses on the role of EPDCs and the expression of this signalling molecule with respect to cardiac fibrous tissue and valve development.

The aim of the studies described in this thesis was to further elucidate the distinct roles of EPDCs in heart development and to explore their relevance for the development of human congenital heart malformations. The studies have been performed using quail and chicken embryos, because of the great resemble between avian and human embryos, with respect to heart development.

In *Chapter 1* human coronary malformations and the possible role for EPDCs therein are described.

In *Chapter 2* the role of EPDCs in coronary development and coronary ingrowth in two avian experimental models was studied. In quail embryos epicardial development was hampered to study the effect of disturbed or

inhibited epicardial outgrowth. In chicken embryos epicardial development was blocked simultaneously with the transplantation of a quail PEO (epicardial rescue model). With these two models we could study the effect of an inhibiton of epicardial outgrowth and of a delay in epicardial outgrowth on myocardial and coronary development.
In *Chapter 3* epicardial quail-chicken chimeras were analyzed to study the migratory patterns of EPDCs. By creating quail-chicken chimeras, quail EPDCs could be traced during chicken embryonic development. Myocardial heterogeneity in permissiveness for EPDCs appeared to determine their migratory behaviour.

In *Chapter 4* the association of EPDCs with Purkinje cell development was further analyzed and described, again by the use of epicardial inhibition and chimera-techniques.

In *Chapter 5* the role of EPDCs as periostin-producing cells in the development of the fibrous heart tissues is described.Co-cultures of periostin-producing mesenchymal cells and cardiomyocytes indicated a mechanism, by which periostin induced transdifferentiation of cardiomyocytes into a mesenchymal/fibroblast-like phenotype.

In *Chapter 6* the rare human congenital heart malformation corrected transposition of the great arteries (CCTGA) is described. CCTGA is a rare condition in which several abnormalities occur where EPDCs could be involved. In our present patient group coronary abnormalities have been studied, that suggest epicardial involvement in the pathogenesis of this human cardiac congenital malformation. The latter hypothesis provides leads for further analysis of epicardial involvement in the pathogenesis of other congenital cardiac malformations.

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Chapter 2

Development of the coronary vasculature and implications for coronary abnormalities in general and specifically in pulmonary atresia without ventricular septal defect

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Abstract

Aim Coronary vascular anomalies are an important factor in congenital heart disease in the neonate. However, our knowledge of the pathomorphogenesis is still defective.
Material and methods 1. study of coronary anomaly variations in congenital heart disease using specimens and 2. study of the role of epicardium derived cells (EPDC) and neural crest cells in coronary vascular formation using quail-chicken chimeras.
Results The clinical and pathology data revealed the existence of ventriculo-coronary-arterial-communications during fetal life before pulmonary atresia was established. This supported a primary coronary developmental anomaly as the origin of some cases of pulmonary atresia as opposed to other cases in which the pulmonary orifice atresia was the primary anomaly. Our experimental work showed the high relevance of the development of the epicardium and epicardium derived cells for the formation or the coronary vasculature and showed the coronary vascular ingrowth into the myocardium and subsequently into the aorta and the right atrium. Absence of epicardium derived cells lead to embryonic death while delayed outgrowth could result in absence of main coronary arteries to pin-point orifice formation. In these cases the circulation was maintained through ventriculo-coronary-arterial-communications. Neural crest cells were important for the patterning of the coronary vasculature. We have extended this knowledge to a number of other heart malformations.
Conclusions Coronary vascular anomalies are highly linked to development of extracardiac contributors like the epicardium and the neural crest. A proper interaction between these cell types and the myocardium and aortic arterial wall are important for normal vascular development.

Introduction

Coronary vascular abnormalities in human neonates and also adults can be divided in main branch anomalies and ventricular intramural anomalies. Main branch anomalies consist of abnormal patterning such as e.g. seen in several congenital heart malformations like transposition of the great arteries (TGA) (1) and for clinical purposes has been simplified by a classification system often referred to as the Leiden

Convention. In a small percentage of cases with TGA a main coronary vessel takes an aortic wall intramural course which complicates the arterial switch surgery (2). This anomaly can also be seen as an isolated phenomenon in otherwise healthy adults and can lead to sudden death in young athletes. A different kind of anomaly refers to ventricular intramural coronary patterning exemplified by ventriculo-coronary arterial communications (VCAC) as seen in pulmonary atresia without ventricular septal defects (3-5). The latter malformation actually presents with two variants. One in which there are no VCAC but severe endocardial fibro-elastosis of the right ventricular wall and a second variant which shows the VCAC but lacks endocardial fibro-elastosis. Ultrasound fetal observations in which VCAC were found in utero preceding development of pulmonary atresia (6) prompted us to re-evaluate coronary vascular formation.

Exciting steps have been made using a quail-chicken chimera approach in which we and others, followed the development of the epicardial layer covering the initially bare myocardium(7-9). These experiments showed that the development of the coronary vasculature is dependent on formation of the epicardium. The epicardium derived cells, so called EPDC, are the source of the smooth muscle cells as well as the fibroblasts of the coronary arteries (10;11) whereas the endothelial cells use the subepicardial space as a scaffold to reach the myocardium (8;12). The process of coronary vascular formation consists of several time dependent stages of ingrowth, first into the myocardium and secondly into the aorta (8;13;14) as well as into the right atrium (15). The ingrowth and normal patterning of the coronary vasculature is also influenced by autonomic nervous system derived from neural crest cells (16;17).

In this paper we will present experimental data on manipulation of the epicardial cells as well as neural crest cells on coronary vascular development and will try to relate these to abnormalities seen in the human situations.

Experimental design

I. *Mechanical inhibition of epicardial outgrowth*

The pro-epicardial organ (PEO) starts to grow out at HH15/16 in a chicken embryo. Thereafter this layer shows epithelial to mesenchymal transformation (EMT) leading to the formation of epicardium derived cells, so-called EPDC. We have followed normal timing of migration and ingrowth of the EPDC by making quail-chicken chimeras in which we implanted a quail PEO next to the host chicken PEO (8;15). The next step was to initiate complete to partial inhibition of outgrowth of the PEO both in chicken and quail embryos at HH15-17 following a technique described by Männer (18). The last approach in this mechanical inhibition experimental set-up was to rescue the phenotype by grafting a quail PEO into the chicken PEO inhibited embryo.

All embryos were studied by serial sectioning and immunohistochemical staining. The quail-specific antibodies QCPN (all quail) and QH1 (anti-endothelial specific) were essential for the evaluation of our data (10).

II. *Anti-sense Ets 1/2 inhibition of epicardial to mesenchymal transformation.*

In these experiments the asEts construct was injected into the embryonic bloodstream which resulted in a diminished EMTof specifically the epicardial layer (19). The phenotype was relatively mild so that coronary vascular formation at HH30 and HH35 could be studied. The technical procedures were as described for the mechanical inhibition model.

III. *Cardiac neural crest ablation embryos*

In these chicken embryos the cardiac neural crest has been ablated at HH10 as designed and described by the group of Kirby (20). In the resultant hearts with a common arterial trunk we studied the coronary vascular pattern as well as the neural crest derived ganglia.

Results

Normal epicardial and coronary development

Normal migration of epicardial cells over the myocardial surface takes place between HH15 and HH24/25 after which there is a complete covering of the heart. At HH24/25 the epicardial cells, that start their outgrowth at the venous pole, have reached the myocardial to arterial boundary of the outflow tract. The arterial pole is covered by a mesothelial layer referred to as arterial epicardium, which is a more cuboidal phenotype as compared to the the squamous venous pole derived epicardium. EMT is seen first in the inner curvature and already at HH19 the first differentiated endothelial cells are encountered in that area. Marked endothelial to myocardial ingrowth over the heart is seen between HH25 to HH29 accompanied by the formation of an intramyocardial network (Figure 1a) that is connected to the sinus venosus of the heart. At HH32 ingrowth of endothelial cells into the aorta is seen, through an adventitial area that is particularly rich in apoptosis (21). At the same time ingrowth is seen ventrally into the right atrium. Both sites show neural crest derived ganglia (22).

After start of the systemic circulation through the coronary vasculature the EPDC are recruited into the smooth muscle cells (SMC) and the fibroblasts of the coronary vasculature during the process of arteriogenesis (10;11).

In all experiments there are scarce connections between the endocardially lined ventricular intertrabecular spaces and the developing coronary vascular system. A schematic overview is presented in Figure 2a-d?

Coronary developmental abnormalities

In the mechanical inhibition model it was shown that complete inhibition resulted in embryonic lethality at HH28/29 before a coronary vasculature was established. The partial inhibition leads to embryos that survived these stages and lived up to HH35 to HH40, clearly showing a more normal coronary vascular phenotype with longer duration of survival. For the present study HH35 embryo were of most interest as they clearly showed both abnormalities of the main coronary arteries as well as of the ventricular intramural vascularisation.

The main coronary arteries presented as a single artery, pin-point orifices or completely absent main artery connections. In the latter cases we showed the existence of smaller and larger VCAC (Figure 1c). In areas with very thin to absent epicardium there was clearly diminished ventricular wall vascularisation (Figure 1b). It is of interest that our so-called rescue model resulted in a similar phenotype as the partial inhibition model that resulted from a delay of the outgrowth of the PEO graft.

The anti-sense Ets 1/2 model effectually showed the same phenotype as the mechanical inhibition model. Also these cases presented with absent main coronary artery connections and a diminished intramyocardial vascularisation. We could, however, find small VCAC, that compensated for the lack of main coronary flow.

In the neural crest ablation group we found, as expected, absent aortic to pulmonary separation resulting in a common arterial trunk overriding a ventricular septal defect. In these cases coronary vascular ingrowth was seen but was abnormal in that both pin point orifices and single main coronary arteries were observed. The neuronal ganglia that under normal circumstances closely adhere to a main coronary artery were not absent but showed an irregular pattern with several small ganglia.

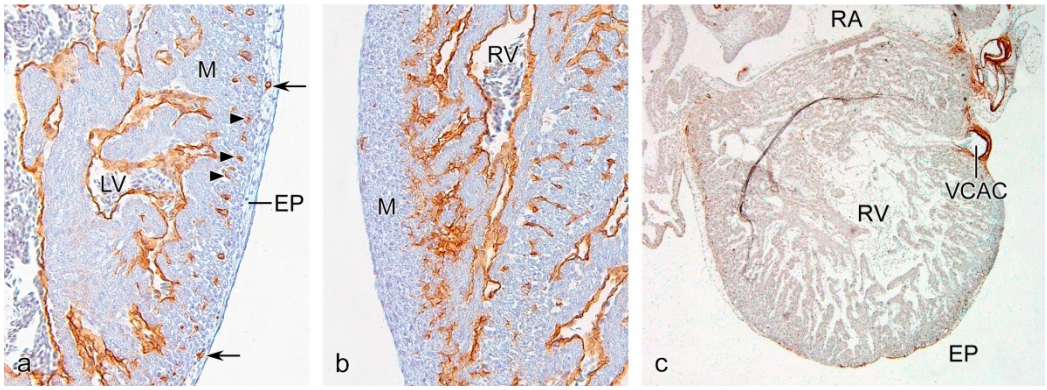

Figure 1. Sections of quail and chicken-quail chimera hearts to show aspects of normal and abnormal coronary vascular formation

a. Sagittal section of the left ventricular wall of a PEO inhibited HH30 quail heart that has been stained with the quail endothelial specific QH1 marker. Overlying the myocardium (M) is the epicardium (EP), containing a few endothelial cells (arrows). The first development of the intramyocardial coronary vasculature is seen as a row of small capillaries (arrowheads) that will form a vascular network. The endocardial lining of the intertrabecular spaces is also QH1 positive. Under normal circumstances there are no connections between the two systems.
b. Sagittal section of the same heart as depicted in (a) but at a site where there is no epicardial covering of the myocardium. The intramyocardial network is not developing at this site and the myocardium is dependent on the intertrabecular spaces for its perfusion
c. Sagittal section through a HH35 chicken heart in which a rescue was performed with a quail PEO after ablation of the chicken PEO. The brown staining is with the all quail QCPN antibody. This shows a thin, quail derived epicardial layer. The coronary vasculature is almost absent and no main coronary arteries were found. The circulation is maintained through a large ventriculo-coronary-arterial-communication (VCAC). It is clear that the wall of this VCAC, inclusive the smooth muscle cells, is made up of epicardium derived cells.

Discussion

Data over the recent years from many studies have shown that development of the coronary vascular system is highly dependent on a proper interaction of epicardium and myocardium. Our studies, that mainly deal with the avian system have clearly shown that hampering epicardial outgrowth is deleterious for normal development (19;23). There are also mouse models that support the phenomena such as the VCAM1-/- (24) and more recently the FOG2 -/- (25) mouse. These models, however, as the phenotype cannot be controlled, lead to early embryonic lethality and have not been able to show the formation of VCAC and aberrant main or absent coronary arteries (19;23).

Coronary vascular ingrowth is of course also dependent on growth factors such as VEGF, PDGF, TGFβ and knock out models show abnormal vascular differentiation with less well stabilized arteries (26) but no phenotypes that compare to the main abnormalities encountered in human congenital heart disease. We therefore assume that mechanisms underlying these abnormalities might in several of these cases clearly relate to insufficient contribution of extracardiac cell sources such as neural crest cells and EPDC.

The following morphogenetic concepts for a number of human coronary abnormalities are devised on this basis and are exemplified below.

1. *Pulmonary atresia without VSD*

In this heart malformation we are most probably dealing with two diseases. First of all those cases that do not present with VCAC. It is expected that during development the pulmonary orifice becomes atretic leading to an increased right ventricular pressure with as resultant marked endocardial fibro-elastosis formation (Figure 2e). This can also lead to myocardial fibrosis and pathology of the subendocardial coronary vasculature but the main coronary arteries have a normal histology (5;27). The function of the right ventricle in dependent on the microvascular pattern and the amount of fibrosis that are clearly altered in these cases (28).This in contrast to cases with pulmonary atresia that do present with marked VCAC. In these cases we have described severe coronary vascular pathology not only at the site of the fistulae but also more distal.

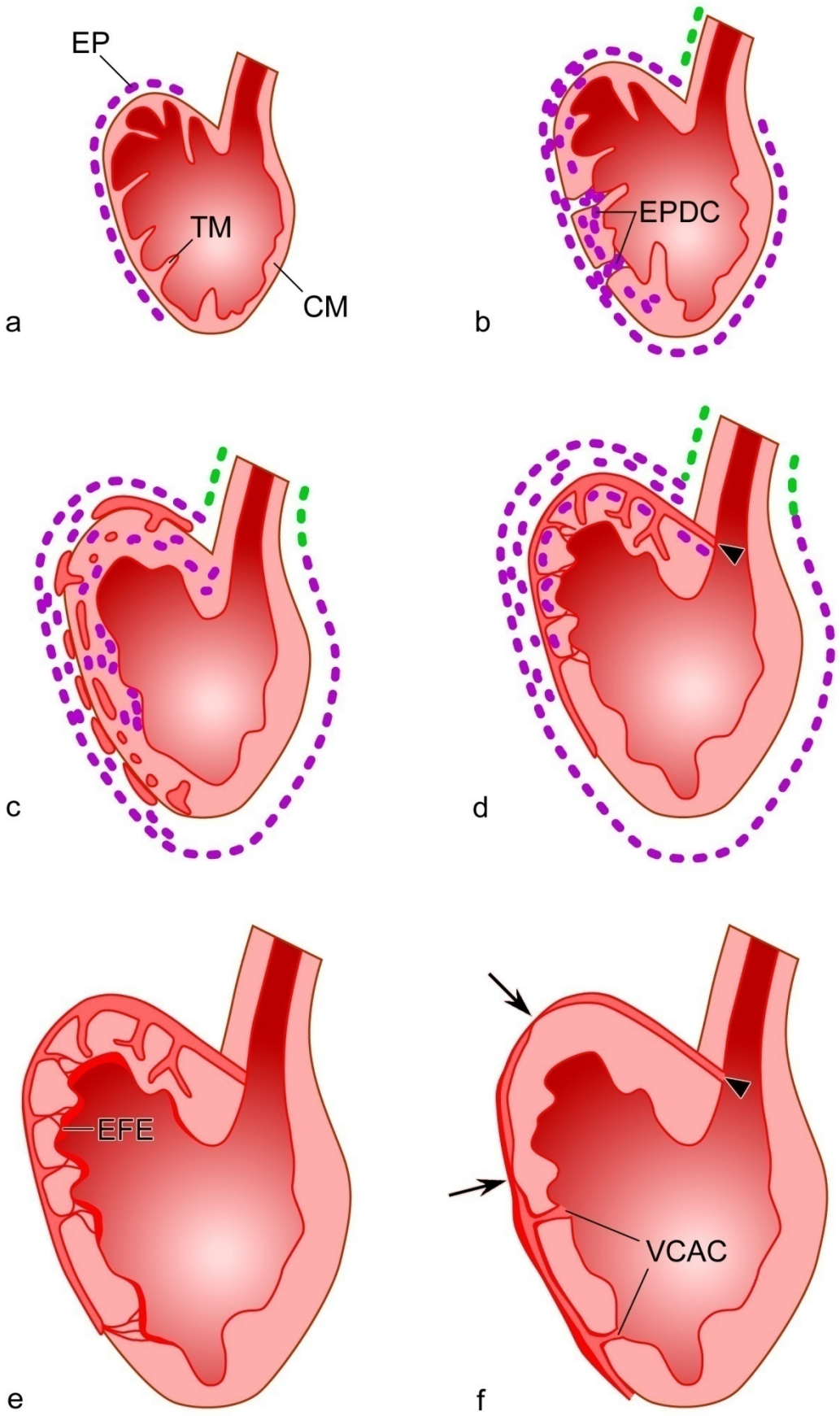
EP
TM
CM
a
EPDC
b
c
d
EFE
e
VCAC
f

Figure 2. Schematic representation of the development of the normal coronary vasculature (a-d) and the situation in the two forms of pulmonary atresia without VSD.

a. Stage HH19: the compact myocardium (CM) is still thin and most myocardium is trabeculated (TM). The overlying developing epicardium is depicted in purple.
b. Stage HH24: through EMT epicardium derived cells (EPDC) migrate into the myocardial layer and through holes in the myocardium into a subendocardial space.
c. Stage HH29: in the subendocardial space and in the myocardium endothelial cells, derived from the PEO are found (red vessel segments). In green the arterial mesothelial collar (arterial epicardium) is indicated. There is no connection yet of the coronary vessels with the aorta. The myocardial holes have disappeared with growth of the compact myocardium
d. Stage HH32: the coronary vasculature has connected to the aorta (arrowhead) and also to the right atrium (not shown). The process of arteriogenesis starts with formation of EPDC derived smooth muscle cells around the arteries. The media of the veins forms much later in time
e. Schematic representation of the ventricle of a case with pulmonary atresia without VSD and no VCAC. The scheme is theoretical as the myocardial pathology shows the situation in the right ventricular wall, while the artery coming of the ventricle is the aorta showing the ingrowth of the main coronary artery. The message is that increased ventricular pressure has lead to development of endocardial fibroelastosis (EFE), while the main and the intramyocardial coronary arteries are not severly damaged. The myocardium of the right ventricle is not optimally perfused as there is a marked increase of fibrosis (not shown).
f. Schematic representation of the ventricle of a case with pulmonary atresia without VSD and VCAC. The scheme is theoretical as the myocardial pathology shows the situation in the right ventricular wall, while the artery coming of the ventricle is the aorta showing the ingrowth of the main coronary artery, which can show abnormalities such as a pin-point orifice(arrowhead). The message is that there is no increased ventricular pressure as the blood can move to and fro through the large VCAC's. The coronary artery wall at the connection site is severly thickened and dependent on peripheral coronary occlusions the circulation becomes VCAC dependent. These cases show less fibrosis in the myocardium

These can comprise complete obliteration of the vessels. It remained an enigma how to explain these two variant of the disease until in-utero fetal diagnostics revealed the existance of cases with VCAC and a still patent pulmonary orifice that became atretic over time (6;29). We therefore postulate that abnormal epicardial and coronary formation is the primary culprit in this disease. Histopathology of the fetal cases showed that the lining of the ventricular intertrabecular connections were actually normal, while the fetal main coronary arteries were diseased and could actually be obliterated (27). To distinguish the two diseases might have implications for the set-up of intrauterine therapy and the possibility to prevent and diminish development of serious coronary vascular disease

2. *Hypoplastic left heart syndrome (HLHS)*

A pathogenetic mechanism leading to this disease is still missing but it is clear that in the cases with mitral stenosis, an atretic aortic orifice and the rudimentary left ventricle there is a high risk for coronary vascular problems. Earlier studies from our group showed that there are most often two main coronary arteries connecting to the hypoplastic aorta and that patterning is essentially normal. In cases with both aortic and mitral atresia the coronary circulation is retrogradely fed through the aorta and within the left ventricle no high pressure is built up. The vasculature in these cases is essentially normal (30). This in contrast to cases with aortic stenosis and mitral atresia where, like in pulmonary atresia without VSD and without VCAC, the high pressure build up leads to marked endocardial fibroelastosis and subendocardial coronary vascular pathology (30). This situation resembles the cases with pulmonary atresia without VSD and no VCAC. We have also looked at the microvasculature of the right ventricle , which is the functional ventricle in the HLHS. In contrast to cases with pulmonary atresia, in the HLHS there are numerous small capillaries and small cardiomyocytes. At birth the myocardium does not show marked fibrosis (31). We assume that in the HLHS the primary problem is not epicardially dominated but is situated within the myocardium and the valve formation.

3. Coronary vascular mainbranch variation in TGA

In TGA we encounter a large number of variations in main coronary artery branching (32). Clinically, knowledge of the variations is very relevant as the arteries need to be translocated during the arterial switch procedure (1). We still have insufficient insight in the pathomorphogenesis of TGA but current models point towards an outflow tract non- rotation problem. It is still unsolved why coronary arteries home to the aorta under all circumstances and the major deviations in main branch positions can be explained by the shortest course from the subepicardial region to the site of ingrowth. These sites are always present in the two facing (towards the pulmonary orfice) sinusses of the aorta. It is remarkable that adjoining the main stems and orifice always a neuronal, neural crest derived, ganglion is formed. There are no data up till now that we know of that indicate

whether neural crest derived ganglia are normal or abnormal in the few mouse models that have been described for TGA (33). The absence of orifices or pin-point orifices can be explained by a disturbed epicardial outgrowth over the non- rotating outflow tract.

4. *Common arterial trunk and coronary abnormalities*

In common arterial trunk in the neonate we are confronted with a common arterial orifice which can harbour a varying number of semilunar valves. In most cases we are dealing with four leaflets and not the embryologically expected six leaflets. From a study of the position of the coronary orifices in these hearts we know that there are often abnormalities such as pin-point orifices and abnormally high take offs. The latter is of significance for surgery where a separate pulmonary trunk is created and the high positioned coronary orifices should be avoided during translocation of the right and left pulmonary artery (34). It is remarkable that a quarter of the orifice circumference is never seen to possess a coronary orifice, which might indicate that even in this anomaly there is an aortic (coronary) and a pulmonary (non-coronary) side of the orifice. This is supported by our experimental studies where we ablated the neural crest in chicken embryos. In these studies a common arterial trunk develops as the outflow tract septum does not or inproperly develop. We have shown that in fact the situation in the human neonate is recapitulated in these embryos, as we find a similar variation in orifice position and hight in these embryos. The main branch anomalies consisted of a single coronary artery to pin-point orifices. As we could aways locate the aortic and the pulmonary side we also could conclude that the coronary orifices were confined to the aortic side (17).

We can speculate about the role of the neural crest cells and the role of the EPDC in these cases. We know that neural crest cells are important for outflow tract development and septation and we could postulate an disturbed epicardial covering of the outflow tract resulting in the main branch coronary anomalies.

In conclusion we can state that we have provided experimental evidence for the important role of EPDC as well as neural crest cells in the formation of the coronary vascular network. Defective interaction of both cell types with each other and/or the underlying myocardium and vascular wall can lead to coronary vascular abnormalities. Our current defective knowledge of the pathomorphogenesis of congenital heart malformations needs to be extended to fully understand the impact of the above findings and perhaps use it in treatment or prevention of malformations.

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Chapter 3

Myocardial heterogeneity in permissiveness for epicardium-derived cells and endothelial precursor cells along the developing heart tube at the onset of coronary vascularization.

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Abstract

The coronary vasculature develops from mesothelial and endothelial precursor cells (EPCs) derived from the proepicardial organ (PEO), that migrate over the heart to form the epicardium. By epithelial-mesenchymal transition, the subepicardium and epicardium-derived cells (EPDCs) are formed. EPDCs migrate into the myocardium where they differentiate into smooth muscle cells and fibroblasts, that stabilize the developing coronary vasculature and contribute to myocardial architecture. Complete PEO-ablation results in embryonic lethality due to cardiac defects, including a looping disorder with a too wide inner curvature.
To investigate the behaviour of early coronary contributors, we analyzed normal quail embryos and found lumenized endothelial vessels in the subepicardium already at stage HH19. Furthermore, EPCs had penetrated into the myocardium of the inner curvature. To confirm that the myocardium of the inner curvature is specifically permissive for EPCs and to study early EPDC migration in more detail, chimeric chicken embryos harbouring a quail PEO were analyzed. Lateral epicardial outgrowth and EMT were observed throughout, but migration into the myocardium was restricted to the inner curvature between HH19-22. The permissive myocardial area expanded to the atrium, atrioventricular canal and trabeculated ventricle at stage HH23-24. In contrast, outflow tract myocardium was never found to be permissive for EPDCs and EPCs untill HH30, not even when the quail PEO was attached directly onto it.
We conclude that early coronary formation starts in the inner curvature, and hypothesize that the presence of PEO-derived cells is essential for the maturation of the inner curvature and subsequent looping of the heart tube.

Introduction

Heart development starts with the formation of a simple tube, consisting of an inner endocardial layer and an outer myocardial layer, with cardiac jelly in between. With increase in size and functioning of the heart, the development of a coronary circulation becomes necessary. Coronary formation is classically divided in primary formation of the endothelial network, during the process of angiogenesis and in a secondary stabilization of the vessel wall by smooth muscle cells (SMCs) and pericytes during the process of arteriogenesis. Both processes are initiated by the development of the proepicardial organ (PEO) and the epicardium during the late looping phase of the tubular heart. The PEO can be seen as villous protrusions from the ventral wall of the sinus venosus, and consists of mesothelial, mesenchymal, and endothelial (precursor) cells[1] . From the proepicardial villi, cells transverse the pericardial cavity along extracellular matrix bridges[2] and attach to the dorsal side of the developing ventricle [3], where they start spreading over the myocardial surface of the heart tube, to form the epicardium [4]. By epithelial-mesenchymal transition (EMT) of epicardial cells, the mesenchymal subepicardium is formed. Cells from this layer, the so-called epicardium-derived cells (EPDCs), migrate and differentiate into coronary smooth muscle cells and adventitial fibroblasts to stabilize the developing coronary vessels [5], and into interstitial fibroblasts to give rigidity to the myocardium [6]. Furthermore, they migrate to the endocardial cushions of the atrioventricular canal [6]. The importance of proper development of the (sub)epicardium and hence, of correct EPDC deposition and differentiation is underlined by an increasing number of studies in which epicardial development was manipulated, either mechanically [7] or genetically [8-12]. The spectrum of cardiac defects ranges from severe abnormalities resulting in embryonic lethality, *e.g.* with very thin myocardium, absence of coronary development and abberant cardiac looping in quail embryos with a completely ablated PEO [7], to milder disturbances as diminished coronary SMC deposition and abnormal patterning of coronary arteries in antisense Ets-treated chicken embryos [12].

Whereas the migration patterns of the proepicardium and their derivative mesenchymal EPDCs have been studied quite extensively (for a recent review see [13], detailed studies on the developmental behaviour of the proepicardial endothelial (precursor) population during early epicardial and coronary development have begun to emerge only recently (see e.g. [14]. We therefore analyzed the initial events of coronary endothelial (precursor) cell migration at the onset of coronary development.

Materials and Methods

Normal and chimeric embryos

Normal endothelial (precursor) cell behaviour was studied in Japanese quail (*Coturnix coturnix japonica*) embryos. To study EPDC and endothelial (precursor) cell migration in more detail, chimeras were generated using White Leghorn chicken (*Gallus domesticus*) embryos as hosts and quail embryos as donors. Embryos were staged according to the criteria of Hamburger and Hamilton [15].

Quail-chicken chimeras were made, basically according to the chimerisation technique as described by Poelmann et al. [16]. In brief, the proepicardium of an Hamburger and Hamilton stage 15-18 (HH15-18) quail embryo was isolated together with a tiny piece of liver tissue, to provide endothelial precursor cells to the proepicardial transplant. The isolated proepicardium and adjacent liver tissue were transplanted into the pericardial cavity of a HH15-18 chicken host embryo through the naturally occurring hiatus in the body wall that exists until HH18. The transplanted tissue was positioned at different sites along the developing heart tube: adjacent to the sinus venosus and atrial region, in the inner curvature or dorsally of the ventricle (Figure 1). Chimeras were harvested between stage HH19-30. The number and characteristics of successful chimerisations, verified by a specific quail nuclear immunohistochemical staining with QCPN (see below), are listed in Table I.

Histological procedures

Unless indicated otherwise, tissue processing and immunohistochemistry were as described earlier [5]. Normal and chimeric embryos were fixed by overnight immersion in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) at 4°C. Serial sections were immunostained with the monoclonal antibodies QCPN (Hybridoma Bank Baltimore, MD, USA; diluted 1:4), QH1 (Hybridoma Bank; diluted 1:500) and HHF35 (Dakopatts M635, Glostrup, Denmark; diluted 1:500) that recognise specifically quail cell nuclear antigen, quail endothelial (precursor) cells and muscle actins, respectively. For the QCPN staining, antigen unmasking was applied, by incubating the sections for 3 x 4 minutes in 0.01 M citrate buffer (pH 6.0) at 98°C in a microwave. Sections were examined by light microscopy. An Olympus AX-70 light microscope in combination with an Olympus DP-12 digital camera was used for microphotography.

Definitions

QH1-positive cells.

The QH1 antibody recognises specifically quail endothelial cells and their precursors. As was defined earlier [16], only QH1-positive cells that line a lumen are considered to be true endothelial cells. Dispersed single or clustered cells located in either mesenchymal or mesothelial regions of the (sub)epicardium, or the myocardium are considered as endothelial precursor cells when they do not line a lumen, but have already a relatively flat, endothelial cell-like appearance. Isolated round and relatively large QH1-positive cells are considered to be cells of the haematopoietic lineage (hemangioblasts) that can give rise to several blood cell types, and to endothelial precursor cells [17]. QH1 also recognises endocardial cells. Thus, it is impossible to discern PEO-derived endothelial (precursor) cells from endocardial cells originating from the primary heart field by staining quail embryonic sections with QH1.

PEO, epicardium, subepicardium and EPDCs

The PEO is defined as the cauliflower-like protrusion that sprouts from the sinus venosus, traverses the pericardial cavity and attaches to the myocardium of the ventricle. The PEO contains a variety of cell and tissue types, *viz.* endothelial (precursor) cells, capillaries, epithelium and mesenchyme. Once the PEO has attached to the myocardial surface, the outer mesothelial layer is defined as the epicardium. The subepicardial mesenchymal cells, originating from the epicardium by EMT, form the subepicardium. EPDCs encompass all cells derived from the epicardium, that is, the subepicardial mesenchymal cells and their derivatives (smooth muscle cells and interstitial fibroblasts).

Results

Early endothelial (precursor) cell migration in normal quail embryos

To study the earliest events in coronary formation, we analysed PEO-associated endothelial cell migration in normal quail embryos between HH18-27. As can be seen in Figure 2A and 2B, endothelial precursor cells were present in the mesothelial epicardium and in the mesenchymal subepicardium already at stage HH17+. The close juxtaposition of the liver primordium and the proepicardium, clearly visible in transverse sections of HH17 and HH21 embryos (Fig. 2A and 2D), suggests that the liver, which is a hemangioblastic organ at this age [18], is the primary source of these proepicardial endothelial precursor cells. Migration of the EPCs through the proepicardial organ results in the formation of lumenized endothelial capillaries already at stage HH19 (Fig. 2, C and D). These early capillaries form in the subepicardium that is located dorsally of the primitive atrioventricular canal and ventricle, at the initial attachment site of the PEO. In this lumenized vessel network erythrocytes were found only sporadically in 6 quail embryos (HH20-22; see Fig. 2D). Blood vessels containing notable amounts of erythrocytes were observed for the first time in the subepicardium covering the dorsal side of the ventricle and atrioventricular canal at HH24.

The earliest myocardial invasion by endothelial precursor cells was observed from HH20 onwards, be it not in the region of this early vessel network, but in the inner curvature (Fig. 3). As was deduced from the position of these endothelial precursor cells in serial sections, they were derived from, or connected to the endothelium of the more caudally located subepicardial capillaries. Because the QH1 antibody stains endocardial cells equally well, it appeared to be difficult to discern PEO-associated endothelial precursor cells in the myocardium from the endocardium-lined ends of the trabecular lumina.

Quail-chicken chimeras

To circumvent the difficulty that endocardial cells closely resemble endothelial (precursor) cells in an immunohistochemical QH1 staining, chimeric embryos were generated, in which the PEO of a quail embryo was transplanted to the pericardial cavity of a chicken embryo of equivalent age. In the resulting quail-chicken chimeras, QH1 could react only with the PEO-associated endothelial cells, leaving the chicken-derived endocardial cells unstained. A developmental series of quail-chicken chimeras (HH19-30) was made, in which the donor PEO was placed at several sites along the developing heart tube at HH16-17 (Fig. 1, Table I), just at the timepoint when proepicardial cells start to transverse the pericardial cavity to reach the heart. The chicken host PEO was fully preserved. Thus, transplantaton did not interfere with the endogenous outgrowth of the chicken PEO and the heart could develop normally [16]. This could also be deduced from the fact that the embryos had normal heart morphology.

With respect to the site of transplantation, we observed that PEO attachment to the heart and epicardial outgrowth succeeded in 31 out of 56 surviving embryos when the PEO was placed adjacent to the sinus venosus or in the inner curvature of the looping heart. In contrast, PEOs transplanted dorso-caudally of the ventricular myocardium did not attach in 15 out of 25 cases, or attached to the liver in the remaining 10 embryos. This may be indicative for a limited capacity of the ventricular myocardium to attract PEO-derived cells to its surface.

The site and extent of quail epicardial outgrowth in the embryos where the PEO had attached well has been summarized in Table II. The presence of a subepicardial mesenchymal layer was a prerequisite for the ingrowth of PEO-derived cells into the underlying myocardium. More surprisingly, we observed that only the myocardium of the inner curvature was permissive for endothelial precursor cells and mesenchymal EPDCs between stage HH19 and HH22 (Fig. 4). The myocardium of the atrioventricular canal and that of the outflow tract were not invaded by PEO-derived cells, not even when a multicellular layer of subepicardial mesenchyme was present. The myocardium of the inner curvature is not covered with cushion tissue at its luminal side. However, PEO-derived cell invasion of the myocardium was not directly related to the absence of cushion tissue, since it was also observed in cushion-bearing myocardium of the atrioventricular canal at later stages of development (not shown). From HH23 onward, PEO-derived cells were also observed in the myocardium of the atria (Fig. 5 A-C) and in the ventricular myocardium (Fig. 5 D and E). The myocardium of the outflow tract was not permissive for EPDCs, at least untill HH26. Only at HH30, EPDCs were observed in the outflow tract myocardium upto its most distal border (Fig. 5H-J).

From our set of quail-chicken chimeras we could not precisely infer whether the endothelial precursor cells and EPDCs reached their intramyocardial locations only via the epicardium on the outside, or also by spreading

within the myocardium. Only sporadically quail-derived EPDCs were observed in the myocardium underneath a layer of chicken-derived epicardium, and most quail EPDCs were deposited in the myocardium beneath a quail-derived QCPN-positive epicardial region. Figures 5D and E illustrate how an epicardium rich in quail cells delivers many quail EPDCs to the underlying myocardium, whereas an epicardium with less quail cells covers a myocardium in which less QCPN-positive EPDCs are present. Thus, endothelial precursor cell and EPDC migration from the epicardial to the myocardial layer seems to be a quite local process. Along the heart tube, the endothelial QH1 staining in the myocardium colocalized with the nuclear signal of the QCPN-positive quail cells in a large majority (95%) of the embryos, indicating that the entrance of endothelial precursor cells into the myocardium might precede that of mesenchymal EPDCs. EPDCs penetrating beyond the compact myocardium, into the trabecular myocardium and subendocardial space, were mostly found underneath those regions of the compact myocardium that were supplied well with endothelial (precursor) cells (Fig. 5, F and G).

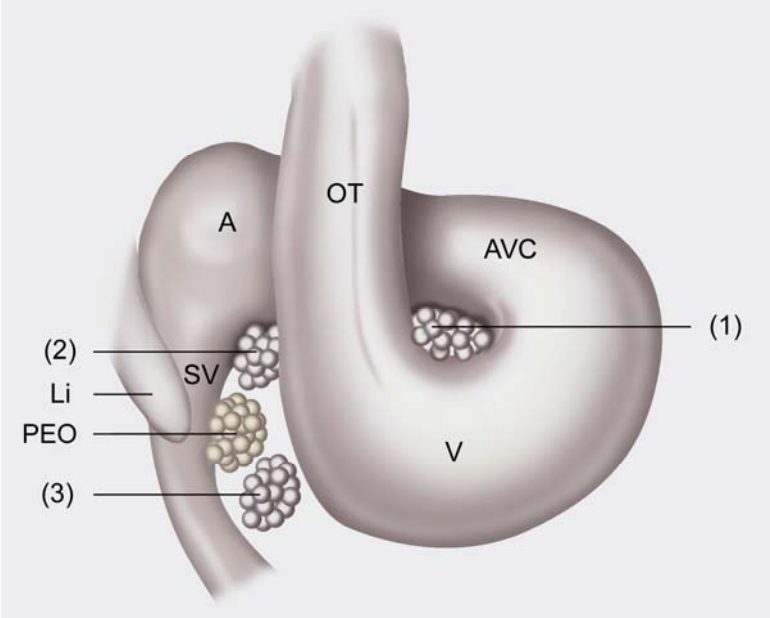

Figure 1: Schematic drawing of the looped heart tube of an HH17 chicken embryo, illustrating the sites to which quail PEOs were transplanted in order to generate the quail-chicken chimeras described in Table 1. PEOs of equivalent age were transplanted to either (1) the inner curvature, (2) a site adjacent to the sinus venosus, "on top" of the endogenous PEO or (3) a site dorso-caudal of the developing ventricle of HH15-17 chicken embryos. A, atrium; AVC, atrioventricular canal; Li, liver; OT, outflow tract; SV, sinus venosus; V ventricle.

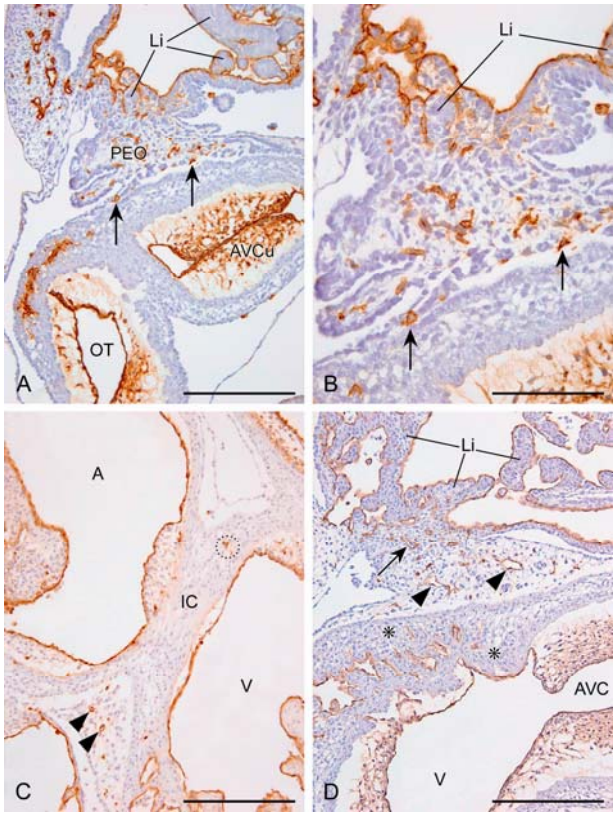

Figure 2: Early angiogenesis visualized by QH1 staining in the epicardium of quail embryos at stage HH17 (transverse sections in ***A*** and ***B***), HH19 (sagittal section in ***C***) and HH21 (transverse section in ***D***). At HH17+ endothelial (precursor) cells, indicated by arrow in ***A*** and ***B***, are already present in the proepicardial organ. Given the close proximity of the liver (Li) and the PEO, (***A***, ***B*** and ***D***), these endothelial cells are most likely derived from the hematopoietic liver tissue. A few endothelial precursor cells have migrated into the myocardium of the inner curvature (IC; encircled in ***C***). Lumenized endothelial vessels are observed from HH19 onwards (arrowheads in ***C*** and ***D***). An arrow indicates a lumenized vessel filled with erythrocytes in a transverse section of an HH21 quail embryo in ***D***. An endothelial capillary network, probably continuous with the developing liver sinusoids, is present in the subepicardium at the site where the PEO attaches to the myocardium. The QH1-positive cells in the myocardium (asterisks) are endocardial cells lining the trabecular lumina. Graft-derived endothelial precursor cells do not enter the myocardium in this region. Bars, 100 μm (***A***, ***C*** and ***D***), 50 μm (***B)***

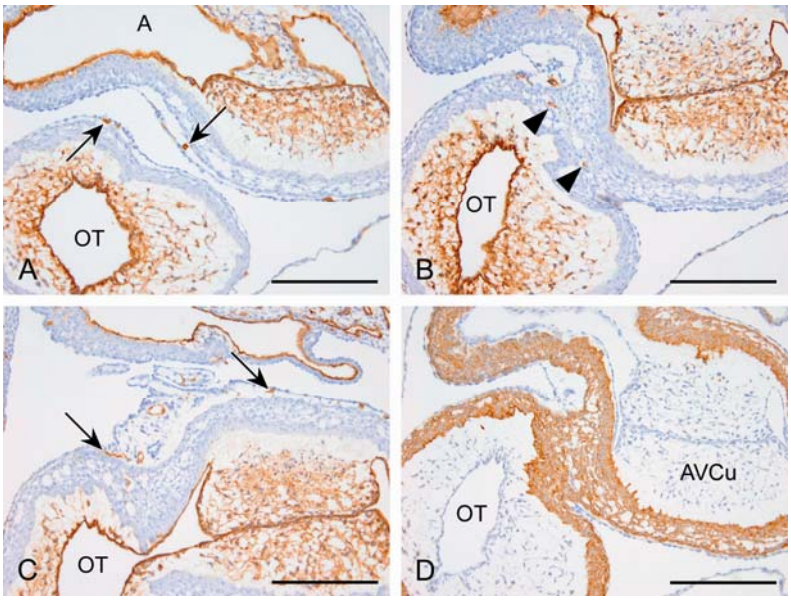

Figure 3: Series of transverse sections of a stage HH20+ quail heart, stained with QH1 to demonstrate endothelial (precursor) cells (***A***, ***B***, ***C***), and with HHF35 to delineate the myocardium (***D***, consecutive section to ***B***). Endothelial precursor cells invading the myocardium were seen only at the site of the inner curvature (arrowheads) are shown in ***B.*** Endothelial precursor cells in the subepicardium surrounding the outflow tract and atrioventricular canal do not enter the myocardium (arrows in ***A*** and ***C***). A, atrium; AVCu, atrioventricular cushion; OT, outflow tract. Bars, 100 μm.

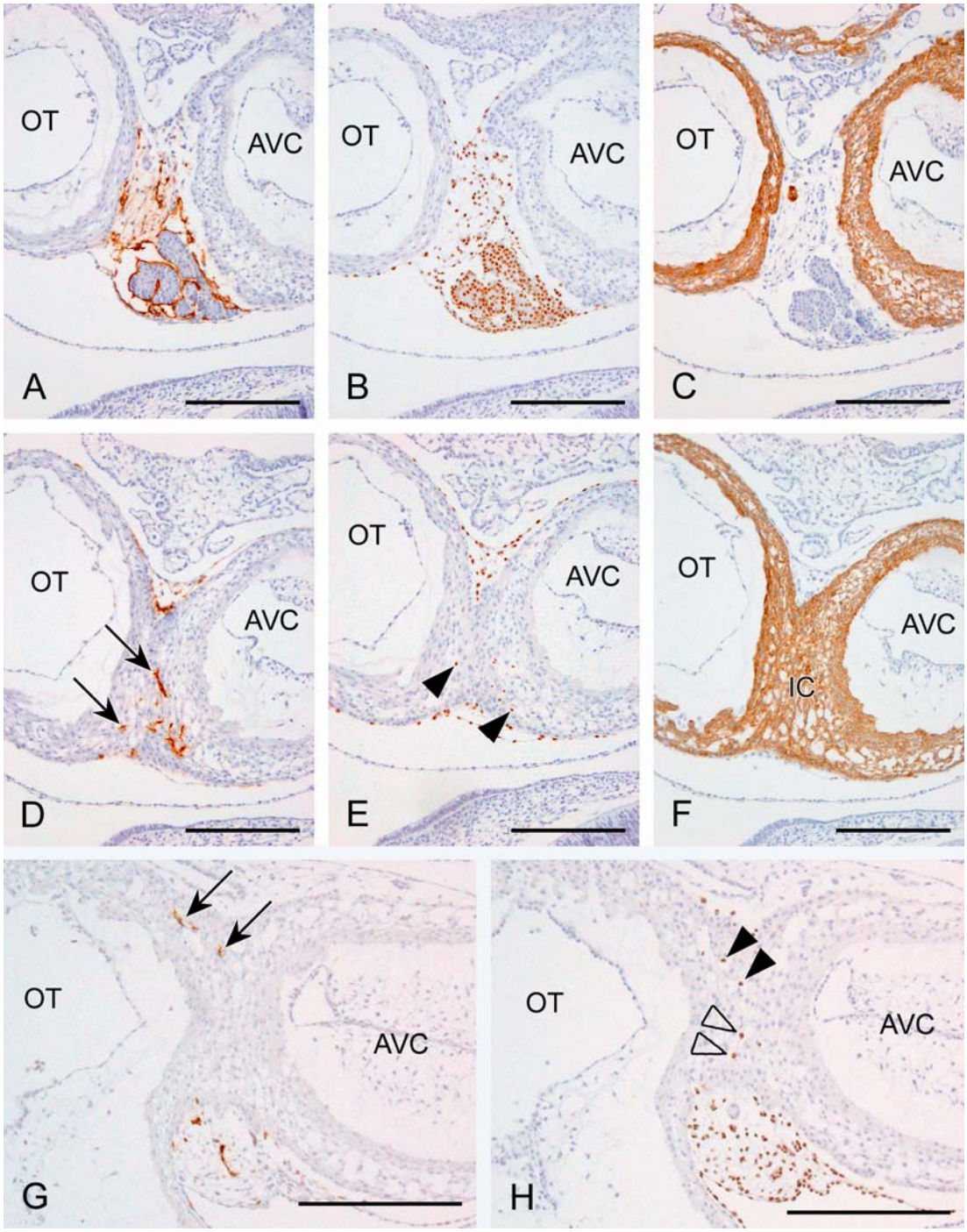

Figure 4: Serial sections of a stage HH19 (***A-F*)** and HH21 (***G,H***) quail-chicken chimeric embryos in which a quail PEO (HH16) was transplanted to the inner curvature of a chicken host (HH15). Sections were stained with QH1 for quail endothelial (precursor) cells (***A***, ***D, G***), with QCPN for quail-derived cells (***B***, ***E, H***) and with HHF35 to demarcate the myocardium (***C***, ***F***). Note that PEO-derived cells do not migrate into the myocardium of the atrioventricular canal (AVC) and that of the outflow tract (OT) in the upper panel. The lower panel depicts serial sections 35 μm more caudally, and shows that only the myocardium of the inner curvature (IC), is permissive for endothelial precursor cells (arrows). QH1 and QCPN staining (arrowheads) mostly colocalize (see text), indicating that the first cells to enter the inner curvature are endothelial precursor cells rather than mesenchymal EPDCs. However, that not only endothelial (precursor) cells invade the myocardium at these early stages of development is shown in panels ***G*** and ***H,*** depicting serial sections of the inner curvature of the HH21 chimeric embryo that received a quail PEO-graft with the most minimal piece of liver. Arrows and closed arrowheads indicate endothelial (precursor) cells; open arrowheads indicate mesenchymal EPDCs. Bars, 100 μm.

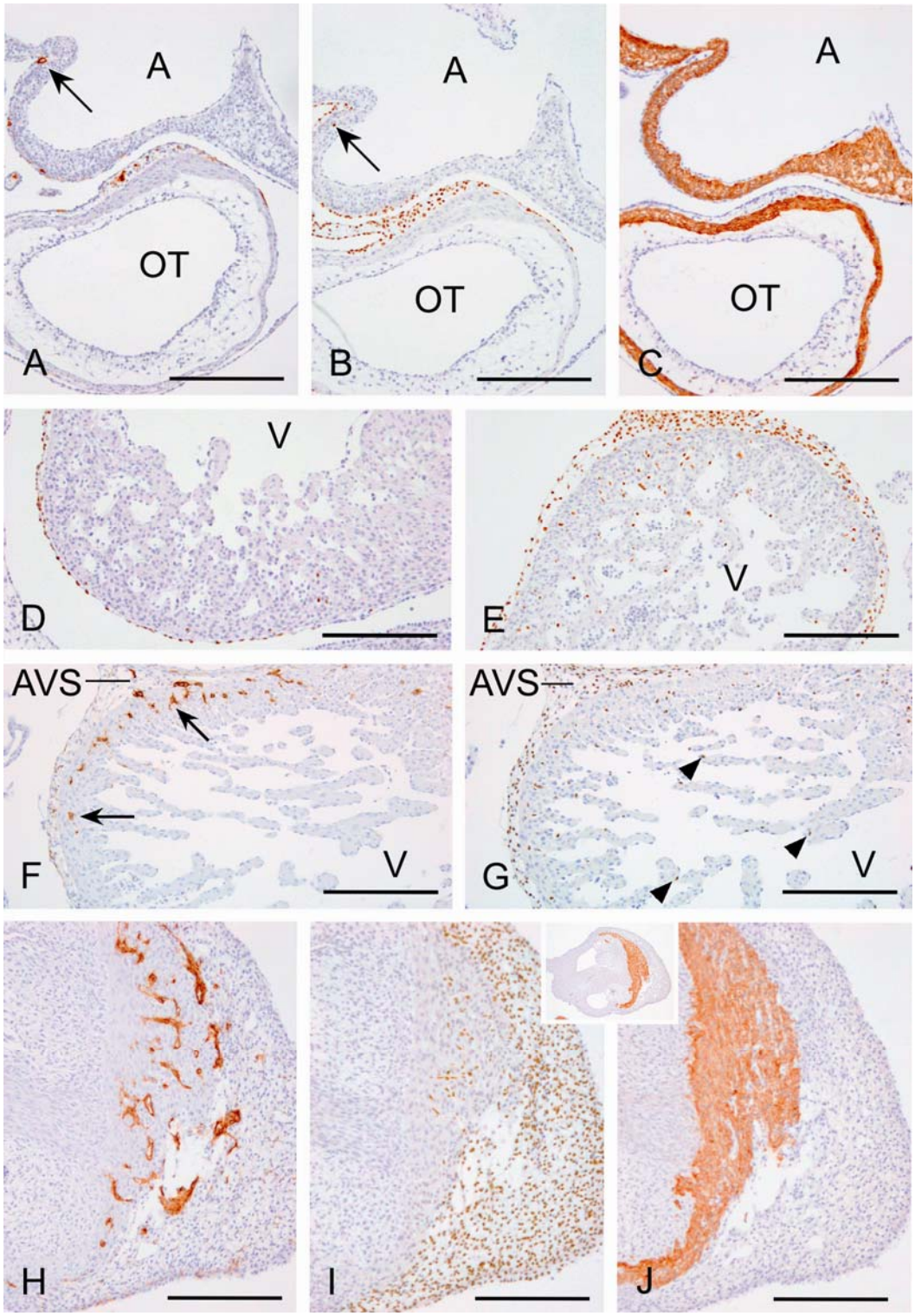

Figure 5: Serial transverse sections of the hearts of stage HH23 (***A-C***), stage HH26 (***D***, ***E***) and stage HH30 (***F-J***) quail-chicken chimeras, stained with QH1 (***A***, ***F, H***), QCPN (***B***, ***D***, ***E***, ***G, I***) and HHF35 (***C***,***J***). Arrows indicate the presence of PEO-derived cells in the atrial myocardium at HH23. The outflow tract myocardium is resistant to invasion by endothelial precursor cells and EPDCs. At HH26, the trabecular myocardium contains EPDCs. This is particularly clearly visible in regions with a well-developed quail-derived subepicardium. Serial sections of the right ventricular wall of an HH30 chimera show that endothelial PEO-derived cells invade the compact myocardium (arrows in ***F***), whereas mesenchymal EPDCs reach also the trabecular myocardium and subendocardial space (arrowheads in ***G***)**.** Only at HH30 quail-derived cells penetrating the outflow tract myocardium were observed (panels ***H-J***). The inset shows that photomicrographs ***H-J*** concern the most distal outflow region, at the myocardial border. A, atrium; OT, outflow tract; V, ventricle. Bars, 100 μm.

Table I. Characteristics of quail-chicken chimeras used in this study. Only chicken embryos in which the PEO was grafted successfully are listed. For the site of transplantation see also Figure 1. Donor PEOs inserted next to the developing ventricle (at position 3 in Fig.1) never attached correctly.

harvesting stage (HH)	stage at time of transplantation (HH)		transplant site	attachment site[1]
	quail PEO	chicken host		
19	16	15	IC	A, AVC, OT
20	17	17	IC	IC, V
20	17	17	IC	OT
20	17	15	IC	A, AVC, OT
20	16	16	IC	AVC, IC, OT
20	15	16	IC	OT
20	15	16	SV	OT
20+	17	16	SV	OT
21	15	15	IC	AVC, IC, OT
21	16	18	SV	A, OT
21	16	16	SV	IC, OT
21	15	18	SV	A
21	18	18	SV	Li
21	17	18	SV	OT
21	15	16	IC	AVC, OT
22	17	18	IC	IC, V
22	17	17	IC	IC,
22+	15	16	SV	A, AVC
23	18	18	SV	A, OT
24	18	15	SV	A, OT
24+	16	17	SV	V
24+	15	17	SV	IC, V
25	17	16	SV	OT
25	15	15	SV	OT
25	17	17	IC	A
25	17	18	IC	IC
25	17	17	SV	AVC, IC, OT, Li,
26	18	16	SV	A, OT
26+	16	16	SV	IC, OT
27	17	17	SV	V
30	16	15	IC	N.D.

[1] A, atrium; AVC, atrioventricular canal; IC, inner curvature; Li, liver; N.D., not determined; OT, outflow tract; SV, sinus venosus; V, ventricle.

Table II. Overview of sites of EMT and PEO-derived cell migration into th e myocardium in the quail-chicken chimeras used in this study. The presence of graft-derived subepicardial mesenchyme (✧) and endothelial (precursor) cell and EPDC migration (●) into the myocardium is indicated. Although a multicellular layer of subepicardial mesenchyme could be present at several sites along the heart tube, before stage 23 ingrowth of PEO-derived cells occurred exclusively in the inner curvature. A, atrium; AVC, atrioventricular canal; IC, inner curvature; OT, outflow tract; SV, sinus venosus; V, ventricle

harvesting stage (HH)	SV	A	AVC	V	IC	OT
19		✧	✧		✧●	✧
20				✧	✧●	
20						✧
20		✧	✧		✧●	✧
20			✧		✧●	✧
20			✧		✧●	✧
20						✧
20+						
21		✧	✧	✧	✧●	✧
21		✧	✧		✧●	✧
21					✧●	✧
21	✧	✧				
21				✧		
21						✧
21			✧	✧	✧●	✧
22				✧	✧●	
22			✧		✧●	
23		✧	✧●		✧●	
23		✧●				✧
24	✧	✧●	✧●	✧●	✧●	✧
24				✧●	✧●	✧
24			✧●	✧●	✧●	
25						✧
25						✧
25		✧●				✧
25		✧●			✧●	✧
25				✧●	✧●	✧
26			✧●	✧●	✧●	✧
26+				✧●	✧●	✧
27			✧●	✧●	✧●	✧
30		✧●	✧●	✧●	✧●	✧●

Discussion

In the present study we analysed the behaviour of early coronary contributors both in normal quail embryos and in proepicardial quail-chicken chimeras. Many aspects of the development of the coronary vasculature have been studied earlier by our group and others (for a recent review see [19]. For example, the intimate topographical relationship of liver primordium and the sinus venosus was documented by Virágh et al. [20]. The liver primordium is highly vascularized with developing capillaries, which, together with the perihepatic mesenchyme, enter the wall of the sinus venosus. It has further been observed that the caudal part of the splanchnic vessel plexus becomes part of the liver sinusoids, and that the more cranial part extends in the direction of the proepicardial organ [21]. Thus, the endocardial cells that make up the capillaries developing in the proepicardial strands can be considered to be continuous with the endothelial cells of the liver sinusoids. Further evidence for a role of liver-derived endothelial (precursor) cells in the development of the coronary vasculature comes from earlier chimera experiments, in which the quail PEO was excised very precisely, and transplanted to the pericardial cavity of a chicken host without any liver tissue. In the resulting embryos, quail-derived endothelial cells were never observed [16]. Although the precise origin of the endothelial (precursor) cells in the PEO is still a matter of debate [14], indirect proof of endothelial (precursor) cell migration through the PEO has been provided recently by the finding that larger numbers of QH1 positive cells were observed at the base of the PEO than in its villous tips in HH17 quail embryos [14]**.**

In earlier studies true vascularization in the subepicardium, defined by the presence of lumenized capillaries, was described not to occur before HH25 [16, 20, 21]. We now show that lumenized endothelial vessels, in tissue sections sometimes observed with an erythrocyte inside, can be found in the subepicardium already at stage HH19. Because some of these were localized close to the developing liver, they may well have been arisen by sprouting from the sinus venosus-liver plexus. Thus, coronary formation at the dorsal side of the developing heart, at the attachment site of the outgrowing PEO, would be initiated by angiogenesis, rather than by vasculogenesis. On the other hand, as was described both in earlier and in more recent publications large hemangioblastic cells were observed in the epicardium, the subepicardium and on the naked heart surface [14, 21]. These are indicative for the *de novo* vasculogenesis that has been shown to be instrumental in coronary development [22].

In the young quail-chicken chimeras (HH19-23), *only* in the inner curvature quail-derived EPDCs were able to migrate further than the subepicardium, and to find a permissive myocardium to migrate into. PEO-transplants located next to the outflow tract, or close to the developing ventricle failed to deposit coronary precursor cells into the myocardial layer of the AV canal, the atria and the ventricles untill HH23/24. To our knowledge, this is the first *in vivo* study demonstrating that the invasion of coronary contributors into the myocardium and thus, the onset of myocardial vascularisation, depends on characteristics of the underlying myocardium. In the avian embryo, the PEO protrudes from the dorsal mesothelium to the dorsal side of the developing common ventricle from where it begins to ensheath the myocardium in the region of the atrioventricular canal. Instead of entering the myocardium in this region, the PEO-associated endothelial cells travel all the way to the inner curvature to initiate the formation of intramyocardial coronary capillaries. In our quail-chicken chimera experiments, quail endothelial precursor cell and EPDC ingrowth started exclusively in the myocardium of the inner curvature, whereas outflow tract myocardium was refractory to this, even when the quail PEO was attached directly onto it. This also confirms the notion that the myocardial microenvironment is an important determinant for endothelial precursor cell and EPDC migration and coronary vessel formation [23, 24]. As for the nature of the myocardial signals we can only speculate. A marked anatomical difference between the myocardium of the inner curvature, compared with that of the atrioventricular canal and the outflow tract, is that there is no endocardial cushion present on the inside. Possibly, angiogenic regulators (inhibitors) secreted by the endocardium or cushion mesenchyme induce the myocardium to be non-permissive for endothelial cells coming from the other side in the AV canal and outflowtract. Good candidates to function as such antiangiogenic regulators are TGFβ2 and TGFβ3. *In vitro* analysis showed that TGFβ1, -2 and -3 inhibit the myocardial signals that regulate epicardial EMT [23]. In chicken embryos, both TGFβ2 and -3 are expressed in the myocardium and endocardium of the AV canal and outflow tract during cushion formation around stage HH17 [25], just preceding the endothelial (precursor) ingrowth of the inner curvature. In mouse embryos strong TGFβ2 expression is seen in the outflow tract and atrioventricular cushions and underlying myocardium between embryonic day (ED) 9.5 and ED12.5, comparable to chicken stage HH20-25. At ED11.5-12.5, epicardial TGFβ2 and -3 expression increases,

especially in the epicardium covering the ventral side of the developing ventricles [26]. This might explain why the subepicardium is relatively thin at this location. After ED 12.5 TGFβ2 expression remains high in the cushion mesenchyme of the outflow tract and AV canal. On the pro-angiogenic side, VEGF becomes upregulated in the atrioventricular field of the heart from ED10.5 onwards [27]. VEGF expression stimulates endothelial proliferation and migration [28], and has been shown to activate the endothelial proteolytic machinery [29]. Thus, the AV region might become prone to invasion by epicardially-derived endothelial and mesenchymal cells, whereas the outflow tract myocardium remains inaccessible until the VEGF signaling routes can be fully employed. In quail embryos, increased endothelial VEGF-R2 and VEGF-R3 expression coincides with the ingrowth of coronary capillaries at the base of the truncus arteriosus to form the coronary plexus that preceeds the formation of the coronary stems [30].

The fact that we did not find myocardial endothelial (precursor) cells and EPDCs of quail origin in regions covered by a chicken epicardium, indicates that myocardial entry of these cells is indeed a very local process, as was already suggested by earlier findings in older chimeric embryos [6]. The local delivery of coronary components only to the underlying myocardium could also be seen in stage HH35 quail embryos in which epicardial outgrowth was partially inhibited. Only the ventricular regions covered with epicardium acquired a compact myocardium with regularly arranged coronary vessels. Naked compact ventricular myocardium was completely devoid of these vessels, indicating that the local presence of coronary precursor cells is a prerequisite for coronary vessel formation (Eralp and Gittenberger-de Groot, unpublished observations).

In the set chimeras that we analyzed, the endothelial (precursor) cells seemed to be the first to enter the myocardium. At later stages, more intramyocardial mesenchymal EPDCs were observed. Only in the ventricular myocardium, where epicardium-derived fibroblasts also contribute to myocardial rigidity, the EPDCs outnumbered the endothelial (precursor) cells from stage HH25/26 onwards. However, in myocardial regions where coronary artery stabilisation is the one of the EPDC functions, many of the proepicardium-derived cells are endothelial cells, which seem to be followed by the mesenchymal EPDCs. It is tempting to speculate that, probably via PDGF-B signaling [31] the endothelial (precursor) cells pave the way for the entrance of the mesenchymal cells that will assist in the formation of stable vessels. Further support for the idea that penetration by endothelial (precursor) cells may facilitate mesenchymal EPDC migration comes from our observation in older chimeras that only in regions where the compact myocardium was supplied with numerous quail endothelial cells, the underlying trabecular myocardium contained many quail-derived EPDCs.

Finally, between HH20-HH24 the inner curvature is not only the site where coronary formation starts, it is also a region that is very much involved in the last phase of the cardiac looping process. During this phase, the proximal two thirds of the outflow tract or primitive conus shifts from the right lateral position to its final position, ventral to the right atrium [32]. In quail embryos in which proepicardial outgrowth was inhibited, cardiac looping was severely disturbed [7]. Although we did not directly address the issue, the present study might give a first concrete clue for a role of the epicardium in the looping process. We hypothesize that the selective ingrowth of proepicardially derived endothelial cells into the inner curvature is the starting signal for the myocardium to rearrange its fibrils and for the conus to shift leftwards.

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Chapter 4

Coronary artery and orifice development is dependent on proper timing of epicardial outgrowth and associated apoptosis patterns

Epicardium mediates coronary development through apoptosis

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Abstract

The proepicardial organ provides differentiated cell types to the myocardial wall and facilitates angiogenesis and arteriogenesis. Ingrowth of the coronary arteries into the aortic wall has recently been linked to apoptosis. This study was set up to examine the effect of an inhibition of epicardial outgrowth on apoptotic patterning and coronary development..
Epicardial outgrowth was blocked at HH15-17 in quail embryos, that survived until HH25-35(n=33). Due to structural defects of the heart, embryos with complete inhibition of outgrowth did not survive after HH29. These embryos presented with thin compact myocardium, devoid of vessels. In embryos with delayed epicardial outgrowth the phenotype was less severe, and surviving embryos were studied up to HH35. In these embryos myocardial vascularization was poor and normally occurring apoptosis in the peritruncal region at HH30 was diminished. Embryos studied at HH35 displayed an abnormal coronary network and absent coronary orifices.
In a further set of experiments (n=10) proepicardial outgrowth was inhibited in chicken embryos at HH15, followed by transplantation of a quail proepicardial organ into the pericardial cavity, in order to rescue the cardiac phenotype. These chimeras were studied at HH29 and HH35. Myocardial development was restored, however in 3 out of 4 rescued embryos (HH35) the coronary orifices were absent.
In the absence of proper timing of epicardial outgrowth, myocardial development and vascularization are disturbed, and apoptosis is diminished, specifically in the peritruncal region. During later development, this leads to defective or absent connections of the coronary system to the systemic circulation.

Introduction In early embryonic life, the primitive heart tube consists of two layers, the myocardium and the endocardium. During development a third layer, called the epicardium, is formed[1]. Epicardial cells are derived from the proepicardial organ (PEO), that protrudes as a cauliflower-like structure from the mesothelial lining of the body cavity near the sinus venosus and primitive liver, towards the inner curvature of the heart[2]. The villi of the PEO reach the heart at the atrial side and the epicardial cells start to spread over the naked heart tube, until they eventually cover the myocardium completely [3-5]. Between the epicardium and the myocardium a subepicardial layer develops. Epicardium-derived cells (EPDCs) cells, that detach from the epicardium by epithelial to mesenchymal transformation (EMT) make up this subepicardial mesenchyme. Both perihepatic endothelial cells from the dorsal mesocardium as well as EPDCs migrate into the subepicardial layer and produce extracellular matrix components [6]. The subepicardial mesenchyme is most prominent at the atrio-ventricular and interventricular grooves. In contrast, the epicardial mesothelium remains in close contact with the free myocardium of the atria and ventricles[7]. The coronary vessel network develops within the subepicardial mesenchyme [7;8]. The initial endothelial network is in open contact with the sinus venosus[9] and starts growing over the heart encircling the AV groove towards the ventral side. The coronary endothelial plexus reaches the ventriculo-arterial junction and forms a peritruncal ring. From here vessels grow into the aorta[10-12] and into the right atrium[9]. This ultimately results in two coronary arteries that connect to the aorta[11].

Part of the EPDCs migrate into the underlying layers to differentiate into several cell types. Smooth muscle cells of coronary arteries, myocardial and subendocardial interstitial fibroblasts and a subpopulation of the mesenchymal cells of the endocardial cushions are derived from EPDCs[13 14-16]. Several mouse models (VCAM-1$^{-/-}$, α_4 integrin$^{-/-}$ and FOG2 null mutants[17-19]) as well as our avian models [20-22] have shown that coronary vessel formation is severely disturbed when the epicardium fails to develop properly.

EPDCs which have migrated into the myocardial layer and differentiated into interstitial fibroblasts have been hypothesized to provide for a signalling function in myocardial development [14;23]. Similarly, epicardially regulated signaling has been hypothesized to be related to apoptosis, which accompanies outflow tract remodelling and coronary ingrowth into the aorta[24;25]. Other studies have related disturbances in epicardial outgrowth with reduced apoptosis in the myocardium of the ouflow tract region[26]. The role of apoptosis could become more clear by studying the effects of absent or disturbed epicardial contribution. In the present study, inhibition and adjusted chimera techniques were used to determine the role of EPDCs in coronary vascular develoment.

Materials and Methods White Leghorn chicken embryos (*Gallus domesticus*) and Japanese quail embryos (*Coturnix coturnix japonica*) were used and staged according to the criteria of Hamburger and Hamilton (HH)[27].Normal controls consisted of developmental series of quail and chicken embryos from stages HH29 to HH35.

Inhibition of epicardial outgrowthOutgrowth of the PEO in quail embryos (HH15 to HH18) was inhibited by a piece of egg shell membrane, basically as described by Männer[20]. After reincubation at 37.5°C (80% humidity), embryos were isolated at stages HH25-HH35. We distinghuished in the 33 harvested embryos between either a complete inhibition (n=15) or a partial inhibition (n=18). The embryos with partial inhibition were mechanistically considered to have a delay in epicardial outgrowth.

Rescue after PEO inhibitionRescued embryos were produced as quail-chicken chimeras, by transplanting a quail PEO of HH15-17 into the pericardial cavity of a chicken host of equivalent age, after inhibiting host PEO outgrowth. After reincubation, the embryos were isolated at stages HH29-HH35.

Preparation of the chicken-quail chimeras

After incubation at 37.5° C (80% humidity) for about 3 days, quail embryos ranging from HH15 to HH18 were used as donors. The chicken host embryos were incubated for about 3 days, and ranged from HH15 to HH18. A quail transplant was inserted into the pericardial cavity of the chicken, which could be reached through the naturally existing body wall hiatus at this stage. The transplant was placed beneath the heart tube adjacent to the sinus venosus or in the inner curvature of the heart loop [8]. After reincubation, the embryos were isolated at stages HH29 and HH30.

Immunohistochemistry

Isolation and processing of embryos for immunohistochemistry were performed as described earlier[9]. Serial sections were subjected to standard immunohistochemical procedures, [5;8;9], including the TUNEL (Roche) staining for apoptosis[28]. For the detection of quail cells in our chimeras, we used an anti-quail nuclear antibody (QCPN, Hybridoma Bank) diluted 1:2, and a quail endothelial marker (QH1, Hybridoma Bank), diluted 1:500. To analyze myocardial and vascular development, we used several specific markers, e.g. the anti-actin muscle marker HHF35 (Dako, Denmark) (1:500), the anti-quail-endothelial antibody QH1 (1:500) and the smooth muscle cell marker 1A4 (Sigma, St. Louis) (1:3000). Whole-mount cytokeratin (Dako, Denmark) staining was performed to analyze epicardial covering of the heart[29].

Analysis of apoptosis frequency and morphometry

We measured the volumes of total myocardium, the ouflow tract cushions and the atrioventricular cushions. We also measured the apoptotic volumes of these specific regions while defining the peritruncal ring as a separate region. We used the volume counting method according to Cavalieri[30;31]. In short, the number of points on a grid hitting the tissue of interest on a series of sections was counted. Volumes could be calculated from the counted numbers by Cavalieri's formula:

$$V = \Sigma[P] \cdot M^{-2} \cdot a \cdot d$$

In this formula, V is the volume in mm^3, $\Sigma[P]$ is the total number of counted points, M is the magnification, a is the point area in mm^2, and d is the distance between the counted sections in mm. Quail hearts (HH30) with a delay in epicardial outgrowth were compared to normal hearts of the same stage The apoptotic volumes obtained in the delay group were normalized for differences in volumes. Results were compared statistically using Student's t-test.

Results

To analyze the regulatory role of EPDCs on myocardial and coronary development, two sets of experimental embryos were generated. We used quail embryos in which we blocked PEO outgrowth and chicken embryos in which inhibition of epicardial outgrowth was rescued by transplantation of an exogenous quail PEO. These two models show a range of heart malformations, that illustrate the importance of properly timed epicardial behaviour for the development of the coronary system and the myocardial architecture.

Inhibition of epicardial outgrowth

We obtained 33 embryos in which epicardial outgrowth was completely (n=15) or partially (n=18) inhibited. The embryos with partial inhibition were mechanistically considered to have a delay in epicardial outgrowth.

In embryos with complete inhibition the malformations were severe and as a result embryos did not survive after HH30. Whole-mount staining with the mesothelial marker anti-cytokeratin showed nearly naked hearts (Figure 1a). In these hearts, there was compensatory outgrowth of cytokeratin positive cells, earlier referred to as a mesothelial collar[21], that covered the great arteries and the myocardium of the outflow tract to as far as the inner curvature (Figure 1a-c).

Cardiac looping and septation were disturbed to a variable extent. All cases presented with a double-inlet to double-outlet configuration. In most embryos a common arterial trunk was found and AV cushion formation was deficient or absent in all embryos (Figure 1b). Interventricular septation was disturbed. The position of the heart was in some cases abnormal, with the apex tilted cranially.

The compact myocardium and trabeculated myocardium in these embryos were abnormally thin (Figure 1d-g).

Early coronary development was severely disturbed. After formation of an initial coronary endothelial plexus sprouting from the sinus venosus (not shown) no vessels had penetrated the myocardium (Figure 1h-k).

Delay in epicardial outgrowth; effects at HH30

In partially inhibited embryos (n=18), the epicardial cells had reached the heart and grown over the myocardium, but, since the egg shell membrane blocked the PEO initially, epicardial outgrowth was delayed by approximately 1 day.

We analyzed embryos at different stages HH30 (n=10) and HH35 (n=8), to study both myocardial vascularization and the development of the coronary orifices, respectively. In this paragraph, the observations in the younger embryos (HH30) are presented before the older embryos (HH35) are discussed.

In the embryos of stage HH30 myocardial architecture was apparently normal (Figure 1d, 2c). Yet, volume measurements showed that the myocardial volume was significantly reduced by 47 % ($p<0.05$) in embryos with delayed epicardial outgrowth compared to normal embryos (Table 1). Moreover, myocardial vascularization was severely disturbed. Immunohistochemical staining with the QH1 antibody, showed that only the myocardium, that was covered by epicardium with a properly developed subepicardial mesenchyme, was vascularized correctly. In other parts, where subepicardial mesenchyme was missing, vascularization was very poor (Figure 2).

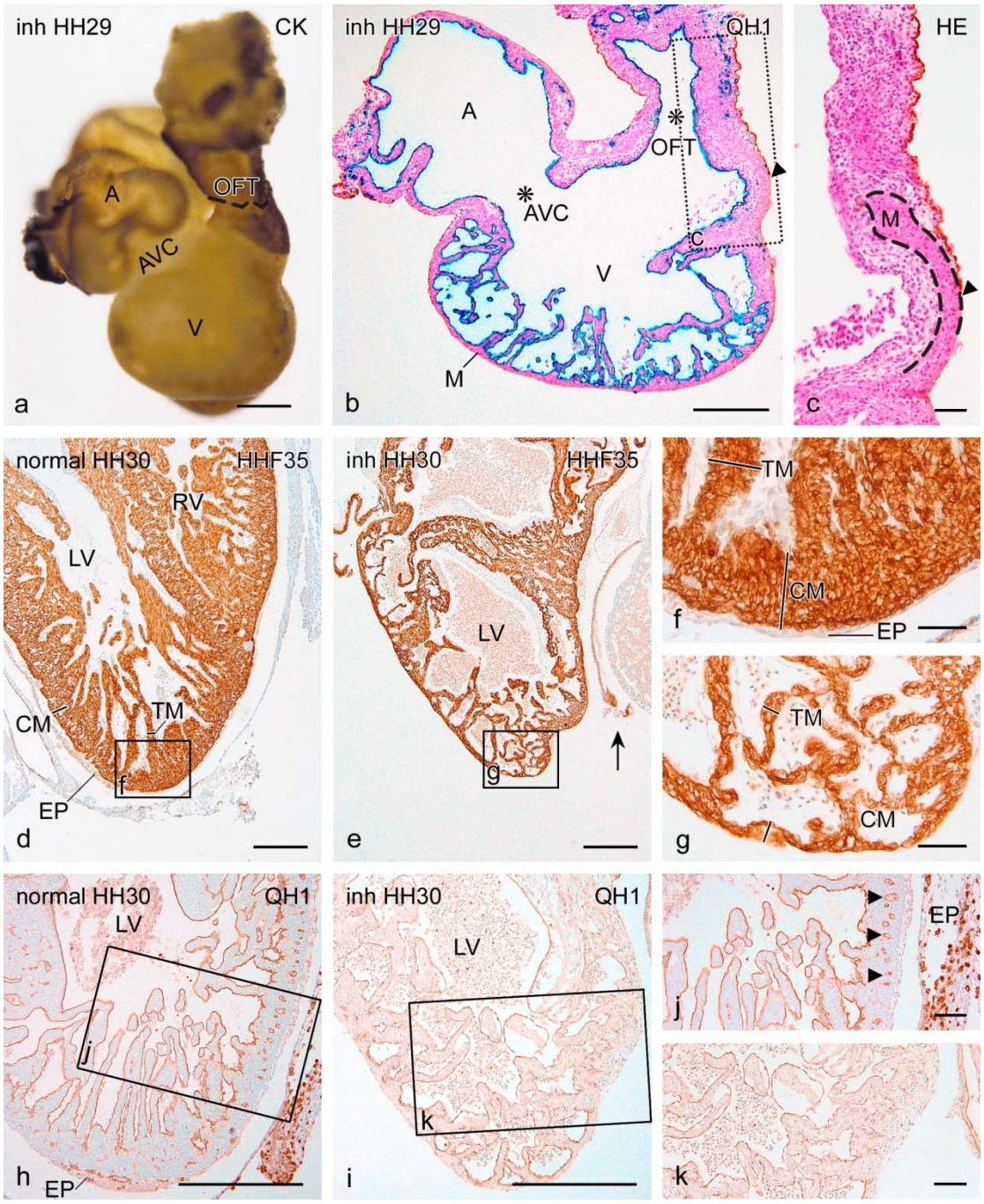

Figure 1: a, lateral view of a quail heart (HH29) with complete inhibition (inh) of epicardial outgrowth over the myocardium. The brown covering (whole-mount cytokeratin staining) represents the mesothelial outgrowth of the arterial epicardium. The entire ventricle and both atria have not been covered by epicardium. The dotted line demarquates the distal border of the outflow tract myocardium. b, Frontal section of the heart depicted in a. Note abnormal looping, deficient cushion formation (asterisks) and thin myocardium (M). The arrowhead indicates the border of the arterial epicardium c, Consecutive section of b, with higher magnification, shows cytokeratine (CK) positive cells (in brown) that have grown over the outflow tract myocardium. d, Sagittal section of a normal quail heart (HH30) stained for muscle actin (HHF35). The myocardium is covered by epicardium (EP). The compact myocardium (CM) and trabeculated myocardium (TM) are normal. e, Sagittal section of a quail heart (HH30) with inhibition of epicardial outgrowth. The epicardium is absent. Both the compact myocardium and the trabeculated myocardium are abnormally thin. The arrow points to the egg shell membrane, placed between the proepicardial organ (PEO) and the primitive heart tube. f, Higher magnification of d. See boxed area. g, Higher magnification of e. See boxed area.
h, Sagittal section of a normal quail heart (HH30) stained for quail endothelium (QH1), shows normal vessel development (arrows) in subepicardial and myocardial layers. i, Sagittal section of a quail heart (HH30) with inhibition reveals that no vessels have developed at all. j, Higher magnification of h. Arrowheads indicate capillaries. See boxed area. k, Higher magnification of i. See boxed area.
A: atrium; AVC: atrioventricular canal; HE: haematoxilin-eosin; LV: left ventricle; OFT: outflow tract; RV: right ventricle; V: ventricle. Scale bars = 250 μm in a,b,d,e,h,i, 50 μm in c,f,g,j,k.

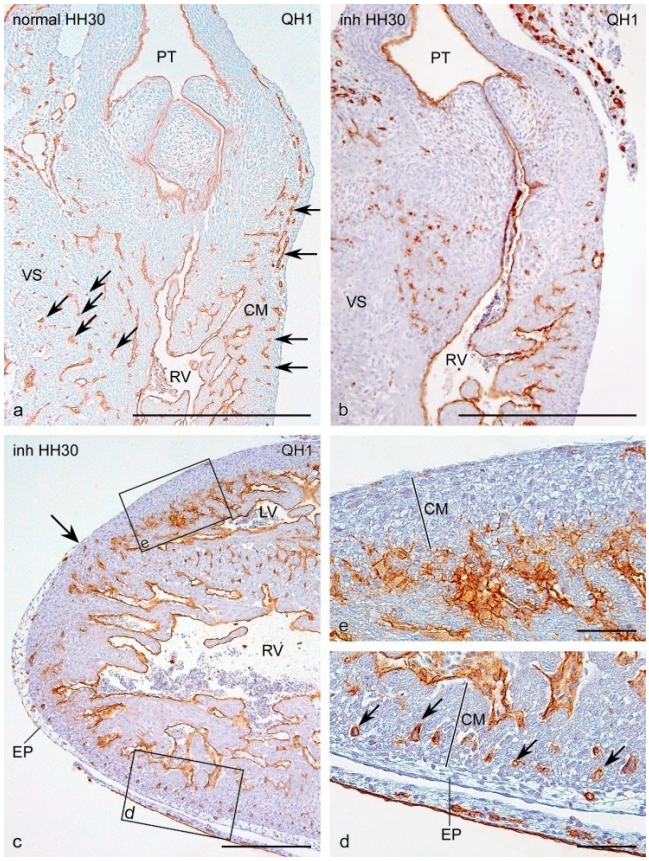

Figure 2: Sagittal sections of quail hearts (HH30) stained for quail endothelium (QH1=brown) showing normal and abnormal development of the vessel network. Also the endothelial lining of the pulmonary trunk (PT) and valve leaflets, as well as the endocardium, lining the ventricles are positive for QH1. a, Section of the right ventricle (RV) of a normal heart with evident presence of coronary vessels in the compact myocardium (CM) and in the ventricular septum (VS). Arrows indicate capillaries. b, Section of the RV of a representative quail heart with inhibition (inh) of epicardial outgrowth, where presence of coronary endothelial cells was remarkably less than in normal hearts. c, Section through apex of a quail heart (HH30) with inhibition, where the myocardium is partly covered with normal epicardium (EP). Arrow indicates border of normal epicardium and epicardium without development of a subepicardial layer. The compact myocardium (CM), where a normal subepicardial layer has developed, is penetrated by coronary vessels (arrows in d), whereas the abnormal myocardium with hardly any formation of a subepicardial layer is nearly devoid of vessels (e).
LV: left ventricle. Scale bars = 250 µm in a to c, 50 µm in d and e.

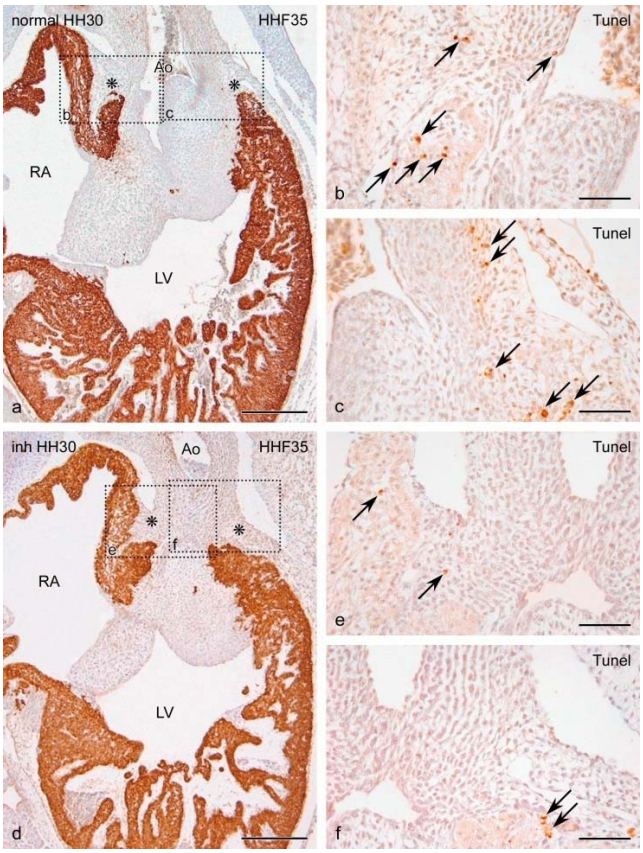

Figure 3: Sagittal sections of a normal quail heart (HH30) (a-c) and a quail heart (HH30) with inhibition (inh) of epicardial outgrowth (d-f) stained for muscle actin (HHF35) and apoptosis (Tunel=brown). It illustrates the difference in apoptotic levels in the peritruncal region. a, Section through the aorta (Ao) of a normal quail heart, stained for muscle actin. The asterisks indicate the peritruncal region. b, and c, are magnifications of a consecutive section stained with Tunel showing apoptosis in the peritruncal region. d, section through the aorta (Ao) of quail heart with inhibition. e, and f, are magnifications of a consecutive section stained with Tunel showing hardly any apoptosis in the peritruncal region.
LV: left ventricle; RA: right atrium. Scale bars= 250 µm in a and d, 50 µm in b,c,e,f

Apoptosis frequency and morphometry

In order to study the relation of epicardial development with apoptosis in the peritruncal ring, we performed a TUNEL staining on embryos of stage HH30, just prior to coronary ingrowth. Apoptosis was markedly diminished in embryos with disturbed epicardial outgrowth (Figure 3 and Table 1). Apoptotic volumes of various areas of the heart were measured and these showed, that in the peritruncal ring, in the outflow tract myocardium, and in the outflow tract cushions, apoptosis was significantly reduced in embryos with epicardial delay compared to normal embryos. Apoptosis was not significantly reduced in the AV cushions. Apoptosis in the peritruncal ring was reduced by 83% ($p<0.001$), in the outflow tract myocardium by 56% ($p<0.01$) and in the outflow tract cushions by 88% ($p<0.05$). When these numbers were normalized for myocardial volume, apoptosis in the peritruncal ring was reduced by 71% ($p<0.01$) and and in the outflow tract cushions by 84% ($p<0.05$) (Table 1).

We did not only examine the incidence of apoptosis, but also the location of the apoptotic cells, concentrating on the ingrowth sites of the coronary arteries. In the control embryos we observed clusters of apoptotic cells. These clusters were at the sites where the main stems of the coronary arteries invade the aorta. Around the pulmonary artery we found only small numbers of randomly scattered apoptotic cells. In contrast, in the embryos with a delay in epicardial outgrowth, we observed not only reduced apoptosis, but also that the apoptotic cells were randomly located. Nevertheless, there was more apoptosis around the aorta than the pulmonary trunk.

Rescue of epicardial inhibition

To study whether the phenotype, observed in embryos with disturbed epicardial outgrowth, could be rescued, we created an experimental set, in which transplantation of an exogenous PEO was performed after inhibition of the endogenous PEO. In this set the obtained embryos (n=10), showed complete epicardial covering, that was of mixed chicken and quail origin.

Myocardial development was in all cases restored to normal already before coronary vascularization was established (Figure 4a). In the rescued embryos the endocardial cushions were well developed and myocardialized. Scattered EPDCs of quail origin were seen throughout the myocardium of the ventricles and atria. Few or no EPDCs were found in the AV-cushions of the younger embryos (HH29/HH30; n=6), whereas the older embryos (HH35; n=4) showed abundant presence of EPDCs in the mitral and tricuspid valve leaflets (Figure 4b-d).

Only a few cardiac abnormalities were seen. One embryo (HH35) presented with a double-inlet left ventricle. Another embryo (HH35) showed a double-outlet right ventricle.

However, close study of the proximal coronary stem development in the 4 older embryos (HH35), showed, that three out of 4 embryos had abnormalities in their coronary to aortic connections. One case had no coronary connections to the aorta. Compensatory ventriculo-coronary-arterial connections (VCAC or fistulae) had developed, one growing into the heart from the transplanted PEO and other fistulae had developed as subepicardial cavities contacting the ventricular lumen with the coronary vessels. The subepicardial cavities were surrounded by cells which expressed smooth muscle actin (Figure 4j). In the second embryo we found only one atretic coronary artery connecting to a pin-point orifice into the the aorta at the left sided sinus (Figure 4e). In the third embryo the coronary branches of the left coronary artery, the left anterior descending and the ramus circumflexus, connected separately to the aorta and thus formed a double orifice (not shown). The cells, that made up the vessel wall of the main coronary arteries, were quail derived (Figure 2g)

Delay in epicardial outgrowth; effects at HH35

In embryos of stage HH35 with partial inhibition of epicardial outgrowth, similar defects of the main coronary stems as in the rescued embryos were observed. Three out of 8 embryos displayed malformations of the proximal coronary stems. One heart had only one single coronary connection to the aorta. In the other two hearts no coronary connections were found at all. Instead very deep trabeculae were observed, possibly indicative for presence of small fistulae. Nevertheless, sizeable fistulae were not seen. In these two hearts, as demonstrated by immunostaining with the QH1 and 1A4 antibodies, coronary vessel network development in the myocardial wall was very poor and no coronary smooth muscle cells were found at all (Figure 4h,i).

The other five hearts displayed various myocardial and coronary abnormalities. In two of these hearts, the coronary branches were abnormally large and irregular of shape. Two further hearts displayed outflow tract malformations. One contained a persistent truncus arteriosus with abnormal cardiac looping and the other showed a double-outlet right ventricle.

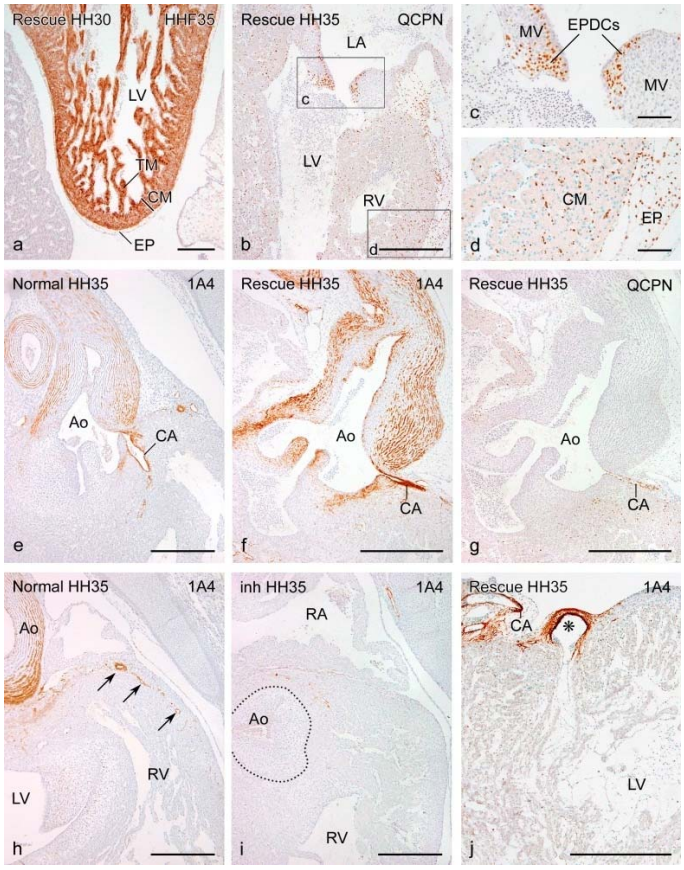

Figure 4: a, Sagittal section of a rescue experiment in a chicken-quail chimera heart (HH30) with transplantation of a quail proepicardial organ (PEO) after inhibition of host PEO. The section is stained for muscle actin (HHF35) showing normal epicardial covering (EP) and restored development of compact (CM) and trabeculated myocardium (TM). b, Sagittal plane of rescued heart (HH35) illustrating the spreading of graft derived epicarium-derived cells (EPDCs), stained with an anti-quail nuclear antibody (QCPN). EPDC migration was observed throughout the entire chicken heart (HH35). Migration into valve leaflets was only seen from HH35 onwards. c, Higher magnification of b. See boxed area. d, Higher magnification of b, showing extensive migration of graft derived EPDCs throughout compact myocardium (CM) and epicardial layer (EP). See boxed area. e, Proximal coronary artery (CA) of a normal quail heart (HH35) in a sagittal section. Staining for smooth muscle cell actin (1A4), shows normal development of the coronary medial layer. f, Pin-point orifice with an atretic proximal CA in a rescued embryo (HH35) in a sagittal section. The medial layer of the proximal coronary stem has developed properly. g, Consecutive section of f, stained for quail cells (QCPN) showing that graft derived EPDCs make up the vessel wall of the proximal coronary stem. h, Sagittal section of the same heart depicted in e, through the atrioventricular (AV) groove, stained for smooth muscle actin (1A4), showing a well developed coronary network with normal formation of the media of the coronary arteries (arrows). i, Transverse section of a quail heart (HH35) with inhibition (inh) of epicardial outgrowth, which lacked both coronary connections to the aorta, was stained for smooth muscle cells (SMC). No coronary SMCs were found throughout the entire heart. Dotted line demarquates aorta (Ao). j, Sagittal section through a rescued heart (HH35) showing ventriculo-coronary arterial connections (VCAC) or fistulae. This heart also lacked both coronary connections and as a compensatory mechanism fistulae had developed, as well as some SMC lined coronary arteries. Staining for smooth muscle actin showed development of SMCs, triggered by contact with the systemic circulation. LA: left atrium; LV: left ventricle; MV: mitral valve leaflet; RA: right atrium; RV: right ventricle. Scale bars= 250 μm in a,b,e to f, 50 μm in c and d.

Table 1. Myocardial and apoptotic volume estimates of normal embryonic quail hearts (HH30) and embryonic quail hearts (HH30) with a delay in epicardial outgrowth. Corrected row indicates apoptotic volumes after correction for diminished myocardial volumes in embryos with epicardial inhibition.

	control mean ± std (n=4)	**delayed mean ± std** (n=4)	**p-value**	**corrected delayed**	**p-value**
myocardial volume mm^3	1.12 ± 0.25	0.65 ± 0.21	**0.029**		
V apoptosis OT cushions $\cdot 10^{-3}$ mm^3	0.50 ± 0.26	0.06 ± 0.02	**0.015**	0.08 ± 0.07	**0.020**
V apoptosis AV cushions $\cdot 10^{-3}$ mm^3	1.06 ± 0.49	0.71 ± 0.26	**0.26**	1.07 ± 0.40	**0.96**
V apoptosis PR $\cdot 10^{-3}$ mm^3	1.00 ± 0.19	0.17 ± 0.19	**0.00086**	0.29 ± 0.32	**0.0098**
V apoptosis OT myocardium& mesenchyme $\cdot 10^{-3}$ mm^3	2.12 ± 0.42	0.94 ± 0.38	**0.0060**	1.60 ± 0.67	**0.232**
V apoptosis total $\cdot 10^{-3}$ mm^3	4.68 ± 0.87	1.87 ± 0.65	**0.0021**	3.20 ± 1.11	**0.082**

Discussion

This study confirms our earlier findings, that after complete inhibition of epicardial outgrowth, embryos die due to structural and severe myocardial and vascular abnormalities. The myocardium does not developly and no endothelial capillary network is formed. Cardiac looping and cushion formation are also unsufficient to form a functional heart[21]. Tevosian et al presented a model with a comparable phenotype in which the FOG-2 gene, which is a cofactor of the GATA-family (GATA1-6) of transcription factors, is essential for epithelial to mesenchymal transformation. These mice displayed disturbed vascularization and myocardial maturation. Reexpression of FOG-2 restores the myocardial phenotype [19].

Similar to their rescue-model, transplanting a quail PEO into the pericardial cavity of a chicken embryo after inhibiting host epicardial outgrowth, results in a rescue of myocardial development[21](this study). These rescued hearts appear normal, because myocardial architecture is restored. However, the transplanted PEO does not attach immediately to the myocardial heart tube and consequently a delay in epicardial outgrowth occurs. The delay is visible in the covering of in particular the outflow tract, which contains a mixed population of chick derived arterial epicardium and quail-PEO derived cells, whereas in normal embryos or chimeras the outflow tract epicardium at HH30 is made up solely by PEO derived cells [5]. In our rescued embryos this state of development is reached only just before stage HH35 (not shown). A similar observation was made by Männer in a quail-chicken chimera model, in which chicken PEO outgrowth was blocked and a quail PEO was simultaneously transplanted into the pericardial cavity of a chicken embyro [16]. The mixed population contains PEO derived donor cells and arterially derived epicardial cells. This is a result of compensatory outgrowth of arterially derived epicardium, also seen in embryos with a complete inhibition of PEO outgrowth where a collar of arterial epicardium grows over the proximal outflow tract [21]. This arterial epicardium, with specific immunohistochemical characteristics has also been described by Perez-Pomares and colleagues[32]. A novel finding in our rescued embryos is the occurrence of coronary vascular abnormalities such as absent connections to the aorta and fistulae in the myocardial wall, indicating, that proper timing of epicardial outgrowth is essential for correct connection of the coronary stems to the aorta.

In earlier work it has been demonstrated that proper development of the main coronary stems is established through ingrowth of endothelial cells into the myocardial wall and the aorta [8;11;33]. It was recently reported that there is a relation between apoptosis and coronary ingrowth[24]. Furthermore, a connection between epicardial outgrowth and apoptotic patterning of the outflow tract, was presented, using the PEO inhibition model [26]. The latter study demonstrated that, when epicardial outgrowth is delayed, apoptosis in the outflow tract is diminished, specifically during the stages in which the coronary vessel network makes contact with the systemic circulation, also providing further leads to investigate the involvement of apoptosis in coronary development. Watanabe and colleagues implied an important function of apoptosis in shortening and remodelling of the outflow tract [25]. Based on the findings, reported both by Rothenberg[26] and Velkey[24], we propose an experimental model in which delayed epicardial outgrowth causes coronary misconnection through diminished apoptosis. Secondary consequences are compensatory development of fistulae and abnormal remodelling of the vessel network.

To test our hypothesis we caused a disturbance in epicardial outgrowth by the technique of Männer[20]. We harvested embryos at two different stages (HH30 and HH35). Stage HH30 was to study apoptotic patterns in the outflow tract and subaortic region, since at that stage a peak in apoptosis occurs in this region [28;34]. Specifically in the peritruncal region, apoptosis was significantly diminished in embryos with PEO inhibition, supporting the hypothesis that proper timing of epicardial outgrowth is essential for normal levels of apoptosis.

Not only apoptosis was abnormal in these embryos. Parts of myocardium that lacked epicardium with a proper subepicardial layer were poorly vascularized, suggesting that correct timing of epicardial outgrowth is essential for the normal development of the coronary network.

Although myocardial architecture seemed normal, the vascular abnormalities, seen at stage HH30, were even more severe in the manipulated embryos at HH35. Hearts of these embryos did not only display insufficient vascular penetration, but the establishment of the proximal coronary stems was also defective. As a result the development of a proper medial layer of the coronary vessels was hampered and the coronary vessels were abnormally large and irregularly shaped, implying a venous phenotype.

Earlier studies have postulated epicardial involvement in apoptosis in the outflow tract and remodelling of the outflow tract[25]. Our study concentrates on the role of epicardium and apoptosis in coronary development.

However, the incidence of outflow tract abnormalitites in these embryos also supports the hypothesis, that timed epicardial outgrowth mediates apoptosis in the outflow tract, which is necessary for remodelling and shortening [25].

Clinical relevance

Abnormal coronary development in animal models has been studied in relation to impaired epicardial functioning. Studies with both genetic (FOG2 -/- in mice[19], antisense-Ets in chicken[22]) approaches, as well as with mechanical disruptions (this study) in epicardial development show a severe range in coronary malformations. This implies an important function for the epicardium in the regulation of coronary development, starting at early stages with initial endothelial ingrowth and still going on during the final connection of the coronary network to the aorta. The malformations as seen in our study and as presented in earlier work reflect on human coronary pathology. For the purpose of understanding, we have distinguished between abnormalities of the main coronary arteries and the maldevelopment of the initial intramural network.

Congenital malformations of the main coronary branches in humans have been documented and classified by Angelini[35]. These include pin-point orifices, double orifices, absence of coronary connections and single arteries. These malformations are also seen in our avian epicardial inhibition model and presented in earlier work by Lie-Venema et al., an Ets-1 and Ets-2 transcription factor blocking model in chicken embryos[22]. Gittenberger-de Groot and colleagues have postulated pathogenetic processes of ventriculo-coronary arterial communications (VCAC) or fistulae in humans, being a compensatory mechanism as a result of deficient coronary connections to the aorta[36]. These findings are also seen in the present study, supporting the hypothesis that, when the coronary system can not make contact with the systemic circulation through aortic orifices, the coronary vessel network searches for other ways to connect with the systemic circulation.

It is still not clear, what drives the coronary system to connect to the aorta and not to the pulmonary trunk. This last phenomenon is a rare malformation as seen in the Bland-White-Garland syndrome[37;38]. However, even in complicated malformations such as congenitally corrected transposition of the great arteries (CCTGA) the coronary arteries connect to the aorta, which is connected to the morphological right ventricle. Our data show that apoptosis in the peritruncal ring around the aorta is not only more prominently present as compared to the pulmonary trunk, but the apoptotic cells are also clustered and located at specific sites were future coronary stems will develop. This implies a role for apoptosis, induced in connection with EPDCs, in establishing connections to the systemic circulation. It needs further investigation, concerning the intrinsic factor that causes the specifically located induction of apoptosis.

In the hearts in our study, we found a severe reduction in vascularization of the myocardium. Nevertheless, these hearts were viable, probably due to various compensatory mechanisms. However, it is imaginable, that in the adult situation, when a heart functions on a fully mature vascularization, the heart has limited recrutable coronary capacity in conditions of ischaemia. This means that the potential for neovascularisation and *de novo* development of collateral vessels may be disturbed.

The impact of poor vascularization in the embryonic or neonatal stage, due to epicardial maldevelopment, on myocardial behaviour during and after conditions of ischaemia in the adult situation, can not be determined from this study. Yet the presented data provide certain leads for further research on epicardial contribution to myocardial vascularisation. Moreover, new light has been shed on epicardially regulated apoptosis in relation to the development of the proximal coronary stems and ingrowth patterning.

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Chapter 5

Epicardium-derived cells (EPDCs) are important for correct development of the Purkinje fibers in the avian heart

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Abstract

During embryonic development, the proepicardial organ (PEO) grows out over the heart surface to form the epicardium. Following epithelial-mesenchymal transformation, epicardium-derived cells (EPDCs) migrate into the heart and contribute to the developing coronary arteries, to the valves and to the myocardium. The peripheral Purkinje fiber network develops from differentiating cardiomyocytes in the ventricular myocardium. Intrigued by the close spatial relationship between the final destinations of migrating EPDCs and Purkinje fiber differentiation in the avian heart, *viz.* surrounding the coronary arteries and at subendocardial sites, we investigated whether inhibition of epicardial outgrowth would disturb cardiomyocyte differentiation into Purkinje fibers.

To this end epicardial development was inhibited mechanically with a membrane, or genetically, by suppressing epicardial epithelial-to-mesenchymal transformation with antisense retroviral vectors affecting Ets transcription factor levels(n=4, HH39-41). In both epicardial inhibition models we evaluated Purkinje fiber development by EAP-300 immunohistochemistry and found that restraints on EPDC development resulted in morphologically aberrant differentiation of Purkinje fibers. Purkinje fiber hyperplasia was observed both periarterially and at subendocardial positions. Furthermore, the cells were morphologically abnormal and not aligned in orderly Purkinje fibers.

We conclude that EPDCs are instrumental in Purkinje fiber differentiation, and we hypothesize that they cooperate directly with endothelial and endocardial cells in the development of the peripheral conduction system.

Introduction

During heart development, the myocardial heart tube is covered by the epicardial mesothelium, derived from the proepicardial organ (PEO). In the chicken and quail embryo, the PEO develops around day three of incubation as a protrusion of the coelomic wall near the sinus venosus [1]. A subset of the epicardial cells undergoes epithelial-mesenchymal transformation (EMT) to generate a mesenchymal cell population occupying the subepicardial layer. These epicardium-derived cells (EPDCs) can migrate into the myocardium and differentiate into interstitial, subendocardial and coronary adventitial fibroblasts, as well as coronary smooth muscle cells [2, 3]. Several studies have demonstrated crucial roles of EPDCs in formation of the compact myocardium [4-7] and coronary development [8], while a morphogenic role for EPDCs in valve development has also been postulated [2, 9].

Retroviral tagging experiments showed that the Purkinje fibers of the peripheral conduction system differentiate from cardiomyocytes, in close spatio-temporal relation to the developing coronary vasculature [10, 11], whereas the subendocardial Purkinje fibers develop in the proximity of endocardial cells. These unique differentiation sites suggested an inductive role both for periarterial and subendocardial EPDCs and for a paracrine signal from the endocardium and arterial beds in the recruitment of cardiomyocytes into the Purkinje fiber network [12, 13]. Recent experimentation showed that Purkinje fiber differentiation is tightly regulated by hemodynamic alterations, while mature endothelin-1 (ET-1) and ET-converting enzyme 1 (ECE1) were identified as inductive molecules [14-16]. Concommittant retroviral expression of mature ET-1 and ECE1 was even sufficient for the ectopic conversion of adjacent cardiomyocytes into Purkinje fibers [14]. Because interstitial EPDCs are present throughout the myocardium [2], even the ectopically converting cardiomyocytes could have been influenced by a juxtaposed EPDC.

In the present study, we tested the hypothesis that EPDCs play a role in the differentiation of the Purkinje fibers by morphologic analysis of the conductive network after mechanical and genetic disturbance of epicardial development.

Materials and Methods

To study the role of EPDCs in the development of Purkinje fibers, we used a mechanical and a genetic experimental model in which EPDC development and differentiation were disturbed. From earlier work we knew that with either model the extent of epicardial disturbance is variable [7, 17]. For close immunohistochemical examination, we specifically chose the embryos (n=7), without large morphological and coronary aberrations, to ensure that our analysis would focus on the influence of disturbed or delayed epicardial outgrowth rather than on the influence of impaired coronary formation on Purkinje fiber development. Normal controls consisted of untreated Japanese quail embryos (*Coturnix coturnix japonica*; n=7) from Hamburger and Hamilton stages 39 to 42 [18].

Mechanical inhibition of epicardial outgrowth

Outgrowth of the PEO in quail embryos (HH15 to HH18; n=15) was inhibited by placing a piece of egg shell membrane between the PEO and the heart tube, as described before [19, 20]. After re-incubation at 37.5°C (80% humidity), embryos were isolated at stages HH40-HH42.

Genetic inhibition of epicardial development

In earlier work we established that epicardial development could be impaired by antisense down-regulation of the Ets-1 and Ets-2 transcription factors in chicken embryos [7]. In this model, Ets-1 and Ets-2 expression were downregulated simultaneously in vivo. In short, white Leghorn chicken embryos (*Gallus domesticus*) were injected with the retroviral CXasetsIZ construct via the the right anterior vitelline vein at developmental stage HH14/HH15 as described earlier [7]. Four embryos of developmental stage HH39-HH41 were analysed.

Immunohistochemistry

Isolation and processing of embryos for immunohistochemistry were done as described earlier [21]. Serial sections were produced and subjected to standard immunohistochemical procedures [21, 22]. For analysis of coronary vessel development we used the alpha-actin smooth muscle antibody 1A4 (Sigma, St Louis, 1:3000). For detection of Purkinje fibers, we used the EAP-300 antibody [23] diluted 1:200. The size of the area occupied by fluorescent EAP-positive cells was determined in 5 vessels of comparable diameter in the interventricular septum of the control and experimental groups (3 embryos per group). To maintain and enhance the signal of the secondary FITC-labeled antibody after immunofluorescent data acquisition, sections of interest were incubated with antifluorescein antibody Fab fragment conjugated with peroxidase (Converter-POD, Roche Diagnostics, Mannheim). In paraformaldehyde-fixed tissues this resulted in better signal-to-background ratios than the use of immunofluorescence alone. Apoptosis in the Purkinje fibers was assessed as described earlier [24].

Statistical analysis

Data were represented as average ± standard deviation. A non-paired , two-tailed student's *t*-test was used for statistical comparison. A p-value less than 0.05 was considered statistically significant.

Results

To assess the role of EPDCs in Purkinje fiber development, we used two independent avian models in which epicardial development was disturbed. The severity of the cardiac abnormalities in these models is closely related to the degree of epicardial outgrowth inhibition. Especially the procedure for mechanical PEO inhibition yields embryos in which epicardial development is disturbed in variable degrees [25]. This results in a broad range of malformations, varying from complete absence of the epicardium and embryonic lethality around stage HH29 [6] to only a delay in epicardial outgrowth with a mild cardiac phenotype [26] and survival until hatching at stage 46. The peripheral Purkinje fiber conduction system develops rather late in embryonic life, from stage HH36 onwards. Thus, the manipulated embryos surviving beyond stage HH39 that were used for this study, were typically those that were only mildly affected by the inhibition procedure; both in the mechanical and in the genetic inhibition model.

Purkinje fiber development in embryos with a mechanical inhibition of epicardial outgrowth.

In the embryos with mechanically inhibited epicardial outgrowth that succeeded to survive beyond developmental stage HH40, severe cardiac malformations were not observed, except for an occasional reduction in heart size. However, immunofluorescent staining with the EAP-300 antibody, specific for avian conduction tissue [23], showed that both the periarterial and the subendocardial Purkinje fibers were affected in these embryos. To quantify the hypoplasia of the periarterial Purkinje fibers (Figure 1), fluorescently stained areas occupied by the periarterially located Purkinje fibers were measured in 15 vessels with a comparable diameter (5 in each of 3 embryos) located in the interventricular septum, at approximately mid-ventricular level in both the experimental and control groups. Because the periarterial occupancy with EAP-300 positive cells may vary between the vessels within the same heart, we analyzed the arteries with the most EAP-300 cells, both in control and experimental embryos. The EAP-300 postitive area was found to be reduced by up to 89% ($p<0.05$; Figure 1 g-i, and Figure 2). Hypoplasia of the periarterial Purkinje fiber network appeared to be the result of a reduction in the number of EAP-300 positive cells, whereas the amount of EAP-300 signal per cell was not altered significantly in the immunofluorescent stainings.

To ensure that our analysis would focus on the influence of disturbed or delayed epicardial outgrowth rather than on the influence of impaired coronary formation on Purkinje fiber development, we selected 5 embryos with a normal distribution of the coronary arteries for a more detailed morphological analysis of the periarterial and subendocardial Purkinje fibers. Whereas we could observe hardly any green fluorescein signal above background by immunofluorescence microscopy (not shown), anti-fluorescein signal enhancement and conventional immunohistochemistry revealed a faint EAP-300 signal around the coronary arteries in these embryos (Figure 3 c and d). The coronaries were surrounded by a normal amount of 1A4-positive smooth muscle cells (not shown). In addition, cellular morphology seemed abnormal, especially in the subendocardially located Purkinje fibers . Whereas in normal embryos cells of the subendocardial Purkinje fibers were aligned in continuous fiber-like strands, in the inhibition embryos they were abnormally large, rounded and had failed to differentiate into a coherent Purkinje fiber network (Figure 3).

Purkinje fiber development in embryos with genetic inhibition of epicardial outgrowth.

To show that the Purkinje fiber hypoplasia observed after PEO inhibition with a piece of eggshell membrane did not depend on this particular inhibition model we confirmed the results in the genetic inhibition model available in our group. Retrovirally delivered antisense Ets-1/2 has been shown to decrease the contribution of EPDCs to heart formation by blocking epicardial epithelial-mesenchymal transformation [7] . In the 4 embryos transduced with the CXasEtsIZ retrovirus (antisense-Ets embryos, HH 39-41), there were no gross morphologic abnormalities, coronary architecture and myocardial thickness were within the normal range, and there was some subepicardial mesenchyme present in the atrio-ventricular groove. By this, they were classified as being only mildly affected by the retroviral manipulation of epicardial EMT. Similar to the observations in the mechanically inhibited embryos, a marked reduction (up to 92%, $p<0.05$) of periarterially located Purkinje fibers was found (Figure 1i and Figure 3). Also resembling those in the mechanically inhibited embryos were the large, rounded subendocardial EAP-300 positive cells that failed to form continuous strands (not shown).

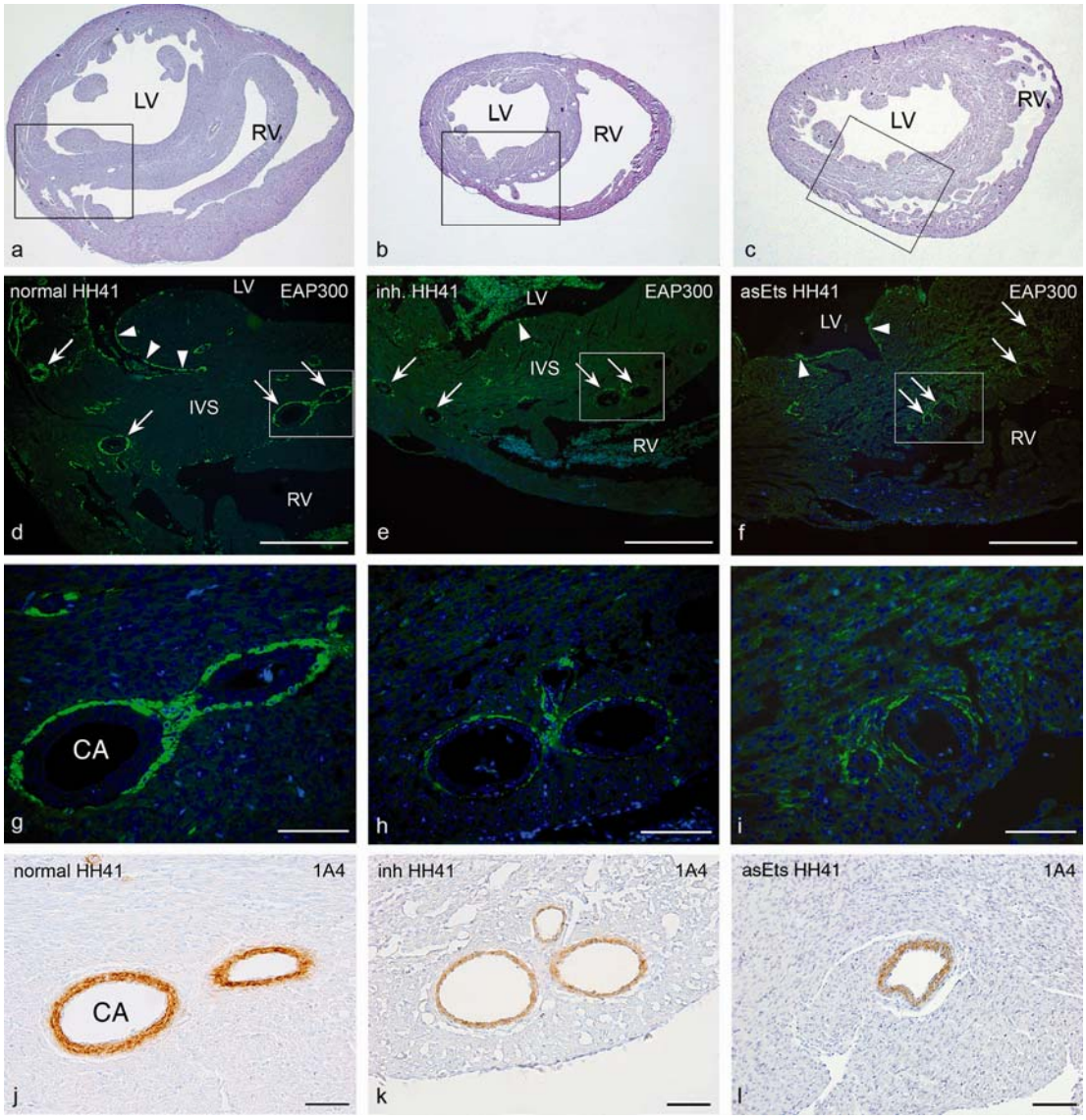

Figure 1. Photomicrographs of representative transverse sections of a normal control quail heart (HH41; **a, d, g,** and **j**), a quail heart with a mechanical inhibition of epicardial outgrowth (inh; HH41; **b, e, h,** and **k**, and a chicken heart with hampered EPDC differentiation due to retrovirally induced down-regulation of Ets-1 and Ets-2 (asEts; HH41; **c, f, i,** and **l**). Panels **a-c** show haematoxylin-eosin stained overviews of the sections, at approximately mid-ventricular level. Boxed areas in **a-c** delineate the regions depicted in **d-f**. Serial sections were stained for Purkinje fiber cells with the EAP-300 antibody (in green) and nuclei were stained with DAPI (in blue). In the control heart, (**d, g**) normal development of the periarterial (arrows) and subendocardial (arrowheads) Purkinje fiber cells was observed. In contrast, in the inhibition embryo (**e, h**), and in the asEts-1/2 embryo (**f, i**), we observed a decrease in Purkinje fiber cell numbers. The details (**g-i**; boxed areas in **d-f**) show periarterially located Purkinje fiber cells. In embryos with disturbed epicardial contribution EAP-300 positive cell numbers were dramatically reduced compared to normal embryos. Quantification of the area occupied by periarterial Purkinje fibers was performed as described in the text under Materials & Methods, in sections like these, comparing coronary arteries of similar diameter. Panels **j-l** show the 1A4 (smooth muscle actin) staining in consecutive sections of **g- i**. The smooth muscle cell layer of the coronary arteries (CA) was slightly thinner in this mechanically PEO-inhibited embryo (**k**). However, this was not a consistent finding. Also the asEts-1/2 embryos displayed coronary arteries with normal thickness of the smooth muscle cell layer (**l**). CA, coronary artery; IVS, interventricular septum; LV, left ventricle; RV, right ventricle. Scale bars, 250 μm in **d-f**; 50 μm in **g-l**.

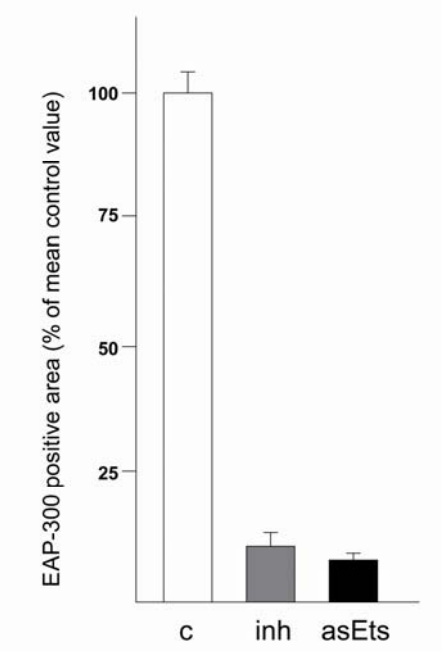

Figure 2. Quantification of periarterial Purkinje fiber hypoplasia in embryos with mechanical and retrovirally induced inhibition of epicardial outgrowth. The size of the area occupied by EAP-300 positive cells was determined as described in the Materials & Methods section. Significant ($p<0.05$) reduction of the periarterial Purkinje fiber area was observed in both experimental groups (mechanical inhibition: 10.4% ± 2.7%; grey bar; genetic inhibition: 8.3% ± 1.4%; black bar) compared to control embryos (100% ± 3.8%; white bar). Error bars indicate standard deviations.

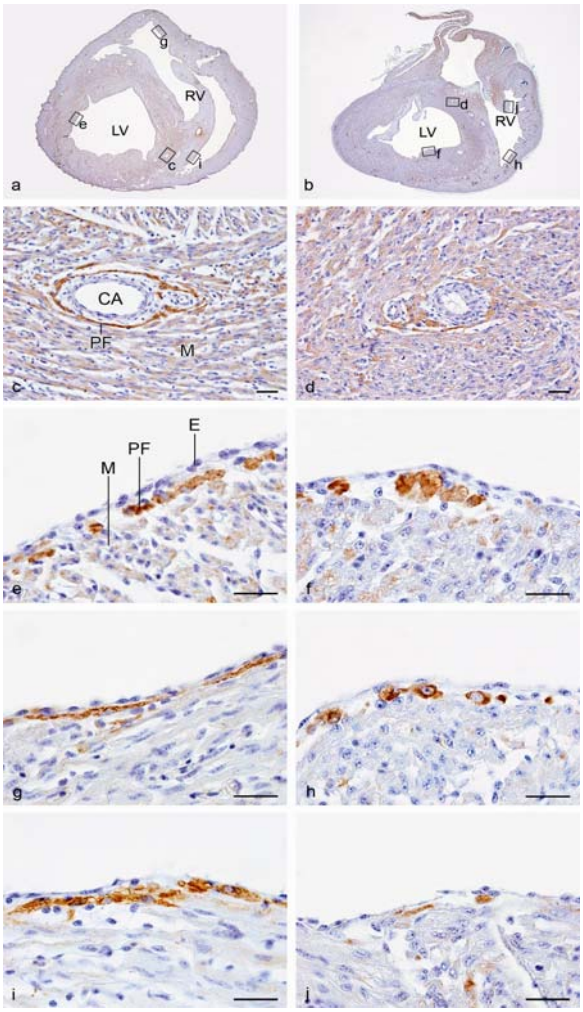

Figure 3. Photomicrographs of representative transverse sections of a normal control quail heart (HH 41; **a, c, e, g,** and **i**) and a quail heart with mechanical inhibition of epicardial outgrowth. (inh; HH 41; **b, d, f, h** and **j**). Sections were stained with immunofluorescent EAP-300 antibody and DAB-converted as described in the Materials & Methods section (**c-j)**. Panels **a** and **b** show the complete sections at approximately mid-ventricular level, where coronary arteries of similar diameter are present. The location of the enlargements in panels **c**-j is indicated. In the mechanically inhibited heart, the periarterial EAP-300 signal is faint and diffuse (**d**) when compared to the signal in the periarterial Purkinje fibers in the normal control heart (**c**). This is consistent with the finding that EAP-300 immunofluorescence did not surpass background in these embryos (Figure 2). Additionally, EAP-300 staining revealed abnormally large rounded cells in the subendocardial compartment of the peripheral conduction system. These cells were not as neatly organized in fibers as the EAP-300 positive cells in controls (**e**, **f**; left ventricular and **g**, **h**; right ventricular ventral free walls). Hyperplasia and absence of fiber-like organization of the subendocardial Purkinje fibers was observed throughout, and is illustrated here by EAP-300 staining in the moderator band of the right ventricle (**i, j**; lateral side of the moderator band). CA, coronary artery, E, endothelium; LV, left ventricle; M, myocardium; PF, Purkinje fiber; RV, right ventricle. Scale bars, 20 μm.

Discussion

Proper timing of the development of the epicardium and sequential transformation of the epicardium into migratory EPDCs is important for cardiac development. It has been shown that EPDCs are essential for formation of the compact myocardium [6, 7, 27, 28], and that proepicardial organ-derived endothelial precursor cells and EPDCs provide the building blocks of the coronary vascular network [3, 22, 29]. Furthermore, EPDCs are involved in sculpting and remodelling of the outflow tract [30, 31] and have a key function in establishing the coronary orifices [32]. The experimental results presented in this study provide the first direct evidence that EPDC are also directly involved in the development of the peripheral Purkinje fiber conduction system.

The recruitment of cardiomyocytes into the Purkinje fiber system has been demonstrated to be related to hemodynamic influences derived from the coronary vasculature and endocardial lining of the ventricular lumen [10, 11, 13], with mature ET-1 and the ECE1 metalloprotease as key inductive factors [14-16]. Earlier work demonstrated dramatic effects on the development of cardiac architecture and coronary vasculature when epicardial differentiation was disturbed [6, 7, 27, 33]. However, the developmental effect of epicardial inhibition, either mechanically or genetically, is variable and the late surviving embryos (>HH39) in the present study were specifically included because they were the least affected. This was not only deduced from the fact that they had been able to survive the critical developmental stage of HH35 (when the coronary ostia must have been formed), but also from their normal coronary and myocardial architecture. Accordingly, except for an occasional difference in the size of the hearts, no gross cardiac abnormalities were observed. We therefore advocate a direct effect of EPDCs on the conversion of cardiomyocytes into conduction cells and do not consider hemodynamic alterations to be the cause of the Purkinje fiber network hypoplasia in our experimental embryos. However, we cannot completely rule out this possibility in a minority of the embryos because of the slight concomitant thinning of the coronary smooth muscle layer. Our reasoning that EPDCs have a direct effect on Purkinje fiber formation is further substantiated by the abnormalities in the subendocardial portion of the peripheral conduction system.

We have not found evidence that increased apoptosis was the cause of the Purkinje fiber hypoplasia. Pruning of conduction tissue development by apoptotic events occurs specifically between stage HH29-32 in the future His bundle and bundle branches, and similar apoptotic pruning was suggested but not shown for the developing peripheral Purkinje fibers [34]. It can not be excluded that the apoptosis in the central conduction system is neural crest cell related [35], whereas the peripheral Purkinje network has a specific relation with EPDCs [12]. In embryos with epicardial outgrowth inhibition we observed decreased, not increased, levels of apoptosis in the ventricular myocardium at stage HH30 [36]. Additionally, in the present study we did not find apoptotic cells in the Purkinje fiber network of both the normal and the PEO-inhibited hearts at stage HH40 (not shown).

We can only speculate on the molecular mechanism by which EPDCs promote Purkinje fiber differentiation. We favour the idea of a direct paracrine effect of EPDCs on the cardiomyocytes to differentiate into Purkinje fibers, supported by the abnormalities of the subendocardial Purkinje fibers in our embryos. We propose a model in which EPDCs cooperate with endothelial cells in the differentiation of conductive tissue from cardiomyocytes (see Figure 4). In our favourite hypothesis, EPDCs cause cardiomyocytes to change into more primitive cardiomyocytes. The fate of these EPDC-induced primitive cardiomyocytes (PCMs) is then determined by local factors, like the ET-1 cleavage product of ECE1, which could as a second 'hit' be delivered by endothelial or endocardial cells, and cause the PCM to finally differentiate into a conductive cell of the Purkinje fiber network. Absence of or a decrease in either the first EPDC-derived, or the second, endothelial/endocardial cell-derived inductive event leads to hypoplasia of the Purkinje fiber network. In earlier studies demonstrating the inductive capacities of endothelin and ECE-1 in Purkinje fiber conversion, the requirement for the initial EPDC-derived signal was fulfilled by the presence of interstitial EPDCs (fibroblasts) in either the cardiomyocyte culture *in vitro* [37], or *in vivo*, in the myocardium [14]. It can also be hypothesized that the endocardial or endothelial cell would signal to the EPDC, which in turn, would impose a directive signal on the neighbouring cardiomyocyte. Alternatively, we can explain our findings with a model in which the levels of mature ET-1 are increased, either indirectly, by a cascade in which the EPDC instructs the endocardial/ endothelial cell to enhance its ET-1 and/or ECE1 production, or directly, by the EPDCs. It has been shown that

cultured EPDCs have ET-1 in their secretion repertoire [38]; it remains to be investigated whether this also applies for EPDCs *in vivo* at the time of Purkinje fiber development.

Whatever mechanism it may turn out to be, the present study indicates that Purkinje fiber development is influenced by EPDCs within a narrow time window. We observed aberrant Purkinje fiber development in embryos with practically no morphological defects. The smooth muscle layer of the coronary arteries is comprised of differentiated EPDCs and because this layer is almost as good as normal in the inhibition embryos used in this study, it can be deduced that a drastic decrease of periarterial EPDCs does not account for the Purkinje fiber hypoplasia. During the procedure of mechanical inhibition of proepicardial outgrowth, we may well have induced only a delay in the time of arrival of the subendocardial and periarterial EPDCs, by destroying the extracellular matrix bridges connecting the PEO to the heart [39] while inserting a piece of eggshell membrane. When subsequently the piece of membrane failed to stay in place, PEO cells may have grown around the membrane and "rescued" the normal phenotype, except for an epicardial outgrowth delay of approximately one day with less noticeable developmental defects as a consequence. A similar phenomenon has been noted in PEO-inhibited chicken embryos rescued with a quail PEO inserted into the pericardial cavity. In these embryos coronary ostia formation was disturbed [40]. Preliminary electrographical experiments (not shown) indicating a concommitant decrease in action potential propagation from endocardium to epicardium, implicate that such minor changes in EPDC behaviour may have functional consequences as well, which is of particular interest from a clinical point of view.

In summary, we found that inhibition of epicardial outgrowth leads to morphological abnormalities of the Purkinje fiber network. We postulate that EPDCs, in close collaboration with endothelial and endocardial cells, play an essential role in the development of the peripheral conduction system of the heart. Our preliminary indications for electrocardiographical defects due to Purkinje fiber hypoplasia provide a basis for further research on the role of EPDCs in the development of congenital conduction deficits.

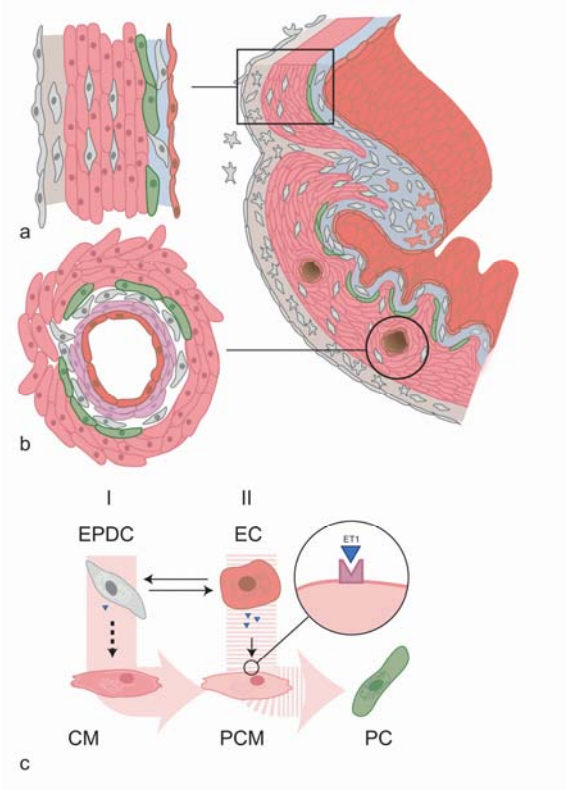

Figure 4. Hypothetical model for the role of EPDCs in the differentiation of Purkinje fibers. EPDCs derive from the epicardial mesothelium (grey outer layer) by epithelial-mesenchymal transition, to form the subepicardial mesenchyme (stellate cells, grey). Following migration, these cells differentiate into periarterial smooth muscle cells (SMCs, in purple) and fibroblasts (spindle-shaped, in grey), myocardial interstitial fibroblasts (grey) and subendocardial fibroblasts (grey). Purkinje fiber cells (green) develop at **a**) subendocardial and **b**) periarterial sites, where both EPDCs (smooth muscle cells and/or fibroblasts) and endocardial /endothelial cells (red) are in close proximity of the cardiomyocytes (pink) that convert into conduction tissue. **c**) Purkinje fiber cell (PC) differentiation occurs after two subsequent inductive 'hits'. Firstly, an EPDC-derived factor causes the cardiomyocyte (CM) to change into a more primitive cardiomyocyte (PCM). Thereafter, hemodynamically determined expression of ET-1 and ECE1 by endocardial / endothelial cells (EC) brings about the final signal that allows the primitive cardiomyocyte to convert into a conduction cell. Alternatively, the endothelial or endocardial cell instructs the EPDC to express one or more inductive factors. Among these, ET-1 is a candidate since it has been shown that cultured EPDCs can express this molecule [38].

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Chapter 6

Periostin expression by epicardium-derived cells (EPDCs) is involved in the development of the atrioventricular valves and fibrous heart skeleton.

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Abstract

The epicardium is embryologically formed by outgrowth of proepicardial cells over the naked heart tube. Epicardium-derived cells (EPDCs) migrate into the myocardium, contributing to myocardial architecture, valve development and the coronary vasculature. Defective EPDC formation causes valve malformations, myocardial thinning and coronary defects. In the atrioventricular valves and the fibrous heart skeleton isolating atrial from ventricular myocardium, EPDCs colocalize with periostin, a matrix molecule involved in remodeling. We investigated whether proepicardial outgrowth inhibition affected periostin expression and how this related to development of the atrioventricular valves and fibrous heart skeleton.

Periostin expression by epicardium and EPDCs was confirmed *in vitro* in primary cultures of human and quail EPDCs. Disturbing EPDC formation in quail embryos reduced periostin expression in the endocardial cushions and atrioventricular junction. Disturbed fibrous tissue development resulted in atrioventricular myocardial connections reflected by preexcitation electrocardiographic patterns.

We conclude that EPDCs are local producers of periostin. Disturbance of EPDC formation results in decreased cardiac periostin levels and hampers the development of fibrous tissue in atrioventricular junction and the developing atrioventricular valves. The resulting cardiac anomalies might link to Wolff-Parkinson White syndrome with persistent atrioventricular myocardial connections.

Introduction

The epicardium develops when cells of the proepicardial organ (PEO), a cauliflower-like protrusion of the sinus venosus at the level of the primordial liver, contact the initially naked heart tube and grow out over the myocardium [1]. Subsequently, part of the epicardial cells loose their epithelial characteristics by epithelial-mesenchymal transformation (EMT) and form a subepicardial layer of mesenchymal epicardium-derived cells (EPDCs;[2]). During further development these EPDCs migrate into the underlying myocardium and differentiate into interstitial, subendocardial and coronary adventitial fibroblasts, as well as coronary smooth muscle cells [2-8]. Procollagen I deposition at the site where EPDCs are present indicate how they might contribute to the formation of the fibrous heart skeleton [2]. Several studies demonstrated crucial roles for EPDCs in the formation of myocardial architecture and coronary development [9-13], whereas a morphogenic role for EPDCs in valve development has also been postulated [2].

After migration to the developing valves, EPDCs intermingle with other populations of cells important for valve morphogenesis when they reach the cushion mesenchyme. Wherever the cushion tissue directly contacts myocardial tissue, remodeling events are engendered that are critical for the establishment of a four-chambered heart. In the proximal region of the outflow tract (below the level of the developing valves), myocardial tissue replaces or grows into the cushion mesenchyme to form the muscular outlet septum by a process called myocardialization [14, 15]. In segments of the primary heart tube where the atrioventricular (AV) valves develop, the continuity of a myocardial covering or sleeve is not maintained. Rather, fibrous tissue replaces the myocardium to form an annular fibrous "skeleton" to which the AV valve leaflets attach. It has been found that sites where the myocardium persists or "disappears" correlate with variations in the expression of periostin, an extracellular matrix (ECM) protein encoded by a gene with homology to the Drosophila fasciclin gene family [16].

Periostin was identified as an important developmental factor [17]. It was implicated in regulating adhesion and differentiation of osteoblasts [18] and has been recognized as an anti-osteogenic molecule [19]. Periostin has been shown to be expressed in developing and mature valves of the heart [17]. In ovarian epithelial cells it regulates adhesion and migration via its binding to the αvβ3 and αvβ5 integrins [20]. In the heart, periostin is expressed initially in all cushion tissues. However, in the proximal outflow tract (conus) cushions, periostin expression is downregulated during development [19, 21] whereas in the distal outflow tract and AV prevalvular cushions, periostin expression is enhanced, particularly at the boundary with the myocardium. Based on this correlation and a similar one that occurs in adult myocardium following ischemic injury [22], periostin appears to have an “anti-myocardial” effect. Also under pathological conditions with increased fibrosis, such as myocardial infarction, cardiac hypertrophy and vascular injury, upregulation of cardiovascular periostin expression occurs [23-25].

In the normal embryonic heart, the spatiotemporal expression of periostin closely resembles the migratory patterns of EPDCs (*Cf.* [2] And [17, 19, 21]) Furthermore, i*n situ* hybridization experiments showed that the epicardium, the EPDCs of the subepicardium and dispersed cells in the myocardium synthesize periostin [21]. The dispersed myocardial expression pattern of periostin is highly reminiscent of the myocardial distribution of EPDCs in qiuail-chicken chimeras [2]. From earlier studies we know khat EPDCs are indispensable for normal AV valve and fibrous heart skeleton development, as well as for proper ventricular myocardial architecture. In this study we studied periostin expression by epicardium and EPDCs in i*n vitro* cultures and investigated whether expression of periostin in the AV region is affected by epicardial outgrowth inhibition. We hypothesized that AV valve differentiation and development of the fibrous AV junction might be disturbed when periostin expression was significantly diminished by inhibition of epicardial outgrowth.

Materials and Methods

Embryonic quail PEO and adult human EPDC cultures

To confirm periostin synthesis by epicardial cells and EPDCs we performed immunohistochemical and immunofluorescent stainings on human adult EPDCs, isolated from the atrial appendages as described earlier [26], and on quail PEO explants isolated at stage HH16 quail embryos and cultured for 1 week on glass slides coated with 0.1 μg/ml fibronectin and 0.1% gelatin. Periostin expression was analyzed in 4 PEO explant cultures and in primary human EPDC cultures from three individuals.

Embryos and mechanical inhibition of epicardial outgrowth.

To study the role of periostin expression by EPDCs in the development of the fibrous tissues of the heart we used a mechanical experimental inhibition model in which EPDC development and differentiation were disturbed in quail embryos. Normal controls consisted of untreated quail embryos of Hamburger and Hamilton stages [27] HH30 (n= 4), HH35 (n=3) and HH41 (n=7).
Outgrowth of the PEO in quail embryos (HH15 to HH18) was inhibited by placing a piece of egg shell membrane between the PEO and the heart tube, as described earlier [12, 28]. After reincubation at 37.5°C (80% humidity), embryos were isolated at stages HH 30 (n=7), HH 35 (n=6) and HH 41 (n=8). Of each stage at least 4 embryos had decreased formation of the epicardium upon routine histochemical staining. These embryos were used for further analysis.

Electrocardiographical analysis

A total of 5 control and 8 mechanically inhibited epicardial outgrowth quail embryos (stage HH 41) were used for electrocardiographical analysis. The embryos were taken out of the eggshell and placed in a plastic Petri dish in warm Tyrode's solution. Surface electrocardiograms (ECGs) were recorded by four platinum electrode wires connected to the Petri dish and stored on an optical disk of a customized acquisition system (Prucka Engineering, Houston TX, U.S.A.) for off-line analysis. Heart rate (RR-interval), AV conduction (PR-interval) and intraventricular conduction (QRS-width) times were measured. A non-paired, two-tailed student's t-test was used for statistical comparison between the ECG data of control and epicardially inhibited quails. A p-value of less than 0.05 was considered statistically significant.

Immunohistochemistry and immunofluorescence

Isolation and processing of embryos for immunohistochemistry were done as described earlier [2]. Serial sections were produced and subjected to standard immunohistochemical procedures [2]. Sections were incubated with antibodies recognizing periostin [19] diluted 1:200), quail endothelial antigen (QH1, Hybridoma Bank; diluted 1:500), αγ muscle actin (HHF35, DAKO; diluted 1:500) and α-smooth muscle actin (1A4, Sigma-Aldrich; diluted 1:3000). Immunohistochemical staining of cell cultures was performed with the periostin antibody (1:200 dilution) and anti-Wilms' Tumor 1 suppressor protein WT-1(Santa Cruz sc-192, diluted 1:1000).
Photomicrographs were recorded using an Olympus-70 light microscope in combination with an Olympus DP-12 digital camera. Immunofluorescent staining of *in vitro* cultures was performed with the periostin antibody (1:200 dilution) followed by incubation with a biotinylated secondary antibody (goat-anti-rabbit BA-1000; Vector Labs) and subsequent incubation with avidin-FITC (A-2001; Vector Labs).

Results

EPDCs produce periostin.

To ascertain that epicardial cells and EPDCs are true producers of periostin, we cultured isolated human adult epicardium as well as embryonic PEO explants of the quail. During culture, epicardial cells undergo epithelial-to-mesenchymal transformation and EPDCs are abundantly present. In both types of cells periostin was detected, both in a punctuate pattern at the cell membrane and in a diffuse intracellular pattern close to the nucleus (Figure 1). In the primary cultures of quail EPDCs (n=4) the expression of periostin at distinct focal sites was observed specifically in the outer rim of the PEO explant (Figure 1a).

We certified for the purity of the human EPDC cultures by a WT-1 staining (Figure 1b). In these EPDCs cultures (n=3), periostin expression seemed to be related to the differentiation status of the individual cells. Especially in cells with a marked spindle-shape, periostin had accumulated in distinct foci (Figure 1c and d), whereas its expression was more diffuse in cells with a less differentiated, cobblestone morphology (Figure 1d).

Morphology and periostin expression in embryos with inhibited epicardial outgrowth; stage HH30.

To investigate whether defective formation of the epicardium would lead to a decrease in periostin expression in the AV region and related defects, cardiac morphology and immunohistochemical patterns of periostin staining in embryos with inhibited epicardial outgrowth and normal controls were compared at three stages during development. We focussed at the sites where EPDCs are normally known to reside with specific emphasis on the AV junction and the AV valves, the subendocardium, the ventricular wall and in the coronary vessel walls.

In normal embryos of developmental stage HH30, periostin immunostaining was high in regions where EPDCs are known to be present. For example in the AV sulcus, periostin was observed in the epicardium and subepicardium (Figure 2a,b). Similarly, high levels of periostin were observed in the developing AV cushions and at atrial and ventricular subendocardial sites (Figure 3a, c). In contrast, in the embryos with inhibited epicardial outgrowth, the expression levels were severely diminished or absent in the AV cushions (Figure 3b, d). Only weak expression was observed in the subvalvular ventricular myocardium. In the ventricle, periostin was present in the subendocardium of the trabeculae in controls, whereas in embryos with inhibited epicardial outgrowth, it was observed at similar trabecular sites but its level of expression was decreased (Figure 4a, b). With respect to its trabecular expression pattern, we found it localized in the subendocardium rather than in the endocardium (Figure 4c,d).

Morphology and periostin expression in embryos with inhibited epicardial outgrowth; stage HH35.

At stage HH35, periostin was expressed in a more differentiated pattern in normal embryos. It was observed in the fibrous continuity in the AV sulcus, reaching from the epicardium to the AV valve (Figure 5a). The periostin expression in the normal left AV valves was restricted to its ventricular aspect. In the epicardially inhibited embryos we observed no continuous band of periostin expression in the AV groove, coincident with the absence of atrioventricular sulcus mesenchyme in this region (Figure 5b). At the sites of absent AV fibrous annulus formation, continuity of atrial and ventricular myocardium was observed. (Compare Figure 5c, d). This myocardial continuity was additional to the normal connection of the common bundle of the conduction system at the crux of the heart (Figure 5a,c). Also, expression in the AV cushions/valves was markedly diminished compared to the developing distal outflow cushions (not shown). Spatially, periostin staining that persisted in the AV cushions of epicardially inhibited hearts was not confined to the ventricular aspect of the cushion (Figure 5b). At its mural side, the myocardium of the left AV cushion tissue had not delaminated. Hereby, the cushion had not developed into a regular mitral valve leaflet (compare Figure 5a and b).

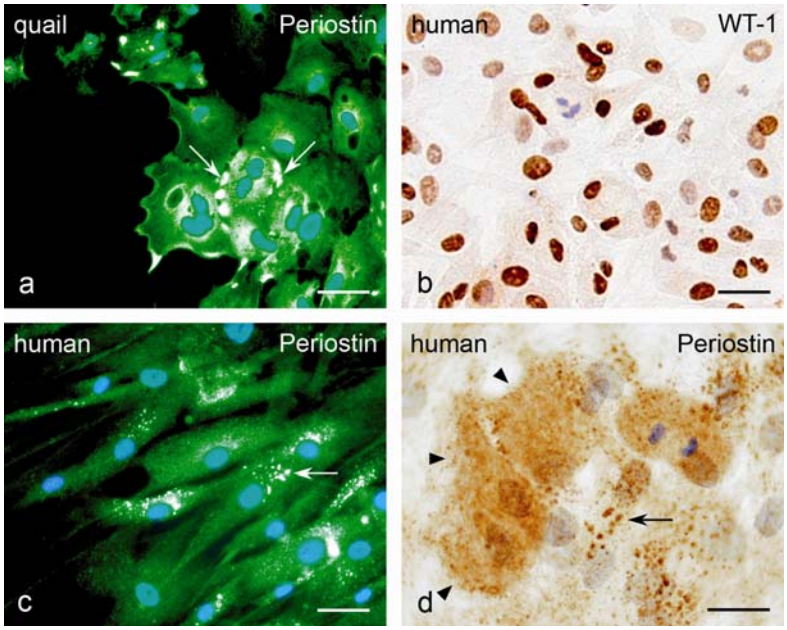

Figure 1: Periostin is expressed by embryonic quail (a) and adult human epicardial cells and EPDCs (b, c,d). In the quail proepicardial organ explant cultures (a) the cells express periostin both as distinct foci at the cellular membrane (highlighted in white; indicated by an arrow) as well as in a diffuse cytoplasmic and perinuclear expression pattern. In adult human epicardium harvested from the atrium, the nuclei of the initially cobblestone cells are positive for the epicardium-specific WT-1 antibody (b). Some nuclei have lost their positivity after epithelial-to-mesenchymal transformation resulting in a spindle-shaped phenotype typical for EPDCs. In human adult EPDCs this spindle-shaped phenotype shows both focal expression (highlighted in white, indicated by an arrow in c and arrow in d) and diffuse perinuclear expression. In a DAB staining of a culture of human EPDCs (d) both cobblestone (arrowheads) as well as spindle-shaped (arrow) cells are present. In the later a more marked punctuate periostin expression is observed. Scale bars, 30 µm (a,b,c); 20µm (d).

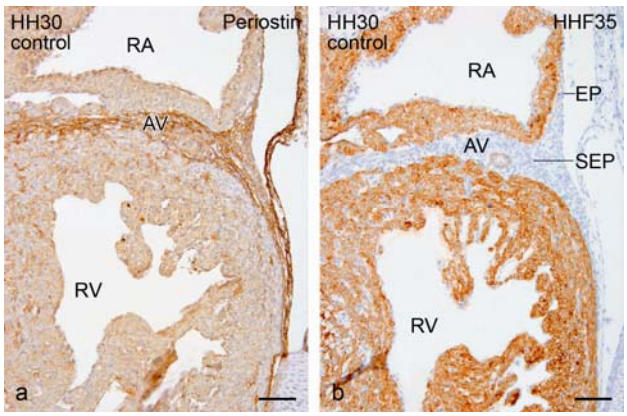

Figure 2: The epicardium and the subepicardium, consisting of EPDCs, express periostin. Photomicrographs of serial sagittal sections through the AV junction (AV) at the level of the right ventricular base of a normal embryonic quail heart at developmental stage HH30. (**a)** High levels of periostin are found in the EPDCs of the subepicardium**,** which is delineated by the absence of actin, as is shown in (**b)**. EP, epicardium; RA, right atrium; RV, right ventricle; SEP, subepicardium. Scale bar, 60 µm (**a,b**)

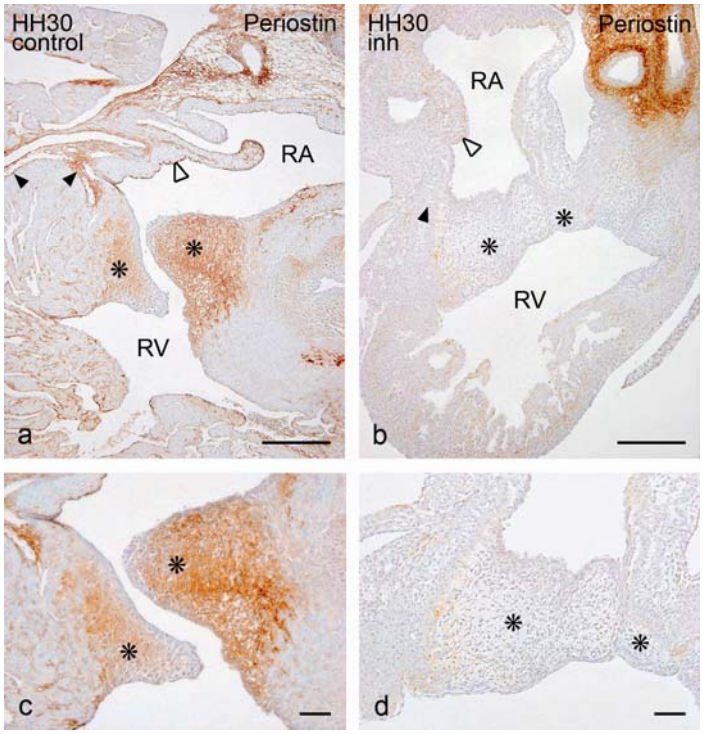

Figure 3: Photomicrographs of representative sagittal sections stained for periostin of a normal quail heart (**a, c**) and an epicardially inhibited quail heart (**b, d**) at developmental stage HH30. Panels **a** and **d** are enlargements of the cushion regions in **a** and **b**, respectively. In the normal heart periostin expression was observed in the AV-cushions (asterisks), in the (sub)epicardium and subepicardially in the AV groove (filled arrowheads), and subendocardially in the atrium (open arrowheads). At these sites periostin expression was absent in the embryos with inhibition of epicardial outgrowth (**b**). RA, right atrium; RV, right ventricle. Scale bars, 200 µm (**a,b**); 60 µm (**c,d**).

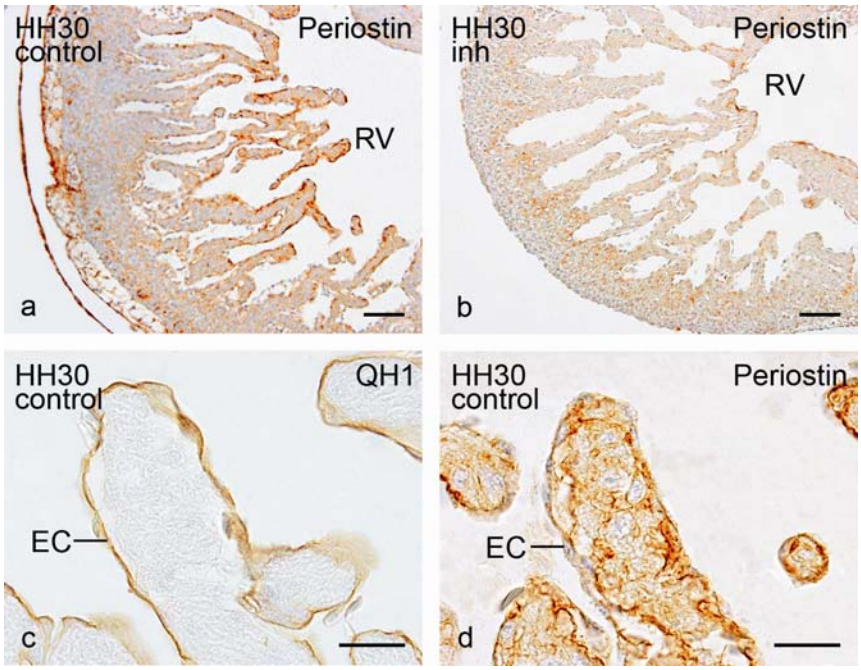

Figure 4: Inhibition of epicardial outgrowth causes a decrease in trabecular periostin expression, as is shown by these photomicrographs of representative sagittal sections stained for periostin of a normal quail heart (**a**) and an epicardially inhibited quail heart (**b**) at developmental stage HH30. With respect to the cells that produce the trabecular periostin signal, we noted that there is no colocalization of the endocardial marker QH1 (**c)** and periostin (**d**) in the trabeculae in serial sections of a normal embryonic quail heart at HH30. EC, endocardium; RV, right ventricle. Scale bar, 60 µm (**a,b**); 20 µm (**c,d**).

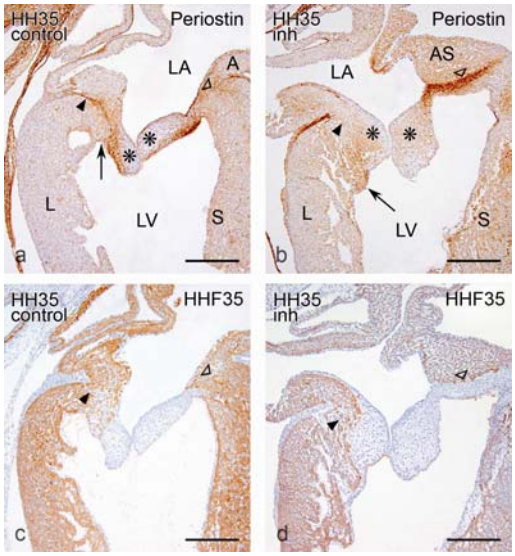

Figure 5: Photomicrographs of representative sagittal sections of a normal embryo (**a,c)** and an embryo with inhibited proepicardial outgrowth (**b,d)** at developmental stage HH35 stained for periostin (**a,b)** and myocardial actin HHF35 (**c,d**). In control embryos the cushions had developed into the valve leaflets of the mitral valve, with condensed mesenchyme (asterisk) and especially **at** their ventricular aspects containing high levels of periostin **(a).** Normal delamination of the mural valve leaflet is indicated by an arrow (**a)**. In epicardially inhibited embryos, the cushions were immature, consisting of loosely organized mesenchyme (asterisk) with a diffuse staining pattern of periostin without signs of valve architecture (**b).** The mural valve leaflet did not delaminate (arrow in **b**). In the fibrous heart skeleton developing at the atrioventricular junction between the sulcus epicardium and the developing AV valve periostin expression was high in the control embryos (arrowhead in **a**), while myocardial actin expression was low in that area (arrowhead in **c).** Periostin expression in the AV sulcus of epicardially inhibited embryos was seen only in the sulcus epicardium but did not reach into the AV valve (**b**, arrowhead).
There is more actin staining tissue connecting the atrial and ventricular myocardium in the inhibited embryos (arrowhead in **d**). The section level at the septal side of both control and inhibited embryos (open arrowheads) cannot be compared for the actin and periostin staining as in the control embryo we are in the region of the atrioventricular myocardial bridging related to the developing conduction system while in the inhibited embryo we are in the fibrous heartskeleton at the base of the atrial septum (AS). A, atrial wall; L, lateral ventricular myocardium; LA, left atrium; LV, left ventricle; S, septal ventricular myocardium. Scale bars, 200 μm.

ECG recordings and morphology in embryos with inhibition of epicardial outgrowth; stage HH41

To investigate whether epicardial outgrowth inhibition and the consequent decrease in atrioventricular subepicardial mesenchyme and periostin expression were reflected by impaired functional insulation between the atrial and ventricular myocardium, the hearts of normal and inhibited embryos were analyzed electrophysiologically. In 3 out of 8 inhibited embryos electrocardiograms (ECGs) pre-excitation over an accessory pathway was revealed (Figure 6). Whereas in normal controls the PR interval was 57 ± 8 ms and the QRS-time 25 ± 2 ms, in these embryos the PR-interval was as short as 30±3ms ($p<0.01$) and the QRS-complex as wide as 43±3ms ($p<0.05$). Morphological sections showed that in these hearts, the myocardium of the ventricles and atria was not separated by fibrous tissue of the atrioventricular annulus. The fibrous insulation was present at this age in normal controls (Figure 7 a, b, e, g). In the embryos with electrocardiographical preexitation patterns, myocardial bridges similar to those observed at stage HH35 had remained between the right atrium and ventricle (Figure 7 c, d, f, h). The diameter of muscular AV connection was approximately 150-200 μm, as was calculated from the section thickness and the number of sections in which we observed AV myocardial bridging. In the present set of stage HH41 embryos with inhibited epicardial outgrowth, we did not find remaining myocardial connections in the left AV junction.

With respect to the valves, a marked decrease in periostin levels could be detected in the mitral valve leaflets (Figure 8a, b). Furthermore, there was diminished expression of periostin in the interstitial spaces of the ventricular myocardium (Figure 8 c, d), and also periarterial expression of periostin was lower in epicardially inhibited embryos than in normal controls (Figure 8e, f).

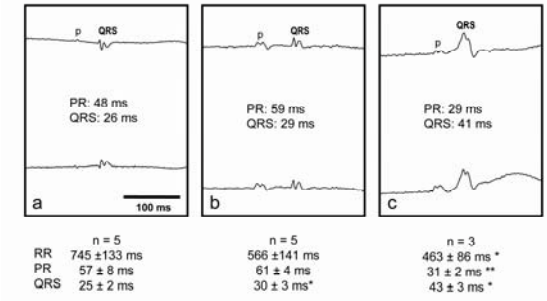

Figure 6: Representative electrocardiograms (ECGs) showing simultaneously recorded tracings of (**a)** a normal quail hearts (HH41), (**b)** a quail heart (HH41) with inhibition of epicardial outgrowth without pre-excitation but with prolonged QRS time, and (**c)** a quail heart (HH41) with inhibition of epicardial outgrowth and pre-excitation. Embryos with pre-excitation showed a short PR interval of 31±2ms (**, $p<0.01$) and a QRS-complex as wide as 43±3ms (*, $p<0.05$).

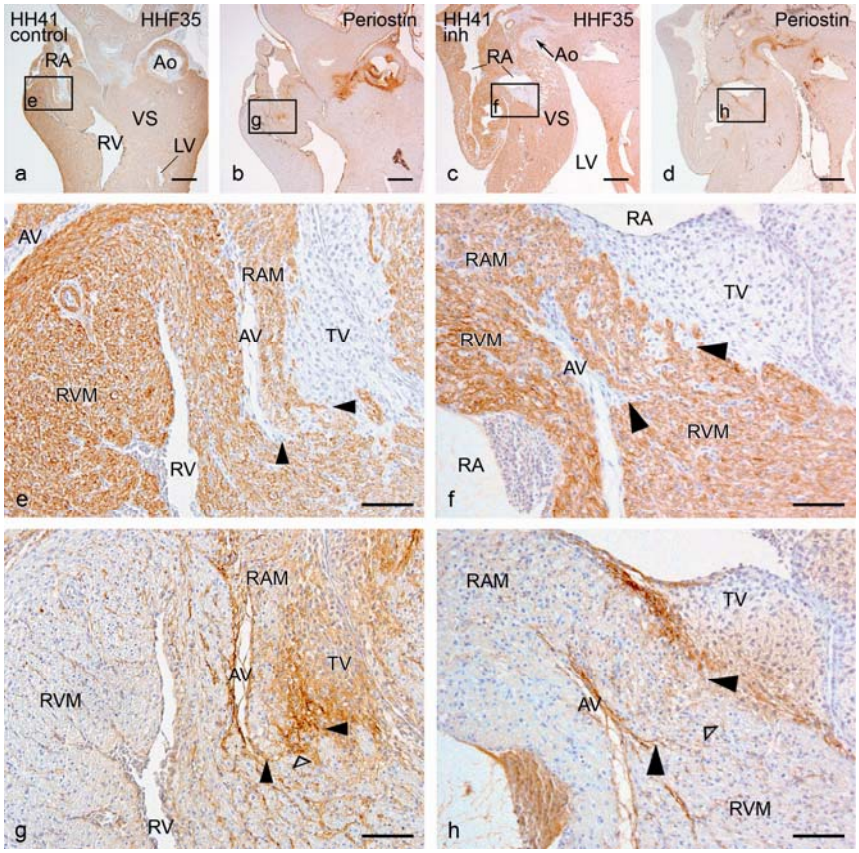

Figure 7: Photomicrographs of representative frontal sections of a normal quail heart (**a, b, e, g)** and a quail heart with inhibition of epicardial outgrowth and pre-excitation in (**c, d, f, h)** at developmental stage HH 41. Sections were stained with the myocardial actin marker HHF35 (**a, c, e, f)** and with periostin (**b, d, g, h)**. The AV junction **(AV)** in the normal heart consisted of a continuous layer of periostin-expressing cells. Even at its thinnest points (open arrowhead and between arrowheads), the periostin-positive, mesenchymal cell layer is not interrupted (**e**, **g)**. In contrast, the mesenchymal insulation between atrium and ventricle in the epicardially inhibited quail heart is bridged by a broad myocardial atrioventricular connection (open arrow head) of approximately 200 µm in diameter (in between the arrowheads in **f),** which is further characterized by the absence of continuous periostin staining (in between the arrowheads in **h**). Ao, aorta; LV, left ventricle; RA, right atrium; RAM, right atrial myocardium; RV, right ventricle; RVM, right ventricular myocardium; TV, tricuspid valve; VS, ventricular septum. Scale bars, 300 µm (**a-d**), 60 µm (**e-h**).

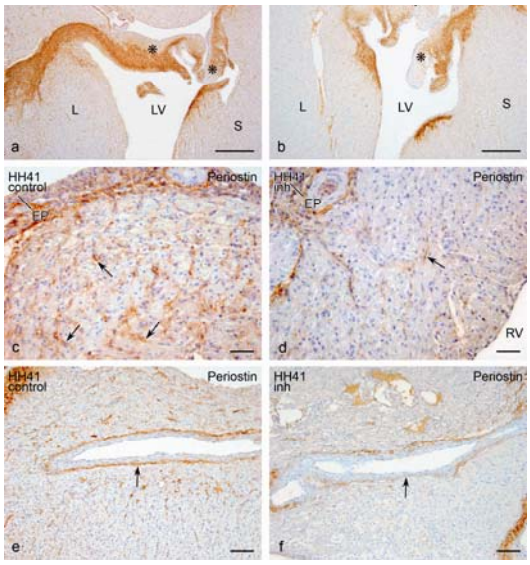

Figure 8: Photomicrographs of representative frontal sections of a normal quail heart (**a, c, e)** and a quail heart with inhibition of epicardial outgrowth (**b, d, f)** at developmental stage HH 41. Compared to control stainings, diminished periostin expression was observed in the mitral valve leaflets (asterisks in **a, b),** in the interstitial space between the cardiomyocytes, as is shown here for the myocardium of the right ventricular base (arrows in **c, d**), and periarterially in the coronary vessel wall (arrows in **e, f)**. L, lateral ventricular myocardium; S, septal ventricular myocardium. Scale bars, 200 µm (**a, b**), 30 µm (**c, d**), 60 µm (**e,f**).

Discussion

During normal development, EPDCs migrate and contribute to a variety of morphologically delineated structures in the heart. Previous tracing experiments and observations after mechanical or genetic inhibition of epicardial differentiation showed their indispensable contribution to the heart as smooth muscle cells and fibroblasts in the coronary media and adventitia, as interstitial fibroblasts contributing to the architecture of the myocardial wall and as mesenchymal cells in the developing cushions (e.g. [2, 4, 11, 12], reviewed by [29] and[30]). In addition, EPDCs were shown to be important for the differentiation of the Purkinje fibers in the developing chicken heart [31].

Whereas the migration routes and the need for the physical presence of EPDCs in heart development have become increasingly clear in the last decade, the signaling routes and instructive signaling molecules used by these multipotent cells are still largely unknown. The spatiotemporal colocalization of the profibrogenic ECM protein periostin [32] and pro-collagen I producing EPDCs [2], triggered us to investigate whether periostin might be one of the factors important for EPDC function. Earlier *in situ* hybridization experiments showed that the epicardium and the subepicardial EPDCs are periostin-synthesizing cells [21]. In the present study we confirm that EPDCs are indeed true producers of periostin. As many EPDCs are destined to become cardiac fibroblasts [2, 6, 8], this is consistent with later findings of periostin-expressing fibroblasts in the heart [22, 23, 33]. Both adult and embryonic isolated EPDCs express periostin both in a punctate pattern and in the cytoplasm. That the presence of EPDCs is also strongly associated with the presence of periostin protein *in vivo* is particularly obvious in the epicardium and subepicardium covering the base of the ventricles and in the subepicardium of the AV sulcus and AV junction. In the myocardium, inhibition of PEO outgrowth resulted in diminished interstitial periostin expression, which may correlate well with limited numbers of interstitial EPDCs. In this respect it is very likely that the subendocardial trabecular periostin expression which has initially been ascribed to the endocardium [19], is in fact produced by the subendocardial trabecular EPDCs [2, 6]. This is further substantiated by our current finding that epicardial inhibition causes a loss of trabecular periostin expression.

The diminished periostin levels in the present study seem to be the direct consequence of the limited epicardial contribution to the heart in embryos with inhibition of proepicardial outgrowth. Although we consider a direct causal relation between loss of periostin-expressing EPDCs and the loss of periostin at sites where they are normally present the most likely, loss of instructive signalling by EPDCs may also account for the decrease in periostin expression after proepicardial inhibition.

During development, the subepicardial space of the AV sulcus becomes populated with epicardium-derived mesenchyme which is initially separated from the endocardium-derived cushion mesenchyme by an intact AV junctional myocardium. Subsequently, EPDCs migrate through the junctional myocardium into the AV cushions. Within the endocardial cushion, the EPDCs favour two positions; one at the myocardial/endocardial cushion interface, and one subendocardially at the site facing the ventricular lumen [2]. Eventually, discontinuities within the AV junctional myocardium permit complete intermingling of the two mesenchyme populations which, over time, collectively form a purely fibrous annulus for the AV valves. This fibrous structure also serves to insulate atrial myocardium from ventricular myocardium to assure that all pacemaker activity is directed through the central conduction system. Although the number of embryos is rather limited, our results clearly indicate that with the lack of EPDCs and consequent decrease in periostin, the incompleted fibrous insulation leads to pre-exitation patterns in the ECGs.

That periostin may be an important signaling molecule in the development of the valves and fibrous heart skeleton has been postulated earlier in descriptions of its expression patterns [16, 17, 19, 21, 32, 34, 35]. Considering the mechanism by which periostin influences the formation of fibrous tissue many questions are still unresolved. Multiple potential cellular mechanisms regulated by periostin could support its function in the formation of a fibrous scaffold. It can modify cell adhesion [36], cell motility [20, 37]. Furthermore, periostin promotes collagen invasion by cushion mesenchymal cells [34] and is indispensable for collagen fibrillogenesis and the maintenance of connective tissue biomechanical properties [32]. Periostin binds to fibronectin, tenascin-C, collagen V and periostin itself [38]. This also fits with the description of the *in vivo* expression of fibronectin [39] and procollagen I [2] at locations were periostin and EPDCs have been reported during valve development.

Our finding that reduced periostin after EPDC withdrawal hampers the correct development of fibrous tissue in the AV region is consistent with a vast body of evidence that peristion exerts a pro-fibrotic role in development and under pathological conditions as after myocardial infarction and pressure overload (e.g. [22, 40,

[41]). Further recent evidence was also provided by the phenotype of a new periostin knockout mouse, showing compromised fibrosis leading to myocardial wall rupture after myocardial infarction [33]. In contrast, periostin overexpression induced cardiac hypertrophy rather than cardiac fibrosis, probably because overexpression was achieved in cardiomyocytes rather than in fibroblasts, which are the natural source of cardiac periostin [33].

The present study describes how loss of EPDCs leads to diminished periostin levels, associated with defects in the maturation of the atrioventricular valves and fibrous annulus. With respect to periostin gene knockout experimentation, two mouse models have described in literature. Their complete cardiac phenotypes have not yet been published, but valve abnormalities that may cause perinatal death exist in a subpopulation of the first periostin knockout mice [42, 43]. Our data also correspond well with those observed in mice with a conditional deletion of the Alk3 gene [44]. In this mouse downregulation of periostin in the AV region was also associated with persistent atriventricular myocardial connections. Clinically, this phenotype is reminiscent of Wolff-Parkinson-White (WPW) syndrome [45]. In this study the incompleted development of a fibrous insulation in the atrioventricular junction caused a persisting atrioventricular connection between atrial and ventricular myocardium with consequent pre-exitation of the ventricle. Similar electrical bypasses consisting of functional myocardium were also found in a model for Mahaim tachycardia [46] and during development when base-to-apex conduction is still predominant [35]. Together with our new data on changes in periostin expression after epicardial inhibition, this suggests that an epicardial outgrowth and/or differentiation deficit may underlie congenital pathogenesis of the atrioventricular fibrous annulus and valves.

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Chapter 7

Surgical implications of coronary arterial anatomy in adults with congenital cardiac disease.

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Abstract

In adults with congenital heart disease coronary arterial anatomy, normal as well as anomalous, may have implications in surgical reconstruction of an underlying cardiac structure.
In addition to the diagnostic imaging, necessary in surgery for adult congenital heart disease, additional information with regard to the spatial relation between the relevant cardiac structure and the coronary arterial system may be required for planning the operation and providing a good outcome. The congenital cardiac surgeon should have the necessary skills in coronary artery bypass techniques. With lack of adequate data, the estimation of mortality due to complications as a result of coronary damage in surgery for adult congenital cardiac disease of below 1% seems fair.

Introduction

In adults with congenital heart disease, the origin and course of the coronary arteries, both normal as well as anomalous, may have implications in surgical reconstruction of an underlying cardiac structure. In isolated congenital coronary arterial anomalies, a wide variety in anatomy as well as pathophysiology and clinical presentation is found [1,2]. Recently this was exemplified in a review on anomalous connection of a coronary artery to the opposite sinus of the aorta, a subgroup of anomalies frequently showing intussusception of the ectopic coronary artery in between the aorta and the pulmonary artery [3]. In addition to the diagnostic imaging, necessary in surgery for adult congenital heart disease, additional information with regard to the spatial relation between the relevant cardiac structure and the coronary arterial system may be required for planning the operation and providing a good outcome. The goal of this presentation is to increase the awareness for the spatial relationship between the coronary arterial system, either normal or anomalous, and underlying structural congenital heart disease and the possible consequences for surgery and outcome in this regard.

Recent findings in coronary embryology

Embryological studies have shown the crucial role of the pro-epicardial organ (PEO) in the development the coronary arterial system [4]. Epicardium Derived Cells (EPDC's) from this PEO migrate to the endocardial cushions, the myocardium and subepicardium, the fibrous skeleton and importantly also to the coronary vasculature [5]. It has been shown that in a process of epithelial mesenchymal transformation, the EPDC's differentiate into myocardial, subepicardial and perivascular fibroblasts and coronary arterial smooth muscle cells [6,7]. The EPDC's are essential for myocardial maturation, myocardial vascularization, stimulation of coronary vascular formation, building up the medial layer of the coronary arteries and for ingrowth of the coronary arteries into the great arteries, usually the aorta [8]. All these studies confirmed that coronary arterial development concentrates in three identifiable rings, the peritruncal ring, containing the coronary arterial orifices, the atrioventricular groove ring and the interventricular septal ring [9].

Coronary arterial anomalies

With regard to the results of the embryological studies, it is not surprising that the vast majority of coronary arterial anomalies are found in the area of the three rings.

Among the anomalies associated with myocardial ischemia are coronary fistula's, the abnormal right or left coronary artery connected to the pulmonary artery (ARCAPA or ALCAPA), congenital coronary stenosis or atresia, anomalous origin from the contralateral sinus [3] and a single orifice with a branch running between the aorta and pulmonary artery [1-3].

Anomalies coronary arteries usually not associated with myocardial ischemia are a left circumflex coronary artery originating from the right sinus and the coronary arteries originating from one sinus with separate orifices [1-3].

The incidence, clinical characteristics and possible therapeutical interventions for these coronary arterial anomalies are well described. Nevertheless, attempts at further classification of coronary anomalies are being produced, a recent contribution being made with regard to the anomalous origin of a coronary artery from the opposite sinus [3].

All of these can be regarded as isolated anomalies [1,2]. Exact figures on their prevalence are not available, presented numbers being dependant on differences in definition, sampling and background cohort [3]. It seems reasonable to assume that the figure in patients is below 1% and in the general population probably below 0.01% [1-3].

Treatment options in isolated congenital anomalies of proximal aortic coronary arteries

Not all cases of isolated congenital anomalies of the coronary arteries warrant treatment [1,3]. For instance the right abnormal coronary artery connected to the opposite sinus much less frequently needs treatment as compared to the left coronary artery connected to the opposite sinus [3]. In symptomatic patients, the treatment options in isolated anomalies of the proximal coronary arteries connected to the aorta are guided by clinical

presentation. Essentially 3 treatment options are relevant: medical or observative treatment, percutaneous intervention with stent deployment or surgical treatment [3].
Surgical options should depend on the local anatomy and the surgical expertise and may consist of reimplantion of the ectopic coronary arterial orifice, unroofing of the intramural coronary segment or creating of an additional aortic orifice at the end of the intramural course of the coronary artery [1,3]

Surgical relevance

In adults with congenital heart disease, the origin and course of coronary arteries have implications in surgical reconstruction of an underlying cardiac structure, The spatial relation between the relevant cardiac structure and the coronary arterial system is essential for planning the operation and providing a good outcome. Three-dimensional representation of the diagnostic data plays an increasingly important role in this regard.
It is important to realize that not only the coronary anomalies pose a risk in cardiac surgery in adults with congenital heart disease, but also injury of the normal coronary artery system.
The diagnostic sequence may therefore start with the usual echocardiography [3]. This will provide spatial information on the structural abnormalities and may provide data on the proximal coronary arterial anatomy as well. If however this information is inconclusive, computed tomography or magnetic imaging is recommend, directed at both cardiac as well as coronary anatomy and their interrelationship [3]. Coronary angiography is important to document the proximal and further course as well as the luminal quality of the coronary arteries, but is less optimal to provide anatomical data on the interrelationship with cardiac structures, even with contemporary contrast injection of cardiac cavities. It is of utmost importance that a final inspection is performed at surgery before irreversible surgical steps are being made.

Surgery near the proximal coronary arteries

The proximal parts of both normal and abnormal coronary arteries are at risk in surgery near the proximal coronary arteries. In any aortotomy the surgeon should be aware of the position of the coronary orifices and the course of the proximal segment of the coronary arteries. Harvesting the pulmonary root in an autograft procedure may damage the left main coronary artery or the first septal branch. In any primary or redo surgical procedure of the right ventricular outflow tract (e.g. tetralogy of Fallot, pulmonary atresia, common arterial trunk) a coronary arterial branch may also be at risk for injury. This may include a left anterior descending coronary artery (LAD) from the right coronary sinus, a single right coronary artery (RCA) with an LAD anterior to the pulmonary trunk, a single left coronary artery with an RCA anterior to the pulmonary trunk or a large infundibular branch from the RCA. In pulmonary root surgery (e.g. allograft implantation) the left main coronary artery at the posterior annular level of the pulmonary orifice may also be at risk. In most of these patients atherosclerotic coronary disease does not play a role, probably because of the younger age at operation
No hard data are available on actual damage, but it seems reasonable to estimate mortality due to complications as a result of coronary arterial damage up to 1%.

Surgery on the coronary orifices

Also the coronary arterial orifices themselves may be at risk for damage in aortic root surgery. Particularly when coronary orifices are replanted in root replacement surgery as in the autograft procedure or during prosthetic valve composite graft root replacement. Especially when the coronary buttons have to be re-excised and re-implanted again, scar tissue and local fibrosis and calcification may pose an additional challenge in the pursuit of successful surgery. In case of surgery in common arterial trunk, the increased variability of the position of the coronary arterial orifices should be adequately appreciated [10]. Despite the fact that coronary arterial anatomy is not any longer considered a risk factor for adverse outcome in the neonatal arterial switch operation, this may not necessarily be the case in the double switch procedure. Adult coronary orifices and proximal coronary arteries are definitely less pliable than in children. In addition, the incidence of anomalies of the proximal coronary arteries in corrected transposition of the great arteries is increased. Because of the young age of the patients atherosclerotic orifice degeneration does not play a role at this moment but may play a role in the future. Although no firm data are available, again it seems fair to estimate mortality due to complications as a result of coronary damage in this regard at up to 1%.

Coronary arteries as means of transport

In all surgical procedures that involve opening of the aortic root, the coronary orifices are at risk of damage due to manipulation. The risk is small, but direct application of cardioplegia into the orifices may be traumatic. In addition there is the risk of coronary embolism by gas (air or carbon dioxide) or debris after completion of the root surgery.

Conclusions

Not only abnormal, but also normal coronary arterial anatomy may have implications in surgery for adult congenital heart disease.

Three-dimensional diagnostic tools demonstrating the spatial relation between coronary arteries and the cardiac region of interest are essential.

The congenital cardiac surgeon should have the necessary skills in coronary artery bypass techniques. With lack of adequate data, the estimation of mortality due to complications as a result of coronary damage in surgery for adult congenital cardiac disease of below 1% seems fair. So far, clinical atherosclerotic coronary disease in adults with congenital cardiac disease is infrequent.

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Chapter 8

General Discussion

General discussion

The studies as presented in this thesis postulate novel roles for EPDCs, which not only add new insights to understanding heart development but may also be, albeit to a lesser extent, important for clinical practice.
It should be noted that the chapters providing the experimental data in this thesis describe results obtained in avian embryos. These share many aspects of heart development with mammals, but a direct comparison is not provided in this chapter. For further reading on comparative cardiac development and morphology please see[1-4].

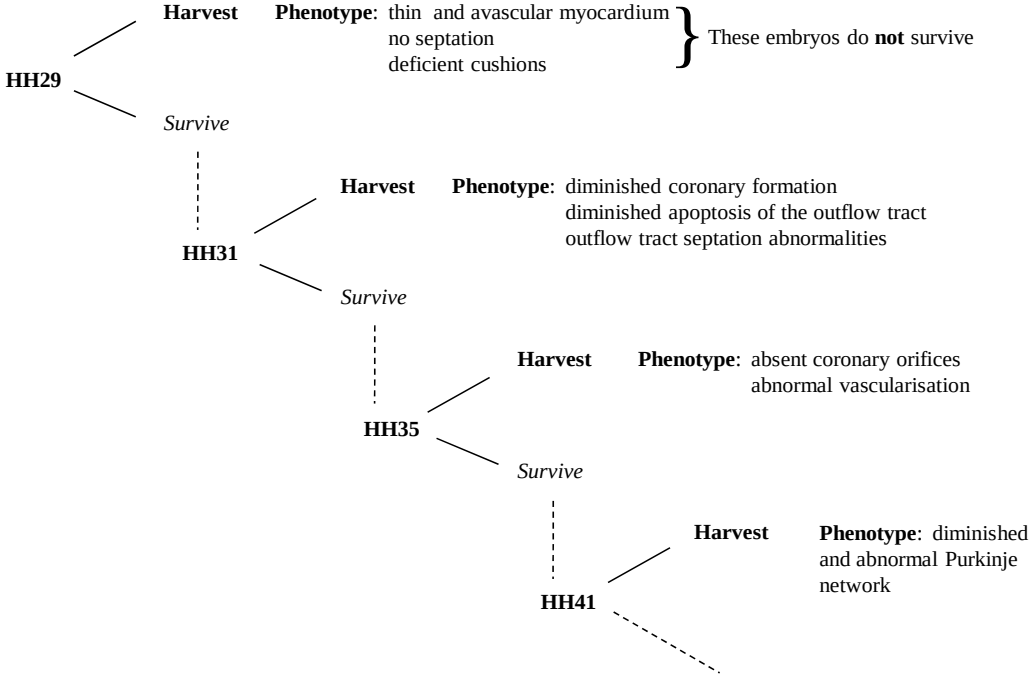
Early epicardial development

As the primitive heart tube, its layers consisting of myocardium and at the luminal side the endocardium, undergoes structural looping changes, a group of cells arises at the dorsal venous entrance side of the heart, just ventral to the primordial liver[5]. This group of cells bulges out as a cauliflower like structure, giving rise to what is defined as the pro-epicardial organ (PEO). During further development this PEO contacts the naked myocardium and upon this attachment cells of the PEO grow out over the heart, eventually covering the entire heart with a mesothelial layer of epicardium[1, 6-8]. This migration takes place in a certain spatio-temporal pattern as described by Vrancken-Peeters[9]. Covering is completed at stage HH25 when PEO-derived epicardium meets arterially derived epicardium just proximal to the outflow tract cushions. It is here where key-processes occur in coronary development and, as presented in Chapter 3, where delay in epicardial outgrowth has great consequences for coronary ingrowth into the aorta[10].
The epicardium that is formed consists of mesothelial cells. Promptly after establishment of the epicardium, a subepicardial space develops into which mesothelial epicardium-derived cells (EPDCs) migrate and transform into a mesenchymal cell type. This latter process is called epithelial-mesenchymal transformation. This population of EPDCs remain in their multi-potent mesenchymal state until they differentiate into a variety cell types as they reach their destination after migrating to various sites of the heart during development[11-14]. The specific spatio-temporal migration of EPDCs into the underlying layers is described and discussed in Chapter 2[15]. The chapters in this thesis concentrate on specific functions of the EPDCs during normal and abnormal development. The experimental study design is based on tracing of EPDCs by chimera-techniques and studying development after mechanical intervention with epicardial outgrowth.
The latter technique, referred to in our studies as the epicardial inhibition model[16], produced embryos with a spectrum of abnormalities, ranging from complete epicardial outgrowth inhibition to only a delay in outgrowth. The resulting phenotypes vary from severe morphological abnormalities resulting in embryonic death at stage HH29 to Purkinje fiber hypoplasia and survival until hatching. In our experiments we found that as the epicardially inhibited embryos are phenotypically allowed to survive longer, the less severe malformations were found. In figure 1 this schematically illustrated.

EPDCs and coronary development

Earlier work has already shown that EPDCs contribute to coronary development[8, 11-13, 15, 17]. In brief, after an endothelial network of vessels is established made up of liver-derived endothelial progenitor cells that have migrated along with the PEO, which contact the systemic circulation, the medial and adventitial layer are formed by recruitment of EPDCs. EPDCs differentiate into smooth muscle cells and adventitial fibroblasts to stabilise the primitive coronary vessel network.
The effects of complete inhibition and delay in epicardial outgrowth on myocardial architecture and coronary development are presented and discussed in chapter 3[10]. The severe maldevelopment of the myocardium with total absence of coronary development confirmed results from earlier epicardial inhibition models[16].
In addition our study for the first time provided a model in which previously described epicardially induced apoptotic processes regulate coronary ingrowth[18, 19]. Apoptosis has shown to be important in structural developmental changes of the cardiac outflow tract. The experiments in chapter 3 not only supported these findings but also added to the understanding of the process of coronary ingrowth into the aorta and the contribution of Fas-ligand (FasL) in EPDC initiated apoptosis. Previous studies had already determined EPDC-induced apoptosis in outflow tract morphogenesis[19].

Epicardial inhibition at HH15/17: stage specific effects



EPDCs and Purkinje fiber development

As less severely affected embryos survive longer during development, other processes in heart development could be studied. The role of EPDCs in Purkinje fiber and heart valve development is presented and discussed in chapters 4 and 5, respectively.

The Purkinje cells which make up the conducting Purkinje fibers of the heart have been shown to originate from cardiomyocytes[20]. Cardiomyocytes are thought to dedifferentiate and develop into Purkinje cells. Endothelin-1, which is expressed by endocardial and coronary endothelial cells under influence of hemodynamic changes, plays a significant role in this process. The Purkinje fibers develop not only periarterially but also subendocardially. Sites, abundant with EPDCs[14]. Our inhibition model showed hampered development of the Purkinje fibers not only histologically, but also in functional experiments[21]. Conduction was studied by ECG-measurements and these presented with widened complexes in the inhibited embryos, showing that ventricular conduction is affected. Our hypothesis on this matter is that EPDCs interact with endothelial cells by inducing cardiomyocytes into a dedifferentiated state, after which differentiation takes place into a Purkinje cell type under influence of ET-1[22]. Periostin is speculated to be a signalling molecule in this process, as was further studied in chapter 5.

Unpublished results of chimera experiments have revealed that EPDCs themselves do not differentiate into Purkinje cells, supporting the model in which cardiomyocytes solely contribute to the Purkinje population[23].

EPDCs and development of the cardiac fibrous tissues

The fibrous tissues of the heart, i.e. the atrioventricular valves and the fibrous junction between atria and ventricles, give structure to the heart and provide for electrical insulation, slowing the electrical signal which is needed in the normal cardiac cycle of diastole and systole.

The initial formation of endocardial cushions at the atrio-ventricular region and outflow tract, starts with invasion of endocardial cells which have undergone EMT under influence of myocardial signalling by BMP-2[23, 24]. Further mesenchymal cells are added by the subsequent migration of EPDCs into these areas. Eventually the proportion of EPDCs populating the AV-valves diminishes[25]. Several hypotheses exist on this temporal abundancy of EPDCs in the AV-cushions. Increase of endocardial mesenchymal cells is supposed to be relatively increased during later embryonic stages. This, however, does not correlate with the idea that EPDCs would have an inhibitory function on EMT of the endocardial cells[26]. Findings that reported TGFß1-3 expression

in the proepicardium, the epicardium and EPDCs in the AV-cushions advocate a signalling role in which EMT is stimulated[27, 28].
Earlier it was hypothesised that EPDCs exerted a morphogenetic role in valve formation, by deposition of fibrous extracellular matrix, based on colocalisation of EPDCs with procollagen I[26]. In our experiments we showed defective valve development in embryos with delayed epicardial outgrowth. Not only valves were abnormal, but also defects in the fibrous AV-ring, or rather persisting myocardial connections between atria and ventricles were found.
During valve and fibrous tissue development a role for the signalling molecule periostin was postulated earlier[29]. Recent studies confirmed defective valve and fibrous tissue formation in periostin null mice[30]. Periostin has also been shown to function in adult hearts as a profibrotic factor, both in normal and pathological conditions[30-33]. Our experiments confirmed[34] expression of periostin by EPDCs and showed diminished expression in epicardially inhibited embryos, which leads to valve defects and persistent myocardial AV-connections. These developmental models are presented in Chapter 5 and point out novel significant roles for EPDCs in cardiac valve and fibrous tissue development.

Conclusive and additional thoughts with perspectives for further research

EPDCs, extracardiac or not?
The idea that EPDCs would be of extracardiac origin is questionnable. In the light of new insights on the developent of the secondary heart field, consisting of anterior and posterior heart field, we have come to the conclusion that the PEO grows out of the posterior heart field and thus can not be considered to be of extracardiac origin. The posterior heart field is the part of the secondary heart field that is found at the dorsal venous entrance side from where cardiomyocytes are added to the heart during further development. This means that only the neural crest cells, which migrate to various parts of the developing hearts, can be seen as true extracardiac contributors to heart development. Figure 2 illustrates this reconsideration schematically.

A schematic representation of EPDC fate and function is shown in figure 3. It gives an overview of the known and novel roles of EPDCs as presented in this thesis. Two recently published studies report differentiation of EPDCs into cardiomyocytes, shown by transgenic labelling with the Tbx18 and WT1 genes, as epicardial markers[35,36]. And although differentiation of cells that express these markers into cardiomyocytes, albeit only a small proportion of the total number of cardiomyocytes, the expression of these genes is switched on at a stage where differentiation of celltypes into the myocardial or epicardial lineage has not yet taken place. These cells are part of the early coelomic epithelium, which eventually will give rise to the development of the posterior heart field. It can therefore still not be stated that EPDCs can differentiate into cardiomyocytes. In fact, to date, all other tracing studies have shown that EPDCs cannot differentiate into cardiomyocytes and support only a signalling role in myocardial development.

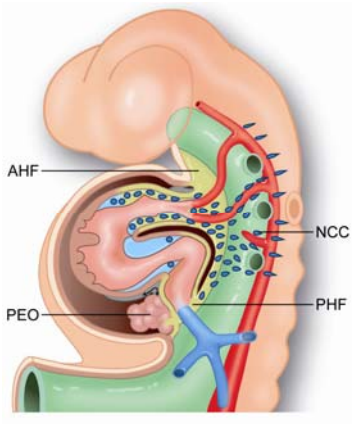


Figure 2: Schematic picture of the continuum of the primary (brown and blue) and secondary (yellow) heart-forming fields. The secondary heart-forming field can be divided into the anterior heart-field (AHF, at the outflow tract region of the heart, and the posterior heart-field, at the inlet region of the heart. The PEO, the cauliflower-like protrusion of the sinus venosus, into the pericardial cavity, develops as a part of the posterior heart field and can thus be regarded as originating from cardiac source. The neural crest cells (NCC) can be considered as true extracardiac contributors to heart development as they migrate from the neural crest along the arterial and venous pole into the heart, to the blue regions originating from the primary heart field. (Adapted with permission from Poelmann and Gittenberger-de Groot)

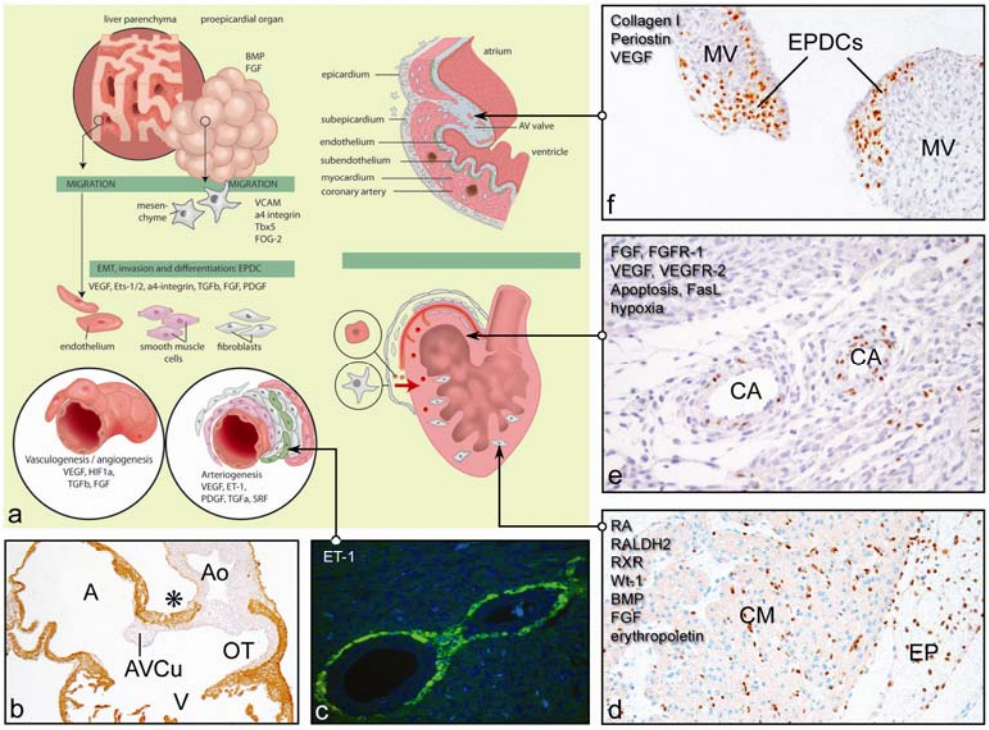


FIGURE 3. Schematic figure of EPDC fate and function. (a) Together with the development of the PEO, endothelial precursor cells of the liver region travel along with the proepicardial cells to the naked heart tube. After migration and epithelial-mesenchymal transformation of the epicardium, the subepicardial mesenchyme is formed, and EPDCs start to migrate into the underlying myocardium (a,f) where they differentiate into SMCs and fibroblasts. Counterclockwise, the photomicrographs (b–f) indicate their migratory fates and functions. Factors known to be involved in these developmental processes are listed in the left upper corner of the photomicrographs (for references, see text). (b) Migration into the myocardium starts in the inner curvature, where EPDCs may well have a function in the late looping process. This photomicrograph shows a far-too-wide inner curvature after mechanical ablation of the PEO ([43], used with permission from the authors). (c) EPDCs induce the formation of the peripheral conduction system; Purkinje fibers, shown here after immunofluorescent staining with the EAP- 300 antibody, will not develop properly in epicardially inhibited embryos ([71], used with permission from the authors). (d) In a proepicardial quail-chicken chimera, tracing with the quail-specific nuclear marker QCPN reveals that a large proportion of the cells of the ventricular myocardial walls are EPDCs ([69], used with permission from the authors). Here, they contribute to myocardial architecture by expression of procollagen I and the extracellular matrix protein periostin (for references see text). (e) This photomicrograph of a quail-chicken chimera demonstrates that in the coronary arteries, EPDCs constitute the SMCs and fibroblasts that stabilize the vessel walls. (f) In the developing atrioventricular valves, many of the fibroblasts in the valve leaflets are epicardium derived, as is shown in this section of a proepicardial quailchicken chimera ([69], used with permission from the authors). A, atrium; Ao, aorta; AVCu, atrioventricular cushion; CA, coronary artery; CM, myocardium; EP, (sub)epicardium; MV, mitral valve; V, ventricle.

development by EPDCs is impeded, functional malformations such as accesory pathways could be found, which

are also seen in the Wolf-Parkinson-White syndrome and in Mahaim's tachycardia[37,38]. Interestingly, the WPW-syndrome often coincides with Ebstein's anomaly[38]. Moreover, in the rare congenital cardiac malformation such as congenitally corrected transposition of the great arteries (CCTGA) all processes in which novel roles for EPDCs were presented in this thesis, are involved in the pathogenesis. Abnormal looping, defective ingrowth of coronary arteries, valve abnormalities and conduction system defects are part of the complex variety of this rare syndrome. The range of malformations, also seen in other epicardial inhibition models[39, 40] supports the idea that disturbed epicardial outgrowth is likely to be involved in the associated development of congenital heart malformations in humans. As indicated in Chapter 7, a better understanding of EPDC involvement in coronary development, also provides an improved insight in coronary anatomy, both normal and anomalous, and their clinical implications in congenital heart disease.

Recommendations for further research should include elaborative studies on Purkinje fiber development as this the least investigated field of cardiac development to date. Further analysis of involvement in and interaction with signalling factors such as ET1 in relation to EPDCs needs attention.

With regard to myocardial maturation, for example in the light of the human cardiac non-compaction syndrome, clearification of EPDC malfunctioning is valuable to explore. And since coronary ischemic disease and subsequent myocardial damage account for the vast majority of cardiac disease, in the light of current stem cell research, significant roles for EPDCs should be considered, as is already performed in our lab with promising results[41,42].

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Corrigenda:

- Op pagina 9, hoofdstuk 1, Introduction, zijn, bij de korte vooruitblikken op de hoofdstukken, Chapter 3 en 4 per abuis omgewisseld. De gedeelten na "In Chapter 3" en "In Chapter 4" dienen omgewisseld te worden.

- Hoewel ik professor Jan Klein uiteraard zeer erkentelijk zou zijn, indien hij zitting zou nemen in de promotie-commissie, dient dit dankwoord zich echter te richten tot zijn vervanger, professor Robert Jan Stolker, tevens opvolger van Jan Klein als hoogleraar Anesthesiologie, Erasmus MC.

- Beste Robert Jan, niet alleen ben je als afdelinghoofd Anesthesiologie altijd enorm betrokken bij hoe de zaken op en buiten de OK gaan, maar heb je ook vaak meegeleefd met de vorderingen omtrent mijn promotie. Je vond dat ik mijn promotie meer moest promoten bij collega's, want dat is iets om trots op te zijn. Trots ben ik dat jij zitting neemt mijn promotie-commisie. Bedankt.

Curriculum Vitae

Ismail Eralp was born on the 22th of August 1979 in Rotterdam, the Netherlands. After finishing the Gymnasium *cum laude* in 1997 at the Grotius College in Heerlen, the Netherlands, he started medical school at the Erasmus University Rotterdam. After finishing his graduation thesis at the department of Cardiothoracic Surgery, Erasmus MC, he started his PhD-project at the department of Anatomy&Embryology of the Leiden University Medical Center in September 2001. In September 2005 he started with medical rotations as to finish his medicine studies. In September 2007 he obtained his medical degree en started working as a resident at the department of Anesthesiology, ErasmusMC. In August 2008 he started his training as an anesthesiologist.