Ets-1 and Ets-2 Transcription Factors Are Essential for Normal Coronary and Myocardial Development in Chicken Embryos

Heleen Lie-Venema, Adriana C. Gittenberger-de Groot, Louis J.P. van Empel, Marit J. Boot, Henri Kerkdijk, Eric de Kant, Marco C. DeRuiter

Abstract—In the development of a functional myocardium and formation of the coronary vasculature, epicardium-derived cells play an essential role. The proepicardial organ contributes to the developing coronary system by delivering mural cells to the endothelium-lined vessels. In search of genes that regulate the behavior of (pro)epicardial cells, the Ets-1 and Ets-2 transcription factors stand out as strong candidates. In the present study, the hypothesis that Ets transcription factors have a role in proper coronary and myocardial development was tested via antisense technology, by targeting Ets-1 and Ets-2 mRNAs to downregulate protein expression in chicken embryos. The results suggest that hereby the development of the coronary system is hampered, primarily by defects in the process of epithelial-mesenchymal transformation of the mesothelia of the primary and secondary heart fields. This was indicated by a lack of periarterial and epicardial mesenchyme, of peripheral coronary smooth muscle cells, and changes in myocardial morphology. A defect in myocardial perfusion caused by the absence of one or both coronary ostia seems to be “solved” by the development of numerous small fistulae connecting the ventricular lumen with the subepicardially located coronary vessels. The presence of coronary vascular aberrations in the antisense-Ets phenotype enabled us for the first time to study abnormal coronary development in a model that is not lethal to the embryo. (Circ Res. 2003;92:749-756.)

Key Words: Ets transcription factors ■ epicardium ■ coronary arteries ■ myocardium ■ development

Initially, the developing heart consists of a myocardial tube lined with endocardium, with cardiac jelly in between. With the increase in myocardial functioning, a separate vasculature of the myocardial wall becomes necessary. The formation of the coronary system is preceded by the outgrowth of the proepicardial organ over the heart to form the epicardium, followed by infiltration of endothelial cells from the septum transversum into the subepicardial space to form a capillary network. Subsequently, epicardial cells transdifferentiate and migrate to form the subepicardial and subendocardial mesenchyme. These mesenchymal cells then differentiate into smooth muscle cells (SMCs) and adventitial fibroblasts of the coronary vessel walls, into myocardial interstitial fibroblasts, subendocardial cells, and cells in the cushion mesenchyme. Quail-chicken chimeras showed that the endocardium of the ventricular lumen does not contribute to the formation of the coronary vasculature. The long held idea that coronary arteries develop by endocardial budding from the trabecular folds could thus be refuted. This implies that the presence of fistulae in some congenital heart malformations like pulmonary atresia without ventricular septal defects are not the result of such persisting luminated embryonic connections. Recent studies indicate that fistulae can in fact be the initial problem in the developing myocardium, and that secondarily, the pulmonary stenosis to atresia develops. This makes it more relevant to study in depth the formation of coronary fistulae.

Inhibition of epicardial outgrowth leads to embryo lethality due to severe myocardial thinning and lack of coronary vessel formation. The epicardial ablation model could not be used to study the role of epithelial-mesenchymal transformation in coronary formation because, due to its experimental nature, migration of the proepicardial cells could not take place. In search of genes involved in epicardial epithelial-mesenchymal transformation, the transcription factors Ets-1 and Ets-2 seemed good candidates. The genes that are known to have and/or use Ets binding sites encode transcription factors, cell-cycle regulators, matrix-degrading proteinases, cytokeratins, cell adhesion molecules, and growth factors. Ets transcription factors contain a variant helix-turn-helix motif used for binding to a common core DNA sequence, and can interact cooperatively with other transcription factors such as Fos/Jun, GATA-1, and other Ets-family members. In the developing heart, Ets-1 immunoreactivity is present throughout, but most prominently so in areas of epithelial-mesenchymal transformation, viz. the subepicardial mesen-
Retroviral Constructs and Transduction of Chicken Embryos

Two retroviral constructs were used to generate transduced chicken embryos (Figure 1a). The proviral pCXIZ vector (kindly provided by Dr T. Mikawa, Department of Cell Biology, Cornell University Medical College, New York, NY12) was used to generate the pCXasetsIZ construct, containing 700 bp of the chicken Ets-1 cDNA,13 from the BglII site at +390 bp to the HindIII site at +1090 bp in the open reading frame. This Ets-1 cDNA fragment is complementary to 700 nt of the Ets-1 mRNA, including 100 nt of the sequence that encodes the DNA binding domain. Because this part is homologous to the sequence that encodes the DNA-binding domain in the mRNA for Ets-2, the antisense Ets-1 cDNA fragment can inhibit translation of both Ets-1 and the Ets-2 mRNA. The Ets-1 cDNA fragment was cloned in antisense orientation in between the LTR and the IRES (internal ribosomal entry site) located upstream of the LacZ gene. Methods for stable transfection of the retroviral construct into the canine packaging cell line D17.2G, virus harvest, and staging according to Hamburger and Hamilton.16

Bloodstream injection with viral suspension of CXasetsIZ was performed in chicken embryos at developmental stage HH14 to HH15 via the right anterior vitelline vein until the heart was filled (n=20). The window was closed with Scotch tape, and the eggs were further incubated until the embryo was between HH35 and HH37. Embryos injected with CXL (n=15), the backbone construct,14 and untreated (wild-type) embryos (n=5) served as controls.

RT-PCR

The thorax specimens of experimental and control animals (HH 32; n=3) were isolated and snap-frozen in liquid nitrogen. Total RNA was isolated using RNAzol B (Campro Scientific, The Netherlands). RNA concentration was determined spectrophotometrically. Concentration and RNA integrity were checked by ethidium bromide staining on agarose gel. First-strand cDNA was prepared from 2 µg total RNA using 0.2 µg random hexamers (Promega), 0.5 µg poly-dT primers, and Ready-to-go first-strand cDNA beads (Amersham) in 33 µL RT-PCR mixture, with 500 bp CXL and GAPDH primers to check equal input of template cDNA (500 bp PCR product). M indicates molecular weight marker. c, Western blot analysis of Et4-expression in total protein extracts of CXL- and CXasetsIZ-infected fibroblasts. Both Ets-1 (54 kDa) and Ets-2 (68 kDa) expression levels were downregulated by infection with the antisense Ets construct (top). Equal loading was checked by Coomassie staining (bottom). Expression was reduced to 72% and 65% of Ets-1 and Ets-2 levels, respectively, in CXL-infected cells.

Transduction of Chicken Embryonic Fibroblasts (CEFs) and Western Blot Protein Analysis

Primary CEFs were isolated from thoraces of HH30 to HH35 chicken embryos. After removal of heart and lungs, minced tissue was cultured for two days in Iscove’s modified DMEM with glutamax-I (Gibco), 5% FCS, and antibiotic-antimycotic cocktail (Gibco). First passage embryonic fibroblasts were grown to subconfluence, and infected overnight with equal amounts of freshly prepared CXL and CXasetsIZ retrovirus in medium containing 20 ng/mL polybrene. The medium was changed and cells were cultured for 2 days. Cells were scraped from the culture plates, centrifuged at 1500g, and snap-frozen in liquid nitrogen until use. Total protein was

Materials and Methods

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extracted in ice-cold RIPA buffer (PBS with 1% (v/v) Igepal-CA630, 0.5% (w/v) sodium deoxycholate, and 0.1% SDS) containing PMSF serine protease inhibitor at a concentration of 1 mmol/L. The total protein content of the extracts was determined using the bicinchoninic acid method (BCA kit, Pierce). Western blot analysis of total protein was performed on extracts from 3 independent experiments using the Western Breeze immunodetection kit (anti-rabbit; Invitrogen). Jurkat nuclear extract (Santa Cruz Biotechnology) served as a positive control. In short, 40 μg of total protein or 20 μg of Jurkat nuclear protein was separated by SDS-PAGE on a 10% gel and blotted onto PVDF membrane (Bio-Rad). Membranes were incubated with polyclonal anti-Ets-1 and anti-Ets-2 antibodies (Santa Cruz Biotechnology; sc-350 and sc-351, respectively, both diluted 1:1500), and chemiluminescence was detected by autoradiography. To check equal loading of the gels, 20 μg of total protein was separated on a 10% gel and stained with Coomassie Brilliant Blue. Ets-specific signals were quantified and corrected for total protein loading by image analysis with an 8-bit CCD camera and LabWorks software (UVP, Cambridge, UK). Data were statistically analyzed as independent groups with the Student’s t test.

**Histological Procedures**

Unless indicated otherwise, tissue processing and (immuno)histochemistry were as described earlier. The thorax specimens of CXasetsIZ-infected and wild-type control embryos were fixed by overnight immersion in 4% paraformaldehyde (PFA) in PBS at 4°C. CXL-infected thorax specimens were immersion-fixed in 2% PFA in PBS at room temperature for 2 hours and stained in toto for β-galactosidase followed by overnight postfixation in 4% PFA at 4°C. Morphology of heart and blood vessels was analyzed in hematoxylin-eosin-stained sections. Sections of specific interest were immunostained with 1A4 anti-smooth muscle α-actin (Sigma) to identify SMCs and with the polyclonal anti-β-actin antibody developed by Dr C. Chaponnier and kindly provided by Dr M.L. Bochaton-Piallat (both from the Department of Pathology, CMU, Geneva, Switzerland). The latter antibody reacts with cytoplasmic β-actin in coronary endothelial cells, but hardly in endocardial cells. Sections were counterstained with nuclear fast red or hematoxylin.

**Results**

To investigate the role of Ets transcription factors in cardiovascular development, Ets protein expression in chicken embryos was targeted by introducing a retroviral antisense Ets construct (CXasetsIZ, Figure 1a) via the bloodstream. Effective transcription of the retroviral CXasetsIZ sequence was demonstrated by RT-PCR on the LacZ gene product in CXasetsIZ-infected hearts at HH 32 (Figure 1b). The functionality of the transcribed antisense molecule was tested in primary cultures of chicken embryonic fibroblasts (CEF). In 3 independent Western blot experiments, total protein extracts of CEFs infected with either CXasetsIZ or the control backbone construct CXL were analyzed for the abundance of Ets protein. Both Ets-1 and Ets-2 protein expression levels were decreased by antisense Ets treatment (Figure 1f). Quantitative and statistical analysis of the data showed that the expression of Ets-1 protein was downregulated to 73±10% (SEM; P<0.05) and that of Ets-2 protein to 65±11% (SEM; P<0.05) of the levels that were found in CEFs transduced with CXL.

Both in embryonic fibroblasts and in the embryo, CXasetsIZ-infected cells could not be visualized by β-galactosidase staining because of an unforeseen lack of function of the IRES. However, the occurrence of in vivo infection and transcription of the CXasetsIZ construct could be inferred from the aforementioned RT-PCR data and from the embryonic phenotype (see Table). Control bloodstream injections with CXL showed that the proepicardial organ was a target for infection (Figure 2a). CXL-positive cells were observed in the cardiac epicardium (Figures 2b and 2d) and in the arterial trunk, the pericardium, and the myocardium (see Table). Also, endocardial and myocardial cells could be infected (not shown). The overall percentage of infected cells varied largely between embryos and was estimated to be between 2% and 20%. The retroviral construct was expressed throughout the embryonic period studied. Although in most embryos regions derived from both the inflow and the outflow tract showed almost equal amounts of β-galactosidase staining, right ventricular fibulization was seen; this observation was confirmed by immunohistochemical staining for cytoplasmic β-actin. ISM α-actin staining was examined in sections of the caudal-ventral region of the right ventricle.

**Defects in Cardiac Morphology of Antisense Ets-Treated Embryos**

Antisense Ets-injected embryonic hearts, examined between developmental stage HH35 and HH38, showed morphological aberrations at 3 different sites: the epicardium, the coronary circulation, and the myocardium (see Table). The malformations coincide with a primary defect in the process of epithelial-mesenchymal transformation that takes place in the cardiac epicardium, covering the myocardium. In CXasetsIZ-injected embryos, the epicardium was thin and consisted of only one or a few layers of densely packed cells (Figure 3d). Normally these cells are loosely organized in a layer of about 5 to 15 cells in thickness (Figure 3e). Lateral outgrowth of the mesothelium was not disturbed (not shown). Also, the mesenchyme around the arterial trunk, the periar-
Coronary Defects
The coronary system was affected by the presence of the antisense Ets RNA; this could be seen macroscopically because subepicardial hemorrhages covered the hearts of many CXasetsIZ-injected embryos (not shown). Microscopic and immunohistochemical analysis revealed four morphological aberrations, mostly concurrently present within one embryo. Probably most striking is the absence of one or both coronary orifices in 40% of the experimental embryos (Figures 4a through 4c) and underdevelopment of the coronary arteries that normally penetrate the aortic vessel wall to form the ostia.\(^{18}\) Second, in the ventricular periphery, the developing coronary arteries were not regularly organized, but showed an irregular distribution over the free-wall myocardium (Figures 3a through 3c). Third, coronary capillaries seemed to contact the ventricular lumen in most of the CXasetsIZ-injected heart. Indeed, when appropriately stained, small fistulae—forming junctions between the coronary circulation and the ventricular lumen could be observed. They could be visualized by staining with a polyclonal antibody directed against cytoplasmic β-actin, discriminating between endothelial cells of the coronary vessels and those of the ventricular endocardium (Figures 4f through 4h). In one embryo, microfistulae were observed in the left ventricular myocardium, but coronary-ventricular junctions were predominantly present in the right ventricular free walls of CXasetsIZ-treated hearts. No microfistulae were observed in the ventricular septum. Lastly, immunohistochemical staining for smooth muscle α-actin demonstrated a lack of SMCs in the peripheral coronary arteries (Figures 4i and 4j). The presence of antisense Ets RNA specifically influenced arterial organization and the number of SMCs in the coronary vasculature, because CXasetsIZ-treated embryos developed aortic and pharyngeal arch arteries with normal morphology and SMC content (not shown). CXL-infected control animals showed that viral infection could take place in these vessels.

Myocardial Defects
As a third afflicted site, the myocardium was abnormal in CXasetsIZ-treated embryos. The ventricular wall was attenuated and trabeculae were broader and fewer in number than in the hearts of in control animals. These phenomena were most prominent in the lateral and ventral free wall myocardium of the right ventricle (Figure 5). Furthermore, the ventricular septum did not close in 25% of the antisense Ets-treated embryos, resulting in subaortic ventricular septal defects.

Discussion
The constellation of cardiac abnormalities found in CXasetsIZ-injected embryos indicated that downregulation of Ets-1 and Ets-2 caused primarily a defect in the process of epithelial-mesenchymal transformation. Antisense Ets injection did not prevent cells of the proepicardial organ to traverse the pericardial cavity and reach the heart, nor did it affect lateral outgrowth of the epicardial cells over the cardiac surface. Yet, the transformation of mesothelial cells to form the underlying loosely organized subepicardial mesenchyme was severely hampered. At sites where the epicardium consisted of more than one cell layer, cells were more condensed, indicating an aberrant development of the epicardial extra-cellular matrix. This suggests a defect in epithelial-mesenchymal transformation, rather than in mesenchymal proliferation, which can be well explained by the fact that Ets transcription factors control the expression of many proteins involved in matrix development and cell-matrix interactions (for example, MMPs and integrins). The defect in epithelial-mesenchymal transformation was seen both in the cardiac epicardium and periarterial epicardium. The latter does not derive from the proepicardial organ, but most likely originates from aortic sac mesoderm\(^8\) derived from the secondary heart field. This heart field mesoderm was originally described by De la Cruz et al\(^{19}\) and is believed to give rise to the outflow tract of the developing heart.\(^{20}\) Both mesothelia meet...
Ligand gene promoter. Therefore, an additional explanation for the impaired orifice formation is that it may result from a decrease in apoptosis at the site of the coronary orifice-to-be. However, it is in this region that the peritruncal capillary plexus is assembled from endothelium originating from the septum transversum. After endothelial penetration of the aortic arch, on the other hand, only a small rim of densely packed epicardial cells was present. CXL-treated hearts are covered with an epicardium that is normal in texture and thickness. The embryos seemed to “solve” the resulting defect in myocardial perfusion by the development of small fistulae connecting the ventricular lumen with subepicardially located coronary vessels; a process that may have been enhanced by the more fragile architecture of the compact myocardium.

Similar to the absence of coronary ostia, the other malformations in the antisense-\textit{Ets}–treated hearts can be explained as being secondary to the hampered process of epithelial-mesenchymal transformation. As was argued, coronary capillaries cannot properly be stabilized by epicardium-derived SMCs and fibroblasts and do not develop into bona fide coronary arteries when EPDCs fail to form via the process of epithelial-mesenchymal transformation. In our antisense \textit{Ets} chicken model, this is illustrated by the diminished SMC deposition in the peripheral ventricular coronary arteries, and may well explain the irregular structure of the coronary vascular network in the myocardium. Similarly, the hypoplasia of the ventricular myocardium and abnormal trabecularisation are indicative of defective outgrowth of EPDCs, known to be necessary for the formation of the fibrous heart skeleton and proper myocardial organization. The thin myocardium resembled that seen after inhibition of epithelial outgrowth. Therefore, we think that the observed myocardial thinning was rather a secondary effect via defective EPDC formation, than a primary effect of CXLs-intervention and \textit{Ets} downregulation in the cardiomyocytes themselves. That VSDs were found in the hearts of the experimental embryos fits well with the observation that epicardium-derived mesenchymal cells are located in the subendocardial layer of the developing interventricular septum and suggests a role for EPDCs in closure of the ventricular septum.

That injection of virus via the anterior vitelline vein leads to infection of (pro)epicardial cells may need some explanation, because the proepicardial organ (PEO) is not vascularized at the time of infection. The PEO is then located just ventrally of the omphalomesenteric vein. This is exactly where blood from the vitelline veins enters the embryonic heart, and where, because of its pulsatile nature, the bloodstream pauses and the virus can settle to infect the surrounding cells. Furthermore, this is a site of extensive angiogenesis at this time, when the splanchnic vessel plexus—still continuous with the liver sinusoids, and connected to the sinus venosus—extends in the direction of the PEO. Rearrangements of the endothelial cell layer will most likely induce leakage and enables the virus to contact the underlying structure, that is, the proepicardial organ. Endothelial contacts are not necessarily always very tight and viral leakage after bloodstream injection has been observed more often at sites where angiogenesis takes place (M.C. DeRuiter, unpublished observations, 2002). That leakage occurs across the endothelial/endocardial barrier can also be inferred from the fact that cardiomyocytes are often infected when virus is delivered via this route.

Myocardial thinning and fistulae formation were seen particularly in the outflow part of the right ventricle after antisense \textit{Ets}-1/2 intervention. The myocardium of this part of the heart evolves from the secondary heart field mesoderm between embryonic stage HH14 and HH22, that is, approximately at the time of injection. Because retroviral
integration will occur in dividing cells only, the right ventricle and outflow tract may therefore be more prone to infection than the left ventricle that originates from the "older" primary heart tube.

In mice with disrupted Ets-1 and Ets-2 genes, cardiac malformations have not been described because heart morphology was not studied and because the null mutation was embryolethal due to defective trophoblast development, respectively. Ets-2−/− embryos could be rescued by aggregation of 6- to 8-cell stage embryos with Ets+/− tetraploid cells that form a functional trophoblast but do not contribute to the embryo. Also, in the rescued Ets-2 knockout mice, displaying epidermal and hair growth abnormalities, heart malformations were not explicitly described. Ets-1 and Ets-2 can act cooperatively to activate transcription, and the two transcription factors are so closely related that they can be functionally redundant. Therefore, it is conceivable that defects in cardiac development will only occur when the expression of both transcription factors is targeted. The antisense Ets-1 construct used in present study is complementary to both the Ets-1 and to the Ets-2 mRNA. Western blot analysis showed that the protein expression of both transcription factors was downregulated in CXasetsIZ-infected cells to an equal extent.

An intriguing observation in the present study is that the epicardial, coronary, and myocardial defects develop in spite of the fact that only a percentage of the cells in the developing heart is targeted by the retroviral gene transfer technique used. However, the many target genes of Ets-1 and Ets-2 that specify secreted proteins and proteins involved in intercellular and cell-matrix interactions (reviewed by Sementchenko and Watson) can explain the relatively broad effect of the antisense Ets-1/2 intervention. Moreover, EPDCs are normally infiltrating large areas of the developing heart. When the epicardium fails to generate sufficient EPDCs, many cells have to miss the epicardial signals that contribute to the development of a healthy heart. From partial ablation experiments, we know that even small disturbances of the epicardial outgrowth can have rather dramatic consequences for coronary development (I. Eralp and A.C. Gittenberger-de Groot, unpublished observations, 2003). Future experiments in which the PEO will be infected selectively and in which Ets- target gene expression will be analyzed are expected to yield more conclusive data on this issue.

The antisense-Ets phenotype in our chicken model is reminiscent to that of the FOG-2−/− mouse in which the expression of the cardiac cofactor Friend-of-GATA-2 was eliminated. In this knockout model, defective epicardial–mesenchymal transformation was believed to result in a thin myocardium and defective coronary outgrowth. Whether and how Ets-1 or Ets-2 transcription factors relate to FOG
cofactors and GATA transcription factors is as yet unknown. GATA-4 mutants in which interaction between FOG-2 and GATA-4 was impaired have a similar phenotype. Although no direct proof for a direct interaction between GATA-4 and Ets-1 or Ets-2 is available, the notion that several genes involved in vascular biology have both GATA and Ets consensus sequences in their promoters (eg, in the tie-231 and Flk-132 genes) and proven cooperative binding between Ets-1 and Ets-2 with GATA-333 may give further clues for the mode of action by which Ets transcription factors influence coronary development.

The coronary defects and myocardial thinning in our antisense-\textit{Ets} chicken model also resemble those in the VCAM-1 and \(\alpha_4\) integrin gene knockout mouse models.34,35 In these mice, epicardial cells do not cover large portions of the heart. Although the phenotype in our chicken model is likely to be milder, because the genetic modification is only partial, the presence of Ets-binding sites in and the interaction of Ets-1 with promoter elements of the VCAM-1 gene36 and the \(\alpha_4\) integrin gene, respectively, may explain the similarity of the experimentally induced abnormalities.

To delineate more precisely the role of Ets-1 and Ets-2 in the epicardial contribution to heart development, our future research will aim at the selective injection of the proepicardial organ with Ets transcription factor–specific antisense constructs to downregulate the protein expression of either Ets-1, Ets-2, or both. Until then, our findings supplement those on the role of Ets transcription factors in angiogenesis and pathological invasive processes.

Together, the data available on expression patterns, target genes, and cardiac malformations after antisense injections indicate that Ets-1 and Ets-2 transcription factors are key regulators in the processes of epicardial differentiation and epithelial-mesenchymal transformation that underlie the formation of a functional coronary vasculature. Our present and future avian models may help to understand the molecular defects in congenital coronary malformations and yield clues for research on revascularization of the failing myocardium.

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