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 periartrial 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 lumized 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 mesench-
chyme. In cushion mesenchyme, a site of endothelial-mesenchymal transformation, both Ets molecules are expressed.

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.

Materials and Methods

Retroviral Constructs and Transduction of Chicken Embryos

Two retroviral constructs were used to generate transduced chicken embryos (Figure 1a). The prerotroviral pCXIZ vector (kindly provided by Dr T. Mikawa, Department of Cell Biology, Cornell University Medical College, New York, NY) was used to generate the CXasetsIZ construct, containing 700 bp of the chicken Ets-1 cDNA, 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 5' 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, virus harvest, and injection techniques are described elsewhere.

Fertilized specified pathogen-free White Leghorn eggs (ID-DLO, Doorn, The Netherlands) were incubated for 54 to 60 hours at 37°C. Embryos (Figure 1a). The proretroviral pCXIZ vector (kindly provided by Dr T. Mikawa, Department of Cell Biology, Cornell University Medical College, New York, NY) was used to generate the CXasetsIZ construct containing 700 bp of the chicken Ets-1 cDNA, and the control CXL vector. 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.

Figure 1. a, Two retroviral constructs were used for bloodstream injections of chicken embryos at HH14: the CXasetsIZ construct containing 700 bp of antisense Ets-1 cDNA and the control CXL vector. b, RT-PCR using LacZ-specific primers and total RNA isolated from the thorax demonstrates transcription of the construct in CXasetsIZ-injected embryos (300 bp PCR product), but not in wild-type controls. Bottom, RT-PCR with GAPDH primers to check equal input of template cDNA (500 bp PCR product). M indicates molecular weight marker. c, Western blot analysis of Ets-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).
extracted in ice-cold RIPA buffer (PBS with 1% (v/v) Igepal-CA630, 0.5% (wt/vol) 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 hematoxylin-eosin–stained sections. Sections of specific interest were immunostained with the polyclonal anti-β-actin antibody developed by Dr C. Chapronnier 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). Quantiative 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 epicardium (Figure 2c). 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 fistulae were seen; this observation was confirmed by immunohistochemical staining for cytoplasmic β-actin. Immunohistochemical staining for cytoplasmic β-actin 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).

**Epicardial Defects**

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).

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).

Figure 2. In CXL-injected embryos, LacZ gene expression (blue staining) could be detected in transversal sections (a) in the PEO at HH20. PEO is attached dorsally (D) to the sinus venosus and liver region. Ventral (V) it protrudes toward the inner curvature of the heart tube (scale bar=50 μm). Transversal sections at HH36 show CXL-infected cells (b and d) in the cardiac epicardium (CEP; scale bars=375 μm and 100 μm, respectively) and (c) in the arterial epicardium (AEP; scale bar=100 μm). LV and RV indicate left and right ventricular lumina, respectively; AAo, ascending aorta; BA, brachiocephalic artery.

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 mesoderm9 derived from the secondary heart field. This heart field mesoderm was originally described by De la Cruz et al19 and is believed to give rise to the outflow tract of the developing heart.20 Both mesothelia meet
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- 
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 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. Therefore, we think that the observed myocardial thinning was rather a secondary effect via defective EPDC formation, than a primary effect of CXasetSZ-infection and 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 endocardial 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 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-1 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-ETS chicken model also resemble those in the VCAM-1 and α5 integrin gene knockout mouse models. 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 gene and the α5 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.

Taken 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.

Acknowledgments

This work was supported by grants NHS 97.065 and NHS 2001.015/20 from the Netherlands Heart Foundation. Jan Lens is kindly acknowledged for his help in preparing the figures.

References


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 and Marco C. DeRuiter

Circ Res. 2003;92:749-756; originally published online March 13, 2003; doi: 10.1161/01.RES.0000066662.70010.DB

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/92/7/749

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/