Double-Outlet Right Ventricle and Overriding Tricuspid Valve Reflect Disturbances of Looping, Myocardialization, Endocardial Cushion Differentiation, and Apoptosis in TGF-β₂-Knockout Mice

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Background—Transforming growth factor-β₂ (TGF-β₂) is a member of a family of growth factors with the potential to modify multiple processes. Mice deficient in the TGF-β₂ gene die around birth and show a variety of defects of different organs, including the heart.

Methods and Results—We studied the hearts of TGF-β₂-null mouse embryos from 11.5 to 18.5 days of gestation to analyze the types of defects and determine which processes of cardiac morphogenesis are affected by the absence of TGF-β₂. Analysis of serial sections revealed malformations of the outflow tract (typically a double-outlet right ventricle) in 87.5%. There was 1 case of common arterial trunk. Abnormal thickening of the semilunar valves was seen in 4.2%. Associated malformations of the atrioventricular (AV) canal were found in 62.5% and were composed of perimembranous inlet ventricular septal defects (37.5%), AV valve thickening (33.3%), overriding tricuspid valve (25.0%), and complete AV septal defects (4.2%). Anomalies of the aorta and its branches were seen in 33.3%. Immunohistochemical staining showed failure of myocardialization of the mesenchyme of the atrial septum and the ventricular outflow tract as well as deficient valve differentiation. Morphometry documented this to be associated with absence of the normal decrease of total endocardial cushion volume in the older stages. Apoptosis in TGF-β₂-knockout mice was increased, although regional distribution was normal.

Conclusions—TGF-β₂-knockout mice exhibited characteristic cardiovascular anomalies comparable to malformations seen in the human population. (Circulation. 2001;103:2745-2752.)

Key Words: genes | growth substances | heart defects, congenital | morphogenesis
Myocardialization. After overnight incubation with the antibody diluted in PBS with 1% ovalbumin and 0.05% Tween-20, the sections were rinsed in PBS and 0.05% Tween-20. Sections were subsequently incubated with rabbit anti-mouse antibody (Dako, P260) conjugated to peroxidase (dilution 1:250), goat anti-rabbit antibody (Nordic) (dilution 1:50), and rabbit peroxidase–anti-peroxidase antibody (Nordic) (dilution 1:500) for 90 minutes each with in-between washing. Thereafter, the sections were rinsed again and then subjected to a standard DAB-H2O2 procedure (400 mg DAB/L Tris maleate, pH 7.6) and counterstained with Mayer’s hematoxylin.

Adjacent sections were subjected to the terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) approach (Boehringer) to study the pattern of apoptotic cells by labeling the fragmented DNA that is characteristically found in high concentrations in the nuclei of cells undergoing apoptosis. Serial sections were deparaffinized in xylene, rehydrated, and rinsed in PBS. After pretreatment with proteinase K in 50 mmol/L Tris-HCl (pH 8.0) for 20 minutes at 37°C, the sections were washed twice in PBS and subjected to the TUNEL reaction at 37°C for 90 minutes. After 2 rinsings in PBS, the fluorescent Fab label was conjugated to peroxidase for 30 minutes, followed by washing in PBS. After DAB-H2O2 staining for 10 minutes, the sections were counterstained with hematoxylin.

The sections of the TGF-β2-null embryos were analyzed by light microscopy and compared with wild-type embryos of the same age. Embryos from day 13.5 onward, when ventricular septation is normally complete, were included for systematic analysis of congenital heart defects. In embryos of 11.5 to 16.5 days, the volume of apoptosis, endocardial cushions, and ventricular myocardium (as an indicator of developmental age) was measured by the Cavalieri method as performed by Bouman et al.10

### Results

#### Cardiovascular Malformations

Abnormalities of the heart and great vessels were diagnosed in 21 of 24 TGF-β2–knockout embryos older than 12.5 days (87.5%). The findings are summarized in Table 1. Malformations of the outflow tract include the ventricular outlet and the arterial orifice level. The term “AV canal” was used for anomalies related to the region of the atrioventricular canal and the ventricular inlet segment. Vascular abnormalities were divided into aortic and pulmonary.

The main anomaly consisted of a type of double-outlet right ventricle (DORV) (Figure 1a). DORV was seen with different relative positions of the arterial orifices, the aortic orifice being posterior (13 of 19), in a side-by-side position (3 of 19), or slightly anterior (3 of 19) to the pulmonary orifice. All hearts with DORV had a bilateral muscular conus. Dextroposition of the outflow tract resulted in a large-outlet ventricular septal defect (VSD) due to the abnormal position of the outflow tract septum relative to the ventricular septum. The VSD was more closely related to the aortic orifice in cases with a posterior position of the latter. In case of a deficient outflow tract septum, the VSD was committed to both arterial orifices. There was 1 case of common arterial trunk with interruption of the aortic arch (Figure 1b and 1c). Abnormal thickening of the leaflets of the pulmonary valve was seen in 2 hearts, including the aortic valve leaflets in 1.

Malformations of the AV canal were found in 15 cases (62.5%; Table 1). An inlet VSD was present in 9 embryos, associated with overriding of the tricuspid orifice in 6 cases (Figure 1d). The insertions of the tension apparatus of the tricuspid valve type B (Figure 1b and 1c). Abnormal thickening of the leaflets of the pulmonary valve was seen in 2 hearts, including the aortic valve leaflets in 1.

Malformations of the AV canal were found in 15 cases (62.5%; Table 1). An inlet VSD was present in 9 embryos, associated with overriding of the tricuspid orifice in 6 cases (Figure 1d). The insertions of the tension apparatus of the tricuspid valve type B (Figure 1b and 1c). Abnormal thickening of the leaflets of the pulmonary valve was seen in 2 hearts, including the aortic valve leaflets in 1.

### TABLE 1. Cardiovascular Abnormalities in TGF-β2–Knockout Mouse Embryos

<table>
<thead>
<tr>
<th>Abnormal Part, Type of Abnormality</th>
<th>No. of Cases</th>
<th>% of Cases</th>
<th>No. of Cases, Summary</th>
<th>% of Cases, Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outflow tract</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DORV</td>
<td>19</td>
<td>79.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Common arterial trunk</td>
<td>1</td>
<td>4.2</td>
<td>21</td>
<td>87.5</td>
</tr>
<tr>
<td>Thickening of PV±AoV</td>
<td>2</td>
<td>8.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AV canal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perimembranous inlet VSD</td>
<td>9</td>
<td>37.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thickening of TV±MV</td>
<td>8</td>
<td>33.3</td>
<td>15</td>
<td>62.5</td>
</tr>
<tr>
<td>Overriding of tricuspid orifice</td>
<td>6</td>
<td>25.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete AV septal defect</td>
<td>1</td>
<td>4.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aorta + branches</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypoplasia of aortic arch±ascending aorta</td>
<td>5</td>
<td>20.8</td>
<td>9</td>
<td>33.3</td>
</tr>
<tr>
<td>Interruption of the aortic arch</td>
<td>2</td>
<td>8.3</td>
<td>9</td>
<td>33.3</td>
</tr>
<tr>
<td>Aberrant RSA</td>
<td>2</td>
<td>8.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Remnant of right dorsal aorta</td>
<td>1</td>
<td>4.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pulmonary arteries</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypoplasia</td>
<td>1</td>
<td>4.2</td>
<td>1</td>
<td>4.2</td>
</tr>
<tr>
<td>No abnormality</td>
<td>3</td>
<td>12.5</td>
<td>3</td>
<td>12.5</td>
</tr>
</tbody>
</table>

PV indicates pulmonary valve; AoV, aortic valve; TV, tricuspid valve; MV, mitral valve; and RSA, right subclavian artery.
hypoplasia of the aortic arch, 2 of which had an interruption of the aortic arch type B with an aberrant right subclavian artery (Figure 1c).

In general, malformations involved >1 segment of the developing cardiovascular system, the association of anomalies of the outflow tract with abnormalities of the AV canal being most common (Table 2).

**TABLE 2.** Association of Anomalies in TGF-β2-Knockout Mouse Embryos Day 13.5 to 18.5 (n=17*)

<table>
<thead>
<tr>
<th>Abnormal Part</th>
<th>Outflow Tract</th>
<th>Aorta+Branches</th>
<th>Pulmonary Arteries</th>
</tr>
</thead>
<tbody>
<tr>
<td>AV canal, n (%)</td>
<td>15 (62.5)</td>
<td>6 (25.0)</td>
<td>1 (4.2)</td>
</tr>
<tr>
<td>Outflow tract, n (%)</td>
<td>...</td>
<td>7 (29.2)</td>
<td>1 (4.2)</td>
</tr>
<tr>
<td>Aorta+branches</td>
<td>...</td>
<td>...</td>
<td>0</td>
</tr>
</tbody>
</table>

Percentages are percent of all cases. Abbreviations as in Table 1.

*Four of the 21 cases with cardiac malformations had abnormalities of the outflow tract only.

**Apoptosis**

In the TGF-β2-knockout mouse embryos, apoptosis was found in the same structures as in the wild-type mice (Figures 2 and 3), even though their relative position within the heart was frequently abnormal as a result of the malformations present. A large number of apoptotic cells was encountered in the endocardial cushions of the outflow tract (Figure 2a...
Through 2d). A cluster of TUNEL-positive cell fragments was also found in the mesenchyme at the base of the atrial septum (Figure 3a through 3c). Scattered apoptotic cells were seen in the cushions of the semilunar and AV valve leaflets. Although apoptosis was present primarily in the mesenchymal tissue, TUNEL-positive cells were occasionally encountered within the myocardium of both wild-type and mutant embryos.

Morphometric evaluation of the volume of apoptotic cells in the outflow tract demonstrated differences in timing and amount of apoptosis between wild-type and TGF-β2−/− knockout embryos (Figure 4a). Apoptotic cells were found in both groups from 11.5 to 16.5 embryonic days, but in the mutant mice, the maximum of apoptosis was higher, occurred ~1 day later (day 14.5), and decreased more slowly than normal (Figures 2, 4a, and 5).

At the vascular level, there was increased apoptosis in the aortic segments deriving from the fourth arch. Details of apoptosis and remodeling of the aortic arches in this model will be part of a separate article.

Abnormalities of Cardiac Septation and Myocardialization

TGF-β2−/−null mice exhibited a variable degree of hypoplasia of the tissues deriving from the outflow tract ridges and the septal parts of the AV cushions.
In the wild-type embryos of 13.5 days, the outflow tract ridges were composed of thick mesenchymal tissue (Figure 6a) separating the right and left ventricular outflow tracts. During the next 2 days, this region became completely myocardialized (Figure 6b), mainly forming the subpulmonary muscular infundibulum.

In contrast, the TGF-β2-null embryos showed a variable degree of underdevelopment of the outflow tract separation not related to the type of heart defect. Because most cases presented with a VSD, this structure could be recognized as an actual outlet septum. Whereas the outflow tract cushions had become a muscular structure of normal size in some of the older hearts (Figure 6c), in other embryos only a small fibrous ridge was found below the semilunar valves (Figure 6d). In some cases, fusion of the proximal cushions was incomplete (Figure 6e).

In the wild-type embryos, the ostium primum was closed at 13.5 days by mesenchymal tissue (Figure 7a). Apoptosis was seen at the border between the myocardial and the mesenchymal components of the atrial septum. At later stages of development, the lower part of the atrial septum had become...
muscular, the only fibrous tissue being found at the level of the AV valves (Figure 7b).

In the mutant mice, development of the atrial septum was normal in all but 1 case. In this 18.5-day-old embryo, the lower part of the atrial septum was still mesenchymal, with a large primum type of atrial septal defect extending below this rim down to the level of the AV valves (Figure 7c and 7d).

In the region involving the ventricular part of the AV canal, incomplete fusion of the endocardial cushions resulted in a small membranous VSD (Figure 7e), whereas an extensive perimembranous inlet VSD was seen in cases in which outflow tract and AV cushions did not meet (Figure 7f).

Measurements of the total endocardial cushion volume gave additional evidence that the development of the mesenchymal tissue was abnormal (Figure 4b). In the wild-type embryos, myocardialization of mesenchyme from day 13.5 to 15.5 correlated with a decrease in endocardial cushion volume. The residual cushion tissue represented mainly the fibrous tissue of the AV and semilunar valves. In the TGF-$\beta_2$–mutant mice, total endocardial cushion volume at 13.5 days was normal. The normal decrease in total endocardial cushion volume in the older embryos, however, was not seen in this group. Total ventricular myocardial volume did not differ significantly between the 2 groups, but in some of the TGF-$\beta_2$–mutant embryos, the myocardium of the right ventricle in particular was more spongy, which appeared to be associated with ventricular dilatation (not shown).

**Abnormalities of Valve Differentiation**

The TGF-$\beta_2$–knockout embryos showed abnormalities of AV and semilunar valve differentiation. Whereas malformation of the outflow tract ridges and the mesenchymal atrial septum resulted in septal hypoplasia, the abnormal valve leaflets were hyperplastic and retained a thick and cushion-like appearance (Figure 8b). The right-sided valves (tricuspid and pulmonary valve) were more frequently abnormal than the left-sided valves (mitral and aortic valve).

**Discussion**

**Cardiovascular Malformations Represented a Range**

Knockout of the TGF-$\beta_2$ gene resulted in a spectrum of cardiovascular malformations, ranging from normal-appearing hearts to specimens with severe abnormalities of several cardiac segments. Anomalies always involved the outflow tract. Malformations due to failure of normal endocardial cushion differentiation may be underrepresented in this study because they could be diagnosed only in the older embryos. The cardiovascular anomalies observed imply a role of TGF-$\beta_2$ in several developmental processes (Figure 9, Table 3).

**Abnormalities of Looping and Wedging**

The hearts of TGF-$\beta_2$–null mice showed various degrees of failure of normal remodeling of the primitive heart. Overriding tricuspid valve and DORV appeared to represent a disturbance of the final phase of the cardiac looping process, with persistence of spatial relations that represent normal intermediate stages during cardiac morphogenesis.11

The mechanisms transforming the normal double-outlet condition of the early embryo into the definite ventriculoarterial alignments are still under investigation. This process requires remodeling of the subarterial infundibulum. In the normal mature heart, only a subpulmonary conus is present,11 which can be attributed in part to the formation of the subpulmonary muscular infundibulum through proper separation of the right and left outflow tracts. This process was clearly disturbed in the TGF-$\beta_2$ mutants. Neither in our wild-type nor in the TGF-$\beta_2$–null embryos could we find a
cluster of myocardial apoptotic cells that might relate to conal absorption as described by Watanabe and coworkers in the chicken embryo.

Abnormalities of Endocardial Cushion Differentiation

Absence of TGF-β2 resulted in abnormalities of the cardiac valve leaflets and septa that derive from endocardial cushion tissue. TGF-β2 has been shown to play a role in the induction of the cushions. It is expressed from day 8.5 to 9.5 postcoitum at particularly high levels in the myocardium underlying the regions in which the endocardial-mesenchymal transformation producing the cushions will take place. During the period of normal mesenchymal differentiation, the expression of TGF-β2 is limited to the cushion tissue. Interestingly, the maximum total endocardial cushion volume in the TGF-β2-deficient mice in our study was not smaller than normal. This suggested that not the process of cushion formation but rather the process of endocardial cushion fusion and differentiation was disturbed in the absence of TGF-β2.

TGF-β2 have been implicated in the migration and homing of neural crest cells. Although intracardiac mesenchymal distribution of apoptotic cells, most of which are thought to be of neural crest origin, in the TGF-β2–knockout embryos was normal, altered neural crest cell migration may account for the absence of neural crest–derived suprasemilunar mesenchyme in the case with common arterial trunk.

TGF-β2 is secreted in a latent form that can be activated by neural crest cell proteolysis, the active form in turn decreasing with increasing neural crest–derived proteolytic activity. The active form in turn decreases with increasing neural crest–derived proteolytic activity. The active form in turn decreases with increasing neural crest–derived proteolytic activity. In the TGF-β2–mutant embryos, apoptosis was increased, and higher levels of fragmented cells could be found during the later stages of development, suggesting disturbances of the feedback mechanisms in the absence of TGF-β2.

During the process of differentiation, the outflow tract ridges normally become the free-standing subpulmonary muscular infundibulum. Myocardialization of the ridges appears to result from migration of myocardial cells into the endocardial cushions, possibly induced by activation of TGF-β2 through neural crest cell apoptosis. The role of mechanical traction in outflow tract septation needs to be reevaluated. In the TGF-β2–null mutant embryos, disturbance of myocardialization with ineffective neural crest cell signaling and increased apoptosis resulted in a fibrous (and hence hypoplastic) subvalvular tissue rim. In accordance with the presence of DORV and the accompanying VSD, the subvalvar fibrous tissue can be seen as an actual hypoplastic outflow septum.

The mesenchymal part of the atrial septum also becomes a muscular structure during normal development, a process that can be disturbed in the absence of TGF-β2. Data are not conclusive as to whether myocardialization in this region results from a process similar to that in the outflow septum. As evidence for possible myocardialization, some apoptotic cells can be found in the mesenchyme of the atrial septum at the border to the muscular part. The atrial tissue at the base of the interatrial septum as well as the top of the ventricular septum, however, has been shown to stain positively for HNK-1 as a marker for the developing conduction system, which may require neural crest cells for differentiation. The role of neural crest cells in the AV region therefore deserves further study. In the mesenchyme on top of the ventricular septum, we did not find any apoptotic cells as evidence for myocardialization. It rather appears that with remodeling of this area, this part of the cushion tissue is taken up into the level of the AV valves.

Comparison of Cardiovascular Anomalies in the TGF-β2–Knockout Mouse With Other Animal Models

The TGF-β2–knockout mouse presents with a characteristic set of cardiovascular malformations that are, however, not specific. Literature data reveal mouse models with some comparable and some additional malformations. A combination of inflow tract and outflow tract anomalies has been reported in the retinoic acid receptor RXR-α–knockout mouse and the trisomy 16 mouse. Information on dysplastic valves is missing, as well as data on mechanistic processes that involve altered apoptosis or myocardialization. To reveal comparable mechanisms, it will be necessary to study the TGF-β2 expression in the above-mentioned models. We have not been able to find a model with anomalies of the semilunar and AV valves as described for the TGF-β2 mutants. The transcriptional factor Sox-4–knockout mouse, with dysplastic semilunar valves as well as outflow tract anomalies, dies already at day 14 postcoitum.

Anomalies comparable to those seen in the TGF-β2–null mutant have been reported in a chicken embryo in which the venous inflow to the heart was altered by ligation of the right vitelline vein. There was failure of myocardialization of outflow tract ridges and a resultant fibrous outflow tract septum. This seemed, however, to be the result of diminished neural crest cells in this area combined with absence of apoptosis, in contrast to what we observed in the TGF-β2–knockout mouse.

These data show that comparable congenital malformations can easily be the result of alterations of different developmental pathways that deserve further study.

In summary, TGF-β2–knockout mice exhibited a range of cardiovascular anomalies that resulted from failure of normal
completion of looping and septation of the outflow tract and the AV canal, as well as abnormalities of valve differentiation and arterial growth. Ventricular remodeling, myocardialization of endocardial cushion tissue, and apoptosis are the processes affected most. On the basis of the morphological similarity of the abnormalities found to heart malformations in humans, it can be speculated that TGF-β plays a role in normal and possibly abnormal cardiac morphogenesis in humans.

Acknowledgments
This project was supported by grants from the Netherlands Heart Foundation (Dr Gittenberger-de Groot) and the Deutsche Forschungsgemeinschaft (Dr Bartram), and National Institutes of Health grants HD-26471 and HL-41496 (Dr Doetschman). We thank Jan H. Lens for his expert assistance with preparing the figures.

References
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Circulation. 2001;103:2745-2752
doi: 10.1161/01.CIR.103.22.2745
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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