Tetralogy of Fallot and Alterations in Vascular Endothelial Growth Factor-A Signaling and Notch Signaling in Mouse Embryos Solely Expressing the VEGF120 Isoform

Nynke M.S. van den Akker, Daniël G.M. Molin, Patricia P.W.M. Peters, Saskia Maas, Lambertus J. Wisse, Ronald van Brempt, Conny J. van Munsteren, Margot M. Bartelings, Robert E. Poelmann, Peter Carmeliet, Adriana C. Gittenberger-de Groot

Abstract—The importance of vascular endothelial growth factor-A (VEGF) and subsequent Notch signaling in cardiac outflow tract development is generally recognized. Although genetic heterogeneity and mutations of these genes in both humans and mouse models relate to a high susceptibility to develop outflow tract malformations such as tetralogy of Fallot and peripheral pulmonary stenosis, no etiology has been proposed so far. Using immunohistochemistry, in situ hybridization, and quantitative RT-PCR on embryonic hearts, we have shown spatiotemporal increase and abnormal patterning of Vegf/VEGF/(phosphorylated) VEGFR-2, (cleaved) Notch1, and Jagged2 in the outflow tract of Vegf120/120 mouse embryos. This coincides with hyperplasia of specifically the outflow tract cushions and a high degree of subpulmonary myocardial apoptosis that, in later stages, manifest as pulmonary stenosis and ventricular septal defects. We postulate that increase of VEGF and Notch signaling during right ventricular outflow tract development can lead to abnormal development of both cushion and myocardial structures. Defective right ventricular outflow tract development as presented provides new insight in the etiology of tetralogy of Fallot. (Circ Res. 2007;100:842-849.)

Key Words: tetralogy of Fallot ■ apoptosis ■ VEGF ■ Notch

The importance of vascular endothelial growth factor-A (VEGF) for angiogenesis is ultimately demonstrated by the early embryonic death of both VEGF heterozygous and VEGF receptor (VEGFR)-2 homozygous knockout mice. Recent data point toward a critical role for VEGF during cardiac development as well. It has been shown in humans that a low expression VEGF haplotype correlates with increased risk for tetralogy of Fallot (TOF), a congenital heart disease consisting of a ventricular septal defect (VSD), pulmonary stenosis, and dextroposition of the aorta. Furthermore, the VEGF165 isoform has been postulated as a modifier of the cardiovascular phenotype in the human DiGeorge syndrome. In addition, mouse models in which genes of proteins involved in VEGF signaling are mutated show comparable cardiac malformations.

During cardiac development, the early outflow tract (OFT) must be divided into the aortic and pulmonary OFT with proper alignment to the left and right ventricle, respectively. Here, the development of both the endocardial cushions and the adjacent myocardium is crucial. Within this process, the cushions must be populated by cells predominantly recruited from the cushion endocardium through epithelial–mesenchymal transformation (EMT). A role for VEGF in atrioventricular cushion EMT has been shown, albeit both stimulating and inhibiting. This led to the hypothesis that VEGF must be expressed within a “physiological window” during cushion development.

The myocardium of the pulmonary OFT, derived from the secondary heart field (SHF), is a distinct population added to the arterial pole after the initial heart tube is formed. SHF addition is critical for OFT development as ablation of the SHF in chicken embryos leads to cardiac malformations such as TOF, in which anomalous OFT development is obvious. Furthermore, upregulated by hypoxia, VEGF seems to play a role in this part of OFT development.

VEGF signaling can upregulate members of the Jagged/Delta-like/Notch family. JAGGED1 mutations in humans are correlated with TOF or pulmonary stenosis. Involvement of other members of Notch signaling in cardiac development has been demonstrated by several mouse mutants. Notch has also been described to stimulate endocardial cushion EMT, implying a potential effect of VEGF on cushion development via Notch signaling.
To investigate the role of VEGF and Notch signaling, we made use of the previously described Vegf120/120 mouse model.\(^5,27\) We demonstrate that Vegf120/120 mouse embryos, which solely express the VEGF120 isoform, are highly susceptible for development of TOF. We hypothesize that this is most likely attributable to spatiotemporal elevations of VEGF and Notch signaling, mainly seen in the SHF-derived right ventricular OFT myocardium.

### Materials and Methods

#### Mouse Embryos and Tissue Processing

All animal experiments were approved by the Animal Ethics Committee of the Leiden University and performed according to the Guide for the Care and Use of Laboratory Animals published by the NIH. An extensive description of mouse experiments can be found in the online data supplement, available at http://circres.ahajournals.org.

#### Immunohistochemistry

Paraffin sections of 5 μm were incubated overnight with primary antibody; after which, sections were incubated with secondary antibody. For the detection of apoptosis, the TUNEL kit was used (1684817, Roche/Boehringer Mannheim, Basel, Switzerland). An extensive description of the technique and of the antibodies used can be found in the online data supplement.

#### In Situ Hybridization

Sense and antisense \(^{35}\)S-radiolabeled Vegf-A RNA probes were transcribed using a 451-bp clone encoding for the mouse Vegf-120 isoform (pVEGF2; kindly provided by Dr G. Breier, University of Technology, Dresden, Germany). Radiolabeled in situ hybridization was performed.\(^28\) A brief description can be found in the online data supplement.

#### Quantitative RT-PCR

RNA was isolated using the RNeasy mini kit (Qiagen). All samples were normalized for input based on β-actin and GAPDH. Data analyses were performed using an Excel spreadsheet based on geNorm (Relative expression with error propagation).\(^29\) Statistic significance was tested using randomization testing, as provided in the REST2005 program.\(^30\) Samples with a probability value of <0.05 were regarded to be significant different between the groups. Primer sequences and detailed description of the technique appear in the online data supplement (supplemental Table II).

### Results

#### Morphology

**Right Ventricular OFT Development Is Impaired**

At E10.5, the development of the common OFT of Vegf120/120 embryos was comparable to wild-type littermates. In E11.5 to E13.5 mutants, we could observe hyperplasia of the proximal OFT cushions (Figure 1a and 1c; Table 1). More striking, in all of these embryos, a large apoptotic ring surrounded the right ventricular OFT, as observed using Mayer’s hematoxylin (Table 1),\(^32\) as well as with TUNEL staining, specifically located in the subpulmonary myocardium up to the level of the developing pulmonary valves (Figure 1b through 1d and 1f through 1h). Stenosis of the right ventricular OFT concomitant with hypoplasia of the pulmonary trunk or pulmonary arteries was apparent in several cases (Figure 1c and 1g; Table 1).

At E14.5 to E19.5, right ventricular OFT abnormalities varied from stenosis of the left pulmonary artery at the level of branching from the pulmonary trunk (Figure 1i and 1m) to...
stenosis of the right ventricular OFT, which was always accompanied by hypoplasia of the pulmonary trunk (Table 2). In cases with severe OFT stenosis, almost complete atresia of the pulmonary trunk was seen (Figure 1j and 1n).

Pulmonary OFT and Aortic Arch Defects
All pharyngeal arch arteries were present in Vegf120/120 embryos of E10.5, excluding anomalous anlage. Already at E11.5, associated with pulmonary OFT stenosis, atresia of the ductus arteriosus (DA) was observed in 2 of 4 embryos. From E14.5 onward, an atretic strand or absence of the DA was seen (Figure 1k and 1o) and coincided in 8 of 9 cases with pulmonary OFT stenosis (Table 2). Other aortic arch malformations observed from E14.5 onward were right or double aortic arch with right dominance and a right DA (Figure 1l and 1p; Table 2) or hypoplasia of the aortic arch (5 of 31).

**TABLE 1. Outflow Abnormalities in Vegf120/120 Embryos**

<table>
<thead>
<tr>
<th>Cardiac Anomaly</th>
<th>No./Total</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis subpulmonary myocardium</td>
<td>14/14</td>
<td>100</td>
</tr>
<tr>
<td>Hyperplasia OFT cushions</td>
<td>8/14</td>
<td>57</td>
</tr>
<tr>
<td>Only</td>
<td>3/14</td>
<td>21</td>
</tr>
<tr>
<td>Plus malposition OFT cushions</td>
<td>5/14</td>
<td>36</td>
</tr>
<tr>
<td>Hypoplasia PT/PA</td>
<td>9/14</td>
<td>64</td>
</tr>
<tr>
<td>Only</td>
<td>3/14</td>
<td>21</td>
</tr>
<tr>
<td>Plus stenosis RV-OFT*</td>
<td>6/14</td>
<td>43</td>
</tr>
<tr>
<td>Plus hyperplasia OFT cushions</td>
<td>2/14</td>
<td>14</td>
</tr>
</tbody>
</table>

Age of embryos was E11.5 to E13.5. PT/PA indicates pulmonary trunk/pulmonary artery(ies); RV-OFT, right ventricular OFT. *These 6/14 embryos represent all aged E11.5 to E13.5 with stenosis of the right ventricular OFT.

**TABLE 2. TOF-Related Abnormalities in Vegf120/120 Embryos**

<table>
<thead>
<tr>
<th>Cardiac Anomaly</th>
<th>No./Total</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSD</td>
<td>13/31</td>
<td>42</td>
</tr>
<tr>
<td>Subaortic</td>
<td>11/31</td>
<td>35</td>
</tr>
<tr>
<td>Plus muscular</td>
<td>2/31</td>
<td>6</td>
</tr>
<tr>
<td>Muscular only</td>
<td>2/31</td>
<td>6</td>
</tr>
<tr>
<td>Subaortic VSD + dextroposition aorta (DORV)</td>
<td>10/31</td>
<td>32</td>
</tr>
<tr>
<td>Plus stenosis RV-OFT (TOF)</td>
<td>9/31</td>
<td>29</td>
</tr>
<tr>
<td>Hypoplasia PT/PA</td>
<td>19/31</td>
<td>61</td>
</tr>
<tr>
<td>Only</td>
<td>9/31</td>
<td>29</td>
</tr>
<tr>
<td>Plus stenosis RV-OFT*</td>
<td>10/31</td>
<td>32</td>
</tr>
<tr>
<td>Plus atresia DA†</td>
<td>8/31</td>
<td>26</td>
</tr>
<tr>
<td>Right aortic arch</td>
<td>4/31</td>
<td>13</td>
</tr>
<tr>
<td>Only</td>
<td>3/31</td>
<td>10</td>
</tr>
<tr>
<td>Plus TOF</td>
<td>1/31</td>
<td>3</td>
</tr>
<tr>
<td>Double aortic arch</td>
<td>2/31</td>
<td>6</td>
</tr>
<tr>
<td>Only</td>
<td>1/31</td>
<td>3</td>
</tr>
<tr>
<td>Plus subaortic VSD</td>
<td>1/31</td>
<td>3</td>
</tr>
<tr>
<td>Thin myocardium</td>
<td>17/31</td>
<td>55</td>
</tr>
</tbody>
</table>

Age of embryos was E14.5 to E19.5. PT/PA indicates pulmonary trunk/pulmonary artery(ies); RV-OFT, right ventricular OFT; DA, ductus arteriosus; DORV, double-outlet right ventricle. *These 10/31 embryos represent all aged E14.5 to E19.5 with stenosis of the right ventricular OFT. †In addition, 1 embryo with isolated DA atresia was found.

Pulmonary/systemic collateral arteries from the dorsal aorta to the lungs were found in embryos with pulmonary stenosis and absence of the DA at later stages of development (data not shown).

Other Cardiac Anomalies
Correct looping of the early heart tube is crucial for proper cardiac septation. In half of the E10.5 and E11.5 Vegf120/120 embryos, looping was diminished (Figure 2a and 2d), leading to a wider inner curvature and a ventral displacement of the OFT. Vegf120/120 embryos of E11.5 to E13.5 showed malposition of the OFT cushions, together with ventral displacement of the OFT (Table 1), which, at later stages (E14.5 to E19.5), was concomitant with subaortic VSDs (Figure 2b and 2e; Table 2). Significant dextroposition of the aorta and a subaortic VSD, which is referred to as a double-outlet right ventricle (Table 2), went along with a side-by-side positioning of the great arteries (Figure 2c and 2f). Often, a combination of a subaortic VSD, dextroposition of the aorta and right ventricular OFT stenosis, TOF, was found (Table 2).

**Figure 2. Heart malformations in Vegf120/120 embryos. Stainings performed are indicated in the upper right corner of each panel. Wild-type and Vegf120/120 mouse embryos are compared as indicated in the upper left corner, together with age. Abnormal OFT looping was observed in mutant embryos (asterisk in a and d). Subaortic ventricular septal defect was encountered in older mutant embryos (b and asterisk in e), as was dextroposition of the ascending aorta and side-by-side positioning of the OFT (c and f). c and f. Three-dimensional reconstructions of α/γMA-stained sections. The pink structure is the aortic arch (AoA), and the green structure is the pulmonary trunk (P). A indicates ascending aorta; α/γMA, α/γ muscle actin; α-SMA, α-smooth muscle actin; LV, left ventricle; RV, right ventricle; V, ventricle. Scale bar: 60 μm (a and d); 200 μm (b and e).**

**Signaling**

Vegf120 mRNA Levels Are Increased and Patterning Is Abnormal
To determine the mRNA levels of the different Vegf isoforms during normal cardiac development, isoform-specific quantitative RT-PCR on normal hearts of E14.5, E16.5, and E18.5 was performed. In this time span, Vegf120 was least prominent compared with the larger isoforms, although a temporal increase was seen at E16.5 (Figure 3a). When comparing total Vegf mRNA levels of wild-type to Vegf120/120 hearts, no significant difference was found (Figure 3b). However, when only the expression of the Vegf120 isoform was compared, a 6- to 16-fold increase was seen in the Vegf120/120 hearts, being most prominent at E16.5 (Figure 3c).
Spatiotemporal changes in Vegf mRNA patterning were investigated in Vegf120/120 embryonic hearts, using radioactive in situ hybridization. Between E10.5 to E14.5, high expression was observed in the OFT myocardium at the level of the OFT cushions, whereas very low expression was seen in the endocardium of the OFT cushions of wild-type embryos (Figure 4a, 4b, 4d, and 4e). Increased Vegf mRNA signal in the endocardial cells of the OFT cushions was found in Vegf120/120 embryos of E10.5, whereas the level in the OFT myocardium was comparable between genotypes at this age (Figure 4d). In Vegf120/120 embryos of E14.5, a highly increased Vegf signal was seen in the subpulmonary myocardium (Figure 4e), up to the level of the OFT valves when compared with wild-type littermates (Figure 4b). In wild-type embryos of E16.5, the highest expression was present at the borderline of compact to trabecular myocardium (data not shown). At E18.5, only scattered Vegf expression was observed (Figure 4c). In Vegf120/120 hearts of E16.5 to E18.5, the OFT myocardial signal was higher (data not shown) and the expression at the borderline of compact to trabecular myocardium was markedly increased (Figure 4f).

**Increased Expression of VEGF, (Phosphorylated) VEGFR-2, (Cleaved) Notch1, and Jagged2 in the OFT**

In accordance with the in situ hybridization data, VEGF levels were increased in the endothelium of the OFT cushions at E10.5 (Figure 5a and 5e). In older embryos (E13.5 and older), the VEGF protein expression was equally distributed throughout wild-type myocardium, whereas the staining intensity was higher in the trabeculae compared with the compact myocardium in Vegf120/120 embryos (data not shown). In wild-type embryos of E10.5, VEGFR-2 expression was present throughout the myocardium and in the endocardium of the OFT. Expression in these cell types was higher in the Vegf120/120 embryos (Figure 5b and 5f). Although in wild types of E18.5 and older the myocardial VEGFR-2 signal had disappeared, the mutants still expressed low levels of VEGFR-2 in the myocardium (data not shown). Furthermore, in the subpulmonary myocardium, in the region of the apoptotic area, an increase in expression of phosphorylated VEGFR-2 expression was observed, indicating locally high levels of VEGF signaling in Vegf120/120 mouse embryos of E11.5 to E14.5 (Figure 5j and 5m).

Spatiotemporally coinciding with Vegf/VEGF and VEGFR-2 expression, we observed both Notch1 expression and presence of cleaved (activated) Notch1 in the OFT endocardium of Vegf+/+ embryos of E10.5 (Figure 5c and 5d). In Vegf120/120 mutants of E10.5, an increased number of endocardial and mesenchymal cells revealed Notch1 expression and presence of cleaved Notch1, whereas no differences were seen for the myocardial expression (Figure 5g and 5h). Expression patterns of Notch2 were unaltered between both genotypes. In wild-type embryos of E11.5 to E15.5, Jagged2 expression was present at low levels in the subpulmonary OFT myocardium (Figure 5k). Jagged2 expression in the mutants embryos was more prominent (Figure 5n), especially for the apoptotic subpulmonary myocardium (Figure 1f through 1h). This increase in Jagged2 expression colocalized with increase of phosphorylated VEGFR-2 expression and higher levels of cleaved Notch1 when compared...
with wild-type littermates (Figure 5i, 5j, 5l, and 5m). Also, increased level of Jagged1 in the OFT myocardium was obvious in Vegf120/120 embryos (data not shown), whereas, again, no differences in Notch2 expression were seen.

Discussion

It has been shown in humans that mutations in the VEGF gene,33 its promoter3 or in JAGGED120–22 increase the risk to develop congenital heart disease, such as TOF and pulmonary stenosis. To unravel the role of VEGF in normal and abnormal OFT development, we investigated several stages of heart development in wild-type and Vegf120/120 mouse embryos that have been reported with TOF.5 We found that spatiotemporal increase of VEGF and subsequent Notch signaling coincides with hyperplasia of the OFT cushions and abnormally high levels of apoptosis in the subpulmonary myocardium. In addition, abnormal size and positioning of the OFT cushions as found in our model are associated with cardiac looping defects, VSD and malpositioning of the great arteries.34,35 Also, changes in endocardial VEGF signaling has been shown to cause heart bending defects.36 The anomalies in pulmonary OFT morphogenesis, as exemplified in the Vegf120/120 mutants, can contribute to the development of TOF, consisting of right ventricular OFT stenosis, dextroposition of the aorta, and subaortic VSD.

Alterations in Vegf Expression in Vegf120/120 Mutants

Although cardiac levels of total Vegf mRNA do not differ between Vegf120/120 and wild-type embryos, expression levels of the Vegf120 isoform are markedly higher in mutants. Because during normal cardiac development, VEGF120 is the least prominent isoform (Figure 3a),37 an adverse effect of overexpression in Vegf120/120 embryos should be considered. Furthermore, this mouse model lacks the heparin- and NP-1–binding VEGF isoforms (ie, VEGF164 and VEGF188) and, hence, a lack of neuropilin-1–mediated VEGFR-2 signaling is expected.38 However, we observe increased levels of Vegf (Figure 4e) and of phosphorylated VEGFR-2 (Figure 5m) in the subpulmonary cardiomyocytes, indicating locally increased, instead of decreased, signaling. Based on quantitative RT-PCR data (Figure 3a) and in situ hybridization comprising all isoforms (Figure 4b), it is expected that in wild-type embryos, the nonsoluble VEGF164 isoform is dominant in the subpulmonary myocardium. In the Vegf120/120 embryos, only the soluble VEGF120 isoform is expressed. This could initially result in decreased signaling.
followed by altered feedback mechanisms in Vegf expression, which then lead to the extreme increase of Vegf120 levels (Figures 3c and 4e). However, as little is known about feedback mechanisms regulating Vegf expression, this remains elusive at this point.

**VEGF and Notch Signaling in Abnormal Cushion Development**

VEGF signaling has been described to play a role in OFT cushion development. Although these processes largely take place before E10.5, we still find expression levels of VEGF and VEGFR-2 in the endocardium and the mesenchyme of the OFT cushions of normal embryos (Figure 5a, 5b, 5e, and 5f) and favor a role of VEGF signaling in cushion expansion as well. VEGF has been reported to increase Notch1 expression. In agreement with earlier observations, agreement with earlier observations,41,43 the observed increase in Notch signaling in the subpulmonary myocardium, as indicated by cleaved Notch1 and Jagged2, could account for the pathological levels of apoptosis found in the Vegf120/120 model. Thus, we conclude that VEGF signaling is protective for the subpulmonary cardiomyocytes, as suggested by Sugishita et al, within a physiological window; when signaling levels are too low or too high (as in the Vegf120/120 mouse model), the cardiomyocytes will undergo (Notch-mediated) apoptosis, leading to congenital heart malformations.

In humans, changes in NOTCH1 signaling by mutations in the JAGGED1 gene can lead to congenital cardiac malformations such as TOF. However, little is known about the alterations in signaling under these conditions. It can be speculated that in these cases, decreased JAGGED1 function favors JAGGED2-related signaling, leading to a comparable condition as in our mouse model. This is only speculative, as little information is available at present regarding the differences in affinities and/or intracellular pathways between the combinations of Notch ligands and receptors. However, as we show that spatiotemporal changes in VEGF and Notch signaling are associated with the development of cardiac abnormalities, we think that our model does provide further insight into the embryonic development of right ventricular OFT anomalies, with a potential function for VEGF and Notch signaling.

It should be mentioned that the affected subpulmonary myocardium has been linked to the SHF. Recent data suggest that this myocardium is a distinct population critically involved in proper positioning of the large OFT vessels. Furthermore, increasing evidence is pointing toward a link between alterations in SHF development and the etiology of OFT anomalies including TOF. We postulate that this SHF-derived subpulmonary myocardium is highly sensitive for VEGF and Notch signaling. The high levels of apoptosis in this myocardial population probably lead to hypoplasia of the pulmonary trunk and the often observed right ventricular OFT stenosis. The occurrence of this phenotype in our model is likely aggravated by the manifestation of the earlier discussed cushion hyperplasia. Furthermore, ablation of this SHF-derived myocardium, in our case by apoptosis, might lead to alterations in proper positioning of the OFT vessels leading to dextroposition of the aorta.

**Apoptosis of Subpulmonary Myocardium Colocalizes With Increased Notch Signaling**

During mouse and chicken development, apoptosis of subaortic cardiomyocytes has been described as a normal phenomenon essential for proper OFT remodeling. In addition, it has been described in chicken embryos that aforementioned apoptosis is associated with hypoxia-induced expression of VEGF. In this research, we show that the subpulmonary Vegf expression coincides with high phosphorylated VEGFR-2 and Jagged2 expression of cardiomyocytes. Although only a very small number of apoptotic cells is seen in the OFT myocardium of wild-type embryos, which is in agreement with earlier observations, Vegf120/120 mutants show remarkable large ring-like apoptotic areas in the subpulmonary myocardium. These areas spatiotemporally overlap with elevated levels of Vegf, phosphorylated VEGFR-2, cleaved Notch1 and Jagged2. This indicates that increased VEGF and Notch signaling is present in the subpulmonary myocardium of Vegf120/120 embryos. It has been described that stimulation of Notch1 by Jagged2 can result in apoptosis, and the observed increase in Notch signaling in the subpulmonary myocardium, as indicated by cleaved Notch1 and Jagged2, could account for the pathological levels of apoptosis found in the Vegf120/120 model. Thus, we conclude that VEGF signaling is protective for the subpulmonary cardiomyocytes, as suggested by Sugishita et al, within a physiological window; when signaling levels are too low or too high (as in the Vegf120/120 mouse model), the cardiomyocytes will undergo (Notch-mediated) apoptosis, leading to congenital heart malformations.

**Abnormalities of the Pulmonary Arteries and Aortic Arch**

The development of vascular anomalies has been linked to altered blood flow and, as such, can develop secondary to cardiac outflow defects. The high frequencies of pulmonary vascular defects (ie, hypoplasia and atresia of the DA and pulmonary arteries) in the Vegf120/120 mutant along with right ventricular OFT obstruction is in agreement with this
assumption. The severe pulmonary outflow or arterial stenosis will impair blood flow to the lungs. We suggest that this leads to local hypoxia and development of collateral vessels originating from the dorsal aorta, as observed in our mouse model as well as in human neonates with severe pulmonary stenosis.

The Vegf120/120 mouse model has been described as a model with overt cardiovascular defects found in patients with DiGeorge syndrome (ie, TOF, common arterial trunk, and aortic arch interruption type B). However, the occurrence of aortic arch malformations in this research seems to differ slightly from earlier published data on this model. This might be explained by the differences in time points analyzed between both studies. As in this research, embryos at several different time points of development were investigated (E10.5 to E19.5 versus E14.5/neonates), the number of anomalies encountered here might be underestimated because of ongoing development.

Conclusions
We conclude that during normal heart development, VEGF and subsequent Notch signaling must be tightly controlled, especially in the SHF-derived myocardium of the right ventricular OFT. In the Vegf120/120 mice, local increase of VEGF signaling in this region leads, likely via changes in the Notch pathway, to hyperplasia of the OFT cushions and apoposis of the SHF-derived subpulmonary myocardium. This working model might explain the development of TOF in the human population as found in individuals with VEGF and JAGGED1 mutations and 22q11 deletions.

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Disclosures
None.

References


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Materials and Methods

Mouse embryos and tissue processing
All animal experiments were approved by the Animal Ethics Committee of the Leiden University and performed according to the Guide for the Care and Use of Laboratory Animals published by the NIH. Vegf+/120 mice were crossed to obtain Vegf120/120 embryos and Vegf+/+ wild type littermates. The morning of the vaginal plug was stated embryonic day (E)0.5. Pregnant females were sacrificed by cervical dislocation. The embryos were isolated and either snap-frozen in liquid nitrogen or fixed in 4% paraformaldehyde/phosphate-buffered saline (0.1 Mol/L, pH7.4), dehydrated and embedded in paraffin. In total, 34 Vegf+/+ and 47 Vegf120/120 embryos ranging from E10.5 to E19.5 were embedded in paraffin and used for microscopic analyses. Of the snap-frozen embryos (12 Vegf+/+ and 13 Vegf120/120 embryos of E14.5, E16.5 or E18.5) hearts were dissected and veins and great arteries were removed and stored in RNaLater (Ambion, Cambridgeshire, UK) at -80°C before use for RT-qPCR.

Immunohistochemistry
Paraffin sections of 5 µM were deparaffinated and antigen retrieval was performed by heating the sections (12 minutes to 98º C) in citric acid buffer (0.01 Mol/L, pH6.0) in several cases (table S1). Inhibition of endogenous peroxidase was performed by incubating the sections for 20 minutes in PBS with 0.3% H₂O₂. Incubation with primary antibody (table S1) was performed overnight, after which sections were incubated for 1 hour with peroxidase-labelled antibody (rabbit-α-mouse; P0260, DAKO, Glostrup, Denmark) or biotin-labelled secondary antibody (goat-α-rabbit (BA-1000), horse-α-mouse (BA-2000) and horse-α-goat (BA-9500), all Vector Labs, Burlingame, USA). In case of the cleaved Notch1 staining, the CSA-II kit (K1497, DAKO, Glostrup, Denmark) was used for amplification. When using biotin-labelled antibody, additional incubation with the Vectastain ABC staining kit (PK-6100, Vector Labs, Burlingame, USA) was performed for 45 minutes. For the detection of apoptosis, the Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL)-kit was used according to manual (1684817, Roche/Boehringer Mannheim, Basel, Switzerland), followed by anti-FITC antibody (1772465, Roche/Boehringer Mannheim, Basel, Switzerland). Visualisation was performed with the DAB-procedure and Mayer's hematoxilin was used as a counterstaining. The sections were mounted with Entellan (1.07961.0100, Merck, Darmstadt, Germany). Differences between genotypes were scored as such when this was shown per immunostaining at least in 3 different embryos per genotype per age-group. An extensive description of the primary antibodies used is listed in table S1. When omitting the primary antibodies, no signal was detected. In case of the Notch1 and Jagged2 antibodies, additional characterisation was performed using preincubation of the primary antibody with competing peptide, which led to loss of signal (see fig S1).

**In situ hybridisation**
Sense and anti-sense $^{35}$S-radiolabelled Vegf-A RNA probes were transcribed using a 451-bp clone encoding for the mouse Vegf-120 isoform (pVEGF2; kindly provided by Dr. G. Breier, University of Technology, Dresden, Germany). To obtain sense and antisense $^{35}$S-labelled riboprobes, the clones were linearized and transcribed with T7 or T3 RNA-polymerase, respectively. Radioactive in situ hybridisation (ISH) was performed\(^1\). In brief, after hybridisation, sections were dehydrated in graded ethanol, air-dried, coated with Ilford G5 emulsion (ILFORD, Ltd., Mobberly, England), and exposed (14 days at 4°C). Subsequently, the slides were developed in Kodak D19 developing solution (Kodak, Chalons-s. Saone, France) for 4 minutes at room temperature, rinsed in distilled water, and fixed in Ilford Hypam fixative (ILFORD, Ltd.). Sections were briefly counterstained with Mayer’s hematoxylin, dehydrated, and mounted with Malinol (Schmid & Co, Stuttgart-Untertürkheim, Germany). The sections were examined by light microscopy using darkfield optics.

\textbf{RT-qPCR}

Prior to total RNA isolation the tissues were disrupted using a micro-pestle (Kleinfeld Labortechnik, Gehrden, Germany) in Qiagen RLT buffer (Qiagen, Hilden, Germany). After samples were homogenized total RNA was isolated using the RNeasy mini kit (QIagen). Samples with sufficient RNA quality (OD\(_{260/280} > 1.9\) and a RIN >7.5) were approved for analysis. A total of 100ng total RNA sample was subjected to reverse transcription (RT). RT-qPCR was performed by using Superscript™III Platinum Two-step qRT-PCR kit and SYBR green (Invitrogen, Paisley, UK) with a primer concentration of 10µM. Primers were designed with Oligoperfect™ Designer (Invitrogen), Primer3 and Mfold (http://www.idtdna.com/scitools/Applications/mfold/) and synthesized by Eurogentec (Seraing, Belgium). qPCR reactions were run on a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Veenendaal, the Netherlands). All samples were
normalized for input based on two normalizing genes, β-actin and GAPDH. The qPCR
efficiency of all primers was tested and included in the data analysis. Data analyses
were performed using an Excel spreadsheet based on geNorm (Relative expression with
error propagation)². Statistic significance was tested using randomization testing as
provided in the REST2005 program³. Samples with a p-value <0.05 were regarded to be
significant different between the groups. Primer sequences are shown in table S2.
References


Figure legends

**Figure S1.** Characterisation of the anti-Notch1 and anti-Jagged2 antibodies using competing peptides. The stainings performed are indicated in the upper right corner. All stainings have been performed on a Vegf+/120 mouse embryo of E13.5. The staining is performed according to the protocols described in this manuscript. Figure a and b are the positive controls whilst figure c and d show sections in which the primary antibody has been omitted. Figure e and f show sections incubated with primary antibody preincubated with 75x competing peptide. Scale bar – 200 µM.
Table S1. Characteristics of primary antibodies used for immunohistochemistry.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Cat. no. (company)</th>
<th>Antigen retrieval</th>
<th>ABC-incubation</th>
<th>CSA-II kit (DAKO)</th>
<th>Reference</th>
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<tr>
<td>α-SMA (1A4)</td>
<td>A2547 (Sigma-Adrich, Missouri, USA)</td>
<td>Yes</td>
<td>No</td>
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<td>Skalli et al⁴</td>
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<td>VEGFR-2</td>
<td>AF644 (R&amp;D Systems, Minneapolis, USA)</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
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<td>p-VEGFR-2</td>
<td>Sc-16628 (Santa Cruz, Santa Cruz, USA)</td>
<td>No</td>
<td>Yes</td>
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<td>Notch1</td>
<td>sc-6014-R (Santa Cruz, Santa Cruz, USA)</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Sestan et al⁶</td>
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<td>cleaved Notch1</td>
<td>2421 (Cell Signaling, Beverly, MA, USA)</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Tanaka et al⁷</td>
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<td>Notch2</td>
<td>sc-5545 (Santa Cruz, Santa Cruz, USA)</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Okuyama et al⁸</td>
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<td>Jagged1</td>
<td>sc-6011 (Santa Cruz, Santa Cruz, USA)</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Sestan et al⁶</td>
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<td>Jagged2</td>
<td>sc-8158 (Santa Cruz, Santa Cruz, USA)</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Rivolta et al⁹</td>
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<td>VEGF</td>
<td>sc-507 (Santa Cruz, Santa Cruz, USA)</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
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<td>α/γ-MA (HHF35)</td>
<td>M0635 (DAKO, Glostrup, Denmark)</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Tsukada et al¹¹</td>
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<td>5’- ccagatctttctccatgtcgt-3’</td>
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<td>5’- cgtagtgagtcatactgg-3’</td>
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<td>5’- gtcagagagcaacatcacca-3’</td>
<td>5’- catctgctgtgcttaggaa-3’</td>
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<td>vegf120</td>
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<td>5’- ggctgtgacatattttctgg-3’</td>
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<td>5’- cgagtcgtgtttttgaggaac-3’</td>
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<td>vegf188</td>
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<td>5’- cgagtcgtgtttttggaggaac-3’</td>
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