Influence of blood flow on shear stress responsive genes in the development of cardiac malformations : The involvement of the endothelin-1 pathway

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Chapter 7

General Discussion

7.1 Influence of Blood Flow on Gene Expression

The aim of this thesis was to determine the effect of changes in shear stress on alterations in gene expression, and the role of this on the subsequent development of cardiovascular malformations.

7.1.1 Gene Expression and Shear Stress

In vitro it had already been demonstrated that genes respond to changes in shear stress[1-3]. *In vivo*, however, it was not clear whether shear stress-related gene expression was important in cardiovascular development. The changes in blood flow, predominantly in the inner curvature of the embryonic heart, and the concomitant cardiovascular malformations caused by venous clip[4,5] strongly suggested that genes that are important in cardiovascular development would respond to alterations in shear. At first, it was not known to which shear stress levels the chicken endocardial cells are subjected. We have previously demonstrated[6] that in the outflow tract (OFT) of Hamburger and Hamilton (HH)[7] stage 15 chicken embryos the maximum wall shear stress is 50 dyne/cm^2, or 5 Pa. This is comparable with adult human shear stress levels in arteries, which can be up to 7 Pa[8]. In addition, due to the low Reynolds number (Re), which indicates the ratio between forces of inertia and forces of viscosity, embryonic blood (Re<1) will have its maximum velocity shifted to the inner curvature of a vessel. This was also shown in the inner curvature of the cardiac OFT[6].

The gene expression patterns of endothelin-1 (*ET-1*), lung Krüppel-like factor (*KLF2*) and endothelial nitric oxide synthase (*NOS-3*) during normal chicken cardiovascular development are very specific, and can be linked to the patterns of shear stress (Chapter 2). The *KLF2* expression pattern from the HH18 heart in Chapter 2 is comparable with the shear distribution pattern demonstrated in the HH14 heart in Chapter 3, confirming that *KLF2* can be used as a high shear stress marker in the chicken embryo. In Chapter 6 we show that this *KLF2* expression is invertedly correlated with the presence of primary cilia, indicating that these cilia are present in low shear areas.

In early development our genes of interest displayed somewhat similar patterns to each other, showing overlap in the atrioventricular canal (AVC) and OFT (Chapter 2). Despite the suggested non-shear-dependent expression of *ET-1* and *NOS-3* in these regions and stages, we demonstrated that an alteration in blood flow caused changes in the expression of these genes (Chapter 3). After venous clip, *ET-1* was decreased in the heart, whereas *NOS-3* was increased like *KLF2*, which confirmed that not only flow patterns were altered, but also shear stress. However, another factor that may be changed by altered hemodynamics is

cyclic stretch, caused by pressure pulsations. Since this mechanical force has no effect on *KLF2* and *ET-1* gene expression[9,10] (Hierck, unpublished data 2005), it can be neglected. Our data suggest that the constitutive expression of the genes in normal early development is more important than shear-regulated expression. When shear stress is suddenly altered, however, the shear-dependent regulation overrules.

In vitro studies have demonstrated that high steady laminar shear stress increases both *KLF2* and *NOS-3*[3,11,12], and that it down-regulates *ET-1*[13,14]. The similar alterations in expression levels after venous clip demonstrate that in the heart shear stress is locally increased. The fact that shear stress may change differently in specific regions can be demonstrated by the temporarily decreased flow in the dorsal aorta after venous clip[15]. The down-regulated *KLF2* and *NOS-3*, and increased *ET-1* expression confirm that shear stress is decreased in this vessel (Chapter 3). Recently, Dekker *et al*.[10] have shown that the flow-regulated expression of *NOS-3* and *ET-1* is highly dependent on *KLF2* in human umbilical vein endothelial cells. Our results from the heart and the dorsal aorta from Chapter 3 show that this may also be the case *in vivo*, since *KLF2* and *NOS-3* react similarly to an increase or decrease in shear, and *ET-1* responds opposite to *KLF2*. However, at one region in the heart the changes in *KLF2* and *ET-1* were not complementary, implying that *ET-1* may also be influenced independently of *KLF2* and directly by shear stress. *KLF2* was decreased in the downstream slope of the AVC, indicating a decrease in shear. But *ET-1* was also down-regulated. Because the Reynolds number of chicken embryonic blood is approximately 0.5, forces of inertia cannot be completely ignored[6]. Since 0.5 is smaller than the value (2100) that determines whether flow is laminar (Re<2100) or turbulent (Re>2100), chicken embryonic blood flow is laminar, but due to inertia disturbances may appear. In combination with the widening geometry of the heart downstream from the narrow AVC, and the pumping function of the heart, laminar vortices or oscillations may occur, resulting in a shear stress gradient with lower mean shear and a concomitant decrease in *KLF2* expression. *ET-1* decreases with both steady and oscillating laminar flow[11], indicating that flow is probably oscillatory or vortical in this particular region after clip. Measurements with micro-Particle Image Velocimetry (μPIV) should provide the precise flow pattern.

7.1.2 Shear Sensing

It is clear that alterations by venous clip cause changes in shear stress and shear-related gene expression, but the question remained how shear stress is sensed by the endothelial cells. Several shear stress sensors were postulated previously, such as cell surface ion channels, cell-cell contacts, cell-matrix molecules, and membrane structures (reviewed by Traub and

Berk[16] and Resnick *et al*.[17]). These potential shear stress sensors are all directly or indirectly linked to the cytoskeleton (Reviewed by Helmke and Davies[18]). Primary cilia have also been described to act as shear sensors on adult kidney epithelial cells[19], and in the early embryonic epithelial cells of Hensen's node[20,21]. In Chapter 6, we have demonstrated that primary cilia are also present on chicken endothelial and endocardial cells. Because these cilia are connected to the cytoskeleton[22], they are considered to be shear sensors in endothelium and endocardium with the cytoskeleton as central transducer (Chapter 6). Cilia are present in low shear areas, such as the atria and ventricles. In high shear regions cilia are disassembled[23]. In the latter areas the endothelial cells are aligned in the direction of the flow with a different composition of the cytoskeleton compared with low flow-exposed endocardial cells[8,24]. Therefore, in low shear areas cilia are needed to sense changes in shear stress and to transmit these changes to the cytoskeleton. In the ventricles, the presence of primary cilia was invertedly related with the expression of *KLF2*. Cilia were present in the deeper parts of the trabeculations, whereas *KLF2* was expressed at the tips of the trabeculations, where shear stress is higher (Chapters 2 and 6). In the atrial septum, however, *KLF2* expression overlapped with the presence of cilia. We suggested that this was because of the micro flow patterns due to the fenestrations in the septum. The presence of primary cilia overlaps with *ET-1* expression in the top of the atrium cranial to the entry of the sinus venosus, and in the most proximal and most downstream part of the AVC (Chapters 2 and 6). This confirms that *ET-1* is expressed in low shear areas. However, cilia are present in the complete atrium and in the cryptes of the ventricular trabeculations, where *ET-1* is sporadically expressed. The level of shear stress may be of such a magnitude that cilia are present, but that *ET-1* is not expressed. A threshold in shear stress levels may exist for genes to respond to. Furthermore, in the sinus venosus *ET-1* is expressed (Chapter 2), whereas cilia are only observed occasionally on these endothelial cells. This difference in the number of cilia on endothelial or endocardial cells was ascribed to the heterogeneity of these cells[25,26] (Hierck, unpublished data 2005; Chapter 6).

In the normal situation no primary cilia are present in the inner curvature, because shear stress is always high in this area, and *KLF2* is expressed (Chapters 2 and 6). After venous clip, shear stress is even increased, implying that cilia are not the shear sensors in this region after this intervention. We propose that the cytoskeleton functions as a shear sensor/transducer in these high shear areas (Chapter 6). In the pharyngeal arch artery (PAA) system, primary cilia were only detected in the proximal part of the 6th pair of PAAs. The restricted presence of cilia in the PAA system can be explained by the regional differences in endothelial cell response to shear stress, but also by the fact that most of the blood from the

embryonic circulation goes to the brain (Chapter 6). Also after venous clip more blood flows to the head (Hogers, unpublished results), suggesting that an increase in the number of cilia in the 4th arch arteries may be present. However, no alterations in gene expression were detected in the PAA system after clip (Chapter 3). In the region where the two cardinal veins enter the sinus venosus, *KLF2* expression is absent, but *ET-1* expression was shifted, possibly due to a local decrease in shear in the right part and an increase in shear in the left part of this area. Therefore, in spite of the preference of primary cilia to be present on endocardial cells instead of endothelial cells, the number of cilia may be increased in the right part of this region, which would be additional proof for altered shear in this area.

7.2 Mechanism of the ET-1 Pathway in the Venous Clip Model

In Chapters 4 and 5, infusion experiments are described, where ET-1 and ET-1-receptor antagonists were infused into the extra-embryonic circulation. These experiments were performed to investigate whether a disturbance in the ET-1 pathway results in similar abnormalities as in venous clip. Infusion experiments are, however, different from the venous clip model, since the infusion experiments cause a bolus of ET-1 or its receptor antagonists, whereas in the venous clip model gene expression is affected for a longer period of time, and more genes will be disturbed. However, we demonstrated that the ET-1 pathway is involved in the venous clip model, since disturbances in this cascade resulted in similar, but less severe, functional and morphological abnormalities (Chapter 4).

It has been described before that the proportion endothelin-A (ETA) and endothelin-B (ETB) receptors may differ between different vascular beds in the adult[27]. In the embryo this is also the case. At HH18, *ETA* receptor mRNA is absent in the vitelline vessel wall, whereas that of the *ETB* receptor is expressed in abundance in both endothelium and media of the vessel wall (Chapter 5). In the embryonic chicken heart both *ETA* and *ETB* receptor mRNAs are strongly present (Chapter 4). This implies that the ET-1 pathway in the peripheral vitelline circulation is mechanistically different from the intra-embryonic circulation (Chapter 5).

Since cardiac malformations arise after venous clip, a possible mechanism for ET-1 in the venous clipped heart will be described (Fig. 7.1). This description is in part specific for a region where cushions are present, because *ET-1* is predominantly decreased in the inner curvature along the AV and OFT cushions after clip (Chapter 3). In Figure 7.1 a cilium is shown. This does not represent the cushion areas, but the low shear regions where these primary cilia are present and sense changes in flow that are transmitted to the cytoskeleton (Chapter 6). The increase in shear stress in cushion areas leads to an increase in *KLF2*

expression[3] (Chapter 3) (Fig. 7.1a). Through *KLF2*, or directly by shear stress, *NOS-3* expression is upregulated and *ET-1* is decreased in the endocardial cells[10,13,28] (Fig. 7.1b). This decrease in *ET-1* mRNA also leads to a down-regulation of ET-1 protein release[9,13,29] (Fig. 7.1c1,2). Normally, ET-1 is predominantly secreted at the abluminal side[30,31] toward the cardiac jelly and myocardium (Fig. 7.1c1).

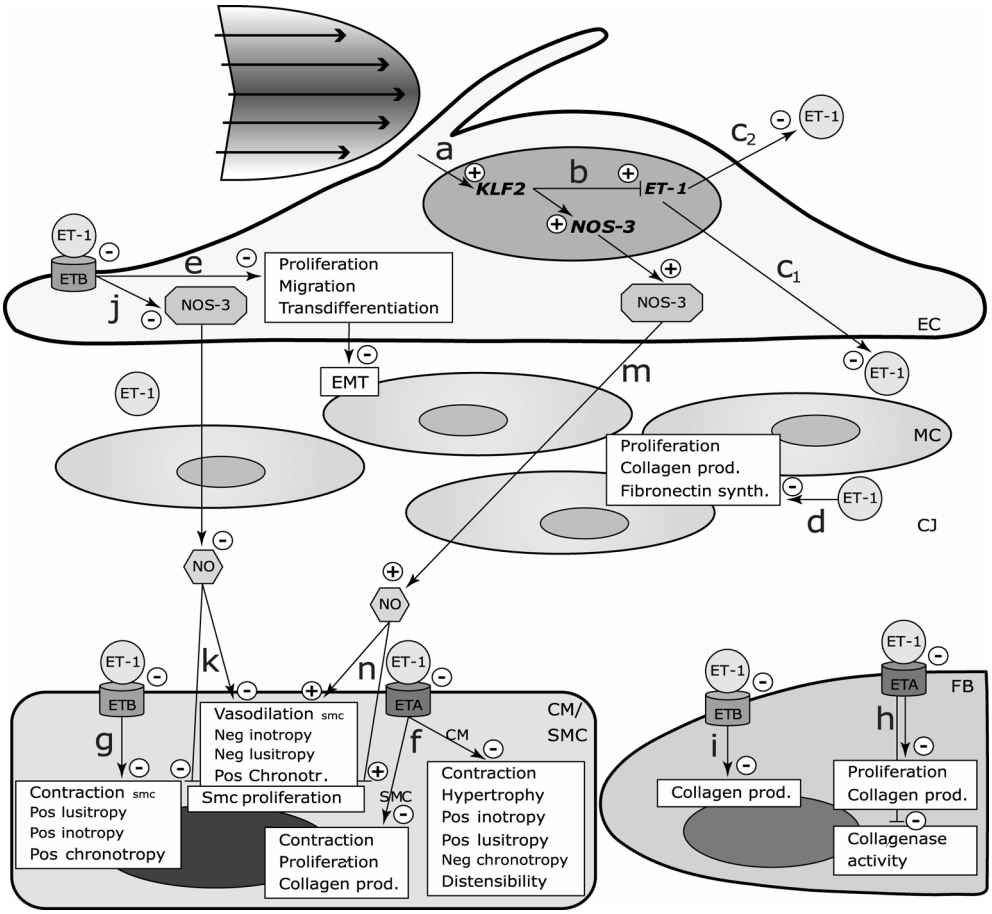

Figure 7.1. Scheme demonstrating the effects of venous clip on shear stress (a), gene expression (b), and cardiovascular processes, predominantly by ET-1 (c-k), and by NOS-3 (m,n). +, processes are stimulated by venous clip; -, processes are inhibited; CJ, cardiac jelly; CM, cardiomyocyte; EC, endothelial/endocardial cell; EMT, epithelial to mesenchymal transformation; FB, fibroblast; SMC, smooth muscle cell.

7.2.1 ET-1 in Cushion Development

It has been reported that ET-1 stimulates the proliferation of mesenchymal cells[32], probably also in the cardiac jelly, where it, in addition, may influence the extracellular matrix by regulating the synthesis of fibronectin[33,34] and collagen[35,36] (Fig. 7.1d). However, this may only occur in the early stages, since *ET-1* mRNA was only present in the endocardial cushions of up to stage HH24, where it still overlapped with *KLF2* expression (Chapter 2). This possible role in cushion development of ET-1 may explain the overlap with *KLF2* and the concomitant apparent non-shear dependent expression.

ET-1 is also secreted luminally (Fig. 7.1c2) and can flow through the complete cardiovascular system, where it is quickly cleared by the ETB receptor[37-39]. This was described for the ETB receptor in the lungs and kidneys of adult guinea pigs and rats. Since these organs are not well developed yet at HH18, ET-1 may bind to the ETB receptor in the complete cardiovascular system in early embryonic development. Because ET-1 can stimulate proliferation and migration through the ETB receptor, it can contribute to neovascularisation[40,41] (Fig. 7.1e). In cushion tissue, the processes of proliferation and migration participate in the formation of the valves. Therefore, and because valve formation is impaired in *Edn*$^{-/-}$ mice, it may be involved in epithelial to mesenchymal transformation (EMT)[42,43], and in transdifferentiation, which was shown for endothelial cells of the dorsal aorta[43] (Fig. 7.1e). However, ETB receptor mRNA, and most likely ETB receptor protein, are not present along cushion tissue from approximately HH22 onward (Chapter 4), implying that ET-1 will not have an influence on the mentioned processes after HH22. Since after venous clip ET-1 is down-regulated, proliferation, migration, EMT, and extracellular matrix synthesis will diminish (Fig. 7.1d,e), resulting in the observed impaired development of the endocardial cushions[44], which was also shown in ET-1$^{-/-}$ mice[42]. In the preseptation stages, hypoplastic AV cushions were the most common malformation in clipped embryos. Less mesenchymal cells were present, which were accumulated directly under the endocardial lining of the AV-cusions[44]. This suggested that proliferation was impaired as well as migration, in which we postulate that the ET-1 pathway played a role. The fact that these malformations were not observed in periseptation stages was ascribed to the high embryolethality between HH22 and HH24. Embryos with severe malformations of the AV cushions were not able to survive, and were selected out at HH24[44]. In the infusion experiments these embryos may have been missed, since the readouts were at HH24 and HH35. Another explanation has been mentioned above; these were bolus-infusions leading to a disturbance of only one pathway. After venous clip, more genes are affected and for a longer period of time.

Another role for ET-1 in cushion development is in the OFT septation. ET-1 is strongly expressed from HH24 to H27 in normal OFT-mesenchyme (Chapter 2), and OFT septation defects are described in the venous clip model[44]. This suggests that ET-1 is involved in defects of OFT septation after venous clip, since a decrease in ET-1, if this expression is shear-dependent, will result in hampered development and myocardialisation of the OFT cusion[40,41,43,45]. However, infusion of ET-1 and receptor antagonists did not result in malformations of OFT septation (Chapter 4). The lack of abnormalities in OFT septation in the infusion experiments may again be due to the limited effects of the bolus infusion. Not only valve and OFT-septation defects were observed after venous clip, also ventricular septum defects (VSDs) were present, an abnormality formed among others by impaired cushion development and fusion. The decrease in ET-1 may also explain these malformations.

7.2.2 ET-1 in the Cardiac Wall

Abluminally secreted ET-1 can, besides affecting cushion development, bind to its receptors on cardiac fibroblasts, smooth muscle cells (SMCs), or cardiomyocytes. Through the ETA receptor it induces contraction of cardiomyocytes[46,47] and SMCs through both ETA and ETB receptors[48] (Fig. 7.1f,g). In addition, through the ETA receptor it stimulates proliferation of cardiac fibroblasts[49,50] (Fig. 7.1h) and SMCs[51-53], and hypertrophy of cardiomyocytes[54-57] (Fig. 7.1f). In vascular smooth muscle cells[35] and cardiac fibroblasts[36], ET-1 induces collagen production (Fig. 7.1f,h). In the latter cell type, collagenase activity is inhibited through the ETA receptor, and the ETB receptor is also involved in production[36] (Fig. 7.1i).

In cardiomyocytes, ET-1 has through the ETA receptor a positive influence on inotropy, lusitropy, and distensibility[58,59] (Fig. 7.1f). These are the contraction force, the rate of relaxation of myocytes, and the amount of stretch or expansion of the myocardium, respectively. However, the ETB receptor also plays a role in inotropy and lusitropy, since the endocardial ETB receptor was demonstrated to elicit a small negative effect of both (Fig. 7.1j,k), and the myocardial receptor a slight positive effect[60] (Fig. 7.1g). The endocardial ETB receptor is, furthermore, involved in vasodilation of SMCs[61]. On chronotropy, i.e., the contraction rate of the heart, ET-1 has an overall positive influence[62,63] (Chapter 5), however, it can exert a negative chronotropic effect through the ETA receptor[63] (Fig. 7.1f), which in chicken embryos of HH18 was not observed in our experimental setup (Chapter 5). Through the ETB receptor, ET-1 elicits a positive effect (Chapter 5), which is desensitised by repetitive application of ET-1, resulting in an overall negative chronotropy[64]. It is not known whether ETB receptors in the endocardium, the myocardium, or both are involved (Fig 7.1g,j,k). In

addition, the chronotropic effects also differ in species, since sinoatrial node cells from the rabbit display negative chronotropic effects induced by ET-1, whereas these cells from guinea pigs and rats showed positive chronotropic effects[64,65]. Infusion of ET-1 in a vitelline vein of chicken embryos, resulted in an increase in heart rate (Chapter 5). Therefore, we pose that ET-1-induced chronotropic effects in chicken embryos are similar to those in rats and guinea pigs (Fig. 7.1f,g,j,k). Because after venous clip ET-1 is decreased in the heart, all these processes may be impaired (Fig. 7.1f-k).

7.2.3 ET-1 in Cardiac Function and Morphology

The immediate effects Stekelenburg-de Vos *et al*.[15] demonstrated after venous clip could not be due to a decrease in *ET-1* mRNA, since mRNA production takes more time, and may come from the immediate increased or decreased release of functional ET-1 protein. Heart rate was decreased for up to 2-3 hours after clip, which means that chronotropy is diminished. Because of the overall positive chronotropic effect of ET-1[62,63] (Fig. 7.1f,g,j,k), the decrease in chronotropy implies that the immediate ET-1 protein release is down-regulated by venous clip. A down-regulation of ET-1 protein may also explain the decreased mean dorsal aortic blood flow, peak acceleration (a measure for cardiac contraction force), and stroke volume, since contraction and inotropy will be impaired as well (Fig. 7.1f,g,j,k).

Due to the flow and shear stress changes, *ET-1* mRNA is down-regulated for at least 3 hours after venous clip, resulting in a decreased protein release as described above. We postulate that the ventricular wall is less developed, since hypertrophy of cardiomyocytes, proliferation of cardiac fibroblasts, and collagen production by cardiac fibroblasts (Fig. 7.1f,h,i) are hampered by decreased ET-1. A decreased thickness of the compact layer of the ventricular wall was shown after clip[44] and after infusion of ET-1 or its receptor antagonists (Chapter 4), confirming the involvement of the ET-1 pathway in the ventricular wall after venous ligation. Furthermore, the heart will have impaired contractile, inotropic and lusitropic responses by diminished ET-1 (Fig. 7.1f,g,j,k). This can explain the trend in enhanced end-diastolic ventricular stiffnes (E_{ED}), or reduced compliance, and the reduced end-systolic ventricular elastance (E_{ES}), which implies a diminished contractility of ventricular myocardium, at HH21[66]. At HH24, the E_{ES} was still decreased, the E_{ED} was significantly increased (Stekelenburg-de Vos, unpublished results), and peak acceleration was down-regulated[67], demonstrating the attenuated inotropy and lusitropy after clip due to sustained decreases in ET-1. In addition, the reduced compliance, distensibility and lusitropy by diminished ET-1, explains the decreased diastolic passive ventricular filling at HH24[68]. In chapter 4 we have confirmed that ET-1 is involved in this function through

blockade of the ETA receptor. However, infusion of ET-1 itself resulted in similar changes. In Chapter 5 we confirmed in chicken embryos that exogenous ET-1 preferentially binds to the ETB receptors in the endothelium and endocardium, instead of those in the myocardium[69]. This results in a greater effect through the endocardial ETB receptor, leading to enhanced negative lusitropy and a diminished effect on distensibility (Fig. 7.1f,j,k). We explained the increased active ventricular filling in the infusion experiments by the increase in receptor mRNA through the positive feedback mechanisms after blocking the receptors, thereby increasing the inotropic effect of the atria. However, after venous clip we encounter a down-regulation in *ET-1* mRNA and not a blockade of receptors. Therefore, it is not known whether a decrease in *ET-1* mRNA will also lead to an up-regulation of its receptors. Furthermore, up-regulating the receptors will not be effective, since the ligand, ET-1, is decreased. In addition, the down-regulation in *ET-1* mRNA was encountered at the inner curvature downstream from the AV canal. In the atria, where the inotropy is expected to be enhanced, *ET-1* was not altered 3 hours after clip (Chapter 3). Therefore, the increased active ventricular filling after venous clip cannot be explained by the early changes of *ET-1*, or its receptors. Due to the developmental abnormalities after the initial effect of altered gene expression, blood flow and shear stress may be changed (Chapter 4), and *ET-1* expression may have been increased in the atria at HH24.

In contrast to the unaltered dorsal aortic blood flow and stroke volume at HH24 after venous clip, dorsal aortic flow velocity, peak systolic and mean volumetric blood flow, and stroke volume were all increased at HH34[67]. Because of the morphological malformations, partly induced by the ET-1 down-regulation, a decrease, or no alteration of these parameters is expected. These increases in hemodynamic parameters also suggest an increase in ET-1 production and circulating ET-1 from HH24 onward by means of compensation, which is also the case in adult humans with heart failure[70,71].

Proper remodelling of the inner curvature is important for normal cardiac looping[72,73], a process that is impaired in the venous clip model. The inner curvature of the heart was not tight enough, thereby preventing the alignment of the individual cardiac septa, resulting in a VSD in 66% of the venous clip embryos[44]. ET-1 was predominantly decreased in the inner curvature, and it is involved in developmental processes (Fig. 7.1). Therefore, ET-1 may also play a role in cardiac looping, and in the disturbed looping after venous clip, resulting, together with impaired cushion development (see above), in VSDs.

7.2.4 Other Mechanisms Involved in Venous Clip

Not ony the ET-1 pathway is disturbed after venous clip, but *NOS-3* is also altered. In the heart *NOS-3* is up-regulated in endocardium by the increase in shear stress (Chapter 3; Fig. 7.1a,b), which results in an enhanced synthesis[11] and possible release of NO (Fig. 7.1m). This stimulates the inhibition of SMC proliferation and stimulates vasodilation, negative inotropy, negative lusitropy and positive chronotropy[58,60,74-76] (Fig. 7.1n). These actions of NO counterbalance the effect of impaired ET-1 through the endothelial ETB receptor[60,61,64] (Fig. 7.1j,k), but enhance the effect of disturbed ET-1 through the myocardial ETA receptor[58,59], which, for inotropy and lusitropy, prevailed over ETB-mediated counter actions (Fig. 7.1f). Inhibition of NOS from day 12 to day 18 chicken embryos resulted in increased biventricular wall area and an increase in the left ventricular wall thickness[77]. This suggests that up-regulated *NOS-3* may be involved in the decreased ventricular wall thickness observed after venous clip (Chapter 4). Furthermore, *Nos-3*$^{-/-}$ mice are hypertensive and display bicuspid aortic valves, heart failure, and ASDs and VSDs[78-81], demonstrating the involvement of *NOS-3* in cardiac development. However, transgenic mice overexpressing *Nos-3* are hypotensive and show a reduced vascular sensitivity to NO[82], which implies that the local overexpression of *NOS-3* after venous clip induces less sensitivity to NO. This suggests that after venous clip mainly the effects of reduced ET-1 through the ETA receptors need to be taken into account, and that up-regulated NOS-3 is not involved in the decreased ventricular wall thickness after venous clip.

KLF2 was also demonstrated to be increased after venous ligation (Chapter 3). Absence of *KLF2* leads to abnormal thinning of the tunica media and concomitant instability of the vessel wall[83]. Whether an increase in *KLF2*, besides its effects through *ET-1* and *NOS-3*, has itself an effect on cardiovascular development is not known.

Other genes will also be involved in the venous clip model. Genes such as platelet derived growth factor-A and -B (*PDGF-A, PDGF-B*), vascular endothelial growth factor receptor 2 (*VEGFR2*/*Flk1*), and most importantly transforming growth factor-β (*TGF-β*) are all involved in cardiovascular development[84-88] and are shear stress responsive[1,2,89,90]. Expression of *TGF-β* was decreased in the OFT and AV cushions, and expression of the TGF-β type III receptor was completely absent at HH24 after venous clip (Hogers, unpublished results). In addition, TGF-β has been demonstrated to be involved in EMT[91], and in other cardiovascular developmental processes that are impaired in venous clip[88]. This puts TGF-β in a position of an additional important factor involved in the development of cardiovascular malformations after venous ligation.

7.3 Future Research

It is clear that ET-1 plays a major role in cardiac dysfunction and morphological malformations after venous clip. Therefore, the possible role for ET-1 in EMT needs to be investigated, including the role of TGF-β in this impaired process after venous clip. In addition, processes resulting in the decreased ventricular wall thickness[44] (Chapter 4), such as hypertrophy and extracellular matrix production or deposition need to be examined. Investigation of whether the morphological impairments lead to the functional disturbances is required as well. Furthermore, the possible role of ET-1 in outflow tract septation has to be analysed.

Since alterations in gene expression are an indirect way to conclude that shear stress is in- or decreased after clip, a direct method of shear stress calculation in the heart and vessels, by means of μPIV measurements, is preferred. In Chapter 5 we have demonstrated that this technique is very sensitive. Therefore, it will be very effective for mapping the shear stress distribution in the cardiovascular system of normal and experimental embryos at increasing stages.

The role of the cytoskeleton and primary cilia in shear sensing and gene expression during embryonic development and maldevelopment also needs attention. Furthermore, it is important to investigate the function of shear sensing and gene expression in atherosclerosis, since atherosclerotic plaques develop at low and unsteady shear areas. Cilia, the cytoskeleton, and shear-related alterations in gene expression may play a role.

References

1. Malek AM, Izumo S. Control of endothelial cell gene expression by flow. *J Biomech*. 1995;28:1515-1528.
2. McCormick SM, Eskin SG, McIntire LV, Teng CL, Lu CM, Russell CG, Chittur KK. DNA microarray reveals changes in gene expression of shear stressed human umbilical vein endothelial cells. *Proc Natl Acad Sci U S A*. 2001;98:8955-8960.
3. Dekker RJ, Van Soest S, Fontijn RD, Salamanca S, de Groot PG, VanBavel E, Pannekoek H, Horrevoets AJG. Prolonged fluid shear stress induces a distinct set of endothelial cell genes, most specifically lung Krüppel-like factor (*KLF2*). *Blood*. 2002;100:1689-1698.
4. Hogers B, DeRuiter MC, Gittenberger-de Groot AC, Poelmann RE. Unilateral vitelline vein ligation alters intracardiac blood flow patterns and morphogenesis in the chick embryo. *Circ Res*. 1997;80:473-481.
5. Hogers B, DeRuiter MC, Gittenberger-de Groot AC, Poelmann RE. Extraembryonic venous obstructions lead to cardiovascular malformations and can be embryolethal. *Cardiovasc Res*. 1999;41:87-99.
6. Vennemann, P., Kiger, K. T., Lindken, R., Groenendijk, B. C. W., Stekelenburg-de Vos, S., ten Hagen, T. L. M., Ursem, N. T. C., Poelmann, R. E., Westerweel, J., and Hierck, B. P. *In vivo* micro particle image velocimetry

measurements of blood-plasma in the embryonic avian heart. *J Biomech*. 2005. In Press. http://dx.doi.org/10.1016/j.jbiomech.2005.03.015

7. Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. *J Morphol*. 1951;88:49-92.
8. Malek AM, Alper SL, Izumo S. Hemodynamic shear stress and its role in atherosclerosis. *JAMA*. 1999;282:2035-2042.
9. Malek AM, Izumo S. Physiological fluid shear stress causes downregulation of endothelin-1 mRNA in bovine aortic endothelium. *Am J Physiol*. 1992;263 (2Pt1):C389-C396.
10. Dekker RJ, van Thienen JV, Elderkamp YW, Seppen J, de Vries CJM, Biessen EA, van Berkel TJ, Pannekoek H, Horrevoets AJG. Endothelial KLF2 links local arterial shear stress levels to the expression of vascular-tone regulating genes. *Am J Pathol*. 2005;167:609-618.
11. Noris M, Morigi M, Donadelli R, Aiello S, Foppolo M, Todeschini M, Orisio S, Remuzzi G, Remuzzi A. Nitric oxide synthesis by cultured endothelial cells is modulated by flow conditions. *Circ Res*. 1995;76:536-543.
12. Nishida K, Harrison DG, Navas JP, Fisher AA, Dockery SP, Uematsu M, Nerem RM, Alexander RW, Murphy TJ. Molecular cloning and characterization of the constitutive bovine aortic endothelial cell nitric oxide synthase. *J Clin Invest*. 1992;90:2092-2096.
13. Sharefkin JB, Diamond SL, Eskin SG, McIntire LV, Dieffenbach CW. Fluid flow decreases preproendothelin mRNA levels and suppresses endothelin-1 peptide release in cultured human endothelial cells. *J Vasc Surg*. 1991;14:1-9.
14. Masatsugu K, Itoh H, Chun TH, Ogawa Y, Tamura N, Yamashita J, Doi K, Inoue M, Fukunaga Y, Sawada N, Saito T, Korenaga R, Ando J, Nakao K. Physiologic shear stress suppresses endothelin-converting enzyme-1 expression in vascular endothelial cells. *J Cardiovasc Pharmacol*. 1998;31(Suppl 1):S42-S45.
15. Stekelenburg-de Vos S, Ursem NTC, Hop WCJ, Wladimiroff JW, Gittenberger-de Groot AC, Poelmann RE. Acutely altered hemodynamics following venous obstruction in the early chick embryo. *J Exp Biol*. 2003;206:1051-1057.
16. Traub O, Berk BC. Laminar shear stress: mechanisms by which endothelial cells transduce an atheroprotective force. *Arterioscler Thromb Vasc Biol*. 1998;18:677-685.
17. Resnick N, Yahav H, Shay-Salit A, Shushy M, Schubert S, Zilberman LC, Wofovitz E. Fluid shear stress and the vascular endothelium: for better and for worse. *Prog Biophys Mol Biol*. 2003;81:177-199.
18. Helmke BP, Davies PF. The cytoskeleton under external fluid mechanical forces: Hemodynamic forces acting on the endothelium. *Ann Biomed Eng*. 2002;30:284-296.
19. Praetorius HA, Spring KR. Bending the MDCK cell primary cilium increases intracellular calcium. *J Membr Biol*. 2001;184:71-79.
20. McGrath J, Somlo S, Makova S, Tian X, Brueckner M. Two populations of node monocilia initiate left-right asymmetry in the mouse. *Cell*. 2003;114:61-73.
21. Yost HJ. Left-right asymmetry: Nodal cilia make and catch a wave. *Curr Biol*. 2003;13:R808-R809.
22. Gordon RE, Lane BP, Miller F. Identification of Contractile Proteins in Basal Bodies of Ciliated Tracheal Epithelial-Cells. *J Histochem Cytochem*. 1980;28:1189-1197.
23. Iomini C, Tejada K, Mo W, Vaananen H, Piperno G. Primary cilia of human endothelial cells disassemble under laminar shear stress. *J Cell Biol*. 2004;164:811-817.
24. Topper JN, Gimbrone MA, Jr. Blood flow and vascular gene expression: fluid shear stress as a modulator of endothelial phenotype. *Mol Med Today*. 1999;5:40-46.

25. Chi JT, Chang HY, Haraldsen G, Jahnsen FL, Troyanskaya OG, Chang DS, Wang Z, Rockson SG, van de Rijn M, Botstein D, Brown PO. Endothelial cell diversity revealed by global expression profiling. *Proc Natl Acad Sci U S A*. 2003;100:10623-10628.
26. Hong R. The DiGeorge anomaly (CATCH 22, DiGeorge/velocardiofacial syndrome). *Semin Hematol*. 1998;35:282-290.
27. Haynes WG, Webb DJ. Endothelin as a regulator of cardiovascular function in health and disease. *J Hypertens*. 1998;16:1081-1098.
28. Ziegler T, Silacci P, Harrison VJ, Hayoz D. Nitric oxide synthase expression in endothelial cells exposed to mechanical forces. *Hypertension*. 1998;32:351-355.
29. Morawietz H, Talanow R, Szibor M, Rueckschloss U, Schubert A, Bartling B, Darmer D, Holtz J. Regulation of the endothelin system by shear stress in human endothelial cells. *J Physiol*. 2000;525(Pt 3):761-770.
30. Yoshimoto S, Ishizaki Y, Mori A, Sasaki T, Takakura K, Murota SI. The Role of Cerebral Microvessel Endothelium in Regulation of Cerebral Blood-Flow Through Production of Endothelin-1. *J Cardiovasc Pharmacol*. 1991;17:S260-S263.
31. Wagner OF, Christ G, Wojta J, Vierhapper H, Parzer S, Nowotny PJ, Schneider B, Waldhausl W, Binder BR. Polar Secretion of Endothelin-1 by Cultured Endothelial-Cells. *J Biol Chem*. 1992;267:16066-16068.
32. Choi KH, Kang SW, Lee SW, Lee HY, Han DS, Kang BS. The effect of lovastatin on proliferation of cultured rat mesangial and aortic smooth muscle cells. *Yonsei Med J*. 1995;36:251-261.
33. Gómez-Garre, D., Ruiz-Ortega, M., Ortego, M., Largo, R., López-Armada, M. J., Plaza, J. J., González, E., and Egido, J. Effects and interactions of endothelin-1 and angiotensin II on matrix protein expression and synthesis and mesangial cell growth. *Hypertension*. 1996;27:885-892.
34. Marini M, Carpi S, Bellini A, Patalano F, Mattoli S. Endothelin-1 induces increased fibronectin expression in human bronchial epithelial cells. *Biochem Biophys Res Commun*. 1996;220:896-899.
35. Rizvi MAD, Katwa L, Spadone D.P., Myers PR. The effects of endothelin-1 on collagen type I and type III synthesis in cultured porcine coronary artery vascular smooth muscle cells. *J Mol Cell Cardiol*. 1996;28:243-252.
36. Guarda E, Katwa LC, Myers PR, Tyagi SC, Weber KT. Effects of endothelins on collagen turnover in cardiac fibroblasts. *Cardiovasc Res*. 1993;27:2130-2134.
37. Sirviö ML, Metsärinne K, Saijonmaa O, Fyhrquist F. Tissue Distribution and Half-Life of I-125 Endothelin in the Rat - Importance of Pulmonary Clearance. *Biochem Biophys Res Commun*. 1990;167:1191-1195.
38. de Nucci G, Thomas R, D'Orleans-Juste P, Antunes E, Walder C, Warner TD, Vane JR. Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. *Proc Natl Acad Sci U S A*. 1988;85:9797-9800.
39. Johnström P, Fryer TD, Richards HK, Harris NG, Barret O, Clark JC, Pickard JD, Davenport AP. Positron emission tomography using 18F-labelled endothelin-1 reveals prevention of binding to cardiac receptors owing to tissue-specific clearance by ET B receptors in vivo. *Br J Pharmacol*. 2005;144:115-122.
40. Morbidelli L, Orlando C, Maggi CA, Ledda F, Ziche M. Proliferation and migration of endothelial cells is promoted by endothelins via activation of ETB receptors. *Am J Physiol*. 1995;269:H686-H695.
41. Dong F, Zhang X, Wold LE, Ren Q, Zhang Z, Ren J. Endothelin-1 enhances oxidative stress, cell proliferation and reduces apoptosis in human umbilical vein endothelial cells: role of ETB receptor, NADPH oxidase and caveolin-1. *Br J Pharmacol*. 2005;145:323-333.
42. Kurihara Y, Kurihara H, Oda H, Maemura K, Nagai R, Ishikawa T, Yazaki Y. Aortic arch malformations and ventricular septal defect in mice deficient in endothelin-1. *J Clin Invest*. 1995;96:293-300.

43. DeRuiter MC, Poelmann RE, VanMunsteren JC, Mironov V, Markwald RR, Gittenberger-de Groot AC. Embryonic endothelial cells transdifferentiate into mesenchymal cells expressing smooth muscle actins in vivo and in vitro. *Circ Res*. 1997;80:444-451.
44. Hogers B, Gittenberger-de-Groot AC, DeRuiter MC, Mentink MMT, Poelmann RE. Cardiac inflow malformations are more lethal and precede cardiac outflow malformations. Chick embryonic venous clip model. Chapter 5: 70-100. In: Hogers B, ed. The role of blood flow in normal and abnormal heart development. Ponsen & Looijen BV, Wageningen, 1998.
45. Brand M, Kempf H, Paul M, Corvol P, Gasc JM. Expression of endothelins in human cardiogenesis. *J Mol Med*. 2002;80:715-723.
46. Kelso EJ, McDermott BJ, Silke B, Spiers JP. EndothelinA receptor subtype mediates endothelin-induced contractility in left ventricular cardiomyocytes isolated from rabbit myocardium. *J Pharmacol Exp Ther*. 2000;294:1047-1052.
47. Shah AM. Decreased myocardial contractility after damage to endocardial endothelium is caused mainly by loss of endothelin production. *Cardiovasc Res*. 1995;30:644-645.
48. Marsault R, Feolde E, Frelin C. Receptor Externalization Determines Sustained Contractile Responses to Endothelin-1 in the Rat Aorta. *Am J Physiol*. 1993;264:C687-C693.
49. Fujisaki H, Ito H, Hirata Y, Tanaka M, Hata M, Lin MH, Adachi S, Akimoto H, Marumo F, Hiroe M. Natriuretic Peptides Inhibit Angiotensin-Ii-Induced Proliferation of Rat Cardiac Fibroblasts by Blocking Endothelin-1 Gene-Expression. *J Clin Invest*. 1995;96:1059-1065.
50. Piacentini L, Gray M, Honbo NY, Chentoufi J, Bergman M, Karliner JS. Endothelin-1 stimulates cardiac fibroblast proliferation through activation of protein kinase C. *J Mol Cell Cardiol*. 2000;32:565-576.
51. Yang Z, Krasnici N, Luscher TF. Endothelin-1 potentiates human smooth muscle cell growth to PDGF: effects of ETA and ETB receptor blockade. *Circulation*. 1999;100:5-8.
52. Hafizi S, Allen SP, Goodwin AT, Chester AH, Yacoub MH. Endothelin-1 stimulates proliferation of human coronary smooth muscle cells via the ETA receptor and is co-mitogenic with growth factors. *Atherosclerosis*. 1999;146:351-359.
53. Zhang YM, Wang KQ, Zhou GM, Zuo J, Ge JB. Endothelin-1 promoted proliferation of vascular smooth muscle cell through pathway of extracellular signal-regulated kinase and cyclin D1. *Acta Pharmacol Sin*. 2003;24:563-568.
54. Ito H, Hiroe M, Hirata Y, Fujisaki H, Adachi S, Akimoto H, Ohta Y, Marumo F. Endothelin ET_A receptor antagonist blocks cardiac hypertrophy provoked by hemodynamic overload. *Circulation*. 1994;89:2198-2203.
55. Shubeita HE, McDonough PM, Harris AN, Knowlton KU, Glembotski CC, Brown JH, Chien KR. Endothelin induction of inositol phospholipid hydrolysis, sarcomere assembly, and cardiac gene expression in ventricular myocytes. A paracrine mechanism for myocardial cell hypertrophy. *J Biol Chem*. 1990;265:20555-20562.
56. Yamazaki T, Komuro I, Kudoh S, Zou Y, Shiojima I, Hiroi Y, Mizuno T, Maemura K, Kurihara H, Aikawa R, Takano H, Yazaki Y. Endothelin-1 is involved in mechanical stress-induced cardiomyocyte hypertrophy. *J Biol Chem*. 1996;271:3221-3228.
57. Ito H, Hirata Y, Hiroe M, Tsujino M, Adachi S, Takamoto T, Nitta M, Taniguchi K, Marumo F. Endothelin-1 induces hypertrophy with enhanced expression of muscle-specific genes in cultured neonatal rat cardiomyocytes. *Circ Res*. 1991;69:209-215.
58. Leite-Moreira AF, Bras-Silva C, Pedrosa CA, Rocha-Sousa AA. ET-1 increases distensibility of acutely loaded myocardium: a novel ETA and Na+/H+ exchanger-mediated effect. *Am J Physiol Heart Circ Physiol*. 2003;284:H1332-H1339.
59. Nagasaka T, Izumi M, Hori M, Ozaki H, Karaki H. Positive inotropic effect of endothelin-1 in the neonatal mouse right ventricle. *Eur J Pharmacol*. 2003;472:197-204.

60. Leite-Moreira AF, Bras-Silva C. Inotropic effects of ETB receptor stimulation and their modulation by endocardial endothelium, NO, and prostaglandins. *Am J Physiol Heart Circ Physiol*. 2004;287:H1194-H1199.
61. Verhaar MC, Strachan FE, Newby DE, Cruden NL, Koomans HA, Rabelink TJ, Webb DJ. Endothelin-A receptor antagonist-mediated vasodilatation is attenuated by inhibition of nitric oxide synthesis and by endothelin-B receptor blockade. *Circulation*. 1998;97:752-756.
62. Ishikawa T, Yanagisawa M, Kimura S, Goto K, Masaki T. Positive chronotropic effects of endothelin, a novel endothelium-derived vasoconstrictor peptide. *Pflugers Arch*. 1988;413:108-110.
63. Ono K, Eto K, Sakamoto A, Masaki T, Shibata K, Sada T, Hashimoto K, Tsujimoto G. Negative chronotropic effect of endothelin 1 mediated through ETA receptors in guinea pig atria. *Circ Res*. 1995;76:284-292.
64. Ono K, Sakamoto A, Masaki T, Satake M. Desensitization of ET(A) endothelin receptor-mediated negative chronotropic response in right atria--species difference and intracellular mechanisms. *Br J Pharmacol*. 1998;125:787-797.
65. Ono K, Masumiya H, Sakamoto A, Christe G, Shijuku T, Tanaka H, Shigenobu K, Ozaki Y. Electrophysiological analysis of the negative chronotropic effect of endothelin-1 in rabbit sinoatrial node cells. *J Physiol*. 2001;537:467-488.
66. Stekelenburg-de Vos S, Steendijk P, Ursem NT, Wladimiroff JW, Delfos R, Poelmann RE. Systolic and Diastolic Ventricular Function Assessed by Pressure-Volume Loops in the Stage 21 Venous Clipped Chick Embryo. *Pediatr Res*. 2005;57:16-21.
67. Broekhuizen MLA, Hogers B, DeRuiter MC, Poelmann RE, Gittenberger-de Groot AC, Wladimiroff JW. Altered hemodynamics in chick embryos after extraembryonic venous obstruction. *Ultrasound Obstet Gynecol*. 1999;13:437-445.
68. Ursem NTC, Stekelenburg-de Vos S, Wladimiroff JW, Poelmann RE, Gittenberger-de Groot AC, Hu N, Clark EB. Ventricular diastolic filling characteristics in stage-24 chick embryos after extra-embryonic venous obstruction. *J Exp Biol*. 2004;207:1487-1490.
69. D'Orleans-Juste P, Labonte J, Bkaily G, Choufani S, Plante M, Honore JC. Function of the endothelin(B) receptor in cardiovascular physiology and pathophysiology. *Pharmacology & Therapeutics*. 2002;95:221-238.
70. Miyauchi T, Masaki T. Pathophysiology of endothelin in the cardiovascular system. *Annu Rev Physiol*. 1999;61:391-415.
71. Giannessi D, Del Ry S, Vitale RL. The role of endothelins and their receptors in heart failure. *Pharmacol Res*. 2001;43:111-126.
72. Gittenberger-de Groot AC, Bartelings MM, Poelmann RE. Overview: Cardiac morphogenesis. Chapter 15: 157-168. In: Clark EB, Markwald RR, Takao A, eds. Developmental mechanisms of heart disease. Proceedings of the fourth international symposium on etiology and morphogenesis of congenital heart disease. Futura Press Mount Kisco, New York, 1995.
73. Gittenberger-de Groot AC, Bartelings MM, DeRuiter MC, Poelmann RE. Basics of cardiac development for the understanding of congenital heart malformations. *Pediatr Res*. 2005;57:169-176.
74. Moroi M, Zhang L, Yasuda T, Virmani R, Gold HK, Fishman MC. Interaction of genetic deficiency of endothelial nitric oxide, gender, and pregnancy in vascular response to injury in mice. *J Clin Invest*. 1998;101:1225-1232.
75. Rudic RD, Shesely EG, Maeda N, Smithies O, Segal SS, Sessa WC. Direct evidence for the importance of endothelium-derived nitric oxide in vascular remodeling. *J Clin Invest*. 1998;101:731-736.
76. Gödecke A, Heinicke T, Kamkin A, Kiseleva I, Strasser RH, Decking UK, Stumpe T, Isenberg G, Schrader J. Inotropic response to beta-adrenergic receptor stimulation and anti-adrenergic effect of ACh in endothelial NO synthase-deficient mouse hearts. *J Physiol*. 2001;532:195-204.

77. Villamor E, Kessels CG, van Suylen RJ, De Mey JG, Blanco CE. Cardiopulmonary Effects of Chronic Administration of the NO Synthase Inhibitor L-NAME in the Chick Embryo. *Biol Neonate*. 2005;88:156-163.
78. Huang PL, Huang Z, Mashimo H, Bloch KD, Moskowitz MA, Bevan JA, Fishman MC. Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature*. 1995;377:239-242.
79. Shesely EG, Maeda N, Kim HS, Desai KM, Krege JH, Laubach VE, Sherman PA, Sessa WC, Smithies O. Elevated blood pressures in mice lacking endothelial nitric oxide synthase. *Proc Natl Acad Sci U S A*. 1996;93:13176-13181.
80. Lee TC, Zhao YD, Courtman DW, Stewart DJ. Abnormal aortic valve development in mice lacking endothelial nitric oxide synthase. *Circulation*. 2000;101:2345-2348.
81. Feng Q, Song W, Lu X, Hamilton JA, Lei M, Peng T, Yee SP. Development of heart failure and congenital septal defects in mice lacking endothelial nitric oxide synthase. *Circulation*. 2002;106:873-879.
82. Ohashi Y, Kawashima S, Hirata K, Yamashita T, Ishida T, Inoue N, Sakoda T, Kurihara H, Yazaki Y, Yokoyama M. Hypotension and reduced nitric oxide-elicited vasorelaxation in transgenic mice overexpressing endothelial nitric oxide synthase. *J Clin Invest*. 1998;102:2061-2071.
83. Kuo CT, Veselits ML, Barton KP, Lu MM, Clendenin C, Leiden JM. The LKLF transcription factor is required for normal tunica media formation and blood vessel stabilization during murine embryogenesis. *Genes Dev*. 1997;11:2996-3006.
84. van den Akker NMS, Lie-Venema H, Maas S, Eralp I, DeRuiter MC, Poelmann RE, Groot ACG. Platelet-derived growth factors in the developing avian heart and maturating coronary vasculature. *Dev Dyn*. 2005;233:1579-1588.
85. Stalmans I, Lambrechts D, Desmet F, Jansen S, Wang J, Maity S, Kneer P, von der Ohe M, Swillen A, Maes C, Gewillig M, Molin DGM, Hellings P, Boetel T, Haardt M, Compernolle V, Dewerchin M, Vlietinck R, Emanuel B, Gittenberger-de Groot AC, Esguerra CV, Scambler P, Morrow B, Driscoll DA, Moons L, Carmeliet G, Behn-Krappa A, DeVviendt K, Collen D, Conway SJ, Carmeliet P. VEGF: a modifier of the del22q11 (DiGeorge) syndrome? *Nat Med*. 2003;9:173-182.
86. Brandenburg H, Steegers EA, Groot AC. Potential involvement of vascular endothelial growth factor in pathophysiology of Turner syndrome. *Med Hypotheses*. 2005;65:300-304.
87. Molin DG, Poelmann RE, DeRuiter MC, Azhar M, Doetschman T, Gittenberger-de Groot AC. Transforming growth factor beta-SMAD2 signaling regulates aortic arch innervation and development. *Circ Res*. 2004;95:1109-1117.
88. Bartram U, Molin DGM, Wisse LJ, Mohamad A, Sanford LP, Doetschman T, Speer CP, Poelmann RE, Gittenberger-de Groot AC. Double-outlet right ventricle and overriding tricuspid valve reflect disturbances of looping, myocardialization, endocardial cushion differentiation, and apoptosis in TGFß2-knockout mice. *Circulation*. 2001;103:2745-2752.
89. Jin ZG, Ueba H, Tanimoto T, Lungu AO, Frame MD, Berk BC. Ligand-independent activation of vascular endothelial growth factor receptor 2 by fluid shear stress regulates activation of endothelial nitric oxide synthase. *Circ Res*. 2003;93:354-363.
90. Braddock M, Schwachtgen JL, Houston P, Dickson MC, Lee MJ, Campbell CJ. Fluid Shear Stress Modulation of Gene Expression in Endothelial Cells. *News Physiol Sci*. 1998;13:241-246.
91. Brown CB, Boyer AS, Runyan RB, Barnett JV. Antibodies to the Type II TGFbeta receptor block cell activation and migration during atrioventricular cushion transformation in the heart. *Dev Biol*. 1996;174:248-257.