Chapter 2

Development-related Changes in the Expression of Shear Stress Responsive Genes \textit{KLF2, ET-1 and NOS-3} in the Developing Cardiovascular System of Chicken Embryos

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Abstract

Blood flow patterns play an important role in cardiovascular development, as changes can cause congenital heart malformations. Shear stress is positively correlated to blood flow. Therefore, it is likely that shear stress is also involved in cardiac development. In this study, we investigated the expression patterns of ET-1, NOS-3, and KLF2 mRNA in a series of developmental stages of the chicken embryo. These genes are reported to be shear responsive. It has been demonstrated that KLF2 is confined to areas of high shear stress in the adult human aorta. From in vitro studies it is known that ET-1 is down-regulated by shear stress, whereas NOS-3 is up-regulated. Therefore, we expect ET-1 to be low or absent and NOS-3 to be high at sites where KLF2 expression is high. Our study shows that in the early stages expression patterns are mostly not shear stress-related, whereas during development this correlation becomes stronger. We demonstrate overlapping expression patterns of KLF2 and NOS-3 in the narrow parts of the cardiovascular system, like the cardiac inflow tract, the atrioventricular canal, outflow tract, and in the early stages in the aortic sac and the pharyngeal arch arteries. In these regions, the expression patterns of KLF2 and NOS-3 exclude that of ET-1. Our results suggest that in the embryonic cardiovascular system KLF2 is expressed in regions of highest shear stress, and that ET-1 and NOS-3 expression, at least in the later stages, is related to shear stress.

Introduction

Blood flow generates various hemodynamic forces that act on the vessel wall, such as hydrostatic pressure, cyclic strain, stretch and fluid shear stress, modulating the endothelial structure and function (reviewed by Gimbrone et al.). It is known that atherosclerotic plaques develop in low and unsteady shear stress areas. Thus, in adults, shear stress plays among other factors a role in atherogenesis. During embryonic development, changes in blood flow are very important. Ligation of a vitelline vein (venous clip) in a chicken embryo results in a change in blood flow patterns through the heart and causes cardiac and pharyngeal arch artery malformations. As shear stress is positively related to blood flow, it would be conceivable that altered shear stress is involved in the development of these anomalies.

Endothelin-1 (ET-1) is a growth hormone involved in vasoconstriction. Nitric oxide (NO) on the other hand is involved in many functions, including vasodilation. ET-1 and endothelial nitric oxide synthase (NOS-3 or eNOS) genes contain shear stress response elements (SSRE)
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in their promoter, and can respond to changes in shear stress. Krüppel-like factor 2 (KLF2 or LKLF), a member of the SP/XKLF family of transcription factors, is also shown to be shear stress responsive. Furthermore, knockout mice for Edn-1, endothelin converting enzyme-1 (Ece-1), and the endothelin-A receptor (Eta) show a spectrum of cardiovascular malformations, resembling the ones from the chicken venous clip model. These findings demonstrate the involvement of genes from the endothelin cascade in heart development. Nos-3-deficient mice display bicuspid aortic valves, heart failure, and atrial and ventricular septal defects, which identifies Nos-3 as an important player in cardiac development. KLF2 expression has been reported to be confined to the endothelium in areas of high shear stress of adult human vascular tissue, and it is important for the formation of the media of the vessel wall.

It has also been shown in vitro that KLF2 mRNA was up-regulated by steady laminar shear stress. In contrast, when endothelial cells are exposed to steady laminar shear stress, the ET-1 mRNA level is decreased, as well as the ET-1 protein level. In addition, Malek and Izumo, and Morawietz et al. showed an early transient increase in ET-1 expression between 0.5 and 1 hr after onset of shear stress, and a dose- or magnitude-dependent decrease below static control level afterward. Malek and Izumo showed that this decrease was reversible. After a period of 6 hours of high shear stress, they abrogated the flow and reported a recovery of ET-1 expression. Thus, there appears to be a short transient up-regulation of ET-1 expression under high steady laminar shear stress conditions, which is followed by a decrease in ET-1 expression. During unidirectional pulsatile flow or shear stress, which occurs under physiological conditions, endothelial cells sometimes react in the same way as under steady laminar flow by a decrease in ET-1 expression, compared with low flow controls. However, they can also react with an increase in ET-1 expression compared with static controls. This reaction is a different response than in the steady laminar shear stress studies. The expression of Nos-3 increases in steady stress-stimulated endothelial cells compared with static controls. Unidirectional pulsatile shear stress also induces an increase in Nos-3 expression compared with static controls. Qiu and Tarbell showed an increase in NO production under steady as well as pulsatile flow conditions compared with static controls, which would mean that Nos-3 expression is increased. However, the pulsatile flow conditions show a lower NO production than the steady flow conditions.

In this study, we investigated the expression patterns of ET-1, KLF2 and Nos-3 in the chicken heart between Hamburger and Hamilton stage (HH) 16 and HH30 of development. KLF2 signals appeared related to areas of expected high shear stress.
Therefore, we used KLF2 as a shear stress marker and compared ET-1 and NOS-3 with this expression. We test the hypothesis that the ET-1 expression is low or absent at the sites where KLF2 expression is high and that NOS-3 expression overlaps with that of KLF2.

Materials and Methods

Embryos
Fertilised White Leghorn eggs (Gallus domesticus) were incubated at 37°C and 60 to 70% relative humidity. Embryos were staged according to Hamburger and Hamilton. The following stages were collected: HH16, 17, 18, 19, 20, 22, 24, 27 and HH30. The embryos were fixed overnight in 4% paraformaldehyde in 0.1M phosphate buffer at 4°C, after which they were dehydrated in graded ethanol and embedded in paraffin. After this procedure, the embryos were sectioned at 5μm and mounted onto 3-triaminopropyl-triethoxy-silane (TESPA)-coated (Sigma, St. Louis, MO) glass slides.

Immunohistochemistry
After deparaffinisation the sections were treated for 12 min with 0.3% H2O2 in phosphate buffered saline (PBS) to quench endogenous peroxidase activity. Routine immunohistochemical staining was performed by using an overnight incubation with the primary antibody HHF35 (DAKO, Denmark) against muscle actin diluted 1:500 in PBS with 0.05% Tween-20 and 1% ovalbumin. After rinsing in PBS, the sections were incubated with horseradish peroxidase-conjugated rabbit anti-mouse antibody (1:200, DAKO, Denmark). After rinsing in PBS, administration of goat anti-rabbit immunoglobulins (GAR/Ig, 1:50, Nordic, Tilburg, The Netherlands), and washing in PBS, the sections were incubated with rabbit peroxidase-antiperoxidase complex (R/PAP, 1:500, Nordic). After thorough rinsing with PBS, the sections were treated with 0.04% diaminobenzidine tetrahydrochloride (DAB)/0.06% H2O2 in 0.05M Tris-maleic acid (pH 7.6) for 10 min at room temperature. The sections were counterstained with Mayer’s hematoxylin, dehydrated, and mounted in Entellan.

Radioactive In Situ Hybridisation
Probes
35S-labelled chicken-specific riboprobes were produced from a 607-bp (nucleotides 339-946) KLF2 fragment, a 619-bp (nucleotides 177-796) ET-1 fragment (kindly provided by
Yanagisawa, UTSWMS, Dallas), and a 498-bp (nucleotides 339-837) NOS-3 fragment, cloned in PCRII (Invitrogen). After linearisation, sense and antisense cRNA was transcribed in transcription buffer, 0.01M dithiothreitol, 0.25mM G/A/CTP mix, 1.4 U/μl RNase inhibitor, and 1.5U/μl of the appropriate RNA polymerase (T7 for KLF2 and ET-1, and SP6 for NOS-3) in the presence of 2.31MB 35S-UTP. Concentration of the probes was normalised to 1x10^5 cpm, and probes were used for in situ hybridisation. All sense probes showed negative hybridisation results (not shown).

In Situ Hybridisation

In situ hybridisation was performed on PFA-fixed tissue as described by Hierck et al.31. Hybridised sections were dehydrated in graded ethanol and air-dried before being coated with Ilford G5 emulsion (ILFORD Ltd., Mobberley, UK) and were exposed for 10-12 days at 4°C. Exposed slides were developed in Kodak D19 developing solution (Kodak, France) for 4 min at room temperature, rinsed, and fixed for 4 min in Ilford fixative (ILFORD Imaging Ltd., Mobberley, UK). Finally, the sections were counterstained with Mayer’s hematoxylin, dehydrated, and embedded in Pertex (Histolab, Göteborg, Sweden). Darkfield photomicrographs were taken from consecutive sections of the same embryo, hybridised to different probes for good comparison.

Three-Dimensional Reconstruction

To combine the expression patterns from the sections, three-dimensional (3D)-reconstructions were created as a visualisation tool. The 3D-reconstructions of the endothelial lining of the heart and vessels of stage HH18, HH24 and HH27 chicken embryos were prepared by using the Amira software package (TGS, San Diego, USA), resulting in a representation of a vascular cast. To generate a 3D-reconstruction, digital photomicrographs were taken of every seventh section used for in situ hybridisation. This means that the point distance in the z-axis for the reconstruction is large compared with the x- and y-axis, and as a consequence, a terrace-like structure was created. In situ hybridisation slides in general show staining patterns ranging from cells completely studded with silver grains to a few grains, not different from background. For purposes of presentation, this wealth of information was reduced to a simple positive-negative pattern. In principle, this method can be done by applying thresholds in grey scales. This strategy turned out to be unwieldy, mainly due to the fine pattern of cell-to-cell differences. This problem was solved by investigating the sections by two independent observers who reconciled for an overall
pattern. The positive areas of KLF2, ET-1, and NOS-3 were added to the reconstructed vascular cast. Restriction of the data sets sometimes leads to an artificial loss of small structures, like the narrowest lumens of the pharyngeal arch arteries at stage HH18 and HH27. The selected figures allow for optimal view of the expression patterns, but as these are retrieved from 3D-data sets, other viewpoints are also possible.

Comparison of Expression Patterns in Heart Segments and Stages

KLF2, ET-1, and NOS-3 expression in chicken embryos from HH16-HH30 have been compared spatially. In Figures 2.2, 2.3, and 2.5, this is shown in stages HH18, HH24, and HH27, respectively. The 3D-reconstructions and transverse in situ hybridisation sections show expression of KLF2 (Figs. 2.2a-d, 2.3a-d, and 2.5a-d), ET-1 (Figs. 2.2e-h, 2.3e-h, and 2.5e-h), and NOS-3 (Figs. 2.2i-l, 2.3i-l, and 2.5i-l), predominantly in the endothelium and endocardium. The expression patterns from the sections are projected on 3D-reconstructions of the endothelial lining of the heart, pharyngeal arch arteries, and dorsal aorta (Figs. 2.2a,e,i, 2.3a,e,i, and 2.5a,e,i) to visualise the patterns. The 3D-reconstructions graphically represent the expression patterns and not the expression levels. The horizontally matched figures of KLF2, ET-1, and NOS-3 (e.g., Figs. 2.2b,f,j, 2.3b,f,j, and 2.5b,f,j) represent consecutive sections. Panel m in Figures 2.2, 2.3, and 2.5 shows the different and labelled structures in the 3D-reconstructions. The levels of the transverse sections in the 3D-reconstructions are shown in Figures 2.2n-p, 2.3n-p, and 2.5n-p. In panels a from Figures 2.2, 2.3, and 2.5, KLF2 expression is demarcated in blue, the expression of ET-1 is shown in panels e in yellow, and in panels i, NOS-3 expression is depicted in green.

Results

Short Description

The early embryonic chicken heart is a simple tube that starts to loop around stage HH10 (approximately 2 days of incubation), atrial septation starts at stage HH19 (approximately 3 days of incubation), and ventricular septation begins at stage HH20 (approximately 3.5 days of incubation). In the mean time, cushions develop in the atrioventricular canal (AV canal) and outflow tract, which will form the atrioventricular valves and the semilunar valves in the aorta and the pulmonary trunk, respectively. The outflow tract and the AV canal are relatively narrow compared with the developing chambers. More upstream, the area where the veins enter the atrium (sinus venosus) is also narrow. Blood flow velocity and fluid shear stress are expected to be high in these areas, because filling a subsequent wider lumen
needs a local increase in flow. Additionally, shear stress is inversely correlated to the diameter of the lumen; thus, the smaller the diameter of the lumen, the higher the shear stress will be on the endothelial lining. The atria and ventricles, being wide parts of the heart, would therefore have low shear stress levels and subsequently show shear-independent gene expression patterns. In the aortic sac, from which the pharyngeal arch arteries that are connected to the dorsal aorta arise, the shear stress is likewise expected to alter. The first, second, and third pharyngeal arch arteries develop successively after which the first regresses and the fourth will develop. The second will also disappear and finally three pharyngeal arch arteries, i.e., the third, fourth and sixth persist, at least partially. Thus, the pharyngeal arch arterial system is subjected to extensive morphometric changes, presumably also involving shear stress.

The in situ hybridisations of the stages studied (HH16 to HH30) demonstrate a concomitant change in expression. In the following description, the expression during development in the different areas of the heart, including the pharyngeal arch arteries, going from inflow to outflow, is analysed. Localisation of the in situ hybridisation signal to the endothelium/endocardium is demonstrated in Figure 2.1. Actin staining identifies the myocardium and the overlying endothelium (Fig. 2.1a). Endothelin-1 mRNA in an adjacent section (Fig. 2.1b) is clearly present in the endothelium and not in the underlying cell layers. This endothelium specificity was also found in the embryonic vasculature (not shown). Note that Figures 2.1-2.7 show the localisation of expression and that Figure 2.8 shows a semi-quantitative representation of the level of expression. Panels a, e and i from Figures 2.2, 2.3, and 2.5 must be regarded for an overall view; conclusions were drawn from the original in situ hybridisations.

![Figure 2.1.](image)

**Figure 2.1.** a,b: Endothelium specificity of the ISH signal is demonstrated by an actin staining (HHF35) of a section through the AV canal of a HH20 chicken embryo (a), and an ISH signal from an adjacent section probed for ET-1 (b). It is clear that the endothelium is positive for ET-1 whereas underlying tissues are not. AVC, atrioventricular canal; E, endothelium; M, myocardium; OFT, outflow tract. Scale bars = 50μm.
Expression Pattern of KLF2

KLF2 expression in the cardiovascular system in the embryo from stage HH16 to HH30 is restricted to the endothelium and endocardium. Early in development, KLF2 is present in parts of the atrium, the AV canal, along the ventricular trabeculations, in parts of the outflow tract, in the aortic sac, and in the pharyngeal arch arteries (Figs. 2.2a-d, 2.7a, 2.8). The level of expression and the pattern in most of these areas remain constant during development from HH16 to HH30, i.e., in the atrium the expression is confined to specific sites. KLF2 mRNA is detected in the endocardium cranial to the site where the sinus venosus enters the right atrium and in the narrow area of the entrance of the sinus venosus (Figs. 2.2c, 2.5c, 2.6a). From stage HH18 onward, the level of expression in the sinus venosus remains constant (Fig. 2.8). During development the interatrial septum develops, along which the endocardium shows high KLF2 expression (Fig. 2.5b). The endocardium lining the AV cushions remains strongly positive (Figs. 2.3a,d, 2.4a, 2.5a,c,d), and expression is slightly increased between stage HH18 and HH27 (Fig. 2.8). The endocardium along the tips of the trabeculations shows focal expression (Figs. 2.3d, 2.5d, 2.7a-c), of which the level remains constant (Fig. 2.8). KLF2 in the outflow tract, aortic sac, and the inner curvature (Figs. 2.3a,c, 2.5a-c) remains also stable. However, the level in the distal outflow tract increases during development (Fig. 2.8).

In the pharyngeal arch arteries, the KLF2 expression pattern changes during development. From at least stage HH16 onward KLF2 is present in the pharyngeal arch arteries (Figs. 2.2a,b, 2.8). Around HH20 it becomes weaker, and at HH27, it is hardly detectable (Figs. 2.3a,b, 2.5a, 2.8). At HH22, the internal carotid arteries, being the two-sided dorsal aortae running into the head, start to show some weak KLF2 expression (results not shown). This contrast is also visible between the distal outflow tract and the pharyngeal arch arteries, which are also in a close distance: the decrease of KLF2 in the arch arteries is the opposite of the increase in the distal outflow tract (Fig. 2.8).

Summarising, KLF2 is expressed in the endothelium and endocardium of narrow parts of the cardiovascular system, e.g., the sinus venosus entering the atrium, the AV canal, the outflow tract, the aortic sac, and during the early stages of development in the pharyngeal arch arteries.

Expression Pattern of ET-1

The expression of ET-1 in the cardiovascular system is not only confined to the endothelium and endocardium, but it is also observed in the endoderm of the pharyngeal pouches, and in the ectoderm and mesodermal core of the pharyngeal arches of young embryos (Fig. 2.2f).
Figure 2.2. a-p: Localization of KLF2, ET-1 and NOS-3 at HH18. Note the presence of KLF2 and NOS-3 and absence of ET-1 in the sinus horns (compare a, e, i). Also note the positive ET-1 and negative KLF2 and NOS-3 expression in the narrow part of the sino-atrial transition, ET-1 is detected both upstream and downstream (arrows). In the AVC (d, h, l) along the inner curvature (as compiled in a, e, i) all three messages are present. h: ET-1 shows expression at the lateral side of the cushion (arrows), the flat top shows weaker expression. i: NOS-3 is detected along the ventricular trabeculations, unlike the other two genes (compare a, e). The proximal OFT shows excluding expression of KLF2 with ET-1 (a, e), NOS-3 is weakly expressed. In the distal OFT, messages overlap with each other (compilation in a, e, i). In the Aos and PAAAs, KLF2 (a, b) and NOS-3 (i, j) are present, in contrast to ET-1 (c, f). ET-1 and NOS-3 are excluding with KLF2 in the DAO (compare a, e, i). The asterisk in m indicates the inner curvature. 2, second pharyngeal arch artery; A, atrium; Aos, aortic sac; AVC, atrioventricular canal; Dao, dorsal aorta; OFT, outflow tract; SV, sinus venosus; V, ventricle. Scale bars = 200µm.
Figure 2.3. a-p: Localisation of gene expression at stage HH24. The SV is almost completely negative for KLF2 and NOS-3, and positive for ET-1 (d, h, l). Expression in the AVC is more distinct as compared with stage HH18. KLF2 (a, d) and NOS-3 (i, l) are both present, although NOS-3 very weak. ET-1 (e, h) is detected adjacent to the AVC in the atrial wall. For boxed areas see Figure 2.4. The compilation figures a, e and i show that the inner curvature is positive for KLF2 and NOS-3 but negative for ET-1. In the ventricle, NOS-3 (i, l) is homogeneously present along the trabeculations, KLF2 (a, d) shows spotted expression, and isolated cells are ET-1 positive (e, h). In the complete OFT, ET-1 (e, g) and KLF2 (a, c) are excluding. NOS-3 (i, k) overlaps with KLF2 (a, c). A sharply bordered part of the OFT-mesenchyme is positive for ET-1 (g). The OFT shows overlapping KLF2 and NOS-3, whereas the Aos, PAAs, and DAo show overlapping ET-1 (e, f) and NOS-3 (i, j), excluding with KLF2 (a, b). The asterisk in m indicates the inner curvature. 3, third pharyngeal arch artery; AVC, atrioventricular canal; Cu, cushion tissue; Dao, dorsal aorta; FG, foregut; LA, left atrium; M, myocardium; OFT, outflow tract; SV, sinus venosus; V, ventricle. Scale bars = 200µm.
Interestingly, a part of the mesenchyme of the AV and outflow tract cushions is transiently positive (Fig. 2.3g). The following description is focussed particularly on the endothelial expression. During early development, endothelin-1 mRNA is present in parts of the sinus venosus, the atrium, AV canal, outflow tract, aortic sac and the dorsal aorta (Figs. 2.2e,g,h, 2.8). The expression in the atrium is confined to specific sites, particularly in the endothelium cranial to the entry of the sinus venosus, and in the wider part of the entry of the sinus venosus into the atrium (Figs. 2.2e, 2.5g arrow, 2.6b), of which the level remains constant during development (Fig. 2.8). In contrast, the expression in the endocardium along the AV cushions is developmentally regulated. At HH16, ET-1 mRNA is observed in the endocardium lining the cushions (Fig. 2.8). From HH18 onward, the expression is diminishing (Figs. 2.2h, 2.8), and at HH24 it is hardly detectable along the cushions (Figs. 2.3h, 2.4b, 2.8); only a small part of the anterior cushion remains positive (Figs. 2.3e,h, 2.4b). ET-1 mRNA cannot be observed along the cushions at HH27 (Figs. 2.5e,g,h, 2.8); however, it is present adjacent to the AV cushions in the ventral walls of the atria (Figs. 2.5e,g arrowheads). In the endocardium lining the ventricular trabeculations, only a few isolated cells are ET-1 positive (Fig. 2.8), and the number increases slightly from stage HH18 to HH27 (Figs. 2.3h, 2.5h, 2.7d-f). In the compact myocardium, ET-1 mRNA is observed at the sites of coronary vessel development from approximately stage HH24 onward (Figs. 2.3h, 2.5h arrows, 2.7f). In the outflow tract, the expression decreases from stage HH18 onward (Fig. 2.8). At HH24, some expression of ET-1 is still observed (Fig. 2.3e); however, at HH27, ET-1 mRNA in the outflow tract is not detectable (Figs. 2.5e-g, 2.8). The decrease in the distal outflow tract is comparable to the decrease in the AV canal (Fig. 2.8).

![Figure 2.4](image_url)

**Figure 2.4.** Higher magnifications of the AV canal from panels d, h and l from Figure 2.3. a-c: Shown are the localisations of expression of KLF2 (a, arrows), of ET-1 (b, arrows), and of NOS-3 (c, arrows). Note that only ET-1 is expressed on the atrial side (b, double arrow), and is not expressed along the dorsal AV cushion (b, arrowhead), where KLF2 (a, arrowhead) and NOS-3 (c, arrowhead) are present. AVC, atrioventricular canal; LA, left atrium. Scale bars = 100μm.
From HH18 onward, the endothelium of the aortic sac is negative for ET-1 (Figs. 2.2e,f). The endothelium of the pharyngeal arch arteries starts to become positive from stage HH19 and remains positive (Figs. 2.3e,f, 2.5e, 2.8). The dorsal aorta remains positive during development from HH16 to HH30 (Figs. 2.2e, 2.3e, 2.5e).
Summarising, ET-1 expression is shown in the sinoatrial transition, in the AV canal and outflow tract in the earlier stages, in the ventricle and pharyngeal arch arteries in the later stages, and in the dorsal aorta. The AV canal becomes negative around HH22, like the distal outflow tract. The ventricle starts to show isolated positive endocardial cells from stage HH18 onward. The arch arteries become positive around stage HH19. It was shown that endothelin-1 is not only expressed in endothelium and endocardium, but also in the endoderm, ectoderm and mesodermal core of the pharyngeal arches, and in the mesenchyme of the cushions.

Expression Pattern of NOS-3
The expression of NOS-3 in the cardiovascular system is restricted to endothelium and endocardium. Early in development NOS-3 mRNA is strongly present in the endothelium and endocardium of the proximal and distal outflow tract, the aortic sac, the proximal parts of the pharyngeal arch arteries, and of the microvasculature in the head (Figs. 2.2j, 2.8). It is observed at low levels in the endothelium and endocardium of the sinoatrial transition, AV
Figure 2.7. a-i: Shown are the localisations of expression of KLF2 (a-c), ET-1 (d-f) and NOS-3 (g-i) in parts of the ventricles at stages HH18 (a, d, g), HH24 (b, e, h) and HH27 (c, f, i). At stage HH18, only a few endocardial cells are KLF2-positive, however, the signal is strong (a, arrows). At HH24 more cells are positive, these are located at the tips of the ventricular trabeculations, not close to the compact myocardium (b, arrows). This finding holds true also for KLF2 at HH27 (c, arrows). ET-1 at stage HH18 shows very few positive endocardial cells along the ventricular trabeculations (d, arrows). Slightly more positive cells are visible at stages HH24 (e, arrows) and HH27 (f, arrows). Note the ET-1 positivity in the myocardium at sites of coronary development (f, arrowheads). NOS-3 shows the same expression in all three stages; along the ventricular trabeculations all endocardial cells are positive (g-i). Scale bars = 50 μm in a,d,g, 125 μm in b,c,e,f,h,i.

canal, ventricle, the medial part of the outflow tract, dorsal aorta, and the internal carotid arteries (Figs. 2.2j-l, 2.7g, 2.8). In most of these areas, the expression patterns remain stable during development from HH16 to HH30 (Fig. 2.8). In the atrium, the narrow part of the sinoatrial transition, and the endocardium cranial to the entry of the sinus venosus into the atrium remain positive for NOS-3 (Figs. 2.5k arrow, 2.6c, 2.8). The endothelial lining of the developing interatrial septum is slightly positive (Fig. 2.5j). The endothelium along the AV cushions shows a constant NOS-3 expression during development from HH16 to HH30 (Figs. 2.3i,l, 2.4c, 2.5i,l, 2.8). The pattern of NOS-3 along the ventricular trabeculations, in the
different parts of the outflow tract, the aortic sac, and the pharyngeal arch arteries remains also constant during development (Figs. 2.3i-l, 2.5i-l, 2.7g-i, 2.8). However, in the distal outflow tract the level of expression increases from stage HH20 onward (Fig. 2.8).

Summarising, NOS-3 expression overlaps with that of KLF2 in the narrowest parts of the cardiovascular system, but, in addition, is more widespread. It was found in the AV canal, the ventricles, outflow tract, aortic sac, pharyngeal arch arteries, internal carotid arteries, and in the dorsal aorta.

Figure 2.8. Diagram showing a semi-quantitative representation of expression levels of KLF2, ET-1 and NOS-3 in the endothelium/endocardium of the sinus venosus (SV), atrioventricular canal (AVC), trabeculated ventricle (TV), distal outflow tract (dOFT), and of the pharyngeal arch arteries (PAA) during chicken development from stage HH16 to HH30. As arch artery remodelling is severe at these stages, the bars represent vessels that are present. Note that levels of expression of KLF2 in sinus venosus, AV canal, and distal outflow tract are comparable during development, all showing a slight increase from stage HH16 onward and a slight decrease around stage HH30. In the trabeculated ventricle, expression of KLF2 and NOS-3 remains stable. ET-1 expression is only present in a few isolated cells from HH18 to HH30 (interrupted line). In the pharyngeal arch arteries, the level of expression of KLF2 drops quickly after stage HH16 to a very low level. In contrast, expression of ET-1 in the pharyngeal arch arteries increases reciprocal to KLF2. As ET-1 expression in these arch arteries comes up (HH18), expression in the AV canal and distal outflow tract decreases. The level of NOS-3 expression remains constant during development in these structures. Only in the distal outflow tract an increase is detectable from stage HH20 onward.
Discussion

Our results are consistent with the hypothesis that changes in blood flow during cardiovascular development can result in morphological changes through shear stress induced altered gene expression. A part of this hypothesis, that intracardiac hemodynamics play an important role in cardiogenesis, is confirmed by Hove et al. Hogers et al. developed a chicken venous clip model, in which a lateral vitelline vein was ligated, and as a result, intracardiac blood flow patterns changed, and cardiac and pharyngeal arch artery malformations developed. Stekelenburg-de Vos et al., by using the same venous clip model, showed that the blood flow decreases transiently for up to 5 hours after setting the clip. This could result in fast changes in gene expression patterns, which eventually can lead to the cardiovascular anomalies found in the venous clip model. In the present study, gene patterns in the embryo correlate with expected differences in levels of shear stress throughout the cardiovascular system. mRNA of ET-1 and of NOS-3, which produces NO, the functional counterpart of ET-1 in the vascular system, were investigated in detail. We correlated the expression patterns of these genes to the expression pattern of KLF2, a transcription factor of which the expression is confined to the endothelium of the adult human aorta at sites of high shear stress. Our hypothesis is that areas with expected high shear stress show high NOS-3 and KLF2 expression, whereas areas with low shear stress show high ET-1 expression. Accordingly, we demonstrate that, especially during late development, areas of presumed highest shear stress are delineated by KLF2 expression, as KLF2 is expressed in areas with a narrow lumen. These sites would be high shear stress areas as can be concluded from the Hagen Poiseuille formula: \( \tau = \frac{4\mu Q}{\pi R^3} \), which states that wall shear stress (\( \tau \)) is dependent on the blood viscosity (\( \mu \)), the volumetric blood flow (\( Q \)), and the lumen radius (\( R \)). The viscosity of the blood is assumed to be constant throughout the vascular system. Therefore, the only parameters that may vary are radius and flow. Shear stress is inversely correlated with the radius, which means that a smaller diameter will result in a higher shear stress. In narrow areas, e.g., the AV canal, shear stress would be higher compared with the adjacent wider atrium and ventricle. The latter two will normally have low levels of shear stress and would therefore show shear stress-independent gene expression. From in vitro studies it is known that ET-1 on the one hand, and KLF2 and NOS-3 on the other hand, respond differently to high steady laminar shear stress, i.e., ET-1 is down-regulated, and KLF2 and NOS-3 are both upregulated. In vitro studies have shown that ET-1 and NOS-3 can both be up-regulated by unidirectional pulsatile shear stress. This is the kind of shear stress that is physiological; the blood is pumped through the
cardiovascular system in a pulsatile manner. Most obviously, this is what is found in the in vivo embryonic situation and will be most pronounced in the narrowest regions, such as the sinoatrial transition, the AV canal and outflow tract. According to the above-mentioned studies, pulsatile flow would increase the ET-1 expression, however, our results show no expression of ET-1 in these regions at the later stages studied. In contrast, the in vitro results of the steady laminar shear stress studies are in line with our observations. During stages of intense cardiovascular remodelling (HH20–HH30), the ET-1 and KLF2/NOS-3 expression patterns exclude each other at the narrow sites. This finding suggests that, during these stages of embryonic development, regulation by steady laminar flow is of more importance than regulation by pulsatile flow. The frequency of the pulse may not be high enough or the level of shear stress may be an important factor as Mattart et al. showed: at 0.3 dyne/cm², pulsed flow gives a higher mRNA induction of ET-1 than non-pulsed flow, however, at 6 dyne/cm² there is no significant difference between the two types of flow. The excluding expression patterns of the three genes also suggests that ET-1 is negatively correlated to shear stress, i.e., high shear stress results in a low ET-1 expression, and that NOS-3 is positively correlated to shear stress. This would provide a mechanism for altered shear stress to tamper with cardiovascular development through shear-related gene expression. However, it is clear that not all areas of endothelial/endocardial NOS-3 and ET-1 expression are shear stress related. This includes the AV canal and outflow tract in the earlier stages. Here, expression becomes shear stress related later in development. The non-overlapping areas of endothelial NOS-3 with KLF2, in, e.g., the dorsal aorta and the ventricles, as well as non-endothelial areas in which ET-1 is present, e.g., the mesenchyme of the outflow tract and AV cushions, and endo-, ecto-, and mesoderm of the pharyngeal arches, appear not shear stress related. Differential expression initiated by other pathways is likely to be an additional mechanism for the regulation of gene activation at these sites. In the pharyngeal arch arteries, the patterns of KLF2 and ET-1 are complementary and also inverted in a time-dependent manner. The arch artery endothelium is positive for KLF2 and negative for ET-1 up to stage HH19. After that stage ET-1 increases and KLF2 decreases. Assuming that the expression is shear stress regulated, this suggests that during development, the shear stress in the pharyngeal arch arteries decreases, probably due to widening of the lumen that balances the increase in flow due to normal development. In the earlier stages, the expression patterns of ET-1 and KLF2 partially overlap in the outflow tract and in the AV canal, whereas at stage HH27 they are entirely alternating. This suggests that during development the influence of high and low shear stress becomes more specific.
to certain areas. Expression patterns may change during development due to remodelling of heart and vessels, changing angles of blood flow, and to increased cardiac performance. Endothelial cells can react to changes in shear stress, although the underlying mechanism is still unknown. Membrane receptors, integrins or cell-surface ion channels are probably involved. Once changes in shear are registered at the cell membrane level, a signal will be sent to the nucleus by means of second messengers. It is known that second messengers such as ionised cytosolic calcium, intracellular lipid products of the polyphosphoinositide pathway, and nitric oxide are generated by flow stimulation (reviewed by Gimbrone et al.). Transcription factors, such as c-fos, Egr-1, Sp1 and NFκB, are subsequently activated and involved in gene regulation. Whether binding to SSREs or transcriptional activation through oxidative stress is responsible for shear mediated transcription is still unclear. Khachigian et al., however, have demonstrated that components of NFκB can interact directly with the SSRE motif in the human platelet-derived growth factor-B promoter, thereby promoting shear-induced gene expression. Negative and positive SSREs are known, such as the TRE (AP-1) site, which binds the transcription factors fos and jun, that inhibits transcription. The Egr-1/Sp1 binding site appears to stimulate transcription in response to activation. Induction of gene expression by the binding of transcription factors to a SSRE would eventually give rise to changes in levels of, e.g., endothelin-1 or NO, which are vasoactive substances in adults. Likewise, exogenous ET-1 in embryos is involved in vasoconstriction. Shear-related gene expression, therefore, is to be expected in embryos.

NOS-3 with or without shear stress regulation is involved in the function and development of the cardiovascular system. NO production by elevated shear stress limits vasoconstriction in the coronary arteries of adult dogs, thereby influencing the coronary microcirculation. Because of the abundance of NOS-3 in the rat heart during late gestation, it is thought that NO would also influence the coronary circulation at this developmental stage. Furthermore, genes and proteins from the NO-cascade are involved in myocardial contraction, and in cardiomyogenesis. Nos-3-/- mice showed that NOS-3 is involved in the formation of the aortic valves, and the atrial and ventricular septa. KLF2 is in some way also involved in heart and vessel development. It has been shown by Kuo et al. that KLF2 is involved in the formation of the media of the vessel wall. Klf2-deficient mice die between E12.5 and E14.5 because of severe haemorrhaging caused by an abnormal vascular tunica media. The function of KLF2 during embryonic development remains to be determined.

The pathway of ET-1 is important in cardiovascular development. Kurihara et al., studying Edn1-/- mice, showed aortic arch malformations and ventricular septal defects. Eta-/- and Ece-
1/- mice demonstrated abnormal regression of the 4th and 6th pharyngeal arch arteries, an enlargement of the 3rd arch artery, and abnormal persistence of the bilateral ductus caroticus and of the right dorsal aorta. These malformations lead to arterial abnormalities similar to human congenital cardiac defects44. Craniofacial abnormalities and defects in the cardiovascular outflow tract in Eta-/- mice were shown by Clouthier et al.12. Ece-1-/- and ETA-/- mice demonstrated ET-1-/- and ETA-/- like craniofacial and cardiac malformations11. The group of Kempf et al.45, using ETA antagonists, demonstrated similar phenotypes as obtained in ET-1-/- and ETA-/- mice, being craniofacial, cardiac, and great vessel defects. Thus, genes from the ET-1/ECE-1/ETA cascade are involved in craniofacial, heart and vessel development and the knockout models show similar cardiovascular defects as observed in the chicken venous clip model.

In summary, we show shear stress related expression of ET-1 and NOS-3. Mouse models with downregulation of our candidate genes show cardiac malformations that are comparable to the abnormalities we have evoked in the venous clip model. In this clip model flow patterns are shifted and as flow is positively correlated to shear stress, shear stress patterns are disturbed resulting in changes in gene expression. This suggests that shear stress and shear stress-induced or -repressed gene expression are important in the normal remodelling of the cardiovascular system.

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References


