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Hemodynamics in Cardiac Development

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Abstract

The beating heart is subject to intrinsic mechanical factors, exerted by contraction of the myocardium (stretch and strain) and fluid forces of the enclosed blood (wall shear stress). The earliest contractions of the heart occur already in the 10-somite stage in the tubular as yet unsegmented heart. With development the looping heart becomes asymmetric providing varying diameters and curvatures resulting in unequal flow profiles. These flow profiles exert various wall shear stresses and as a consequence different expression patterns of shear responsive genes. In this paper we investigate the morphological changes of the heart after changes the blood flow by ligation of the right vitelline vein in a model chicken embryo and analyze the extended expression in the endocardial cushions of the shear responsive gene Tgfbeta receptor III. A major phenomenon is the diminished endocardial-mesenchymal transition resulting in hypoplastic (even absence of) atrioventricular and outflow tract endocardial cushions, that might be lethal in early phases. The surviving embryos exhibit several cardiac malformations including ventricular septal defects and malformed semilunar valves related to a malposition of the aortopulmonary septum and the enclosed neural crest cells. We discuss the results in the light of the interactions between several shear stress responsive signaling pathways including Vegf, Notch, Pdgf, Klf2, eNos, Endothelin and Tgfβ/Bmp/Smad.

Key words: cardiogenesis, endocardial cushions, neural crest, hemodynamics, shear stress, semilunar valve, outflow tract septum, Klf2, growth factors, Transforming growth factor

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Introduction

Heart development requires complex interactions starting with the mesodermal cardiac crescent and resulting in mammals in the fully septated four-chambered beating heart. This complexity relies strongly on the concerted spatio-temporal regulation of many genes. It is evident that a rather limited number of cardiac transcription factors, more specifically Tbx5, Nkx2.5 and the Gata-family, govern major developmental steps, and mutations in these genes have been reported to lead to congenital heart defects (CHD). The pathways reveal highly complex backgrounds including e.g. dose-dependency, histon modifications, copy number variants and post-transcriptional regulation by e.g. microRNAs [1]. The majority of CHD (~80%), however, is considered multifactorial, implying that other, probably environmental, factors are also involved. These include cholesterol metabolism , homocysteine, maternal diabetes, hyperglycemia and hemodynamics.

Simple changes in the venous blood flow to the model chicken heart [2] result in a variety of ventricular septal defects (VSDs) and pharyngeal arch arterial malformations. Altered gene expression patterns have been found for flow-dependent Et1 (endothelin), eNos and Klf2 [3], bound to the lining layers of the cardiovascular system, i.e. the endocardium and the endothelium.

Obviously, heart development engages not only the shear stress-sensitive endothelial/ endocardial inner lining but also myocardial, epicardial, smooth muscle and neuronal cells, and fibroblasts derived from their precursors. These involve development and differentiation of the pharyngeal endoderm, first heart field (FHF), second heart field (SHF) and cardiac neural crest (NC), interacting harmoniously to construct the various components and compartments of the heart. Transmission of hemodynamic cues to the underlying layers (fibroblast, myocardium, smooth muscle cell) must be an important function of the endothelium and endocardium, and this has been confirmed at least for the Et1 and eNOS pathways [4].

An important morphogenetic event in heart development is the differentiation of the endocardium into mesenchymal cells, the so-called endocardial-mesenchymal transition [5] (EMT), by which both the atrioventricular (AV) and the outflow tract (OFT) endocardial

cushions will be formed. These cushions, located prominently in the bloodstream, function as backflow-preventing structures only transforming into definitive valvular leaflets in later phases. Furthermore, they are instrumental in septation processes in atrium, ventricle and outflow tract. As a histological consequence, the underlying cells derived from endocardium, SHF or NC, are in intimate contact with the overlying endocardium (in the case of the heart) and endothelium (in the case of the aortic sac and pharyngeal arch arteries).

Besides being influenced by hemodynamics, genes might also be caught in webs of other interactions exemplified by the Sonic hedgehog (Shh)-Gli pathway, that is shear stress dependent being part of the shear sensing primary cilium [6], but also involved in cholesterol metabolism [7]. To carry this even further interactions between Shh-Gli with Zic3, Pitx2 and Nodal are important in left-right asymmetry [8] while Nodal-Pitx2 interactions are also involved in asymmetrical development of the pharyngeal arch arteries This systemt changes the blood flow in the aortic sac leading to differential signalling by shear stress responsive genes such as Pdgf Receptor as well as Vegf Receptor-2 for further downstream arterial remodelling [9].

Essential for unraveling the influence of hemodynamics is that normal flow patterns can be investigated and modeled in vitro and in vivo during development and compared to structural alterations as performed in accessible embryos like chicken [10—13] and zebrafish [14, 15].

In this paper we will provide new information on the effect of ligating a vitelline artery in an experimental chicken embryo on the morphogenesis of the endocardial cushions and the expression of a shear stress responsive gene (Transforming growth factor Receptor III), The ensuing behavior of endocardial and neural crest cells is analyzed , which leads to malformations in the heart under hemodynamic challenging.

We will blend the results of shear stress sensitivity on changes in gene expression patterns in a broader sense as brought to us by several different approaches. These include i) Cell culture systems, ii) In vivo transgenic technology, iii) The survey of (cardiovascular) patient populations and iv) Direct (surgical) manipulations in chicken embryos and zebrafish.

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Cardiac anomalies after vitelline vein ligation.

Materials and methods

Ligation procedures. Fertilized white leghorn eggs were incubated (70 hrs) reaching Hamburger Hamilton stage (HH) 17. The egg shell was windowed and the vitelline membranes removed above the most proximal part of the right lateral vitelline vein. A small incision was made to expose the vein and a nickel microclip was clamped around it. Cessation and subsequent rerouting of blood flow was confirmed. Eggs were resealed, re-incubated and sacrificed at successive stages. Shams (n=14) lacking the clip and normal eggs (n=10) served as controls. Gross morphology and heart morphology and function were evaluated.

Immunohistochemistry. Embryos were fixed (4°C, overnight) in 98% ethanol containing 2% glacial acetic acid, dehydrated in ethanol, embedded in paraffin and serially sectioned at 5µm. The sections were mounted, air-dried, rinsed twice (15 min) in phosphate buffered saline (PBS) and once in PBS supplemented with 0.05% Tween-20. Ten clips and 6 controls were studied for the expression of Tgfβ Receptor III (TBRIII) used as shear stress marker. Staining was performed with the primary antibody TBRκ, anti Tgfβ Receptor type III (kindly provided by Dr. J.V.Barnett, Nashville, Tennessee, USA), 1:50 diluted in PBS/Tween-20 and 1% ovalbumin. The sections were rinsed twice (15 min) in PBS and once in PBS/Tween-20 (15 min) and incubated (2 hrs) with the 2nd antibody, 1:300 diluted rabbit anti-mouse horseradish complex, rinsed (3x10 min) in PBS and exposed (10 min) to 0.04% diaminobenzidine tetrachloride in 0.05 M TRIS-Maleic acid (pH 7.6) with 0.07% imidazole and 0.06‰ H_2O_2. The reaction was stopped in PBS. The sections were counterstained with Mayer's hematoxylin (5 sec) and covered in Entellan.

Scanning electron microscopy (SEM). The right lateral vitelline vein of 68 embryos was ligated and investigated in preseptation (HH 18-24) and postseptation embryos (HH34 and older). The hearts were perfusion-fixed with a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.1M sodium cacodylate (pH 7.2, 4˚C) and stored overnight. Preseptated hearts were opened frontally and septated hearts were opened transversally immediately caudal to the atrial appendages. The hearts were rinsed (0.1M cacodylate buffer, pH 7.2) and postfixed (1% OsO_4, same buffer, 4˚C), and dehydrated in graded ethanol. Preparations were critically point dried over CO_2 by conventional methods, mounted on aluminium stubs, sputter-coated (Balzers MED 010) with gold (3 min) and studied with the Philips SEM 525M.

Labeling of neural crest cells. NC cell tracing has been described elsewhere [16]. In brief, we used specific pathogen free eggs, free of helper virus. The eggs were windowed at HH stage 9-10. The open neural groove was flushed gently with a solution containing the polycation polybrene (80-100µg/ml, Sigma, St Louis, MO) and a replication-incompetent retrovirus containing the bacterial LacZ reporter gene). The eggs were resealed and reincubated until HH17. Only normal embryos were used further for vitelline vein ligation (see above). The embryos were reincubated until HH 34-37. Embryos (n=13) were fixed in paraformaldehyde 4% and stained overnight with X-gal [16]. Non-ligated retrovirally infected embryos (n=16) served as controls.

Apoptosis. To investigate the presence and distribution pattern of apoptotic (NC) cells we subjected retrovirally infected embryos to the TUNEL approach (Tdt-mediated dUTP nick end labeling) using a commercially available kit (Boehringer, Mannheim) to detect fragmented DNA [16]. After counterstaining, sections were dehydrated and mounted in Entellan.

Survival rates. The survival rate after venous ligation was almost 79%. We observed that stages HH22-24 were critical in relation to survival.

Results

Impaired development in preseptation stages and TBRIII expression

Normal hearts showed a relatively short atrioventricular (AV) junction (Fig. 1a) compared to ligated embryos (Fig.1b). After ligation the inner curvature was wider creating a larger distance between outflow tract and AV area (compare Figs. 1a and 1b). In normal and sham operated embryos numerous mesenchymal cushion cells resulting from EMT were seen and the endocardium covering the cushions was squamous. In ligated embryos, the cushions lacked many cells, where they accumulated directly under the cuboidal lining. Hypoplastic atrioventricular cushions were the most common malformations (38%) in ligated embryos (N=63) in stages HH18-24 (compare Figs. 1c and 1d). The superior cushion was affected more frequently than the inferior one. Hypoplastic outflow tract cushions were observed in 17% of the ligated embryos (Fig. 1d).

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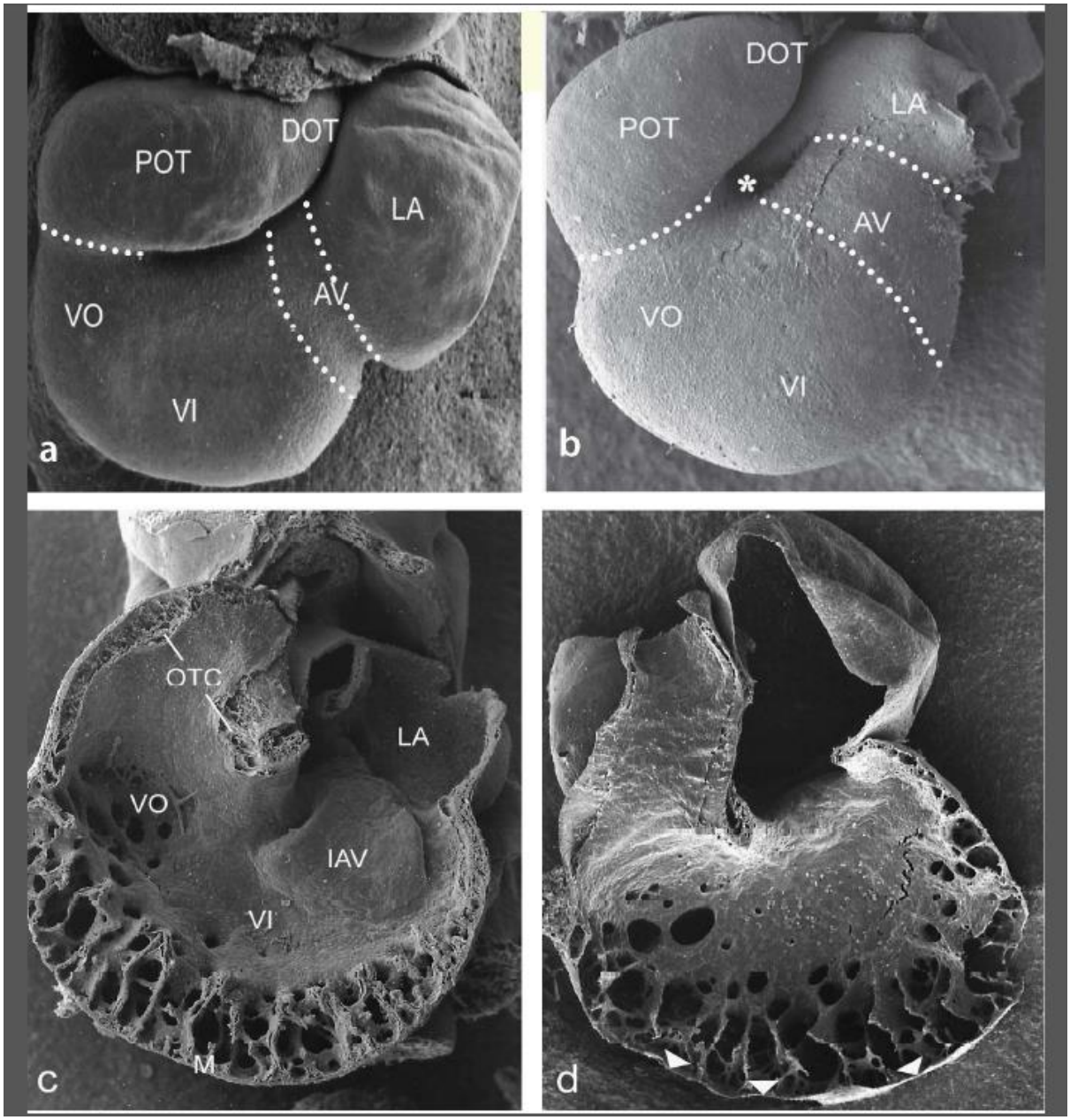

In normal HH18 embryos, TBRIII expression was observed in the endothelium of the dorsal aorta, cardinal vein, pharyngeal arch arteries, the endocardium covering the AV and OFT cushions, the epicardium and mesonephros. From HH20 onwards the expression in cushion endocardium was absent, while mesenchymal cells continued to express TBRIII although at a lower level than that seen in ventricular endocardial cells. Venous ligation resulted in HH20-24 in expansion of TBRIII expression toward additional endocardial cells including those lining the atrial septum (compare Figs. 2a, 2b), the floor of the left atrium and the ventricular trabeculae (Figs. 2e, 2f). Furthermore, there was sustained expression in the AV cushion endocardium, especially in the inferior AV cushion beyond HH20 (Figs. 2c, 2d).

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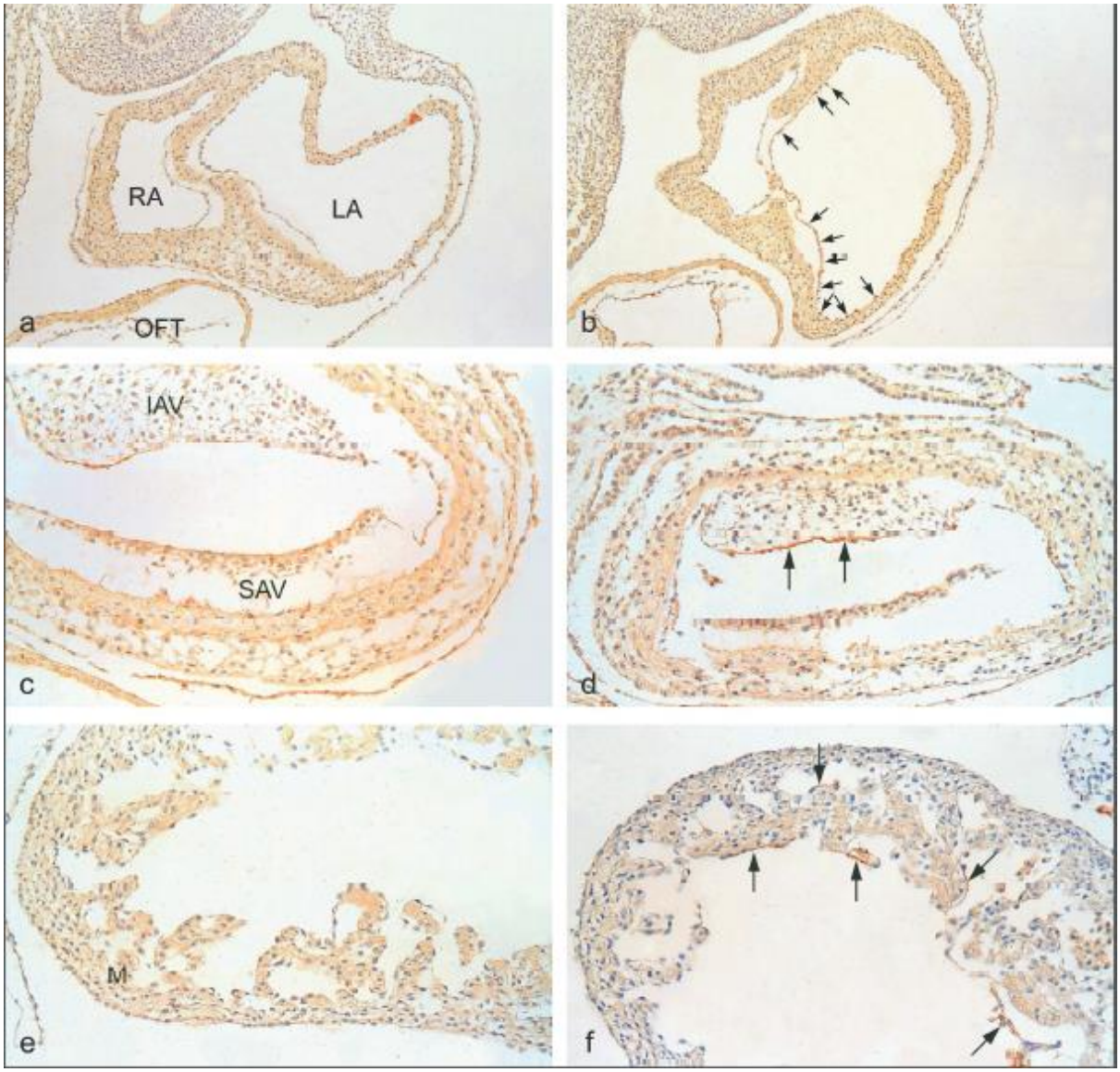
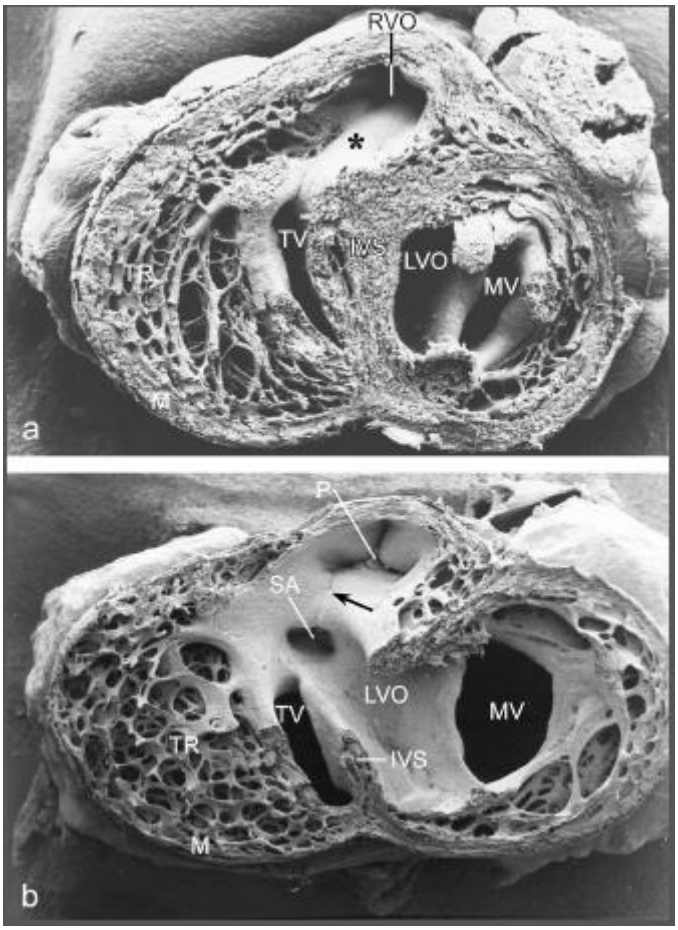


Peri and postseptation stages

In HH34 the muscular OFT septum is continuous with the interventricular septum by which the left and right ventricular outflow are fully separated (Fig. 3a). After ligation a ventricular septum defect results in 66% of the embryos (Fig. 3b). Both arterial orifices may be above the right ventricle (double outlet right ventricle). The aorta is stenotic and the mitral valve leaflets are abnormal. The myocardium is severely affected, as seen by a thin interventricular septum, a thin compact layer and abnormal trabeculations (compare Figs. 3a, 3b). After serial sectioning and LacZ tracing the histology makes this even more clear (Fig.4). In normal embryos the left and right ventricular outflow tract are separated by the aortopulmonary septum harboring among others NC cells that can be traced using the retroviral-LacZ method (Figs. 4a, 4e). Many of the NC cells go into apoptosis (Fig. 4b). After hemodynamic challenging by the venous clip complete septation did not occur in 66% of

the investigated embryos in HH27-45. In embryos with a subarterial VSD these were doubly committed and characterized by the most distal border of the VSD being at the semilunar valve level (Fig. 4c). The condensed mesenchyme of the aortopulmonary septum, separating the orifices of the aorta and pulmonary valve at the level of the commissures, was ventrally displaced but could still contain many NC cells (Fig. 4d). Incomplete cardiac looping, already observed in preseptation stages (Fig. 1b) resulted in dextroposed aorta in 41% of the surviving ligated abnormal embryos. We observed a continuous spectrum of deficient myocardialization of the outflow tract septum. Semilunar valve malformations were frequently encountered (25/70) in combination with a VSD (Figs. 4c, 4f).



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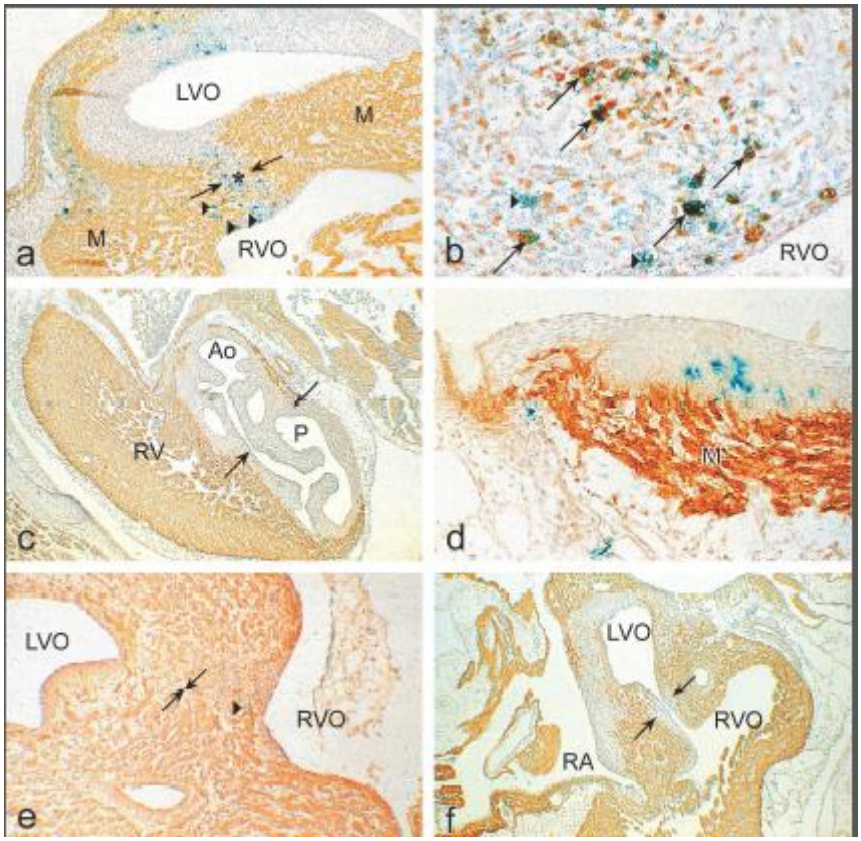

Discussion

We observed hypoplastic and even absent AV cushions in 38% of the survivors of all preseptation stages (HH18-24). Normally, the cushions fulfill a valve function preventing backflow. We suppose that ligated embryos with severe hypoplastic AV-cushions were not able to maintain appropriate cardiac output and died before or near HH22-24. The severe cases have selected themselves out by HH24, which would explain the rare presence of AV-anomalies in postseptation stages.

Proper AV and OFT endocardial cushion formation starts with EMT of the overlying endocardium in which many gene pathways are involved [5]. Tgfß signaling [17] is a shear stress dependent mechanism initiating and supporting this transformation [18]. The involvement of TBR III suggests a role for Tgfß2, since this ligand requires TBRIII for high affinity binding [19]. A functional role for Tgfß2 is further supported by valve defects and a non-myocardialized OFT septum in a Tgfß2 null mouse [20,21]. Since venous ligation results in alteration in endocardial morphogenesis and valve formation, the finding of persisting TBR III expression supports its role in diminished cushion formation. Normally TBR III is downregulated as the cushions have been seeded with mesenchymal cells [22]. Here, this downregulation does not take place together with diminished seeding of the cushions.

Hypoplastic AV cushions, myocardial wall thinning and abnormal ventricular trabeculation correlated well with previous hemodynamic measurements [23]. Hearts probably reacted to decreased function by ventricular dilation which in turn impaired cardiac looping, a key mechanism in the formation of VSDs [1].

Peri/postseptation stages. During development the types of the observed malformations changed. All ligated embryos were abnormal immediately after ligation, while from HH24 onwards, also apparently normal embryos were encountered. Moreover, a number of VSDs closed spontaneously, which can also be observed in human preclinical care.

After arrival in the AP-septum all NC cells become apoptotic [16, 24], and subsequently the mesenchyme is replaced by myocardium [25]. Abnormalities in the aortopulmonary septum after ligation were recognizable as a ventral displacement of the central mass of the condensed mesenchyme [2], as supported by LacZ tracing of NC cells (this study). Furthermore, in ligated embryos we could hardly observe apoptosis explaining the continued presence of NC cells. These phenomena lead to an exclusively mesenchymal OFT septum suggesting that apoptosis of NC cells is important for myocardialization [26]. The programmed death of NC cells in a limited time frame [24, 26] still presents an enigma but we favor the following chain of events. Normally, NC cells migrate into the assigned cardiac regions and become apoptotic, thereby changing the (extracellular) microenvironment. In vitro NC cells produce a latent form of Tgfß [27], also abundantly present in the matrix of embryonic hearts [28] that could be activated by NC proteolysis, and is diminished after venous ligation because of a diminished apoptosis. Tgfß signaling is important in OFT myocardialization as migration [29] and differentiation [30] of cardiomyocytes is controlled by Tgfß. In Tgfß-2 null mice the OFT septum is not myocardialized [20, 21].

The semilunar valve malformations after venous ligation are difficult to compare with other models as those have rarely been described. The most severe semilunar valve malformations in our model are combined with allegedly NC-related subarterial VSDs, confirming the involvement of NC cells in semilunar valve development [31].

Ciliary Mechanosensing

Changes in the cellular environment are perceived by many cell types through e.g. integrins [32] and receptive organelles such as non-motile monocilia or primary cilia [33], important for various aspects of shear stress-dependent signaling during embryonic development

[34]. Monocilia are not only involved in shear stress mediation but in many other processes, revealing diverse regulatory inputs [35]. Mechanical activation of the cilium evokes trafficking along the ciliary membrane and the enclosed microtubular system, resulting in intracellular signaling [36]. Several ciliary genes themselves are shear stress responsive, such as Aurora, Intraflagellar transporters and the homodimeric Platelet derived growth factor receptor (PdgfRαα) [37]. In the cardiovascular system endothelial and endocardial cells present monocilia [38 39], particularly in areas of low shear stress [40, 41].
Monocilia are relatively abundant in the trabecular sinuses and in those curvatures where the flow and the ensuing shear stress is low, such as the outer curvature of the aortic arch [42, 43] and even in adult mouse where monocilia are also abundant in the aortic valve sinuses and more downstream near branching points of e.g. the carotid arteries. In Apo3-/- mice the monocilia are found on the shoulders of atherosclerotic lesions in areas of turbulent flow, suggesting a relation between hemodynamics, monocilia and lesion formation [42]. Experiments using cultured endothelial and endocardial cells in flow chambers demonstrate that the (dis)appearance of monocilia is highly dynamic and subject to many factors [18].

Gene expression patterns in mechanosensing

It is obvious that an embryo does not exist as a separate entity, but is connected to its yolk sac (and in mammals also the placenta), the blood volume of these embryonic/fetal organs differs in development but is certainly not negligible. Therefore, it is no surprise that manipulating blood flow coming from the yolk sac will have major implications on cardiovascular development and may even be embryo lethal [44]. Ligation of yolk sac vessels results in altered hemodynamics [11, 23], succeeded by upregulation of Klf2 and Nos3, but by downregulation of Et-1 [3]. Increasing the hemodynamic load of the developing heart by outflow tract banding [45] resulted in increased cellularity of the OFT cushions and changes in the extracellular matrix combined with altered expression of factors known to be markers of EMT. Among these were Notch, Tgfβ, VegfR and Gata4 [45], all involved not only in matrix metabolism but also in many other aspects of cardiovascular development. In a meta-study flow-related signalling is confirmed for ~1650 shear stress responsive genes expressed in human umbilical vein endothelial cells (HUVECs) cultured in

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vitro [37] regulating genes in 24 signalling pathways. In this paper we concentrate on a subset of factors and signalling pathways that have also been identified in vivo during cardiac development. We will focus on Vegf, Notch, Pdgf, Klf2, Nos3, Endothelin , and Tgfβ/Bmp.

Vascular endothelial growth factor (Vegf) signaling

The Vegf/Vegf Receptor family contains several ligands (Vegf-A, B, C, D and PlGF), binding to Vegf Receptor1 (also named Flt1), R2 (also named Flk1/Kdr), R3, and cofactors as Neuropilin-1 and -2 [46]. Both Vegf-A and Vegf-B are shear responsive in HUVEC [37]. VegfR1 and 2 are expressed on endothelial cells and bind Vegf-A. All Vegf isoforms bind VegfR2, although presence of a co-factor seems involved in regulating specific effects (reviewed in [47]). Several additional pathways are involved such as hypoxia-signaling [48] and homocysteine metabolism [49] to keep the phenotype in balance. Vegf polymorphisms and redundant pathways are involved as shown by the various possible outcomes. These include Tetralogy of Fallot, valvular and septal defects and left ventricular outflow obstruction. It is interesting to realize that all cardiac malformations reported in VEGF polymorphisms can be traced back to developmental disorders in endocardium, epicardium or NC cells and their epithelium-to-mesenchymal transitions (EMT) [47, 48}. Furthermore, one of the main downstream networks of Vegf/VegfR2 is the Notch-signaling pathway [50].

Notch signaling.

Several members of the endothelial [37, 51, 52] and endocardial [53] Notch signaling pathway are shear stress sensitive including Dll4 (involved in trabeculation and coronary vessel formation), Jag1 and Jag2 (involved in chamber maturation and compaction) and downstream genes such as Hes and Hairy [54]. Perturbation of the signaling balance severely interrupts cardiac chamber formation. Dll4 mutant embryos showed more than 2000 affected genes [53]. Notch signaling in the SHF mediates interactions with the homing NC cells involved in proper outflow tract development [55].

In a human subpopulation mutations in Jagged1, also involved in Alagille syndrome, result in cardiac defects, including bicuspid aortic valve and Tetralogy of Fallot with dysmorphic pulmonary valve, overriding aorta, VSDs and right ventricular hypertrophy [56]. In animal

models the outcome is complicated due to redundancies in the pathway, while penetrance of the phenotype highly depends on the genetic background [57].

Platelet derived growth factor (Pdgf) signaling.

Pdgf isoforms consist of homodimers and heterodimers of four chains (A-D). They bind to the Pdgf receptor α and β subunits with varying affinity. Various members of the Pdgf pathway are shear stress responsive, including PdgfRαα, that in fibroblasts is even localized to the primary cilium itself [58]. Dimer signaling often involves intermediate transducers such as (shear stress responsive) Ras and PI3K. Shear stress applied to bovine endothelial cells produces enhanced PdgfRα activation giving a chemotactic response to smooth muscle cells [59]. Dysregulation of the human PdgfA gene is associated with total anomalous pulmonary venous return providing evidence that this gene is involved in proper formation of the cardiac inflow tract. This is confirmed in mouse and chicken embryos studying the PdgfRα and its ligand PdgfA [60, 61]. PdgfA, -C and its receptor α are involved in remodeling of the compact and trabeculated myocardium as well as development of the AV-valves through epicardium-myocardial interaction [61].

Krüppel-like factor-2

In adult vessels the mechanical force of shear stress is a strong inducer of Klf2 [37, 62, 63]. In HUVEC Klf2 regulates the transcription of many downstream factors in e.g. the Tgfβ signaling pathway [64] and also aquaporin-1, a nitric oxide transporter [65]. Klf2 is expressed in the endocardium of mouse and chicken and heavily involved in normal cardiogenesis [66]. It is engaged in regulating endocardial cell morphology during chamber ballooning. Cell-specific conditional Klf2 knock out mice demonstrated endothelial loss of Klf2, resulting in lethal embryonic heart failure (Lee 2006). Klf2 ablation results in reduced Sox9, UDP-glucose dehydrogenase (Ugdh), Gata4 and Tbx5 mRNA in the AV canal [66]. In the chicken embryonic heart its expression has been shown particularly in areas of high shear forces, which is the inner curvature of the heart, and at narrow regions such as the atrioventricular canal and the outflow tract [4]. It has to be kept in mind that high shear areas are nearly devoid of primary cilia as these are abundant specifically in low shear areas [42]. Endocardial differentiation defined by expression of Klf2 and Notch1 is dependent on blood flow within the ventricle and the atrioventricular canal.

Endothelin signaling

Endothelin is a small peptide derived from prepro-Et-1 mRNA that becomes translated into a 203-amino acid precursor, converted by endopeptidases into big-Et-1 which becomes cleaved by endothelin-converting-enzyme (ECE) into the functional endothelin. This exerts its activity through two main classes of receptors, Et-A and Et-B, present on e.g. smooth muscle and endothelial cells. ECE and Et-B are both shear stress responsive [37, 68] and expression is down-regulated upon applying shear forces [69].

Endothelin is an endogenous vasoconstrictor but has also vasodilator properties mediated by nitric oxide and prostacyclin release through activation of the endothelial Et-B receptor. Furthermore endothelin is a growth factor involved in the proliferation of fibroblasts and smooth muscle cells through the Et-A receptor and the proliferation of endothelial cells through the Et-B receptor (reviewed by [4]). Et-1 mRNA and protein production are regulated by wall shear stress, although the mechanisms are controversial [70]. In early chicken embryos Et-1 is expressed in the lining of the endocardial cushions, where during development it becomes complementary to that of Nos3 and Klf2 [71]. Et-1 knock out mice display similar cardiovascular defects as seen in chicken embryos after ligation of the vitelline vein [44, 72]. Involvement of Et-1 in the induced abnormalities found after venous ligation is proven by systemic application of endothelin or antagonists of Et-A and Et-B showing also direct hemodynamic changes[71].

Nitric oxide (NO) signaling

Nos3**,** also called endothelial nitric oxide synthase (eNos), is the major isoform in the vascular system, and also expressed in cardiomyocytes, the functional counterpart of Et-1. Both eNos and Nostrin (nitric oxide trafficker) are shear stress responsive [37]. In vitro laminar shear stress promotes NO-formation and increases the expression of Nos3 [73, 74]. The synthase is mainly localized in plasmalemmal vesicles, caveolae, where it is involved in the production and release of the bioactive NO. Nos3 katalyses the conversion of L-arginine and oxygen to L-citrullin and NO. Expression depends also on e.g. hypoxia, whereas the balance between NO and Et-1 is physiologically important for maintaining vascular homeostasis. In vivo studies showed that Nos3 expression overlaps with the high shear marker Klf2 [3]. In Nos3 deficient mice smooth muscle cell proliferation in a carotid

artery ligation model is suppressed [75] and they display bicuspid aortic valve, heart failure, VSD and ASD [76]. Furthermore, these mice show hypoplastic coronary arteries already early in development (where it is probably related to the rise in blood pressure and flow at the onset of arterial irrigation), followed by postnatal myocardial infarction. The underlying mechanism is complex as lack of Nos3 results in down-regulation of Gata4, Wilms tumour-1, Vegf, Fgf and erythropoietin and furthermore in inhibited migration of epicardial cells [77]. The epicardium is important for coronary formation and differentiation [78]. In chicken embryos its expression increases after ligation of a vitelline vein, just like Klf2 [3, 4].

Tgfβ/Bmp signaling

Members of the Tgfβ superfamily include activins and inhibins, Bmps (bone morphogenetic proteins) and others. Shear responsiveness is confirmed for but not limited to Tgfα, Tgfβ-1, TgfβR-1, latent Tgf binding proteins, Bmp1, 2 and 4 and the Bmp endothelial regulator [37]. Tgfβ1, 2, and 3 exhibit a myriad of regulatory, proliferative, and inductive functions in sometimes distinct but also overlapping spatial and temporal patterns in development as well as in the adult. The Tgfβs are secreted as latent complexes and become activated by e.g. metalloproteinases, and have interaction with integrins, reactive oxygen species, retinoids and others. Three classes of receptors are distinguished, Tgfβ-RI (Alk 1-7), -RII (including TgfβR2, BmpR2 and activin receptors), and -RIII (including betaglycan and endoglin) with varying degrees of affinity to the ligands [80. 81]. The TGFβ/BMP downstream signaling pathway involves activated Smad proteins.

In vitro applied shear stress resulted in alignment of endothelial cells, diminished apoptosis and proliferation, and increase in Tgfβ3, Klf2, phosphorylation of Nos3 and NO-release. EMT of the endocardium is coordinated by Bmp2 through the activation of genes that regulate intercellular communication, cell adhesion and extracellular matrix deposition [82]). In the outflow tract not only endocardial cells migrate into the cushion, but also NC cells expressing TgfßR2 [83], that modulate outflow tract septation and cushion remodeling. Knockdown of Tgfß2 results in a variety of cardiac malformations [20, 21] while knock down of Tgfβ3 prevented the induction of Klf2 [84]. In embryonic endothelial cells shear stress activates Tgfβ/Alk5 signaling, while induction of Klf2 is Alk5 dependent [43].

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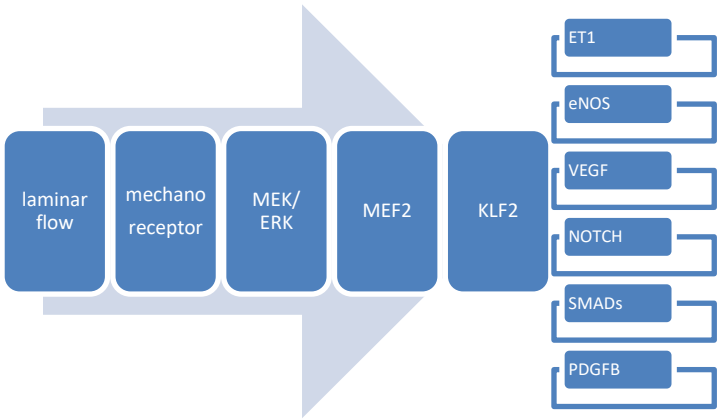
Knock out mice present many phenotypes in major organs including the cardiovascular system (see for an overview of phenotypes [81]) often involving the cardiac jelly and the cardiac cushions.

Interactions between flow-responsive genes.

The above featured signaling pathways after flow bear many more influences from other factors such as hypoxia (Vegf and eNos), and are part of myriad cascades that are not solely flow responsive (Vegf and Notch, Tgfß signaling). Furthermore, interactions might be reciprocal as for instance in Pdgf-B and Klf2 in embryonic aortic smooth muscle maturation [85]. The key factor in flow-responsiveness in the adult vessel wall seems to be Klf2 governing a gene transcription profile of >1000 genes [86] involved in cell migration, vasomotor function, hemostasis, inflammation and morphology changes and we propose that Klf2 likewise plays an important role in embryonic development of the cardiovascular system. An all-inclusive scheme showing the (potential) ramifications is hard to conceive, therefore, we provide a simplified version of the main flow-dependent interactions in cardiovascular development (Fig.5).



Consequences for human embryonic development

It is evident that shear stress responsive genes share pathways in the genesis of malformation complexes. We have shown that endocardial and NC cells take part in the chicken venous ligation phenotype. Whether alteration in placental blood flow in mammals will lead to a similar spectrum of anomalies remains to be determined with advanced ultrasound and Doppler techniques. Retrospective research showed a correlation of human placental anomalies with intrauterine growth retardation [87]. Human cardiovascular

malformations are related to low birth weight, body length and head circumference. Furthermore, abnormal circulation may result in growth retardation[88]. It was demonstrated that fetuses with intrauterine growth retardation displayed increased umbilical, placental and utero-placental resistance, decreased end-diastolic flow velocities in the descending aorta and umbilical artery, and decreased peak systolic flow velocities at cardiac level [89]. By prenatal diagnosis using Echo-Doppler techniques it has been shown that malformations specifically of the outflow tract and aortic arches can aggravate due to diminished blood flow through stenotic regions. Prenatal surgical interventions to relieve the stenosis results in restoration and limitation of the cardiac malformation [90]. Thus evidence is present for a role of hemodynamic factors in the human fetus in growth retardation as well as the emergence of congenital (cardiac) malformations.

Figure Legends

Fig. 1. Cardiac looping in normal and ligated embryos HH20. SEM of ventral views. **Fig a.** Normal embryo with cardiac segments indicated. **Fig b**. Ligated embryo. The retarded looping resembles that of a HH17 embryo with an open inner curvature (*). The AV canal is relatively long. **Fig c and d.** Interior view of dorsal heart halves. **Fig c.** The inferior AV cushion and the OFT cushions are well developed, ventricular trabeculations have formed. **Fig d.** AV and OFT cushions are non-existent, trabeculations are abnormal and the compact myocardium is thin (arrowheads). AV: Atrioventricular groove, DOT: distal outflow tract, IAV inferior AV cushion, LA: left part of atrium, M: compact myocardium, OTC: outflow tract cushions, POT: proximal outflow tract, VI ventricular inlet, VO: ventricular outlet, * inner curvature, arrowheads: thin compact myocardium.

Fig. 2. Expression of TGFβ type III receptor. **Fig a**. Normal left and right atrium of HH20. No TBRIII expression in atrial endocardium. **Fig b.** Ligated embryo with ectopic TBRIII expression along the atrial septum and atrial floor (arrows). **Fig c.** AV cushions of normal HH22 embryo, TBRIII expression is downregulated. **Fig d.** Ligated embryo with prolonged endocardial TBRIII expression (arrows). **Fig e.** Normal HH20 embryo, ventricular trabeculations lack TBRIII. **Fig f.** Ectopic TBRIII expression of the endocardium lining ventricular trabeculations (arrows). IAV: inferior AV cushion, LA: left atrium, OFT: outflow tract, RA: right atrium, SA: superior AV cushion.

Fig. 3. SEM of HH34 postseptation embryos viewed from apex to base. **Fig a**. Normal heart. The muscular OFT septum (*) is continuous with the interventricular septum (IVS). **Fig b**. Ligated embryo with a subaortic VSD. The line of the fused OFT cushions is indicated (arrow). Both arterial orifices (SA and P) are situated above the right ventricle (double outlet right ventricle). The aorta is stenotic (SA) and the mitral valve leaflets (MV) are abnormal. The myocardium is severely affected, as seen by a thin IVS and compact layer (M) and abnormal trabeculations (TR). IVS: interventricular septum, LVO: left ventricular outflow tract, M: compact myocardium, MV: mitral valve, P: pulmonary orifice, RVO: right ventricular outflow tract, SA: stenotic aorta, TR: trabeculations, TV: tricuspid valve.

Fig. 4. LacZ tracing and apoptosis of neural crest cells immunostained for actin. **Fig a.** Proximal outflow tract region of a retrovirus infected, non-ligated HH31 embryo. Myocardialization of the OFT is nearly complete (*) as the opposing parts of the myocardium (M) almost touch (arrows). NC cells (blue) are present in the mesenchyme and myocardium (arrowheads). **Fig b.** Adjacent section with magnified aorto-pulmonary septum, subjected to TUNEL for apoptotic cells (brown). Most of the apoptotic (brown) cells are also blue, indicating NC cells. After apoptosis X-gal granules give way the position of the original NC cell (arrows). **Fig c**. HH37 ligated embryo with a subarterial VSD, showing confluence of the semilunar valve leaflets without myocardialization (arrows far apart). **Fig d.** Retrovirus infected, ligated HH37 embryo. Numerous blue NC cells in the ventral prong of the AP-septum. Adjacent TUNEL stained sections (not shown) presented no apoptotic cells. **Fig e.** Retrovirus infected, non-ligated HH37 embryo with full myocardialization (arrows meet each other) only a single blue NC cell (arrowhead) has been registered. **Fig f.** Ligated HH37 embryo showing a subaortic VSD with substantial myocardialization of the AP septum (compare with Fig c). Ao: aorta, LVO: left ventricular outflow tract, M myocardium, P: pulmonary trunk, RA: right atrium, RV: right ventricle, RVO: right ventricular outflow tract.

Fig. 5. Alteration in laminar flow invokes changes in mechanoreceptors such as monocilia and integrins, followed by activation of e.g. the Mek/Erk- Mef2 cascade. Mef2 activates nuclear expression of Klf2 thereby influencing Et-1, eNos, Vegf, Smads (particularly P-Smad2, and Smad4/7) and probably Pdgf-B. Upregulation of Klf2 by changes in laminar flow (but also by Angiopoietin-activation of the Tie2 receptor and by statins) involves down-regulation of most of the latter genes. It is interesting to note that oxidative stress and cytokine but also oscillatory flow not sensed by monocilia downregulate Klf2 expression. Data mainly based on [3, 4, 63, 86].

Author contributions R.E.P and A.C.G-de G. both conceived the concepts, gathered the material, wrote, discussed and approved the paper.

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