The pro-inflammatory signalling regulator Stat4 promotes vasculogenesis of great vessels derived from endothelial precursors

Zhao-Zheng Meng¹,*, Wei Liu¹,*, Yu Xia¹, Hui-Min Yin¹, Chi-Yuan Zhang¹, Dan Su¹, Li-Feng Yan¹, Ai-Hua Gu² & Yong Zhou¹

Vasculogenic defects of great vessels (GVs) are a major cause of congenital cardiovascular diseases. However, genetic regulators of endothelial precursors in GV vasculogenesis remain largely unknown. Here we show that Stat4, a transcription factor known for its regulatory role of pro-inflammatory signalling, promotes GV vasculogenesis in zebrafish. We find stat4 transcripts highly enriched in nkx2.5⁺ endothelial precursors in the pharynx and demonstrate that genetic ablation of stat4 causes stenosis of pharyngeal arch arteries (PAAs) by suppressing PAAs 3–6 angioblast development. We further show that stat4 is a downstream target of nkx2.5 and that it autonomously promotes proliferation of endothelial precursors of the mesoderm. Mechanistically, stat4 regulates the emerging PAA angioblasts by inhibiting the expression of hdac3 and counteracting the effect of stat1a. Altogether, our study establishes a role for Stat4 in zebrafish great vessel development, and suggests that Stat4 may serve as a therapeutic target for GV defects.

¹ Key Laboratory of Stem Cell Biology, Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences & Shanghai Jiao Tong University School of Medicine, Shanghai 200031, China. ² State Key Laboratory of Reproductive Medicine, Institute of Toxicology, School of Public Health, Nanjing Medical University, Nanjing 211166, China. * These authors contributed equally to this work. Correspondence and requests for materials should be addressed to A.-H.G. (email: aihuagu@njmu.edu.cn) or to Y.Z. (email: zhouyong@sibs.ac.cn).
GREAT VESSEL DEFECTS (GVDs) ARE ONE OF THE MAJOR CAUSES OF CONGENITAL CARDIOVASCULAR DISEASES, WHICH LEAD TO THE DEATH OF MILLIONS OF INFANTS AND CHILDREN ANNUALLY. GVDs INCLUDE CAROTID ARTERY ANOMALIES, ABERRANT SUBCLAVIAN ARTERIES, INTERRUPTED AORTIC ARCH, COACTIONS OF THE AORTA, PATENT DUCTUS ARTERIOSUS, PROXIMAL PULMONARY ARTERY HYPOPLASIA AND OTHERS. CELLULAR AND GENETIC MECHANISMS OF GREAT VESSEL MALFORMATIONS REMAIN LARGELY UNKNOWN, PARTLY DUE TO THE INTRICATE STRUCTURE AND TRANSFORMATION OF GVS IN FETAL GROWTH. INVESTIGATION OF THE VASCULOGENESIS OF GREAT VESSELS MAY HELP CLARIFY GVD ETIOLOGIES. DURING VASCULOGENESIS, ANGIOBlasts, WHICH ORIGINATE FROM ENDOTHELIAL PRECURSORS OF THE MESODERM, COALESCE AND ASSEMBLE INTO THE PRIMARY STRUCTURE OF GREAT VESSELS SUCH AS THE AORTIC ARCH ARTERIES (ALSO KNOWN AS PHARYNGEAL ARCH ARTERIES, PAAS). THE PAAS connect the blood flow from the heart to the dorsal aorta in the embryo, and they provide an indispensable contribution to the carotid arteries and great vessels of the heart.

RECENT STUDIES HAVE FOUND THAT THE ENDOTHELIUM OF GREAT VESSELS IS DERIVED FROM MESODERMAL PRECURSORS EXPRESSING HEART FIELD MARKERS, SUCH AS Nkx2.5. IT HAS BEEN REPORTED THAT SUBPOPULATIONS OF Nkx2.5+ CELLS IN THE ANTERIOR LATERAL PLATE MESODERM (ALPM) CONDENSE TO FORM FOUR PAIRS OF CLUSTERS THAT GIVE RISE TO PAAS 3–6, BUT THE FUNCTION OF Nkx2.5 IS NOT REQUIRED UNTIL THE ACTIVATION OF THE VASCULOGENESIS PROGRAMME OF GREAT VESSELS. THIS FINDING SUGGESTS THAT SPATIOTEMPORALLY SPECIFIC MEDIATORS REGULATE THE ANGIOBlast FATE OF Nkx2.5+ ENDOTHELIAL PRECURSORS TO FORM GREAT VESSELS. WE PREVIOUSLY FOUND THAT REPRESSION OF THE SECOND HEART FIELD (SHF) REGULATOR Tltbp3 led to great vessel anomalies. It is reported that the deletion of the SHF regulator FGF8 in the cardiogenic mesoderm also causes PAA phenotypes in mice. Tcf21, together with Nkx2.5, marks a dispensable group of PAA progenitors for great vessel formation in zebrafish. Although Tbx haploinsufficiency causes aortic arch defects in mice, it is involved in endoderm–mesenchyme interactions but not angioblast formation in the mesoderm. Despite the roles of these cardiogenic regulators in the development of great vessels, the specific regulators of Nkx2.5+ endothelial precursors of great vessels have not been revealed.

USING THE PROTOZEBRAS CRYSTAL EMBRYO MODEL, WE ARE ABLE TO VISUALIZE THE PROCESS OF GREAT VESSEL VASCULOGENESIS, WHICH IS DIFFICULT TO STUDY IN MICE AND HUMANS DUE TO GESTATION IN THE UTERUS. This vasculogenesis programme in zebrafish lasts from 28 h post-fertilization (hpf) to 48 hpf, which is comparable to similar processes that occur in the fourth week of human embryo development. USING THIS MODEL, WE CONDUCT A TRANSCRIPTONAL MICROARRAY ANALYSIS OF Nkx2.5+ CELLS AT 30 HPF AND IDENTIFY SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION FACTOR 4 (Stat4), WHICH IS HIGHLY EXPRESSED, AS A POTENTIAL REGULATOR OF Nkx2.5+ ENDOTHELIAL PRECURSORS. AS A PRO-INFLAMMATORY SIGNALLING MODERATOR, Stat4 has long been shown to direct the development of fully functional T HELPER 1 CELLS AND IS CANONICALLY ACTIVATED BY INTERLEUKIN (IL)-12 RECEPTOR-ASSOCIATED JAK KINASE.

IN THIS STUDY, WE GENERATE A stat4 MUTANT LINE USING THE CRISPR/CAS SYSTEM, AND DESCRIBE A PIVOTAL ROLE OF Stat4 IN REGULATING THE PROLIFERATION OF ENDOTHELIAL PRECURSORS DURING GREAT VESSEL VASCULOGENESIS. FURTHERMORE, WE UNCOVER THAT stat4 IS DOWNSTREAM OF Nkx2.5 AND PROMOTES ANGIOBlast FORMATION BY SUPPRESSING hdac3 AND stat1a. OUR FINDINGS ECLUCIDATE A SURPRISING AND IMPORTANT FUNCTION OF Stat4 IN THE ESTABLISHMENT OF GREAT VESSELS.

RESULTS

Conservation of Stat4 and its expression in the pharynx. The orthologues of all mammalian STAT genes in zebrafish have been identified with the high primary sequence conservation. Zebrafish Stat4 encodes a 731-aa protein, which contains four functional domains and shares 68.7% sequence similarity with human STAT4. Stat4 is located on Chromosome 9 in zebrafish, and no paralogs have been identified. The genes flanking zebrafish Stat4 are syntenic with the STAT4 locus in humans. AS EVIDENT FROM THE DOMAIN STRUCTURE AND AMINO-ACID SEQUENCE ALIGNMENT, ZEBRAFISH Stat4 IS CLOSERLY RELATED TO THE CORRESPONDING MAMMALIAN HOMOLOGUES (Fig. 1a and Supplementary Fig. 1).

THE EXPRESSION LEVEL OF stat4 WAS FOUND TO BE 18-FOLD HIGHER IN nKX2.5+ CELLS BY TRANSCRIPTONAL MICROARRAY ANALYSIS OF THE Tg(nkx2.5:ZsYellow) LINE AT 30 HPF THAN IN SOMATIC CELLS (Fig. 1g). Pathway enrichment analysis showed that Jak-Stat signalling was a major upregulated pathway in nKx2.5+ cells (Supplementary Fig. 2A). THE ACTIVATED GENES OF THE Jak-Stat PATHWAY WERE LISTED IN THE HEATMAP AND SCATTER PLOT, AMONG WHICH stat4 WAS THE MOST HIGHLY ACTIVATED (Supplementary Fig. 2B,C). IN SITU HYBRIDIZATION SHOWED THAT EXPRESSION OF stat4 WAS ENRICHED IN THE ALPM AT 28 H POST FERTILIZATION (hpf) AND 48 hpf (Fig. 1b–d and Supplementary Fig. 3A–G). Stat4 transcripts were also distributed in the pharyngeal arch area at 60 hpf (Supplementary Fig. 3H). AS SHOWN IN Supplementary Fig. 3E–I, stat4 IS NOT EXPRESSED IN THE HEART AT 24 hpf OR 48 hpf, BUT WE COULD OBSERVE THE WEAK EXPRESSION OF stat4 IN THE HEART AT 60 hpf.

SIMILAR TO stat4, nKx2.5 TRANSCRIPTS WERE CO-LOCALIZED IN THE PHARYNGEAL CLUSTERS OF THE ALPM AT 28 hpf, WHICH SUBSEQUENTLY DIFFERENTIATES INTO AORTIC ARCH ANGIOBlasts IN THE PHARYNX, AS VISUALIZED BY THE ANGIOBlast MARKER tcf1 (Fig. 1e,f). LATER, nKX2.5+ ZsYellow POPULATIONS FROM THE MESODERM FORMED THE PRIMARY GREAT VESSELS, THE THIRD TO SIXTH PAAS (PAAs 3–6) VISUALIZED BY THE ZsYellow+/mCherry+ POPULATION OF Tg(nkx2.5:ZsYellow);Tg(flk1:mCherry) EMBRYOS (Supplementary Fig. 4A–C). THE Stat4 PROTEIN CO-LOCALIZED WITH Nkx2.5 PROTEINS IN PAAs 3–6 AT 60 hpf, AS REVEALED BY DOUBLE IMMUNOFLOUORESCENT STAINING (Fig. 1h–j).

Loss of stat4 causes stenosis of the primitive great vessels. A splice morpholino, targeting the splice acceptor site for exon 3 of stat4, was employed to suppress normally spliced stat4 transcripts in zebrafish embryos, generating stat4 morphants. Reverse transcription PCR showed that the transcript was efficiently knocked down. Whereas control embryos expressed normal stat4 transcripts, the morphants expressed improperly spliced stat4 messenger RNAs (mRNAs) without exons 3 and 4 (Supplementary Fig. 5A). THE stat4 MOPHANTS DID NOT SHOW OBVIOUS DEVELOPMENTAL ABNORMALITIES OTHER THAN THE STENOSIS OF PAAs 3–6.

THE CRISPR/Cas SYSTEM WAS EMPLOYED TO GENERATE stat4 ZEBRAFISH MUTANTS BY TARGETING EXON 3 (Supplementary Fig. 5B). After CRISPR/Cas RNA INJECTION, WE DETECT THE F1 generation heterozygotes and found robust induction of targeted insertion/deletion mutations by assessing the frequency of altered alleles (Supplementary Table 1). THE SELECTED HOMOZYGOUS stat4 MUTANT OF THE F3 GENERATION SHOWN IN Fig. 2 HAD A TWO-BASE PAIR DELETION THAT CAUSED A FRAME-SHIFT MUTATION AND A PREMATURE STOP CODON IN EXON 3, LEADING TO A TRUNCATED PROTEIN WITH 58 AMINO ACIDS (Fig. 2A). THE HETEROZYGOS AND HOMOZYGOS stat4 MUTANTS WERE BORN AT NORMAL MENDELIAN RATIOS AND SHOWED ATRIOPHARYNGEAL REGIONS AND A SLIGHTLY SMALL HEAD AT 72 hpf COMPARED WITH THE WILD-TYPE SIBLINGS. THE MUTANTS HAD FEWER AND SHORTER ARTERIES THAN THEIR WILD-TYPE SIBLINGS, WHICH HAVE FULL-LENGTH ARTERIES AND PLUMP NECKS. THERE WERE NO APPARENT DEFECTS IN THE OTHER TISSUES OR THE DEVELOPMENT OF HOMOZYGOTES (Supplementary Fig. 6A–C). THE stat4 MUTANT AND HOMOZYGOTE EMBRYOS HAD THE...
same phenotype, but the deficiency of mutants was stronger than that of morphants.

The defect of PAAs 3–6 can be seen in pathological sections of the mutants. The arch arteries in mutants lack lumen endothelium, but the surrounding smooth muscle of the vasculature did not have any evident abnormality compared with wild-type controls. The deficiency of PAAs 3–6 in the mutants led to atrophy of the pharynx, and the jaw defect can also be observed in tissue sections (Fig. 2b,c). The stenosis of PAAs 3–6 caused by stat4 deficiency can be clearly visualized by the stat4−/−; Tg(kdr:mCherry) lines (Fig. 2d,e). Furthermore, the shunted blood flow labelled by gata1− was undetectable in PAAs 3–6 vessels in the stat4−/−; Tg(gata1:DsRed) line, while the major vessels such as the lateral dorsal aorta and common cardinal vein retained robust blood flow (Fig. 2f,g). The stat4−/−; Tg(fli1:EGFP); Tg(gata1:DsRed) line indicated that the narrow and short PAAs 3–6 in the mutants were unable to support blood flow (Fig. 2h,i and Supplementary Fig. 7A). The lack of blood flow was due to stenosis of the arch artery lumens, visualized in the stat4−/−; Tg(kdr:muEGFP) line. The cell count numbers revealed an ~58% decrease in differentiated endothelial cells of PAAs 3–6 in stat4 mutants compared with the control (Fig. 2j-l). As shown in Supplementary Fig. 7B,C, the yellow cells remained in PAAs 3–6 of stat4 morphants in the Tg (nkx2.5:yellow); Tg(kdr:mCherry) line, indicating that the remained structure of PAAs 3–6 was derived from nkx2.5+ cells. However, an insufficient number of nkx2.5+ cells led to the deficient growth of the arch arteries in the morphants compared with the controls.

Taken together, suppression of stat4 caused agrowth deficiency of PAAs 3–6, while the first pair of aortic arch arteries that is established before the initiation of circulation were not impaired in either the control or stat4 mutant/morphant embryos. The model of stenosis of the primitive great vessels (PAAs 3–6) caused by stat4 deficiency is shown in Fig. 2m.

Lack of Stat4 prevents emergence of PAA angioblasts. In stat4 mutants, the lack of tie1 transcripts in arch arteries 3–6 of the stat4 mutants suggested a decline in the vasculogenesis programme of the nkx2.5+ cells derived from PAAs 3–6 compared with the control group at 60 hpf (Fig. 3a,b). The resin sections showed that the tie1 probe labelled the four cords of PAAs angioblasts in the control, while only minimal tie1+ angioblasts were observed in PAAs 3–6 of stat4 mutants (Fig. 3c,d). The vasculogenesis defect in stat4 mutants was observed as early as emergence of the four pairs of angioblastic cords (ACs) (as indicated by the asterisk and bracket). Loss of stat4 inhibited the emergence of tie1+ labelled ACs, the number of which was significantly decreased in mutants at 44 hpf. The stat4 morphants phenocopied the tie1+ PAA angioblast deficiency seen in the mutants. The recruitment of stat4 mRNA specifically restored all four pairs of ACs in the morphants (Fig. 3e–h,k). Evaluation of endothelial precursors by etv2 and scl at the top of
the signalling hierarchy of angioblast formation demonstrated that suppression of stat4 inhibited the emergence of both etv2 and scl clusters in stat4 mutants (Fig. 3i,j,m–o). The myoblast marker tcf21 was not affected in the mutants at 28 hpf (Fig. 3l,p) or in the Tg(tcf21:GFP) stat4 morphants at 30 hpf (Supplementary Fig. 8S,T). Evaluation of flk1 expression, the endothelial marker, showed the loss of ACs 3–6 at 38 hpf (Supplementary Fig. 7D–F). Angiogenesis represented by flk1+ inter-segmental vessels was not affected in the stat4 morphants (Supplementary Fig. 7G–I). Evaluation of the vascular mesoderm by etv2 demonstrated that other vasculatures such as the cranial21 or somatic vasculatures were not significantly impaired by suppression of stat4 (Supplementary Fig. 7J,K).

Embryonic haematopoiesis in the intermediate cell mass was not evidently disturbed by scl probe detection in morphants (Supplementary Fig. 8A,E). The gata1-labelled somatic blood flow was normal in stat4 morphants compared with the control group (Supplementary Fig. 8B,F). Rag1-labelled T cells of the thymus and the foxn1-labelled thymus epithelia are not interrupted in stat4 morphants compared with the control embryos (Supplementary Fig. 8C,D,G,H). Neural crest-derived pharyngeal mesenchyme is unaffected in stat4 morphants, as revealed by hand2 and dlx2a expression patterns (Supplementary Fig. 8I,J,M,N). The normal tbx1 expression pattern in morphants demonstrated that the loss of stat4 did not alter pharyngeal ectoderm or endoderm at 28 hpf, comparable to the controls (Supplementary Fig. 8K,O). Additionally, tbx1 expression in the pharyngeal mesoderm is not significantly impaired in the mutants at 28 hpf (Supplementary Fig. 8Q,R).

Meanwhile, the gata4+ cardiac progenitors in the ALPM remain normal in stat4 morphants (Supplementary Fig. 8L,P) at 18-somite stage (ss). Both the nkx2.5+ cardiac progenitors and the formation of the linear heart tube are normal in stat4 morphants (Supplementary Fig. 9A,B,D,E). No obvious differences are found in the lbp3+ SHF of the control embryos and the morphants (Supplementary Fig. 9I,L). The heart morphology from 48 hpf to 60 hpf is not impaired in the embryos lacking stat4 compared with the control using the Tg(nkx2.5:ZsYellow) line (Supplementary Fig. 9C,F).

**Stat4 promotes proliferation of nkx2.5+ endothelial precursors.** Morphogenesis of the four clusters of nkx2.5+ endothelial precursors in the ALPM remained unchanged, despite the lack of stat4, as shown in the transverse sections of Tg(nkx2.5:ZsYellow) embryos at 30 hpf (Fig. 4a,c). However, the BrdU cooperation assay showed that the quantity of the co-localized BrdU+ (red) and nkx2.5+ (green) cells was significantly reduced in the stat4 morphants compared with the control embryos (Fig. 4a–d,g, yellow). Cellular apoptotic evaluation of nkx2.5+ clusters by TdT-mediated
dUTP nick end labelling (TUNEL) revealed that the lack of stat4 did not impair endothelial precursor survival (Fig. 4e,f,h).

Cell-dye was used to label the nkg2.5+ endothelial precursors to further investigate their proliferation and migration in the absence of stat4. Tg(nkg2.5:ZsYellow) embryos were injected with CellTracker Red in the ZsYellow+ pharyngeal cluster region at 28 hpf. After culturing for 4 h, the single cell was labelled and imaged at 32 hpf as the starting point. At this time, the labelled cells in the control and morphant embryos did not show any obvious differences. The same embryos were imaged again at 55 hpf. As shown in Fig. 5a,b,d,e, the labelled cell forms multiple dye+ cells in the PAAs of control embryos, while the labelled cell remains in its original location without proliferation in the stat4 morphants, indicating repressed proliferation and migration of endothelial precursors in stat4 morphants.

We also wondered whether stat4 regulated the fate equilibrium between the endocardium and great vessel endothelium derived from nkg2.5+ endothelial precursors. No labelled cells migrated to the heart tube in morphants, as shown in Fig. 5e. Using the Tg(fl:h: nucEGFP) line, we counted the endocardium cell numbers in the heart and outflow tract at 55 hpf and found no obvious changes in cell numbers in the morphants compared with the controls (Fig. 5c,f,h). Hence, stat4 was not involved in the fate determination between the great vessel endothelium and the endocardium. Evaluation of myod indicated no defects in the PAA- and facial-muscle lineage or in the somatic skeletal muscle of mutants (Fig. 5k,l). Only the distance between PAA- and facial-muscle lineage or in the somatic skeletal muscle in stat4 mutants was shorter than that in the control embryos.

The specification of endothelial precursors in the ALPM was tested by comparing the morphogenesis of the four pairs of nkg2.5+ pharyngeal cluster region at 55 hpf, found no obvious differences in cell numbers in the morphants compared with the controls (Fig. 5c,f,h). Hence, stat4 was not involved in the fate determination between the great vessel endothelium and the endocardium. Evaluation of myod indicated no defects in the PAA- and facial-muscle lineage or in the somatic skeletal muscle of mutants (Fig. 5k,l). Only the distance between PAA- and facial-muscle lineage or in the somatic skeletal muscle in stat4 mutants was shorter than that in the control embryos.

The specification of endothelial precursors in the ALPM was tested by comparing the morphogenesis of the four pairs of nkg2.5+ pharyngeal cluster region at 55 hpf, found no obvious differences in cell numbers in the morphants compared with the controls (Fig. 5c,f,h). Hence, stat4 was not involved in the fate determination between the great vessel endothelium and the endocardium. Evaluation of myod indicated no defects in the PAA- and facial-muscle lineage or in the somatic skeletal muscle of mutants (Fig. 5k,l). Only the distance between PAA- and facial-muscle lineage or in the somatic skeletal muscle in stat4 mutants was shorter than that in the control embryos.

The specification of endothelial precursors in the ALPM was tested by comparing the morphogenesis of the four pairs of nkg2.5+ pharyngeal cluster region at 55 hpf, found no obvious changes in cell numbers in the morphants compared with the controls (Fig. 5c,f,h). Hence, stat4 was not involved in the fate determination between the great vessel endothelium and the endocardium. Evaluation of myod indicated no defects in the PAA- and facial-muscle lineage or in the somatic skeletal muscle of mutants (Fig. 5k,l). Only the distance between PAA- and facial-muscle lineage or in the somatic skeletal muscle in stat4 mutants was shorter than that in the control embryos.

The specification of endothelial precursors in the ALPM was tested by comparing the morphogenesis of the four pairs of nkg2.5+ pharyngeal cluster region at 55 hpf, found no obvious differences in cell numbers in the morphants compared with the controls (Fig. 5c,f,h). Hence, stat4 was not involved in the fate determination between the great vessel endothelium and the endocardium. Evaluation of myod indicated no defects in the PAA- and facial-muscle lineage or in the somatic skeletal muscle of mutants (Fig. 5k,l). Only the distance between PAA- and facial-muscle lineage or in the somatic skeletal muscle in stat4 mutants was shorter than that in the control embryos.

The specification of endothelial precursors in the ALPM was tested by comparing the morphogenesis of the four pairs of nkg2.5+ pharyngeal cluster region at 55 hpf, found no obvious differences in cell numbers in the morphants compared with the controls (Fig. 5c,f,h). Hence, stat4 was not involved in the fate determination between the great vessel endothelium and the endocardium. Evaluation of myod indicated no defects in the PAA- and facial-muscle lineage or in the somatic skeletal muscle of mutants (Fig. 5k,l). Only the distance between PAA- and facial-muscle lineage or in the somatic skeletal muscle in stat4 mutants was shorter than that in the control embryos.

The specification of endothelial precursors in the ALPM was tested by comparing the morphogenesis of the four pairs of nkg2.5+ pharyngeal cluster region at 55 hpf, found no obvious differences in cell numbers in the morphants compared with the controls (Fig. 5c,f,h). Hence, stat4 was not involved in the fate determination between the great vessel endothelium and the endocardium. Evaluation of myod indicated no defects in the PAA- and facial-muscle lineage or in the somatic skeletal muscle of mutants (Fig. 5k,l). Only the distance between PAA- and facial-muscle lineage or in the somatic skeletal muscle in stat4 mutants was shorter than that in the control embryos.

The specification of endothelial precursors in the ALPM was tested by comparing the morphogenesis of the four pairs of nkg2.5+ pharyngeal cluster region at 55 hpf, found no obvious differences in cell numbers in the morphants compared with the controls (Fig. 5c,f,h). Hence, stat4 was not involved in the fate determination between the great vessel endothelium and the endocardium. Evaluation of myod indicated no defects in the PAA- and facial-muscle lineage or in the somatic skeletal muscle of mutants (Fig. 5k,l). Only the distance between PAA- and facial-muscle lineage or in the somatic skeletal muscle in stat4 mutants was shorter than that in the control embryos.

The specification of endothelial precursors in the ALPM was tested by comparing the morphogenesis of the four pairs of nkg2.5+ pharyngeal cluster region at 55 hpf, found no obvious differences in cell numbers in the morphants compared with the controls (Fig. 5c,f,h). Hence, stat4 was not involved in the fate determination between the great vessel endothelium and the endocardium. Evaluation of myod indicated no defects in the PAA- and facial-muscle lineage or in the somatic skeletal muscle of mutants (Fig. 5k,l). Only the distance between PAA- and facial-muscle lineage or in the somatic skeletal muscle in stat4 mutants was shorter than that in the control embryos.

The specification of endothelial precursors in the ALPM was tested by comparing the morphogenesis of the four pairs of nkg2.5+ pharyngeal cluster region at 55 hpf, found no obvious differences in cell numbers in the morphants compared with the controls (Fig. 5c,f,h). Hence, stat4 was not involved in the fate determination between the great vessel endothelium and the endocardium. Evaluation of myod indicated no defects in the PAA- and facial-muscle lineage or in the somatic skeletal muscle of mutants (Fig. 5k,l). Only the distance between PAA- and facial-muscle lineage or in the somatic skeletal muscle in stat4 mutants was shorter than that in the control embryos.

The specification of endothelial precursors in the ALPM was tested by comparing the morphogenesis of the four pairs of nkg2.5+ pharyngeal cluster region at 55 hpf, found no obvious differences in cell numbers in the morphants compared with the controls (Fig. 5c,f,h). Hence, stat4 was not involved in the fate determination between the great vessel endothelium and the endocardium. Evaluation of myod indicated no defects in the PAA- and facial-muscle lineage or in the somatic skeletal muscle of mutants (Fig. 5k,l). Only the distance between PAA- and facial-muscle lineage or in the somatic skeletal muscle in stat4 mutants was shorter than that in the control embryos.

The specification of endothelial precursors in the ALPM was tested by comparing the morphogenesis of the four pairs of nkg2.5+ pharyngeal cluster region at 55 hpf, found no obvious differences in cell numbers in the morphants compared with the controls (Fig. 5c,f,h). Hence, stat4 was not involved in the fate determination between the great vessel endothelium and the endocardium. Evaluation of myod indicated no defects in the PAA- and facial-muscle lineage or in the somatic skeletal muscle of mutants (Fig. 5k,l). Only the distance between PAA- and facial-muscle lineage or in the somatic skeletal muscle in stat4 mutants was shorter than that in the control embryos.

The specification of endothelial precursors in the ALPM was tested by comparing the morphogenesis of the four pairs of nkg2.5+ pharyngeal cluster region at 55 hpf, found no obvious differences in cell numbers in the morphants compared with the controls (Fig. 5c,f,h). Hence, stat4 was not involved in the fate determination between the great vessel endothelium and the endocardium. Evaluation of myod indicated no defects in the PAA- and facial-muscle lineage or in the somatic skeletal muscle of mutants (Fig. 5k,l). Only the distance between PAA- and facial-muscle lineage or in the somatic skeletal muscle in stat4 mutants was shorter than that in the control embryos.
precursors was not prevented by the suppression of stat4 (Fig. 5i,j), consistent with the results in Fig. 4a–d and Supplementary Fig. 9G,H,J,K. The schematic illustration in Fig. 5m shows the fate of nkx2.5+ cells in the ALPM, and the loss of stat4 can inhibit the proliferation of nkx2.5+ endothelia precursor clusters 3–6 to cause insufficient assembly of the primitive great vessels.

**Stat4 acts downstream of Nkx2.5 to regulate PAA angioblasts.** Notably, nkx2.5 knock down reduced the number of PAAs ACs, which showed a similar phenotype as the stat4 morphant embryos, consistent with the previous study12. Co-suppression of stat4 and nkx2.5 by half dosages of morpholinos caused a total depletion of ACs 3–6. Overexpression of nkx2.5 transcripts by injecting *in vitro* synthesized full-length nkx2.5 mRNA did not restore the tie1+ AC quantity of stat4 morphants. In contrast, replenishment of stat4 mRNA rescued the reduction in the number of ACs in nkx2.5 morphant embryos, as shown by the increase in the percentage of embryos with over two pairs of ACs 3–6 to control levels (Fig. 6a–f).

The chromatin immunoprecipitation (ChIP) assay showed that Nkx2.5 can bind to the promoter region of stat4, and the binding can be inhibited by nkx2.5 knock down. The Nkx2.5 DNA-binding site (NKE)22 was found in the binding enrichment region at the stat4 locus using primer 5, suggesting that stat4 was a direct target of Nkx2.5 (Fig. 6g).

Cellular autonomous analysis of Stat4 was carried out by utilizing an nkx2.5 promoter and the tol2 transposon to generate chimeric embryos with transient gene expression in nkx2.5+ cells, as shown in Fig. 6h,n. Driven by the nkx2.5 promoter, full-length (f) or N-terminal truncated forms (Δ) of Stat4 were expressed with high fidelity in nkx2.5+ cells, as indicated by the mCherry fluorescence, within bilateral ACs at 32 hpf. The *tie1 in situ* hybridization of Opsin-treated ACs showed a dominant-truncated Stat4 in nkx2.5+ cells and the negative embryos (ANG) reveals that Opsin embryos displayed a reduced number of ACs compared with ANG and controls.

Overexpression of full-length Stat4 in nkx2.5+ cells (POS) can sufficiently reverse the reduced number of ACs caused by the nkx2.5 morpholino (NG) (Fig. 6h–q).

**Stat4 regulates angioblasts by suppressing Stat1a and Hdac3.** To understand the downstream signalling of Stat4 in this process, a small molecule lisofylline, the canonical inhibitor of IL-12-mediated *stat4* activation23, was used to treat the embryos. The *tie1 in situ* hybridization of Lisofylline-treated embryos showed that the number of PAA angioblast cords did not decline, indicating that IL-12 signalling was not involved in the angioblast formation. However, overexpression of the Stat4-specific inhibitor Socs3a/b24 did reduce the number of *tie1*-expressing cords. Similarly, overexpression of another Stat4-specific inhibitor, Pias2 (previously known as Piasx25), also resulted in a decrease of *tie1*-expressing cords, mimicking the phenotype of stat4 mutants (Fig. 7a–e).

Many studies have confirmed the association of the Stat4-Stat1 locus in chronic inflammation diseases26, and Stat4 can counteract the function of Stat1 in T cells27. Hence, we knocked down stat1a and stat1b in *stat4* mutants and found that the knockdown of *stat1a* can partially rescue the loss of ACs but not *stat1b* (Fig. 7i,j). It has been reported that Hdac3 deacetylates Stat1, thus permitting its phosphorylation and ability to inhibit proliferation. Notably, HDAC type I and II inhibitor Trichostatin A (TSA)-treated embryos rescue the PAA angioblast cluster loss seen in mutants. It has been reported that HDACs 3 is responsive to TSA treatment in vascular endothelium28. We found that the injection of *hdac3* mRNA can efficiently inhibit vasculogenesis of the great vessels. Moreover, morpholino-mediated *hdac3* knock down can rescue the PAAs phenotype of the *stat4* mutant (Fig. 7f–h,k).

The ChIP assays showed that Stat4 can target the promoter regions of *hdac3* and *stat1a*, and the binding was mostly enriched in the *hdac3* promoter region identified by primer 3 (Fig. 7l–n). Furthermore, the levels of Hdac3 and Stat1 proteins were upregulated in *stat4* mutants, as shown in the western blot, while...
the Nkx2.5 protein level was not affected (Fig. 7p and Supplementary Fig. 10). Expression level changes of these genes were confirmed by quantitative PCR (Q-PCR) results. The cell proliferation-related gene cdk2 was significantly downregulated, whereas the expression levels of its inhibitors cdkn2a/b and atrip were elevated in the absence of stat4 (Fig. 7o). Based on the described results, we show a schematic representation of the signalling pathway regulated by stat4 and involved in PAA angioblast formation (Fig. 7q).

Discussion

The deficiency of primitive great vessels is a key predisposing factor for cardiovascular disease. Unlike the relatively advanced knowledge of endothelial development, little information about the regulation of endothelial precursors of the great vessels has been available to date. Our findings demonstrate that Stat4 has a critical role in promoting vasculogenesis of the great vessels. Several lines of evidence support this conclusion: (1) stat4 transcripts are located specifically in the pharynx and persisted throughout PAA vasculogenesis; (2) loss of stat4 selectively causes the stenosis of PAAAs 3–6 by suppressing angioblast emergence of the great vessels in mutants; (3) lack of stat4 inhibits the proliferation but not the apoptosis, fate equilibrium or specification of nkh2.5 endothelial precursors; (4) Stat4 expression is regulated by Nkh2.5 to autonomously promote PAA angioblast formation; and (5) Stat4 promotes PAA angioblast emergence by inhibiting...
epigenetic regulator \(hdac3\) expression and countering the effects of \(stat1\).

PAA endothelial progenitors derive from the caudal \(nkx2.5^+\) clusters in the ALPM\(^{12}\). We found that the proliferation of these progenitors is \(Stat4\) dependent. The \(nkx2.5^+\) PAA endothelial progenitors could still differentiate into arch artery endothelium without \(Stat4\). However, lack of \(stat4\) prevents proliferation of \(nkx2.5^+\) cells and eventually results in stenosis of the primitive GVs. A previous study showed that \(tcf21\), together with \(nkx2.5\), marked a group of PAA progenitor cells, but the \(tcf21^+ nkx2.5^+\) progenitors were not required for PAA formation\(^{10}\). In this study, we found that genetic ablation of \(stat4\) did not affect the \(tcf21\)-expressing PAA cells. The PAA- and facial-muscle also remained normal in \(stat4\) mutants, indicating that the muscle cell lineage of PAA was not affected by the loss of \(stat4\). This evidence implicated that \(stat4\) might specifically regulate the
PAA endothelial cell lineage rather than other cell lineages of PAA.

Although Stat4 has been most extensively investigated as a critical mediator for several pro-inflammatory cytokines and chemokines such as IL-12 and type I interferon in T lymphocytes, it was unknown whether it had any role in vasculogenesis. Several studies have provided clues that the Stat4-mediated inflammation is involved in cardiovascular diseases. STAT4 has been
demonstrated to be one of the strongest genetic susceptibility factors for systemic lupus erythematosus, and notably, patients with systemic lupus erythematosus with the *STAT4* risk allele have a strikingly increased risk of cardiovascular disease. *STAT4* has been found to be involved in the development of cardiac allograft vasculopathy in mice. Also, PIAS2, the specific inhibitor of *STAT4*, has a critical role in human umbilical vein endothelial cells. This evidence implicates a genetic predisposition via *STAT4* to some cardiovascular diseases.

Some of the *Stat4*-related pro-inflammatory factors may participate in the vasculogenesis of great vessels. Recent studies have highlighted a paradigm where the endogenous mechanisms of pro-inflammatory factors function to maintain normal tissue homeostasis. Pro-inflammatory cytokines and chemokines are also reported to be expressed in the hearts of infants with congenital heart disease with GVDs. Low levels of *STAT4* are expressed in cultured human umbilical vein endothelial cells and are tyrosine-phosphorylated by interferon but not IL-12 (ref. 36). We show here that canonical IL-12-Stat4 signalling does not participate in *Stat4*-mediated PAA vasculogenesis, however, *Stat4*-specific inhibition by Pias2, Socs3a and Socs3b suppresses PAA angioblasts formation. Most SOCS proteins act in a classical negative-feedback loop to inhibit cytokine signal transduction. Socs3 can specifically inhibit the activation of Stat4 by binding to the Stat4 docking site in its upstream receptor through the SH2 domain. In addition, socs3a and socs3b are found to be highly expressed in *nkx2.5* cells, together with the upregulation of *stat4* in this study (Supplementary Fig. 2).

Since previous studies have confirmed the association of *STAT4*-STAT1 locus on chromosome 2 (Fig. 1a) in inflammatory diseases and *stat1a* was enriched in the *nkx2.5* cells in our microarray, we speculated that Stat1 may be involved in the Stat4 signalling network. Indeed, repression of *stat1a* can partially rescue GVDs caused by the absence of *stat4*. Previous studies have shown that *stat1* has anti-proliferation roles in pro-inflammatory cells and vasculogenesis. Moreover, *stat4* acts as a key molecule in overcoming the Stat1-dependent inhibition of proliferation in T cells. Hence, it is possible that *stat4* can activate the proliferation of PAA by inhibiting Stat1a. It is reported that HDAC can modulate the pro-inflammatory response, thus, it was conceivable to hypothesize that HDAC may be involved in the Stat4 signalling in GV vasculogenesis. Indeed, we show that treatment with the HDAC type I and II specific inhibitor TSA can reverse the PAA phenotype of *stat4* mutant embryos, and *Hdac3* has an important role in PAA vasculogenesis downstream of Stat4. A recent study showed that TSA could enhance vascular repair by recruiting human endothelial progenitors and increasing the expression of SCL-dependent genes, which may support our findings here.

It is reported that HDAC3 inhibitor has no significant effect on the heart. We also confirmed that *stat4* deficiency did not have evident effects on the heart during PAA formation. Meanwhile, Stat1 can be deacetylated by Hdad3, thus permitting its phosphorylation. Based on our data and the previous reports, *Hdac3* seems to be an important target of *stat4* in mediating the proliferation of the endothelial precursor of great vessels.

In summary, our study provides insights into a potential role of the pro-inflammatory regulator Stat4 in great vessel establishment and endothelial precursor proliferation. Therefore, Stat4 can possibly serve as a diagnostic marker or a therapeutic target for GVD.

**Methods**

**Zebrafish strains maintenance.** Zebrafish embryos were raised at 28.5 °C and morphologically staged. The wild-type lines AB/Tubingen, Tg(nkx2.5:ZsYellow), Tg(stat1a:DRed), Tg(fli1:GFP), Tg (tc21: GFP) and Tg(fli1:nuocGFP) lines have been previously reported. Embryos or tail fins were collected for genotyping. All zebrafish in this study were treated in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The experimental protocols were approved by the Review Board on the Ethics of Animal Experiments of Institute of Health Sciences, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences (Shanghai, China).

**FAC sorting and microarray analysis.** The embryos of Tg(nkx2.5:ZsYellow) at 30 hpf were dissociated by incubation in 1 × PBS supplemented with 0.125% dispase (Life Technologies, NY, USA) for 30 to 45 min at 28.5 °C. The cells were washed and resuspended in ice-cold 0.9 × PBS plus 5% FBS and were passed through a filter with a 40 μm pore size. Samples were sorted twice into lysis buffer via a triple-LASER fluorescence-activated cell sorting instrument (FACSaria, BD Biosciences, CA, USA) to ensure cell purities of >95%, maximal RNA integrity, and minimal loss of material. Two independent sets of purified RNA from *nkx2.5* cells and *nkx2.5* somatic cells were prepared and labelled according to the manufacturer’s instructions (Affymetrix, Santa Clara, CA, US). Samples were then hybridized onto a Zebrafish Oligonucleotide Microarray (AffymetrixGeneChip). The Affymetrix(R) software and GeneSpring software were used to analyse the data and visualize differential expression, respectively.

**Construction and microinjection of CRISPR/Cas.** The *stat4* target sites of CRISPR/Cas were determined online (http://crispr.mit.edu/). For the efficient induction of mutagenesis in *stat4*, one target site was finally selected from the candidate sites. *Stat4* guide RNA (gRNA) was targeted to exon 3 (3’- ATAGGGCAT CAAACCATGAATCTAGT-5’ and reverse) with forward (5’-TAAAGGCAT CAAACCATGAATCTAGT-3’) and reverse (5’-TAAAGGCAT CAAACCATGAATCTAGT-3’) primers. *Stat4* gRNA was obtained by in vitro transcription with the MAXISCRIP T7 Kit (Ambion, Thermo Fisher Scientific Inc., US) and then purified using the mirVana miRNA Isolation Kit (Ambion) following the manufacturer’s instructions. CRISPR cycled nls-zCas-nls RNA was synthesized using SP6 mMessage mMACHINE Transcription Kit (Ambion). A mixture of RNAs (500 ng/ml each of nls-zCas-nls RNA and 90 ng/ml of hRNA) in 0.05% phenol red and 120 mM KCl was injected into the cellular portion of one-cell stage embryos, resulting in an ~60% rate of deformity at 24 hpf.

**Generation of zebrafish *stat4* mutant lines.** The surviving embryos after injection with CRISPR/Cas RNAs were raised to adulthood and screened for

---

**Figure 7** | *stat4* promoted the PAA angioblast programme by suppressing stat1a and *hdac3*. (a-k) In situ hybridization analysis of *tie1* transcripts in the control (a), *stat4* mutants (b), embryos with 50 ng/ml *pias2* mRNA injection (c), embryos with 50 ng/ml *socs3a* and *socs3b* mRNAs injection (d), embryos treated with 85 μM lisofylline (e), *stat4* mutants with 0.2 μM TSA treatment (f), embryos with 50 ng/ml *hdac3* mRNA injection (g), *stat4* mutants with 4 ng/ml *hdac3* morpholino (h), *stat4* mutants with 4 ng/ml *socs3a* morpholino (i), *stat4* mutants with 4 ng/ml *socs3b* morpholino (j) at 44 hpf. Scale bars, 50 μm. (k) Proportional quantification of indicated *tie1* expressing cords number (sd / *n* : *stat4* mutants, Kruskal–Wallis test with the Dunn’s multiple comparison test, **P < 0.01, n > 30 embryos per each group. (l-n) The *ChIP* analysis with anti-Stat4 antibody for the promoter regions of *hdac3* and stat1a in the wild-type control and *stat4* mutants at 44 hpf across six experimental replicates (*n = 30 embryos/replicate in each group, analysis of variance (ANOVA) test with multiple comparison post-hoc test, html : **P < 0.01, ***P < 0.001). (m) The binding enrichment regions at the *hdac3* and *stat1a* locus are indicated by primers (P1-P6). (o) Q-PCR results of related genes expression in the control and *stat4* morphants at 44 hpf across six experimental replicates (*n = 10 embryos/replicate in each group. Error bars indicate s.d., unpaired two-tailed Student’s *t*-test, **P < 0.05, ***P < 0.01). (p) Protein levels of *Nkx2.5*, *Hdac3* and Stat1a in the wild-type control and *stat4* mutants detected by western blots at 44 hpf. β-actin is used as the internal control. (q) A network is drawn to illustrate the *stat4* pathway in controlling PAA proliferation. Red: activation; Blue: suppression.
founders with germ-line mutations in the \textit{stat1} locus. The T7 endonuclease I (T7E1) enzyme was used to screen for mutations (New England Biolabs, MA, USA) according to the manufacturer’s instructions. After the outcross between the founders and wild-type zebrafish, the outcross between the F1 and the wild-type, and the subsequent incross between the heterozygous F2 generation, we obtained several mutant lines for further investigation.

**Microinjection of zebrafish embryos.** Knockdown of genes was achieved by the injection of corresponding anti-sense morpholinos into one-cell-stage embryos. Morpholinos were purchased from Gene Tools (USA). Nkx2.5 morphants were obtained via the injection of 1 nl of Nkx2.5 morpholino (4 ng\(^{-1}\) lnl) targeting the \textit{nks2.5 splicing site}43. To generate \textit{stat4} morphants, one-cell stage embryos were injected with 1 nl of anti-sense morpholino (6 ng\(^{-1}\) lnl) targeting the third-intron fourth exon splice acceptor sequence (\textit{M\textsuperscript{stat4}}\textsuperscript{44}; 5'-GTATTGCATTAGTGTGGCGAGAT AAGAAGA-3'). To validate the effectiveness of \textit{M\textsuperscript{stat4}}-mediated \textit{stat4} splicing inhibition, obtained adults with the same phenotype were used to produce first-strain complementarity cDNAs (cDNA) by reverse transcription from control and \textit{M\textsuperscript{stat4}}-embryos and performed PCR to amplify the sequence spanning the \textit{M\textsuperscript{stat4}} target region with forward (5'-TTACGACAGCGTATTAGGCTCTTG CAA-3') and reverse (5'-TGAAAGTCTCCTTATACA GCGC-3') primers. Then, agarose gel electrophoresis of the PCR products was used to visualize the bands.

Capped tol2 transposase and \textit{nks2.5}, \textit{stat4}, \textit{socs3a}, \textit{socs3b} and \textit{pias2} mRNA were synthesized in vitro from linearized plasmids by SP6 Message MMachine Transcription Kit (Ambion, Supplementary Table 2). Rescue assays were carried out by co-injection of 50 ng\(^{-1}\) mRNA into one-cell-stage embryos. A 4.8-kb \textit{nks2.5} promoter was prepared from Tubingen genome using the forward (5'-GTAGACTGAGTATTCGTTGCGTTAT-3') and reverse (5'-CTAAGGCGGTGGTCTTATACATTAAT-3') primers. The \textit{nks2.5} mRNA was injected into the cellular portion of the one-cell-stage embryos at 50 ng\(^{-1}\) lnl.

**RNA probe synthesis and whole mount in situ hybridization.** Zebrafish wild-type cDNA was used for amplifying \textit{stat4}, \textit{tie1}, \textit{gata4}, \textit{etv2}, \textit{tki}, \textit{thbx1}, \textit{foxi1} and \textit{rgl} sequences that were subcloned into pCS2\textsuperscript{+} vector (Supplementary Table 2). T3 RNA polymerase (Roche Applied Science, IN, USA) was used to generate RNA probes transcripts from linearized DNA templates. Whole-mount in situ hybridizations were performed according to the DIG labelled anti-sense RNA probes (Roche Applied Science, Mannheim, Germany). Embryos were collected and fixed in 4% paraformaldehyde. Following dehydration and rehydration in a decreasing methanol series, the embryos were digested with 0.5 ng\(^{-1}\) lnl of CellTracker Red stock solution and photographed.

The TUNEL assay was performed with the In Situ Cell Death Detection Kit TMR red (Roche). The morpholino injected Tg(nks2.5;ZsYellow) embryos and wild-type embryos at 28 hpf were fixed and stored in 100% methanol at –20°C. After rehydration, Proteinase K digestion and aceton treatment, embryos were permeated with a permeabilization solution (0.5% Triton X–100, 0.1% sodium citrate in PBS) at room temperature for 1 h. Then, the embryos were stained by the enzyme solution and photographed.

**Cell tracker red labelling.** Cell Tracker Red dye was purchased from Invitrogen and diluted to 10 \textmu M in egg water. Tg(nks2.5;ZsYellow) embryos at 28 hpf were injected with an 1 nl drop of dye in the pharyngeal ZsYellow region outside the heart tube. Following the injection, images were taken at ~32 hpf and again at 55 hpf.

**Chromatin immunoprecipitation (ChIP).** Chromatin was prepared from zebrafish embryos at 44 hpf and fragmented by sonication with Fisher Scientific’s Sonic Dismembrator (6% amplitude, pulse for 10 s on and 30 s off for a total sonication ‘on’ time of 2 min 40 s) to produce fragments ranging from 100 to 500 bp. Then, five percent chromatin was removed from each sample and used as the input control. ChIP was performed using antibodies specific for Nkx2.5 (PA3-49431, Thermo Fisher, US), Stat4 (WH006775M1, Sigma, US) and IgG control (2719p, Cell Signaling, US) at dilutions of 1:100, 1:100 and 1:1,000, respectively. The bound DNA and mock DNA were used for SYBR green Q-PCR analyses.

**Western blot analysis.** Western blot was performed with the standard protocols. Protein was prepared from zebrafish embryos at 44 hpf and dissolved in 4 x protein SDS–polyacrylamide gel electrophoresis Loading Buffer (Takara). Nkx2.5, Stat1a and Hdc3 were detected with anti-Nkx2.5 (PA5–49431, Thermo Fisher, US), anti-Stat1a (SABS005364, Sigma, US) and anti-Hdac3 (ab32369, Abcam, US) at the dilution of 1:1,000, followed by incubation with an anti-rabbit IgG horse-radish peroxidase antibody (ab97969, Abcam, US) at a dilution of 1:5,000. \textbeta-actin was used as the internal control using an anti-\textbeta-actin antibody (A2228, Sigma, US), followed by an anti-mouse IgG antibody (62-6520, Invitrogen, US) diluted 1:1,000 and 1:5,000 in the block solution, respectively. Full scans of all western blots are presented in Supplementary Fig. 10.

**Confluent fluorescent imaging.** Whole mount transgenic embryos were prepared in 3% agarose in a glass-bottomed culture dish, and the images were captured using an Olympus FV10-ASW confocal microscope system (Olympus, Tokyo, JP)\textsuperscript{45}. EGFp was excited with a 488-nm Argon laser and imaged through a 505-536-nm filter. ZsYellow protein was excited with a 514-nm Argon laser and imaged through a 530-nm long pass filter. DsRed was excited with a 543-nm HeNe laser and imaged through a 560-nm long pass filter. mCherry was excited with a 564-nm HeNe laser and imaged through a 595-nm filter. The cells were counted in 2-stack confocal images using the Imagel\textsuperscript{46} software.

**Quantitative PCR analysis.** Total RNA was reverse transcribed into cDNA with SuperScript III Reverse Transcriptase (Invitrogen). Quantitative real-time PCR (qPCR) was performed with SYBR Green (TOYOBO) on a 7900HT Fast Real-Time PCR System (Applied Biosystems). The relative RNA amount was calculated with the AACT method and normalized with \textit{gpaD} (the primers are listed in Supplementary Table 2).

**Statistical analysis.** All biological experiments were performed at least three times. Data were analyzed using Graphpad Prism 6 software. Quantitative data were presented as the mean ± s.e.m. The normality of data was examined by the Shapiro-Wilk test. The data with normal distributions were analysed by the unpaired Student’s two-tailed \textit{t}-test between two groups, while the analysis of variance test with the multiple comparison \textit{post-hoc} test was used to compare three or more groups. The data of non-normal distributions were analysed by the Kruskal-Wallis test with adjustments for multiple comparisons\textsuperscript{69}. Statistical significance was denoted by *\textit{P}<0.05, **\textit{P}<0.01, ***\textit{P}<0.001. The number of ACs (1 to 4) on the same side of the pharynx was counted to evaluate the deficiency of PAA3s – 6. Dysmorphic animals due to unknown developmental delays were excluded equally from the control and experimental groups.

**Data availability.** Microarray data have been deposited in ArrayExpress with the accession number E-MTAB-5406. All relevant data supporting the findings of this study are available within the paper and its supplementary information files, or from the authors on request.

**References**

1. The Global Burden of Disease Study C. Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013. \textit{Lancet} 386, 743–800 (2015).
2. van der Linde, D. et al. Birth prevalence of congenital heart disease worldwide: a systematic review and meta-analysis. J. Am. Coll. Cardiol. 58, 2241–2247 (2011).

3. Smiseth, O. A. et al. The heart: implications for congenital heart disease. Annu. Rev. Physiol. 63, 451–469 (2001).

4. Berguer, R. Function and Surgery of the Carotid and Vertebral Arteries 1st edn (LWW, 2013).

5. Brown, M. L. et al. Coercation of the aorta: lifelong surveillance is mandatory following surgical repair. J. Am. Coll. Cardiol. 62, 1020–1025 (2013).

6. Congdon, E. D. Commitment of the aortic-arch system during the development of the human embryo. Contrib. Embryol. 14, 49–U47 (1922).

7. Graham, A. Development of the pharyngeal arches. Am. J. Med. Genet. A 119A, 251–256 (2003).

8. De Val, S. & Black, L. B. Transcriptional control of endothelial cell development. Dev. Cell 16, 180–195 (2009).

9. Schmidt, A., Brixius, K. & Bloch, W. Endothelial precursor cell migration during vasculogenesis. Circ. Res. 101, 125–136 (2007).

10. Nagelberg, D. et al. Origin, specification, and plasticity of the great vessels of the heart. Curr. Biol. 25, 2099–2110 (2015).

11. Moore, K. L., Persaud, T. V. N. & Torcia, M. G. The Developing Human: Clinically Oriented Embryology 10th edn (Saunders, 2015).

12. Paffett-Lugassy, N. et al. Heart field origin of great vessel precursors relies on nkx2.5-mediated vasculogenesis. Nat. Cell Biol. 15, 1362–U1225 (2013).

13. Zhou, Y. et al. Latent TGF-β binding protein 3 identifies a second heart field in zebrafish. Nature 474, 645–648 (2011).

14. Watanabe, Y. et al. Role of mesodermal FGF8 and FGF10 overlaps in the development of the aortic pole of the heart and pharyngeal arch arteries. Circ. Res. 106, 495–U410 (2013).

15. Zhang, Z. et al. Tbx1 expression in pharyngeal epithelia is necessary for pharyngeal arch artery development. Development 132, 5307–5315 (2005).

16. Vokes, S. A. & Krieg, P. A. Endoderm is required for vascular endothelial tube formation, but not for angioblast specification. Development 129, 775–785 (2002).

17. Hiruma, T., Nakajima, Y. & Nakamura, H. Development of pharyngeal arch arteries in early mouse embryo. J. Anat. 201, 15–29 (2002).

18. Korman, B. D., Kastner, D. L., Gregersen, P. K. & Remmers, E. F. STAT4: genetics, mechanisms, and implications for autoimmunity. Curr. Allergy Asthma Rep. 8, 398–403 (2008).

19. Watford, W. T. et al. Signaling by IL-12 and IL-23 and the immunoregulatory roles of STAT4. Immunity 20, 139–156 (2004).

20. Gorissen, M., de Vrieze, E., Flik, G. & Huisings, M. O. STAT genes display differential evolutionary rates that correlate with their roles in the endocrine and immune system. J. Endocrinol. 209, 175–184 (2011).

21. Proux, K., Lu, A. & Sumanas, S. Cranial vasculature in zebrafish forms by angioblast cluster-derived angiogenesis. Dev. Biol. 348, 34–46 (2010).

22. Ferdous, A. et al. Nkx2.5 transactivates the Ets-related protein 71 gene and specifies an endothelial/endocardial fate in the developing embryo. Proc. Natl Acad. Sci. USA 106, 814–819 (2009).

23. Coon, M. E., Diegel, M., Leshinsky, N. & Klaus, S. J. Selective pharmacologic inhibition of murine and human IL-12-dependent Th1 differentiation and STAT4 and the risk of rheumatoid arthritis and systemic lupus erythematosus. Nat. Med. 15, 977–986 (2009).

24. Gil, M. P. et al. Regulating type 1 IFN effects in CD8 T cells during viral infection. Nature 454, 717–725 (2013).

25. Wang, L. et al. STAT4 signaling during neutrophil activation. Nature 454, 717–725 (2008).