CHAPTER V

Identification of P38α target genes involved in the physiological angiogenesis

Hanan Rian, Shuning He, Herman. P Spaink and B. Ewa Snaar-Jagalska

Abstract

Developmental angiogenesis is one of the physiological processes were P38α MAPK signalling is found to be crucial. The formation of intersegmental blood vessels (ISVs) in the zebrafish embryos serves as a paradigm to study angiogenesis in vivo. Here we investigated the role of P38α in the embryonic angiogenesis and further focussed on the identification of the target genes.

To study the role of P38α in angiogenesis we performed knock-down experiments with gene specific morpholinos. Unsaturated knock-down of P38α in zebrafish resulted in a delayed formation of intersegmental vessels and expansion of the intermediate cell mass. Scanning confocal microscopy of Tg(fli:EGFP) embryos revealed absence of filopodia on the tip cells of growing ISV indicating defective endothelial cell migration in P38α morphants. The P38 morpholino phenotype with defective angiogenesis was confirmed by the P38 inhibitor BIRB796. When applied at the 4-8 somite stage the inhibitor completely blocked sprouting of the ISV. To identify P38α target genes involved in physiological angiogenesis we compared the P38α knock-down transcriptome with the GFP+ enriched gene set of Tg(fli:EGFP). T-cell activation RhoGTPase activating protein b (Tagapb) was affected by P38α morpholino and among the pool of GFP+ enriched genes. The vascular endothelial zinc finger 1a (Vezf1) affected by P38 morpholino, was not specifically expressed in the vasculature however was reported previously to play a role in angiogenesis. Furthermore the runt-related transcription factor 1 (Runx) transcription factor was upregulated in P38 morphants which plays an important role in definitive hematopoesis.

Introduction

The virulence component of anthrax lethal toxin lethal factor (LF) is an zinc-metalloprotease that cleaves the NH(2) termini of mitogen-activated protein kinase kinase 1 and 6 (Mekk) causing severe vascular damage.[1,2] This association of MAPK with vascular morphogenesis is further supported by numerous *in vitro* and *in vivo* studies which lead to the overall conclusion that Erk2, Erk5 and P38α MAPK are separately indispensible for angiogenesis: the branching of vasculature from preexisting blood vessels. In contrast, MAPK have not been reported to be involved in vasculogenesis: the formation of blood vessels from endothelial cell precursors (angioblasts). P38α is in particular involved in regulation of cell migration and permeability of endothelial cells[3]. Targeted P38α deletion causes placental angiogenesis defects and subsequent cardiovascular defects in the embryo resulting in lethality starting at E10.5.[4,5]

The P38α MAPK, and the other isoforms β, δ, γ of the P38 MAPK subfamily, are also known as stress activated protein kinases since activity is induced by stressors such endotoxins, hypoxia, cytokines, DNA damaging factors etc.[6] However P38α activity can be stimulated by growth factors and besides a role in stress signaling is also involved in physiological and developmental processes[7]. P38α and P38β, show a wide tissue expression and can be activated by Mek3 and Mek6 which are in turn activated by an Mapkkk (Mekk) [7-10]. Multiple Mekks are identified to function in the Mekk-Mek3/6-P38α/β cascade[11]. Mekk3, functional in both the P38α as Mek5-Erk5 cascade, was identified as an essential gene for vascular morphogenesis. Knock-out of Mekk3 in mice is lethal, starting at E11, due to extensive defects in blood vessel formation in the embryo, placenta and yolk sac [12].

Several *In vitro* studies have contributed to identification of the molecular signaling events of angiogenesis depending on P38α. P38α activation is triggered by the angio-proliferative factors as vascular endothelial growth factor (Vegf) or fibroblast growth factor 2 (Fgf2) which regulate different aspects of angiogenesis: endothelial tip cell sprouting, proliferation, lining, migration and tube formation. P38α regulates the expression of Vegf post transcriptionally by increasing mRNA half-life and is functional downstream of Vegf in endothelial cells as well [13]. Phosphorylation of the Tyrosine-1214 residue of Vegfr2 (kdrl, flk1), through ligand binding, leads to activation of P38α and subsequent Mapk activated protein kinase 2 (Mk2)-Hsp27 to drive actin remodeling in stress fibers [14,14-16]. Furthermore, Nck, Fyn, Pak2 and Cdc42 are found to function upstream of P38α when stimulated by Vegfr2 [17]. Alternatively, Raftk/Pyk2 and Src have been reported to increase P38α activity and regulate migration of endothelial cells in response to Vegf[18]. A negative feedback signal has also been reported. Vegfr2 induces expression of MiR-20a which blocks

expression of Mekk3 mRNA leading to decreased P38α activity and eventually inhibition of endothelial cell migration[19]. The Vegf-A bound Vegfr2 stimulates P38α activation when complexed with the co-receptor Neuropillin-1 (Nrp1) leading to vessel branching in subcutaneous matrigel plugs. Excessive vessel branching in matrigel plugs was observed after stimulation with Vegf-A165 a splice variant that complexes Vegfr2 with both coreceptors Nrp1 and heparin sulphate proteoglycan (Hspg) also leading to P38α activition[20]. Vegf-A165 induced endothelial cell organization and pericyte association was disturbed in subcutaneous matrigel plugs treated with the P38 inhibitor SB203580.

On the contrary, P38 stimulated with Fgf2 in endothelial cells has been suggested to have a negative effect on angiogenesis. Fgf2 caused a sustained P38 activity in vascular endothelial cells in vivo in the chick chorioallantoic membrane and inhibition with the P38 inhibitor SB202190 enhances neovascularisation. Based on this experiment Fgf2 stimulated P38 is suggested to inhibit endothelial cell survival, proliferation and differentiation in collagen gel cultures of bovine capillary endothelial cells [21].

Furthermore, P38 is likely to be involved in inflammation linked angiogenesis mediated by Interleukin 1β[22]. Endothelial tube formation and migration induced by Interleukin 1β was depending on Traf6 complex formation with caveolin-1 and subsequent P38-MK2 activation. TRAF6 activation of P38-Mapk is could have been mediated via the transforming growth factor (Tgf)-β-activated kinase 1 (Tak1)/Tak1-binding protein 1 (Tab1)/Tab2 signaling complex [23].

Targeted knock-out studies in mice have clearly demonstrated that the P38α is essential for angiogenesis and could not be compensated by the other P38 isoforms. Most studies of P38α dependant angiogenesis, tough mostly performed *in vitro* using non-selective P38 inhibitors, have identifyed some of the molecular events upstream of P38. Since P38α is able to affect the activity of many substrates including transcription factors the downstream signalling and gene expression during angiogenesis is still an unexplored area. At 1 day post fertilization the zebrafish forms the intersegmental vessels (ISV) allowing study of angiogenesis that can be visualized in detail. The growth of ISV along the somite boundaries is often assayed as measure for physiological angiogenesis. Since we have recently developed P38α specific antisense oligonucleotides (morpholinos) to knock-down P38α, we used this new tool to study the role of P38α in embryonic angiogenesis.

Material & Methods

Zebrafish husbandry

Zebrafish (Danio rerio) wild type line was maintained under standard conditions and guidelines given in the zebrafish book (Westerfield 200). Embryos were kept at 28°C or 31°C to adjust required speed of development for experiment.

Morpholino microinjection and life imaging

Tg(fli:EGFP)^{y1} (Lawson et al., 2002) embryos were injected between 0- and 1- cell stage with either P38αA translational blocking morpholino (ATG) 5'GTGGGTCTTTCTTTCTGCGACATGC3', or standard control morpholino 5'CCTCTTACCTCAGTTACAATTTATA3'(Genetools). Morpholino's where dissolved in 1XDanieau's buffer [58mM NaCl, 0.7mM KCl, 0.4mM $MgSO_4$, 0.6mM $Ca(NO_3)_2$, 5.0mM HEPES pH7.6] containing 1% phenol red solution. Injected embryos were kept at 28°C for live imaging.

Chemical Inhibitor treatment of *Tg(fli1:egfp)^{y1}*

The Pan-P38 inhibitor Birb-796 was dissolved in DMSO and used to block P38 activity in *Tg(fli:EGFP)^{y1}* embryos. Because the inhibitor cannot pass through the chorion sufficiently, embryos have been mechanically dechorionated in Danieau buffer into agarose coated dishes. 100 µM Birb796 was added at 4-7 somite stage before sprouting of ISVs (around 24somite stage). Embryos were kept at 28°C and staged in hours post fertilization (hpf) according to (Kimmel et al. 1995) for live imaging.

Protease dissociation of *Tg(fli1:egfp)^{y1}* and FACS

GFP+ cells were isolated from untreated *Tg(fli:EGFP)^{y1}* of 24hpf kept o/n at 28°C or 31°C. Embryos have been bleached and dechorionated prior to deyolking and dissociation. Dechorionation was performed by enzymatic digestion with Pronase (2 mg/ml Roche Applied Science) for 7 mintutes with subsequent washing in 1XDanieau's buffer. Dissociation was performed as previously reported (Covassin et al. 2006). On average 220 embryos per condition was sufficient for $1x10^7$ cells which on average would be sufficient for 1.4 µg of GFP^+ RNA. Dissociated cells were centrifuged for 5 min at 500 RCF and resuspended in Leibovitz medium L15 without phenol red, 1% fetal calf serum, 0.8 mM CaCl2, penicillin 50 U/mL and streptomycin 0.05 mg/mL. The cell suspension was filtered through a 50 µm CellTrics filter (Partec) for FACS (BD FACS AriaII cell sorter using a 70-100 µm nozzle). sorted GFP+ and GFP- cells were collected in 1/3 volume L15, 0.8 mM CaCl2, 10% fetal calf serum, 10% zebrafish embryo extract, penicillin 50 U/mL and streptomycin 0.05 mg/mL. Cells were immediately collected and lysed in QIAzol reagent for RNA isolation. On average 200 embryos per condition was sufficient for $1x10^7$ GFP^+ cells for a yield of 1.4 µg of RNA.

RNA isolation for q-PCR or transcriptome analysis

1/5 of chloroform was added to the Qiazol cell lysis, mixed and transferred to phase-lock gel heavy (5-Prime) containing eppendorf tubes of 1.5ml. The RNA liquid phase (top transparent solution) was separated from the rest of the sample by 15min at 12000 g and purified with rneasy minelute cleanup kit (Qiagen) according to manufacture protocol.

50ng of RNA was used for cDNA synthesis for qPCR expression analysis of fli and flt4 (primers Covassin et al.2006) relative to 18S rRNA (primers Tang et al. 2007).RNA samples were delivered to the microarray department University Amsterdam www.microarray.nl/ who performed a RNA quality check with the Agilent 2100 bioanalyzer using eukaryote total RNA pico series II protocol.

A two-color microarray-based gene expression analysis with treatment (GFP+) labelled Cy3 hybridized against control (GFP-) labelled Cy5 on an Agilent 180k chip. For analysis, data files (MAGE and JPEG files of each hybridized chip position) were imported into Rosetta Resolver 7.2 (Rosetta Inpharmatics LLC). Three biological replicate intensity profiles were combined using the default intensity experiment builder which is implemented in the Rosetta Resolver system. 28˚C GFP+ against GFP- (252823310038_2, 252823310039_4, 252823310040_2) and 31˚C GFP+ against GFP- (252823310038_3, 252823310039_1, 252823310040_4).

0.1pmol P38α morpholino or standard control morpholino was injected in zebrafish embryos and RNA was isolated at 24hpf (31˚C). RNA samples were used for transcriptome analysis with a common reference design (common reference=pool of all P38α morpholino and standard control samples) A two-color microarray-based gene expression analysis with treatment (P38α morpholino or standard control morpholino) labelled Cy5 hybridized against control (common reference) labelled Cy3 on an Agilent 180k chip. Three biological replicate intensity profiles were combined using the default intensity experiment builder P38α morpholino against common reference (P38α morpholino against common reference 252823310118_1, 252823310118_3, 252823310119_1), (standard control morpholino against common reference 252823310118_2, 252823310118_4, 252823310119_2).

Results

P38α regulates growth of ISV and is essential for zebrafish angiogenesis

P38αA is widely expressed in the most tissues and has an early developmental function in epiboly. Saturated knock-down with gene specific translational blocking morpholinos causes severe morphological abnormalities and lethality (**chapter IV**). Therefore we studied the role of P38α in embryonic angiogenesis under unsaturated knock-down conditions. We performed a titration experiment in Tg(fli:EGFP) with our previously designed morpholino to obtain the pool of embryos displaying vascular defects and minor morphological defects for further analysis. In the wild type embryos the intersegmental vessels (ISV) in the trunk arise from the lateral posterior mesoderm (LPM), and migrate to the dorsal aorta. From the dorsal aorta the ISV grow along the vertical somite boundaries dorsally at 20 somite stage. Reaching the dorsal most portion of the trunk the ISV form a T shape. Tips of neighbouring vessels connect into the dorsal longitudinal anastomotic vessels (DLAV) which runs horizontally. Unsaturated knock-down of P38α resulted in a significant delayed growth of ISV **Fig1a**.

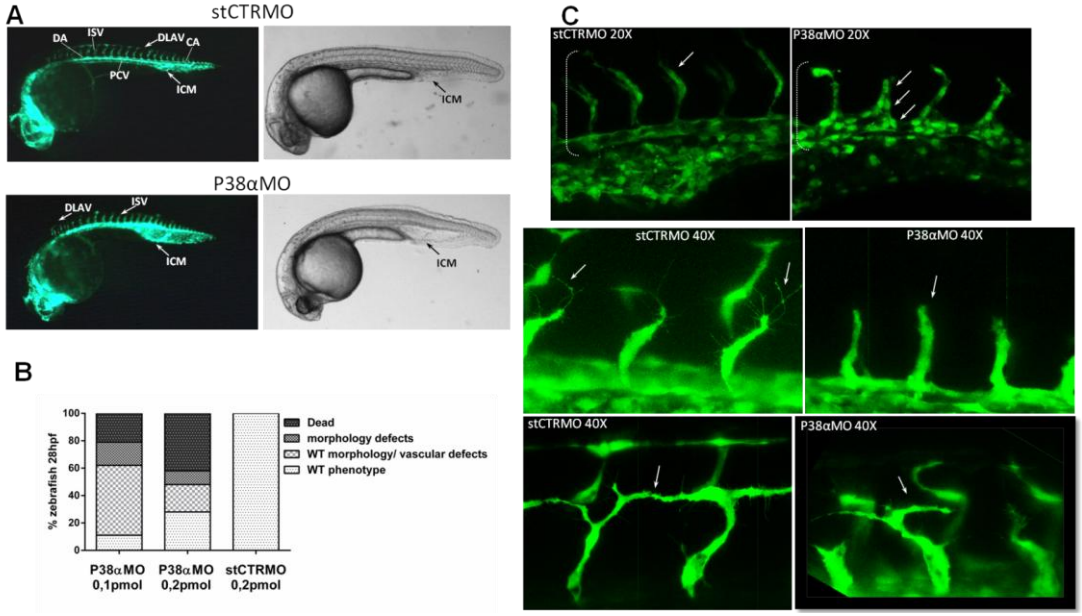

Fig1. Unsaturated morpholino knock-down of P38α delays development of ISV. **A:** Transmission and florescent stereo images of 1,5dpf (28˚C) Tg(Fli:eGFP) embryos injected with P38α or standard control morpholino. **B:** Optimization of unsaturated knock-down to obtain the pool of embryos displaying vascular defects and minor morphological defects. **C:** Confocal images showing ISV of Tg(Fli:eGFP) embryos injected with standard control morpholino **left** or P38α morpholino **right** 20X magnification: **upper panel**. Endothelial cells of P38α morphants display an aberrant morphology (more packed and less stretched). 1,5dpf P38α morphs have sprouted ISV that lack filopodia: **middle panel**. Formation of DLAV is delayed in P38α morphs: **lower panel**.

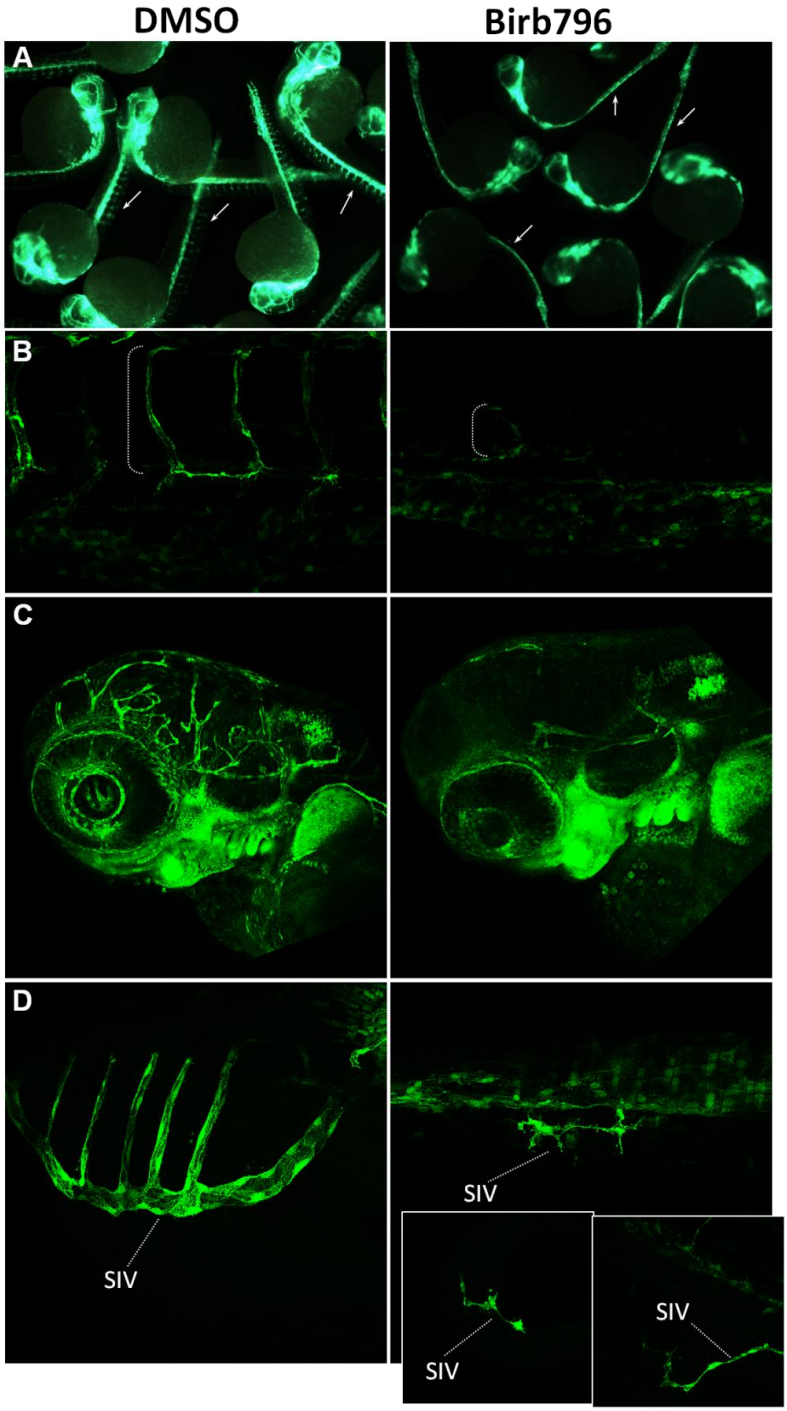

Fig2. The P38 inhibitor Birb798 blocks angiogenesis in the tail, head and formation of the SIV complex. Left: DMSO treated embryos, **Right**: Birb796 treated embryos. **A-B** Tg(Fli:eGFP) embryos treated with Birb796 lack sprouting of ISV at 1,5dpf. **A** Stereo images and **B** Confocal images 40X magnification **C** At 3,5dpf also brain, eye vasculature where underdeveloped 10X magnification and **D** the SIV was absent or malformed. 20X magnification.

With 0.1 pmol P38α morpholino a significant pool of embryos can be distinguished displaying morphological defects where the ISV have not grown into DLAV compared to standard control morpholino injected embryos **Fig1b**. In addition, an enlargement of the intermediate cell mass (ICM) was clearly visible in P38α morpholino injected embryos. Scanning confocal microscopy revealed absence of the filopodia on the tip cells of growing ISV indicating defective endothelial cell migration in P38α morphants **Fig1c**. Endothelial cells of ISV appeared also more stretched in control embryos than in P38α morphants. The P38 morpholino phenotype on sprouting ISV was confirmed by the pan P38 inhibitor BIRB796 **Fig2**. When applied between 4-7 somite stage the inhibitor blocked sprouting of ISV from the dorsal aorta **Fig2a-b**. BIRB796 was also applied at 2dpf resulting in undeveloped head/eye vasculature. After this treatment the subintestinal vein (SIV) complex was malformed or completely absent at 3,5dpf **Fig2c-d**.

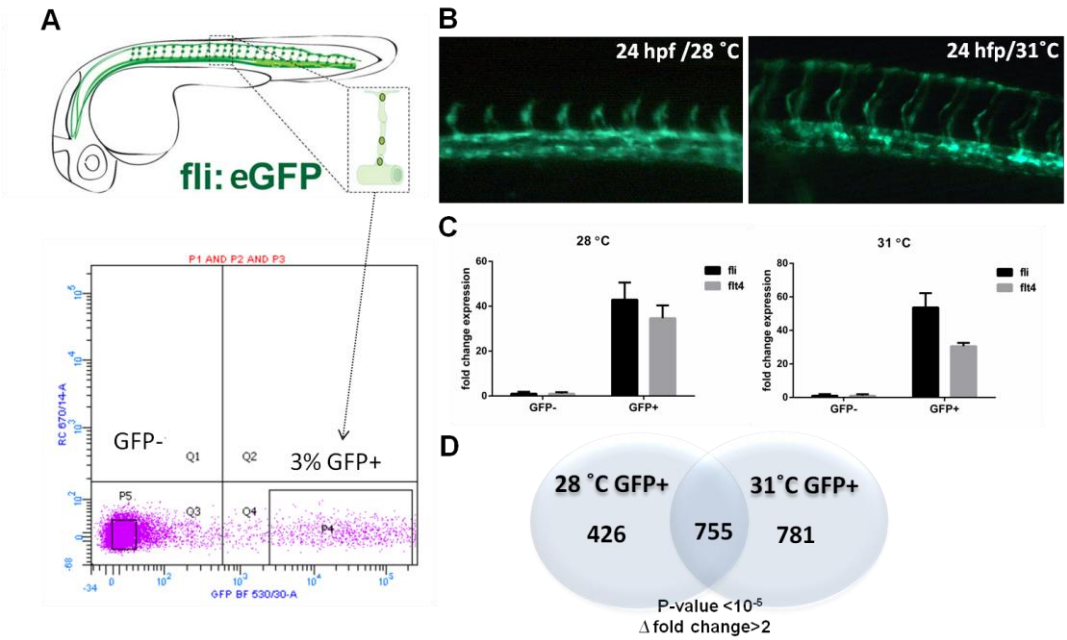

Fig3. GFP+ cells are FACs sorted from embryos for transcriptome profiling to discover endothelial genes important for physiological angiogenesis. **A** Single unattached cells were sorted (P1, P2 and P3) of which approximately 3% was GFP+ (P4) and 97% GFP- (P5). **B** WT 24hpf dissociation time point**;** Tg(Fli;GFP) embryos were kept o/n at either 28˚C or 31˚C. **C** Bar graph of Q-pcr results comparing fli and flt4 expression in RNA samples from sorted cells. Graphs are calculated from the mean of a biological duplicate. **D** number of transcripts identified and the number of overlapping transcripts. Threshold settings fold change >2 and p-value $<10^{-5}$.

Transcriptional analysis of fli expressing cells isolated from Tg(Fli:GFP)

To characterize the transcriptome of Fli expressing cells RNA from FACS sorted GFP+ cells was hybridized against GFP- RNA from Tg(Fli:GFP) on a 180k Agilent chip **Fig3**. Two stages of ISV development were analyzed at 24hpf. The first pool of embryos was kept overnight at 28˚C and at 24hpf the ISV were still growing and did not connect to form the DLAV. In contrast, the second pool was kept overnight at 31˚C and at 24hpf the ISV were fully grown and DLAV was formed **Fig3b.** Expression analysis of Fli1 and Flt4 with Quantitative PCR, comparing GFP+ against GFP-, confirmed successful sorting of endothelial cells and RNA quality used for transcriptome analysis **Fig3c**. In the 28˚C grown embryos a total of 1181 genes were expressed with a fold change above 2 ($p<10^{-5}$) in GFP+ cells compared to GFP- cells. The transcriptome of the 31˚C grown embryos consist of 1536 genes of which 755 overlapped with the 28˚C embryo transcriptome.

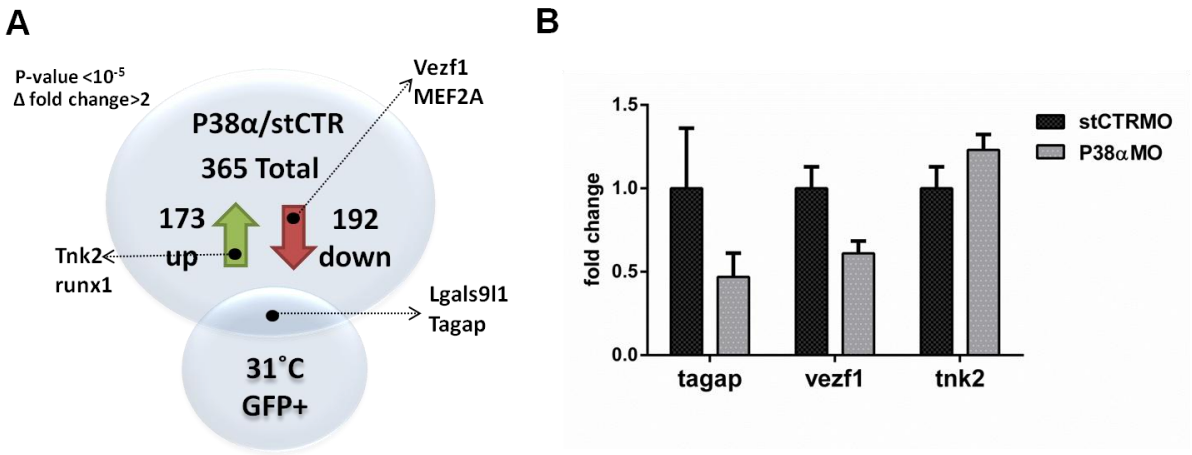

Fig4. Transcriptome profiling of P38α morphants displaying vascular defect. A Number of identified genes in 0.1pmol P38α morpholino injected embryos with some selected genes of interest. results are from a biological triplicates. **B** Bar graph of Q-PCR expression analysis of Tagap, Vezf1 and Tnk2. Means are calculated from a biological triplicate. Expression levels are normalized against the 18s reference gene.

Identification of P38α target genes involved in physiological angiogenesis

To identify the P38α target genes involved in physiological angiogenesis P38α morphants with delayed growth of ISV were snap-freezed in liquid nitrogen for transcriptome analysis. Standard control morpholino injected embryos were taken as a control. Under this unsaturated knock-down condition a total of 365 genes were identified as P38α dependant of which 173 upregulated and 192 downregulated compared to standard control morpholino. Tyrosine kinase, non-receptor, 2 (Tnk2) and runt-related transcription factor 1 (Runx1) were identified among the upregulated genes. The downregulated genes contained vascular

endothelial zinc finger 1 (Vezf1) and myocyte enhancer factor 2A (Mef2A). Embryos in this experiment were kept overnight at 31˚C. The P38α transcriptome could therefore be compared with the 31˚C transcriptome of sorted Fli:GFP+. The overlap contained 2 genes lectin, galactoside-binding, soluble, 9 (galectin 9)-like 1 (Lgal29l1) and T-Cell Activation GTPase-Activating Protein b (Tagapb) both downregulated. Results were validated with Q-PCR which was successful for all genes except Runx1 which is located at the telomere of chromosome 1 and specific primers could not be designed. Lgals9l1 and Mef2a displayed inconsistency between biological triplicates and Tnk2 showed a small fold change of 1,2. Tagap and Vezf1 were significantly downregulated in P38α morphants compared to standard control morpholino.

Discussion

The zebrafish contains all of the 14 MAPK identified so far in mammals including the four P38α isoforms. As the rodent and human cell culture experiments indicated previously, the angiogenesis process depends on P38α activation. In this study, we investigated the role of P38α in zebrafish embryonic angiogenesis. We observed that targeted knock-down of P38α, with gene specific morpholino in Tg(fli:EGFP) delayed the growth of intersegmental vessels significantly. Birb796, a chemical inhibiting all P38 isoforms, abolished the sprouting of ISV as well and was even more potent in inhibiting angiogenesis then the morpholino. The concentration of the morpholino was downscaled in order to prevent morphological abnormalities and therefore the downregulation of P38α mRNA was only partly effective. In addition, the possibility exists that all four P38 isoforms redundantly regulate angiogenesis in the zebrafish and therefore the phenotypes after Birb796 treatment were more severe. With transgenic zebrafish technology using a dominant negative mutant of P38α (and other isoforms) the specificity of the angiogenic phenotype can be further investigated. Also, by using promotors or control elements of endothelial specific marker expression such as fli, kdrl the question can be answered whether the defective growth of ISV is a result of P38α knockdown in the endothelial cells or due to knockdown in the surrounding tissue.

When visualizing the ISV in more detail we found in P38α morphants the lack of filopodia sprouts that are normally present on the tip cells. Since P38α influences the activity of transcription factors we performed transcriptome analysis to characterize this defect at the level of gene expression. Sorting out GFP+ cells from Tg(fli:EGFP) for transcriptome analysis against GFP- provided a gene list of hematopoietic, pharyngeal and endothelial marker genes. This list obtained using 180k Agilent chip is more complete then previously published list by group of Lawson and can be used as reference in the future research[24]. From comparison

of the P38α morpholino transcriptome with the GFP+/GFP- transcriptome we selected Tagapb as the endothelial expressed gene (zfin in situ data) downregulated by P38α morpholino.
Interestingly we also found the transcription factor Runx1 to be 6 fold upregulated in P38α morphants. Runx1 is critical for definitive haematopoiesis and known to prime the hemangioblast (blood precursor) or hemogenic endothelium for differentiation into hematopoietic stem cells [25-27]. Hemogenic endothelium is a unique population of vascular cells that could give rise to blood cells. Perhaps the increase of Runx1 expression is related to the expansion of the intermediate cell mass we observed in P38α morphants. In zebrafish at 24hpf Runx1 expression is present in the dorsal aorta, posterior blood island (ICM) but also in the nervous system. Explaining why we did not find Runx1 mRNA enriched in GFP+ cells sorted from Tg(fli:EGFP). Mammalian Runx1 has previously been found functional in neuronal axon outgrowth and guidance a process which greatly resembles the navigation of blood vessels during angiogenesis[28]. Multiple axonal guidance cues, such as the semaphorins, are also involved in the blood vessel guidance[29]. Since P38α is expressed in the most tissues and also associated with neurite outgrowth it is likely to regulate downstream genes critical for angiogenesis even tough not exclusively expressed in endothelial cells[30]. Vezf1 is selected from the P38α morpholino transcription profile and are interesting for further functional studies in zebrafish. Vezf1 is a zinc finger transcription factor that regulates endothelial sprouting and organizes the vascular network [31].

Conclusion

Similarly as in mammals, zebrafish angiogenesis depends on P38 activity. However the endothelial cell autonomous specific function of P38α needs to be further addressed. P38α morpholino reduces the expression of Tagapb of which expression is concentrated in endothelial cells. Furthermore, the expression of Runx1 is increased in P38α morphants. P38α signalling leads therefore to the suppression of this gene in endothelial, hematopeotic or hematopeitic endothelial cells. Since the molecular mechanisms of hemogenic endothelium differentiation is not well defined yet it is worth investigating the role of P38α in this process.

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