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A zebrafish model for functional screening of flow-responsive genes

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ABSTRACT

Objective: Atherosclerosis is initiated at branches and bends of arteries exposed to disturbed blood flow that generates low shear stress. This mechanical environment promotes lesions by inducing endothelial cell (EC) apoptosis and dysfunction via mechanisms that are incompletely understood. Although transcriptome-based studies have identified multiple shear-responsive genes, most of them have an unknown function. To address this, we investigated whether zebrafish embryos can be used for functional screening of mechanosensitive genes that regulate EC apoptosis in mammalian arteries.

Approach and Results: We firstly demonstrated that flow regulates EC apoptosis in developing zebrafish vasculature. Specifically, suppression of blood flow in zebrafish embryos (by targeting cardiac troponin) enhanced that rate of EC apoptosis (approximately 10%) compared to controls exposed to flow (approximately 1%). A panel of candidate regulators of apoptosis were identified by transcriptome profiling of ECs from high and low shear stress regions of the porcine aorta. Genes that displayed the greatest differential expression and possessed 1-2 zebrafish orthologues were screened for regulation of apoptosis in zebrafish vasculature exposed to flow or no-flow conditions using a knockdown approach. A phenotypic change was observed in 4 genes; p53-related protein (*PERP*) and programmed cell death 2-like protein functioned as positive regulators of apoptosis, whereas angiopoietin-like 4 and cadherin 13 were negative regulators. The regulation of *perp, cdh13, angptl4* and *pdcd2l* by shear stress and the effects of *perp* and *cdh13* on EC apoptosis were confirmed by studies of cultured EC exposed to flow.

Conclusions: We conclude that a zebrafish model of flow manipulation coupled to gene knockdown can be used for functional screening of mechanosensitive genes in vascular endothelial cells, thus providing potential therapeutic targets to prevent or treat endothelial injury at atheroprone sites.

ABBREVIATIONS

ANGPTL4, angiopoietin-like 4
CDH13, cadherin 13
CFD, computational fluid dynamics
EC, endothelial cell
EGFP, enhanced green fluorescent protein
HUVEC, human umbilical vein endothelial cell
MO, morpholino
MRI, magnetic resonance imaging
OSI, oscillatory shear index
PAEC, porcine aortic endothelial cell
PDCD2L, programmed cell death 2-like protein
PERP, p53-related protein
siRNA, small interfering RNA
WSS, wall shear stress

INTRODUCTION

Endothelial cell (EC) responses to wall shear stress (WSS), a force exerted on the endothelium by flowing blood, play a crucial role in vascular homeostasis and also contribute to arterial disease. Plaque formation occurs at branches and bends exposed to disturbed blood flow which generates sites of hemodynamic stasis and WSS with low magnitude and variations in direction (oscillations).[1, 2] These hemodynamic conditions promote atherosclerosis by inducing EC apoptosis and dysfunction.[3, 4] This is highly relevant to atherosclerosis pathophysiology because EC apoptosis initiates lesion development at sites of low WSS[5-13] and promotes plaque erosion[1, 14] via mechanisms that are only partially understood.[9-13, 15] By contrast, regions of arteries exposed to uniform blood flow are protected because high WSS at these sites maintains EC in a quiescent state. Previous studies of the EC transcriptome revealed that flow alters the expression of hundreds of genes, but the function of the majority of them is unknown.[16-19] Therefore, new strategies to identify the function of flow-modulated genes and their role in vascular physiology are urgently required. Screening gene function represents a powerful and unbiased approach used widely to study cellular responses to biochemical signals. However, to our knowledge, functional screening of cells exposed to mechanical force has not been reported.

The zebrafish is a unique vertebrate model that combines advantages characteristic of invertebrate models (small size, powerful genetic tractability, high fecundity, ease of maintenance and relatively low cost) with a high degree of evolutionary conservation with mammals. Thus zebrafish are invaluable not only for studying vertebrate development and physiology, but also for modelling human diseases. While zebrafish embryos have been used extensively to study the effects of flow on angiogenesis and other developmental processes,[20] they have been used less frequently to study adult vasculature. Here we tested the hypothesis that flow regulates EC physiology through mechanisms that are, at least in part, conserved between in adult mammalian arteries and developing zebrafish vasculature. If correct, then zebrafish embryos could provide a valuable model for studying the function of genes that are co-expressed in adult mammalian arteries. We tested this by examining whether zebrafish embryos can be used to identify genes that regulate apoptosis in adult mammalian arteries under different hemodynamic conditions. Our studies revealed that flow is a potent regulator of EC apoptosis in developing zebrafish vasculature. A panel of flow-sensitive input genes expressed in mammalian arteries was assembled by transcriptome profiling of ECs from high and low WSS regions of the porcine aorta. Gene silencing of this panel in zebrafish embryos led to the identification of four genes that regulated apoptosis: p53-related protein (*perp*) and programmed cell death 2-like protein (*pdcd2l*) which functioned as positive regulators of apoptosis under conditions of flow cessation, whereas *cdh13* and *angptl4* exerted anti-apoptotic function. The regulation of PERP, PDCD2L, CDH13 and ANGPTL4 by shear stress was confirmed by *en face* staining of the murine endothelium and by using EC exposed to flow. The ability of PERP and CDH13 to regulate EC apoptosis was confirmed by gene silencing studies in cultured human EC. We conclude that mechanosensitive pathways that control EC apoptosis are partially conserved between zebrafish embryos and mammalian systems. Thus zebrafish embryos may provide a useful model for functional screening of mechanosensitive pathways.

MATERIAL AND METHODS

Materials and Methods are available in the online-only Data Supplement.

RESULTS

A zebrafish model to study endothelial apoptosis regulation by hemodynamic forces

We wished to know whether flow regulates EC apoptosis in the vasculature of zebrafish embryos. This was addressed by manipulating flow which normally commences with cardiac contraction at approximately 24 hours post fertilisation (hpf). To study hemodynamic responses in embryos, blood flow was blocked either by using *silent heart* (*sih*) morpholino antisense oligonucleotide (MO), which targets cardiac troponin T2 leading to a non-beating heart,[21] or by treating embryos with the anaesthetic tricaine to stop cardiac contraction. It should be noted that embryos lacking blood flow remain viable for up to 5 days, since a sufficient supply of oxygen and nutrients is provided by diffusion.[22] Consistent with this, we did not observe a hypoxic response in *sih* embryos during development using a hypoxia reporter line *phd3:GFP*[23] (Fig. S1) and zebrafish embryos lacking blood flow did not upregulate hypoxia responsive genes.[24]

EC apoptosis was assessed in transgenic *flk1:EGFP-NLS* embryos[25] (GFP^+ EC nuclei) by active caspase 3 immunohistochemistry (Fig. 1A-C) or by TUNEL assay (Fig. S2). Increased EC apoptosis was observed at 30-32 hpf in the aorta and caudal vein plexus of embryos lacking blood flow (Fig. 1A-D and Fig. S2), while total EC numbers were comparable to controls (Fig. 1E). At 48 hpf, apoptosis was almost completely resolved in embryos lacking flow, but EC numbers were decreased and caudal vein geometry was less complex than controls at this time point (Fig. S3). We conclude that suppression of flow triggers a transient wave of EC apoptosis accompanied by EC loss and altered vascular remodelling. On the other hand, blood flow drives EC survival during zebrafish development.

Identification of putative shear-responsive regulators of apoptosis by transcriptional profiling of the porcine aorta

Given that enhanced EC apoptosis was observed in embryonic zebrafish vasculature exposed to static conditions (Fig. 1) and in adult mammalian arteries exposed to low WSS[26] we hypothesised that zebrafish embryos may be used for screening of mechanosensitive genes that regulate apoptosis in adult arteries. To test this, we generated a panel of candidate regulators of apoptosis by transcriptome profiling of ECs from low and high WSS regions of the porcine aorta. Healthy pigs aged 6 months were used to allow identification of genes that predispose low WSS sites to disease. Although pigs have been used extensively to study focal atherogenesis, WSS in the porcine aorta has not been defined previously. We therefore used magnetic resonance imaging (MRI) and computational fluid dynamics (CFD) modelling to characterise flow and WSS in the porcine aortic arch (Fig. 2, Figs. S4 and S5). Steady state simulations revealed velocity profiles skewed towards the outer wall and rotated towards the anterior wall leading to higher WSS on the outer wall compared to the inner wall (Fig. 2 and Fig. S4). Unsteady state simulations were run over multiple cardiac cycles using a single geometry and periodicity was reached at the fourth cycle (Fig. S5A). Velocity profiles were computed at multiple locations of the aortic arch at four representative time points of the cardiac cycle (Fig. S5B). The curvature of the arch introduced a rotation of the velocity profile clearly visible in the deceleration and diastolic phase. At peak systole, the presence of the branches caused a reflection of the incoming high velocity flow that was directed towards the inner wall of the arch. It is also important to note the presence of retrograde flow (see arrow in Fig. S5B) starting at late systole and reaching the maximum at peak

diastole, as previously observed in human studies.[27, 28] Time-averaged WSS was similar to steady simulations with the inner curvature of the arch being exposed to WSS with low magnitude (Fig. S5C) and high oscillatory shear index (OSI) (Fig. S5D).

Based on our CFD model, ECs were isolated from high and low time-averaged WSS regions of the porcine aorta (Fig. 2D) using collagenase prior to extraction of RNA. The integrity of RNA samples was confirmed (Fig. S6A and B) and qPCR revealed high expression of CD31 (EC marker) and negligible quantities of smooth muscle cell (α-SMA) or macrophage (CD14) markers (Fig. S6C). RNA samples were labelled and hybridised against GeneChip® Porcine Genome Arrays (Affymetrix) which revealed 867 genes to be shear responsive (Table S1). Functional annotation found that 494 genes have a known or putative function, and molecules with an inferred or known role in the regulation of apoptosis showed maximal enrichment (Table S2). The expression of putative apoptosis regulators at high and low WSS sites was visualised using a heat map (Fig. 3A) and we selected the 20 genes with greatest differential expression for further analysis. Out of these, we validated differential expression of 14 genes by qPCR in an independent cohort of pigs (Fig. 3B) whereas differential expression was inconsistent for 6 genes (data not shown). Thus 14 genes with validated differential expression were selected as candidate regulators of apoptosis for functional screening in zebrafish.

Functional screening of apoptotic regulators in zebrafish

We further selected genes for functional screening based on the existence of 1-2 orthologues in zebrafish. From the 14 candidate apoptotic regulators, two genes (*CCL2* and *CSF2*) were excluded because they have no orthologues in zebrafish. By contrast, *LGALS1* has three zebrafish orthologues (Table S3) and was excluded from further analysis due to possible redundancy between the three paralogues, which would make functional analysis difficult. One of the candidate genes, *CD74*, had two zebrafish orthologues, *cd74a* and *cd74b,* and both of these were included in the functional screening study. Therefore, a total of 12 zebrafish genes were selected for functional screening (Table S3). To test whether the candidate genes are expressed in the zebrafish endothelium, we isolated ECs from 26 hpf *flk1:EGFP-NLS* embryos using fluorescence-activated cell sorting (Fig. S7A). The vascular identity of purified GFP^+ cells was confirmed by enriched expression of the EC marker *cdh5* (vascular endothelial cadherin), while sorted GFP^- cells were used as a control (Fig. S7B, upper panels). We detected endothelial expression of all 12 candidate genes, with some genes being particularly abundant (i.e. *angptl4*, *perp*, *tnip1*) (Fig. S7B, centre and lower panels).

Antisense morpholinos (MOs) were used to transiently knock down the expression of candidate genes in order to assess their function in zebrafish embryos. We initially performed dose-response experiments to determine an optimal dose of each MO for gene knockdown and to assess gross effects on embryogenesis. Knockdown of three genes (*fadd*, *igf1*, *tnfsf10*) resulted in embryonic abnormalities even at a relatively low MO dose (Fig. 4 K, M and W). Due to difficulties in distinguishing between direct and indirect effects of gene knockdown in these embryos, *fadd*, *igf1* and *tnfsf10* were excluded from further analysis. For the remaining nine genes, knockdown embryos showed normal morphology comparable to control embryos (Fig. 4). The efficiency of splice-blocking MOs was determined by RT-PCR and qRT-PCR (Fig. S8). Alternatively spliced transcripts in MO-injected samples were observed as a band shift after gel electrophoresis of RT-PCR products (Fig. S8A) and these transcripts were confirmed by sequencing to contain a frameshift leading to a premature stop codon (data not shown). For *angptl4*, *fadd*, *perp* and *tnip1*, a

reduced level of the wildtype transcript was observed in the MO-injected samples (Fig. S8A) and qRT-PCR was consequently used to assess the efficiency of the knockdown (Fig. S8B).

Following knockdown of specific genes using MOs, EC apoptosis was assessed in embryos by staining of active caspase 3 in the presence (control MO) or in the absence of flow (Fig. 5). Knockdown of five genes (*cd74a*, *cd74b*, *jun*, *ptgis* and *tnip1*) did not modify EC apoptosis (Fig. 5) or EC numbers (Fig. S9 B, C, E, H and I) either in the presence or in the absence of blood flow.

Knockdown of *cdh13* resulted in an increase in EC apoptosis in the absence of flow (Fig. 5 and Fig. S9D) whereas depletion of *angptl4* increased apoptosis in the presence of flow (Fig. 5 and Fig. S9A). These data suggest that although *cdh13* and *angptl4* exert anti-apoptotic effects, they function under different mechanical conditions. By contrast, depletion of *perp* or *pdcd2l* led to a profound decrease (over 30%) in EC apoptosis in the absence of flow (Fig. 5 and Fig. S9F and G) suggesting that these genes are pro-apoptotic.

In order to confirm the observed phenotypes, an alternative non-overlapping MO was used for each gene (called MO2) (Fig. S10A). The efficiency of the MO2-mediated knockdown was confirmed by RT-PCR (*angptl4*, *cdh13* and *pdcd2l*, Fig. S10B), qRT-PCR (*angptl4*, Fig. S10C) or by phenotypic analysis (*perp*, Fig. S10D). Injection of the second, non-overlapping *angptl4* MO2 resulted in 2.8 fold increase in EC apoptosis in the presence of flow (Fig. 6A), whereas injection of *cdh13* MO2 led to approximately 50% increase in EC apoptosis in the absence of flow (Fig. 6B). On the other hand, injection of *pdcd2l* MO2 or *perp* MO2 resulted in an approximately 40% decrease in EC apoptosis in the absence of flow (Fig. 6 C and D). Thus the second MO recapitulated the effects of the initial MO for all four genes studied. Taken together, these results indicate that *cdh13* and *angptl4* play a protective role in the endothelium, while *perp* and *pdcd2l* promote EC apoptosis in response to static conditions.

In vivo **mechanistic studies**

To elucidate mechanisms that drive EC apoptosis in response to hemodynamic forces we focused on two positive regulators of this process identified in our screening, *perp* and *pdcd2l.* To establish the mechanism by which they promote EC apoptosis we returned to the porcine model and analysed interrelations between apoptotic regulators identified by EC transcriptome profiling using Ingenuity Pathway Analysis. This assessment revealed p53 as a potential central regulator (Fig. S11). Therefore, we tested whether the p53 pathway is involved in flow-regulated EC apoptosis in zebrafish embryos. Knockdown of *p53* resulted in a decrease in EC apoptosis in the absence of flow that was comparable to *perp* or *pdcd2l* knockdown (Fig. 6E; compare 3, 4, 5). To dissect the potential cross-talk between *p53, perp* and *pdcd2l*, we performed combined knockdowns. Co-knockdowns of *perp* and *pdcd2l*, *p53* and *perp* or *p53* and *pdcd2l* gave similar rates of apoptosis to single knockdown of each gene (Fig. 6E; compare 6-8 with 3-5), suggesting that these molecules belong to a shared signalling pathway.

Expression and functional studies in mammalian ECs

Since screening models can generate false positives we attempted to confirm the expression and function of CDH13, PERP, ANGPTL4, PDCD2L in cultured EC exposed to flow and (where suitable antibodies were available) by *en face* staining of the aorta using mice.

CDH13

En face staining of the murine aorta using anti-Cdh13 antibodies revealed that Cdh13 was expressed at higher levels in the high shear (outer curvature) compared to the low shear (inner curvature) region (Figure 7). A major portion of the Cdh13 pool localised to the plasma membrane (Fig. 7B) suggesting that it is expressed in an active form in EC exposed to high shear. By contrast, control IgG did not produce a signal from murine arteries (Fig. S12). Additionally, we compared gene expression in porcine aortic endothelial cells (PAECs) exposed to flow patterns that model the *in vivo* situation (high uniform and low oscillatory WSS) using two complementary flow systems, an orbital shaker and an ibidi pump system. In the orbital shaking platform, the cells were exposed to flow using 6 well-tissue culture plates which were orbited to generate high unidirectional shear stress in the periphery of the well, and low shear with greater variation in direction at the centre.[29] On the other hand, the ibidi pump system involved seeding cells onto specialised µ-slides and the generation of WSS using a computer-controlled syringe pump to generate flow of desired magnitude and frequency. *CDH13* expression was enhanced in EC exposed to high shear using the ibidi system but not in cells exposed to orbital flow (Fig. S13A) possibly reflecting differences in the mechanical conditions generated by these two systems.

We next wished to know whether CDH13 influences apoptosis in ECs cultured under flow. ECs exposed to low, oscillatory shear stress *in vitro* exhibited increased levels of EC apoptosis, as determined by active caspase 3 staining (Fig. 8) and TUNEL assay (Fig. S14). Notably gene silencing in cultured HUVECs using siRNA revealed that knockdown of *CDH13* (validated by qPCR; Fig. S15A) resulted in significantly increased rates of apoptosis in EC exposed to high or low shear stress (Fig. 8). Interestingly, although *CDH13* knockdown in zebrafish enhanced apoptosis under conditions of flow cessation it did not influence EC exposed to flow, possibly because of compensatory mechanisms that protect sheared EC in developing fish. Nevertheless, the *in vitro* data broadly confirm those obtained by studying zebrafish embryos by indicating that CDH13 exerts anti-apoptotic effects in EC.

PERP

En face staining of the murine aorta revealed that Perp was expressed at higher levels in the low shear (inner curvature) compared to the high shear (outer curvature) region (Fig. 9). PERP localised predominantly to the plasma membrane which is consistent with its localisation in other tissues (Fig. 9B).[30] Similarly, the expression of PERP was elevated in cultured PAECs exposed to low shear compared to cells exposed to high shear using either the ibidi parallel plate system or an orbital platform (Fig. S13B). Knockdown of *PERP* (validated by qPCR; Fig. S15B) resulted in significant reduction in EC apoptosis (Fig. 10). These data confirm those generated by screening genes in zebrafish embryos and indicate that PERP is expressed under low shear conditions where it promotes EC apoptosis in both *in vitro* and *in vivo* systems.

ANGPTL4 and PDCD2L

Studies of cultured PAECs revealed that *ANGPTL4* and *PDCD2L* were expressed at higher levels in cells exposed to low compared to high shear stress using the orbital system (Fig. S13 C and D) which is consistent with their enrichment at the low shear region of arteries (Fig. 3). However *ANGPTL4* was not enriched under low shear stress using the ibidi platform possibly reflecting differences in the mechanical environments generated by the orbital and parallel-plate systems. Silencing of *ANGPTL4* or *PDCD2L* using siRNA (validated by qPCR; Fig. S15 C and D) did not influence apoptosis in cultured EC exposed to high or low WSS (Fig. S16). Thus although both of these molecules regulated apoptosis in zebrafish embryonic EC

exposed to flow they did not influence the viability of cultured mammalian EC, possibly reflecting differences in the developmental stage, mechanical environment or species studied.

Taken together, our studies of murine arteries and cultured EC exposed to different shear stresses confirm observations made using zebrafish embryos that PERP and CDH13 function as shear-responsive regulators of endothelial apoptosis. By contrast, the effects of ANGPTL4 and PDCD2L on EC apoptosis observed in zebrafish embryos were not recapitulated *in vitro*. The study demonstrates the utility of our zebrafish platform for functional screening of shear-responsive genes and also emphasises the potential divergent functions of some genes in *in vivo* and *in vitro* systems.

DISCUSSION

The molecular mechanisms underlying the effects of WSS on EC physiology and atherosclerosis are not fully understood but are known to involve transcriptional changes. While transcriptome analysis of EC exposed to different flow parameters under *in vitro* or *in vivo* conditions[16-19, 31, 32] has identified multiple mechanoresponsive genes the majority of them have not been studied at a functional level. To address this challenge, we established a zebrafish platform to allow functional screening of flow-responsive genes. To our knowledge, this is the first *in vivo* system developed for screening of mechanosensitive genes.

The zebrafish embryo model that we used diverges from vascular response to flow in mammalian arteries in several important respects including species, scale and hemodynamics. Nevertheless, there are a number of parallels between endothelium exposed to static conditions in zebrafish on one hand, and endothelium exposed to disturbed flow and low, oscillatory WSS in mammals on the other. For example, the athero-protective transcription factor *KLF2*/*klf2a* was suppressed by static conditions in embryos and by low WSS conditions in adult vertebrates.[33] In addition, EC cilia are present mainly in areas of low WSS in mammalian arteries and in static conditions in zebrafish embryos and were shown to disassemble upon exposure to laminar shear stress in both systems.[34-36] Finally, a recent study found considerable overlap in the transcriptome of EC exposed to low shear or static conditions.[37] Because of these considerations we hypothesized that manipulation of genes in developing zebrafish vessels may provide information on the pathways that regulate mammalian arterial physiology. Consistent with this notion, our study revealed that flow regulates EC apoptosis via mechanisms that are, at least in part, conserved between embryonic and adult arteries.

We assessed the ability of the zebrafish model to identify mechanosensitive genes that control EC function by focussing on apoptosis regulators that are controlled by flow. To generate a panel of putative flow-sensitive apoptosis genes we carried out microarray transcriptome analysis of high and low shear stress regions of the porcine aorta. Functional annotation revealed apoptosis regulators as the most highly enriched group which is consistent with the hypothesis that multiple genes regulate EC apoptosis at regions of disturbed flow. Our data build on pioneering studies from Peter Davies' group revealing enrichment of pro-inflammatory and stress response molecules at the inner curvature of the porcine aorta.[10, 16, 38] Notably, our study revealed multiple WSS-related genes that were not identified as differentially expressed previously. This discrepancy may be related to technological differences since the commercial porcine gene arrays used in the current study contained >10000 genes more than the custom arrays used previously,[10, 16] and analysis of our data benefitted from sequencing and annotation of the porcine genome (http://www.ensembl.org/Sus_scrofa/Info/Index). More fundamentally, there is only a partial overlap between the anatomical sites studies by the Davies group and those studied currently. For example, whereas we used outer curvature of the aortic arch as the source of ECs exposed to athero-protective flow, previous studies focussed on the descending thoracic aorta,[16] carotid artery and renal artery.[10, 16] Of note, although only a proportion of genes were found to be consistently differentially expressed in the current and previous studies of porcine arteries, the majority of them showed a similar pattern of expression in low and high shear areas (Table S4). It is also interesting to compare the current study with elegant experiments that revealed a causal relationship between WSS and the EC transcriptome in the murine carotid artery.[17] Here, WSS was modified in the left carotid artery by partial ligation prior to assessment of EC gene expression. This approach revealed that WSS regulates multiple genes in murine ECs including those involved in inflammation and immunity. Interestingly, although our current study of the porcine aorta identified

several genes that were also regulated by WSS in the murine carotid artery,[17] the relationship with WSS was not conserved between models (Table S4). Several important biological differences can potentially explain this disparity including differences in vascular bed, variation in fluid dynamics, species and the time that EC were exposed to disturbed flow. We also compared our microarray data to the list of >1600 shear-responsive genes obtained by meta-analysis of published microarray studies performed *in vitro* on HUVECs (Table S5).[39] We found that less than 50% of genes shown to be mechanically regulated in both data sets exhibit the same relationship with WSS. This observation further emphasises the sensitivity of EC to physiological and mechanical stimuli which vary between *in vitro* and *in vivo* systems.

Following our EC transcriptome analysis, we selected the putative apoptosis regulators that displayed the greatest differential expression for functional screening in zebrafish. Comparison between human and zebrafish reference genomes shows that approximately 70% of human genes have at least one obvious zebrafish orthologue.[40] Therefore, not all human genes can be studied in zebrafish, which was the case for *CCL2* and *CSF2*. Additionally, due to a whole-genome duplication which occurred early during the evolution of ray-finned fishes, many human genes have more than one orthologue in the zebrafish genome. Since functional redundancy between zebrafish paralogues can present a challenge when studying zebrafish physiological processes, we excluded from our study genes with more than two orthologues. Gene function in zebrafish has been studied using transient knockdown via MOs or by targeted gene disruption and generation of mutants. For functional screening of a large number of genes, generation of mutants is costly, labour-intensive and time-consuming (zebrafish and mice have similar generation times). Therefore, our approach was to use MOs for the functional screening. One drawback of using MOs is the lack of spatiotemporal control since they are injected into embryos at the 1-cell stage and therefore reduce expression in all embryonic cells. Because of this, genes important for early embryonic development cannot be studied using this model. This was the case for three genes in our study, *fadd*, *igf1* and *tnfsf10*, where knockdown caused gross morphological defects. Nevertheless, we were able to study vascular responses to flow following knockdown of 75% genes where embryonic morphology was normal. Overall of 12 genes studied we detected two anti-apoptotic (*angptl4*, *cdh13*) and two pro-apoptotic molecules (*pdcd2l*, *perp*) that were enriched at mechanically-distinct regions of the aorta. In order to confirm the specificity of the MO-mediated knockdown phenotype, we used two separate, non-overlapping MOs for each gene of interest.

To confirm our observations from functional screening in zebrafish embryos we carried out a series of experiments using cultured EC exposed to flow. Firstly, to understand their mechanism of differential expression, we examined whether CDH13, PERP, *PDCD2L* and *ANGPTL4* respond to WSS in cultured ECs. We used two complementary platforms, an orbital shaker and an ibidi pump system. In the orbital shaker system, the cells in the centre of the well are exposed to WSS with a constant low mean magnitude and rapid changes in direction, whereas in the periphery of the well the WSS magnitude is relatively high and with relatively uniform flow direction.[29] This contrasts with the ibidi parallel plate system which uses a syringe pump to generate high uniform and low oscillatory (1Hz bidirectional) WSS. Two genes, *PERP* and *PDCD2L*, were expressed at higher levels in EC exposed to low WSS conditions in both *in vitro* systems suggesting that their focal expression *in vivo* is maintained by local hemodynamics. On the other hand, *CDH13* expression was enriched by high WSS only in the ibidi system, while *ANGPTL4* upregulation under low WSS was only observed in the orbital system. These observations may reflect differences in the mechanical environment of the orbital and ibidi systems, for example the presence of secondary flows which are generated exclusively by the

orbital platform[29], and they emphasise the importance of studying mechanosensitive gene function using multiple experimental models. Secondly, we assessed the effects of silencing our genes of interest in cultured EC exposed to flow. This study confirmed that *PERP* is a positive regulator of EC apoptosis under low WSS conditions, and that *CDH13* has protective effects (shown schematically in Figure S17). Interestingly, although *CDH13* reduced apoptosis in cultured EC exposed to low or high WSS, its protective effects in zebrafish embryos were only observed in EC exposed to static conditions. This discrepancy may arise from compensatory mechanisms present in embryonic but not adult vessels exposed to flow. Further studies including genetic deletion of Cdh13 in mice are therefore required to determine whether CDH13 exerts protective effects under low and high WSS conditions in adult mammalian arteries. Our study also indicated that although *angptl4* and *pdcd2l* regulate apoptosis in zebrafish endothelium, they did not influence EC viability in cultured human cells exposed to low or high WSS. Therefore, these molecules may regulate apoptosis specifically in developing EC, or their function may require anatomical or physiological features that are found *in vivo* but not in cultured cells, or there may be species-based differences. Further work is required to distinguish between these possibilities. Although we provide the first demonstration that *perp, cdh13, angptl4* and *pdcd2l* are involved in hemodynamic control of apoptosis, they have been linked previously with apoptosis in other systems. PERP was previously identified as a p53 target gene by subtractive cloning of mouse embryonic fibroblasts and was shown to be expressed exclusively in apoptotic cells.[30] Interestingly, PERP can feedback to p53 to promote its activity[41] which is consistent with our observation that PERP and p53 co-operate to promote apoptosis in ECs. Our findings also resonate with previous studies that revealed post-translational modifications of p53 playing a role in disturbed flow-mediated EC apoptosis and contributing to atherosclerotic plaque formation.[13, 15] CDH13 has been shown to protect ECs from oxidative stress-induced apoptosis,[42] and ANGPTL4 acts as a survival factor in the endothelium.[43] Finally, although little is known about the function of PDCD2L, its paralogue PDCD2 promotes apoptosis in a number of human and mammalian cell lines and tissues.[44]

While zebrafish embryos recapitulate some of the pathways that are regulated by flow in adult arteries, there are also limitations. One notable example is the hypoxia-inducible factor 1α (HIF1α) pathway which was enriched at the inner curvature of the the porcine aorta (Fig. 3) but was not activated in zebrafish embryos under static conditions (Fig. S1). This discrepancy may be attributable to differences in oxygen transport and inflammation between these two systems. In the porcine aorta, oxygen delivery to luminal EC relies on transport of red blood cells through the vessel lumen. Computational modelling and empirical measurements suggest that this process can be influenced by secondary flows, which convect oxygen away from the vessel wall to generate regions with reduced oxygen levels[45,46]. By contrast, oxygenation of zebrafish embryos (up to 72 hpf) does not rely on circulation because diffusion is sufficient because of their small size[22-24]. Thus although sites of disturbed flow in the aorta may have lower oxygen tensions leading to enhanced HIF1α activation, this pathway is not activated in zebrafish in response to flow cessation because diffusion prevents the development of a hypoxic environment. Moreover, HIF1α can also be induced by inflammatory signalling pathways[47] which are known to be activated constitutively at the inner curvature of the aortic arch[47,16] but have an uncertain role in zebrafish embryo responses to flow. Thus we conclude that although zebrafish embryos provide a useful *in vivo* model to assess vascular responses to WSS, other

systems are required to study the effects of altered oxygen transport on the vasculature.

In summary, we have established a zebrafish-based model for functional screening of flow-sensitive genes and have used this system to identify novel regulators of EC apoptosis in response to disturbed flow. The study provides an additional mechanism to explain the focal distribution of EC injury and dysfunction in arteries and suggests that this involves p53-PERP-mediated EC apoptosis at atheroprone sites and CDH13-mediated EC survival at atheroprotected sites.

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None.

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HIGHLIGHTS

- Atherosclerosis develops at branches and bends of arteries exposed to disturbed blood flow.

- We investigated whether functional screening of flow-sensitive genes can be achieved using zebrafish, a vertebrate model that can be genetically modified with ease.

- Manipulation of flow altered endothelial cell apoptosis in developing zebrafish vessels and this process was modified by knockdown of a panel of flow-sensitive genes.

- We conclude that a zebrafish model of blood flow manipulation coupled to gene knockdown can be used for functional screening of mechanosensitive genes.

Fig. 1.

Flow cessation induces EC apoptosis in zebrafish embryos. (A) Whole-mount active caspase 3 (red) staining of 30 hpf *flk1:EGFP-NLS* zebrafish embryos (green EC nuclei) in the presence (control) or absence of flow (*sih* MO, tricaine). The region outlined with the white box is shown in higher magnification in (B); white arrows indicate apoptotic ECs (yellow). (C) Zebrafish embryo at 30 hpf. The region outlined with blue box represents the region that is studied in (A). The percentage of EC apoptosis (D) and EC numbers (E) in *sih* MO-injected and tricaine-treated embryos compared to controls was quantified and mean values are shown with standard deviation; n≥15 from three independent experiments, **p<0.01 using one-way ANOVA. (A-C) Lateral view, anterior to the left, dorsal up. Scale bars: 50 μm (A), 15 μm (B), 500 μm (C).

Fig. 2.

Steady state fluid dynamics in the porcine aorta. Steady state fluid dynamics in the porcine aorta were studied using MR imaging and CFD. Five animals were studied and representative data are shown from a single animal. (A) The surfaces of the aortic arch were reconstructed from MR images, smoothed with a low-pass filter and extended with cylindrical flow extensions at the outlets. (B) Three-dimensional velocity contours are shown over six representative planes. (C) A time-averaged WSS map was calculated and mapped onto the aortic geometry. High WSS is represented in red and low WSS in blue. (D) The time-averaged WSS map was unwrapped via a computational incision over the outer aortic wall and the two-dimensional WSS map was visualised with the endothelial layer facing upwards (left panel). The aorta was cut along the outer curvature to expose the lumen and low and high WSS regions were identified by reference to the WSS map (indicated with boxes).

Fig. 3.

Transcriptome profiling of the porcine aorta. The EC transcriptome was studied at low and high WSS regions of the porcine aorta using microarrays. Five pigs were studied. Aortae were cut along the outer curvature to expose the lumen and ECs were isolated from low and high WSS regions. (A) Genes with a known or putative role in apoptosis are presented as a heat map representing expression patterns at low and high WSS sites. Red indicates enrichment in gene expression, whereas green indicates suppression. (B) Validation of microarray data by quantitative RT-PCR. The twenty most differentially regulated apoptotic genes were selected for validation in an independent cohort of pigs. Transcript levels were quantified by qRT-PCR using gene-specific primers. Mean values are shown with standard deviation; n=5, *$p<0.05$, **$p<0.01$, ***$p<0.001$ using an unpaired two-tailed test.

Fig. 4.

Morphology of MO-injected embryos. (A-Z) Zebrafish embryos were injected with 3 ng (left column) or 6 ng (right column) of gene-specific or non-targeting control MO (indicated on the left). Embryo morphology was observed during development and is shown here at 30 hpf. Lateral view, anterior to the left, dorsal up. Scale bar: 500 μm.

Fig. 5. Zebrafish functional screening of putative apoptotic regulators. (A) Zebrafish embryos (*flk1:EGFP-NLS* embryos; green EC nuclei) were injected with MOs targeting candidate genes or a non-targeting control MO (indicated on the left of each row). EC apoptosis was studied in the presence (control MO) or in the absence (*sih* MO) of flow by whole-mount active caspase 3 staining (red). Apoptotic ECs (yellow) were monitored at 30 hpf. Lateral view, anterior to the left, dorsal up. Scale bar: 50 μm. (B) The proportion of apoptotic ECs (number of apoptotic ECs divided by the total number of ECs) normalised to *sih* MO-injected embryos was calculated and mean values are shown with standard error of the mean. n≥15 from three independent experiments. *p<0.05, **p<0.01, ***p<0.001 using one-way ANOVA.

Fig. 6.

Validation of knockdown phenotypes and mechanistic studies of pro-apoptotic regulators. *Angptl4* (A), *cdh13* (B), *pdcd2l* (C) and *perp* (D) knockdown phenotypes were validated by injecting zebrafish embryos (*flk1:EGFP-NLS*) using a second non-overlapping MO (termed MO2) or a non-targeting control MO. (E) The potential cross-talk between *perp, pdcd2l* and *p53* was studied by injecting zebrafish embryos (*flk1:EGFP-NLS* embryos) using MOs targeting *perp, pdcd2l* and *p53* either singly (bars 3-5) or as double knockdowns (bars 6-8) or with a non-targeting control MO. (A-E) EC apoptosis was studied in the presence (control MO) or in the absence (*sih* MO) of flow by whole-mount active caspase 3 staining at 30 hpf. The proportion of apoptotic ECs (number of apoptotic ECs divided by the total number of ECs) normalised to *sih* MO-injected embryos was calculated and mean values are shown with standard error of the mean. n≥15 from three independent experiments. *p<0.05, ***p<0.001 using one-way ANOVA; ns, non-significant.

Fig. 7.

Cdh13 expression was enriched at a high shear stress region of the murine aorta. (A) Expression levels of Cdh13 in ECs were assessed by *en face* staining of low oscillatory WSS (inner curvature) or high WSS (outer curvature) of the aortic arch of C57BL/6 mice (red). ECs were identified by co-staining with anti-CD31 antibodies conjugated to Alexa Fluor 488 (green). Cell nuclei were identified using To-Pro-3 (DNA, blue). The region outlined with the white box is shown in higher magnification in (B). (C) Graph showing quantitation of Cdh13 expression (mean fluorescence intensity with

standard error of the mean). Data were pooled from five independent experiments. **p<0.01 using an unpaired two-tailed t-test.

***Fig. 8 CDH13* protected cultured ECs exposed to flow from apoptosis.** (A, B) HUVECs were transfected with scrambled sequences or *CDH13* siRNA or remained untransfected and incubated for 24 h. Cells were exposed for 72 h to low oscillatory (centre) or high uniform (periphery) WSS using the orbital system. Apoptotic cells were measured by immunofluorescent staining using antibodies that detect cleaved caspase-3 (green) and counterstaining nuclei using To-Pro-3 (purple; DNA). Apoptotic ECs are indicated with white arrows. Data from at least three independent experiments were pooled and the proportion of apoptotic cells are shown with standard error of the mean (B). ** $p<0.01$, ***$p<0.001$ by two-way ANOVA.

Fig. 9.

Perp expression was enriched at a low shear stress region of the murine aorta. (A) Expression levels of Perp in ECs were assessed by *en face* staining of low oscillatory WSS (inner curvature) or high WSS (outer curvature) of the aortic arch of C57BL/6 mice (red). ECs were identified by co-staining with anti-CD31 antibodies conjugated to Alexa Fluor 488 (green). Cell nuclei were identified using To-Pro-3 (DNA, blue). The region outlined with the white box is shown in higher magnification in (B). (C) Graph showing quantitation of Perp expression (mean fluorescence intensity with standard error of the mean). Data were pooled from five independent experiments. ***$p<0.01$ using an unpaired two-tailed t-test.

Fig. 10.

***PERP* promoted apoptosis of cultured EC exposed to low WSS.** HUVECs were transfected with scrambled sequences or *PERP* siRNA or remained untransfected and incubated for 24 h. Cells were exposed for 72 h to low oscillatory (centre) or high uniform (periphery) WSS using the orbital system. Apoptotic cells were measured by immunofluorescent staining using antibodies that detect cleaved caspase-3 (green) and counterstaining nuclei using To-Pro-3 (purple; DNA). Apoptotic ECs are indicated with white arrows. Data from at least three independent experiments were pooled and the proportion of apoptotic cells are shown with standard error of the mean. * $p<0.05$, by two-way ANOVA.