A zebrafish model for functional screening of flow-responsive genes

Jovana Serbanovic-Canic¹,²,³, Amalia de Luca⁴, Christina Warboys⁴, Pedro F. Ferreira⁵, Le A. Luong⁷, Sarah Hsiao⁷, Ismael Gauci¹, Marwa Mahmoud⁷, Shuang Feng¹, John-Paul Ashton¹,³, Henning Walczak⁸, David Firmin⁵, Rob Krams⁶, Justin C. Mason⁴, Dorian O. Haskard⁴, Spencer Sherwin⁷, Victoria Ridger¹,², Timothy JA Chico¹,²,³, Paul C. Evans¹,²,³

Department of Infection, Immunity and Cardiovascular Disease¹, INSIGNEO Institute for In Silico Medicine² and the Bateson Centre³, University of Sheffield, Sheffield S10 2RX, UK.
Departments of Cardiovascular Science⁴, Imaging⁵, Bioengineering⁶, and Aeronautics⁷ Imperial College London, W12 0NN, UK.
⁸Cancer Institute, Faculty of Medical Sciences, University College London, London WC1E 6BT, UK.

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*Address for correspondence:
Professor Paul C Evans,
Department of Cardiovascular Science,
Medical School, University of Sheffield,
Beech Hill Road,
Sheffield S10 2RX,
UK.

Tel: +44 (0) 114 271 2591
Fax: +44 (0) 114 271 1863
e-mail: paul.evans@sheffield.ac.uk
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.

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.

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
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). These hemodynamic conditions promote atherosclerosis by inducing EC apoptosis and dysfunction. 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. 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. Here we examined whether zebrafish embryos can be used for functional screening of mechanosensitive genes. To test this, we focussed on the identification of regulators of EC apoptosis under different hemodynamic conditions. This is highly relevant to atherosclerosis pathophysiology because EC apoptosis initiates lesion development at sites of low WSS and promotes plaque erosion via mechanisms that are only partially understood. Our studies revealed that flow is a potent regulator of EC apoptosis in developing zebrafish vasculature. A panel of flow-sensitive input genes were identified 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, including p53-related protein (PERP) and programmed cell death 2-like protein (PDCD2L) that functioned as positive regulators, demonstrating the utility of the zebrafish model to identify components of mechanosensitive apoptotic pathways.
MATERIAL AND METHODS
Materials and Methods are available in the online-only Data Supplement.
RESULTS
Establishing a zebrafish model for studying endothelial responses to haemodynamic 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, 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. Consistent with this, we did not observe a hypoxic response in sih embryos during development using a hypoxia reporter line phd3:GFP (Fig. S1) and zebrafish embryos lacking blood flow did not upregulate hypoxiaresponsive genes.

EC apoptosis was assessed in transgenic flk1:EGFP-NLS embryos (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 the 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 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 in studies of atherogenesis, WSS in the porcine aorta has not been studied 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. 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 774 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 (Fig. S8A) and qRT-PCR (Fig. S8B). 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 either in the presence or in the absence of blood flow (Fig. 5 and Fig. S9 B, C, E, H and I). Depletion of angptl4 led to increased EC apoptosis in the presence but not the absence of
flow (Fig. 5 and Fig. S9A), suggesting that this gene plays a flow-dependent role in EC survival. In the absence of flow, knockdown of *cdh13* resulted in a modest increase in EC apoptosis, whereas embryos lacking *pdc2l* or *perp* expression showed a profound decrease (over 30%) in EC apoptosis (Fig. 5 and Fig. S9 D, F and G). Taken together, these results indicate that *angi4* and *cdh13* play a protective role in the endothelium, while *pdc2l* and *perp* 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, *pdc2l* and *perp*. 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 (*pdc2l*, Fig. S10B) or by phenotypic analysis (*perp*, Fig. S10C). Injection of the second, non-overlapping *pdc2l* MO2 or *perp* MO2 resulted in an approximately 40% decrease in EC apoptosis in the absence of flow (Fig. 6A), thus recapitulating the knockdown effects using the initial MO. Taken together, these results confirm that *pdc2l* and *perp* are positive regulators of EC apoptosis in the absence of flow.

It was next of interest to establish the mechanism by which *pdc2l* and *perp* regulate EC apoptosis in response to hemodynamic forces. To gain an insight into this mechanism, 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 *pdc2l* or *perp* knockdown (Fig. 6B; compare 3, 4, 5). To dissect the potential cross-talk between p53, *perp* and *pdc2l*, we performed combined knockdowns. Co-knockdowns of *pdc2l* and *perp*, p53 and *perp* or p53 and *pdc2l* gave similar rates of apoptosis to single knockdown of each gene (Fig. 6B; compare 6-8 with 3-5), suggesting that these molecules belong to a shared signalling pathway.

**In vitro validation in mammalian ECs**
To validate observations made using the zebrafish model, we firstly assessed whether *ANGPTL4*, *CDH13*, *PDCD2L* and *PERP* were expressed in cultured ECs and if their expression levels were modified by flow. We compared gene expression in ECs 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 platform, the cells were exposed to flow using 6 well-tissue culture plates placed on an orbital shaker to generate high unidirectional shear stress in the periphery, and low shear with greater variation in direction at the centre. On the other hand, the ibidi pump system involved seeding cells onto specialised µ-slides and the generation of high or low oscillatory WSS using a computer-controlled syringe pump. Studies were carried out using porcine aortic ECs (PAEC) to replicate the cell type studied in vivo, and using human umbilical vein ECs (HUVEC) which are amenable to gene knockdown using siRNA. *ANGPTL4* and *CDH13* were not regulated consistently by flow using orbital and ibidi systems (Fig. S12) and therefore were not pursued further. By contrast, low oscillatory WSS induced *PERP* and *PDCD2L* expression in PAEC and HUVEC using either the orbital or ibidi system (Fig. 7) and we therefore selected both genes for silencing studies in cultured EC. Thus we tested the ability of *PDCD2L* and *PERP* to modify apoptosis induction by low WSS by transiently knocking down gene expression in HUVECs using siRNAs in the orbital shaker or ibidi pump system. ECs exposed to low, oscillatory WSS exhibited increased levels of EC apoptosis, as determined by active caspase 3 staining (Fig. 8A) and TUNEL assay (Fig. S13), compared to cell exposed to high WSS. Depletion of *PDCD2L* expression (validated by qPCR; Fig. S14B) did not have any effect on low WSS-induced EC apoptosis (Fig. S14A). On the other hand, knockdown of *PERP* (validated by qPCR; Fig. 8C) resulted in significant reduction in EC
apoptosis using either of the two flow systems (Fig. 8A and B). Finally, by performing en face staining, we found that increased PERP protein expression correlated with EC apoptosis in the low WSS region of the porcine aorta (Fig. 9). Taken together, our data indicates that the p53 target PERP functions as a shear-responsive apoptotic regulator in the endothelium and demonstrates the utility of our zebrafish platform for functional screening of shear-responsive genes.
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\(^5\)\(^-\)\(^8\)\(^,\)\(^31\)\(^-\)\(^34\) has identified multiple shear stress-responsive 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.\(^24\) 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.\(^25\)\(^-\)\(^27\) 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.\(^5\)\(^,\)\(^14\)\(^,\)\(^35\) 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,\(^5\)\(^,\)\(^14\) 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,\(^5\) carotid artery and renal artery.\(^5\)\(^,\)\(^14\) 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 correlation with flow (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.\(^6\) 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,\(^6\) 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).\(^34\) 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. 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. We were able to study vascular responses to flow following knockdown of 75% genes where embryonic morphology was normal. Our study revealed 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 and also validated our findings using cultured human ECs.

To understand their mechanism of differential expression, we examined whether angptl4, cdh13, pdcd2l and perp 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. On the other hand, the ibidi parallel plate system uses a syringe pump to generate high uniform and low oscillatory (bidirectional) WSS. Two genes, PDCD2L and PERP, were induced by low oscillatory WSS conditions that approximated the mechanical environment found at sites of expression in vivo, suggesting that their focal expression in vivo is maintained by local hemodynamics. On the other hand, ANGPTL4 and CDH13 expression in culture conditions did not replicate the pattern of their focal expression in vivo. It is plausible that the latter genes are regulated in vivo by specific flow patterns not generated in vitro or by non-mechanical factors e.g. inflammatory cells, developmental history.

Although we provide the first demonstration that ANGPTL4, CDH13, PDCD2L and PERP are involved in haemodynamic control of apoptosis, they have been linked previously with apoptosis in other systems. ANGPTL4 acts as a survival factor in the endothelium, and CDH13 has been shown to protect ECs from oxidative stress-induced apoptosis. Although not much is known about the function of PDCD2L, its paralogue PDCD2 promotes apoptosis in a number of human and mammalian cell lines and tissues. Our study indicated that PDCD2L promotes apoptosis in zebrafish endothelium in static conditions, but it did not influence EC viability in cultured human cells exposed to disturbed flow. Therefore, PDCD2L may promote apoptosis specifically in conditions of flow cessation (but not in response to oscillatory flow), or the function of PDCD2L 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. By contrast, PERP positively regulated apoptosis in EC exposed to flow cessation (in zebrafish) or flow disturbance (in human cells). 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.\textsuperscript{33} Interestingly, PERP can feedback to p53 to promote its activity\textsuperscript{42} 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.\textsuperscript{17, 19}

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 apoptosis in arteries and suggests that p53-PERP signalling could be a therapeutic target to prevent or treat early atherogenesis.
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None.
REFERENCES


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. 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. The data are representative of those obtained from n≥15 embryos studied in three independent experiments.
Fig. 6. Perp and pdcd2l promote EC apoptosis in response to flow cessation via p53. (A) perp and pdcd2l knockdown phenotypes were validated by injecting zebrafish embryos (flk1:EGFP-NLS embryos; green EC nuclei) using a second non-overlapping MO (termed MO2) or a non-targeting control MO. (B) 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, B) EC apoptosis was studied in the presence (control MO) or in the absence (sih MO) of flow by whole-mount active caspase 3 staining. 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 (lower panel). n≥15 from three independent experiments. ***p<0.001 using one-way ANOVA; ns, non-significant.
Fig. 7. Induction of **PERP** and **PDCD2L** by low WSS in cultured endothelial cells. (A) PAECs or (B) HUVECs were cultured in 6-well plates and exposed to orbital shaking (upper panels) to generate low oscillatory WSS (4.8 dyne/cm²) at the centre of the well and high uniform WSS (13 dyne/cm²) at the periphery. Alternatively, they were grown on ibidi microslides (lower panels) and exposed to low oscillatory WSS (±4 dyne/cm², 1 Hz) or high uniform WSS (13 dyne/cm²). After 72 h, 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 using an unpaired two-tailed t-test.
Fig. 8. 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 then exposed for 72 h to low oscillatory (centre) or high uniform (periphery) WSS using the orbital system (A, C), or low oscillatory (± 4 dyne/cm^2, 1Hz) WSS using the ibidi microslide system (B, C). (A, B) Apoptotic cells were measured by immunofluorescent staining using antibodies that detect cleaved caspase-3 (green) and counterstaining nuclei using ToPro-3 (purple; DNA). Data from at least three independent experiments were pooled and the proportion of apoptotic cells are shown with standard deviations. (C) PERP mRNA expression was measured by qPCR. * p<0.05, **p<0.01 by two-way ANOVA (A) or using an unpaired two-tailed t-test (B).
Fig. 9. PERP expression correlated with apoptotic EC at a low WSS region of the porcine aorta. Levels of cleaved caspase-3 (upper panels) and PERP (lower panels) were assessed in EC at low and high WSS regions of the porcine aorta by *en face* staining (green). ECs were identified by co-staining with anti-CD31 antibodies (red) and cell nuclei were identified using ToPro-3 (purple; DNA). Representative images are shown. Data were pooled from 6 animals and the proportions of apoptotic cells or PERP-positive cells are shown with standard deviations. *p<0.05, **p<0.01 using an unpaired two-tailed t-test.