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# **Shear stress regulated gene expression and angiogenesis in vascular endothelium**

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## **Abstract**

The behavior of vascular endothelial cells is greatly altered in sites of pathological angiogenesis, such as a developing tumour or atherosclerotic plaque. Until recently it was thought that this was largely due to abnormal chemical signaling, i.e. endothelial cell chemotransduction, at these sites. However, we now demonstrate that the shear stress intensity encountered by endothelial cells can have a profound impact on their gene expression and behaviour. We review the growing body of evidence suggesting that mechanotransduction, too, is a major regulator of pathological angiogenesis.

This fits with the evolving story of physiological angiogenesis, where a combination of metabolic and mechanical signalling is emerging as the probable mechanism by which tight feedback regulation of angiogenesis is achieved *in vivo*.

## **Introduction**

The endothelial lining of blood vessels is subject to numerous local environmental influences, which profoundly affect its behavior. *In vivo* influences on endothelial response include; tissue deformation from muscular strain or wound healing<sup>33</sup>; haemodynamic forces such as vessel wall tension or blood flow induced shear stress; as well as molecular influences including hypoxia and availability of soluble factors including vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF) or nitric oxide (NO), which can all promote angiogenesis<sup>14</sup>.

For the most part, the endothelium is highly metabolically active, but mitotically quiescent under healthy conditions<sup>20</sup>. In the circulation, endothelial cells (EC) are continually exposed to shear stress, as a result of blood flow, to compression by blood pressure, and to tension from strain in the extracellular matrix. Intracellular signalling pathways and gene expression can be induced or modulated by exposure to each of these forces. The physical environment is therefore an important modifier of EC functions such as proliferation, apoptosis, adhesion, motility, and matrix deposition or degradation. Endothelial cells secrete both a luminal glycocalyx that may protect from viscous drag or damage caused by the shearing effect of blood perfusing the vessel, and an abluminal basement membrane that acts as both a physical support and a location for sequestered growth factors<sup>13</sup>.

At sites of angiogenesis and vasculogenesis such as embryonal muscle development, a remodeling muscle undergoing adaption to activity or a tumour, the physical environment is altered to such an extent that endothelial cells are activated and become proliferative, migratory and committed to an angiogenic cascade of coordinated events that leads to an expanded microcirculation. A contributing factor to this change is the alteration of the shear stress experienced by the endothelium. In both the developing/ remodelling muscle, where there is heightened shear stress induced by hyperaemia associated with high metabolic rate during anabolism, and in the tumour, with poor blood flow and low shear stress, angiogenesis is promoted *via* a synergistic relationship between mechanical and biochemical factors<sup>32</sup>. Therefore, the two opposite ends of the spectrum of shear stress both activate angiogenesis.

The level of shear stress experienced by vascular cells in physiologically normal environments can vary widely. In an artery for example normal physiological shear stress falls within the range of 1-7 Pa, whereas venous shear stress ranges from 0.1-0.6 Pa<sup>40,41</sup>. Despite the level of 'normal' shear stress being highly dependant on what vessel is being referred to, the endothelium lining the these vessels tend to be mitotically quiescent under healthy conditions. Therefore it can be speculated that it is not the absolute level of high or low shear stress that induces angiogenesis, but rather how the level varies from normality. This suggests that endothelial cells may be primed to sense variation in shear stress and then respond accordingly.

### **Mechanosensory signaling in shear stress**

The mechanism by which the endothelium senses shear stress remains unclear, as the identity of all the mechanosensing molecules involved in the process is not known<sup>21</sup>.

The activation of ion channels<sup>1,47,48,51</sup>, cation channels<sup>63</sup> and stretch sensitive channels<sup>48</sup> have been observed in endothelial cells immediately after exposure to shear stress. There is also a transient increase in intracellular  $Ca^{2+}$ <sup>1</sup> as well as a considerable increase in nitric oxide production. These factors could play a role in signalling to the cell that it is under shear and eliciting a response, but the molecular identity of these channels and the mechanism by which they are activated by shear stress is unknown.

It has been suggested that shear stress is sensed by a mechanosensory complex located at the cell-cell junction, with CD31 (PECAM) thought to be the primary mechanotransducer. The suggested mechanism involves CD31 being tyrosine phosphorylated and thus directly stimulated by force to initiate signalling<sup>53</sup>. VE-cadherin functions as an adapter and brings VEGFR2 into the proximity of CD31, where it is activated by a src family kinase to recruit PI3-kinase, and thus activate downstream pathways<sup>21,75</sup>(Figure 1).

Emerging evidence suggests that CD31 is not the only mechanosensing factor. In an *in vitro* 'wound' healing migration assay, laminar flow across the culture tends to align migration with the direction of flow, therefore the closure of the wound may be

slower when compared to endothelial cells cultured in static conditions. In both human and mouse endothelial cells, CD31 knock down had no effect on the behavior of endothelial cells in this assay<sup>24</sup>. However, it was observed that the loss of CD31 reduced the sensitivity of certain genes (such as eNOS) to shear stress<sup>24</sup>. This evidence suggests that CD31 is not required for endothelial cell shear stress sensitivity in the wound healing migration assay, although it does play a role in mediating the shear stress sensitivity of certain genes. Consistent with this, CD31<sup>-/-</sup> mice show attenuated shear stress-induced angiogenesis, but retain full capacity for stretch-induced capillary growth<sup>15</sup>. Other mechanisms and mechanotransducers must therefore play some role in sensing the shear stress environment. A few candidates will be discussed.

### Glycocalyx

The glycocalyx is a dynamic polymeric meshwork coating the endothelial luminal surface consisting of proteoglycans, glycosaminoglycans, glycoproteins, and adherent plasma proteins<sup>74</sup>. This complex is thought to play an integral role in shear stress mechanosensing. Enzymatic removal of the dominant glycosaminoglycan in the glycocalyx, heparin sulfate, resulted in a significant loss of shear stress induced NO production in cultured endothelial cells<sup>20</sup>. Likewise enzymatic removal of fellow glycocalyx components, hyaluronic acid<sup>44</sup> and sialic acid<sup>54</sup> also reduces shear stress induced NO production. This suggests that shear stress induced NO production is mediated, at least in part, by signaling from components of the glycocalyx.

### Endothelial nitric oxide synthase (eNOS)

Another component that appears to be important in mediating the shear stress induced response in endothelial cells is eNOS. As has been mentioned, a characteristic endothelial response to elevated shear stress is the production of NO. This is thought to be mediated by a shear stress induced increase in eNOS activity, transcription and mRNA stability<sup>4,11,38</sup>. It is therefore possible that eNOS operates immediately downstream of other mechanosensing components transducing their signaling into an effect.

### **Nitric oxide in shear stress-induced angiogenesis**

A recent study implicated the eNOS-NO pathway as being important for the maintenance of physiological quiescence. *In vitro* HUVEC migration along a VEGF concentration gradient is greatly reduced in venous physiological shear stress (0.3 Pa) relative to static conditions (pathologically low shear stress) <sup>69</sup>. This correlates with an increase in NO in the cells under shear. However, when cells are treated with a pan-NOS inhibitor NG-monomethyl-L-arginine monoacetate (L-NMMA) the rate of endothelial migration becomes insensitive to shear stress <sup>69</sup>. This data points at a role for NO in the maintenance of vascular endothelial cells in a non-angiogenic state and is supported by the observations that L-NMMA treatment increases vascular density in the *in vivo* chick chorioallantoic membrane (CAM) assay <sup>58</sup>.

In contrast, when the level of shear stress is experimentally increased *in vivo*, endothelial cells re-enter a state of activated angiogenesis. This too appears to be mediated by the NOS-NO pathway. For example, administration of prazosin, an  $\alpha_1$ -adrenergic receptor antagonist, causes vasodilatation and increased capillary shear stress. It has been found that this leads to increased capillary to fiber ratios (C:F) and capillary density (CD) in the muscles of treated rats <sup>82</sup> and mice <sup>78</sup>. This is therefore a model of high shear stress-induced angiogenesis. This effect appears to be dependent on NOS activity. In both eNOS knockout mice and mice treated with the NOS inhibitor *N*<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME), the effect of prazosin administration on muscle C:F or CD was found to be either ablated or greatly reduced <sup>3</sup>.

Interestingly the basal C:F and CD was found to be 20% higher in the muscle of eNOS<sup>-/-</sup> mice compared to wild type <sup>3</sup>. This also supports the assertion that at physiological shear stress eNOS-NO pathway is operating to maintain vessel quiescence. The eNOS-NO pathway is therefore extremely important in mediating the entry of endothelial cells into an active angiogenic state in both low and high shear stress environments, whilst maintaining vessel quiescence in physiological shear stress.

### **Physiological and high shear stress in skeletal muscle**

There is a growing body of evidence to suggest that much physiological angiogenesis may be a combination of metabolic and mechanical signalling<sup>27</sup>, e.g. during skeletal muscle remodelling the endothelium is acting as a mechanotransducer. Despite fibre type heterogeneity within mixed muscles, endurance exercise stimulates remarkably targeted capillary growth in the vicinity of aerobic fibers, while after electrical stimulation a similar specificity occurs around glycolytic fibres<sup>2,27</sup>. In addition to an allometric scaling response, where increased fibre girth induces a greater number of capillaries in contact with the sarcolemma, exercise induces a substantial increase in blood flow (functional hyperaemia)<sup>15</sup>. As the endothelium is subjected to increased shear stress, wall tension, and deformation during the duty cycle it is difficult to unravel the proximal stimuli for exercise induced angiogenesis<sup>13</sup>. We have therefore developed animal models that emphasize the individual chemical factors postulated to be important. For the current purpose, we shall concentrate on microvascular response to increased shear stress induced by chronic vasodilatation.

As mentioned, the  $\alpha_1$ -adrenoreceptor antagonist, prazosin, provides a reasonably well targeted response in the rodent hind limb with minimal effects on central cardiovascular status such as blood flow or heart rate. In comparison with another element of the complex exercise signal, that of muscle stretch, a similar degree of expansion in the capillary bed is possible. This occurs with a combination of unique and common signalling pathways, in particular shear stress induced angiogenesis *in vivo* is critically dependent on expression of eNOS<sup>78</sup>, and results in a novel form of capillary growth, that of longitudinal splitting<sup>82</sup>. This form of capillary growth involves little cellular proliferation and may therefore be energetically efficient<sup>14</sup>. As there appears to be little or no abluminal effects, leaving the basement membrane intact, the internal separation of the capillary may be a biomechanical necessity.

There is published data from rats<sup>82</sup>, mice<sup>78</sup>, horses<sup>71</sup> and unpublished pilot data from humans (Figure 2) to suggest that such a phenomenon is widespread, and likely a common response to high shear stress, i.e. a conserved property of the mammalian microcirculation.

### Shear stress regulated genes

In addition to behavioural changes the shear stress intensity encountered by endothelial cells has been found to have a profound impact on their transcriptome<sup>8,50,72,83</sup>. A recent microarray expression profiling of Human Umbilical Cord Vein Endothelial Cells (HUVEC) exposed to differing levels of laminar flow shear stress (1.5 Pa vs. 0 Pa for 24 hours), identified ~350 genes regulated by shear stress. 190 genes (constituting 247 transcripts) were upregulated in laminar flow and 166 genes (constituting 300 transcripts) down-regulated compared to static control cells (unpublished data) (Figure 3). Several flow regulated genes were found to be pro-angiogenic or pro-migratory (summarised in Tables 1 and 2). Strikingly, the pattern of pro-angiogenic gene expression in endothelium experiencing stasis vs. laminar flow shear stress appear to mirror one another, with different members of the same family of genes serving similar roles in each condition. For instance VEGF-A is upregulated in flow whilst VEGF-B is upregulated in stasis. This suggests that angiogenesis is controlled in both conditions by different, but broadly analogous VEGF mediated mechanisms, albeit *via* different isoforms of VEGF.

A similar experiment where HUVEC were exposed to 1.2 Pa vs. 0 Pa for 24 hours identified 35 microRNAs (miR) upregulated and 26 miRs downregulated in flow compared with cells cultured in static conditions<sup>72</sup>. Among the miRs upregulated in flow was miR-19a, which is known to target both cyclin D1 (a mediator of laminar flow growth arrest) and PPAR $\alpha$  (which promotes flow-induced vascular inflammation). It would therefore appear that endothelial cells react to a changing shear stress environment *via* the induction of shear stress regulated factors, many of which are angiogenic.

It can only be speculated how this experiment relates to the situation *in vivo*. 1.5 and 1.2 Pa exceeds the 'normal' shear stress levels for both cultured cells (0 Pa/ stasis) and venous vessels (0.1-0.6 Pa) and so perhaps could be regarded as a relative high shear stress condition. Static culture replicates conditions only experienced in terminal pathology e.g. a solid tumour *in vivo*, and therefore could be said to model the shear stress environment of a tumour to some extent. It would be of interest to

investigate whether separate groups of genes are induced by varying shear stress within the venous physiological range (0.1-0.6 Pa) to ascertain whether there is a switch whereby the endothelial cell senses that shear stress has varied above or below an accepted normal level and thus signals two separate responses, or whether endothelial response is gradated depending on the level of shear stress.

From a clinical point of view it is the genes induced by a relative low shear stress environment that hold the most interest. Both cancer and atherosclerosis are diseases characterised by the presence of low shear stress microenvironments, and there is growing evidence that the endothelial response to low shear stress contributes to the progression of these diseases. From a physiological perspective, however, much of the angiogenesis associated with tissue remodeling may be associated with elevated shear stress, and hence those genes that are upregulated by shear are also of interest.

### **The tumour microenvironment and shear stress**

The tumour vasculature is highly abnormal and functionally and morphologically distinct from normal vessels<sup>35</sup>. Due to dysregulated pathological angiogenesis, tumour associated vessels are highly chaotic in nature composed of tortuous, dilated and elongated vessels with blind ends, bulges, leaky sprouts and considerable variability in diameter<sup>67</sup> (Figure 4). The endothelium itself is exposed to extreme conditions. These include hypoxia, low pH, excessive exposure to pro-angiogenic growth factors such as VEGF produced by the tumour cells, as well as poor blood flow contributing to a low shear stress environment. Exposure to these factors result in a highly abnormal vascular expression pattern and the production of several cell surface markers barely detectable on normal or quiescent vascular endothelial cells<sup>76</sup>. These markers are of considerable interest as potential ligands for the specific targeting of the tumour microenvironment with therapeutics<sup>80</sup>. The role shear stress plays in promoting their production is an emerging field of study and will be addressed in the section entitled ‘Shear stress regulated genes in pathology.’

### **Characterisation of tumour microvessel shear stress**

A tumour possesses an extremely heterogeneous environment, with variable blood flow rates and areas of relative high and low shear stress<sup>77</sup>. The transcriptome and behaviour of endothelium within the tumour, influenced by shear stress among other

factors, is likely to be similarly variable. An investigation of the extent to which this influences tumour endothelial marker heterogeneity is warranted. The issue, which has up until now prevented this investigation, is that traditional techniques for analysing tumour blood flow do not discriminate between different subtypes of vessels but instead allow the measurement of an average flow through the tumour or parts of the tumour. A technique that allows the analysis of shear on an individual vessel basis has recently been published<sup>31</sup>. This technique uses confocal or multiphoton microscopy to track the movement of labelled red blood cells and allows the generation of three dimensional flow profiles within individual vessels and networks<sup>31</sup> (Figure 5). This technique will allow us to understand how shear stress varies within tumours and other vascular networks, and start to determine the extent to which this influences endothelial behavior *in vivo*.

### **Atherosclerosis and shear stress**

Atherosclerosis is another site of pathological angiogenesis. As an atherosclerotic plaque thickens the diffusion capacity of oxygen declines and the resulting increase in angiogenic factors promotes vessel formation from the luminal surface, sustaining plaque growth<sup>45</sup>. Atherosclerotic plaques in both man and animal models develop preferentially at arterial branch points, bifurcations and the lesser curvature of the aorta<sup>9</sup>. Each of these sites are characterized by the presence of disturbed non-laminar flow resulting from flow separation and reattachment, flow reversal and reciprocating flow during the cardiac cycle<sup>9</sup>. This is thought to result in reduced shear stress at these sites<sup>23,41</sup> Figure 6. There is therefore considerable interest in analysing the role low shear stress, resulting from non-laminar flow, plays in promoting the production of atherosclerotic plaques. A multi-organ microarray expression profiling of coronary artery disease identified six genes consistently enhanced in the atherosclerotic arterial wall, associated visceral fat and the carotid stenosis (listed in Table 3)<sup>26</sup>. Notably, five out of the six genes are also cancer-associated, with putative roles in tumour angiogenesis (Table 3). Low shear stress is a major common factor between cancer and atherosclerosis and there is a possibility that the shear stress environment plays a role in the presence of these markers, though this needs to be investigated further. One gene where this does appear to be the case is CLEC14A. Its association with low shear stress environments, angiogenesis, cancer and atherosclerosis mark it out as an interesting therapeutic candidate.

### **Shear stress regulated genes in pathology**

Several shear stress regulated genes have been directly linked with the progression of vascular linked pathologies such as cancer and atherosclerosis. Examples of these include CLEC14A, ROBO4 and TIE1. These molecules will be discussed in this section.

#### CLEC14A

CLEC14A is a vascular specific member of the C-type lectin sub family 14 and is highly upregulated in a number of solid tumours including ovarian, bladder, liver and breast cancers (Mura et al., 2012). Functionally, CLEC14A appears to be highly pro-angiogenic, it promotes endothelial cell migration and tube formation *in vitro*, and also regulates zebrafish vascular development *in vivo*<sup>46</sup>. Of particular interest, however, is that CLEC14A appears to be shear stress regulated. CLEC14A expression is 10 fold enriched in HUVEC cultured in static conditions compared to those exposed to a laminar shear stress of 2Pa for 24 hours<sup>46</sup>. CLEC14A expression therefore appears to be regulated by shear stress, is highly angiogenic, and is implicated in pathological angiogenesis in both tumour and atherosclerotic plaque development.

#### ROBO4

Another shear stress regulated tumour endothelial marker is ROBO4. Its expression is ~50 fold enhanced in HUVEC cultured in static conditions compared to those exposed to a laminar shear stress of 2 Pa for 24 hours<sup>46</sup>. ROBO4 is a vascular specific member of the roundabout axon guidance receptor family. It was first identified as an endothelial specific gene by ‘*in silico* cloning,’ using a subtractive algorithm to screen publically available sequence tag expression data as a method to identify novel endothelial specific genes<sup>28</sup>. The angiogenic properties of ROBO4 are, however, the subject of some controversy. In activated endothelium, *in vitro* studies, using siRNA knockdown of ROBO4 in HUVEC, demonstrate that, like CLEC14A, ROBO4 plays a pro-angiogenic role in endothelial migration and tube formation<sup>66</sup>. Other studies have shown that ROBO4 interacts with UNC5B, a vascular netrin receptor, and promotes resting (or unstimulated) vessel integrity by inhibiting the downstream signaling of VEGF<sup>34</sup>. Function blocking antibodies against ROBO4 and

UNC5B increase angiogenesis and disrupt vessel integrity. In addition ROBO4<sup>-/-</sup> knockout mice have enhanced blood vessel growth and vessel barrier defects<sup>43</sup>, which are rescued by treatment with soluble ROBO4 protein<sup>34</sup>.

This evidence taken together suggests that the role of ROBO4 is context-dependent. In the quiescent endothelium of resting vessels, ROBO4 counteracts VEGF signalling, acting in an anti-proliferative manner to maintain the endothelial barrier. In activated endothelium, however, ROBO4 is expressed in angiogenic tip cells and promotes filopodia, cell migration and tube formation<sup>66</sup>. It is possible that the role ROBO4 plays in endothelial cell behavior is shear stress-dependent, as it appears to be pro-angiogenic in static *in vitro* conditions, but anti-angiogenic in active flow *in vivo*.

### TIE1

Tie1 is an endothelial specific tyrosine kinase receptor<sup>56,60</sup> found to be upregulated in pathologic tumour angiogenesis, arthritis and atherosclerosis<sup>30,39,65,79</sup>. Tie1 appears to be regulated by shear stress, as levels of the gene are decreased by laminar flow *in vitro* and *in vivo*<sup>59,79</sup>. Additionally Tie1 expression is increased in areas of proatherogenic low shear stress, specifically at bifurcations of aortic branches<sup>79</sup>. Tie1 is thought to play a role in endothelial activation during angiogenesis. Tie2, another endothelial specific tyrosine kinase receptor, is ubiquitously expressed in vascular endothelium<sup>12</sup> and is thought to promote endothelial integrity and quiescence<sup>55</sup>. Tie1, when present, is thought to heterodimerise with Tie2 in an inhibitory manner, reducing its ability to maintain endothelial quiescence<sup>64</sup> and thus promote endothelial activation.

Tie1 is also thought to play a key role in the formation of atherosclerotic lesions. An endothelial specific tomoxifen induced Tie1 knockout mouse strain was crossed with Apoe<sup>-/-</sup> mice, a strain with a characteristic atherosclerotic plaque production profile, to form a double knockout. It was found that a 65% reduction in Tie1 expression, induced by moderate tomoxifen treatment, resulted in a 35% and 38% reduction in atherosclerotic lesions in 24 and 49 week-old mice, respectively. Strikingly, an 80% reduction in Tie1 expression, induced by a higher dosage of tomoxifen, resulted in a 68% and 70% reduction in lesion production in 12 and 24 week old mice, respectively

<sup>79</sup>. This suggests that atherosclerotic plaque formation is dependant on Tie1 expression in a dose dependant manner.

### **Summary**

The key points contained within this review can be summarised thus:

- (i) Angiogenesis is regulated by a combination of chemical and mechanical signalling.
- (ii) Shear stress has a profound effect on the transcriptome and behavior of endothelial cells *via* signal transduction from mechanosensing molecules.
- (iii) NO is an important factor in mediating shear stress-induced angiogenesis.
- (iv) High shear induces gene expression that makes endothelial cells less atherogenic, and possibly less motile, but promotes an angiogenic phenotype.
- (v) The detection of low shear stress by the endothelium appears to play a role in atherogenesis and cancer associated vessel abnormalities.

### **Perspectives**

Shear stress appears to play an important role in regulating pathological angiogenesis as well as physiological angiogenesis, in part by regulating a large number of endothelial genes. By investigating further the effect shear stress has on vasculature undergoing pathological angiogenesis we can further understand the molecular mechanisms by which tumours vascularise and atherosclerotic plaques form, with the aim of developing therapies that specifically target these mechanisms. Similarly, understanding physiological angiogenesis may be important for development of effective rehabilitation strategies.

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**Table 1:** Genes upregulated in flow

<b>Gene Name</b>	<b>Role</b>
VEGF-A	Pro-angiogenic growth factor. <sup>5,19</sup>
Neuropilin-2	Co-receptor for VEGF and semaphorin <sup>7,18</sup>
Hyaluronoglucosaminidase 2	Extracellular matrix degradation enzyme thought to be involved in cell proliferation, migration and differentiation <sup>22</sup> .
P2Y (purinergic) receptor G-protein coupled 1	Regulator of vascular tone in response to changes in blood flow and hypoxia <sup>16</sup> .
Aquaporin	Angiogenic molecule important for endothelial cell invasion <sup>62</sup> .
MMP1	Extracellular matrix degradation enzyme, key for vascular invasion and angiogenesis <sup>70</sup> .
MMP14	Extracellular matrix degradation enzyme, key for vascular invasion and angiogenesis <sup>84</sup> .

**Table 2:** Genes down regulated in flow

<b>Gene Name</b>	<b>Role</b>
Angiopoietin 2	Context-dependent angiogenesis modulator <sup>17</sup> .
Neuropilin-1	Co-receptor for VEGF and PDGF <sup>42,68</sup>
Purinergic receptor P2X, ligand-gated ion channel 7	Regulator of vascular tone in response to changes in blood flow and hypoxia <sup>49</sup> .
CDH13 (T-cadherin)	Involved with cell-cell signaling and migration. Upregulated in sites of pathological angiogenesis including atherosclerosis <sup>57</sup> .
HIF3	Hypoxia induced response gene with similarities to the pro-angiogenic transcription factor HIF1 $\alpha$ <sup>37</sup> .
MMP11	Extracellular matrix degradation enzyme, key for vascular invasion and angiogenesis <sup>61</sup> .
VEGF-B	Pro-angiogenic growth factor vital for vessel survival <sup>81</sup>

**Table 3:** Genes expressed in the atherosclerotic arterial wall, associated visceral fat and carotid stenosis <sup>26</sup> are also associated with cancer progression and angiogenesis.

Gene name	Role in cancer
LDB2	Some association as an estrogen receptor cofactor in breast cancer <sup>29</sup>
EDG1	Strongly induced in endothelial cells during tumour angiogenesis <sup>6</sup> .
CDH5 (VE-Cadherin)	Promotes tumour progression by contributing to tumour angiogenesis and enhancing tumour cell proliferation <sup>36</sup> .
GPR116	Regulator of breast cancer metastasis <sup>73</sup> .
CLEC14A	Vascular specific tumour endothelial marker with pro-angiogenic properties <sup>46</sup>
C20orf160	None known

**Figure 1:** A schematic of the possible mechanism for mechanosensing in endothelial cells, *via* a signalling complex involving PECAM, VEGFR2 and VE-cadherin. In state **A** the endothelium is under no mechanical stress and the intracellular region of PECAM-1 is in a closed state, meaning that Src-family kinases cannot access its phosphorylation sites. In state **B** the endothelium is under mechanical strain from the blood. The actin cytoskeleton is stretched and this force is transduced to PECAM-1 via a cytoskeletal linker complex of unknown type. PECAM-1 is pulled into a linear state allowing Src-family kinases access to phosphorylation sites on the PECAM-1 intracellular region, leading to downstream signalling via the Ras-Raf-Mek-Erk pathway. Additionally VE-Cadherin brings VEGFR2 into contact with PECAM-1 forming a complex and allowing Src-family kinases to phosphorylate VEGFR2 leading to downstream signalling via Akt and MAP kinase pathways. <sup>10,21,52,57,75</sup>

**Figure 2.** Pilot data for capillary to fibre ratio (**A**) and capillary density (**B**) showing the likely pro-angiogenic response to chronic vasodilatation (by kind permission of Prof H. Hoppeler, unpublished data). *Vastus lateralis* biopsies taken from 2 healthy males (36 & 37 yo) following 8 weeks oral prazosin (2 mg.d<sup>-1</sup>). Statistical treatment of the data is inappropriate for few subjects, but the results parallel observations in

rodents treated similarly.

**Figure 3.** Differential genomic responses in shear stress. Microarray heat map showing the top 20 up and downregulated genes in HUVEC exposed to 1.5 Pa vs. 0 Pa lamina flow for 24 hours. Numbers: genes upregulated under flow / associated with high shear stress are highlighted in red; genes downregulated under flow / associated with low shear stress are highlighted in blue. More intense colour indicates a greater fold change in gene expression levels (unpublished data).

**Figure 4.** Abnormal vascular organisation results in a low shear stress environment within the tumour. Microvascular corrosion casts of human (A) normal ascending colon and (B) colorectal carcinoma. A normal shear stress environment is the result of regular vascular topography (A), while heterogeneous and low shear accompanies a dysregulation of the microvascular network in pathological conditions such as within a tumour (B). Figure adapted from <sup>35</sup>.

**Figure 5.** Two methods of tracking the three dimensional flow profiles of individual vessels in a network. The blood flow within a field of vessels in a murine glioma was tracked by (A) residence time line scanning (RTLS) and (B) relative velocity field scanning (RVFS). Both methods use multiphoton laser scanning microscopy (MPLSM). Red blood cells (RBCs) were labelled *ex vivo* with a far-red lipophilic fluorescent dye. The labelled RBCs were then introduced into the mouse circulation. RTLS involves scanning along a single line which intersects the vessel(s). This allows the direct analysis of flow velocity by generating fluorescence intensity data along the scanning line over time. By patching together data collected from multiple different angles a map of the flow rate within a network can be generated. RVFS allows a full field analysis of flow by utilising a moving scan line. RVFS is based on the analysis of the length of time a given cell spends in the scan line. The laser line takes a finite amount of time to scan across the given field of view and by analysing the proportion of the time a given cell is within the scan line, the relative movement of the cell (or blood flow rate) in the plane of the scan can be determined. To accurately measure the flow rate of vessels within a field, the data from multiple scans in different planes and at different speeds must be patched together and

analysed.

The two methods do give slightly different results indicated by the white arrows.

Figure adapted from <sup>31</sup>.

**Figure 6:** Disturbed flow at arterial branch points is thought to result in a relatively low shear stress environment. In linear regions of the artery the mechanical force exerted by the flow of blood on the vessel is unidirectional (blue regions). At arterial branch points however the flow is separated and in some places reversed resulting in some regions of the vessel wall experiencing disturbed, multidirectional and relatively low flow (red regions). Figure adapted from <sup>25</sup>.

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