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New developments in Mechanotransduction: Cross talk of the Wnt, TGF- β and Notch signalling pathways in reaction to shear stress

Aliah Abuammah^{a,b,d}, Nataly Maimari^a, Leila Towhidi^a, Jenny Frueh^a, Yean Kok Chooi^c, Christina Warboys^a and Rob Krams^c

^aDepartment of Bioengineering, Royal School of Mines, Imperial College London, London, SW7 2AZ, United Kingdom

^bDepartment of Medicine, Imperial College London, London, SW7 2AZ, United Kingdom

^cDepartment of Bioengineering, Queen Mary London, Queen Mary University of London Mile End Road London, E1 4NS, United Kingdom

^dDepartment of Medicine, King Saud University, King Fahad Cardiac Centre, Riyadh, Saudi Arabia

Aliah Abuammah aliah.abuammah11@imperial.ac.uk

Yean Kok Chooi yeank.chooi06@imperial.ac.uk

Christina Warboys c.warboys@imperial.ac.uk

Nataly Maimari n.maimari@gmail.com

Leila Towhidi l.towhidi@imperial.ac.uk

Rob Krams r.krams@qmul.ac.uk

Corresponding author:

Professor Rob Krams

Bioengineering

School of Engineering and Material Science

Room 2.14

Queen Mary University

Mile End, E1 4NS

Email: r.krams@qmul.ac.uk

Abstract

Mechanotransduction, the ability of cells to detect and react to mechanical forces, is increasingly playing a critical role in a variety of physiological and pathophysiological processes. While the focus has previously been on the MAPK, NF- κ B and ROS generating pathways, ancient embryological pathways have reached little attention. Recently, a surge of new studies have been published on these pathways and their role in mechanotransduction and this review paper aims to provide a concise overview on the latest studies and brings them in to a larger perspective. Special emphasis is on the non-canonical aspects of the Wnt, TGF-beta and Notch pathways and their role in flow.

Introduction

Mechanotransduction, the ability of cells to detect and react to mechanical forces, is increasingly understood to play a critical role in endothelial function and dysfunction. The reaction of cells to mechanical forces is complex and consists of >1600 genes, organised in a large gene network consisting of >10,000 interactions^{1,2}. In order to meaningfully study mechanotransduction, studies often focus on parts of single signalling pathways, however, it is becoming increasingly clear that pathways have a large amount of crosstalk necessitating further studies into the interaction between mechanosensitive signalling pathways^{1,2}. In a recent study of endothelial gene expression following exposure to shear stress where we merged multiple, single laboratory microarray studies to increase their power, we surprisingly identified three signalling pathways as shear sensitive: Wnt, TGF- β , and Notch. Here we will describe the current findings on the sensitivity of the individual pathways to shear stress and evaluate the meaning of their cross talk with respect to endothelial function.

Mechanotransductive forces and the Wnt pathway

The Wnt signalling pathway regulates a diverse range of cellular processes including proliferation, survival, differentiation, polarity, migration and repair and has been reviewed extensively elsewhere^{3,4}. The Wnt pathway is highly complex due to the presence of 19 Wnt ligands, 10 Frizzled receptors and multiple co-regulators. The key components of the canonical and non-canonical Wnt signalling pathways are summarised in Figure 1.

Both the canonical and non-canonical pathways have been shown to react to mechanical forces in mesenchymal stem cells⁵ and osteocytes⁶ and to play a role in vessel maturation and repair in zebrafish⁷. During lymphatic valve formation oscillatory shear stress (OSS) increases the activation of β -catenin and the expression of β -catenin dependent genes that direct valve formation⁸. In endothelial cells of developing cardiac valves, it has similarly been shown that KLF-2 regulates Wnt-9a expression in reaction to increments in shear stress⁹. In contrast, in endothelial cells under unidirectional, laminar shear stress (LSS), the non-canonical pathway (Wnt-5a, Wnt-11) has been shown to inhibit axial polarization and reduce flow sensitivity, independent of downstream effects of KLF-2/KLF-4¹⁰. Therefore, it seems that lymphatic, valvular and vascular endothelium respond differently to shear stress in terms of Wnt-regulation.

To further study the role of Wnt signalling in adult endothelial cells under flow, we merged multiple, microarray studies to increase the power of these studies and detect more subtle changes in Wnt signalling in response to shear stress². Our study identified shear regulation of members of the canonical pathway (Porc, Wnt-1, Frizzled-1, TCF/LEF, and cycD), members of the non-canonical Wnt-5a/Ca²⁺ pathway (Frizzled-1, PLC and PKC) and members of the non-canonical Planar Cell Polarity (PCP) pathway (Frizzled, Prickle, and RAC). Subsequently, we evaluated whether KLF-2 was upstream of this

activation, by inhibiting KLF-2 with siRNA in HUVECs subjected to 48 hours of high shear stress (2Pa). In this study, we identified Wnt-10b, Fzd-1, TCF-7-L1 (transcription factor 7-like 1), IL-8, NLK (Nemo-Like Kinase), PAI-1 (plasminogen activator inhibitor), CTNNB-1P-1 (β -catenin interacting protein-1) and endothelin to be KLF-2 dependent (unpublished data). However, in above studies the level of shear stress on Wnt activation was not evaluated and it may still be the case that a different shear stress sensitivity exists for canonical vs. non-canonical pathways.

Mechanotransductive forces and the Notch pathway

Notch is a highly conserved pathway that plays an important role during vascular development and arterial specification, sprouting angiogenesis and in endothelial quiescence¹¹. The signalling pathway is summarised in Figure 2. As with the Wnt pathway multiple combinations of delta-like/Jagged ligands with Notch receptors adds complexity and evidence of non-canonical Notch pathways are also emerging¹². Recently, a non-canonical Notch pathway was identified in endothelial cells involving the transmembrane domain of Notch that regulates the adherens junction and endothelial permeability¹³.

Several studies have demonstrated that the Notch pathway is regulated by shear stress in endothelial cells. The majority of studies report that the expression of Notch ligands, Notch and the nuclear accumulation of NICD increases in response to LSS in a dose-dependent manner^{14–18}. Our own study confirmed these findings; Fringe, Delta, DeltaX, SMRT and Hes1/5 were upregulated with time in adult endothelial cells under LSS². Conversely, another study reported a contradictory observation that the Notch pathway was activated preferentially by low/OSS and was unaffected by LSS, although here the cells were exposed to shear stress for 1h only and so the significance of these findings is limited¹⁹. Similarly another study reported that LSS (2 dynes/cm² for 24h) reduced Notch expression although this effect was observed in lymphatic endothelium; this effect was not observed in vascular endothelium possibly since the magnitude of shear stress was too low to elicit a response²⁰. Another study also reports that Notch is activated by low shear stress and has atherogenic functions²¹ although an absence of adequate controls for example, use of a non-constrictive carotid cuff to control for the effects of flow modification or inflammation/scarring and the lack of *in vitro* data for high shear stress over the same time course limit the interpretation of this study. A recent comprehensive study looked at the shear-dependent regulation of Notch in adult endothelial cells²². LSS increased the expression of Notch-1 in a dose and time-dependent fashion²². Furthermore, the authors propose that Notch has atheroprotective functions since silencing of Notch with siRNA increased proliferation and inflammation and destabilised cell-cell junctions whilst genetic deletion of Notch in endothelial cells increased atherosclerosis in protected (high shear) regions of the vasculature in hypercholesterolemic mice²². Intriguingly, Notch can also be regulated by KLF4²³ but the role of this signalling axis in shear conditions is unknown. The mechanoactivation of the non-canonical Notch pathway in endothelial cells has received little attention to date and is an important area for future study.

Mechanotransductive forces and the TGF- β pathway

The TGF- β signalling pathway is involved in a variety of cellular including cell growth, cell differentiation, apoptosis, and quiescence. The pathway is reviewed in detail elsewhere²⁴ and summarised in Figure 3. TGF- β signalling regulates the activation state of endothelial cells through its opposing actions on ALK-1 and ALK-5 receptors; TGF- β promotes quiescence via activation of ALK-5, whereas activation of ALK-1 increases proliferation.

Shear stress affects the TGF- β pathway in multiple ways. LSS increased the expression of active and latent TGF- β 3 (but had no effect on TGF- β 1) and increased activation of Smad2²⁵. Furthermore, inhibition of TGF- β 3 or ALK5 was found to reduce the shear-dependent increase in KLF-2 expression

and NO production suggesting that the TGF- β 3/ALK5/Smad2 axis is atheroprotective²⁵. Furthermore, LSS also reduced the pro-inflammatory effects of TGF- β 1 through increased expression of inhibitory Smads and inhibition of TAK-1²⁶. On the other hand, OSS has also been shown to increase Smad2 activation which was responsible for the increased NF- κ B activation under these conditions. Interestingly these effects were much more pronounced in cells cultured on fibronectin suggesting interaction of integrin signalling with the TGF- β pathway²⁷. OSS also increased TGF- β 1 expression in aortic valve endothelium which may be linked to increased endothelial-to-mesenchymal transition observed in these cells²⁸. In addition, the expression of BMPs is also regulated by shear stress; it has long been recognised that OSS mediates pro-inflammatory signalling via increases in BMP-4²⁹.

Our study, based on an extended microarray analysis, confirmed the above findings and extended them by showing that the following components of the canonical TGF- β pathway were regulated by shear stress: BMPRII, SMAD1/5/8, SMAD1/2, SMAD6/7, P107 and FST².

Crosstalk between Wnt, Notch and TGF- β pathways

Despite their largely overlapping functions in the vasculature, only a few studies have focussed on the interaction of the above pathways in endothelial cells. The findings are summarised here with a focus on research in vascular cells in the last 5 years

Wnt and Notch

Crosstalk between these two pathways occurs at many levels, through protein interactions, formation of transcriptional complexes and post-translational modifications. In many cases Wnt/ β -catenin acts in synergy with Notch; β -catenin directly associates with and co-activates NICD forming a transcriptional complex in arterial cells that drives the expression of genes that regulate arterial specification³⁰. Evidence also suggests that NICD can be phosphorylated by GSK3 β and hence regulated by the Wnt signalling pathway³¹. Notch signalling can also be inhibited following activation of the Wnt pathway due to an inhibitory interaction of Dishevelled with Notch³². Wnt signalling can also be modulated by the Notch pathway; Notch can bind to and sequester unphosphorylated (active) β -catenin at the membrane, preventing nuclear localisation and transcriptional activity, therefore acts to negatively regulate Wnt signalling^{33,34}. Crosstalk between Wnt and Notch has been shown to play a crucial role in vascular remodelling and angiogenesis. During vascular development, β -catenin promotes Notch activity in endothelial cells by binding to the Dll4 promoter and up-regulating the transcription of Dll4 and increasing Notch signalling³⁵.

Wnt and TGF β

The crosstalk between Wnt and TGF β is well documented and has been shown to play an important role in development. The two pathways act synergistically to drive patterns of gene expression that are important in differentiation and determination of cell fate although crosstalk between the two pathways has rarely been studied in endothelial cells. Recently it was shown that TGF β 1/Smad3 increased the expression of Frizzled-7 in pulmonary fibroblasts and that knockdown of Frizzled-7 attenuated TGF β 1-induced increases in the expression of ECM proteins³⁶. In adult mesenchymal stem cells TGF β 1 induced the rapid translocation of β -catenin to the nucleus in a Smad3-dependent manner³⁷. TGF β /Smad3 also increased the expression and secretion of canonical Wnt ligands in smooth muscle cells³⁸. TGF β can also promote canonical Wnt signalling in fibroblasts by decreasing the expression of DKK-1³⁹. Crosstalk may be mediated by direct molecular interactions between transcription factors associated with the two pathways to form unique transcriptional complexes. For example, Smad2, Smad3 and Smad4 can directly associate with LEF-1 which can bind to the promoter

regions on LEF-1 target genes independently of β -catenin⁴⁰. Smad7 can also interact with membrane-bound β -catenin to stabilise cell-cell junctions⁴¹. Crosstalk can also occur in the cytosol via interaction of Smad3 with Axin⁴².

TGF β /BMP and Notch

Crosstalk between the Notch and TGF β /BMP signalling pathways has been well described and reviewed in detail elsewhere^{43,44} and therefore for the purposes of this review we will focus on the interaction of these pathways in endothelial cells. Synergy between these two pathways plays an important role in angiogenesis and vascular biology, promoting quiescence and vessel stability. For example, in a mouse retinal development model increased BMP9/ALK-1 signalling promotes the expression of the Notch target genes, Hey1 and Hey2 which inhibits VEGF-induced angiogenesis and plays an important role in vascular morphogenesis⁴⁵. Similarly, Smad1/Smad5 is required for Dll4/Notch-induced signalling in stalk cells to regulate lateral inhibition in developing mouse embryos⁴⁶. Notch and TGF β signalling pathways also co-operate to maintain blood-brain barrier integrity. Stimulation of cerebrovascular endothelial cells with TGF β promotes the interaction of Smad4 with RBP- to form a transcriptional complex that binds to and activates the N-cadherin promoter which plays an important role in regulating endothelial-pericyte interactions⁴⁷. BMP also acts to enhance Notch signalling in mouse embryonic endothelial cells to inhibit migration and limit angiogenesis; BMP increases the expression of Herp2 through formation of a transcriptional complex comprised of NICD and Smad⁴⁸. Numerous studies have shown that the Notch and TGF β signalling pathways also synergise to promote endothelial-to-mesenchymal transition (End-MT) in rat⁴⁹ and mouse⁵⁰ endothelial. It has also been shown that Smad1 interacts with NICD in response to BMP2 in human aortic smooth muscle cells. This complex binds to the Msx2 promoter resulting in increased gene expression leading to increased vascular calcification⁵¹.

Summary & Conclusions

In summary, the Notch, TGF β and Wnt signalling pathways are known to be up-regulated by laminar shear stress and to play a role in endothelial function and dysfunction. Although these pathways are shear-sensitive the mechanisms of mechanoactivation are unknown and may be mediated by i) upstream mechanosensor(s) that regulate the expression of the components of the pathways, ii) known mechanosensitive pathway e.g. MAPK5-KLF2 pathway, or iii) paracrine and autocrine release of co-factors that may modulate the pathways. It is important to note that the vast majority of these studies have compared one type of shear stress (high or low/oscillatory) with static conditions. Since static culture does not represent a good physiological reference it will be important to directly compare responses between atheroprone and atheroprotective flow types to increase our understanding of the roles of these pathways in endothelial mechanosignalling. Similarly, the vast majority of the studies on crosstalk between these pathways in endothelial cells have been targeted towards understanding vascular development and angiogenesis. The role of crosstalk between these pathways in adult endothelial cells is largely unknown. Furthermore, there have been no studies on the interaction between these pathways in combination with shear stress and as such this is an important area of future study.

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Legends

Figure 1: Schemes of the canonical (left panel), the non-canonical Wnt/ Ca^{2+} (middle panel) and the non-canonical Wnt/PCP pathways (right panel). All three pathways are activated by binding a Wnt-protein ligand to a Frizzled family receptor, which passes the biological signal to the Dishevelled protein inside the cell. The canonical Wnt pathway leads to regulation of gene transcription, the Wnt/Calcium pathway to calcium release and the Wnt/PCP regulates cellular polarisation through its effect on the cytoskeleton. As interaction between the canonical and non-canonical pathways have been increasingly identified the integration of Wnt as a single pathway has been proposed, although whether this occurs in endothelial cells is unknown

Figure 2: The Notch pathway is an ancient pathway that allows to modify genes in neighbouring cells. When endothelial cells are brought under high shear stress the sender cell expressed Notch ligands, which activate the NOTCH1 receptor. As a consequence, the canonical pathway is activated leading to endothelial quiescence. Under low shear stress the non-canonical pathways are regulated leading AKT, PI3K and mTOR activation. Note that Ror2 is sensitive to shear stress activating JNK and inflammation. There are four receptors Notch-1-4 which are activated by 5 mammalian ligands (Delta-like or DLL1, 3, 4 and Jagged 1, 2). After receptor ligation, a series of coordinated protease steps are initiated leading to the release of the intracellular domain of Notch, the Notch Intracellular Domain or NICD. The NICD translocates into the nucleus and binds with RBPJ (Recombination signal-binding protein 1 for J-Kappa), MAML (Master-mind like), p300, and co-activators (CoA) to activate Notch-responsive genes. In the absence of NICD, RBPJ is bound by co-repressors (Cora) and cannot activate Notch-responsive genes. Quiescent endothelium expresses Delta-like1 (DLL-1), DLL-4, Jagged-1, Notch-1 and Notch-4 and uses predominantly the DLL4-Notch-1 signalling pathway to regulate endothelial function.

Figure 3: The TGF-beta pathway reacts to physiological shear stress by activating the canonical pathway leading to growth arrest, and quiescence. In low shear stress both the BMP and TGF-beta receptors are activated leading to NF- κ B activation and to an mTOR dependent proliferation. TGF- β superfamily (Bone Morphogenetic Proteins (BMP), growth and differentiation factors (GDF), TGF- β , Activin, Nodal) ligands bind to a type II receptor (serine/threonine receptor kinase), which recruits and phosphorylates a type I receptor. Each ligand binds to its own type II receptor conferring a level of specificity []. Once activated the type I receptor phosphorylates receptor-regulated r-SMADs (SMAD-1, -2, -3, -5, -8) allowing them to form complexes with common partner (co)-SMADs i.e. SMAD-4. Activated r-SMAD/coSMAD complexes dissociate from the type II receptor translocate to the nucleus where they regulate the transcription of target genes. Besides, positive regulation by ligand-receptor interaction, negative regulation occurs at a variety of levels in the signalling cascade. Firstly, the TGF- β ligands are secreted as latent complexes and their activation is regulated by a multitude of factors (pH, integrins, ROS) []. Secondly, inhibitory or pseudo receptors and inhibitory SMADs (i-SMAD) exist that may prevent or inhibit existing activity []. Thirdly, fine tuning of SMAD concentration occurs by increased/decreased ubiquitination through E3 ligases []. Specificity is therefore regulated by different mechanism, often in combination.

Figure 4: Crosstalk between the Notch and Wnt pathways: 1) β -catenin promotes Notch activity in endothelial cells by binding to the DLL4 promoter and up-regulating the transcription of DLL4 and increasing Notch signalling during vascular development, . 2) β -catenin directly

associates with and co-activates NICD forming a transcriptional complex that binds to the Hes1 promoter and increases Notch signalling. **3)** NICD can form a complex with β -catenin in arterial cells and by localising to RBP-J binding sites can drive the expression of genes that regulate arterial specification. **4)** NICD interact with the LEF-1 Wnt pathway transcription factor to form a transcriptional complex. This complex drives the expression of a unique subset of genes, distinct from those regulated by Notch or Wnt/ β -catenin. **5/6)** NICD regulated by the Wnt signalling pathway through GSK3 β phosphorylation. **5)** Phosphorylation of NICD1 leads to increased stability, enhanced nuclear translocation and increased Notch activity. **6)** Phosphorylation of NICD2 results in reduced transcriptional activity of Notch and expression of Notch target genes. **7)** Notch signalling can be inhibited following activation of the Wnt pathway due to an inhibitory interaction of Dishevelled with Notch. **8)** The expression of Frizzled is increased by Notch in dendritic cells. **9)** Notch induces the expression of Nrarp (*Notch-regulated ankyrin repeat protein*) that binds to and stabilises LEF-1 increasing Wnt-mediated transcriptional activity and promoting stalk cell proliferation.

Figure 5: Crosstalk between Wnt and TGF β : **1)** TGF β 1 induces the rapid translocation of β -catenin to the nucleus in a Smad3-dependent manner; Smad3 is thought to act as a chaperone that promotes stability and facilitates nuclear accumulation. **2)** TGF β /Smad3 increases the expression and secretion of canonical Wnt ligands (Wnt2b, Wnt5a, Wnt9a and Wnt **4)** in smooth muscle cells that increases proliferation. **3)** high concentrations of TGF β 1 inhibited β -catenin expression which may indicate a role in scar re-modelling/maturation. **4)** TGF β can promote canonical Wnt signalling in fibroblasts by decreasing the expression of DKK-1. **5)** Wnt has been shown to regulate the expression of Nodal, a member of the TGF β superfamily, that plays a critical role in left-right determination in the chick embryo. **6)** Smad2, Smad3 and Smad4 can directly associate with LEF-1 which can bind to the promoter regions on LEF-1 target genes independently of β -catenin. **7)** Smad4 can form a transcriptional complex with β -catenin and LEF-1 that mediates co-operative signalling between the two pathways during embryonic development **8)** In mouse embryonic stem cells the BMP-dependent regulation of Msx2 requires the formation of a complex between Smad4 and LEF-1. **9)** Axin promotes ubiquitination and degradation of Smad3 attenuating TGF β signalling.

Figure 6: Crosstalk between the Notch and TGF β /BMP signalling pathways in endothelial cells: **1)** Increased BMP9/ALK-1 signalling promotes the expression of the Notch target genes Hey1 and Hey2 ,in a mouse retinal development model, which inhibits VEGF-induced angiogenesis and plays an important role in vascular morphogenesis. **2)** Smad1/Smad5 is required for Dll4/Notch-induced signalling in stalk cells to regulate lateral inhibition. **3)** The expression of the Notch target gene Herp2 is potentiated by BMP through formation of a transcriptional complex comprised of NICD and Smad that requires the presence of RBP-Jk. This complex binds to the Herp2 promoter and increases gene expression. **4)** The Notch inhibitor DAPT significantly inhibited TGF β induced End-MT in rat corneal endothelial cells. **5)** Smad1 interacts with NICD in response to BMP2 in human aortic smooth muscle cells. This complex binds to the Msx2 promoter resulting in increased gene expression leading to increased vascular calcification.

Wnt/β-catenin signaling pathway after shear

Notch signaling pathway in shear

The novelty of the paper may be summarised as follows

- Previously pathways of the domain of embryology (Notch, Wnt, TGF-beta) have been shown to be shear sensitive.
- Especially their non-canonical variants seem to be important for blood flow related diseases like atherosclerosis.
- These three pathways seems to be interconnected forming a functional overarching network, which is sensitive to shear stress.