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$P2Y_2$ and G_q/G_{11} control blood pressure by mediating endothelial mechanotransduction

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1. ABBREVIATIONS

°C degree Celsius

µ micro

ATP adenosine triphosphate

ADP adenosine diphosphate

BAECs bovine aortic endothelial cells

BSA bovine serum albumin

D-PBS Dulbecco's phosphate buffer saline

EC endothelial cell

eNOS endothelial nitric oxide synthase

ERK1/2 extracellular-signal-regulated kinase-1/2

FAK focal adhesion kinase

FBS fetal bovine serum

Gα G-protein α subunit

Gβγ G-protein βγ subunit

GAP GTPase-activating proteins

GEF guanine nucleotide exchange factor

GPCR G-protein coupled receptor

H hour

HBSS Hank's buffered salt solution

HEK human embryonic kidney

HUVEC human umbilical vein endothelial cells

HUAEC human umbilical arterial endothelial cells

Ig immunoglobulin

1. Abbreviations

KO knockout

Kd knockdown

KLF Kruppel-like factor

M molar

m milli

MAPK mitogen-activated protein kinase

Min minute

NF-κB nuclear factor kappa B

NO nitric oxide

P2Y2 P2Y purinergic receptor 2

PCR polymerase chain reaction

PECAM-1 platelet endothelial cell adhesion molecule 1

PFA paraformaldehyde

pH potential hydrogenii

PI3K phosphoinositol 3-kinase

PKA protein kinase A

PKC protein kinase C

PLC phospholipase C

PTX pertussis toxin

Rac1 RAS-related C3 botulinum substrate-1

RhoA Ras homolog gene family: member-A

RTK receptor tyrosine kinase

S1P sphinogosine-1-phosphate

TIE-2 tunica internal endothelial cell kinase-2

VEGF vascular endothelial growth factor

2. SUMMARY

Hypertension is a primary risk factor for cardiovascular diseases including myocardial infarction and stroke. Major determinants of blood pressure are vasodilatory factors such as nitric oxide (NO) released from the endothelium under the influence of fluid shear stress exerted by the flowing blood. Defects in flow-induced NO formation go along with endothelial dysfunction, initiation and progression of atherosclerosis as well as with arterial hypertension. Previous work has identified several mechanotransducing signaling processes involved in fluid shear stress-induced endothelial effects. But how fluid shear stress initiates the response is poorly understood. Here, I show in human and bovine endothelial cells that the G-protein G_q/G_{11} and the purinergic receptor $P2Y_2$ mediate fluid shear stress-induced endothelial responses such as Ca^{2+} release, nitric oxide (NO) formation and the phosphorylation of platelet-endothelial-cell-adhesion-molecule-1 (PECAM-1), vascular endothelial growth factor-2 (VEGFR-2) and Akt kinase as well as activation of the endothelial NO synthase (eNOS). $P2Y_2$ receptor is activated by adenosine triphosphate (ATP) which is released from endothelial cells under the influence of fluid shear stress. Arteries with $P2Y_2$ or $G\alpha_q/G\alpha_{11}$ deficiency have impaired flow-induced dilatation. Mice with induced endothelium-specific deficiency of $P2Y_2$ or $G\alpha_q/G\alpha_{11}$ develop hypertension which is accompanied by reduced eNOS activation. My data identify $P2Y_2$ and G_q/G_{11} as a critical endothelial mechano-signaling pathway located upstream of mechanotransducing processes described so far. Moreover, I demonstrate that $P2Y_2$ and G_q/G_{11} are required for basal endothelial NO formation, vascular tone and blood pressure.

3. ZUSAMMENFASSUNG

Bluthochdruck ist ein Hauptrisikofaktor für Herz-Kreislauferkrankungen wie zum Beispiel Herzinfarkt oder Schlaganfall. Hauptkomponenten zur Regulierung von Blutdruck sind vasodilatorische Faktoren wie Stickstoffmonoxid (NO), welche aus dem Endothel unter dem Einfluss des Blutstromes und den daraus resultierenden Scherkräften freigesetzt werden. Defekte in Scher-induzierter NO-Bildung werden häufig mit endothelialer Fehlfunktion, der Entstehung und Entwicklung von Atherosklerose, sowie Bluthochdruck in Verbindung gebracht. Frühere Arbeiten haben zwar einige mechanotransduzierende Signalwege identifiziert, welche in der Regulierung von Scher-induzierten Effekten in Endothelzellen involviert sind. Wie Scherkräfte diese Signalwege jedoch induzieren, bleibt ungeklärt. In dieser Arbeit zeigen wir in menschlichen und bovinen Endothelzellen, dass der purinerge G_q/G_{11}-gekoppelte $P2Y_2$-Rezeptor eine Reihe von Scher-induzierten Signalwegen in Endothelzellen auslöst: Ausschüttung von Calcium, Bildung von NO, Phosphorylierung von PECAM-1, VEGFR-2 und Akt, sowie die Aktvierung der NO-Synthase eNOS. Dabei wird $P2Y_2$ durch ATP aktiviert, welches von Endothelzellen nach Strömungs-Stimulation und den dadurch entstehenden Scherkräften sezerniert wird. Des Weiteren weisen isolierte Arterien ohne $P2Y_2$ oder $G\alpha_q/G\alpha_{11}$ eine verminderte Strömungs-induzierte Gefäßweitung auf, und Mäuse mit endothelzell-spezifischem Verlust von $P2Y_2$ oder G_q/G_{11} weisen erhöhten Bluthochdruck und verminderte eNOS-Aktivität auf. Zusammenfassend kann man sagen, dass unsere Arbeit $P2Y_2$ und G_q/G_{11} als wichtige „upstream“-Komponenten der Scher-induzierten Signaltransduktionskette in Endothelzellen identifiziert, und, darüber hinaus, dass $P2Y_2$ und G_q/G_{11} für die basale NO-Bildung in Endothelzellen, sowie für die Regulierung des Gefäßtonus und des Blutdruckes erforderlich sind.

4. INTRODUCTION

4.1 Endothelial cells are mechanosensitive

All cells and organisms, from the embryonic state to the end of life, are physically subjected to mechanical forces and able to translate mechanical stimuli into biochemical signals, a cellular process known as mechanotransduction (DuFort et al., 2011). Extensive research in the field of mechanotransduction has expanded the initial focus from sensory cells to diverse cell types such as myocytes, endothelial cells and smooth muscle cells (Jaalouk and Lammerding, 2009). In the cardiovascular system, endothelial cells and smooth muscle cells are the major cell types of blood vessels and permanently subjected to mechanical forces due to the pulsatile nature of the blood flow caused by the beating heart. Vascular smooth muscle cells (VSMCs) are the major component of the vessel wall and they are primarily subjected to the circumferential stretch resulting from pulsatile changes in blood pressure (Fig. 4-1). Vascular endothelial cells, located between the circulating blood and the vessel wall, are constantly exposed to the frictional force often known as fluid shear stress due to flowing blood (Fig. 4-1).

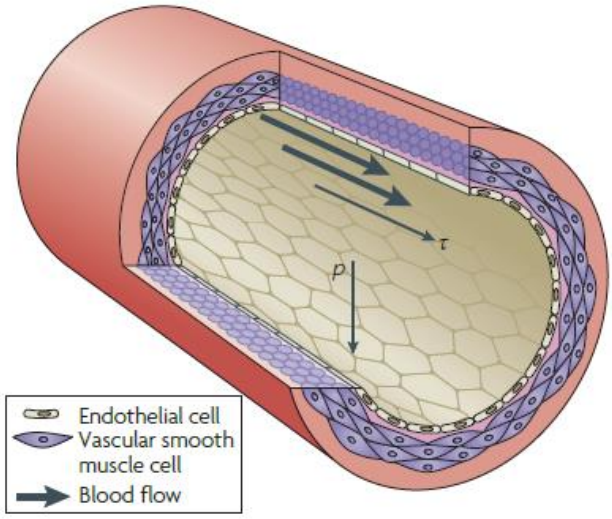

Figure 4-1: Flow and forces on the vessel wall.
Endothelial cells are located in the innermost layer of blood vessel and align longitudinally, and vascular smooth muscle cells form the outer layers of vessel

and align circumferentially. Hydrostatic pressure (*p*) is applied to the vessel wall, which results in the circumferential stretching of the vessel wall. Fluid shear stress (*τ*), the frictional force per unit area, is parallel to the vessel wall and is exerted longitudinally in the direction of the blood flow (Hahn and Schwartz, 2009).

The endothelial cells also are subjected to circumferential stretch and hydrostatic pressure, but it was well accepted in the scientific community that those two physical stimuli are much less important than shear stress (Hahn and Schwartz, 2009).

The very first piece of evidence demonstrating that endothelial cells could sense and respond to fluid shear stress was found by the German pathologist Rudolf Carl Virchow about 150 years ago. In his pioneer study, Virchow observed that endothelial cells morphology in the artery was correlated to the blood flow pattern and that alterations in the blood flow resulted in vascular endothelial injury and thrombosis (Virchow, 1847). Since then, a considerable amount of information on endothelial mechanotransduction has been accumulated from both *in vivo* and *in vitro* studies, and there is now a general consensus that vascular endothelial cells are mechanosensitive (Hahn and Schwartz, 2009; Wozniak and Chen, 2009). The most direct evidence to support this notion is the fact that both under *in vivo* and *in vitro* conditions endothelial cells are elongated, spindle-shaped and uniformly orientated with the long axis parallel to the direction of the flow (Chiu and Chien, 2011; Li et al., 2005). For example, Flaherty et al. reported that endothelial cells from canine thoracic artery were oriented parallel to the direction of blood flow *in vivo* (Flaherty et al., 1972). In addition, they re-implanted a segment of canine thoracic aorta at 90°C to its original orientation and observed that within 10 days after surgery the nuclei of endothelial cells realigned with the direction of the flow (Flaherty et al., 1972). By contrast, when subjected to turbulent flow, the endothelial cells aligned to random directions and had much rounder shapes (Chiu and Chien, 2011; Conway et al., 2010).

In addition to structural responses, endothelial cells also respond to fluid shear stress by changing their functions e.g. proliferation, migration, permeability, and

apoptosis (Ando and Yamamoto, 2009). It has been shown that laminar shear stress causes a reduction of the rate of endothelial cells proliferation (Levesque et al., 1990). In contrast, disturbed flow-induced a higher turnover rate in endothelial cells (Davies et al., 1986; Levesque et al., 1990). Studies by Sprague et al. have shown that endothelial cells migration in wound healing is significantly enhanced by laminar shear stress (Sprague et al., 1997). Hermann and coworkers have found that shear stress suppresses endothelial cells apoptosis induced by treatments such as with TNF-α and/or exogenous oxygen radicals (Hermann et al., 1997). Recently, a study by Satchell and colleagues showed that fluid shear stress reversibly increases endothelial cells permeability via activation of endothelial nitric oxide synthase (eNOS or NOS3) (Bevan et al., 2011).

In addition to behavioral and functional changes, shear stress has been found to regulate gene expression in endothelial cells. Using a DNA microarray technique, McCormick found 52 genes with significantly altered expression under shear stress (McCormick et al., 2001). A more recent study demonstrates that fluid shear stress-dependent epigenetic DNA methylation regulates endothelial gene expression and atherosclerosis (Dunn et al., 2014). All those data point towards the conclusion that endothelial cells are mechanosensitive; they respond to fluid shear stress by changing their morphology, function and gene expression.

4.2 Mechanotransduction of fluid shear stress in endothelial cells

Application of fluid shear stress to endothelial cells stimulates numerous receptors and molecules at both the luminal and abluminal membranes as well as at cell-cell junctions, followed by the activation of numerous signaling pathways within the cell cytosol, a process referred to as mechanotransduction (Fig. 4-2) (Ando and Yamamoto, 2009; Hahn and Schwartz, 2009; Zhou et al., 2014a).

Within seconds, shear stress activates several ion channels, e.g. the TRPV channels, whose activation is one of the earliest responses induced by shear

stress (Kwan et al., 2007). It has been reported that TRPV1, TRPV3 and TRPV4 are expressed in the endothelium and that their activation by shear stress induces a Ca^{2+} influx into endothelial cells (Kwan et al., 2007). A study by Hoger et al. demonstrated that the onset of flow induces an increase of the K^+ ion current in endothelial cells as a result of the activation of Kir2.1 channels (Hoger et al., 2002). Other ion channels like Cl^- channels are also activated shortly after shear stress stimuli (Gautam et al., 2006). The importance of ion channels for shear stress signaling is emphasized by their ability to change the electrophysiological properties of endothelial cells, leading to an increase in $[Ca^{2+}]_i$, and thereby triggering multiple Ca^{2+} dependent signaling pathways. Another important event occurring with the onset of flow is the activation of Src family kinase. Chen and Tzima, for instance, report in several studies that they observed Src to be phosphorylated within a few seconds after the onset of flow, which in turn led to further phosphorylation of platelet endothelial cells adhesion molecule 1 (PECAM-1) and several other tyrosine kinases such as VEGFR-2 and Tie2 (Chen et al., 1999; Takahashi et al., 1997; Tzima et al., 2005).

Within minutes, fluid shear stress triggers the activation of many signaling molecules including integrins (Chen et al., 1999), Rho family GTPases (Tzima et al., 2001), scaffolding protein Gab1 (Dixit et al., 2005), the tyrosine phosphatase SHP2 (Dixit et al., 2005), Jun N-terminal kinase (JNK) (Tzima et al., 2002), extracellular-signal-regulated kinase (ERK) (Jo et al., 1997), protein kinase A (PKA) (Boo, 2006; Boo et al., 2002) and protein kinase B (Akt) (Dimmeler et al., 1999; Fulton et al., 1999). As a result, multiple pathways are activated in this phase, leading to alterations in endothelial cell morphology and function, and to the activation of various transcription factors.

For quite a while now, it has been well accepted that fluid shear stress regulates a number of gene expressions in endothelial cells. Using DNA microarray technology, Benjamin and coworkers identified genes involved in endothelial cells survival and angiogenesis (Tie-2 and VEGFR-2) and vascular remodeling (matrix metalloproteinase 1) that were upregulated in human aortic endothelial cells after 24h of shear (Chen et al., 2001a). In contrast, tyrosine kinase receptor Tie1 expression was reduced by laminar flow. While at regions with

atherogenic disturbed flow, Tie1 expression was upregulated, which was accompanied by decreased IkBα expression but increased ICAM levels, indicating that atherogenic shear stress-induced Tie1 expression may play a novel pro-inflammatory role in atherosclerosis (Woo and Baldwin, 2011; Woo et al., 2011).

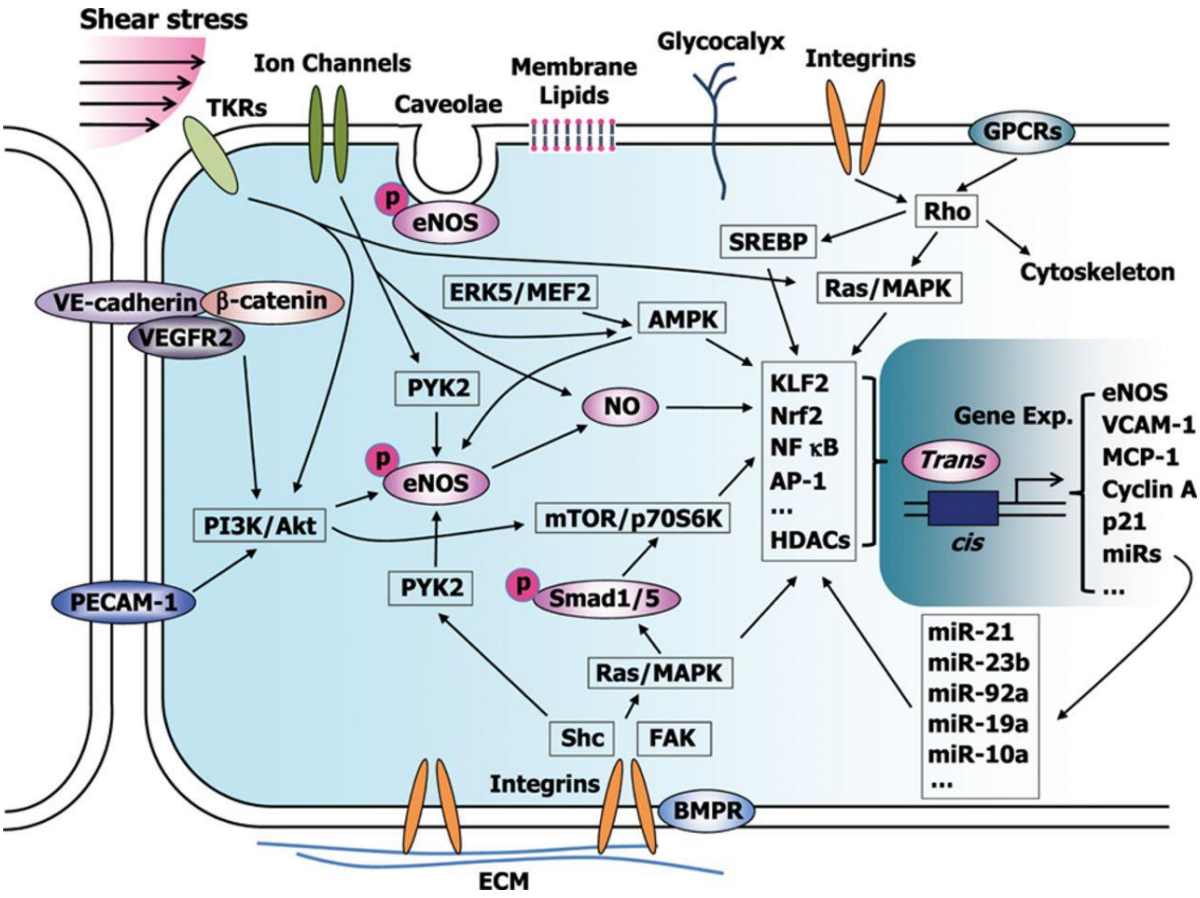

Figure 4-2: Endothelial mechanotransduction induced by fluid shear stress.
Fluid shear stress are capable of activating multiple factors including tyrosine kinase receptors (TKRs), G proteins and G-protein–coupled receptors (GPCRs), ion channels, that lead s to activation of various signaling pathways and many transcription factors. VE-cadherin, vascular endothelial cadherin; PECAM-1, platelet endothelial cell adhesion molecule 1; VEGFR2, vascular endothelial growth factor receptor-2; PI3K, phosphatidylinositol-3-kinase; MAPKs, mitogen-activated protein kinases; eNOS, endothelial nitric oxide synthase; KLF2, Kruppel-like factor 2; NF-κB , Kruppel-like factor 2; VCAM-1, vascular cell adhesion protein 1; MCP-1, monocyte chemoattractant protein-1; AMPKs, AMP-activated protein kinases; BMPR, bone morphogenetic protein receptor; ERK,

extracellular-signal-regulated kinase; FAK, focal adhesion kinase; HDAC, histone deacetylase; MEF2, myocyte enhancer factor-2; MPAKs, mitogen-activated protein kinases; mTOR, mammalian target of rapamycin; PYK2, proline-rich tyrosine kinase 2; SREBP2, sterol regulatory element binding protein 2 (Zhou et al., 2014b).

Shear stress also regulates the expression of various transcription factors, including AP-1, NF-κB, Egr-1, SP-1, GATA 6, and Kruppel-like factor 2 (KLF2). For example, KLF-2 mRNA expression from human umbilical vein endothelial cells (HUVECs) exposed to flow for 24h was increased 5-fold compared to static controls (SenBanerjee et al., 2004). The laminar flow-induced KLF-2 upregulation is believed to be beneficial for endothelial cells due to its inhibiting effects on the expression of various pro-inflammatory genes such as vascular cell adhesion molecule-1 (VCAM-1) and endothelial adhesion molecule E-selectin (Fledderus et al., 2007; van Thienen et al., 2006; Wang et al., 2010a). Thus, endothelial cells responses to shear stress consist of complex cascades of gene responses with different temporal profiles and shear stress regulates endothelial gene expression transcriptionally and post-transcriptionally (Ando and Yamamoto, 2009).

4.3 Potential mechanosensors for fluid shear stress in endothelial cells

The mechanisms governing the mechanosensation in endothelial cells have been an area of intensive investigation, but much about the subject remains obscure. Numerous signaling pathways have been identified to be activated by fluid shear stress. However, it still remains an open issue which of those are primary pathways and which are secondary pathways, and it is still poorly understood, exactly which primary sensor(s) or sensing mechanism(s) recognize(s) shear stress stimuli. At present, several membrane and junction molecules have been suggested to be sensors for fluid shear stress including ion channels, receptor tyrosine kinases, PECAM-1, VE-cadherin, G-proteins, G-protein–coupled receptors (GPCRs), caveola, integrin (Ando and Yamamoto, 2009; Hahn and Schwartz, 2009; Li et al., 2005) (Fig. 4-3). As shear stress acts at the apical surface of endothelial cells, a few local membrane structures like

primary cilia (Nauli et al., 2008), glycocalyx layer (VanTeeffelen et al., 2007) and membrane lipid bilayer (Yamamoto and Ando, 2013) have also been suggested to be primary fluid shear stress sensors (Fig. 4-3).

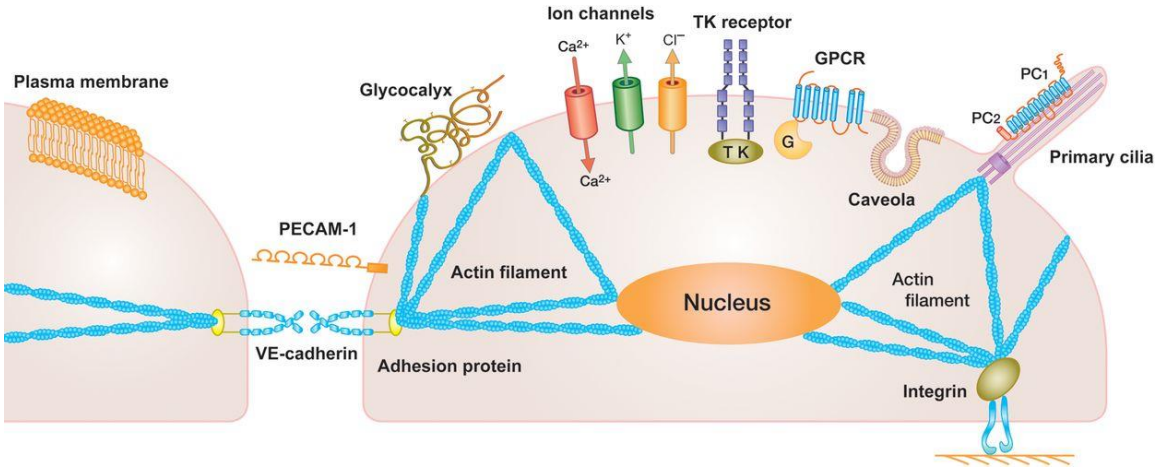

Figure 4-3: Candidates for fluid shear stress sensors.
Fluid shear stress is sensed by luminal endothelial mechanosensors, such as ion channels, tyrosine kinase (TK) receptor, GPCRs, G-proteins, caveolae, adhesion proteins, glycocalyx, primary cilia, integrin, plasma membrane (Yamamoto and Ando, 2013).

4.3.1 Ion channels

Several endothelial ion channels have been shown to be activated by fluid shear stress and been listed as flow sensor candidates. For instance, K^+ and Cl^- ion channels are found to be opened by fluid shear stress resulting in plasma membrane hyperpolarization (Gautam et al., 2006; Hoger et al., 2002). Fluid shear stress also triggers the opening of several TRP channels, which causes a Ca^{2+} influx into endothelial cells (Kohler et al., 2006; Patel et al., 2010). A series of studies from Jo's lab demonstrated that shear stress-induced Ca^{2+} influx into endothelial cells is due to the opening of the Ca^{2+} permeable cation channel $P2X_4$ (Yamamoto et al., 2000a; Yamamoto et al., 2006). Endothelial cells from $P2X4^{-/-}$ mice do not show any normal fluid shear stress-induced Ca^{2+} influx or production of nitric oxide (NO). The study also suggested that ATP is involved in endothelial mechanotransduction since fluid shear stress-induced $P2X_4$

activation requires ATP (Yamamoto et al., 2006). However, the factors that govern fluid shear stress-induced ATP release have yet to be fully defined.

Recently, transmembrane proteins Piezo1 and Piezo2 have been identified as novel mechanosensitive ion channels in many cell types including endothelial cells (Coste et al., 2010). Piezo1 is expressed in mouse endothelial cells of developing blood vessels and its activation by shear stress plays an essential role in embryonic vascular development (Ranade et al., 2014). The importance of Piezo1 channels as sensors of blood flow was also shown in Jing Li's study, in which they found that Piezo1 is required for the shear-stress-evoked ionic current, calcium influx and polarity of endothelial cells (Li et al., 2014). Endothelial-specific disruption of mouse Piezo1 profoundly disturbed the developing vasculature and was lethal for the embryo; these findings suggest that Piezo1 channels function as pivotal integrators in vascular biology (Li et al., 2014).

4.3.2 Integrins

Integrins are transmembrane receptors composed of α and β subunits. When the extracellular domains binding the specific ligands such as extracellular matrix (ECM), fribronectin and collagen, integrins trigger the activation of interior signaling pathways such as focal adhesion kinase, c-Src, and talin via their cytoplasmic domain. Studies have provided evidence that activation of integrins in endothelial cells is controlled by shear stress without a ligand (Bhullar et al., 1998; Goldfinger et al., 2008). When subjected to shear stress, endothelial integrin was shown to bind to WOW-1 in association with the adaptor protein Shc, leading to the rapid activation of FAK, paxillin, c-Src, Fyn and p130CAS (Shyy and Chien, 2002). The shear-induced integrin activation is transient, starts within 1 min after the onset of flow and becomes undetectable after 2 hours (Chen et al., 1999). Integrin activation is directly associated with members of the Rho small GTPase family, including RhoA, Cdc42, and Rac, which have distinct functions in the regulation of actin-based cytoskeletal contractility, alignment and structure. Blocking integrin with antibodies completely abrogated shear stress-induced ERK, JNK and IκB activation,

indicating that integrin operates upstream of those kinases. Moreover, pretreatment of integrins with RGD peptide abrogated shear stress–induced cellular functions such as the secretion of basic fibroblast growth factor (Katsumi et al., 2004; Shyy and Chien, 2002; Tzima et al., 2001). By using a magnetic twisting device to apply shear stress directly to cell surface integrins, Wang et al. and Chen et al. demonstrated that integrins directly transmitted physical signals to the cytoskeleton (Chen et al., 2001b; Wang et al., 1993). Thus, it is well accepted that integrins are key sensing elements involved in endothelial mechanotransduction in vascular cells; however it remains unclear whether integrins are the primary shear stress sensor, since the activation of integrins is observed to happen at the basal side of endothelial cells, whereas shear stress is directly applied to the apical side of endothelial cells.

4.3.3 Receptor tyrosine kinase (RTK)

The receptor tyrosine kinase VEGFR-2 is rapidly activated by shear stress in a ligand-independent manner (Jin et al., 2003). VEGFR-2 is predominantly expressed on endothelial cells and is critical for both normal vascular development and pathological angiogenesis (Sawamiphak et al., 2010). Studies done by Jin showed that fluid shear stress-induced tyrosine phosphorylation of VEGFR-2 within 5 min to 30 min, a phenomenon which did not affect by the specific VEGF-neutralizing antibody 577B11, indicating that fluid shear stress-induced activation of VEGFR-2 is ligand-independent. In addition, Jin's study also suggested that fluid shear stress-induced VEGFR-2 is associated with phosphoinositide 3-kinase (PI3K), which is upstream of Akt and the subsequent activation of eNOS (Jin et al., 2003). An independent study from Chen showed shear stress-induced a transient VEGFR-2 association with an adaptor protein called Shc within 1 min after onset of flow and the association disappeared after 30 min. Interruption of the VEGFR-2 association with a specific Shc blocker inhibits ERK and JNK activation induced by shear stress (Chen et al., 1999). Recent work by Tizma shows that tyrosine Y801 and Y1175 in the VEGFR-2 cytoplasmic tail are the major phosphorylation sites activated by shear stress, and that inhibition of the tyrosine phosphorylation sites of VEGFR-2 abrogates shear stress-induced actin polymerization and cell stiffness (Tzima et al., 2005).

Moreover, VEGFR2 expression was regulated by mechanical tension, as evidenced by VEGFR2 mRNA and protein levels to be higher in cells on the stiffer gels with increased extracellular matrix (ECM) stiffness (Mammoto et al., 2009).

Although it is clear that VEGFR-2 is involved in multiple shear stress signaling pathways, it has not been delineated yet how VEGFR-2 directly senses shear stress. A recent study argued that the autocrine VEGFR-2 activation may occur intracellular and therefore is not affected by treatment with VEGF-neutralizing antibody. The authors also proposed that fluid shear stress-induced VEGFR-2 might be VEGF-dependent (dela Paz et al., 2013). In addition, several other studies report that shear stress-induced VEGFR-2 phosphorylation and its association with PI3K and Shc are blocked by Src or integrin inhibitors, indicating Src kinase and integrins to maybe be upstream of VEGFR-2 for shear stress sensation (Chen et al., 1999; Jin et al., 2003; Tzima et al., 2005).

4.3.4 PECAM-1

PECAM-1, also known as CD31, is a 130-kDa immunoglobulin transmembrane protein mainly localized in the cell-cell junction sites of endothelial cells and it plays a key role in leukocyte extravasation during the inflammatory response (Woodfin et al., 2007). A novel role for PECAM-1 in mechanosensation was first suggested by Fujiwara. In his studies, endothelial PECAM-1 tyrosine phosphorylation was observed within 1 min after application of flow (Osawa et al., 1997). Not only shear stress but also cyclic stretch induces PECAM-1 phosphorylation in endothelial cells (Chiu et al., 2008). In addition, PECAM-1 tyrosine phosphorylation was found by direction force application on PECAM-1 by magnetic beads coated with PECAM-1 antibody (Osawa et al., 2002; Tzima et al., 2005), thus indicating a direct role of PECAM-1 in transmitting force to intracellular signals. PECAM-1 phosphorylation is reported to be essential for shear stress-induced ERK activation, SHP-2 phosphorylation, VEGFR-2 transactivation and NO production (McCormick et al., 2011; Osawa et al., 2002). Therefore it is not surprising that multiple vascular phenotypes were found in PECAM-1 knockout mice. For example, vessels from PECAM-1

knockout mice show reduced collateral artery growth and impaired flow-induced vasodilatation (Bagi et al., 2005). When crossed with the commonly used atherosclerosis model $LDL^{-/-}$ mouse, PECAM-1 influenced initiation and progression of atherosclerosis both positively and negatively in a site-specific manner (Goel et al., 2008). In accordance with this finding, the activation of ICAM-1and NF-κB at branch points of aorta, where flow is disturbed, are absent in the PECAM-1 knockout mouse (Tzima et al., 2005). Those results from *in vitro* and *in vivo* studies point to a crucial role for PECAM-1 in endothelial mechanosensation, and it might serve as a primary sensor in endothelial mechanotransduction.

4.3.5 Primary cilia and Glycocalyx

Primary cilia are small membrane-enclosed, hair-like structures that extend from the cell's apical surface. Through the basal body primary cilia are connected to the center of cytoskeletal microtubules which enables the cilia to transduce any mechanical stimuli throughout the cell (Freund et al., 2012). Primary cilia are expressed in various cell types including epithelial cells, endodermal cells and various endothelial cell types such as human aortic endothelial cells, HUVECs and embryonic endothelial cells (Abdul-Majeed et al., 2012; Iomini et al., 2004; Van der Heiden et al., 2008). Recently, Clapham et al. proposed that primary cilia are specialized calcium signaling organelles regulated by a heteromeric TRP channel, PKD1L1–PKD2L1, in mice and humans (Delling et al., 2013). It has been hypothesized that primary cilia function as fluid shear stress sensors (Nauli et al., 2008). Nauli's study suggests that shear stress induces bending of cilia, thus resulting in a transient increase of intracellular Ca^{2+} concentration and nitric oxide production. In particular the ciliary Ca^{2+} channels polycystin-1 and polycystin-2 have been identified as key molecules mediating cilia mechanosensation (Nauli et al., 2003; Nauli et al., 2008). Endothelial cells with polycystin-1 deficiency are not able to translate fluid shear stress stimulation into signals like Ca^{2+} response and formation of NO. In addition, endothelial cells generated from autosomal dominant polycystic kidney disease (ADPKD) patients are unable to sense fluid shear stress due to the absent expression of polycystin-2 in the cilia (Nauli et al., 2003; Nauli et al., 2008). Primary cilia have also been proposed to be involved in atherogenesis, since its expression is

often restricted to the atherosclerotic predilection sites where flow is disturbed (Van der Heiden et al., 2008). By contrast, a recent study argues that primary cilia are disassembled by high fluid shear stress (Iomini et al., 2004) and that many endothelial cells still respond to flow normally without cilia, indicating there must be an alternative flow sensation mechanism.

The apical surface of endothelial cells is coated with a thin, hydrated structure, the glycocalyx (GCX) layer. It is now well established that the endothelial GCX layer plays important roles in cell-cell communication and signaling. Degraded GCX promotes endothelial dysfunction which leads to atherosclerosis (Nieuwdorp et al., 2005). Especially due to its location on the most apical side of endothelial cells, GCX has been proposed as a flow sensor translating fluid shear stress stimuli into biochemical responses through the cytoskeleton (Ando and Yamamoto, 2009; Hahn and Schwartz, 2009). The heparin sulfate proteoglycan of GCX is present as a random coil under no-flow conditions; but with increasing flow it becomes unfolded into a filament structure. This conformational change may trigger many signaling pathways (Siegel et al., 1996). By using the specific enzyme heparinase III, Florian et al. removed the heparan sulfate from endothelial cells and found that flow-induced NO formation was completely blocked (Florian et al., 2003). Using the same technology, Yao et al. observed that cultured bovine aortic endothelial cells (BAECs) without GCX layer no longer aligned to the flow direction and that expression of vascular endothelial cadherin was reduced in the cell-cell junctions (Yao et al., 2007). *Ex vivo* studies showed that in arteries with degraded GCX flow-induced NO formation and vasodilatation were inhibited (VanTeeffelen et al., 2007). A more recent study provides evidence that glypican-1 from GCX mediates flow-induced eNOS activation by its association with caveolae. The GCX core protein syndecan-1 is connected to the cytoskeleton and mediates flow-induced endothelial cells structural changes such as cytoskeleton reorganization, alignment and elongation. Therefore the authors proposed that glypican-1 acted as a centralized mechanotransmission agent and that syndecan-1 functioned in decentralized mechanotransmission (Ebong et al., 2014; Zeng et al., 2013).

4.4 *In vivo* significance of the actions of fluid shear stress

In vivo, hemodynamic forces throughout each cardiac cycle have been suggested be crucial factors involved in the regulation of blood vessel development and structure, by influencing vascular pathology such as hypertension, atherosclerosis, aneurysms, postenotic dilation and arteriovenous malformations (Malek et al., 1999). Due to the geometry of blood vessels and the complexity of cardiac circulation, all flow in arteries and veins is unsteady. In regions spared by atherosclerosis, the blood surges during the cardiac cycle at an increasing then decreasing velocity as the contraction subsides, resulting in unsteady but unidirectional laminar flow (Fig. 4-4).

The laminar flow is atheroprotective and has been proved to be the most important stimulus for relaxing vascular smooth muscles, a process known as flow-induced dilatation (Smiesko and Johnson, 1993). The vascular endothelium releases a variety of vasoactive factors in response to fluid shear stress (Feletou et al., 2010), such as nitric oxide (NO) produced by eNOS which plays a predominant role in flow-induced vasodilation (Fleming, 2010). The eNOS inhibitor L-NAME almost abrogated flow-induced dilation, thereby supporting the hypothesis that dilation is NO-dependent. Resistant vessels have been found to be particularly sensitive to flow-dependent dilation; however, this kind of dilation also occurs in many of the capillaries, larger arteries and even some conduit vessels.

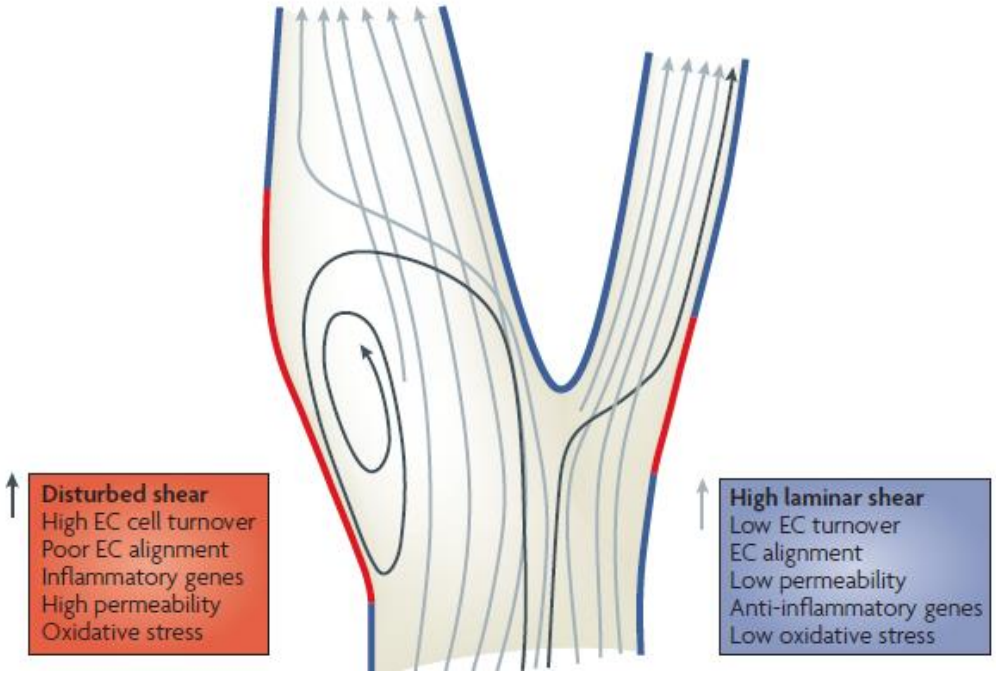

Figure 4-4: The heterogeneous endothelial responses to various shear stress patterns.
In the straight regions of blood vessel, the blood flow always is the same direction with high shear and patterns are laminar (blue segments). Endothelial cells in regions of laminar flow have a quiescent, anti-inflammatory phenotypes such as alignment in the direction of flow, expression of anti-inflammatory genes and low levels of oxidative stress, cell turnover and permeability. These regions are protected from atherosclerosis. In regions where arteries divide or curve sharply, disturbed flow patterns develop (red segments). The disturbed flow is oscillatory, with low average shear stress and constantly changing gradients of shear stress. Endothelial cells in regions of disturbed shear have an activated, pro-inflammatory phenotype characterized by alterations in endothelial cells morphology and structure, increased turnover, increased oxidative stress, increased permeability and expression of inflammatory genes, thereby contributing to the development of atherosclerosis (Hahn and Schwartz, 2009).

More important, impaired flow-dependent dilatation in arteries was found in spontaneously hypertensive rats (Izzard and Heagerty, 1999) as well as in eNOS knockout mice with a hypertensive phenotype (Huang et al., 2001). In the clinical context, flow-mediated dilation is widely perceived as a non-invasive index of endothelial function and vascular health (Gori et al., 2012). A recent study by Mari suggests that flow-induced dilation is impaired in human hypertensive subjects (Nishizaka et al., 2004). Therefore, laminar flow-induced

dilation is a fundamental autoregulatory function of the blood vessel that adapts the vessel diameter to blood flow and critically contributes to basal vascular tone and blood pressure (Davies, 1995).

Compared to laminar flow, disturbed flow at vessel branches and curve sites is a significant local risk factor for atherosclerosis (Fig. 4-4). Flow in these regions is complicate, often reduced in gradients of shear stress and at times reverse direction during the cardiac cycle (Fig. 4-4). The endothelium lining of these regions has increased permeability to plasma macromolecules, increased turnover, proliferation, apoptosis and adhesiveness for monocytes that attach and migrate into the arterial wall, with subsequent alterations in endothelial cells morphology and structure (Zhou et al., 2014a)**.** In addition, disturbed flow suppresses the expression of atheroprotective factors like eNOS, KLF2 and KLF4. It triggers the production of reactive oxygen species and activates inflammatory pathways like NF-κB. It also increases the endoplasmic reticulum (ER) stress, increases leukocyte adhesion and thereby initiates and maintains inflammation within the vessel wall. When additional systemic risk factors are present, atherosclerosis-promoting foam cells might develop (Hahn and Schwartz, 2009; Zhou et al., 2014a).

The correlation between the location of atherosclerotic lesions and regions of disturbed flow is well documented throughout the arterial system, from carotid artery bifurcation to the coronary, intrarenal and femoral artery vasculatures (Chiu et al., 2009). The non-random local manifestation of atherosclerotic lesions holds true for various experimental atherosclerosis models (dietary and/or genetic models) across multiple animal species (monkeys, rabbits, pigs, and rodents) and also for the natural history of atherosclerosis processes in humans (Chiu et al., 2009). Thus, shear stress is a critical determinant of regulating normal physiology functions like basal vascular tone as well as pathologic processes like inflammation and atherosclerosis. A major challenge in this field is the precise measuring of the complex arterial haemodynamics present *in vivo*, the identification of useful biomarkers for atherosclerosis, and the formulation of useful therapeutic interventions for atherosclerosis (Cunningham and Gotlieb, 2005; Davies et al., 2013).

4.5 GPCRs and G-protein mediated signaling in endothelial cells

4.5.1 GPCRs

G-protein coupled receptors (GPCRs) consist of more than 800 members and represent by far the largest family of transmembrane receptors which account for >2% of the total genes encoded by the human genome (Wettschureck and Offermanns, 2005). All GPCRs share a basic core structure consisting of seven transmembrane α-helical segments separated by alternating intracellular and extracellular loop regions. The majority of GPCRs in the mammalian organism belong to the group of olfactory, pheromone and taste receptors which respond to exogenous stimuli (Wettschureck and Offermanns, 2005). About 450 receptors are nonsensory and are activated by ligands like hormones, neurotransmitters, or paracrine factors. For more than 200 of these GPCRs, the endogenous ligands are known. GPCRs for which no endogenous ligand has been found yet are called “orphan” GPCRs (Offermanns, 2003).

Binding of an agonist to a GPCR results in a conformational change which allows the receptor to convey the signal to heterotrimeric G-proteins, which in turn regulate the activity of various effector molecules such as enzymes or ion channels. GPCRs are involved in many physiological and pathological processes, including neurotransmission, hormone and enzyme release from endocrine and exocrine glands, immune responses, cardiac- and smooth-muscle contraction and blood pressure regulation (Offermanns, 2003; Wettschureck and Offermanns, 2005). Their dysfunction contributes to some of the most prevalent human diseases, as reflected by the fact that GPCRs are the target, directly or indirectly, of 50–60% of all current therapeutic agents (Dorsam and Gutkind, 2007).

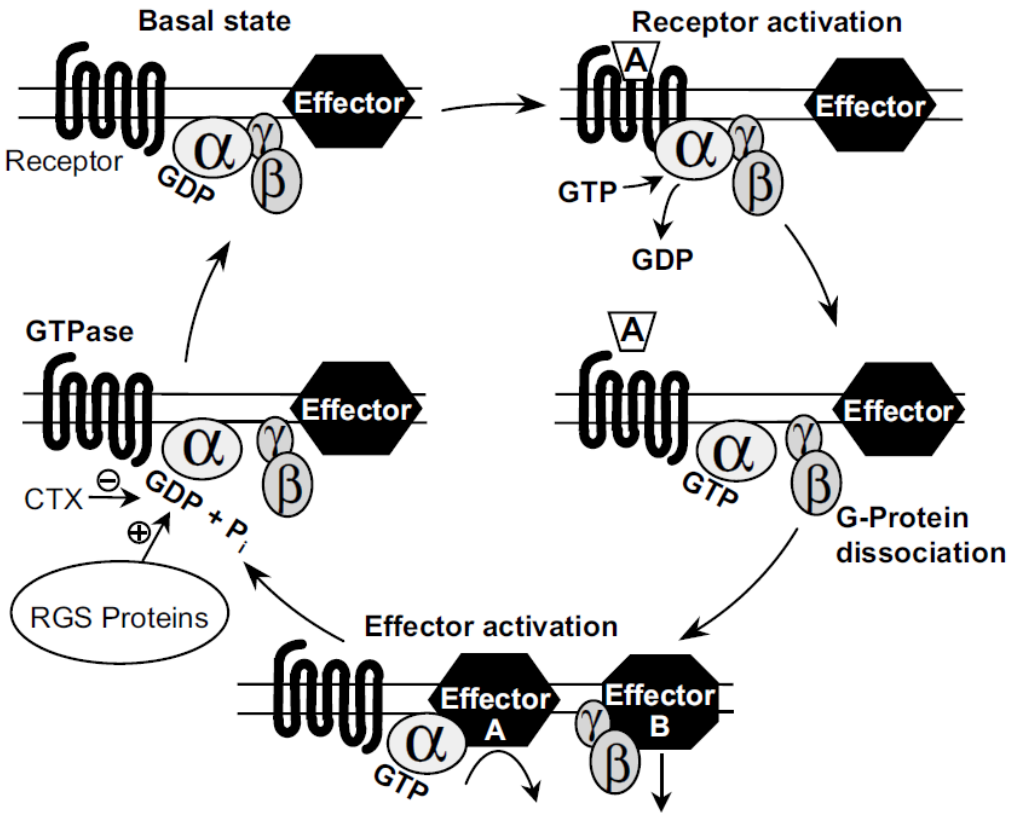

Figure 4-5: Functional cycle of G-protein activity.

The complex of a 7-transmembrane domain receptor and an agonist (A) promotes the release of GDP from the α-subunit of the heterotrimeric G protein resulting in the formation of GTP-bound Gα and Gβγ dissociation. GTP-Gα and dissociated Gβγ are able to modulate effector functions. The spontaneous hydrolysis of GTP to GDP can be accelerated by various effectors as well as by regulators of G protein signaling (RGS) proteins. GDP-bound Gα then reassociates with Gβγ, which in turn inactivates the βγ-complex. CTX, cholera toxin (Offermanns, 2003).

4.5.2 G-proteins

Heterotrimeric guanine nucleotide-binding proteins (G-proteins) are signal transducers that turn on intracellular signaling cascades in response to the activation GPCRs. Heterotrimeric G proteins are composed of a α subunit and a βγ complex. To dynamically transduce signals from receptor to effectors, the heterotrimeric G-protein undergoes an activation-inactivation-cycle (Offermanns, 2003) (Fig. 4-5). In the basal state, the βγ complex and the GDP-

bound α subunit are associated so that they can be recognized by an appropriate activated receptor. Coupling of the activated receptor to the heterotrimer promotes the exchange of GDP for GTP on the α-subunit. Binding of GTP induces dissociation of the α-subunit from the activated receptor as well as from the βγ complex, and both the α-subunit and the βγ-complex are now free to modulate the activity of downstream effector proteins. The signal is terminated by the hydrolysis of GTP by GTPase activity, resulting in binding of the α-subunit to GDP and in its re-association with the βγ-complex to enter a new cycle (Offermanns, 2003). Based on the sequence homology of their α subunits, G-proteins can be grouped into four families, G_s, G_i, G_q/G_{11} and G_{12}/G_{13} (Fig.4-6). The expression pattern of each family is often very specific. Members of same family are structurally similar and often share some of their functional properties.

The G proteins of the G_s family are ubiquitously expressed and they couple to many receptors to activate adenylyl cyclase, a key enzyme which produces cyclic AMP (cAMP) (Fig.4-6) (Freissmuth et al., 1991; Sunahara et al., 1996). On the contrary, activation of G_i-type G-proteins has been shown to mediate receptor-dependent inhibition of various types of adenylyl cyclases (Sunahara et al., 1996). The G-proteins G_{12} and G_{13} are widely expressed and share 67% of their amino acid identity. The important cellular functions of G_{12}/G_{13} are to regulate the formation of actomyosin-based structures and to modulate their contractility by increasing the activity of the small GTPase RhoA (Fig.4-6) (Strathmann and Simon, 1990). The guanine nucleotide exchange factors (GEFs) for Rho, p115RhoGEF, PDZ-RhoGEF, and LARG, have recently been shown to serve as direct effectors that interact with G_{12} and G_{13} (Suzuki et al., 2003). Both G_{12} and G_{13} interact with the cytoplasmic domain of cadherins and cause the release of the transcriptional activator β-catenin (Meigs et al., 2002; Meigs et al., 2001).

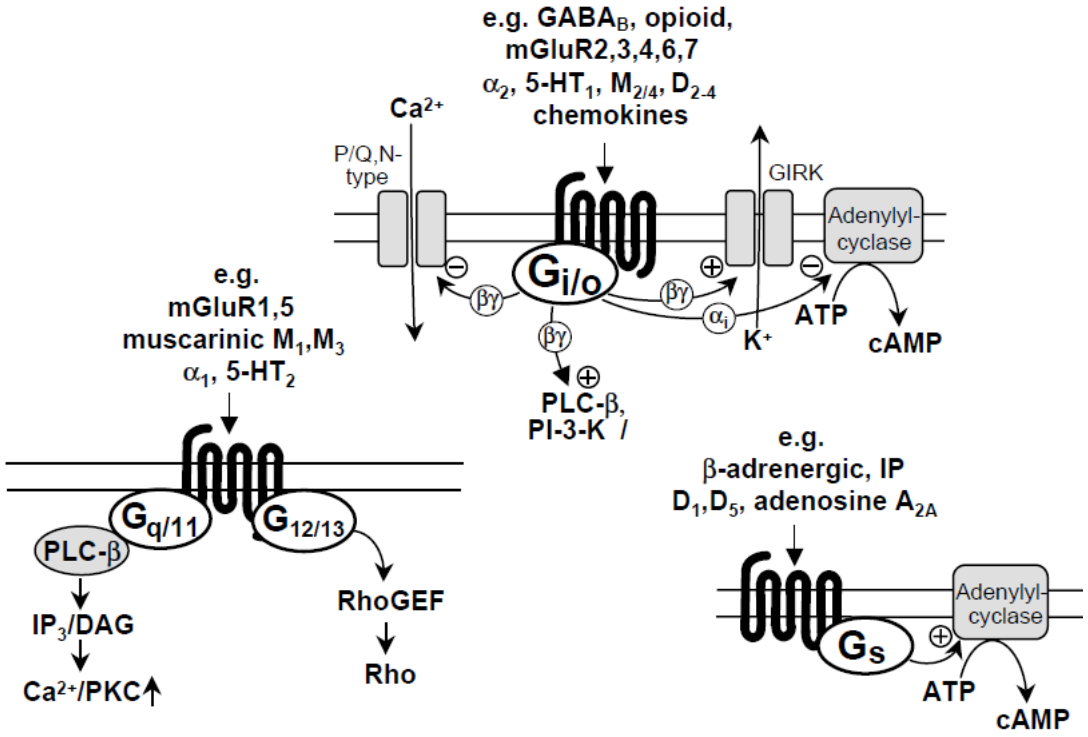

Figure 4-6: Common patterns of receptor-G-protein coupling.
Although there are many exceptions, three basic patterns of receptor-G protein coupling have been found which critically define the cellular response after ligand-dependent receptor activation. PLC-β; phospholipase C-β; PI-3-K, phosphoinositide 3-kinase; IP3, inositol 1,4,5 trisphosphate; DAG, diacylglycerol; GIRK, G-protein regulated inward rectifier potassium channel; PKC, protein kinase C; RhoGEF, Rho guanine nucleotide exchange factor (Offermanns, 2003).

The G-protein family G_q/G_{11} consists of four members, G_q, G_{11}, G_{14} and G_{15}/G_{16}. While G_q and G_{11} are widely expressed, G_{15}/G_{16} is only expressed in the hematopoietic system and G_{14} is restricted to kidney, lung and spleen (Amatruda et al., 1991; Wilkie et al., 1991). The α subunits of the G_q/G_{11} family are known to regulate phospholipase C (PLCβ), which cleaves the phospholipid phosphatidylinositol 4,5-bisphosphate into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). IP3 is released into cytosol to activate IP3 receptors, leading to an increase in calcium. Calcium and DAG work together to activate protein kinase C (PKC), which then phosphorylates other molecules, leading to altered cellular activity (Fig.4-6) (Offermanns, 2003).

Although the function of G_{14} and G_{15} is still largely unknown, the importance of G_q and G_{11} has been well established with knockout mice models. $G\alpha_q$-deficient mice have typical motor discoordination symptoms and suffer from cerebellar ataxia (Offermanns et al., 1997a). They show reduced platelet activation and a prolonged bleeding time (Offermanns et al., 1997b). In addition, $G\alpha_q$-deficient mice have a high risk of perinatal death in newborns (Offermanns et al., 1997b) and a reduced survival rate at postnatal day 30 (Gu et al., 2002) . By contrast, $G\alpha_{11}$-deficient mice were shown to be viable and fertile, without any apparent morphological or functional defects (Offermanns et al., 1998).

Mice deficient in both $G\alpha_q$ and $G\alpha_{11}$ die around embryonic day 10.5 due to myocardial hypoplasia (Offermanns et al., 1998). To circumvent double-knockout embryonic lethality, Cre/LoxP-mediated recombination was used to conditionally inactivate the $G\alpha_q$ gene in a $G\alpha_{11}$-deficient ($gna11^{-/-}$) background (Wettschureck et al., 2001). Deletion of $G\alpha_q$ and $G\alpha_{11}$ specifically in cardiomyocytes prevented the development of cardiac hypertrophy caused by pressure overload (Wettschureck et al., 2001). B-cell-specific inactivation of $G\alpha_q$ and $G\alpha_{11}$ resulted in impaired glucose tolerance and insulin secretion in mice (Sassmann et al., 2010). Thyrocyte-specific $G\alpha_q$ and $G\alpha_{11}$ deficiency impairs thyroid function and prevents goiter development (Kero et al., 2007). In smooth muscle, $G\alpha_q$ and $G\alpha_{11}$ have been shown to be fundamental for smooth muscle contraction; deletion of $G\alpha_q$ and $G\alpha_{11}$ almost abrogates agonist-induced vasoconstriction. Mice lacking $G\alpha_q$ and $G\alpha_{11}$ in vascular smooth muscle develop hypotension and are resistant to salt-induced hypertension (Wirth et al., 2008). Furthermore, smooth muscle-specific $G\alpha_q$ and $G\alpha_{11}$ deficiency blocks the activation of extracellular signal-regulated kinase 1/2, resulting in a reduced VSMC dedifferentiation in response to flow cessation or vascular injury (Althoff et al., 2012).

The endothelial specific $G\alpha_q/G\alpha_{11}$ double knockout mouse recently generated by our lab showed impaired endothelial barrier function and reduced nitric oxide formation brought about by various inflammatory mediators as well as by local anaphylaxis. Mice with endothelium-specific $G\alpha_q/G\alpha_{11}$ deficiency are protected

from the fatal consequences of passive and active systemic anaphylaxis (Korhonen et al., 2009).

Most lately, it has been shown that approximately 80% of unveal melanoma harbor somatic-activating mutations in $G\alpha_q/G\alpha_{11}$ genes (Yu et al., 2014). Feng et al. and Yu et al. proposed that YAP mediates the oncogenic activity of mutant $G\alpha_q/G\alpha_{11}$ in unveal melanoma development, and that the YAP inhibitor verteporfin blocks tumor growth of unveal melanoma cells containing $G\alpha_q/G\alpha_{11}$ mutations, providing evidence for an essential role of the Hippo-YAP pathway in G_q/G_{11}-induced tumorigenesis (Feng et al., 2014; Yu et al., 2014). A major implication of these findings is that traditional GPCR signaling through PLCβ may not be the only, or even the most important, mechanism for propagating $G\alpha_q/G\alpha_{11}$ activity (Field and Harbour, 2014).

4.5.3 GPCRs and G-proteins in endothelial mechanotransduction

Given the importance of GPCRs and G-proteins in the regulation of the endothelium, it is not surprising that GPCRs and G-proteins participate in the transduction of mechanical forces in the endothelium. Several GPCRs and G-proteins have been suggested to be primary mechanosensors (Ando and Yamamoto, 2009; White and Frangos, 2007; Wozniak and Chen, 2009). However, since shear stress-induced activation of GPCRs does not involve a traditional receptor–ligand interaction; the identification of the molecule(s) responsible for fluid shear stress sensing proved tricky (Dai et al., 2004).

G-proteins were shown to be activated by shear stress as quickly as 1s after the onset of flow (Gudi et al., 1998). Blockage of G-proteins by GDPβs abrogates the shear stress-induced NO as well as cGMP production in a dose-dependent manner (Kuchan et al., 1994). Transient transfection with antisense into $G\alpha_q$ blocks shear stress-induced Ras-GTPase activation, while transfection of Gβγ enhances shear stress-induced Ras-GTPase activation (Gudi et al., 2003). In addition, pre-treatment with the G_i inhibitor pertussis toxin blocks shear stress-induced ERK1/2 phosphorylation (Jo et al., 1997). More recently, the G-proteins G_q/G_{11} have been shown to interact with PECAM-1 and to mediate fluid shear

stress-induced Akt phosphorylation (Melchior and Frangos, 2014; Otte et al., 2009).

Up to now, several GPCRs have been shown to be activated by mechanical stimuli in a ligand independent manner. Studies from Komuro's lab have demonstrated the activation of the angiotensin (AT1) receptor by mechanical stretch through an ligand-independent mechanism, as a neutralizing antibody directed against angiotensin II did not attenuate AT1R-dependent stretch-induced signaling (Zou et al., 2004). Later studies reported that mechanical stretch induces an anticlockwise rotation of the AT1 receptor and a shift of transmembrane (TM) 7 into the ligand-binding pocket (Yasuda et al., 2008), leading to the opening of TRP channels, as well as to (PLC) activation and production of inositol phosphates (Mederos y Schnitzler et al., 2011; Mederos y Schnitzler et al., 2008; Storch et al., 2012).

GPCRs can also be activated by fluid shear stress stimuli. By employing time-resolved fluorescence microscopy and GPCR conformation-sensitive Förster resonance energy transfer (FRET), Chachisvilis and coworkers found that fluid shear stress-induced a conformational switch of bradykinin B2 receptor in the absence of its ligand bradykinin, and that this response can be blocked by a B2-selective antagonist (Chachisvilis et al., 2006). In a more recent study, the sphingosine 1-phosphate (S1P) receptor-1 has be suggested to be involved in shear stress-induced ERK, Akt phosphorylation as well as in the cell alignment towards the direction of flow (Jung et al., 2012). However, in most cases these mechanotransducing processes have been studied *in vitro* only, and in isolated endothelial cells, leaving in the dark their *in vivo* role as well as potential upstream regulatory mechanisms.

5. AIM OF THE STUDY

The vascular endothelium is constantly exposed to mechanical forces including fluid shear stress exerted by the flowing blood. The ability of the endothelium to sense fluid shear stress and to translate this information into an intracellular signal is a fundamental function of the endothelial cell layer of blood vessels. Shear stress sensing and transduction controls vascular tone and morphogenesis and affects the susceptibility to diseases, such as atherosclerosis and arterial hypertension. Previous work has identified several mechanotransducing signaling processes in endothelial cells (Hahn and Schwartz, 2009; Tarbell et al., 2014a). But how fluid shear stress initiates these mechanotransducing responses and their *in* vivo roles as well as the upstream regulatory mechanisms remains largely mysterious. The aim of this study was to...

1. ...investigate the role of the G-protein G_q/G_{11} in fluid shear stress mechanotransduction in endothelial cells and its *in vivo* roles.
2. ...identify the potential GPCRs operating upstream of G_q/G_{11} in endothelial mechanotransduction.
3. ...dissect the molecular mechanisms underlying impaired flow sensation and elevated blood pressure in mice with endothelial G_q/G_{11} deficiency.

6. RESULTS

6.1 G_q/G_{11} is required for shear stress-induced Ca^{2+} release

To test the role of G_q/G_{11} in fluid shear stress-induced endothelial responses, I determined the effect of siRNA-mediated knock-down of the α-subunits of G_q and G_{11}, namely $G\alpha_q$ and $G\alpha_{11}$, on shear stress-induced $[Ca^{2+}]i$ response. Transfection of HUVECs and BAECs with siRNA directed against $G\alpha_q$ and $G\alpha_{11}$ significantly suppressed $G\alpha_q/G\alpha_{11}$ protein expression (Fig. 6-1A) as well as $G\alpha_q/G\alpha_{11}$ mRNA expression (Fig. 6-1B).

As soon as endothelial cells were exposed to fluid shear stress, $[Ca^{2+}]i$ increased in correlation with the intensity of applied shear stress (Fig. 6-1C, 6-1D, 6-1E), suggesting that endothelial cells have the ability to accurately convert information on shear stress intensity into changes in intracellular Ca^{2+} concentrations. However, after knock-down of $G\alpha_q/G\alpha_{11}$, shear stress-induced increases in $[Ca^{2+]}i$ were almost abolished (Fig. 6-1C, 6-1D). This finding is consistent with the loss of shear stress-induced $[Ca^{2+}]i$ response in primary mouse lung endothelial cells (MLECs) isolated from endothelium-specific $G\alpha_q/G\alpha_{11}$-deficient mice (EC-q/11-KO) (Fig. 6-1E).

Of note, knock-down of $G\alpha_q/G\alpha_{11}$ also reduced thrombin, which activates G_q/G_{11}-coupled protease receptors (PARs), induced effects but had no effect on ionomycin-induced increases in $[Ca^{2+}]i$ (Fig. 6-1F), indicating that the $[Ca^{2+}]i$ response in $G\alpha_q/G\alpha_{11}$-deficient cells *per se* was not affected. Together, these experiments suggest that G_q/G_{11} is likely an important link for shear stress and Ca^{2+} response in endothelial cells.

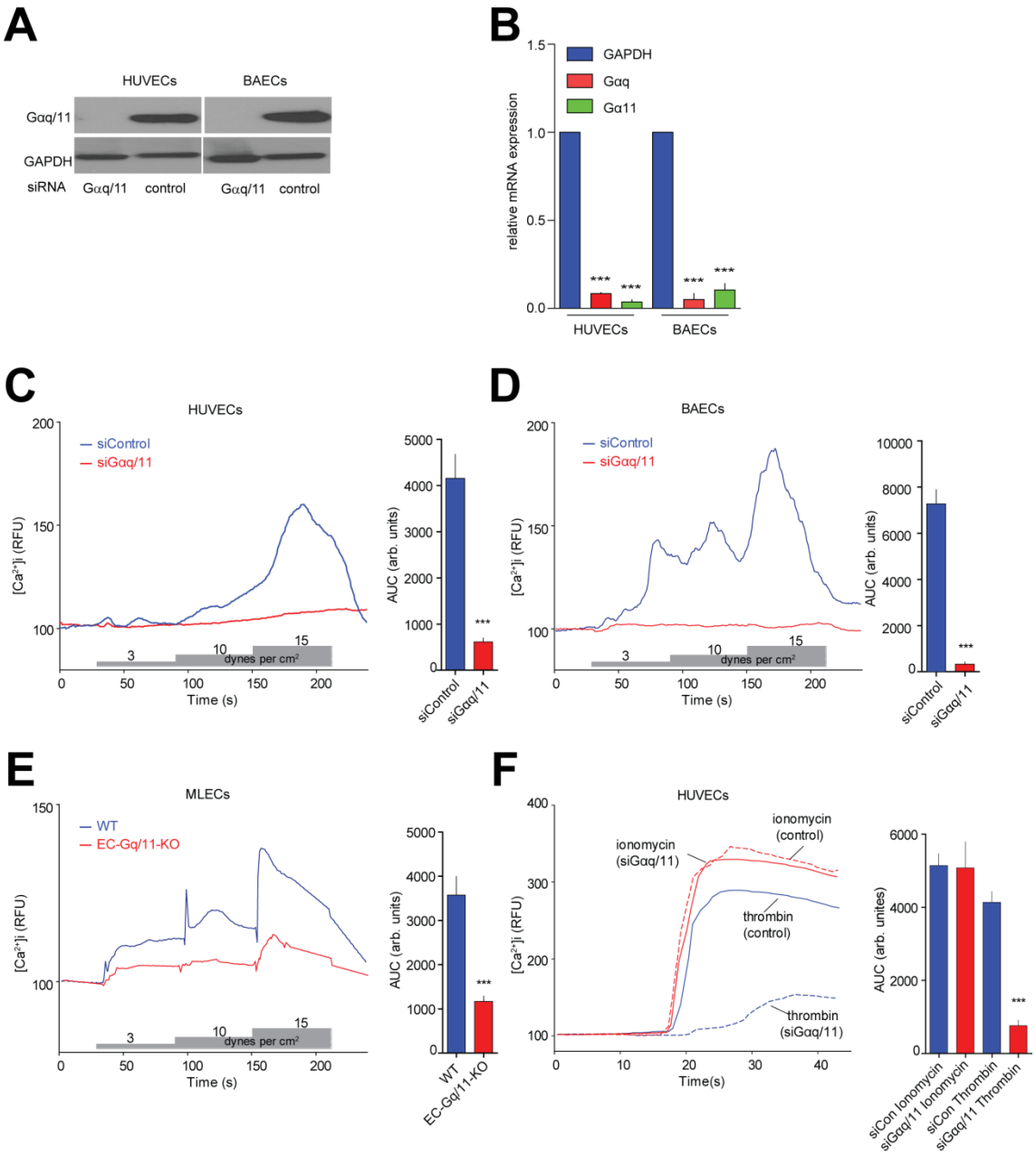

Figure 6-1: G_q/G_{11} is required for shear stress-induced Ca^{2+} response

Indicated cells were transfected with scrambled (control) siRNA or siRNA directed against $G\alpha_q$ and $G\alpha_{11}$. Knock-down of $G\alpha_q/G\alpha_{11}$ was verified by immunoblotting (A) or qRT-PCR (B) (n=3). Fluo-4-loaded HUVECs (C), BAECs (D) and MLECs (E) were exposed to the indicated shear forces, and $[Ca^{2+}]_i$ was determined as fluorescence intensity (RFU, relative fluorescence units). F, ionomycin- and thrombin-induced $[Ca^{2+}]_i$ response in HUVECs. Bar diagrams show areas under the curve (AUC); n=16-25 cells in 3 independent experiments. Shown are means -/+ s.e.m.; ***, P≤0.001.

6.2 G_q/G_{11} is required for shear stress-induced NO formation

Fluid shear stress-induced formation of nitric oxide (NO) is a key signaling molecule for endothelial cells (Dimmeler et al., 1999). When BAECs are exposed to shear stress, a considerable amount of NO is released into the flowing medium, as indicated by the NO metabolite nitrate accumulation (Fig. 6-2A). Interestingly, I found $G\alpha_q/G\alpha_{11}$ knock-downed BAECs failed to generate NO during fluid shear stress stimulation (Fig. 6-2A). Consistent with this finding, fluid shear stress-induced phosphorylation of eNOS at serine1179, a major pathway for NO synthesis is inhibited by the suppression of $G\alpha_q/G\alpha_{11}$ expression (Fig. 6-2B).

It has been shown that Akt is the major upstream regulator for shear stress-induced eNOS phosphorylation (Dimmeler et al., 1999), thus I next studied the Akt response. As with eNOS, while in control BAECs, Akt was activated by shear stress, but in $G\alpha_q/G\alpha_{11}$-knock-downed BAECs Akt was not phosphorylated by shear stress (Fig. 6-2B). Moreover, our studies on a different cell line, HUVECs, showed the same loss of shear stress-induced eNOS and Akt activation upon suppression of $G\alpha_q/G\alpha_{11}$ expression (Fig. 6-2C).

Furthermore, to circumvent the loss of endothelial cell properties during static cell culture and to mimic *in vivo* constant flow conditions, I cultured BAECs under low shear (5 dyne/cm^2) for 15 hours. Applied high shear (35 dyne/cm^2) for 30 min (Fig. 6-2D) to BAECs cultured under low shear also induced strong phosphorylation of Akt and eNOS. Interestingly, loss of $G\alpha_q/G\alpha_{11}$ strongly inhibited both eNOS and Akt activation induced by high shear (Fig. 6-2D). Those experiments demonstrated that $G\alpha_q/G\alpha_{11}$ is essential for fluid shear stress-induced activation of Akt-eNOS pathway and required for fluid shear stress-induced NO formation.

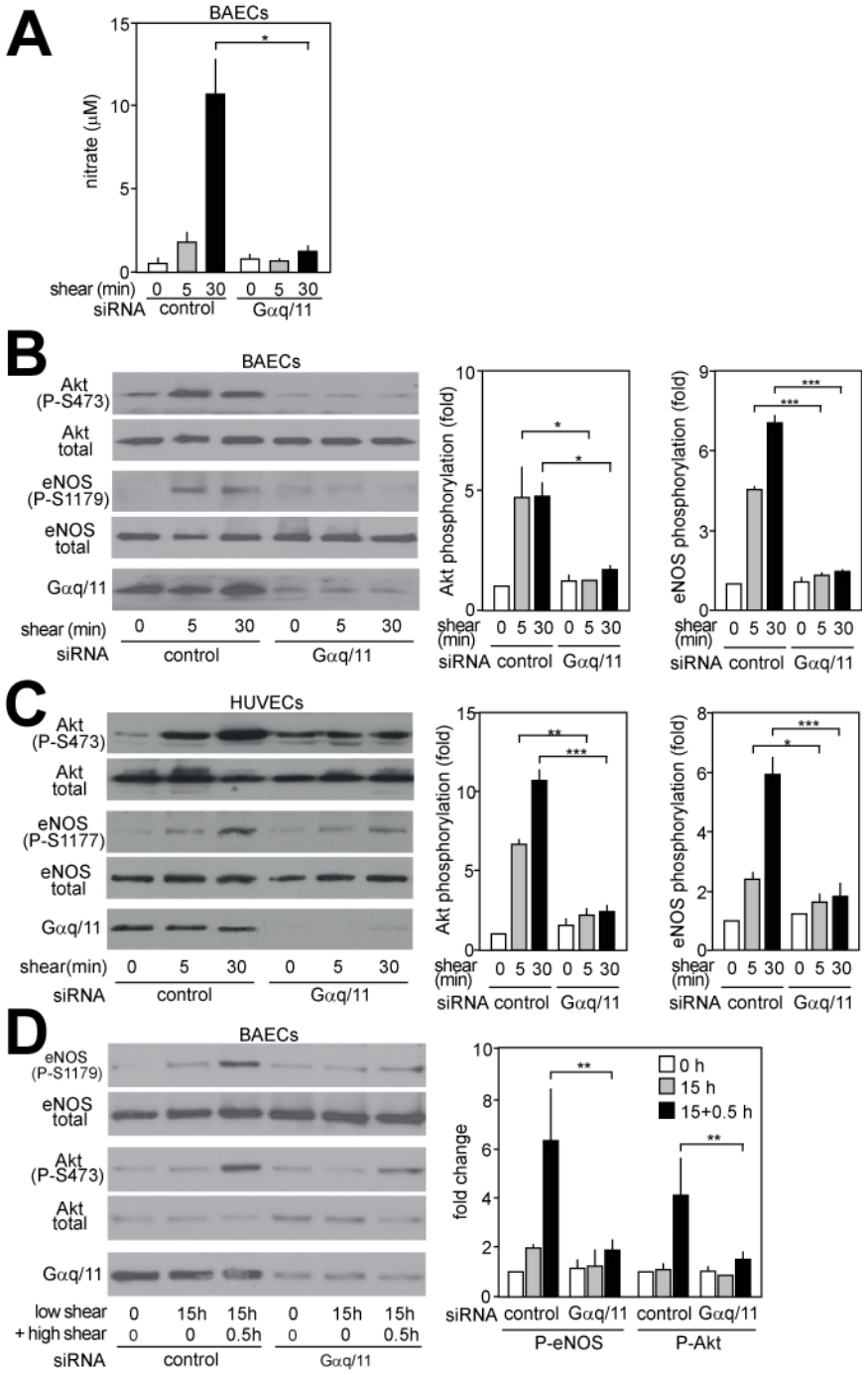

Figure 6-2: G_q/G_{11} is required for shear stress-induced NO release.

Transfected HUVECs and BAECs were exposed to fluid shear (12 and 20 dynes/cm^2, respectively) for the indicated time. A, Shear stress-induced nitrate formation in BAECs medium (n=3). B,C, Shear stress-induced Akt, eNOS phosphorylation in BAECs (B) and HUVECs (C) cultured under static conditions. D, High shear (35 dynes/cm^2) induced Akt, eNOS phosphorylation in BAECs cultured under low-shear (5 dynes/cm^2) for 15 hours. Knockdown of $G\alpha_q/G\alpha_{11}$ was verified by anti-$G\alpha_q/G\alpha_{11}$ immunoblotting. Bar diagrams show the

densitometric evaluation; n=3-5. Shown are means -/+ s.e.m.; n=5-8; **, P≤0.01; ***, P≤0.001

6.3 G_q/G_{11} is required for VEGFR-2 complex activation

Previous work has shown a role of Src, VEGFR-2 and PECAM-1 in endothelial mechanosensation and that this complex seems critical for shear stress-induced Akt, eNOS phosphorylation (Tzima et al., 2005). Therefore, it is important to know whether $G\alpha_q/G\alpha_{11}$ regulate the Akt-eNOS pathway through this complex.

Consistent with previous findings, I observed the tyrosine phosphorylation of VEGFR-2 and PECAM-1 within 5 minutes after exposure to fluid shear stress (Fig.6-3A). Also I found the maximum activation of Src kinase within 15 seconds after the onset of flow (Fig. 6-3B). Moreover, blockade of Src and VEGFR-2 kinase activity with PP2 and Ki8751, respectively, abrogated both shear stress-induced eNOS and Akt phosphorylation in BAECs (Fig. 6-3A), which further support the idea that the Src kinase and VEGFR-2 complex is the upstream of Akt-eNOS pathway in response to fluid shear stress.

Surprisingly, the fluid shear stress-induced tyrosine phosphorylation of Src, PECAM-1 as well as VEGFR-2 was markedly reduced after knock-down of $G\alpha_q/G\alpha_{11}$ (Fig. 6-3B), indicating that G_q/G_{11} operates upstream of these signaling molecules in endothelial mechanotransduction. Of note, eNOS and Akt phosphorylation induced by carbachol, which activates G_q/G_{11}-coupled muscarinic receptors, was prevented after knock-down of $G\alpha_q/G\alpha_{11}$ expression (Fig. 6-3C). However, the phosphorylation of eNOS and Akt induced by insulin, which acts independently of G_q/G_{11}, was unaffected, indicating that the cells had not lost the ability to respond to all stimuli (Fig. 6-3C).

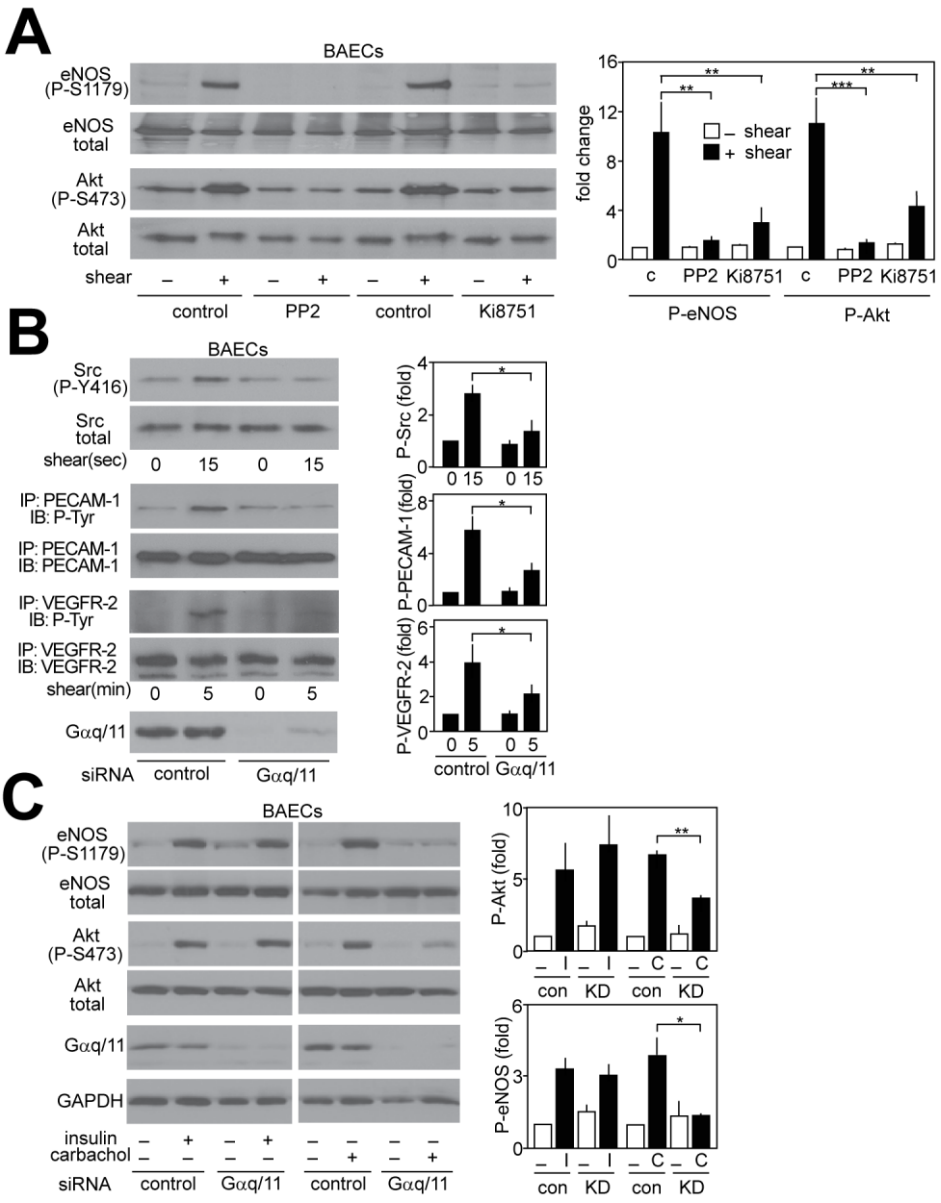

Figure 6-3: G_q/G_{11} is required for shear stress-induced Src, PECAM, VEGFR-2 activation

A, Shear stress-induced eNOS, Akt phosphorylation in the absence (-) or presence of 10 mM PP2 or 500 nM Ki8751. B, Src, PECAM-1 and VEGFR-2 activation by shear stress in transfected BAECs. Src activation was determined by Western blotting for phosphorylated Src tyrosine 416. PECAM-1 and VEGFR-2 activation was determined by immunoprecipitation and Western blotting for tyrosine-phosphorylated PECAM-1 and VEGFR-2. C, Insulin and carbachol induced eNOS, Akt phosphorylation. Knock-down of $G\alpha_q/G\alpha_{11}$ was verified by anti-$G\alpha_q/G\alpha_{11}$ immunoblotting. Bar diagrams show the densitometric evaluation; n=3-5. Shown are means -/+ s.e.m.; *, P≤0.05; **, P≤0.01; ***, P≤0.001.

6.4 G_q/G_{11} is involved in endothelial cell alignment under flow

It is well known that endothelial cells adapt to chronic laminar shear stress by aligning to the direction of flow and elongating the cell shape (Tzima et al., 2005). To understand whether G_q/G_{11} is required for this long term flow-induced endothelial response, I compared the morphology of endothelial cells with or without $G\alpha_q/G\alpha_{11}$ knock-down cultured under long term flow. Human umbilical artery endothelial cells (HUAECs) were subjected to laminar flow up to 36 hours. The control siRNA treated HUAECs showed dominant orientation in parallel to the flow direction (Fig. 6-4A left, Fig. 6-4B). By contrast, loss of $G\alpha_q/G\alpha_{11}$ expression leads to defect in endothelial cells alignment and elongation in response to long term flow (Fig. 6-4A right, Fig. 6-4B).

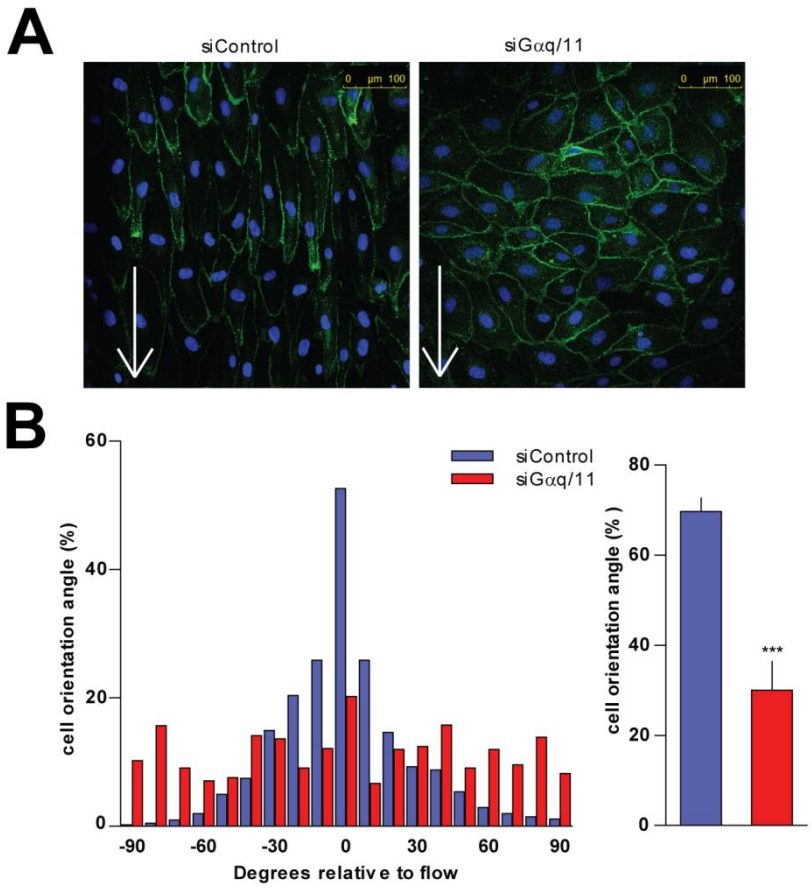

Figure 6-4: G_q/G_{11} is involved in endothelial cell alignment under flow

A, HUAECs were transfected with scrambled (control) siRNA or siRNA directed against $G\alpha_q$ and $G\alpha_{11}$ and were exposed to fluid shear (20 dynes/cm^2) for 36 h as indicated by white arrow. CD31 (green), nuclei (blue). B, quantification of cell orientation (angle measurements), 0° is parallel to the flow chamber and -90° and

90° are perpendicular. Bar diagrams show percentage of orientations in each 10° (n=205, control; n=209, G$\alpha_{q/11}$; 3 independent experiments). B right, percentage of cells which oriented between -30° to 30° to the direction of flow (n=201, control; n=198, G$\alpha_{q/11}$; 3 independent experiments). Shown are means -/+ s.e.m.; ****, P≤0.001.

6.5 G_q/G_{11} is required for flow-induced vasodilatation

To test the physiological relevance of G_q/G_{11} in endothelial flow sensing, we generated a inducible endothelium-specific $G\alpha_q/G\alpha_{11}$-deficient mice (EC-q/11-KO) by crossing $Gnaq^{flox/flox}$; $Gna11^{-/-}$ mice with Tie2-$CreER^{T2}$ mice (Korhonen et al., 2009).

In order to mimic the *vivo* blood perfusion along the endothelium layer, I mounted the isolated artery in a perfusion myograph chamber, then perfused the vessel intraluminally under a constant pressure. The U46619 precontracted mesenteric arteries prepared from WT mouse developed a step wisely relaxation during the increased flow perfusion (Fig. 6-5A, 6-5B). By contrast, in vessels isolated from EC-q/11-KO mice the flow-induced dilation was almost completely lost (Fig. 6-5A, 6-5B).

Also acetylcholine, an agonist activates G_q/G_{11}-coupled muscarinic receptors, induced vasodilation was strongly inhibited in EC-q/11-KO mice (Fig. 6-5D), functionally confirmed the loss of $G\alpha_q/G\alpha_{11}$. However, vessels from EC-q/11-KO mice developed normal myogenic tone like the WT mice (Fig. 6-5C). They are also fully responsive to the endothelium-independent vasodilator sodium nitroprusside (Fig. 6-5E) and the vasoconstrictor phenylephrine (Fig. 6-5F), indicating that contractile and vasodilator function per se were not affected in the knockout mice.

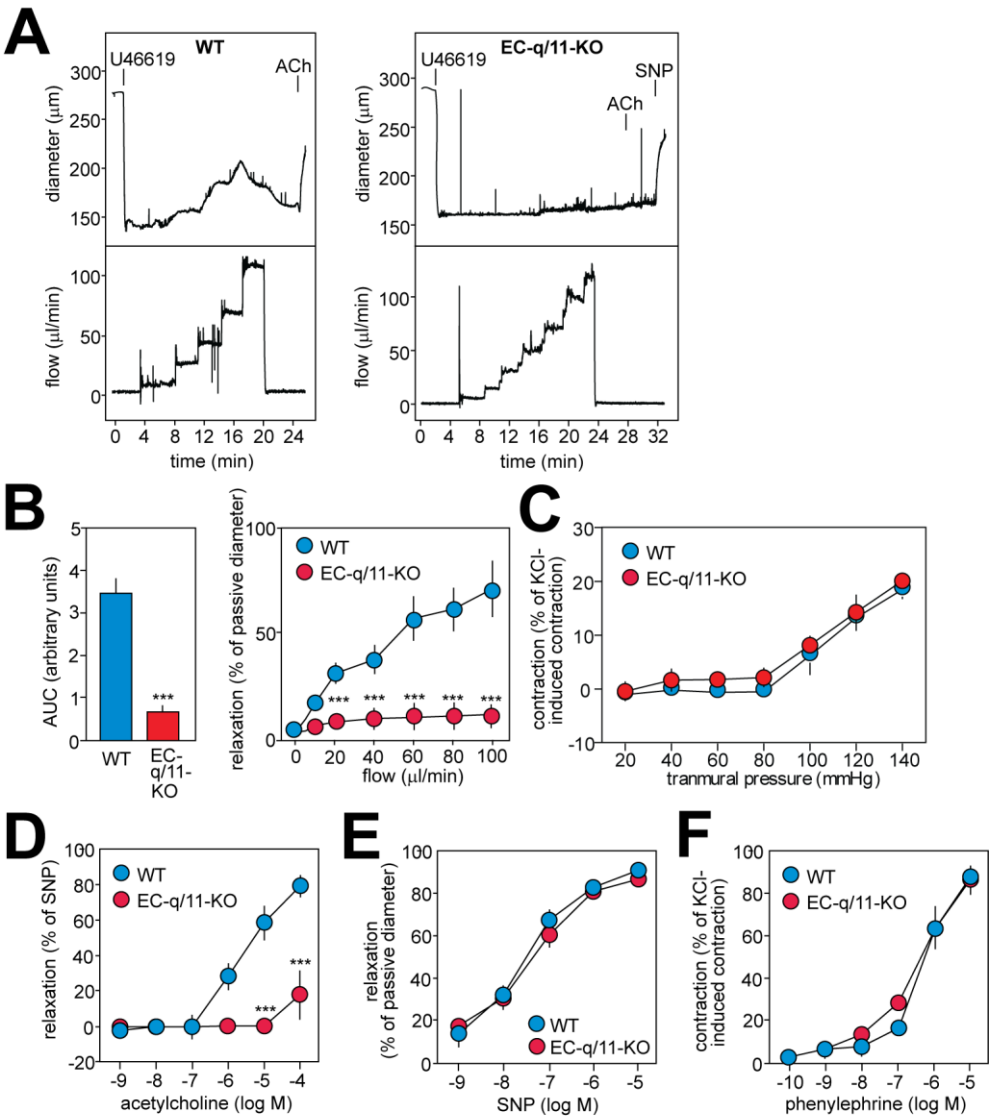

Figure 6-5: G_q/G_{11} is required for flow induce vasodilatation

Mesenteric arteries from tamoxifen-treated wild-type mice (WT) or EC-q/11-KO mice were exposed to flow and various agents (A to F). A, Effect of a stepwise increase in perfusion flow on the diameter of vessels precontracted with 100 nM of the thromboxane A_2 analogue U46619. After flow was stopped, 10 mM acetylcholine (ACh) and 100 mM sodium nitroprusside (SNP) were added. B, Shown is the evaluation of the area under the curve (AUC) and the time course of flow-induced vasorelaxation as percent of the passive vessel diameter. C, Myogenic tone induced by increased pressure. D,E,F, Effect of acetylcholine (D), SNP (E) and phenylephrine (F) on vascular diameter. Shown are means -/+ s.e.m.; n=5-8; ***, P≤0.001.

To further confirm those phenotypes were due to endothelium-specific $G\alpha_q/G\alpha_{11}$ deletion, I first verify the endothelial Tie2 Cre activity with a mT/mG reporter mouse line (Muzumdar et al., 2007). After 5 days of tamoxifen induction, a high efficiency of Cre-mediated recombination of endothelial cells was observed only in the endothelium layer indicated by GFP fluorescence (Fig. 6-6A). Furthermore, the freshly isolated endothelial cells from EC-q/11-KO mice after induction did not express $G\alpha_q/G\alpha_{11}$, indicated by anti-$G\alpha_q/G\alpha_{11}$ immunoblotting (Fig. 6-6B). These results strongly suggest that the specificity and efficiency of the tamoxifen- induced endothelial $G\alpha_q/G\alpha_{11}$ deletion in our EC-q/11-KO mice.

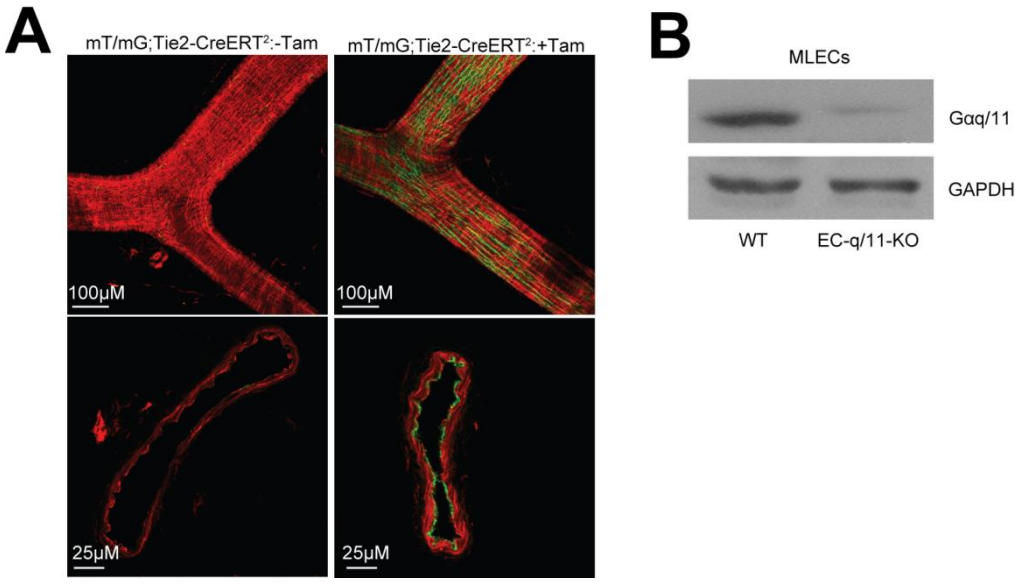

Figure 6-6: Efficiency of tamoxifen-induced Cre recombination

A, Cre recombination in the mouse mesenteric arteries was determined in mT/mG; Tie2CreERT^2 double positive mice, tomato red indicate non-recobined cells while GFP fluorescence indicates recombined cells. Whole mounted vessel, A upper panel; cryosection of vessel, A lower panel. B, Loss of $G\alpha_q/G\alpha_{11}$ expression in primary MLECs was verified by anti-$G\alpha_q/G\alpha_{11}$ immunoblotting after 5-day tamoxifen induction.

6.6 Induction of endothelial G_q/G_{11} deficiency results in hypertension

Flow-induced vasodilation is regarded as a basic mechanism critically contributing to blood pressure regulation (Hahn & Schwartz, 2009). Therefore I next investigated whether blood pressure is affected by endothelial $G\alpha_q/G\alpha_{11}$ deletion. While treatment of wild-type mice with tamoxifen had only a small and transient effect on mean arterial blood pressure, the induction of endothelial $G\alpha_q/G\alpha_{11}$ deficiency in EC-q/11-KO mice by tamoxifen resulted in a significant increase of approximately 20 mmHg within a few days (Fig. 6-7A). Although, the heart rate in the EC-q/11-KO mice is not different from the wild-type mice (data not shown).

Having shown in *vitro* situations G_q/G_{11} is required for fluid shear stress induced NO formation, it is important to know whether the *vivo* blood pressure phenotype is due to the reduced NO formation. Remarkably, I found induction of endothelial $G\alpha_q/G\alpha_{11}$ deficiency in EC-q/11-KO mice by tamoxifen resulted in a significant reduction of plasma nitrate levels, which is parallel to the development of hypertension (Fig. 6-7B).

Accordingly, I found the murine mesenteric artery from the EC-q/11-KO mice showed attenuated phosphorylation of eNOS on serine 1176, indicated by immunoblotting (Fig. 6-7C). In consistent with this finding, the enface staining of endothelium from isolated mesenteric arteries revealed the phosphorylation of eNOS is strongly reduced in the EC-q/11-KO mice after tamoxifen induction (Fig 6-7D).

Taken together, these results indicate that acute loss of endothelial G_q/G_{11}-mediated signaling strongly reduces eNOS activity in vivo resulting in reduced NO formation, increased vascular tone and arterial blood pressure. Thus, the G_q/G_{11}-mediated signaling pathway in endothelial cells is constantly activated to sustain endothelial NO formation and to mediate fluid shear stress-induced vasodilation.

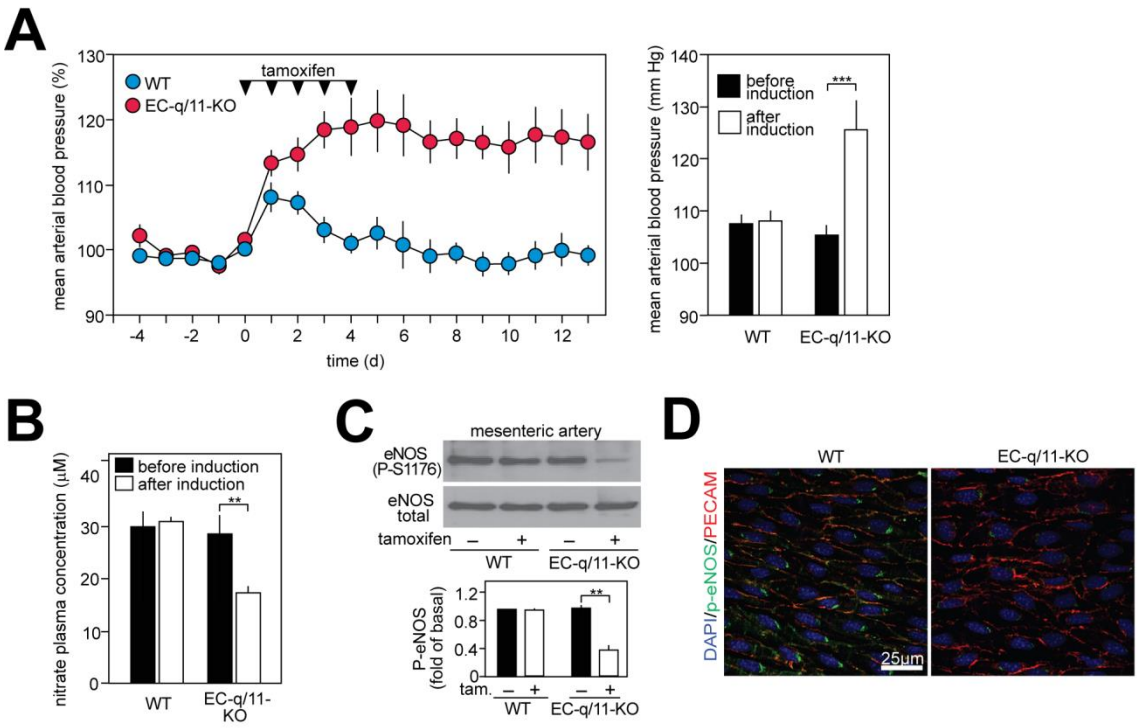

Figure 6-7: Induction of endothelial G_q/G_{11} deficiency results in reduced eNOS activation, hypertension

A, Telemetrically recorded blood pressure of wild-type and EC-q/11-KO mice.. Average blood pressure 5 days before induction was set to 100%. Bar diagram shows mean arterial blood pressure 4 days before tamoxifen treatment and in the 2nd week after induction (n=8-9). B, Plasma nitrate levels in wild-type (WT) and EC-q/11-KO mice. C, Phosphorylation of eNOS at S1176 in lysates of mesenteric arteries prepared before (tamoxifen-) or 3 days after induction (tamoxifen+). Bar diagram shows the densitometric evaluation (n=4). D, Enface staining of phospho-eNOS (serine 1176, green), PECAM (red), DAPI (blue) in mesenteric arteries prepared after 5 days tamoxifen induction. Shown are means -/+ s.e.m.; *, P≤0.05; **, P≤0.01.

6.7 Identification of $P2Y_2$ as a receptor mediating shear stress signaling

To identify potential G-protein coupled receptors operating upstream of G_q/G_{11}, the systemic expression of GPCRs in HUVECs and HUAECs was investigated. A customized qPCR primer and probe library which targeting 418 GPCR genes was built for the expression profiling. 102 receptors were found to be expressed in HUVECs and 113 receptors were found to be expressed in HUAECs. Moreover, 42 receptors were expressed in both cell types (Fig. 6-9A).

Next, an siRNA mediated loss of function assay was performed. By using shear stress-induced Akt phosphorylation at serine 473 as readout, I did an siRNA-mediated screen on those 42 receptors found to be expressed in both HUVECs and HUAECs. While most receptor knock-down did not affect shear stress-induced Akt phosphorylation, knock-down of the purinergic $P2Y_2$ receptor had by far the strongest inhibition effect on fluid shear stress-induced Akt phosphorylation (Fig. 6-9B). Of note, the effect of $P2Y_2$ siRNA is close to the effect of positive control with $G\alpha_q/G\alpha_{11}$ knock-down (Fig. 6-9B).

To rule out the off-target effect, 2 alternative siRNAs were used. Similar to the screening results, both alternative siRNAs strongly inhibited the Akt phosphorylation induced by shear stress (Fig. 6-9C). Interestingly, we also found $P2Y_2$ is abundant expressed in both HUVECs and HUAECs (Fig. 6-9A). These results pointed to an important role of the purinergic $P2Y_2$ receptor in fluid stress signaling.

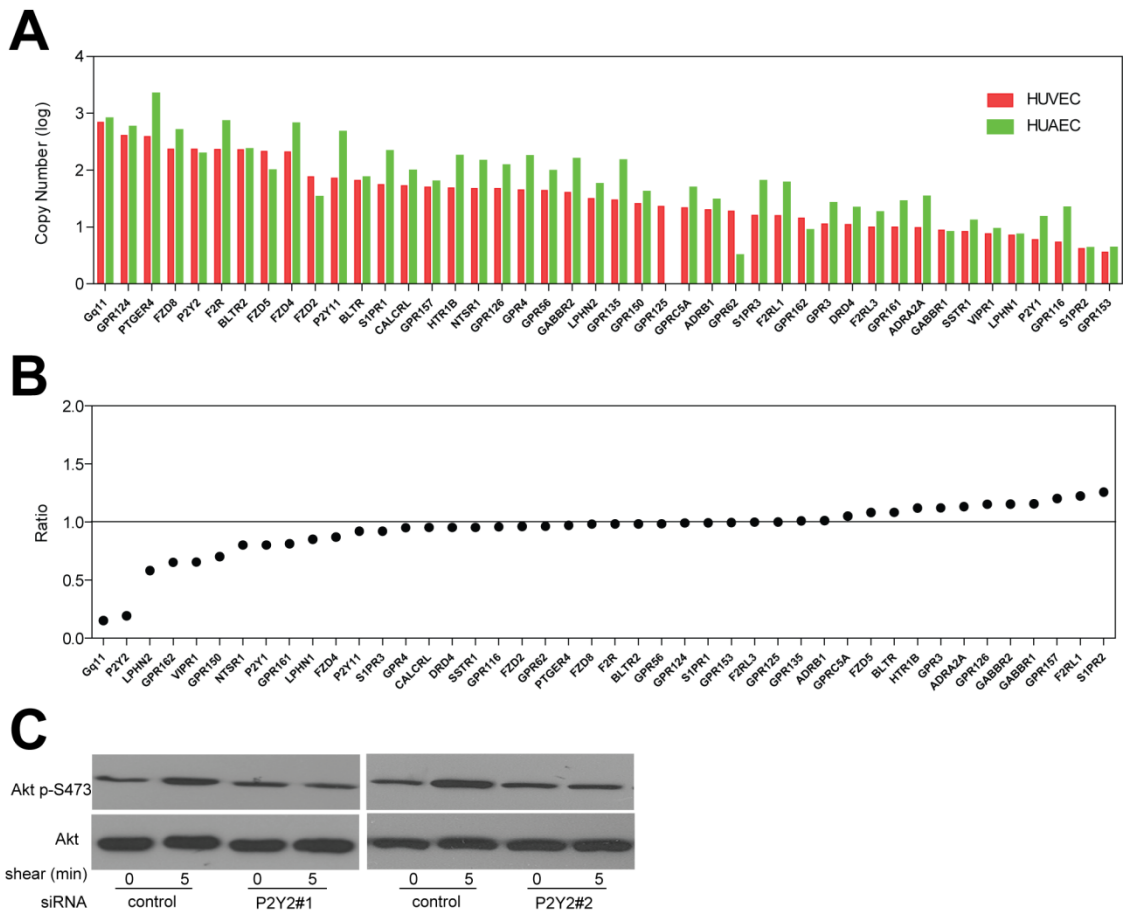

Figure 6-8: Identification of $P2Y_2$ as a receptor mediating fluid shear stress-induced Akt phosphorylation in HUVECs.

A, Expression of non-olfactory GPCRs found both in HUVECs and human umbilical artery endothelial cells (HUAECs). Expression was determined by quantitative RT-PCR. B, SiRNA screen to identify GPCRs mediating fluid shear stress-induced increases in Akt phosphorylation in HUVECs. Shown is the ratio of the fluid shear stress effect on Akt phosphorylation in cells transfected with an siRNA pool against a particular human GPCR and cells transfected with control siRNA. siRNAs directed against $G\alpha_q$ and $G\alpha_{11}$ served as a positive control. C, Representative western blot showed inhibition of shear stress induced Akt phosphorylation by $P2Y_2$ siRNAs.

Consistent with the Akt screening results, suppression of $P2Y_2$ also attenuated shear stress-induced $[Ca^{2+}]i$ response in BAECs (Fig. 6-10A) and HUVECs (Fig. 6-10B), suggesting $P2Y_2$ is required for the acute Ca^{2+}elevation evoked by shear stress. Moreover, the $P2Y_2$ agonists ATP and UTP-induced $[Ca^{2+}]i$ release was reduced (Fig.6-10C) after knockdown of $P2Y_2$ (Fig. 6-10D). These results indicating $P2Y_2$ is the major purinergic receptor in human endothelial cells which mediates ATP and UTP response.

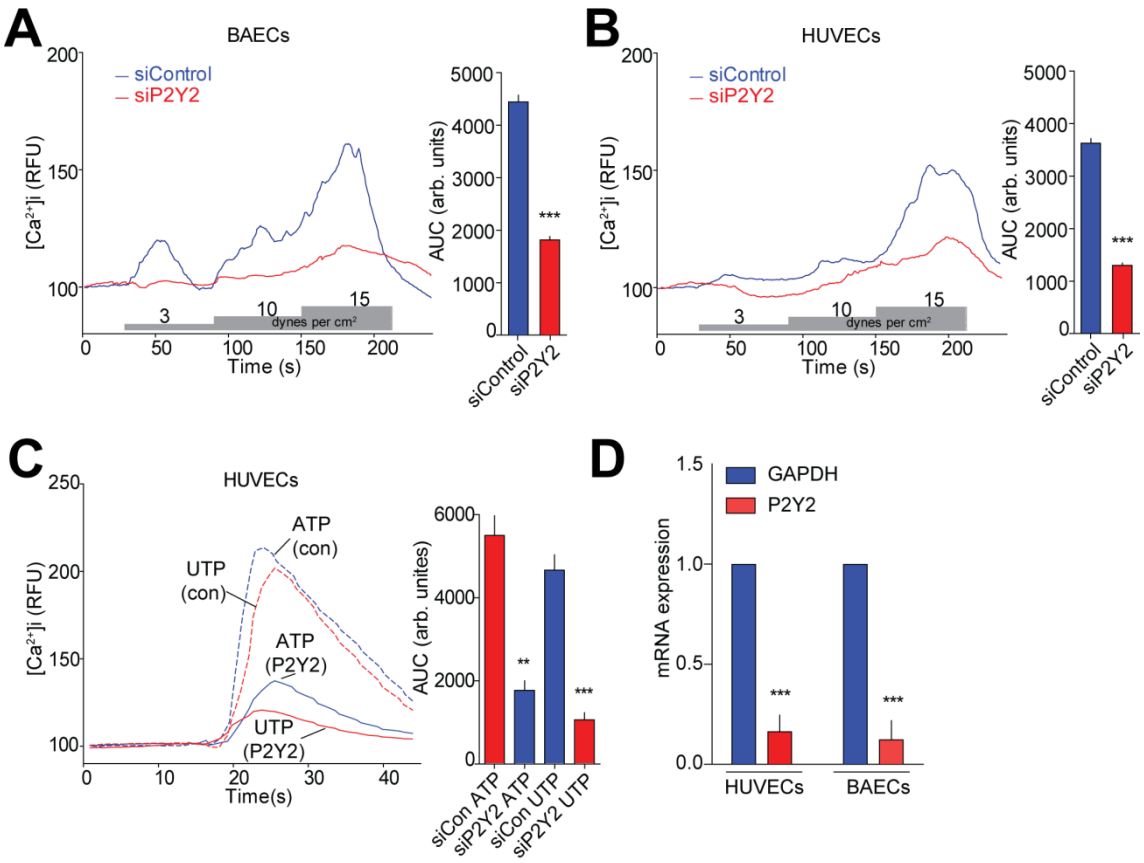

Figure 6-9: $P2Y_2$ mediates endothelial Ca^{2+} response

A, B. the indicated cells were transfected with scrambled (control) siRNA or siRNA directed against $P2Y_2$. Fluo-4-loaded BAECs (A) and HUVECs (B) were exposed to the indicated shear forces, and $[Ca^{2+}]_i$ was determined as fluorescence intensity (RFU, relative fluorescence units). C, 10µM ATP or UTP induced $[Ca^{2+}]_i$ response. Bar diagrams show areas under the curve (AUC); n=16-25 cells in 3 independent experiments. D. Efficiency of siRNA-mediated knock-down of $P2Y_2$ compared to control treated cells (control was set as 1) (n=3). Shown are means -/+ s.e.m.; *, P≤0.05; **, P≤0.01; ***, P≤0.001.

6.8 $P2Y_2$ is require for endothelial mechanotransduction

I next investigated the role of $P2Y_2$ in fluid shear stress-induced NO formation in cultured endothelial cells. As expected, in control siRNA treated cells fluid shear stress increased NO formation by time. By contract, knock-down of $P2Y_2$ significantly inhibited shear stress-induced NO formation (Fig. 6-10A).

To further understand the role of $P2Y_2$ in fluid shear stress activated signaling, I block $P2Y_2$ activation by its antagonist AR-C118925. Inhibition of $P2Y_2$ by AR-C118925 strongly reduced the phosphorylation of Akt and eNOS in response to shear stress (Fig. 6-10B). In consistent with this finding, knock-down of $P2Y_2$ by siRNA also significantly inhibited shear stress-induced Akt and eNOS activation (Fig. 6-10C).

Having shown that shear stress-induced Src and VEGFR-2 activation is upstream of Akt-eNOS pathway, I next determined the role of P2Y2 in Src and VEGFR-2 activation in response to shear stress. Knock-down of P2Y2 greatly attenuated the phosphorylation of Src and VEGFR-2 as well as the activation of PECAM-1 (Fig. 6-10C). Those results point to a crucial role for $P2Y_2$ in endothelial mechanotransduction and indicate that $P2Y_2$ is operating on the upstream of those mechanotransducing pathways.

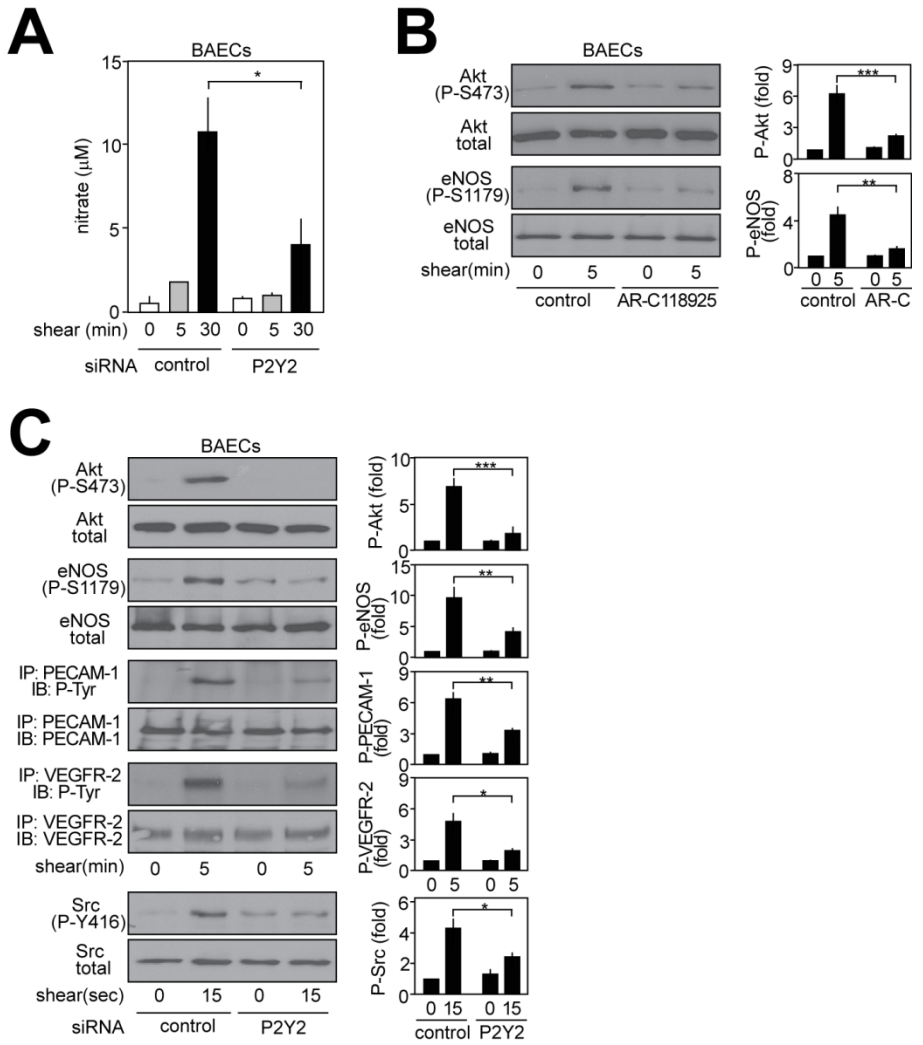

Figure 6-10: $P2Y_2$ is require for shear stress-induced signaling

A, Decreased flow induced nitrate formation in $P2Y_2$ knock-downed BAECs. B, Effect of $P2Y_2$ antagonist AR-C 118925 on flow induced Akt, eNOS activation. C, $P2Y_2$ knock-downed BAECs showed decreased Akt, eNOS, Src, PECAM-1 and VEGFR-2 activation. Bar diagrams show the densitometric evaluation; KD, knock-down; n=3-5. Shown are means -/+ s.e.m.; *, P≤0.05; **, P≤0.01; ***, P≤0.001.

Also ionotropic purinergic receptors have been shown to involve in flow-dependent endothelial effects, and in particular $P2X_4$ is suggested to mediate ATP-induced Ca^{2+}-influx in endothelial cells as well as flow-dependent control of vascular tone ((Yamamoto et al., 2006). Therefore it is important to know whether $P2X_4$ is involved in fluid shear stress induced Akt-eNOS signaling. Unlike $P2Y_2$, knock-down of the main endothelial ionotropic purinergic receptor $P2X_4$ had no effect on fluid shear stress induced phosphorylation of eNOS and Akt (Fig. 6-11A). To further confirm the specificity of the siRNA and rule out the off-target effects, the $P2Y_2$ dependent and independent responses was compared. While the $P2Y_2$ full agonist ATP-induce eNOS and Akt activation is blocked by $P2Y_2$ knock-down (Fig.6-11B), but insulin-induced Akt and eNOS phosphorylation is not affected by $P2Y_2$ knock-down (Fig.6-11C), suggesting $P2Y_2$ is the major purinergic receptor mediates ATP response and there is no general defect with $P2Y_2$ deficiency.

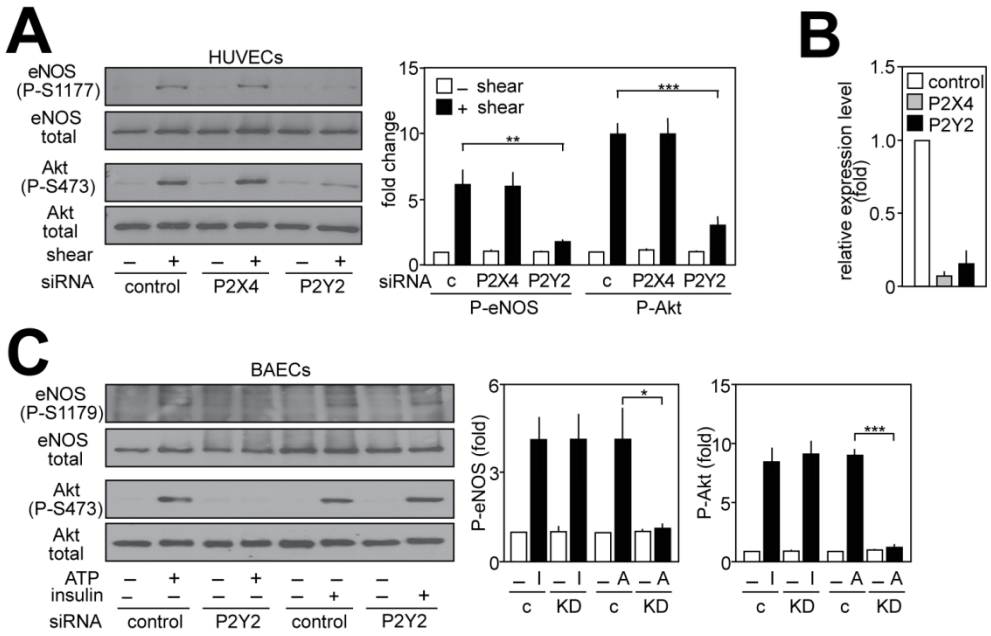

Figure 6-11: $P2Y_2$ is the major purinergic receptor mediates ATP response

A, $P2X_4$ knock-down did not affect flow-induced Akt and eNOS activation. B, Efficiency of siRNA-mediated knock-down of $P2Y_2$ and $P2X_4$ compared to control treated cells (control was set as 1). C, ATP and insulin-induced Akt and eNOS activation in control or $P2Y_2$ siRNAs transfected BAECs. Bar diagrams show the densitometric evaluation; KD, knock-down; n=3-5. Shown are means -/+ s.e.m.; *, P≤0.05; **, P≤0.01; ***, P≤0.001.

Consistent with previous data, we found that fluid shear stress-induced the release of considerable amounts of ATP from endothelial cells (Fig. 6-12A). To further understand whether the fluid shear stress-induced ATP release is the mechanism of $P2Y_2$ activation in response to fluid shear stress, the ATP degrading enzyme apyrase effect was determined. Interestingly I found the fluid shear stress -induced NO formation was strongly reduced by apyrase treatment in BAECs (Fig. 6-12B). Apyrase also significantly inhibited the activation of Akt and eNOS in response to fluid shear stress (Fig. 6-12C), while in insulin-induced Akt and eNOS phosphorylation was not affected in the presence of apyrase (Fig. 6-12D). Based on those results, it is very likely that the fluid shear stress-induced ATP release is the primary mechanism of $P2Y_2$ activation in response to fluid shear stress.

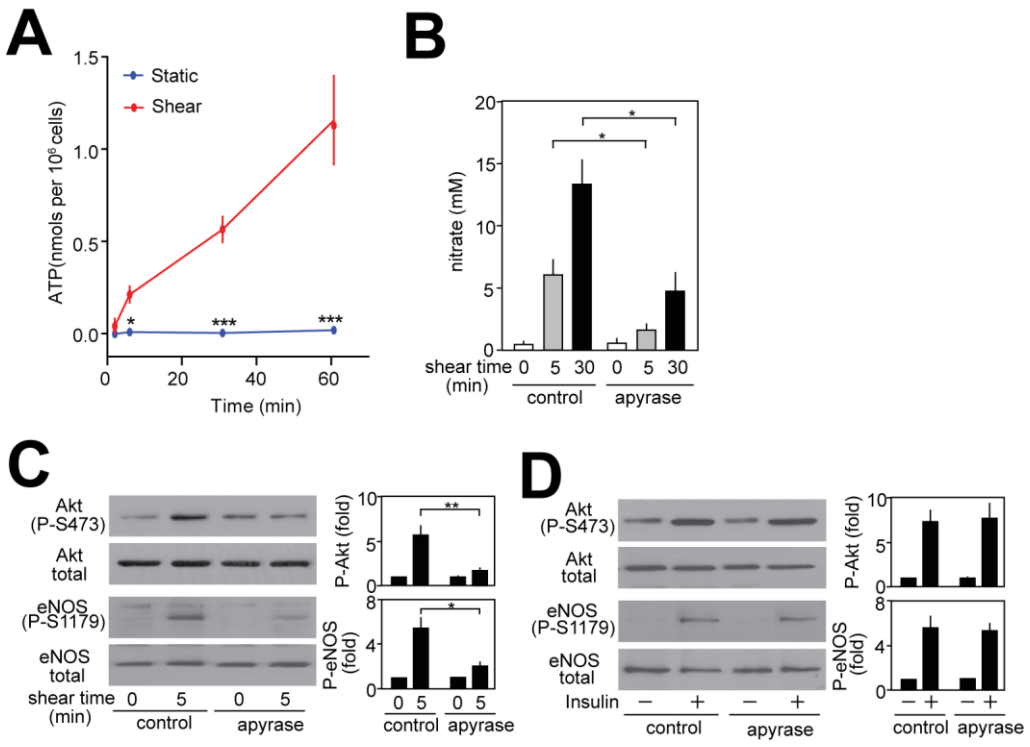

Figure 6-12: Fluid shear stress induced ATP release.

A, Amount of ATP in the supernatant of BAECs kept under static conditions or under flow (20 dynes/m2) for the indicated time (n=4). B, Flow-induced nitrite formation in the supernatant (n=4). C-D, BAECs (n=4) were exposed to fluid shear (C) or to insulin for 10 min (D) in the absence or presence of 10 U/ml apyrase. Akt and eNOS activation was determined by Western blotting for

phosphorylated and total Akt and eNOS . Bar diagrams show the densitometric evaluation. Shown are means -/+ s.e.m.; *, P≤0.05; **, P≤0.01; ***, P≤0.001

6.9 Inducible endothelial P2Y2 KO mice generation

To investigate the physiological role of $P2Y_2$ in endothelial mechanotransduction, I then generated inducible endothelium-specific $P2Y_2$ knock out mice (EC-P2Y2-KO) by crossing the $P2Y2^{flox/flox}$ mice with Tie2-$CreER^{T2}$ mice. The targeting was verified by PCR (Fig. 6-13A-D) and the Cre-mediated loss of $P2Y_2$ was confirmed by no detection of $P2Y_2$ mRNA in primary endothelial cells from EC-P2Y2-KO (Fig. 6-13E)

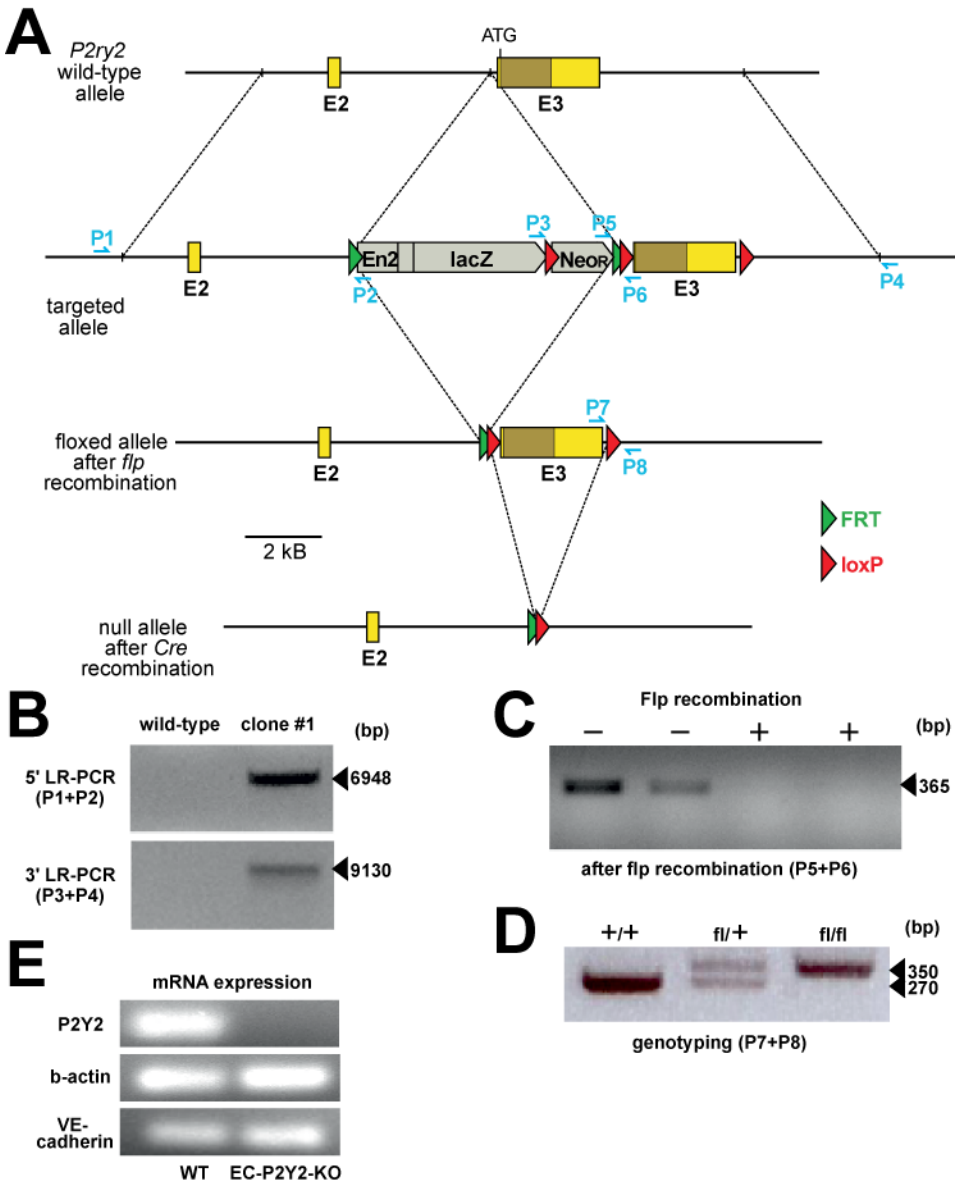

Figure 6-13: Inducible endothelial $P2Y_2$ mice generation

A, Shown are exon 2 and 3 of the P2ry2 gene (E2 and E3) with the coding region in exon 3 shaded. Blue arrows indicate position of primers used to verify the alleles and to genotype mice. B, Verification of ES cell clone HEPD0557 7 B07. C, Verification of Flp-mediated recombination. D, Genotyping of mice without or with one or two floxed $P2Y_2$ alleles. E, Expression of $P2Y_2$ in endothelial cells isolated from skeletal muscle derived from wild-type and induced EC-P2Y2-KO mice as analyzed by qPCR.

6.10 $P2Y_2$ deficient mice developed hypertension

Interestingly, U46619-precontracted mesenteric arteries from EC-P2Y2-KO mice only have mild relaxation response to increases in flow while they fully responded to acetylcholine (Fig. 6-14A, 6-14B). Of note, intraluminal addition of the $P2Y_2$ receptor agonist, ATP, induced vasodilation in the WT mesenteric arteries in a dose-dependent manner, but the relaxation curve is right shifted in arteries from EC-P2Y2-KO mice (Fig. 6-14C), suggesting the involvement of endothelial $P2Y_2$ receptor in ATP-induced relaxation.

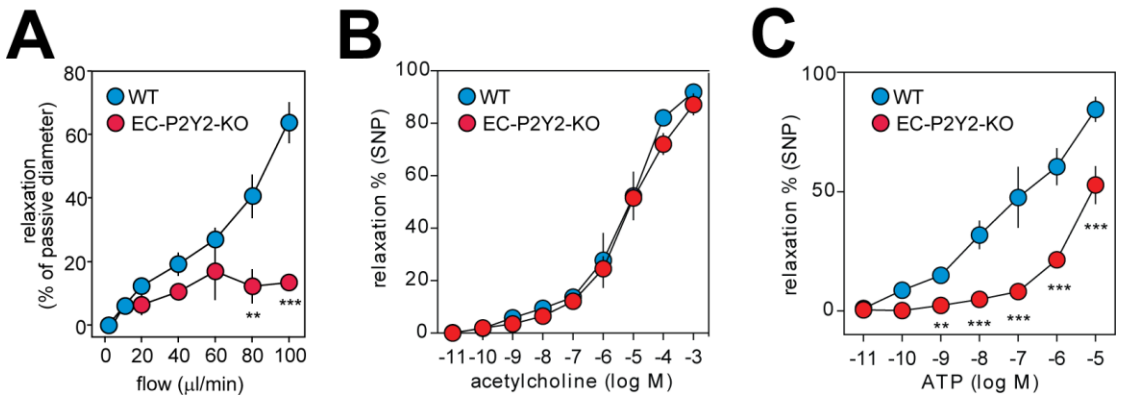

Figure 6-14: $P2Y_2$ deficient mice had impaired flow-induced dilatation

A, Flow-induced dilation in mesenteric arteries isolated from WT or EC-P2Y2-KO mice. B, Acetylcholine induced vasorelaxation. C, ATP induced vasorelaxation. n=8-10. from wild-type and EC-P2Y2-KO mice. Shown are means -/+ s.e.m.; *, P≤0.05; **, P≤0.01; ***, P≤0.001

To investigate whether the endothelium-specific $P2Y_2$ knock-out mice recapitulate the hypertension phenotype of the EC-Gq/11-KO mice, I measured the blood pressure of EC-P2Y2-KO mice via telemetry system.

EC-P2Y2-KO mice similar basal blood pressure compared to wild-type mice before the tamoxifen induction (Fig. 6-15A). However, after the application of tamoxifen, the EC-P2Y2-KO mice showed a 20 mmHg increase in the arterial blood pressure (Fig. 6-15A), while the wild-type control mice had change of arterial blood pressure after induction (Fig. 6-15A).

Similar to EC-Gq/11-KO mice, after the tamoxifen induction the mesenteric artery from the EC-P2Y2-KO mice showed decrease phosphorylation of eNOS on serine 1176, indicated by immunoblotting (Fig. 6-15B).

In addition to the hypertension phenotype, 10 weeks after the tamoxifen induction, the EC-P2Y2-KO mice developed heart hypertrophy as indicated by significant increased heart to body weight ratio (Fig. 6-15C). It is likely that the hypertrophy phenotype is the long term consequence of the elevated blood pressure.

Thus, tamoxifen induction of endothelium-specific $P2Y_2$ deficiency in vivo recapitulated the phenotype of induced endothelial $G\alpha_q/G\alpha_{11}$-deficiency resulted in defect in eNOS activation, loss of flow-induced vasodilatation and elevated blood pressure. Those data strongly indicate $P2Y_2$ is the upstream receptor of G_q/G_{11} in the flow dependent regulation of NO-formation, vascular tone and blood pressure.

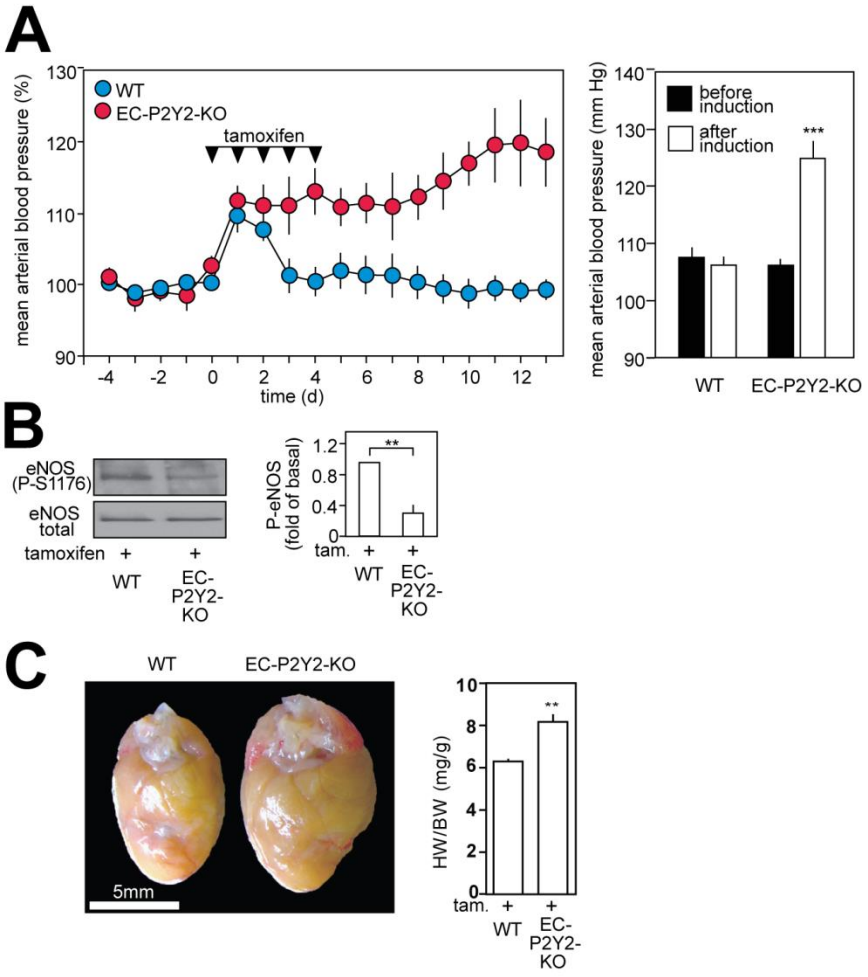

Figure 6-15: $P2Y_2$ deficient mice developed hypertension

A, Blood pressure in wild-type and EC-P2Y2-KO mice before, during and after induction. Average blood pressure 5 days before induction was set to 100%. Bar diagram shows mean arterial blood pressure 4 days before tamoxifen treatment and in the 2nd week after induction (n=8-12). B, Phosphorylation of eNOS at S1176 in lysates of mesenteric arteries prepared 3 days after induction from wild-type and EC-P2Y2-KO mice. Bar diagram shows a densitometric evaluation (n=4). C, Representative images of whole heart prepared 5 weeks after induction from wild-type and EC-P2Y2-KO mice. Bar diagram shows heart weight to body weight ratio (n=6-10). Shown are means -/+ s.e.m.; **, P≤0.01; ***, P≤0.001

7. DISCUSSION

One of the fundamental functions of blood vessels is their ability to sense the mechanical forces of the blood stream on their inner surface, which is covered with endothelial cells. The fluid shear stress exerted by the blood flow on the surface of endothelial cells activates eNOS to produce NO, which is a central determinant of vascular tone (Corson et al., 1996; Fisslthaler et al., 2000; Nauli et al., 2008). Defects in flow-induced NO formation go along with endothelial dysfunction, initiation and progression of atherosclerosis as well as with arterial hypertension (Chiu et al., 2009; Davies et al., 2013). Despite the key role fluid shear stress plays in the physiology and pathophysiology of the vascular system, it is still unclear how endothelial cells sense fluid shear stress.

Several intermediary signaling components linking fluid shear stress to eNOS activation have been described in the last two decades, but their upstream regulation has remained elusive. Although the molecular identity of the primary mechanosensor has yet to be fully defined, a protein complex composed of VEGFR-2, PECAM-1 and VE-cadherin was shown to be critical for fluid shear stress responses in endothelial cells (Tzima et al., 2005). PECAM-1 formed a complex with vascular endothelial cadherin (VE-cadherin), which, following the shear stress-induced tyrosine phosphorylation of PECAM-1, recruits VEGFR-2. VEGFR-2 in turn becomes tyrosine phosphorylated and activates downstream signaling events including the activation of Akt kinase (Conway and Schwartz, 2012; Tzima et al., 2005). Furthermore, this complex is also required for Src phosphorylation and endothelial cells alignment. Biophysical evidence indicate that VEGFR-2, PECAM-1 and VE-cadherin are not a primary mechanosensors, but that are controlled by upstream mechanisms which have remained elusive (Conway et al., 2013; Hur et al., 2012). Other mechanotransducers suggested so far include mechanosensitive ion channels (Barakat et al., 2006), the endothelial glycocalyx layer (Tarbell et al., 2014b; Weinbaum et al., 2007) and the primary cilium (Egorova et al., 2012).

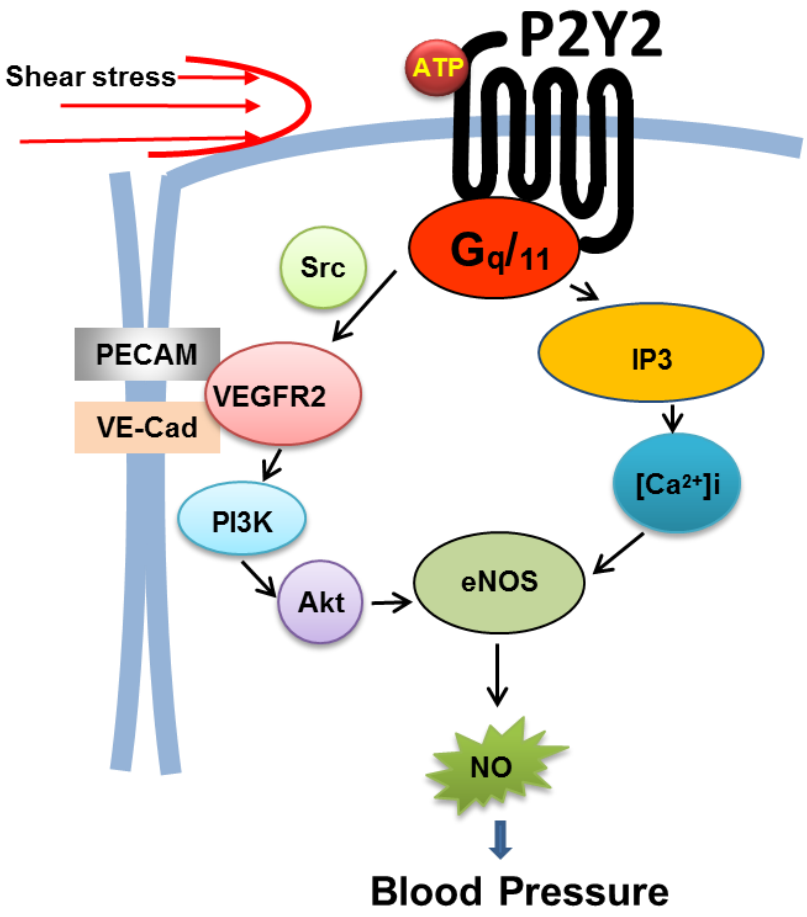

Figure 7: Schematic summary of $P2Y_2$ and G_q/G_{11} controlling blood pressure via endothelial mechanotransduction.

In this manuscript, I report on the identification of the G-protein $G\alpha_q/G\alpha_{11}$ and its coupled purinergic $P2Y_2$ receptor as upstream regulators of mechanosensitive signaling pathways in endothelial cells (Fig. 7). $P2Y_2$ is activated by ATP shown to be released from endothelial cells under the influence of fluid shear stress. By studying human and bovine endothelial cells in flow chambers, I demonstrate that $P2Y_2$ and G_q/G_{11} mediate fluid shear stress-induced phosphorylation of PECAM-1, VEGFR-2, Src and Akt kinases as well as activation of eNOS and the subsequent NO formation (Fig. 7). In mice, I show that the induced and endothelium-selective deletion of the genes encoding

$P2Y_2$ or $G\alpha_q/G\alpha_{11}$ abrogates the ability of flow to activate eNOS and to produce NO, resulting in loss of flow-induced vasodilation and in the development of arterial hypertension. I believe that we have discovered the long-sought upstream mechanism linking fluid shear stress to downstream signaling resulting in eNOS activation, vascular tone regulation and blood pressure control.

Previous studies have shown that shear stress-induced, ligand-independent VEGFR-2 activation is the key step for endothelial mechanotransduction, as it triggers the activation of many downstream proteins, such as PI3K, Akt, eNOS and MAP kinases (Jin et al., 2003). Indeed, I observed tyrosine phosphorylation of VEGFR-2 after 5min flow and, more importantly, the VEGFR-2 inhibitor Ki8751 almost blocked flow-induced eNOS and Akt phosphorylation. My data show that $P2Y_2$ and G_q/G_{11} are the upstream regulators for shear stress-induced tyrosine phosphorylation of VEGFR-2. Data from another group suggest that activation of $P2Y_2$ by UTP induces a rapid tyrosine phosphorylation of VEGFR-2, which results in the activation of Rho family GTPases like Vav2, RhoA and Rac1 (Seye et al., 2004). Moreover, deletion or mutation of two Src homology-3-binding sites in the C-terminal tail of $P2Y_2$ or inhibition of Src kinase activity abolished the $P2Y_2$-mediated transactivation of VEGFR-2 (Weisman et al., 2005). Thus, it is likely that the crosstalk between the $P2Y_2$ and VEGFR-2 signaling pathways is mediated by Src kinase. Two recent studies by Hla and Betscholz suggest that VEGFR-2 signaling is controlled by another GPCR, the SIP1 receptor, and possibly mediated by VE-cadherin (Gaengel et al., 2012; Jung et al., 2012). Alternatively, GPCRs or G-proteins may directly interact with VEGFR-2. Indeed, Zeng and colleagues have proposed that G_q/G_{11} proteins are required for VEGFR-2-mediated RhoA activation as well as HUVEC migration and proliferation. More importantly, they demonstrate that G_q/G_{11} proteins directly interact with VEGFR-2 *in vivo* and that VEGF-induced VEGFR-2 tyrosine phosphorylation requires G_q/G_{11} (Zeng et al., 2002; Zeng et al., 2003).

Secondly, I also found shear stress-induced PECAM-1 phosphorylation to be regulated by $P2Y_2$ and G_q/G_{11}. PECAM-1 is by far the most likely candidate as a

primary flow sensor, as it is essential for many shear stress signaling events, such as Akt, eNOS, Erk , SHP-2 phosphorylation, VEGFR-2 transactivation and NO production (Bagi et al., 2005; Conway et al., 2013; Fleming et al., 2005; Tzima et al., 2005). Also, multiple vascular phenotypes were found in PECAM-1 knockout mice; for instance, vessels from PECAM-1-deficient mice have impaired dilation in response to flow (Bagi et al., 2005). Yeh and coworkers found G_q/G_{11} formed a complex with PECAM-1 in HEK293 cells. Therefore the authors proposed that endogenous G_q/G_{11}-coupled GPCRs expressed by HEK293 cells bridge the interaction between PECAM-1 and G_q/G_{11} (Yeh et al., 2008). In addition, Otte et al. observed a co-localization of G_q/G_{11} and PECAM-1 at the cell–cell junction in the atheroprotected section of mouse aortae. In contrast, G_q/G_{11} was absent from junctions in atheroprone areas as well as in all arterial sections from PECAM-1 knockout mice (Otte et al., 2009). Furthermore, in primary human endothelial cells, temporal gradients in shear stress led to a rapid dissociation of the G_q/G_{11}–PECAM-1 complex within 30 seconds, whereas fluid flow devoid of temporal gradients did not disrupt the complex, suggesting that G_q/G_{11} PECAM-1 might form a mechanosensitive complex (Otte et al., 2009). To date there is no direct evidence showing the crosstalk between PECAM-1 and $P2Y_2$; however, both stretch- and flow-induced PECAM-1 phosphorylation require ATP, which is a full $P2Y_2$ agonist (Chiu et al., 2008).

During the onset of flow, Src phosphorylation was observed in many studies (Chen et al., 1999; Tzima et al., 2005). In my study, I observed maximum Src phosphorylation within 15 seconds after application of flow. In agreement with previous findings, I found that blockage of the activity of Src kinase with PP2 abolished flow-induced eNOS and Akt activation, suggesting Src is upstream of them. In addition, studies from Tizma, Cheng and Fleming, respectively, suggest that Src kinase is even upstream of VEGFR-2 and PECAM-1, since their activation by flow is later than that of Src and, in addition, prevented by PP2 (Chen et al., 1999; Fleming et al., 2005; Tzima et al., 2005). Even though there is now a general consensus that Src kinase activation is the earliest and perhaps most important event in responses to shear stress, it is not clear, how Src kinase is activated. My studies indicate that $P2Y_2$ and G_q/G_{11} are required

for flow-induced Src activation, as demonstrated by the fact that both in HUVECs and BAECs flow-induced Src phosphorylation is prevented by knockdown of either $P2Y_2$ or $G\alpha_q/G\alpha_{11}$. Recent studies have identified two proline-rich SH3 binding sites in the carboxyl-terminal tail of the human $P2Y_2$ receptor that directly associate with the tyrosine kinase Src in protein-binding assays (Liu et al., 2004). Furthermore, Src co-precipitated with the $P2Y_2$ receptor in 1321N1 astrocytoma cells stimulated with the $P2Y_2$ receptor agonist UTP (Liu et al., 2004). Of note, whether the crosstalk between $P2Y_2$ and Src kinase is G-protein-independent or -dependent remains to be investigated. Also, several studies indicate that Src kinase can be regulated by G_q/G_{11} via direct or indirect mechanisms (Luttrell and Luttrell, 2004). For example, in neuronal and hematopoietic cells, Pyk2, a FAK family nonreceptor tyrosine kinase, is activated in response to the rise in intracellular calcium and to PKC activity upon G_q/G_{11} activation. Activated Pyk2 then binds and activates Src (Giannotta et al., 2012; Luttrell and Luttrell, 2004; Xiang et al., 2012). Although Pyk2 has been shown to be activated by shear stress in BAECs (Tai et al., 2002), it is not clear whether Pyk2 provides a link between G_q/G_{11} and Src activation during shear stress.

Another key finding of my studies is that $P2Y_2$ and G_q/G_{11} are essential for eNOS activation and the subsequent NO formation. In cultured cells, I found knockdown of $P2Y_2$ and G_q/G_{11} prevented flow induced eNOS phosphorylation. In addition, I also found that $P2Y_2$ and G_q/G_{11} are required for ATP but not for insulin-induced eNOS activation. Since there is no supporting evidence for a direct interaction of $P2Y_2$ or G_q/G_{11} with eNOS, it is likely that $P2Y_2$ and G_q/G_{11} control eNOS signaling through its upstream regulator. Consistent with this hypothesis, I found $P2Y_2$- or G_q/G_{11}-deficient cells to lack flow-induced activation of Akt, a well-known kinase responsible for shear-induced eNOS activation in endothelial cells. Of note, blockage of G_i by PTX and knockdown of G_{12}/G_{13} did not affect flow-induced Akt phosphorylation (unpublished data), indicating that the involvement of the G-proteins G_i and/or G_{12}/G_{13} in shear stress signaling is rather unlikely. In addition, acute flow induces a transient increase in $[Ca^{2+}]i$, which then leads to Ca^{2+}/calmodulin-dependent eNOS

activation (Fleming and Busse, 1999).In cells lacking $P2Y_2$ and G_q/G_{11}, the flow-induced Ca^{2+} response is diminished, thus further explaining the loss of eNOS activation in $P2Y_2$- and G_q/G_{11}-deficient cells.

Moreover, the reduced flow-induced eNOS phosphorylation seemed accompanied by a loss of flow-induced production of nitrate, a major metabolite for NO. Given the importance of NO in the vasculature, I further studied the vascular tone of vessels from $P2Y_2$ or G_q/G_{11} KO mice. I observed the arteries from $P2Y_2$ or G_q/G_{11} KO mice have normal structure and basal diameter and they fully responded to phenylephrine, KCl induced contraction like the WT vessel. They also preserved a normal myogenic response as well as a complete relaxation to SNP, indicating that contractile and vasodilatory functions per se were not affected. Interestingly, a stepwise increase of flow in WT mesenteric artery induced a significant relaxation of said artery; however, the artery from the $P2Y_2$ or G_q/G_{11} KO mice did not respond to flow anymore. Consistent with the fact that $P2Y_2$- or G_q/G_{11}-deficient BAECs are not able to generate NO during flow stimuli, it is likely that the endothelial cells in the arteries from KO mice have the similar defect. Even though there is no appropriate technique for measuring NO release from blood vessels, I took an indirect approach by measuring eNOS phosphorylation to verify my hypothesis. Indeed, the small mesenteric arteries freshly isolated from $P2Y_2$ or G_q/G_{11} KO mice showed significantly reduced eNOS phosphorylation compared to the WT controls. Thus, the $P2Y_2$- and G_q/G_{11}-mediated signaling pathway in endothelial cells is constantly activated to sustain endothelial NO formation and to mediate fluid shear stress-induced vasodilation. However, I cannot exclude the possibility that $P2Y_2$ and/or G_q/G_{11} regulate endothelial NO formation and flow-induced vasodilation via other pathways such as PKA or PKC.

It is well known that increased shear stress on endothelial cells induces the release of the $P2Y_2$ agonist ATP and that ATP can activate $P2Y_2$ in isolated endothelial cells (Bodin et al., 1991; Burnstock and Ralevic, 2014; John and Barakat, 2001; Yamamoto et al., 2011). Thus, flow-induced endothelial release of ATP through so far poorly understood mechanisms is the most likely flow-dependent activation mechanism of $P2Y_2$. Vascular endothelial cells have been

proposed to release ATP via vesicular exocytosis (Bodin and Burnstock, 2001), ABC-transporters (Schwiebert, 1999), connexin hemichannels (Stout et al., 2002), pannexin channels (Godecke et al., 2012), and by direct synthesis at the extracellular plasma membrane from a cell surface F1/F0-ATP synthase (Yamamoto et al., 2007). Extensive work has demonstrated that shear stress can induce ATP release from several types of cells including endothelial cells (Lohman et al., 2012a). Our current study adds to that by showing significant accumulation of ATP in BAECs medium after flow application and degrading ATP by apyrase inhibited shear stress responses. However, it remains unclear, by which mechanism shear stress triggers endothelial ATP release. Recently, Yamamoto et al. developed a novel chemiluminescence imaging method to visualize the ATP release dynamics by using cell-surface-attached firefly luciferase (Yamamoto et al., 2011). They observed that shear stress stimulated a localized ATP release and Ca^{2+} wave mainly at caveolin-1-rich regions of the cell membrane and that this could be blocked by caveolin-1 knockdown. The authors proposed that localized ATP release at caveolae triggers shear stress-dependent Ca^{2+} signaling in endothelial cells, but were unable to clarify why the localized ATP release occurs preferentially at caveolin-1-rich regions (Yamamoto et al., 2011). In another study from the same group, they found that cell surface ATP synthase is distributed in lipid rafts and that it co-localizes and physically associate with caveolin-1. The cell surface ATP synthase is activated by shear stress and pretreatment with its inhibitor or antibody blocks shear stress-induced ATP release. Moreover, disrupting the association between ATP synthase and caveolin-1 resulted in a marked reduction in shear stress-induced ATP release (Yamamoto et al., 2007). Thus, it is likely that the caveolae localized ATP synthase activation is the primary mechanism for shear stress-induced ATP release. Yet, a considerable amount of ATP was also released outside of the caveolae region (Yamamoto et al., 2011), indicating that other ATP releasing pathways cannot be excluded. A very interesting alternative candidate would be the Panx1 channels.

It has recently been demonstrated that PAR-1 receptor stimulation via thrombin induces ATP release from HUVECs, which is mediated by Panx1 channels

(Godecke et al., 2012). Given the fact that Panx1 and multiple isoforms of purinergic receptors are highly expressed in endothelial cells (Lohman et al., 2012b; Lohman and Isakson, 2014), it is very likely that Panx1 could participate in the endothelial-specific vascular response to flow. In addition, our unpublished data suggested the known pannexin channels inhibitor carbenoxolone to reduce flow-induced Ca^{2+} response in HUVEC. Therefore, one scenario meriting further investigation would be that endothelial shear stress mechanotransducing signaling processes are initiated by ATP.

Purinergic receptors are classified into P1 and P2 receptors families. P1 receptors are G-protein-coupled receptors and further subclassified as A_1, A_{2A}, A_{2B}, or A_3 receptors. They signal through either G_s or G_i and selectively bind the nucleoside adenosine to modulate cellular levels of cAMP (Burnstock and Ralevic, 2014). The P2 receptors are subclassified as either ionotropic P2X receptors, of which seven isoforms have been characterized to date (P2X1–7), or metabotropic P2Y receptors, which contain eight isoforms (P2Y1, 2, 4, 6, 11 – 14) (Burnstock and Ralevic, 2014). The P2X receptors are ligand-gated ion channels activated by ATP and control the influx of extracellular cations, including Na^+ and Ca^{2+}, leading to cellular depolarization. The P2Y family receptors are G-protein-coupled receptors that differentially bind to ATP, ADP, UTP and UDP, and their activation changes with the concentration of intracellular cAMP or Ca^{2+}. While numerous purinergic receptor isoforms have been identified in vascular cells, it has been shown that binding of ATP to $P2X_1$ receptors localized on VSMCs causes an influx of Ca^{2+} resulting in the constriction of the blood vessel (Lamont et al., 2006). The dominant purine receptors on both animal and human endothelial cells are $P2Y_1$, $P2Y_2$, and $P2X_4$ nucleotide receptors and A_{2A} and A_{2B} adenosine receptors (Burnstock, 2009; Gourine et al., 2009), but there are vessel- and species-specific differences in receptor subtype expression (Burnstock and Ralevic, 2014). Vascular endothelial cells also weakly express other types of metabotropic P2Y receptors, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$, although their functions are not clear.

ATP in the blood vessel lumen has been shown to activate $P2Y_1$ and $P2Y_2$ receptors. This triggers the production of inositol triphosphate (IP3) and the release of Ca^{2+}, which then results in the activation of eNOS and subsequent production of NO and, ultimately, induces vascular smooth muscle relaxation (Filippi et al., 1999; Shalev et al., 1999). By contrast, studies by Yamamoto et al. suggest that $P2X_4$ mediates an ATP-induced Ca^{2+}-influx in endothelial cells as well as a NO-dependent control of the vascular tone (Yamamoto et al., 2000b; Yamamoto et al., 2006). However, a more recent study indicates that ATP-induced Ca^{2+} mobilization and NO production *in vitro* involve $P2Y_2$ rather than P2X receptors (Raqeeb et al., 2011). My studies showed that both $P2X_4$ and $P2Y_2$ were abundantly expressed in primary mouse endothelial cells as well as in HUVECs. However, $P2X_4$ was not required for ATP-induced Akt and eNOS phosphorylation, while knockdown of $P2Y_2$ abrogated ATP-induced Akt and eNOS phosphorylation. Furthermore, knockdown of $P2X_4$ did not affect flow-induced Akt or eNOS phosphorylation, while blockade of $P2Y_2$ with the specific antagonist AR-C118925 or knockdown of $P2Y_2$ expression inhibited flow-induced Akt and eNOS phosphorylation. These data indicate that both $P2X_4$ and $P2Y_2$ are involved in acute fluid shear stress-induced Ca^{2+} release, but only $P2Y_2$ seems responsible for sustaining shear stress signaling through Akt and eNOS activation. Furthermore, the globally $P2X_4$-deficient mice are hypertensive and flow-induced dilation impaired and less NO was found in the urine from those mice (Yamamoto et al., 2006). However, it remains unclear whether the elevated blood pressure and defective adaptive vascular remodeling seen in a global, non-conditional knock-out of the $P2X_4$ gene in mice can be attributed to the loss of $P2X_4$ in endothelial cells or other cells.

Previous work has attempted to define the molecular details of the shear stress sensor in endothelial cells, especially the molecular identity of the primary sensor. Performing *in vitro* and *in vivo* experiments, firstly I identified G_q/G_{11} to be essential for endothelial fluid shear stress mechanotransduction, as it links fluid shear stress to downstream signaling processes to maintain eNOS activation, vascular tone and blood pressure. To identify potential G-protein-coupled receptors operating upstream of G_q/G_{11}, I performed an siRNA-

mediated GPCR screen and determined the $P2Y_2$ receptor to be the most promising hit. However, due to the scale of our screen, I cannot exclude the possibility that other GPCRs upstream of G_q/G_{11} are involved in shear stress response. It is also possible that G_q/G_{11} directly senses the flow without other GPCRs involved. Alternatively, since $P2Y_2$ and G_q/G_{11} are both likely to be expressed in the apical side of endothelial cells, where the shear force is initiated, $P2Y_2$ or G_q/G_{11} might have a force-sensing structure which can convert biomechanical forces into biochemical signal without any ligand. It is therefore of interest and merits further investigation to understand whether and how fluid shear stress affects the conformational dynamics of $P2Y_2$ and/or G_q/G_{11}.

The ability of the endothelium to sense fluid shear stress and to transmit this information into an intracellular signal is a fundamental function of the endothelial cell layer of blood vessels. Shear stress sensing and transduction controls vascular tone and morphogenesis and affects the susceptibility to diseases, such as atherosclerosis and hypertension. I have identified a critical mechanosensing pathway consisting of $P2Y_2$ and G_q/G_{11}, which is required for flow-dependent NO-formation and the regulation of vascular tone *in vitro* and *in vivo*. This pathway appears to be upstream of all the mechanotransducing pathways described so far, and it may be interesting to investigate its function in other responses of endothelial cells to fluid shear stress as well as in vascular diseases such as hypertension. Given the fact that the human $P2Y_2$ gene is shown to associate with essential hypertension (Wang et al., 2010b), a more targeted strategy for the treatment of hypertension could involve the development of compounds that selectively interfere with $P2Y_2$ and G_q/G_{11}.

8. MATEIRAL AND METHOD

8.1 Reagents

Acetylcholine	Sigma, Deisenhofen
Apyrase	NEB biolabs
ATP	Sigma, Deisenhofen
ATPγS	Sigma, Deisenhofen
AR-C118925	Christa Müller (Bonn)
BSA, bovine serum albumin	Sigma, Deisenhofen
Carbachol	Sigma, Deisenhofen
Collagenase Type II (Worthington)	CELLSYSTEM
Dispase	BD Biosciences, Heidelberg
Dynabeads	Invitrogen, Karlsruhe
EDTA, disodium ethylenediaminetetraacetate	Merck, Darmstadt
EGTA, ethylenedioxy-bis-(ethylenenitrilo)	Merck, Darmstadt
Elastase, Type III	Sigma, Deisenhofen
Ethanol absolute	AppliChem, Darmstad
Fibronectin, human	BD Pharmingen, Heidelberg
Fluo-4	Invitrogen, Karlsruhe
Foetal bovine serum	Invitrogen, Karlsruhe
HBSS, Hank's balanced salt solution	Invitrogen, Karlsruhe
Insuline	Invitrogen, Karlsruhe
Ionomycin	Sigma, Deisenhofen
Ki8751	Tocris
LY294002	Sigma, Deisenhofen
Magnesium chloride	Merck, Darmstadt
β-2-mercaptoethanol	Merck, Darmstadt
Methanol	Merck, Darmstadt
Milk, nonfat dry	AppliChem, Darmstad
Mineral oil	Sigma, Deisenhofen
O.C.T. freezing medium	Leica Microsystems, Nussloch
PBS	Invitrogen, Karlsruhe
Potassium chloride	Sigma, Deisenhofen
Proteinase K from Tritirachium album	AppliChem, Darmstad
Protein A/G PLUS-Agarose	Santa Cruz
SDS, sodium dodecyl sulfate	Fluka, Deisenhofen
Sodium chloride	AppliChem, Darmstad
Sodium nitroprusside (SNP)	Alexis
Tamoxifen	Sigma, Deisenhofen
TEMED	AppliChem, Darmstad

Thrombin	Sigma, Deisenhofen
Tris, tris-(hydroxymethyl)-aminomethan	Carl Roth, Karlsruhe
Triton X-100	Sigma, Deisenhofen
Trypsin-EDTA	Invitrogen, Karlsruhe
Tween-20	AppliChem, Darmstad
SmartLadder	Eurogentec, Köln
PageRuler Prestained Protein Ladder	Thermo Scientific, USA
PP2	Sigma, Deisenhofen
Phenylephrine (PE)	Sigma, Deisenhofen
UTP	Sigma, Deisenhofen
U46619	Cayman Chemical

8.2 Antibodies

Primary antibodies		
Mouse Anti-Alpha tubulin	Sigma	T9020
Rabbit anti-Akt	Cell signalling	#9272
Rabbit Anti-CD31 (PECAM-1)	Cell signalling	#3528
Rat Anti-CD31 (PECAM-1)	BD Bioscience	550274
Rat Anti-CD144 (VEcad)	eBioscience	12-1441-80
Rabbit Anti-c-Src	Santa Cruz	sc-18
Mouse Anti-eNOS	BD Biosciences	610296
Rabbit Anti-Flk-1	Santa Cruz	sc-505
Rabbit Anti-GAPDH	Cell signalling	#2118
Rabbit Anti-Gαq/Gα11	Santa Cruz	sc-392
Anti-Factin/Phalloidin	Invitrogen	A12379
Rabbit Anti-Phospho Src 416	Cell signalling	#2113
Rabbit Anti-phospho-eNOS S1177	Cell signalling	#9571
Rabbit Anti-phospho-Akt (S473)	Cell signalling	#4060
Mouse Anti-phospho-tyrosine (4G10)	Millipore	#05-1050X
Rabbit Anti-VEGFR-2	Cell signalling	#2479
Secondary antibodies		
Doncky anti- rat lgG	Alexa Fluor-488	A-21208
Doncky anti- rat lgG	Alexa Fluor-594	A-21209
anti- rabbit IgG	Alexa Fluor-488	A-11034
anti- rabbit IgG	Alexa Fluor-594	A-11012
Anti-mouse IgG	Alexa Fluor-488	A-11029

8.3 Cell culture

Human umbilical venous endothelial cells (HUVECs), Human umbilical arterial endothelial cells (HUAECs) and bovine aortic endothelial cells (BAECs) were obtained from Lonza. HUVECs and HUAECs were cultured on collagen-I coated dishes and supplied with endothelial growth medium EGM-2 (Lonza). BAECs were cultured on gelatin coated dishes and grown with EGM-2-MV medium (Lonza). Confluent cells at passages < P4 were used for all experiments.

8.4 Mouse primary endothelial cell isolation and culture

Mouse lung or skeletal muscle were minced into small pieces and washed in PBS then filtered with a 70 μm mesh cell sieve (BD Falcon). The tissues were digested with collagenase II, elastase, dispase and DNAse in 37°C with gentle shaking for 1 hour. ECs were isolated using CD31-labelled dynabeads and further purified by CD144-PE labelled FACS sorting. Cells were recovered and seeded on fibronectin coated dishes supplied with mouse endothelial grown medium from PromoCell. Purity of endothelial cells was determined by CD31 staining and qPCR analysis of CD31 and VE-Cadherin expression.

8.5 Flow experiments

For live cell imaging and alignment studies, cells were placed in a microfluidic plate (Fluxion). The BioFlux Pressure Interface (BioFlux200, Fluxion) connects a highly precise and accurate electropneumatic pump to the well plates to initiate controlled laminar shear stress. For biochemical experiments, cells were seeded in a parallel-plate flow chamber μ-Slide I Luer$^{0.4}$ from ibidi (Germany). Unidirectional laminar flow was generated by a computer-controlled setup containing an air-pressure pump and a two-way switching valve (Ibidi pump system). To prepare large amount protein for immunoprecipitation experiments, a cone-plate viscosimeter (BTF, Germany) was used. During the application of a fluid shear stress, paired static controls were cultured under the same environmental conditions as the stimulated samples.

8.6 Ca^{2+} measurements

Changes in intracellular Ca^{2+} levels were determined by fluorescent Ca^{2+}-sensitive dye Fluo-4 AM (Molecular Probes, Invitrogen). Confluent cells were incubated with 3 µM Fluo-4-AM (Molecular Probes) in HBSS-HEPEs buffer for 20 min at 37°C. Cells were then washed 3 times with HBSS-HEPEs buffer. Living fluorescent images were acquired every 500 ms by Olympus IX81 microscope. Excitation wavelength was set to 488 nm, and emission was recorded at 535 ± 15 nm. The fluorescence intensity per cell was calculated using Olympus Cell^M software.

8.7 NOx measurements

Nitrate and nitrite (NOx) levels in plasma were measured with a highly sensitive HPLC system (ENO-20 Eicom, Japan) as described previously (Jansson et al., 2008). Blood was collected from retro-orbital sinus and immediately centrifuged at 6800 g for 5 minutes at 4° C. Five days before the experiments commenced and then throughout the observation period, all animals were put on a standard chow specifically selected for its low NOx content (Lantmännen, Sweden). Flow-induced NOx release from endothelial cells was measured by using the Nitrate/Nitrite fluorometric assay kit from Cayman as per the manufacturer's instructions. Briefly, the cell supernatant was diluted with assay buffer and incubated with cofactor and nitrate reductase at room temperature for 3 hours to convert nitrate to nitrite. The samples were run in triplicate and the total nitrite fluorescence was read using an automated fluorescent plate reader (Flexstation-3; Molecular Devices) (excitation = 360 nm; emission = 430 nm).

8.8 Determination of ATP concentration

Cells were kept under static conditions or were exposed to fluid shear stress, and the ATP concentration in the supernatant was determined at the indicated time points using a bioluminescence assay (Molecular Probes, A22066) according to the manufacturer's instructions. Luminescence intensity was measured with a Flexstation-3 (Molecular Devices). ATP amounts were calculated using a calibration curve constructed using ATP standards. Parallel

determination of lactatdehydrogenase (LDH) activity (LDH Cytotoxicity Assay Kit, Cayman, 10008882) showed no LDH activity excluding that cell damage contributed to the increased ATP release under fluid shear stress.

8.9 Immunostaining

Cells or freshly isolated mouse mesenteric arteries were fixed with 1% PFA for 15 min and blocked with 5% fetal bovine serum (FBS)plus 0.3% Triton™ X-100 in PBS for 1 hour. Samples were incubated with blocking buffer containing the following primary antibodies at 4°C overnight: anti-eNOS (1:200), anti-phospho-eNOS (S1176, 1:100), anti-PECAM-1 (1:200), Alexa Fluor 594 conjugated anti-phalloidin (1:500) antibodies. After washing with PBS for 5 times, samples were labelled with DAPI and specific florescent conjugated secondary antibodies (Alex fluor 488, Alex Fluor 594 and Alex Fluor Cy5). Images were acquired using a confocal laser scanning microscope (Leica SP5).

8.10 Western blot and immunoprecipitation

Cells were lysed in Triton X-100 buffer supplemented with protease and phosphatase inhibitors. Isolated mesenteric arteries were lysed by RIPA buffer. Soluble supernatants were incubated with appropriate antibodies and protein A/G PLUS-agarose (Santa Cruz) overnight at 4°C. The beads were washed 5 times with lysis buffer. Immunoprecipitates or total cell lysates were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Nitrocellulose membranes were probed with primary and horseradish peroxidase conjugated secondary antibodies (Cell Signaling) and then developed using the ECL detection system (Pierce).Protein band intensity was analyzed by imageJ software (NIH). If subsequent detection of another protein was necessary the membrane was washed with stripping buffer from Pierce and re-blotted as described above.

8.11 Animal models

All procedures of animal care and use in this study were approved by the local animal ethics committees (Regierungspräsidia Karlsruhe and Darmstadt,

Germany). Inducible endothelial-specific Gαq/Gα11-deficient mice (Tie2-CreER^{T2}; Gnaq$^{fl/fl}$; Gna11$^{-/-}$) was generated by intercrossing Tie2-CreER^{T2} with Gnaq$^{fl/fl}$ and Gna11$^{-/-}$ (7th generation backcross toC57BL/6) as described previously (Korhonen et al.2009). In order to induce Cre-mediated recombination, 6-10 weeks littermate were injected intraperitoneally with 1 mg/day tamoxifen dissolved in 50 µl Miglyol oil on five consecutive days. Cre activity was verified by crossing Tie2-CreER^{T2} with mT/mG reporter mouse line (Muzumdar et al., 2007) and Cre recombination was confirmed by western blot analysis $G\alpha_q$/$G\alpha_{11}$ expression in primary isolated mouse lung endothelial cells. JM8A1.N3 mouse embryonic stem (ES) cells generated from the strain C57BL/6N carrying the shown targeted allele of the gene encoding $P2Y_2$ (*P2ry2*) were obtained from EUCOMM (ES cell clone number: HEPD0557 7 B07). ES cells were used to generate chimeric mice, and germ-line transmission was verified after crossing high-percentage chimeras with a Flp deleter mouse line (Rodríguez et al., 2000). Mice carrying the indicated floxed allele were then used to generate inducible endothelium-specific $P2Y_2$-deficient mice (Tie2-CreER^{T2};*P2ry2*$^{flox/flox}$). Deletion of P2Y2 in endothelium was confirmed by qRT-PCR analysis of P2Y2 expression in freshly isolated endothelial cells.

8.12 Pressure myography

Animals were sacrificed by cervical dislocation, and the mesenteric arterial bed was removed and placed in ice-cold Krebs buffer (118.1 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 9.3 mM glucose, and 0.026 mM EDTA, pH 7.4 supplemented with 1% BSA). Arteries of the third or fourth branch were removed from the mesentery by gently peeling away the adventitia and were mounted in the proper proximal-distal orientation between two glass micropipettes seated in a chamber (Danish Myo Technology, Denmark). The inflow cannula was fixed, while the outflow cannula allowed for length adjustment along the longitudinal axis. The artery was secured on both ends by a 10.0 nylon suture and perfused with low flow (5 µl*min^{-1}) to remove any blood from the vessel lumen. The external diameter of the artery was visualized and recorded with a CCD camera using MyoView software.

Temperature of the chamber was kept a constant 37 °C. The mounted artery was initially pressurized to 20 mmHg under no flow conditions and incubated for 30-40 min. Pressure was then increased to 70 mmHg and incubated for 10 min to allow the vessel to reach a steady-state diameter. Vessels were contracted with 50-150 nM U46619 to 40-50% of the passive diameter. After reaching a stable baseline, flow was increased in a stepwise manner by changing the pressure of the inflow- and outflow side inversely, thereby creating a pressure difference across the arteriole without altering the intraluminal pressure. The viability of the vessel was verified at the end of the experiment by dilating the vessel with acetylcholine (10 µM) or sodium nitroprusside (10 µM). Arteries showing less than 60% relaxation were considered damaged and were omitted from further analysis. Vasodilatation to flow was calculated as a percentage of the U46619-induced contraction by the following equation: *% relaxation* = 100 x $(D_F - D_{U46619})/(D_{PD} - D_{U46619})$ where *D* represents the external diameter of vessels; D_F is the vessel diameter during flow; D_{U46619} is the diameter after U46619 contraction; D_{PD} is the passive diameter without any treatment.

8.13 Telemetric blood pressure measurements

A radiotelemetry system (PA-C10; Data Sciences International) was used to monitor blood pressure in conscious, unrestrained mice as described previously (Wirth et al., 2008). Briefly, mice were anesthetized with ketamine (i.p. 100 mg/kg) and xylazine (i.p. 10 mg/kg), and the neck was shaved and disinfecte. The left common carotid artery was isolated carefully from connecting tissue and the vagal nerve parallel to the artery. Position the first ligation suture (6/0 FST) just proximal to the bifurcation of the interior and exterior carotid arteries and tie a secure knot around the artery to ligate the carotid artery. Another temporary occlusion ligation was made by placing a metal clip (Beimer Gefässclip) as close to the clavicle as possible thus isolating at least 6 mm of the artery. A tiny incision was made in the carotid artery next to anterior suture and the pressure-sensing catheters were inserted into carotid artery until its tip sensing region was place in the aorta arch. The loose ends of the occlusion suture around the artery were tied to seal the artery wall around the catheter stem, and the transducer unit was inserted into a subcutaneous pouch along

the right flank. The incision in the neck was closed with surgical thread (5/0, Marlin Violet, Catgut GmbH). After a recovery period of at least one week, pressure recordings were collected, stored and analyzed with Dataquest A.R.T. software version 4.0 (Data Sciences International). We collected data for basal blood pressure and heart rate measurements with a ten second scheduled sampling every five minutes and used a 24-hour mean value for analysis.

8.14 GPCR expression and siRNA screening

We designed a Human GPCR qRT-PCR library which contained 936 primers and probes for 418 non-olfactory GPCRs. The library was synthesized in ten 96-well plates by Sigma for high-throughput analysis. Expression of GPCRs in HUVECs and HUAECs were determined by quantitative RT-PCR. Gpr133 intron spanning primers were used for genomic contamination control and GAPDH primers were used as loading control in all plates.

Cells were transfected with siRNAs using Opti-MEM and Lipofectamine RNAiMAX (Invitrogen). Diluted siRNA and RNAiMAX were mixed gently and incubated for 30 mints at room temperature. The mixture was added to cells and medium was changed after 6 hrs. A repeated knockdown was performed next day and assays were started at 48 hours after second knockdown. SiRNAs against $G\alpha_q$, $G\alpha_{11}$, $P2Y_2$ and $P2X_4$ were from Qiagen. siRNAs used for screen were pools of siRNAs of an siRNA library directed against 514 genes including 407 non-olfactory human GPCRs and 86 olfactory human GPCRs (Qiagen) targeting the same RNA.

8.15 Expression analysis

RNA isolation was performed using Qiagen RNeasy min or micro kit. Reverse transcription was done by using the Transcriptor high fidelity cDNA synthesis kit from Roche. Primers (Table below) were designed with the online tool provided by Roche and quantification was performed using the LightCycler 480 Probe Master System (Roche). Genomic DNA was used as a universal standard to calculate gene copy number per nanogram of RNA. Relative expression levels were obtained by normalisation with GAPDH or 18S values.

qPCR primer sequences

Gene	Forward 5' → 3'	Reverse 5' → 3'
Gnaq (human, bovine, mouse)	tgggtcgggctactctgat	taggggatcttgagcgtgtc
Gna11 (human, bovine, mouse)	gagcacgttcatcaagcaga	gatgttctggtacaccagtttgg
GAPDH (bovine)	tcaccagggctgcttttaat	gaaggtcaatgaaggggtca
GAPDH (human)	gcatcctgggctacactga	ccagcgtcaaaggtggag
P2Y2 (human)	taacctgccacgacacctc	ctgagctgtaggccacgaa
P2Y2 (mouse)	tggtactggccgtcttcg	agtagagggtgcgcgtga
P2Y2 (bovine)	agaactccaggggacaga	cagccagatgtccttagtgtca
P2X4 (human)	agccccatcaaagaacagag	tctctggggtgatgtggtg
18S (mouse)	gcaattattccccatga	gggacttaatcaacgcaagc
VE-Cadherin (mouse)	ccatgatcgacgtgaagaaa	gatgtgcagtgtgtcgtatgg
β-actin (mouse)	aaatcgtgcgtgacatcaaa	tctccagggaggaagaggat

8.16 Statistics

Data are presented as means ± s.e.m.. Comparisons between two groups were performed with unpaired Student's t test, and multiple group comparisons were performed by ANOVA followed by Bonferroni post hoc test. Comparisons between multiple groups at different time points were performed by repeated ANOVA followed by Bonferroni post hoc test. $P \leq 0.05$ was considered to be statistically significant.

8.17 Buffer and stock solutions

Phosphate- buffered saline (PBS) per liter

NACl (137 mM) 8 g

KCl (2.7 mM) 0.2 g

Na2HPO4 (10mM) 1.44 g

KH2PO4 (2nM) 0 .24 g

Dissolved in 800 ml of distilled H2O. Adjest the pH to 7.4 with HCl. Add H2O to 1 liter and sterilization in autoclaving.

Tris EDTA (TE) 10X

Tris-Cl (pH 7.6) 100 mM

EDTA (pH 8.0) 10 mM

TAE Buffer (1X)

Tris-acetate 40 mM

EDTA 1 mM

Tail digestion buffer:

EDTA (pH 8.0) 0.1M

SDS 0.5 %

Tris pH (8.0) 50mM

Proteinas K 0.5 mg/ml

Polymerase chain reaction (**PCR**)

PCR buffer 10X: 200mM Tris, 500mM KCl. Set pH 8.4 with HCl

dNTPS :dATP+ dGTP+dCTP+dTTP (25 µmol)

MgCl2: 50 mM

Target DNA amplifications & mutation PCR -Invitrogen PCR kits (cat no. 11708-013).

PCR reactions (50µl): Buffer 10x: 5 µl, MgCl2: 1.5 µl, dNTPs: 0.5 µl, Taq polymerases: 1 µl, primer (Forward & reverse): 2 µl, DNA: 1-2 µl

and makeup with PCR grade water.

DNA purification - (Qiagen: 28106)

DNA precipitation buffer:

Ammonium acetate (7.5M) 100 µl

Ethanol (99-100%) 600 µl

Cell culture Medium

Medium for cell line:

DMEM+NAA +4.5 mg/l D-glucose (450 ml) 1X (Gibco:10938-025)

L-Glutamine(100X) (Gibco: 25030-081) 5 ml

Pyruvate (100X) (Gibco: 11360088) 5 ml

Penicillin-streptomycin(100X) (Gibco: 15140-122) 5 ml

Fetal bovine serum (10%) (Gibco: 16000044) 50 ml

PBS (CaCl2-, MgCl2 -) (Gibco: 14190-094)

Medium for HUVECs and HUAECs

EGM2 medium (Lonza: CC-3156)

EGM2 supplements (Lonza: CC-4176)

Medium for BAECs

EGM2-MV medium (Lonza: CC-3156)

EGM2 supplements (Lonza: CC-4147)

Primary lung Endothelial cells medium (500 ml)

DMEM/F12 (Gibco cat: 21041-025) 400ml

FCS (20%) 100 ml

Penicillin-streptomycin (100X) 5 ml

ECGS-H (Promocell cat: C30120) 8µl/ml- 4ml

Western blot Buffers

RIPA lysis buffer (RIPA 4X)

NaCl (5M) 12ml

Tris pH 7.4 (2M) 10ml

EDTA pH 8 (0.5 M) 4ml

SDS (20%) 2 ml

Na-DOC (10%) 20ml

Triton X-100 4 ml

Distilled water was added to final volume 100 ml. Solution was stored at 4°C.

Prepared 1X RIPA buffer for 50 ml. Added freshly Protease inhibitor cocktail
AEBSF (100 mM) 50µl
Leupeptin (10 mg/ml) 50µl
Aprotinin (10 mg/ml) 50µl
Pepstatin A (10 mg/ml) 50µl

Triton X-100 lysis buffer

(Need final concentration of 0.25% Triton X-100, 10mM EDTA, 10mM Tris.HCl[pH8.1], 10mM NaCl, 1X protease inhibitor)
Triton X-100 (10%) 2.5 ml
EDTA (0.5 M) 2.0 ml
Tris HCl (p.H 8.1) 1M 1.0 ml
NaCl (1M) 1.0 ml
Distilled water was added to final volume 100 ml. Added freshly Protease inhibitor
cocktail.

SDS PAGE separating gel 7.5% (10 ml)

H2O 4.85 ml
Tris pH 8.8 (1.5M) 0.4%
SDS 2.6 ml
Acrylamind/bisacrylamid30% w/v 2.5 ml
APS (10%) 50 µl
TEMED 5 µl

SDS PAGE stacking gel 4% (5 ml)

H2O 3.05 ml
Tris pH 6.8, 0.4% 1.5M
SDS 1.3 ml
30% w/v Acrylamind/bisacrylamid 65 ml
10% APS 50 µl
TEMED 5 µl

Sample buffer for reducing conditions (6x)

SDS (12%) 3.6 g

Tris-HCl, pH 6.8 300 mM

Tris(1.5M) 6 ml

DTT (600 mM) 2.77 g

BPB (0.6%) 0.18 g

Glycerol (60%) 18 ml

Distilled water was added to 30 ml. Buffer was stored in 0.5 ml aliquots at -20°C.

Add 50 µl β-mercaptoethanol in 1 ml of 4x buffer.

Electrophoresis buffer (5x) Stock

Tris base 154.5 g

Glycine 721 g

SDS 50 g

Distilled water was added to 10 l. Buffer was stored at RT.

Protein transfer buffer (1x)

Tris base 25 mM

Glycine 192 mM

Methanol 20%

PBS-Tween (PBST)

1x PBS

0.1% Tween®20

Solution was kept at RT.

Blocking solution

Skim milk (5%) 5 g

PBST 100 ml

Solution was prepared freshly just before use.

Stripping buffer

Sodium Phosphate buffer (pH 7-7.4) 5 mM

SDS 2%

Freshly before use, 10 µl of β-Mercaptoethanol was added to 50 ml of the buffer.

For mild stripping, Restore Western Blot Stripping Buffer (46428) from Thermo was used

Buffer III

(Final concentration of 0.25M LiCl, 1% NP-40, 1% deoxycholate, 1mM EDTA, 10mM Tris.HCl [pH8.1])

LiCl (4M) 6.25ml

NP-40 (10 %) 10 ml

Deoxycholate (10 %) 10 ml

EDTA (0.5 M) 200 µl

Tris.HCl[pH8.1] 1M 1.0 ml

Distilled water was added to final volume 100 ml.

TE buffer for washing beads

(Need final concentration of 2mM EDTA, 10mM Tris.HCl [pH8.0])

EDTA (0.5 M) 400µl

Tris.HCl[pH8.0] 1M 1 ml

Distilled water was added to final volume 100 ml.

TE buffer for dissolving DNA

(Need final concentration of 1mM EDTA, 10mM Tris.HCl[pH8.0])

EDTA (0.5M) 200µl

Tris.HCl [pH8.0] 1M 1 ml

Distilled water was added to final volume 100 ml.

Elution buffer

(Final concentration of 1% SDS, 10mM EDTA, 50mM Tris.HCl[pH8.1])

To prepare 100ml, need

SDS (10%) 10 ml

EDTA0.5M 2.0 ml

Tris.HCl[pH8.1] 1M 5ml

Distilled water was added to final volume 100 ml

Immunofluorescence staining

Fixation: 4% PFA

Wash buffer I: 50mM Glycine in PBS (RT)

Wash buffer II: PBST (1X)

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CV

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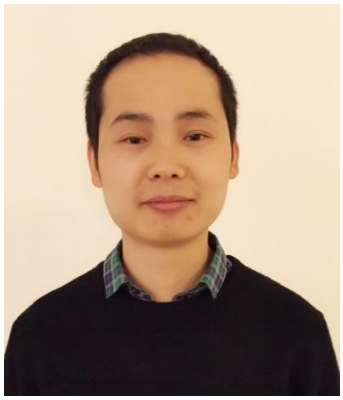

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Schriftliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin der Johann Wolfgang Goethe-Universität Frankfurt am Main zur Promotionsprüfung eingereichte Dissertation mit dem Titel

$P2Y_2$ and G_q/G_{11} control blood pressure by mediating endothelial mechanotransduction

Am Max-Planck-Institut für Herz- und Lungenforschung unter Betreuung und Anleitung von Prof. Dr. Stefan Offermanns ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe. Darüber hinaus versichere ich, nicht die Hilfe einer kommerziellen Promotionsvermittlung in Anspruch genommen zu haben.

Ich habe bisher an keiner in- oder ausländischen Universität ein Gesuch um Zulassung zur Promotion eingereicht. Die vorliegende Arbeit wurde bisher nicht als Dissertation eingereicht.

Vorliegende Ergebnisse der Arbeit werden in folgendem Publikationsorgan veröffentlicht:

Wang, S., Iring, A., Strilic, B., Kaur, H., Burbiel, J., Müller, C., Fleming, I., Lundberg, J., Wettschureck, N., Offermanns, S.
$P2Y_2$ and G_q/G_{11} control blood pressure by mediating endothelial mechanotransduction (manuscript in revision).

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(Ort, Datum) (Unterschrift)