TIE1 ATTENUATION REDUCES ATHEROSCLEROSIS IN A DOSE DEPENDENT

AND SHEAR STRESS SPECIFIC MANNER

By

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To my parents, Simon and Jessie

and

To my best friend, Ana Luisa

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LIST OF ABBREVIATIONS

4OHT	4-hydroxy-tamoxifen
Ang	Angiopoietins
Ao	Aorta
AoV	Aortic Valve
AP-1	Activator protein-1
ApoE	Apolipoprotein E
β-gal	Beta-galactosidase
BAEC	Bovine aortic endothelial cell
BMP-4	Bone morphogenic protein-4
csf	Colony stimulating factor
DiI-AcLDL	1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate-labeled acetylated low-density lipoprotein
ECM	Extracellular matrix
EDRF	Endothelial derived relaxation factor
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cell
ERK	Extracellular signal-regulated kinase
ER^T	Estrogen receptor - tamoxifen inducible
E-selectin	Endothelial-selectin
ET-1	Endothelin-1
Flk1	Fetal liver kinase 1
FN	Fibronectin
FRET	Förster resonance energy transfer / fluorescence resonance energy transfer
GPCR	G protein-coupled receptor
HCAEC	Human coronary artery endothelial cell
HDL	High density lipoprotein
HMG-CoA	2-hydroxy-3-methylglutaryl coenzyme A
HUVEC	Human umbilical vein endothelial cell
IAP	Inhibitor of apoptosis
IFN	Interferon
Ig	immunoglobulin
IL-1β	Interleukin-1β
LCA	Left carotid artery
LDL	Low density lipoprotein
Lu	Lumen
JNK	c-Jun N-terminal kinase
KLF2	Kruppel-like factor 2
LNMA	L-NG-monomethyl arginine
MAEC	Mouse aortic endothelial cell
MAPK	Mitogen activated protein kinase

MCP-1	Monocyte chemotactic protein-1
MEK	MAPK kinase
MEKK	MAPK kinase kinase
MnSOD	Manganese superoxide dismutase
NADPH	Nicotinamide adenine dinucleotide phosphate oxidase
nf-κB	Nuclear factor – κB
nSSRE	Negative shear stress response element
PDGFR	Platelet derived growth factor receptor
PECAM	Platelet endothelial cell adhesion molecule
PI3K	Phosphatidyl inositol 3-phosphate
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13 acetate
RCCA	Right common carotid artery
rpm	Revolutions per minute
SCL	Stem cell leukemia (aka Tal1)
SHP2	SH2 domain-containing tyrosine phosphatase 2
SREBP1	Sterol regulatory element binding protein 1
SSRE	Shear stress response element
SV40	Simian virus 40
TAg	T antigen
TCFA	Thin cap fibroatheroma
Tie1	Tyrosine kinase with immunoglobulin-like and EGF-like domains 1
Tie2	Tyrosine kinase with immunoglobulin-like and EGF-like domains 2
TLR2	Toll-like receptor 2
TNF-α	Tumor necrosis factor – α
TRE	TPA response element
TrkA	Neurotrophic tyrosine kinase, receptor, type 1
TPA	Tetra-decanoyl phorbol acetate
VECadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

CHAPTER I

INTRODUCTION

Overview

Blood flow inflicts on the vascular endothelial surface a frictional force per unit area known as hemodynamic shear stress and the transcriptional response of endothelial cells exposed to non-laminar flow with low shear stress similar to atherosclerosis prone areas is distinct from that elicited by atheroprotective laminar flow with high shear stress. However, the critical mediators of endothelial cell mechanotransduction involved in atherosclerosis have not been clearly delineated. Tie1 is an orphan receptor tyrosine kinase expressed almost exclusively in endothelial cells and there is recent evidence to suggest that its expression is regulated by atherogenic shear stress making it a possible component of the endothelial mechanotransduction machinery. However, ablation of Tie1 expression results in embryonic lethality, thus defining a role for Tie1 signaling in atherosclerosis has been elusive. We therefore hypothesized that Tie1 plays an essential role in endothelial response to atherogenic shear.

The primary focus of my research has been to map the expression profile of Tie1 in adult macrovasculature, evaluate the effect of Tie1 receptor attenuation on atherosclerosis progression *in vivo* and delineate this effect on shear stress-modulated endothelial cell biology *in vitro*. Chapter I will describe the genetic and structural aspects of Tie1 and its role in early embryonic development. It will also summarize the roles that Tie1 plays in *in vitro* endothelial cell biology. Chapter I will go on to provide pertinent background

information on fluid dynamics and the different models of shear stress mechanotransduction *in vitro*. The effect of shear stress on tissue responses and subcellular activity will also be discussed. Finally, Chapter I will evaluate the role of shear stress in atherogenesis and the tripartite argument involving Tie1, shear stress and atherosclerosis will be presented. Chapter II will define the expression of Tie1 *in vivo* and compare Tie1 promoter activity in the aortas of immature 4 week-old mice to those of 12 week-old adult littermates in atherosclerosis-prone and atherosclerosis-resistant regions of the vasculature. Also, Chapter II will demonstrate the use of a unique, *in vivo* shear stress modifying cast to evaluate the effect of different flow profiles and shear stress magnitudes on Tie1 promoter activity. Next, Chapter III focuses on the effect of Tie1 genetic dose reduction on atherosclerosis progression. It also demonstrates the differences in atherosclerosis burden between regions of the aorta that are distinguished in part by their local shear stress profiles. In Chapter IV, a novel method of isolating murine aortic endothelial cells will be presented using a transgenic mouse model that allows for conditional inactivation of Tie1. It will detail the methodology used and discuss the benefits of this *in vitro* model. Chapter V will report assessments of shear stress-induced alterations in Tie1 expression. It will evaluate the effect of *in vitro* Tie1 deletion on flow-mediated endothelial responses. Last, in Chapter VI I conclude by discussing the significance of Tie1 in a shear stress-regulated environment, specifically in the context of atherosclerosis, and I will highlight future experiments to explore the effects of biomechanical forces on the role of Tie1 in endothelial biology.

The Tie1 Receptor Tyrosine Kinase

The Tie (*T*yrosine kinase with *I*g and *E*GF homology) family of receptor tyrosine kinases, comprising of Tie1 and Tie2, was first reported in 1992 (Partanen *et al.*, 1992). To date, studies have shown that Tie1 is almost exclusively expressed only in endothelial cells and cells of hematopoietic lineage (Partanen *et al.*, 1992; Shahrara *et al.*, 2002; Puri & Bernstein, 2003; Antonescu *et al.*, 2009). Although the sister receptors share a high degree of structural homology, the subsequent discovery of angiopoietins (Ang), specific ligands for Tie2 but not for Tie1 has thwarted efforts to elucidate its function. However, *in vivo* gene disruption studies have provided some insight into the role of Tie1 during development. While investigators speculate that Tie1 may serve only to modulate Tie2 signaling, recent evidence suggests a Tie2 independent role for Tie1.

The Role of Tie1 in Development

Tie1 is an orphan receptor tyrosine kinase that is expressed almost exclusively in endothelial cells (Partanen *et al.*, 1992). It is the last receptor tyrosine kinase expressed during embryonic vascular development and ablation of Tie1 expression results in embryonic lethality after embryonic day 13.5 due to severe edema, hemorrhages and loss of microvessel integrity (Figure 1.1.1) (Puri *et al.*, 1995; Sato *et al.*, 1995; Qu *et al.*, 2010). Puri et al (Puri *et al.*, 1995) and Sato et al (Sato *et al.*, 1995) both showed that at e13.0, Tie1-/- embryos have a normal vascular network suggesting that Tie1 is not essential for vasculogenesis. Interestingly, studies using chimeric embryos (mixed Tie1 null and wildtype cells) revealed reduced representation of Tie1 null endothelial cells in the vasculature of mature organs due either to increased cell death, reduced survival or

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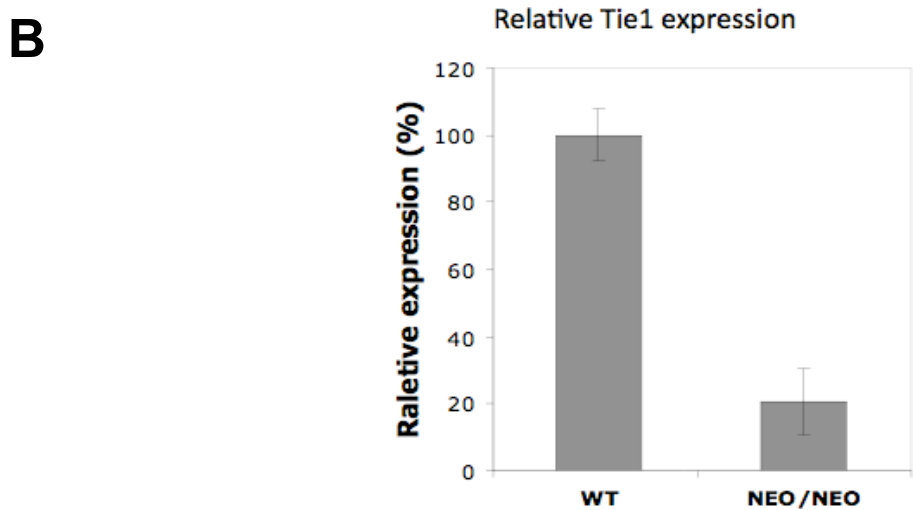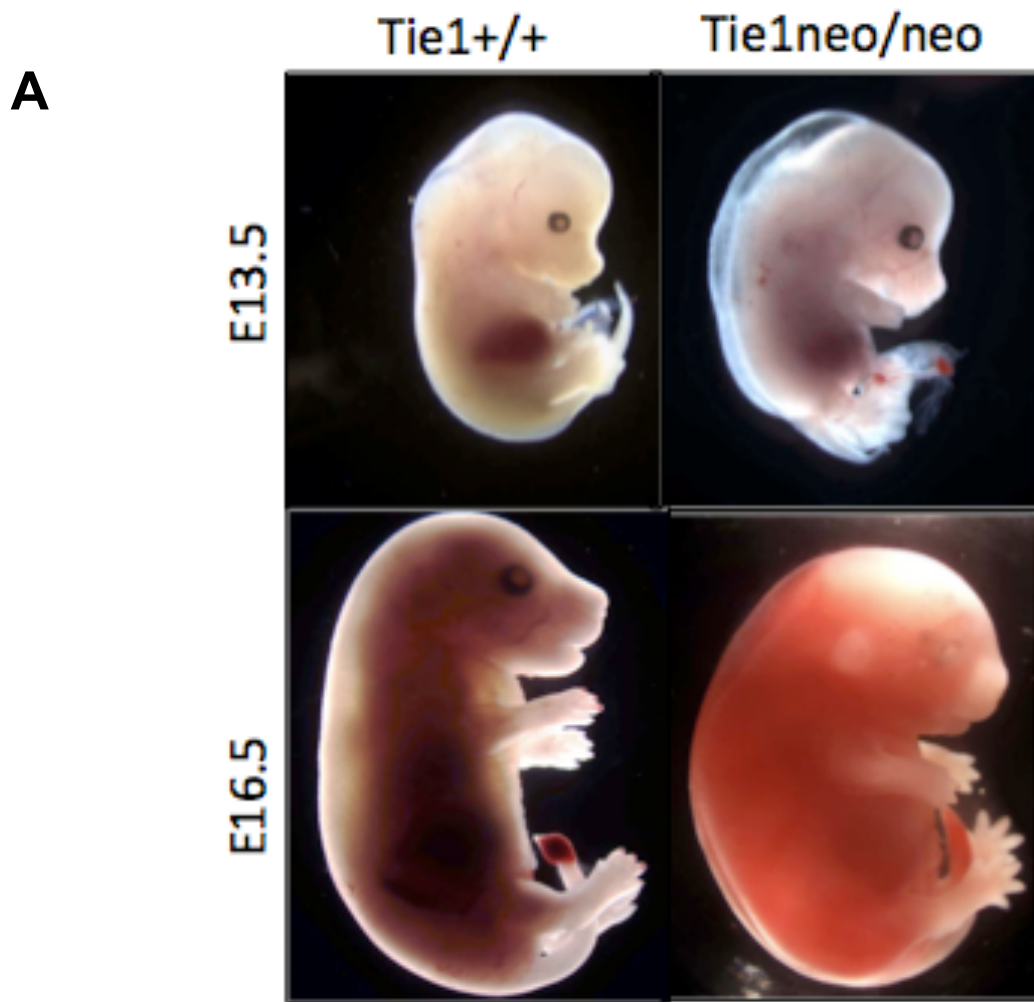
Figure 1.1.1 *Loss of Tie1 leads to early embryonic lethality.* (**A**) Attenuation of Tie1 expression causes edema and massive hemorrhage at e13.5 and early embryonic death by e16.5. (**B**) Insertion of neomycin selection cassettes (neo/neo) significantly reduces Tie1 expression. Figure modified from Qu (2009).

replacement by competitive proliferation of normal endothelial cells, supporting a role for Tie1 in endothelial cell survival (Partanen *et al.*, 1996).

Tie1-/- embryos display increased vascular density and hyperactive endothelial cells (Patan, 1998). These Tie1 knockout cells have increased intra- and trans-cellular vacuoles suggesting a requirement for Tie1 in inhibiting endothelial cell stretching and allowing cell maturation (Figure 1.1.2). Additionally, Tie2 is expressed before Tie1 and its activation enhances endothelial cell activation, hence leading some investigators to postulate that Tie1 may function to counteract Tie2, and loss of inhibition by Tie1 prevents maturation of endothelial cells (Patan, 1998). Taken together, these results suggest that Tie1 is not required for vasculogenesis, but it is required during embryonic development for the integrity and survival of vascular endothelial cells.

The Expression Profile of Tie1

Tie1 expression is driven by an octamer element (5'-ATGCAAAT-3') similar to Tie2 (Figure 1.1.3) (Boutet *et al.*, 2001). Boutet et al showed that mutations in the element decreased Tie1 promoter activity indicating that the promoter functions as a positive regulator. Further, they showed that the promoter binds the ubiquitous transcription factor Oct1 and an unidentified co-factor that was not found in control non-endothelial cells. Identification of this promoter element and insertion of a LacZ reporter has facilitated investigations in Tie1 expression.

Tie1 expression varies in different organs pre- and post-natally (Taichman *et al.*, 2002). Taichman et al found Tie1 is expressed in the heart, kidneys, liver, lungs and brain between embryonic day 13.5 and birth (Figure 1.1.4). At 6 weeks after birth, Tie1
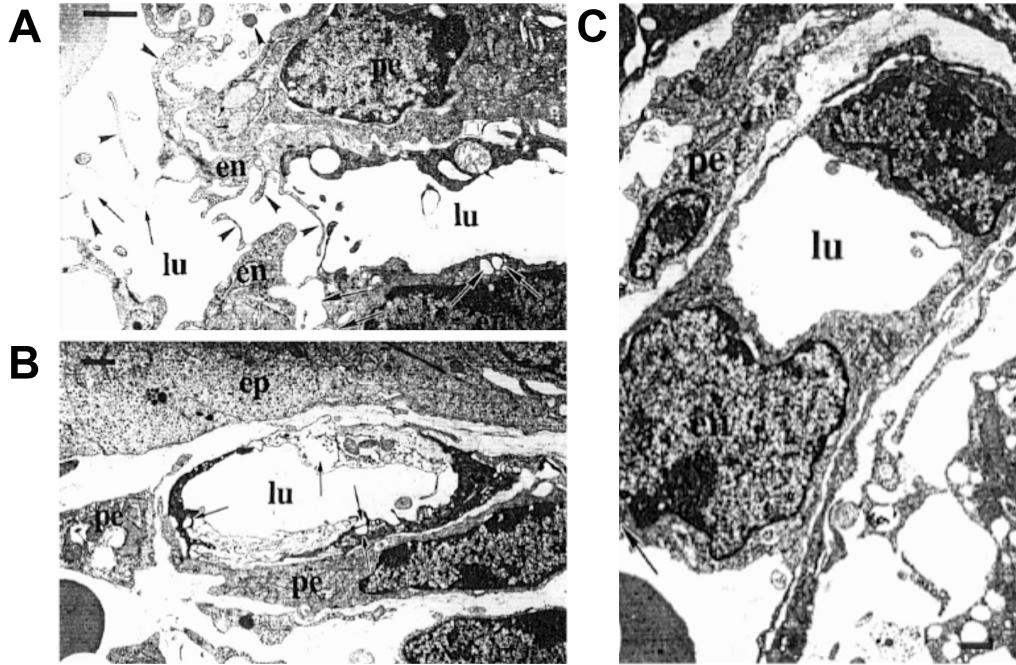
Figure 1.1.2 *Ultrastructure of Tie1-/- endothelial cells in the capillaries of embryonic small intestine mucosa.* (**A, B**) Capillaries from Tie1-/- embryo showing "active" endothelial cells lining the lumen. Cellular extensions and filopodia invade the lumen (arrowheads), and endothelial cells show numerous intracellular and transcellular holes (arrows). (**C**) Endothelial cells from wild-type embryo have less extensions. Adapted from Patan (1998).

Endothelial cell, en; epithelial cell, ep; vessel lumen, lu; periendothelial cell, pe.
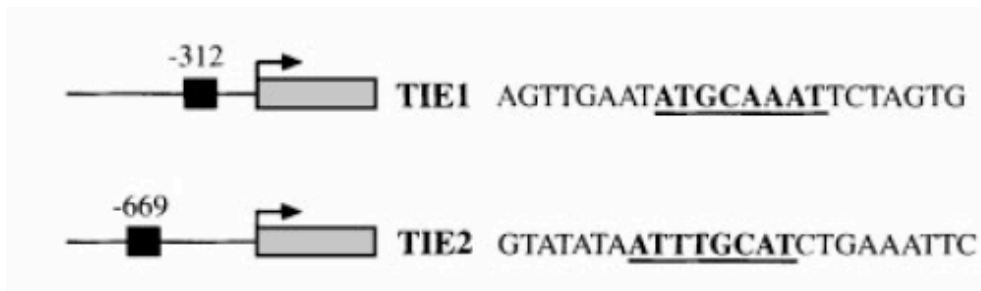
Figure 1.1.3 *Tie1 and Tie2 promoter sequences.* Illustration of murine Tie1 and Tie2 promoters. The consensus octamer element (bolded and underlined) of Tie1, 5'-ATGCAAAT-3', and Tie2 are located at -312 bp and -669 bp respectively. Black boxes indicate the promoter sequence position; arrows indicate transcription start site and the first exon of the gene is represented by a grey box. Modified from Boutet (2001).
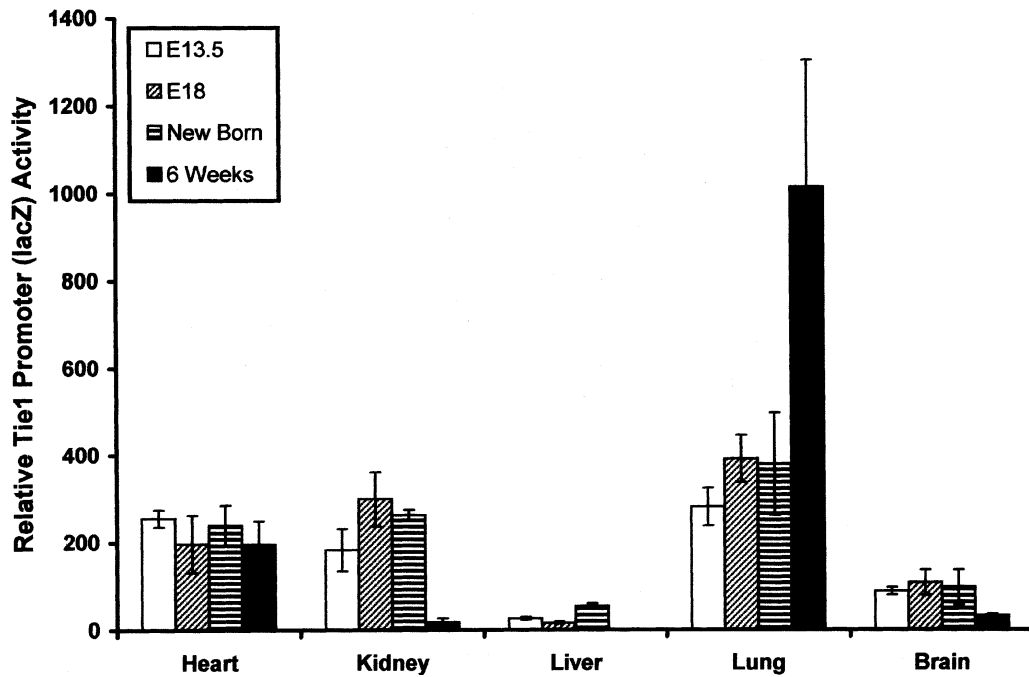
Figure 1.1.4 *Comparison of Tie1 promoter activity pre and postnatal mice. Tie1* promoter activity was determined in the organs of mice expressing the *lacZ* reporter gene under the regulatory domains of *Tie1*. Whereas the heart showed stable expression, and expression declined in the kidney, liver and brain after 6 weeks, *Tie1* promoter activity significantly increased in the adult lungs. Adapted from Taichman (2003).

expression in the heart remains the same, but expression in the kidneys, liver and brain is almost eliminated. Interestingly, lung expression of Tie1 increased up to 4-fold consistent with a role of Tie1 in formation and maturity of the pulmonary vascular bed. Hence, Tie1 transcriptional activity is increased during stages of developmental angiogenesis and the heterogeneity of Tie1 expression in different organs suggests a context dependent role for this receptor in endothelial function.

Tie1 expression is not just increased in adult normal physiology but also in pathological angiogenesis (Korhonen *et al.*, 1992; Kaipainen *et al.*, 1994). Tie1 is expressed in endothelial cells that re-populate the denuded surface of carotid arteries (Fujikawa *et al.*, 1999). Immunohistochemical analyses of balloon-injured rat carotid arteries found increased Tie1 along with Tie2 expression in recently re-endothelialized carotid arteries. Fujikawa et al also showed that the intensity of Tie1 expression increased during the later post-injury time points, correlating with an increase in the density of endothelial cells. They further confirmed this *in vitro*, showing maximal Tie1 expression in human umbilical vein endothelial cells only when they reach 100% confluency. This study suggests that expression of Tie1 correlates with expansion of the endothelial cell population induced by vascular injury.

Interestingly, expression of Tie1 is increased in a number of tumors and its expression has been found to correlate inversely with survival of gastric cancer patients (Lin *et al.*, 1999). Immunocytochemistry analyses has revealed that Tie1 is expressed in epithelial tumor cells in breast (Cance *et al.*, 1993; Tseng *et al.*, 2001; Yang *et al.*, 2003; Uruno *et al.*, 2004), colon (Yang *et al.*, 2003), gastric (Lin *et al.*, 1999) and thyroid (Ito *et al.*,

2004) cancers. Importantly, the addition of Ang1 to breast and colon epithelial tumor cells phosphorylated Tie1 (Rees *et al.*, 2007).

In the vasculature, besides endothelial cells, Tie1 is also expressed in platelets (Tsiamis *et al.*, 2000). Platelet Tie1, however, is different from endothelial Tie1, western blot analyses showed that the platelet form to be 110kDa in contrast to the endothelial form at 135/125 kDa doublet. Unlike the endothelial form of Tie1, the platelet form of Tie1 cannot be cleaved by phorbol ester, suggesting to the investigators that one of its functions may be to competitively bind any yet unidentified Tie1 ligands (Tsiamis *et al.*, 2000).

Structure of the Tie Family Receptors

The extracellular portion of the Tie receptors consists of multiple domains and as described by Macdonald et al, the extracellular fragment of Tie1 folds into a "globular head and a short rod-like stalk" (Macdonald *et al.*, 2006). The "globular head" consists of three immunoglobulin (Ig)-like domains separated by three epidermal growth factor (EGF)-like repeats (Figure 1.1.5) (Macdonald *et al.*, 2006). The stalk domain has been suggested to function as a "spacer" between the ligand binding site and the cell membrane and consists of three β-sheet fibronectin (FN) type III repeats (Partanen *et al.*, 1992). This specific combination and sequence of domains is unique to the Tie family of receptors (Sato *et al.*, 1993). The cytoplasmic portion is comprised of two tyrosine kinase domains that are highly conserved between Tie1 and Tie2. Although earlier studies reported 2 Ig repeats in the extracellular domain (Figure 1.1.5A) (Partanen *et al.*, 1992), Macdonald et al recently confirmed the presence of a third Ig repeat, further bridging the
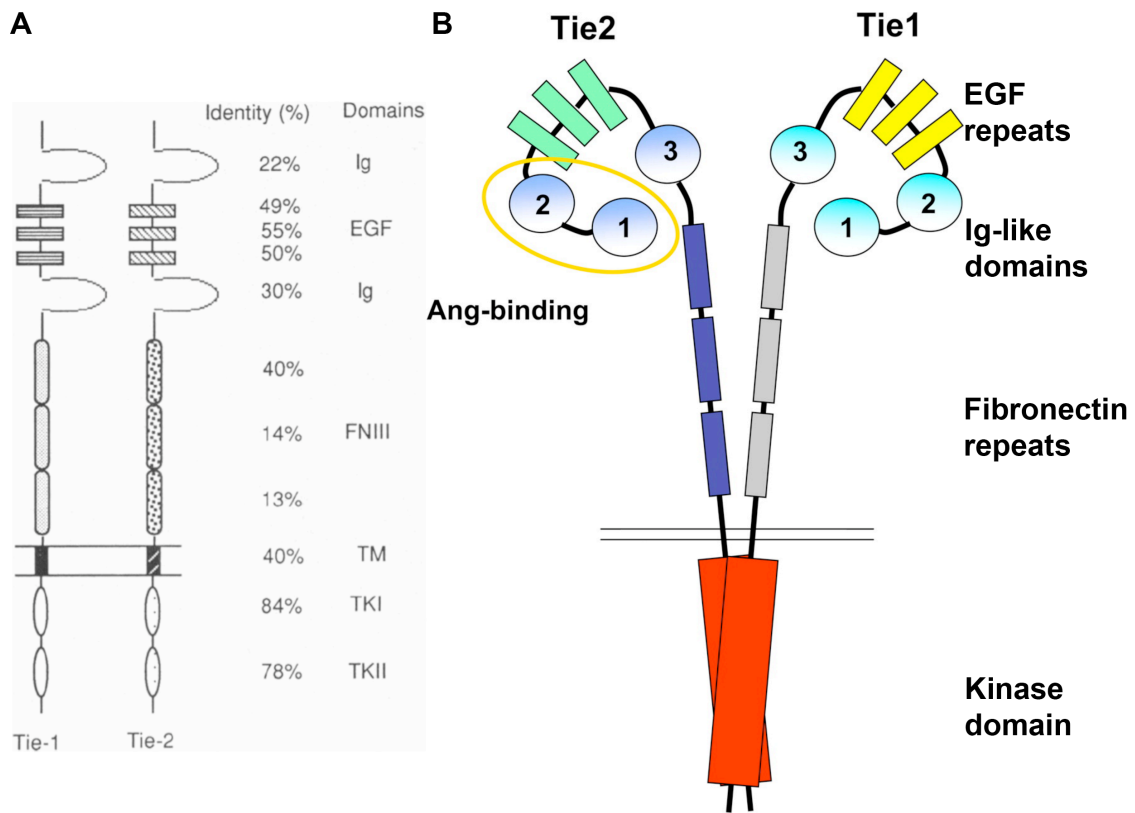
Figure 1.1.5 *Model of Tie1 and Tie2 structures.* (**A**) Early prediction of Tie1 and Tie2 structure with domain homology comparisons. (**B**) Updated model of Tie1 structure. Tie receptors comprise of intracellular kinase domain, the extracellular fragment consists of 3 fibronectin type III repeats, and 3 Ig-like domains separated by 3 EGF-like repeats. These receptors dimerize and contact at the intracellular kinase domains. No interaction in the extracellular domains has been reported. Adapted from Sato (1993) and MacDonald (2006).

Immunoglobulin-like, Ig; epidermal growth factor, EGF; Fibronectin Type III, FNIII; transmembrane, TM; Tyrosine Kinase domain, TKI and TKII.

similarity of Tie1 to Tie2 (Macdonald *et al.*, 2006). In fact, Macdonald et al postulated that the kinase domain and not the Fc fragment is responsible for the heterodimerization of Tie1 with Tie2.

Although the intracellular domains of Tie1 and Tie2 share an overall amino acid identity of approximately 80%, the extracellular domains are more divergent, with an amino acid identity of less than 40% (Dumont *et al.*, 1992; Partanen *et al.*, 1992; Sato *et al.*, 1993). In the extracellular region, the highest identity conservation between the two receptors is found at the EGF repeats, an area that is critical for ligand binding (Macdonald *et al.*, 2006), providing a potential explanation for the selectivity of angiopoietin binding to Tie2 but not Tie1.

Mechanism of Tie1 Signaling

Despite a high degree of homology with Tie2 and extensive efforts, a ligand for Tie1 still has not been found. Hence, the function of the Tie1 orphan receptor remains enigmatic.

Tie1 is Capable of Signaling in the absence of Tie2

Although Tie1 can be activated upon stimulation with Ang1 and Ang2, no direct receptor-ligand interaction has been found (Davis *et al.*, 1996; Maisonpierre *et al.*, 1997; Valenzuela *et al.*, 1999). Since a specific ligand for Tie1 has not been found, two studies utilized a chimeric receptor approach to investigate the signaling mechanism and function of this orphan receptor. Marron et al fused the ectodomain of the TrkA to the transmembrane and intracellular domains of Tie1. However, the addition of the TrkA

ligand, neurotrophin growth factor (NGF), did not elicit phosphorylation of the Tie1 endodomain (Marron *et al.*, 2000). Although chimeric Tie1 receptor experiments did not demonstrate Tie1 autophosphorylation (Marron *et al.*, 2000), evidence of its association with SH2 domain containing tyrosine phosphatase 2 (Shp2) (Marron *et al.*, 2000) suggests that the phosphorylated Tie1 state may be transient.

In a similar study, Kontos and colleagues used a cell line stably expressing csf-Tie1 (colony stimulating factor receptor-Tie1) chimeric receptor, which allowed ligand activation with colony-stimulating factor 1 (Kontos *et al.*, 2002). They showed that the kinase domain is active and causes Tie1 auto-phosphorylation. Notably, they also found that the Tie1 endodomain associates with the p85 subunit of phosphatidylinositol 3-kinase (PI3K) *in vitro*. Moreover, Kontos et al demonstrated that in the absence of Tie2, Tie1 auto-phosphorylation *in vitro* increased production of 3-phosphoinositides and subsequent phosphorylation of Akt by PI3K. This study provided evidence that Tie1 is capable of signaling and that Tie1 may share a common signaling pathway with Tie2.

Proteolytic Cleavage of Tie1

Several cell surface receptors such as the epidermal growth factor receptor (EGFR) and the platelet derived growth factor receptor (PDGFR) undergo cleavage to modulate their mechanism of action (Prenzel *et al.*, 1999; Nemoto *et al.*, 2005). While release of the ectodomain may be required to halt ligand stimulated signaling, the extracellular domain of some receptor tyrosine kinases suppress the signaling capabilities of their intracellular domains (Vecchi *et al.*, 1996). Furthermore, endodomain expression of similar receptor tyrosine kinases results in ligand independent activation of their

signaling activity (Chantry, 1995). Tie1 is also subject to extracellular proteolytic cleavage in the presence of either protein kinase C (PKC) (Yabkowitz *et al.*, 1997), vascular endothelial growth factor (VEGF) (Tsiamis *et al.*, 2002), phorbol 12-myristate 13 acetate (PMA) (Marron *et al.*, 2000) or tumor necrosis factor-α (TNF-α) (Yabkowitz *et al.*, 1999) generating a membrane bound receptor fragment comprising the intracellular and transmembrane domains. Marron et al showed that the truncated Tie1 product persists in the cytosol for several hours (Marron *et al.*, 2000), after which a γ-secretase mediated cleavage of the endodomain from the membrane initiates the lysosomal degradation of the internalized fragment (Marron *et al.*, 2007). The cleaved Tie1 product was also found associated with tyrosine phosphorylated proteins, such as the tyrosine phosphatase and adaptor protein Shp2 (Marron *et al.*, 2000). Hence, the prolonged existence of the cleaved Tie1 intracellular fragment in the cytosol and its association with secondary messengers suggest that this Tie1 endodomain may have further intracellular signaling functions (Marron *et al.*, 2000).

Truncation of Tie1 by VEGF may require tyrosine phosphorylation of the receptor (Tsiamis *et al.*, 2002). Singh et al showed that this truncated Tie1 was tyrosine phosphorylated (Singh *et al.*, 2009). Tsiamis et al showed that, addition of the tyrosine kinase inhibitor genistein, blocked VEGF induced Tie1 cleavage but use of the tyrosine phosphatase inhibitor pervanadate, had no effect. A metalloprotease inhibitor also blocked VEGF induced Tie1 cleavage. Hence, VEGF induced cleavage of Tie1 involves a tyrosine kinase regulated and metalloprotease mediated process.

PMA induced cleavage of Tie1 also causes tyrosine phosphorylation of the truncated endodomain (Marron *et al.*, 2007). While truncation and tyrosine phosphorylation of Tie1

by VEGF was shown to be Tie2 independent (Singh *et al.*, 2009), loss of Tie2 inhibited Tie1 tyrosine phosphorylation. Furthermore, addition of PMA enhanced Ang1 induced Tie1 phosphorylation, suggesting that Tie2 is involved in tyrosine phosphorylation of cleaved Tie1.

Tie1 Associates with Tie2

Tie1 is commonly found associated with Tie2 (Marron *et al.*, 2000; Saharinen *et al.*, 2005; Yuan *et al.*, 2007). Saharinen et al reported that stimulation with Ang1 induces Tie2 translocation to endothelial cell-cell contacts, and when Tie1 is co-expressed, it also translocates to the cell borders. Although Tie1 is found in heterocomplexes with Tie2 in human umbilical vein endothelial cells (HUVECs) (Marron *et al.*, 2000; Kim *et al.*, 2006), in endothelial progenitor cells (EPCs), the receptors were not bound to each other (Kim *et al.*, 2006). Hence, *in vitro* Tie1 is bound to Tie2, and the regulation of Tie2 signaling by Tie1 may be context dependent.

Association of Tie1 with Tie2 may modulate Tie2 signaling. Kim et al demonstrated that association of cell surface Tie1 with Tie2 inhibited angiopoietin (Ang)-2 mediated Tie2 activation (Kim *et al.*, 2006). Another study also reported that the proteolytic processing of Tie1 reduces the accumulation of phosphorylated Tie1 at the cell surface and enhances the responsiveness of Tie2 to Ang1 induced activation (Marron *et al.*, 2007). Interestingly, both Tie1 holoreceptor and endodomain bind with Tie2 (Marron *et al.*, 2000; Tsiamis *et al.*, 2002). These studies together suggest that either the Tie1 holoreceptor or endodomain may play a role in regulating ligand activated Tie2 signaling through the formation of heterodimeric complexes.

Angiopoietin activation of Tie1 requires Tie2

In support of ligand activated Tie1 signaling, Saharinen et al demonstrated that Tie1 can be activated by an Ang1 chimeric protein (COMP-Ang1), as well as native Ang1 and Ang4, but not by Ang2 or Ang3 (Saharinen *et al.*, 2005). In this study, COMP-Ang1 was able to induce tyrosine phosphorylation of Tie1 within 5 minutes and achieving maximum activation at 1 hour. These experiments were performed in immortalized HUVECs overexpressing Tie1 and the level of Tie1 phosphorylation was very low as compared to Ang induced Tie2 phosphorylation. Notably, co-expression with Tie2 was required for robust Tie1 activation (Saharinen *et al.*, 2005; Yuan *et al.*, 2007). In the absence of Tie2, however, COMP-Ang1 induced Tie1 phosphorylation was significantly reduced (Marron *et al.*, 2007). Additionally, Yuan et al showed that a kinase-dead Tie1 mutant was phosphorylated by Ang1 only in the presence of Tie2 and Ang1 failed to activate Tie1 when co-expressed with a kinase-defective Tie2, suggesting a requisite role for Tie2 in Ang1 induced Tie1 activation (Yuan *et al.*, 2007).

Tie1 Function In vitro

Tie1 Inhibits Apoptosis in the Absence of Tie2

On the other hand, using a chimeric receptor approach *in vitro*, Kontos et al demonstrated that in the absence of Tie2, activation of Tie1 induced PI3K mediated Akt phosphorylation, reducing UV-irradiation activated caspase-3, and consequently inhibited apoptosis (Kontos *et al.*, 2002). The inconsistency of these results to the work of Yuan et

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al (Yuan *et al.*, 2007) may be due to the different cell systems used, while Kontos et al overexpressed the Tie1 chimeric receptor in NIH3T3 cells, Yuan et al assessed endogenous receptor expression in HUVECs. Taken together these studies suggest that in the absence of Tie2, phosphorylation of Tie1 activates a similar PI3K mediated pathway, as does Tie2. This Tie1 signal transduction pathway leads to Akt phosphorylation and reduction of cleaved caspase-3 levels, and results in an anti-apoptotic effect not unlike Ang1/Tie2 signaling.

Tie1 modulates Tie2 Activity

Studies show that cleavage of Tie1 modulates Ang induced Tie2 signaling. VEGF induced phosphorylation of Tie2, and VEGF enhancement of Ang1 induced Tie2 activation, both required association of phosphorylated Tie1 endodomain with Tie2 (Singh *et al.*, 2009). Marron et al also showed that cleavage of Tie1 augmented Ang1 induced Tie2 phosphorylation (Marron *et al.*, 2007). Thus regulated processing of Tie1 regulates Tie2 activity.

A potential effect of Tie2 modulation by Tie1 is the inhibition of Ang1 induced pro-survival signaling. Yuan et al found that loss of Tie1 *in vivo* led to increases in capillary density due to increased endothelial cell numbers (Yuan *et al.*, 2007). They affirmed this finding *in vitro* by downregulating Tie1 in HUVECs, and showing potentiated levels of phospho-Akt and mitogen activated protein kinases (MAPK). Thus, either downregulation of Tie1 (Marron *et al.*, 2007; Yuan *et al.*, 2007) or shedding of the Tie1 ectodomain (Marron *et al.*, 2007) augments Ang1 induced Tie2 phosphorylation. This

study suggests that Tie1 may prevent endothelial cell survival by inhibiting Ang1 induced Tie2 signaling.

Tie1 Promotes Endothelial Cell Activation

Ang1 induced Tie2 activation promotes endothelial integrity and inhibits apoptosis (Kwak *et al.*, 1999; Papapetropoulos *et al.*, 2000; Harfouche *et al.*, 2002). Hence, Tie1 may activate endothelial cells by inhibiting the ability of Tie2 in maintaining endothelial quiescence. Recent studies have indicated an expression of Tie1 in inflammatory tissue of rheumatoid arthritis and osteoarthritis patients (Shahrara *et al.*, 2002). Overexpression of Tie1 in HUVECs augments inflammatory markers vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and endothelial (E)-selectin potentially via a p85 mediated pathway (Chan *et al.*, 2008). Conversely, analyses of Tie1 siRNA transfected HUVECs revealed decrease in inflammatory markers such as ICAM-1, VCAM-1, E-selectin, toll-like receptor 2 (TLR2), interleukin-1β (IL-1β) but had no effect on Tie-2, eNOS or transforming growth factor-β (TGF-β) expression (Chan & Sukhatme, 2009). Therefore, Tie1 may play a role in endothelial cell activation and promote inflammation.

Summary: Divergent Roles for Tie1

In summary, the Tie family of tyrosine kinase receptors is critical to maintenance of vessel integrity. Although both Tie1 and Tie2 share great homology in the intracellular regions, their extracellular domains are vastly different, hence the Tie2 specific ligand, angiopoietin has not been found bound to Tie1. Despite the absence of a Tie1 specific

ligand, efforts to elucidate the Tie1 signaling mechanism has yielded different results, largely due to the co-expression of Tie2 (Figure 1.1.6). Phosphorylation and activation of Tie1 by native or synthetic angiopoietins can be achieved, but only with co-expression of Tie2. It appears that in the presence of Tie2, Tie1 functions to attenuate the effect of its downstream signaling, that is, reducing pro-survival and anti-apoptotic signaling. However, in the absence of Tie2, studies suggest that Tie1 assumes the role of Tie2 with a resultant effect of inhibiting apoptosis, an effect opposite to that seen when Tie2 is co-expressed. Hence, in further studies with overexpression of Tie1 and in the presence of Tie2, Tie1 appears to have a pro-inflammatory effect, potentially via excessive inhibition of Tie2. Conversely, downregulation of Tie1 by siRNA showed a distinct reduction of pro-inflammatory molecules potentially due to unfettered Tie2 signaling. While Tie1 is predominantly expressed in endothelial cells, its expression in several breast and colon epithelial cancer cell lines support the idea that Tie1 may play a role in cell activation.

The Role of Hemodynamic Shear Stress on the Endothelium

Biomechanical Forces Exerted by Blood Flow on the Vessel Wall

The Endothelium

The endothelial monolayer exists as a dynamic interface between the circulating hematologic components and the vascular wall. Endothelial cells experience a wall shear stress associated with the viscous flow of blood, and studies have shown vascular
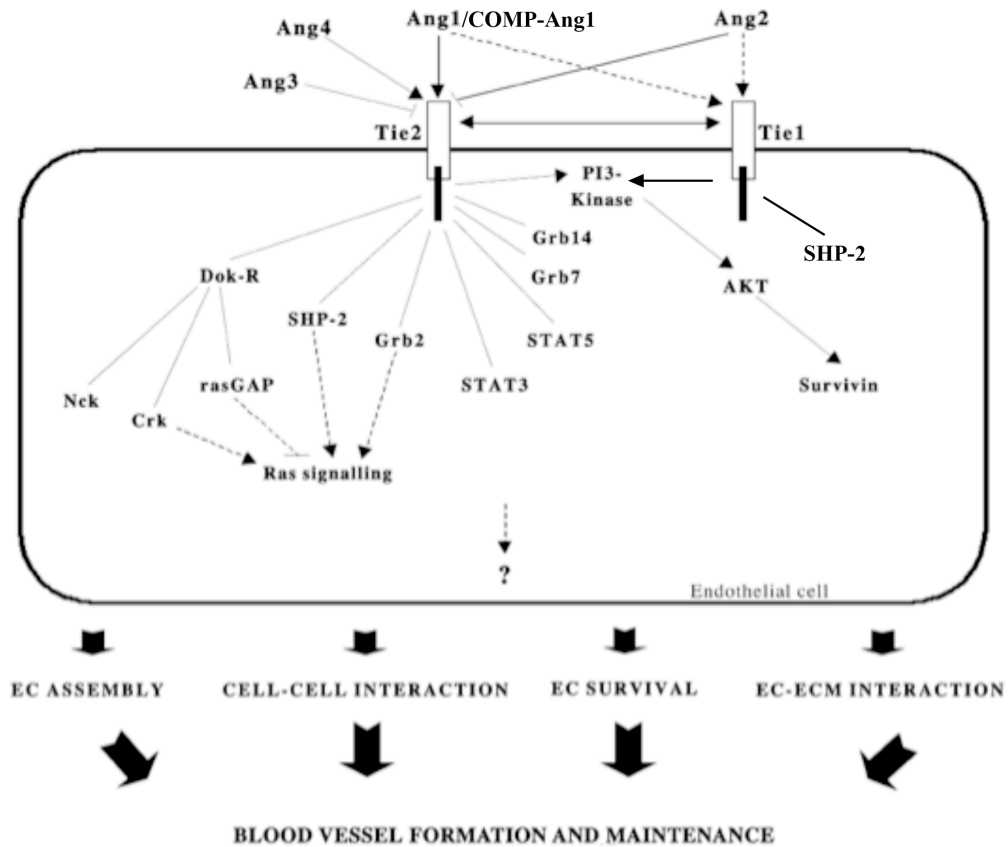
Figure 1.1.6 *Signaling pathways of Tie1 and Tie2.* Summary of the effect of angiopoietins on Tie1 and Tie2 signaling. Numerous functions have been implicated downstream of Ang-Tie signaling. Modified from Loughna (2001).

Angiopoietin, Ang; endothelial cells, EC; extracellular matrix, ECM.

endothelial biology to be influenced by shear stress (Dewey *et al.*, 1981; Levesque & Nerem, 1985; Ives *et al.*, 1986; Levesque *et al.*, 1986). Disruption to the integrity and permeability of this monolayer allows the localized influx of low-density lipoproteins and other inflammatory molecules into the sub-endothelium (White & Frangos, 2007). Vascular endothelial cells reside in a hemodynamically imposed mechanical environment, so it is important to recognize the details of the dynamics of blood flow in the vascular system.

Fluid Mechanics

Due to the nature of its viscosity and the pressures required for its delivery to the extremities, as blood flows through a vessel it exerts a physical force on the vessel wall. This force can be resolved into primary vectors in three dimensions (Figure 1.2.1). First, a force in the longitudinal direction parallel to the vessel wall and to the direction of blood flow is defined as shear stress. This represents the frictional force that blood flow exerts on the endothelial surface. Second, a circumferential force tangential to the circumference of the vessel is termed cyclic stretch. This represents the effect of hemostatic pressure on distension of the vessel wall, with effects on endothelial and smooth muscle cells and extracellular matrix components. Third, hydrostatic pressure also exerts a radial force perpendicular to the vessel wall, compressing the endothelium and vessel wall components. This dissertation focuses on the effect of wall shear stress on the endothelium.

It is important to distinguish fluid shear stress from fluid shear rate, two physical concepts that are characteristics of the local flow dynamics. Fluid shear rate relates to the
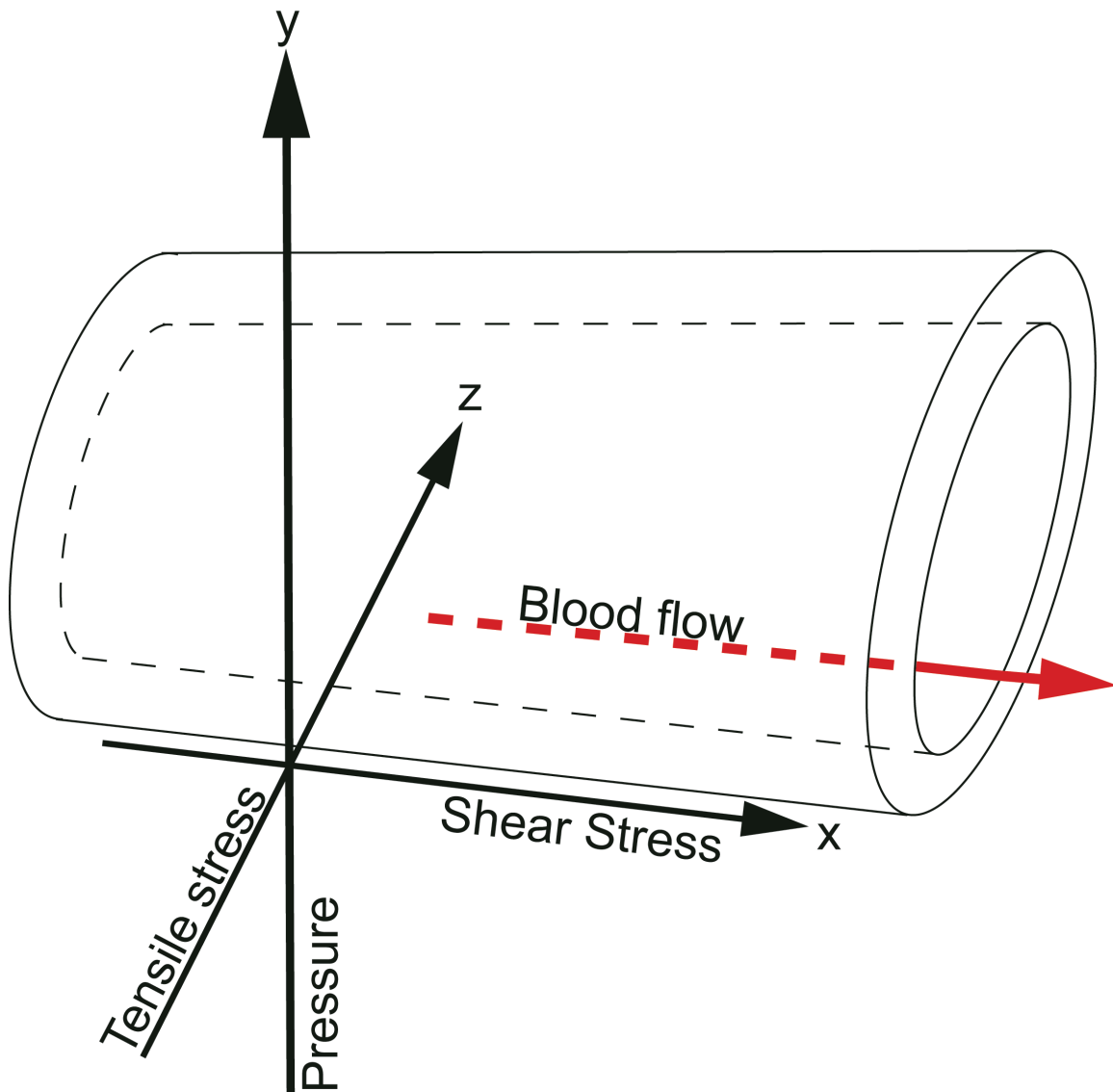
Figure 1.2.1 *3-dimension vectorial representation of hemodynamic forces on the wall of a blood vessel.*

velocity of fluid particles with respect to its perpendicular distance from the endothelial surface. Fluid shear stress is the force in the direction of flow exerted by the fluid components as they pass adjacent to the endothelial cell. Fluid shear stress can be defined as a function of fluid shear rate.

In regions where blood flow is unidirectional without recirculating flow patterns, the time averaged mean shear stress value is positive (i.e. blood flows forward). Shear stress values in humans vary from 1-6 dynes/cm^2 in the venous system and 10-70 dynes/cm^2 in the arterial trees (Figure 1.2.2) (Malek *et al.*, 1999).

$$1 \text{ Dyne} = 1 \times 10^{-5} \text{ Newtons}$$

Following Newton's 2nd Law of Motion,

$$\text{Force} = \text{Mass} \times \text{Acceleration}$$

To qualitatively describe the value of force, 1 Newton is the force required to move 1 kilogram of mass at an acceleration of 1 meter/second^2 (m/s^2). Hence, 1 Dyne is the force required to move 1×10^{-5} kilograms of fluid mass (blood) at an acceleration of 1 m/s^2.

Calculation of shear stress (τ_ω) *in vivo* can be performed utilizing Poiseuille's Law (Westerhof *et al.*, 2005):

$$\tau_\omega = (4 \cdot \mu \cdot Q) / (r^3 \cdot \pi)$$
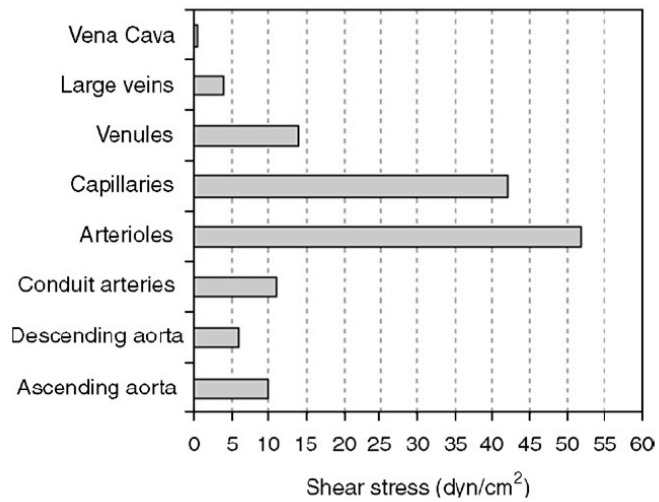
Figure 1.2.2 *Illustration of typical shear stress magnitudes in human vasculature.* Adapted from Papaioannou (2006) and Cheng (2007).

where μ = blood viscosity, Q = blood flow rate, r = internal vessel radius and π = the mathematics constant (3.14159265). The following assumptions have to be made in order to apply the law for the estimation of shear stress:
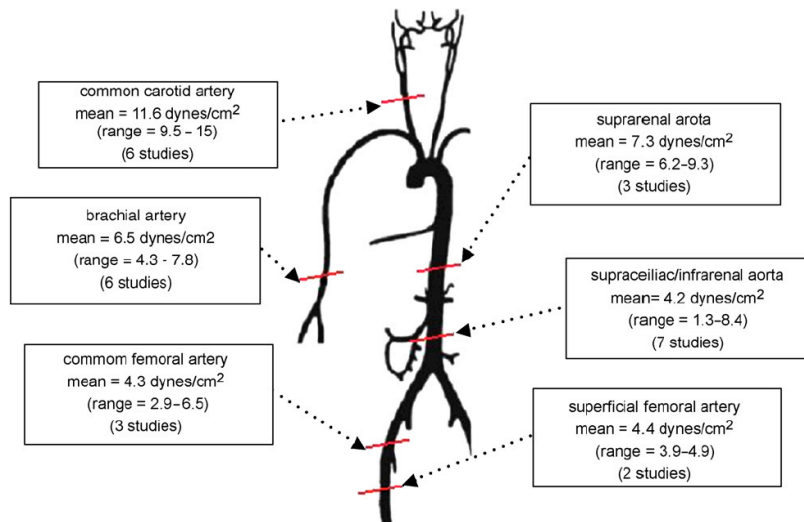
(i) blood is considered a Newtonian fluid*,

(ii) the cross-sectional area of the vessel is cylindrical,

(iii) the vessel is straight with inelastic walls,

(iv) blood flow is steady and laminar, and

(v) there are no slip boundary conditions (i.e. velocity at the wall is zero).

(*Definition of Newtonian fluid – a fluid in which the shear stress is directly proportional in a linear fashion to the velocity gradient in the direction normal to the plane of shear, and this relation is guarded by a constant, the fluid viscosity. The viscosity of the fluid is only dependent on temperature, pressure and chemical composition. *In vivo*, blood viscosity is directly related to hematocrit.)

Qualitatively, Poiseuille's Law describes the change in pressure (force) with radial distance (Figure 1.2.3). The equation assumes that the velocity vector profile of blood flow is parabolic such that fluid next to the wall essentially has zero forward force. This relation highlights the fact that relatively small decreases in vessel diameter at a constant flow rate can markedly increase shear stress at the endothelial surface. Clearly, blood vessels do not remain perfectly cylindrical and blood flow velocity is not always parabolic, nevertheless, Poiseuille's Law can be used to relate pressure drop to shear stress.

P

SS

r
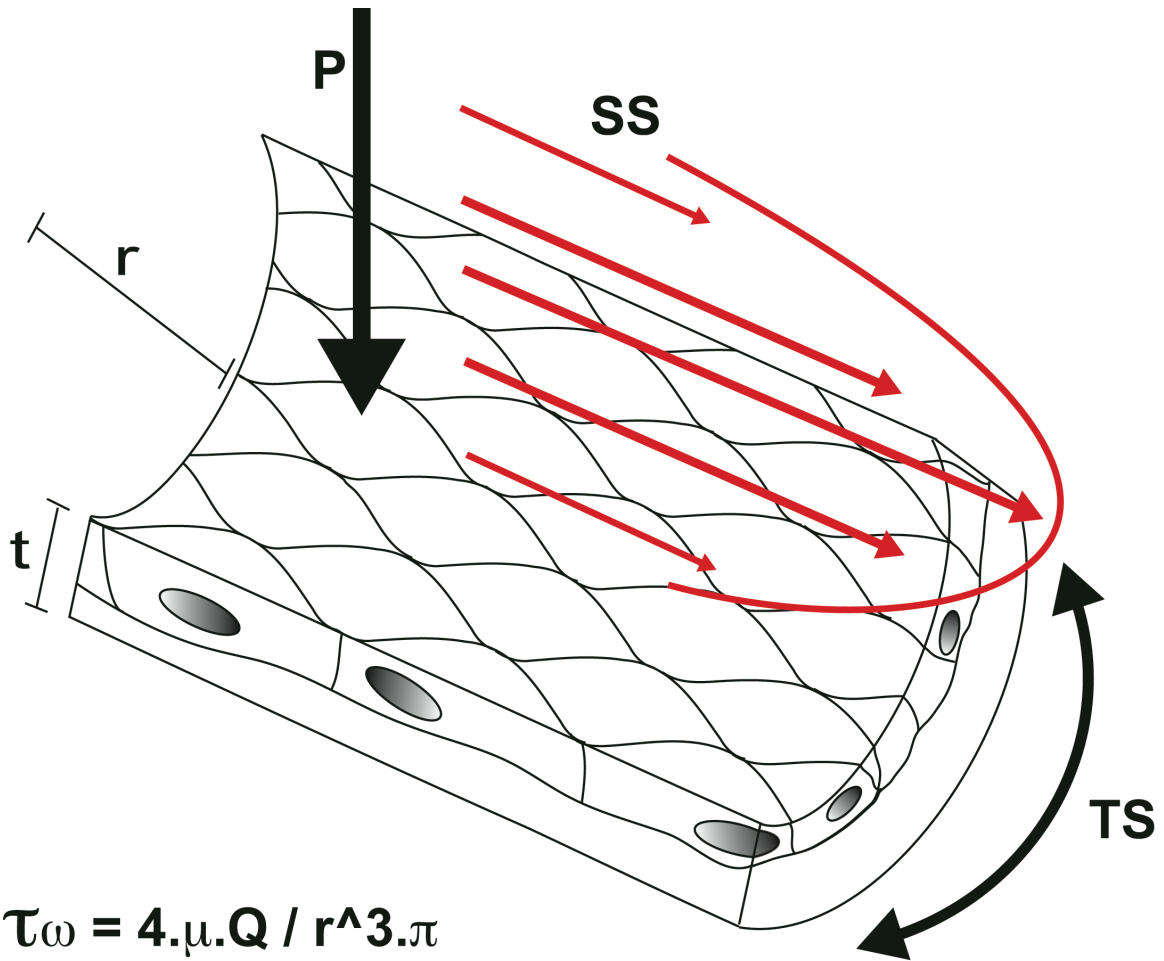
t

$$\tau\omega = 4.\mu.Q / r^3.\pi$$

TS

Figure 1.2.3 *Hemodynamic forces in the vasculature.* Major hemodynamic forces include flow-derived shear stress (SS), radial pressure (P) and tension-derived tensile stress (TS, stretch).

Types of Physiological Flow Profiles

Movement of fluid has two basic components, a directional scalar and a magnitude vector. Flow patterns *in vivo* have been broadly separated into ***laminar*** and ***non-laminar*** flow. The unidirectional movement of fluid and its component particles comprises laminar flow. It is also important to clarify that non-laminar flow (also commonly referred to as disturbed flow (Asakura & Karino, 1990; Chiu *et al.*, 1998; Davies *et al.*, 1999; Gimbrone *et al.*, 2000; Chien, 2003; Li *et al.*, 2005; Chien, 2008)) is a term for a group of flow profiles that include ***recirculatory*** and ***turbulent*** flow (Motomiya & Karino, 1984; Fukushima *et al.*, 1988; Peacock, 1990; Davies, 1995; Davies *et al.*, 1999).

Recirculatory flow is characteristic of vascular branch points (Davies *et al.*, 1999) such as the vertebral, celiac, mesenteric and renal artery branches of the aorta, and the most studied of which are branches of the aortic arch (Suo *et al.*, 2007) and the carotid artery bifurcations (Motomiya & Karino, 1984; Fukushima *et al.*, 1988). Due to the pulsatile nature of blood flow, the outer wall of the branching vessel experiences bidirectional blood flow with the resultant effect of a low time-averaged shear stress (Figure 1.2.4) (Asakura & Karino, 1990).

The complex profile of turbulent flow is not to be confused with recirculatory flow. Turbulence implies characteristics of chaotic flow, encompassing amongst others, two distinguishing qualities: (1) an unsteady flow pattern with spinning vortices, superimposed with (2) random movement of fluid components (Peacock, 1990; White & Frangos, 2007). This highly unique flow profile is found almost exclusively in the aortic sinuses. Although, in rare circumstances, it is possible to achieve turbulent flow under laminar flow conditions if the Reynold's number (ratio of fluid velocity to viscosity)
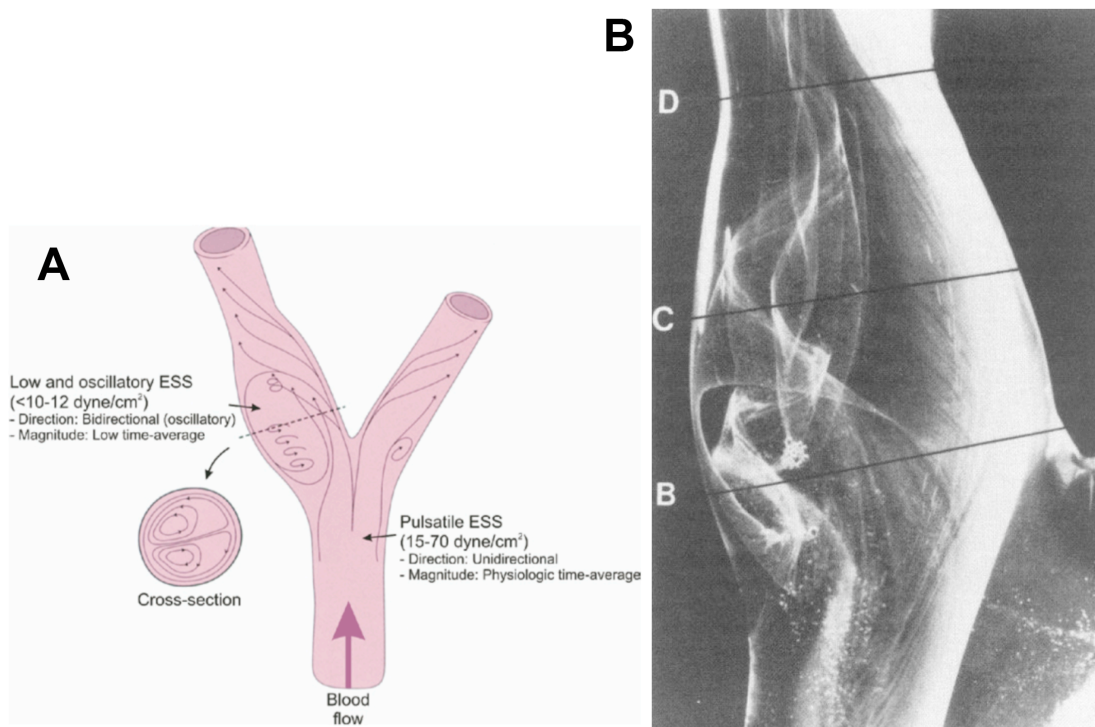
Figure 1.2.4 *Flow divergence at vessel bifurcations.* (**A**) Illustration of flow profiles at a vessel bifurcation. Low shear stress magnitude and oscillatory flow patterns are common at the outer wall. (**B**) Glass model of human carotid sinus visualized by gas bubbles. In regions B and C, there is separation of laminar flow creating a region of flow recirculation that reattaches to the main flow downstream in region D. Also note absence of flow separation on wall of the flow divider. Adapted from Davies (1995) and Ku (1985).

attains an uncharacteristically high value. However, this is extremely rare and will not be discussed further (Peacock, 1990). To date there is not an *in vitro* flow model that reliably mimics the chaotic flow patterns.

Even though flow is laminar, it is still extremely complex in character. Velocity profiles are asymmetric due to the complicated geometry of the vasculature with the result that wall shear stress is not only temporally variable but also spatially variable. There is still a rather large variation in wall shear stress of arterial segments experiencing laminar flow. Several *in vitro* models have been devised and successfully employed to study the flow characteristics and endothelial responses to laminar flow.

In vitro Systems of Fluid Shear Stress

Although it is important to understand the complexity of the *in vivo* hemodynamic environment, the systems that have been developed for use in cell culture studies of hemodynamic effects on endothelial biology have been much simpler. The modeling of *in vivo* fluid flow dynamics has been a pursuit of the medical and engineering disciplines for over two decades. In general, the systems devised up to now have focused on each mechanical force component separately. Thus, there are methods for the investigation of flow effects and methods for the study of cyclic stretch. The systems employed for the investigation of flow effects are addressed in this dissertation.

To understand shear stress, we need to address fluid dynamics. We will assume ideal conditions of (a) no-slip conditions at the boundary, (b) fluid that is incompressible (Newtonian fluid). Fluid can be characterized by its Reynold's number, *Re*:

$Re = (2 \cdot r \cdot u) / \mu$

where r is the radius of the vessel, u is the fluid velocity and μ is the fluid viscosity (Peacock, 1990).

Qualitatively, the Reynold's number is a dimensionless parameter describing the ratio of fluid velocity to its viscosity. When $Re \ll 1$ fluid flow is capable of reaching steady state (Truskey *et al.*, 2009), however when $Re > 1$ fluid flow conditions tend toward turbulence.

We will first address steady flow conditions as this is most commonly encountered in vascular hemodynamics.

1. <u>Cone and Plate Shear Stress apparatus</u> (Figure 1.2.5) (Bussolari *et al.*, 1982)

    The pioneer model was designed by Dewey, Bussolari and Gimbrone in 1982 (Bussolari *et al.*, 1982). Their cone and plate viscometer system is still widely used today. The apparatus utilizes a modified cone and plate viscometer. Rotation of the inverted cone drives movement of the fluid in an azimuthal direction over a monolayer of endothelial cells cultured on a stationary plate. Given a fluid with Reynold's number, $Re \ll 1$, the shear stress can be calculated by the following formula:
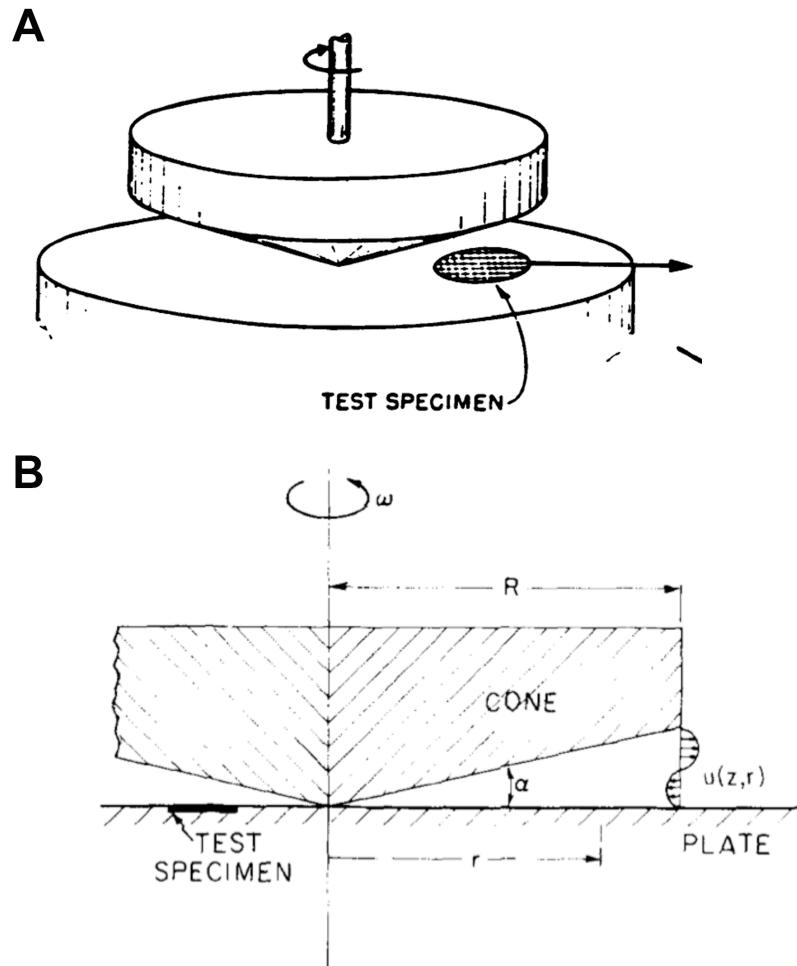
Figure 1.2.5 *Geometry of cone and plate shear stress apparatus.* (**A**) Original design of apparatus showing sample of apparatus. (**B**) Geometry of apparatus and variables pertinent to the calculation of shear stress. Adapted from Bussolari (1982) and Davies (1984).

The azimuthal velocity, υ is:

$$\upsilon = (\omega \cdot z) / \alpha$$

where ω is the angular velocity of the rotation, z is the azimuthal height from the plate surface and α is the cone angle in radians.

Calculating the rate of change of azimuthal velocity with respect to the distance of flow particles from endothelial surface would give us the fluid shear rate:

$$\delta\upsilon/\delta z = \delta(\omega \cdot z/\alpha) / \delta z = \omega/\alpha$$

The fluid shear stress τ_ω can then be calculated,

$$\tau_\omega = \mu \cdot (\delta\upsilon/\delta z)$$

or, substituting the formula of fluid shear rate,

$$\tau_\omega = \mu\omega/\alpha$$

where, μ is the viscosity of the fluid (~0.0101 poise).

The cone and plate apparatus, as utilized in our experiments, has the advantage of a well-defined flow field without the interference of pressure gradients and it also permits a wider dynamic range of shear stresses. Additionally, turbulent flow conditions can also be achieved with fluid of $Re>1$ in this system, or by increasing the cone angle to 1° radians (Bussolari *et al.*, 1982).

2. <u>Parallel Plate Shear Stress model</u> (Figure 1.2.6)

The parallel plate chambers were first reported from a laboratory at Georgia Institute of Technology (Levesque & Nerem, 1985). The key feature of the parallel plate system utilizes the generation of a continuous flow loop to deliver a predetermined and constant flow rate across a cultured endothelial monolayer in a geometrically uniform flow chamber. The cells in the chamber are exposed to a specific shear stress condition as defined by the dimensions of the flow chamber and the pressure drop across the chamber. Studies have modified this chamber with a step system creating a secondary recirculating flow system downstream of the step (Figure 1.2.6B). These modified step flow systems offer qualitative analyses of molecular responses to recirculating flow. Pulsatile flow and its variations have been conducted using the parallel plate flow chamber (Helmlinger *et al.*, 1991; Helmlinger *et al.*, 1995). Additionally, parallel plate flow chambers have been adapted to fit under microscopes for continuous observation of cellular responses during experiments.
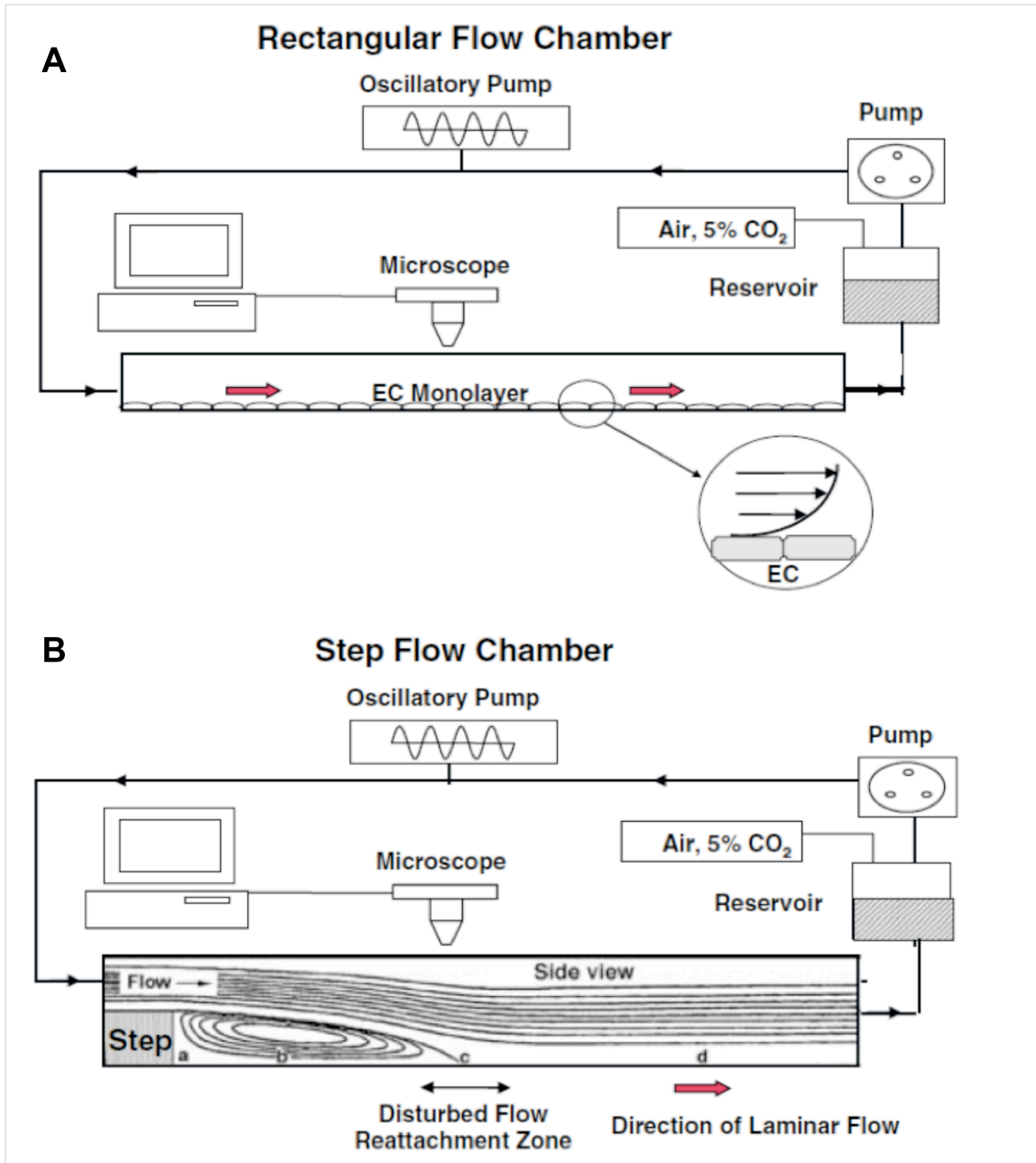
Figure 1.2.6 *Parallel plate flow chamber.* (**A**) Endothelial cells seeded on a tissue culture treated chamber are subjected to media flow (red arrow). Media exiting the chamber returns to a buffer reservoir that is aerated with air + 5% CO_2 mix. A peristaltic pump delivers media to the chamber at a controlled flow rate, and the flow can be modified with an oscillatory pump to simulate bidirectional flow. The parallel plate chamber has the advantage of size and can be built using optically permissive material for real-time recording of cellular responses with a microscope. (**B**) The parallel plate flow chamber may be modified with a "step", creating a zone of disturbed flow immediately distal to the obstacle (a-b), a reattachment zone (c), and resumption of laminar flow (d). Adapted from Chien (2008).

3. Orbital shear stress model

First reported by Tsao et al in 1995 (Tsao *et al.*, 1995), orbital shear stress is applied to confluent cell cultures by using an orbital shaker positioned inside the incubator (Asada *et al.*, 2005; Dardik *et al.*, 2005; Kudo *et al.*, 2005). Endothelial cells are seeded onto matrix-coated 6-well culture plates. Different fluid velocities and shear stresses are found at the center and periphery of the well (Dardik *et al.*, 2005). These center and periphery regions were observed to have different endothelial cell morphology. Studies with selective seeding of endothelial cells at different regions of the well revealed differences in replication rate and nitric oxide (NO) production (Dardik *et al.*, 2005).

With all systems, temporally and spatially uniform fluid shear stress profiles can be achieved (reviewed in (White & Frangos, 2007)). Temporal shear stress gradients are defined as changes in shear stress over a small period of time at a defined location. The gradient is a function of time and hence directly proportional to the rate of change of flow. The value of temporal shear stress gradients is that they can be designed to mimic pulsatile flow conditions *in vivo*. Spatial shear stress gradients are defined as changes in shear stress between two predetermined points on the endothelial monolayer. As a point of note, spatial shear stress gradients can also be defined as two points on a cell when used in atomic force microscopy devices.

On the other hand, *in vitro* temporal shear stress gradients may be undesirable especially in experiments evaluating rapid response molecules such as PDGF, where

changes are observed within 5 minutes (Aromatario *et al.*, 1997; Hu *et al.*, 1998; Sumpio *et al.*, 1998; Bao *et al.*, 1999). However, temporal shear stress gradients at the onset of fluid flow can be resolved by allowing at least 30 seconds for flow conditions to reach a steady state (White & Frangos, 2007).

Types of in vitro Shear Stress Patterns

A variety of shear stress patterns have been used to replicate *in vivo* flow, including laminar flow, pulsatile laminar flow, step flow, oscillatory flow. Steady state flow conditions, simply defined as the application of one flow rate for the duration of the experiment, is a common methodology of *in vitro* shear stress experiments. The steady state laminar flow system offers the attractiveness of a one variable simplicity in experiments. However, pulsatile laminar flow addresses the cyclic nature of *in vivo* blood flow. The step flow system offers the additional quality of conditioning the cells for a period of time before subjecting them to a different shear stress magnitude to assess endothelial responses. Chen-Konak et al. used such a system to investigate the effect of abrupt shear stress changes on the transcription of Tie1 (Chen-Konak *et al.*, 2003). Last, oscillatory shear stress systems have been used as a close representation of recirculatory flow at bifurcations and to a lesser degree, an approximation of turbulent flow as seen at the aortic sinuses. The accepted methodology of this system involves alternating fluid direction at 1 hertz (1 cycle per second) and at the rate of +/- 5 dynes/cm^2 (Sorescu *et al.*, 2003).