CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

Our study revealed at least 4 major new insights into the role of Tie1 in atherosclerosis. First, in the adult aorta Tie1 is exclusively expressed in regions of atherogenic shear stress, such as those with disturbed flow. Second, our data indicate a dynamic downregulation of Tie1 expression *in vivo* under conditions of laminar flow with high shear stress. Third, in conjunction with the unique expression profile of Tie1 at atherosclerosis-prone areas, we found that Tie1 reduction attenuates atherosclerosis progression in a dose dependent manner. Fourth, our *in vitro* data support a pro-inflammatory role for Tie1 at least in part via activation of the nf-κB signaling molecule, p50.

Biomechanical regulation of Tie1

Role of Tie1 in disturbed shear stress

The specific expression of Tie1 at locations of disturbed flow (Chapter II) together with the downregulation and cleavage of Tie1 after alterations in shear stress affirm that Tie1 expression and activity is modulated by hemodynamics. The discovery of a shear stress response element in the promoter region of Tie1 also, suggests that its expression may be under the regulation of an upstream shear stress mediator. However, Chen-Konak et al reported that onset of flow induces the cleavage of Tie1 releasing an endodomain

(Chen-Konak *et al.*, 2003) raising the possibility that this receptor may transduce the luminal physical force into a biochemical signal via its intracellular domain. Thus, it is important to determine whether activation of Tie1 by altered shear stress results in a direct effect on signal transduction (a true "mechanosensor") or whether proteolytic cleavage and subsequent interaction with intracellular proteins is required for the shear stress response (i.e. a shear stress response gene).

Shear stress causes structural changes to cell surface proteins resulting in downstream signaling. Chachisvilis et al had previously shown that shear stress changes the conformation of bradykinin receptor (a G protein-coupled receptor) in a similar manner that the bradykinin agonist would elicit (Chachisvilis *et al.*, 2006). Using Förster resonance energy transfer (FRET; also known as fluorescence resonance energy transfer) they showed that insertion of fluorescent proteins in the third cytoplasmic loop and the C terminus resulted in similar spectral changes either when bradykinin was added or when shear stress was applied. Further, Wang et al showed that shear stress induced phosphorylation of Flk-1 in the same way that addition of VEGF would (Wang *et al.*, 2007). However, unlike VEGF treatment, the application of shear stress did not result in the binding of the Nckβ adapter to the Shc domain, suggesting that these two stimuli cause different conformational changes. Hence, similar studies in Tie1 utilizing FRET technology or targeted mutations in Tie1 extracellular domains will provide valuable information in the specific role of Tie1 in the shear stress-mediated endothelial cell function independent of proteolytic cleavage.

Stretch

The biomechanical force imposed by blood flow *in vivo* comprises three components: shear, stretch and compression (Chapter I) and is possible that any, or all of these components, effect Tie1 mediated signal transduction. We are currently repeating our *in vitro* studies in a model that allows for manipulation of stretch (Zheng *et al.*, 2004). We will determine its effect on Tie1 expression in wild-type cells and, we will also assess transcriptional responses in Tie1 attenuated endothelial cells. However, a limitation of studies in the field and the *in vitro* method employed by our investigation is the one-dimensional treatment of endothelial cells. Currently, there is not any apparatus reported that engages more than one force component. To better mimic *in vivo* conditions, our laboratory has initiated efforts in collaboration with Dr. David Merryman's group to build a device that is capable of regulated shear and stretch simultaneously.

Van Epps et al (Van Epps *et al.*, 2009) had recently shown that arterial segments exposed to cyclic flexure displayed increased apoptosis and macromolecular permeability at the endothelium. In chapter II, we showed that the increased Tie1 expression in the aortic sinus was more pronounced on the aortic valve leaflet that experiences stretch and shear stress. Mirroring our results, Zheng et al (Zheng *et al.*, 2004) showed that cyclic stretch *in vitro* upregulated expression of Tie1 rat coronary endothelial cells. Additionally, we noted in Chapter II that the increased Tie1 expression on the aortic valve leaflets were specific only to the fibrosa surface (facing the aortic sinus) whereas the ventricularis surface (facing the ventricles) did not show any Tie1 expression. This raises the interesting possibility that, despite an induction of Tie1 response by stretch, the effects of laminar flow experienced on the ventricularis surface may take precedence and

downregulate Tie1 expression. In light of these results, it would be beneficial to the understanding of aortic valve biology to elucidate the response of Tie1 to the combined effects of stretch and shear stress.

The Dichotomous role of Tie1

The hemizygous Tie1 knockout mouse ($Tie1^{+/-}$) does not have an apparent endothelial phenotype. Still, as we showed when crossed to the ApoE deficient background, Tie1 heterozygous mice have attenuated atherosclerosis plaque burden (Chapter III), ostensibly through a muted inflammatory response (Chapter V). Patan et al proposed that Tie1 deletion augments an "activated endothelium" state (Patan, 1998). In their analysis of Tie1 deficient endothelial cells in e18.5 embryonic capillary beds, they found cellular projections and filopodia extending into the lumen suggesting an immature endothelial state compared to Tie1 wildtype embryos. Tie1 receptor in HUVECs form heteromeric complexes with Tie2 while in endothelial precursor cells the majority of Tie1 receptors were not associated with Tie2 (Kim *et al.*, 2006) suggesting differential roles for Tie1 in a context dependent manner. While loss of embryonic Tie1 leads to active endothelial cells, our data suggests that post-natal Tie1 deletion decreases inflammation, thus providing support for context dependence of Tie1 function. Our laboratory has undertaken the task of generating Tie2-floxed mice to delineate the contentious role of Tie1 in conjunction with its sister receptor Tie2.

Since Tie1 has been shown to modulate Tie2 signaling, shear stress regulation of Tie1 function may offer an alternate pathway for regulation of Tie2 activity. Activation of Tie2 by laminar shear stress has been demonstrated to prevent serum-starvation induced

apoptosis (Lee & Koh, 2003). Interestingly, both Tie1 holoreceptor and endodomain bind with Tie2 (Marron *et al.*, 2000; Tsiamis *et al.*, 2000), however, neither Tie1 forms are trans-phosphorylated in the presence of activated Tie2. Thus, one of the functions of Tie1 endodomain may be to modulate Tie2 signaling by recruiting proteins to the Tie2 complex.

In summary, in the presence of Tie2, Tie1 functions to inhibit its anti-apoptotic signaling and promote endothelial cell activation. However, when expressed without Tie2, Tie1 tends to promote pro-survival signaling. These results suggest that Tie1 may have a dichotomous role in endothelial cell biology dependent on the presence of Tie2.

Tie1 mediated atherosclerosis progression

Tie1 is Attenuated at Shear Specific Regions that Develop Stable Plaques

Low shear stress is a key factor in the transition of immature plaques to high risk, rupture-prone thin cap fibroatheromas that account for two-thirds of acute coronary syndromes (Koskinas *et al.*, 2009). We noted in Chapter II that Tie1 expression is maintained in similar regions of intrinsic lower shear vessels (carotid, vertebral, celiac, superior and inferior mesenteric arteries) as well as manipulated lower shear regions as evidenced with the shear stress modifying casts. Since Tie1 may play a role in local endothelial inflammation (Chapter V), a process that is critical for the evolution of early plaques into rupture-prone atheromatas, we opine that Tie1 signaling may mediate the development of these high risk plaques. As we report in Chapter III, Tie1 suppression

results in a significant reduction in atherosclerosis, the effect of Tie1 attenuation on the transformation of thin cap fibroatheromatas has yet to be determined.

Role of Tie1 in Atherosclerosis may be Tie2 Independent

Ahmed et al recently showed that the Tie2 antagonist angiopoietin-2 protects against atherosclerosis. Infection of $ApoE^{-/-}$ mice with adenoviral-Ang2 increased nitric oxide levels inhibiting LDL oxidation, which resulted in 38% lesion reduction in controls (Ahmed *et al.*, 2009). Also, by vaccinating mice against Tie2 Hauer et al showed a modest but significant 33% reduction in atherosclerotic lesions of $LDLR^{-/-}$ mice (Hauer *et al.*, 2009). Several studies have postulated that Tie1 modulates Tie2 receptor activity (Marron *et al.*, 2000; Saharinen *et al.*, 2005; Kim *et al.*, 2006) while other reports suggest that the cleavage of Tie1 receptor is required for regulation of Tie2 function and endothelial cell survival(Yabkowitz *et al.*, 1997; McCarthy *et al.*, 1999; Yabkowitz *et al.*, 1999; Marron *et al.*, 2000; Tsiamis *et al.*, 2002; Marron *et al.*, 2007). In these Tie2 targeted investigations where a reduction in atherosclerosis was observed the effect was significantly less than those seen in our studies. Taking into consideration that Tie1 may modulate Tie2 activity, and that our data suggests a Tie2 independent role for Tie1 signaling in atherosclerosis, further genetic studies with dual Tie1 and Tie2 manipulations need to be performed to reveal the co-existing role of these sister receptors in atherosclerosis. To this end, our laboratory has initiated efforts to generate a Tie2-floxed mouse that can be used to induce simultaneous dual-gene deletion.

Role of Tie1 in Endothelial Cell Survival

Endothelial cell survival plays an important role in atherogenesis, the loss of endothelial integrity sparks a cascade of inflammatory and clotting events that lead to the formation of a lesion. Using a chimeric receptor approach, Kontos and colleagues previously stimulated Tie1 phosphorylation, augmenting phospho-Akt and decreasing cleaved caspase-3 levels, suggesting a pro-survival role for Tie1 (Kontos *et al.*, 2002). On the other hand, Yuan et al showed that loss of Tie1 increased phospho-Akt, reduced cleaved caspase-3 levels and rescued HUVECs from serum starvation apoptosis (Yuan *et al.*, 2007). The differing results may be due to experimental approaches. While Kontos and colleagues serum starved NIH 3T3 cells overexpressing chimeric Tie1 receptor, deletion experiments in this study were performed in MAECs assessing intrinsic Tie1 expression. The effect of Tie1 deletion on endothelial cell survival under both serum starvation and shear stress conditions remain to be investigated.

Atherosclerosis at the Aortic Sinus vs. Aortic Branch points

We showed in Chapter III that the progression of atherosclerosis at the aortic sinus is different from that in the rest of the aorta. In fact, the degree of atherosclerosis in Tie1 deleted mice appear to be increased at the aortic sinus as compared to controls. Besides the differences in embryonic origins of the cells, a more exigent disparity lies in the shear stress profiles experienced at each location. Although several investigators have classified the flow pattern in the aortic sinus as "turbulent" (Peacock, 1990; Gimbrone *et al.*, 2000), a very limited number of studies have attempted to dissect its effect on endothelial cell signaling *in vitro* (Freyberg *et al.*, 2001; Dian *et al.*, 2003), whilst there are numerous reports on the effects of recirculatory or oscillatory flow (Surapisitchat *et*

al., 2001; Porat *et al.*, 2004; Sucosky *et al.*, 2009). Hence, it would be of tremendous value to further evaluate the effect of turbulent flow on endothelial responses and specifically Tie1 signaling.

Potential Mechanism of Tie1 Signaling

To date, only a limited number of studies have investigated the signaling mechanism of Tie1 and its effect on gene regulation. In Chapter II, our observation that Tie1 can be rapidly modulated *in vivo* by shear stress alterations also provides an excellent opportunity to directly examine the genetic response of this phenomenon. We are currently performing experiments to pursue the shear stress specific gene response by expression profiling arteries implanted with tapered and non-tapered casts in Tie1 attenuated mice as compared with wild-type controls. We will collect comprehensive data sets that will enable us to determine which regulatory networks are operative during atherosclerosis development.

In conclusion, Tie1 is expressed in specific regions experiencing proatherogenic shear stresses and reduction of Tie1 expression in these regions attenuates atherosclerosis progression in a dose dependent manner. This prophylactic alleviation of atherosclerosis burden by the reduction of Tie1 expression and signaling suggests that Tie1 might prove to be a strong and attractive candidate for a targeted therapeutic approach.