Results

Tie1 Suppression Reduces Distal Aorta Atherosclerosis Progression in ApoE Null mice

To determine the role of Tie1 on atherosclerosis, $Tie1^{+/-}$:$ApoE^{-/-}$ female mice (n=49), fed a regular chow diet, were evaluated for atherosclerotic plaque burden and compared to control $Tie1^{+/+}$:$ApoE^{-/-}$ female littermates (n=41). Mice were assessed at early (12 week-old), intermediate (18 week-old) and advanced (24- and 49 week-old) stages of atherosclerosis. There were no statistically significant differences in serum cholesterol and triglyceride levels between experimental and control mice (Table 1). Western blot and qRT-PCR analyses of pulmonary endothelia respectively showed 40% and 45% reductions in Tie1 protein and mRNA expression of $Tie1^{+/-}$ mice (Figures 3.1A, B).

In the atherosclerosis-prone, disturbed flow regions of the aorta, en face analysis showed a 37.9% suppression of atherosclerotic lesions in 24 week-old mice ($Tie1^{+/+}$:$ApoE^{-/-}$ mice, 1.690±0.244%; $Tie1^{+/-}$:$ApoE^{-/-}$ mice, 1.047±0.200%, $p<0.05$) (Figure 3.1D). This difference persisted for 6 months (49 weeks of age), when the area covered by lesions was 16.134±2.806% in $Tie1^{+/+}$:$ApoE^{-/-}$ mice and 10.572±1.495% in $Tie1^{+/-}$:$ApoE^{-/-}$ mice (34.4% reduction; $p<0.05$) (Figures 3.1C, D). Interestingly, no statistically significant differences were found in the early and intermediate disease stage groups, although trends were confirmatory of what was seen at later disease stages (0.618±0.149% vs. 0.496±0.053% in the 12-week group; 1.522±0.842% vs. 0.558±0.056% in the 18-week group) (Figure 3.1D).

Table 3.1

Plasma cholesterol and triglyceride for ApoE$^{-/-}$ mice

Genotype (12wo)	Cholesterol (mg/dL)	Triglycerides (mg/dL)
Tie1$^{+/+}$:ApoE$^{-/-}$, n=4	208.8	174.7
Tie1$^{-/+}$:ApoE$^{-/-}$, n=8	185.8	153.2
Genotype (18wo)	**Cholesterol (mg/dL)**	**Triglycerides (mg/dL)**
Tie1$^{+/+}$:ApoE$^{-/-}$, n=9	233.5	101.4
Tie1$^{-/+}$:ApoE$^{-/-}$, n=14	275.3	102.0
Genotype (24wo)	**Cholesterol (mg/dL)**	**Triglycerides (mg/dL)**
Tie1$^{+/+}$:ApoE$^{-/-}$, n=20	290.1	115.7
Tie1$^{-/+}$:ApoE$^{-/-}$, n=18	316.4	123.9
Genotype (49wo)	**Cholesterol (mg/dL)**	**Triglycerides (mg/dL)**
Tie1$^{+/+}$:ApoE$^{-/-}$, n=7	254.9	88.2
Tie1$^{-/+}$:ApoE$^{-/-}$, n=9	300.1	90.0

Tie1 reduction caused no discernible effect, no significant differences were found between groups.

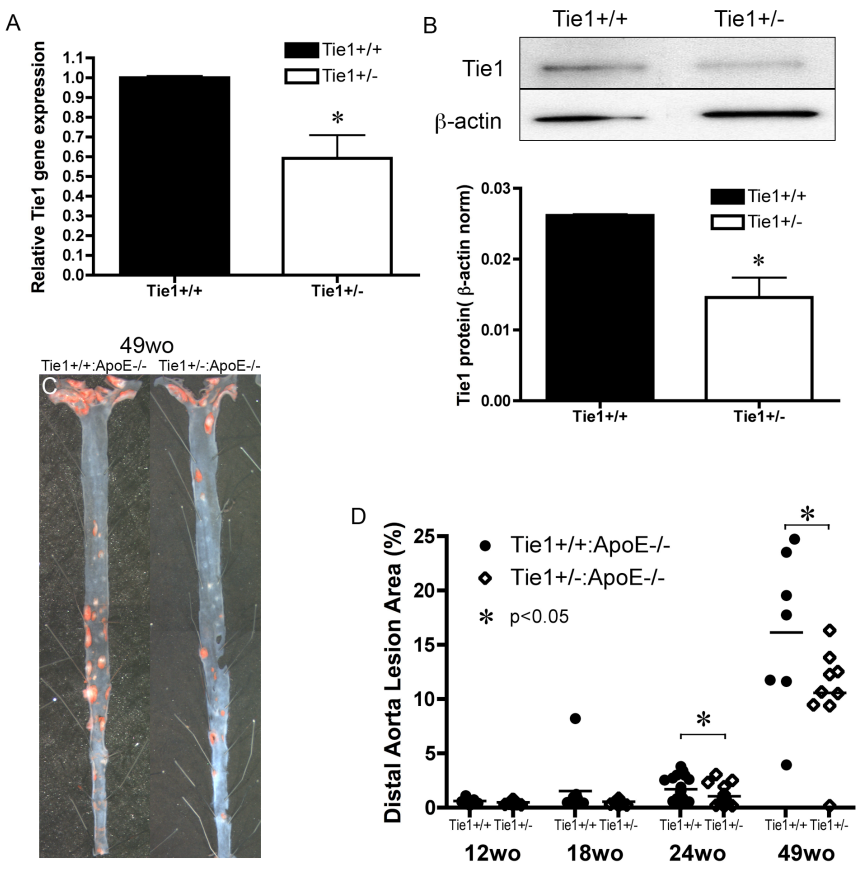

Figure 3.1 ***Tie1 Heterozygousity Reduces Advanced Stage Atherosclerosis Burden***
Quantitation of Tie1 levels in Tie1$^{+/-}$ mice by (**A**) RT-PCR (45% reduction, $p<0.05$) and (**B**) western blot (40% suppression, $p<0.05$). (**C-D**) Effect of reduced Tie1 expression on atherosclerosis. (**C**) Representative images of sudan IV stained aorta (aortic arch, thoracic and abdominal aorta) from 49 week-old Tie1$^{+/+}$:ApoE$^{-/-}$ and Tie1$^{+/-}$:ApoE$^{-/-}$ mice. (**D**) Graph comparisons of atherosclerotic lesion area in Tie1$^{+/+}$:ApoE$^{-/-}$ vs. Tie1$^{+/-}$:ApoE$^{-/-}$ mice analyzed at 12, 18, 24 and 49 weeks (12 week-old: 0.618 ± 0.149% vs. 0.496 ± 0.053%, NS; 18 week-old: 1.522 ± 0.842% vs. 0.558 ± 0.056%, NS; 24 week-old: 1.690 ± 0.244% vs. 1.047 ± 0.200%, 37.9% reduction, $p<0.05$; 49 week-old: 16.134 ± 2.806% vs. 10.572 ± 1.495%, 34.4% reduction, $p<0.05$). Statistically significant differences in atherosclerosis were observed at 24 and 49 weeks (*$p<0.05$).

In the proximal aorta, we found no statistically significant differences in the extent of lesion area between Tie1$^{+/+}$:ApoE$^{-/-}$ and Tie1$^{+/-}$:ApoE$^{-/-}$ mice at any stage (12 week-old: 40030±15311μm^2 vs. 51719±6094μm^2; 18 week-old: 156155±40275μm^2 vs. 152210±25157μm^2; 24 week-old: 286433±16160μm^2 vs. 334760±41861μm^2; 49 week-old: 426680±40877μm^2 vs. 529025±52711μm^2) (Figure 3.2A). The trend toward increased lesions in Tie1$^{+/-}$:ApoE$^{-/-}$ mice, which is opposite to the effects seen in the distal aorta, is interesting but does not warrant further comments given the lack of statistical significance.

Endothelial Specific Tie1 Deficiency Attenuates Atherosclerosis Progression in a Dose Dependent Manner

We next assessed the effect of endothelial specific Tie1 deletion on development of atherosclerotic lesions in ApoE$^{-/-}$ mice. Since Tie1 deletion leads to early embryonic lethality (Puri *et al.*, 1995; Sato *et al.*, 1995), we utilized Cre-Lox technology to delete Tie1 in a spatially and temporally defined manner. We used SCL-ER^T-Cre mice to achieve endothelial specific, tamoxifen-mediated induction of Cre expression (Gothert *et al.*, 2004). To determine an efficient tamoxifen induction dosage, SCL-ER^T-Cre mice were bred to Rosa-LacZ reporter background. We found robust X-gal staining on the aortic valve endothelium and in the microvasculature of the endocardium (Figure 3.3A). Tie1$^{-/flox}$ mice were bred with SCL-ER^T-Cre mice to determine efficiency of Tie1 deletion. In the resulting mice with a null Tie1 allele, a floxed Tie1 allele and endothelial specific Cre (Tie1$^{-/flox}$:SCL-ER^T-Cre), tamoxifen treatment (at 2mg every 48 hours, for 14

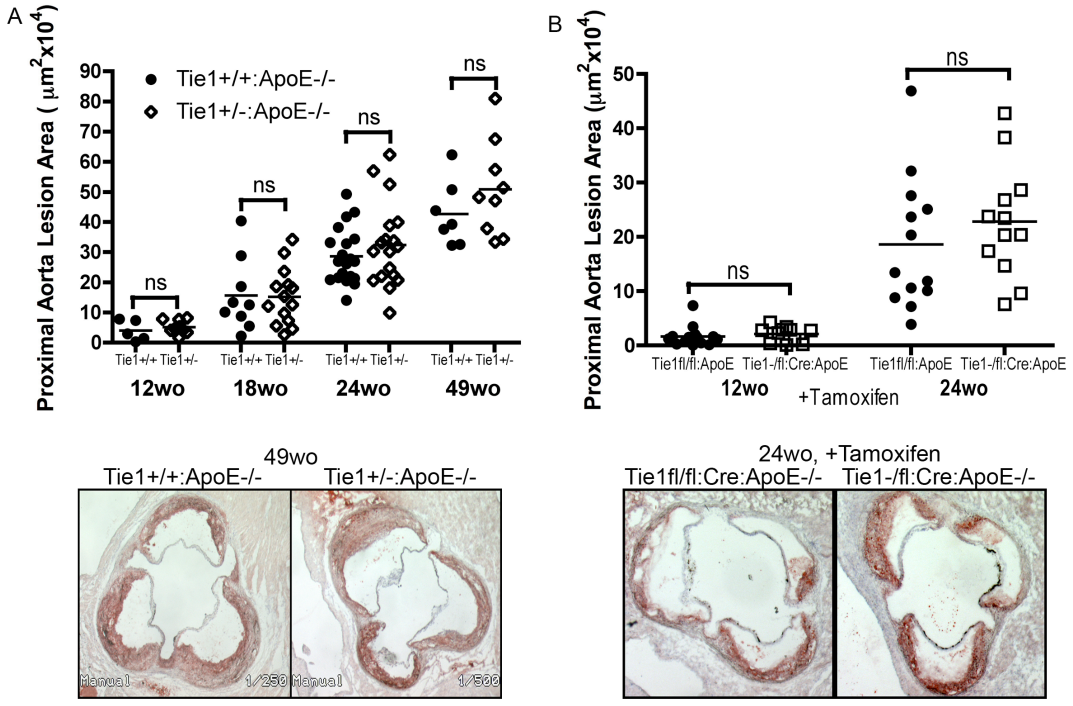

Figure 3.2 ***Tie1 Reduction Does Not Alter Atherosclerosis in the Aortic Valves***
(A) Graphical analyses of atherosclerosis in aortic valves of Tie1+/+:ApoE-/- vs. Tie1+/-:ApoE-/- mice at 12, 18, 24 and 49 weeks. (12 week-old: 40030±15311μm2 vs. 51719±6094μm2; 18 week-old: 156155±40275μm2 vs. 152210±25157μm2; 24 week-old: 286433±16160μm2 vs. 334760±41861μm2; 49 week-old: 426680±40877μm2 vs. 529025±52711μm2). Representative images of oil red o stained aortic valves from 49 week-old Tie1+/+:ApoE-/- and Tie1+/-:ApoE-/- mice. (B) Graphical analyses of atherosclerosis in aortic valves of Tie1flox/flox:ApoE-/- vs. Tie1-/flox: SCL-ERT-Cre:ApoE-/- tamoxifen treated mice (12 week-old: 16607 ± 5004 vs. 21126 ± 4274 μm2, NS ; 24 week-old: 22816 ± 3019 vs. 18587 ± 33856 μm2, NS). Representative images of oil red o stained aortic valves from 24 week-old tamoxifen treated Tie1-/flox:SCL-ERT-Cre:ApoE-/- vs. Tie1flox/flox:ApoE-/- mice.

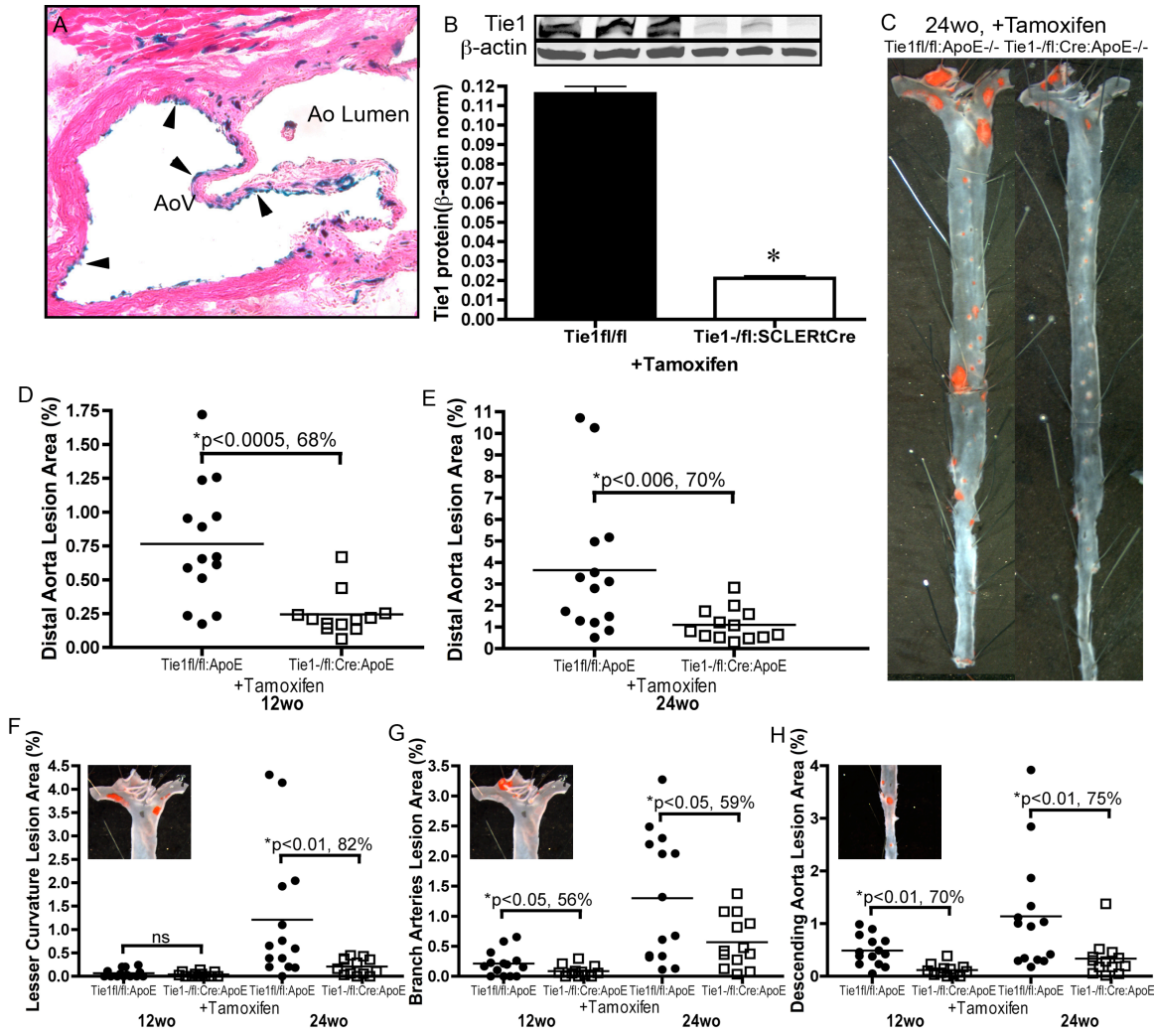

Figure 3.3 ***Endothelial Specific Tie1 Deletion Reduces Atherosclerosis Burden***
(**A**) Representative image of H&E stained aortic valve showing endothelial specific LacZ expression (arrowheads) from tamoxifen treated SCL-ER^{T}-Cre:Rosa26R-LacZ mouse (100x magnification). (**B**) Western blot analysis of pulmonary Tie1 levels from tamoxifen treated Tie1$^{-/flox}$:SCL-ER^{T}-Cre mouse (0.116 ± 0.003 vs. 0.0211 ± 0.001, 81% reduction, *p<0.0001). (**C**) Representative images of sudan IV stained distal aorta from Tie1$^{-/flox}$: SCL-ER^{T}-Cre:ApoE$^{-/-}$ and Tie1$^{flox/flox}$:ApoE$^{-/-}$ mice depicting lesion reductions in Tie1 deleted mice. Graphical comparisons of mean aortic lesion area in tamoxifen treated Tie1$^{-/flox}$: SCL-ER^{T}-Cre:ApoE$^{-/-}$ and Tie1$^{flox/flox}$:ApoE$^{-/-}$ mice analyzed at (**D**) 12 weeks (0.765 ± 0.114% vs. 0.244 ± 0.046%, p<0.0005) and (**E**) 24 weeks (3.644 ± 0.865% vs. 1.108 ± 0.207%, p<0.006). (**F-H**) Comparisons of atherosclerotic lesion areas in Tie1 deleted mice as assessed in three regions of the aorta showing reduction of

atherosclerosis in all regions of disturbed flow. Analyses of mean atherosclerotic lesion area at (**F**) lesser curvature of the aortic arch (12 week-old: 0.066 ± 0.023% vs. 0.038 ± 0.014%, NS; 24week-old: 1.20 ± 0.379% vs. 0.206 ± 0.048%, $p<0.01$), (**G**) aortic arch branch arteries (including brachiocephalic, left common carotid and left subclavian arteries) (12 week-old: 0.211 ± 0.055% vs. 0.086 ± 0.026%, $p<0.05$; 24 week-old: 1.29 ± 0.283% vs. 0.568 ± 0.119%, $p<0.05$) and (**H**) descending aorta (12 week-old: 0.487 ± 0.074% vs. 0.118 ± 0.032%, $p<0.01$; 24 week-old: 1.13 ± 0.291 vs. 0.333 ± 0.097%, $p<0.01$). (Inset figures illustrate respective regions of lesion analysis)

days) had induced an 81% reduction in pulmonary endothelial Tie1 protein levels (Figure 3.3B).

To assess the effect of Tie1 deletion on atherosclerosis progression, $Tie1^{-/flox}$: SCL-ER^T-Cre mice were bred onto the $ApoE^{-/-}$ background. $Tie1^{-/flox}$: SCL-ER^T-Cre:$ApoE^{-/-}$ female mice and control $Tie1^{flox/flox}$:$ApoE^{-/-}$ female littermates were injected with tamoxifen. Since tamoxifen administration has been associated with attenuation of atherosclerosis following chronic and unfettered oral intake, we determined whether acute and limited exposure to tamoxifen had any effect in our experimental model. In fact, we noted a protective effect (46% decrease) 4 weeks after treatment (12 week-old: 1.416±0.222% vs. 0.765±0.114%, $p<0.01$, Figure 3.4A, B), which was not maintained at the 16-week time-point (24 week-old: 4.326±0.54% vs. 3.644±0.865%, NS). Tamoxifen treatment temporarily decreased serum cholesterol in $Tie1^{flox/flox}$:$ApoE^{-/-}$ mice as compared to untreated controls 4 weeks after treatment (12 week-old: 456.44±40.58mg/dL vs. 291.43±19.36mg/dL, $p<0.001$) but this effect was lost 16 weeks after treatment (24 week-old: 331.54±28.54mg/dL vs. 345.20±24.61mg/dL, NS) (Table 2). Tamoxifen treatment also reduced serum triglycerides of 12 and 24 week-old animals (12 week-old: 186.8±8.00mg/dL vs. 147.98±7.38mg/dL, $p<0.01$; 24 week-old: 200.64±9.81mg/dL vs. 141.95±9.44mg/dL, $p<0.01$) (Table 2). However, the tamoxifen-induced reduction in serum triglycerides was not associated with an atheroprotective effect at 24 weeks.

Consistent with our previous studies, en face analysis of the aorta showed a statistically significant reduction in atherosclerotic lesions. In 12 week-old mice with early disease progression we found a 68% decrease in lesions of Tie1 deleted mice

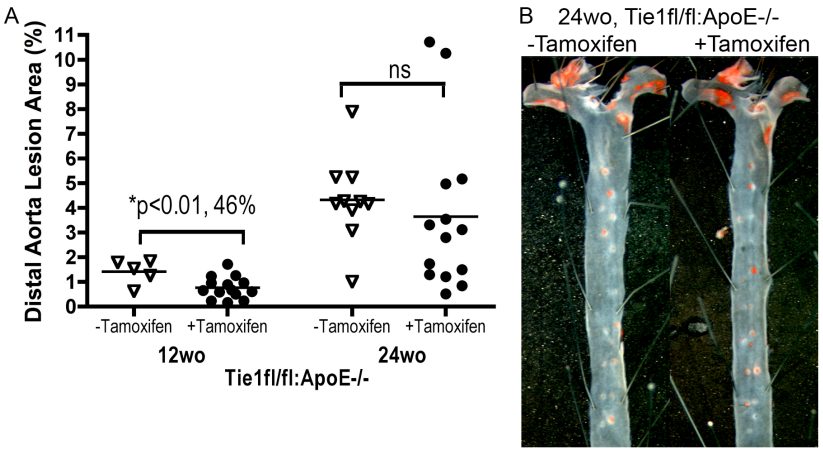

Figure 3.4 ***Tamoxifen Treatment Temporarily Reduces Atherosclerosis in the Short Term***

(A) Comparisons of atherosclerosis burden in untreated and tamoxifen treated Tie1flox/flox:ApoE-/- mice 4 weeks post treatment (12 week-old: 1.416±0.222% vs. 0.765±0.114%, $p<0.01$,) and 16 weeks post treatment (24 week-old: 4.326±0.54% vs. 3.644±0.865%, NS) . (B) Representative images of Sudan IV stained distal aortas from 24 week-old Tie1flox/flox:ApoE-/- untreated and tamoxifen treated mice.

Table 3.2
Plasma cholesterol and triglyceride for tamoxifen treated ApoE$^{-/-}$ mice

Genotype (12wo)	Cholesterol (mg/dL)	Triglycerides (mg/dL)
Tie1$^{flox/flox}$:SCL-ERT-Cre:ApoE-/- No Tamoxifen, n=5	456.4*	186.8$^{\#}$
Tie1$^{flox/flox}$:ApoE$^{-/-}$ + Tamoxifen, n=14	291.4*	147.9$^{**,\#}$
Tie1$^{-/flox}$:SCL-ER^{T}-Cre:ApoE$^{-/-}$ + Tamoxifen, n=12	293.9	172.9**
Genotype (24wo)	**Cholesterol (mg/dL)**	**Triglycerides (mg/dL)**
Tie1$^{flox/flox}$:SCL-ERT-Cre:ApoE-/- No Tamoxifen, n=10	352.1	200.6$^{\#}$
Tie1$^{flox/flox}$:ApoE$^{-/-}$ + Tamoxifen, n=17	345.2	141.9$^{**,\#}$
Tie1$^{-/flox}$:SCL-ER^{T}-Cre:ApoE$^{-/-}$ + Tamoxifen, n=13	331.5	188.6**

Tamoxifen temporarily decreased cholesterol levels 4 weeks post treatment but effect was lost by 24 weeks ($^{*}p<0.05$). Tamoxifen treatment decreased triglycerides at 12 and 24 weeks ($^{\#}p<0.05$). Tie1 deletion decreased triglycerides ($^{**}p<0.01$)

compared to controls (0.765 ± 0.114% vs. 0.244 ± 0.046%, $p<0.0005$, Figure 3.3D). The decrease in atherosclerosis persisted in mice up to 24 weeks, when Tie1 deletion resulted in a 70% decrease in plaque progression as compared to controls (3.644 ± 0.865% vs. 1.108 ± 0.207%, $p<0.006$, Figure 3.3C, E). However, similar to the atherosclerosis data of Tie1 heterozygous mice, we observed no significant difference in the degree of atherosclerosis at the proximal aorta between control $Tie1^{flox/flox}$:$ApoE^{-/-}$ and $Tie1^{-/flox}$: SCL-ER^{T}-Cre:$ApoE^{-/-}$ mice (12 week-old: 16607 ± 5004 vs. 21126 ± 4274 μm^2; 24 week-old: 22816 ± 3019 vs. 18587 ± 33856 μm^2, Figure 3.2B).

Tie1 deletion ($Tie1^{-/flox}$:SCL-ER^{T}-Cre:$ApoE^{-/-}$ + tamoxifen, Table 2) had no effect on serum cholesterol levels as compared to controls ($Tie1^{flox/flox}$:$ApoE^{-/-}$ + tamoxifen). Serum triglycerides in Tie1 deleted ($Tie1^{-/flox}$:SCL-ER^{T}-Cre:$ApoE^{-/-}$ + tamoxifen, 173 mg/dL) mice were slightly increased to controls ($Tie1^{flox/flox}$:$ApoE^{-/-}$ + tamoxifen, 148 mg/dL) but similar to those of non-treated controls ($Tie1^{flox/flox}$:SCL-ER^{T}-Cre:$ApoE^{-/-}$, no tamoxifen, 187 mg/dL, Table 2), serum triglyceride levels were not different. These results suggest that deletion of endothelial Tie1 has a protective effect attenuating the rate of atherosclerosis progression independent of effects on serum cholesterol or triglyceride levels.

Reduction of Atherosclerosis Due to Tie1 Deletion is Location Specific

To determine whether Tie1 deletion has an effect on atherosclerosis distribution based on location and type of shear stress, we analyzed lesions at (a) bifurcations of the aortic arch branch arteries, (b) the lesser curvature of the aortic arch, and (c) the descending aorta. Lesion sizes at all locations were decreased as a consequence of Tie1

deletion (Figure 3.1F-H), suggesting that Tie1 plays a role in disturbed flow mediated atherogenesis at each of these locations.

Discussion

Since we found that Tie1 is specifically expressed at regions of atherogenic shear stress, and a previous study reported Tie1 expression on both the surface and subendothelium of atherosclerotic lesions (Porat *et al.*, 2004), we postulated that attenuation of Tie1 might modulate atherosclerosis progression. We found that moderate reductions in Tie1 ($Tie1^{+/-}$:$ApoE^{-/-}$) elicited 35% and 38% reductions in atherosclerotic lesions of 24 and 49 week-old mice respectively. Remarkably, further reduction of Tie1 by up to 80% in $Tie1^{-/flox}$:SCL-ER^{T}-Cre:$ApoE^{-/-}$ mice resulted in 68% and 70% reduction in lesions of 12 and 24 week-old mice, respectively. Together, these results demonstrate a unique dose dependent amelioration of atherosclerosis progression conferred by attenuation of Tie1 expression.

We also note that attenuation of Tie1 did not significantly alter the extent of atherosclerotic lesions in the aortic valve region. Interestingly, Tie1 attenuation elicits a trend of increased lesions at the aortic sinus, an effect opposite to that observed in the distal aorta. Two recent studies highlighted regional differences in atherosclerotic plaque reduction of PECAM knockout mice (Goel *et al.*, 2008; Harry *et al.*, 2008). Goel et al showed that PECAM deletion in mice on the LDL receptor knockout background significantly increased lesion formation in the aorta, however they observed decreased lesions specifically at the lesser curvature of the aortic arch. Harry et al used PECAM deleted mice on an ApoE knockout background and reported an overall reduction in

atherosclerotic lesions in the aorta. They too found reduced lesions at the lesser curvature of the aortic arch, but not at the descending aorta. The aortic valve also experiences a unique and complex shear stress profile not evidenced elsewhere in the vasculature. Peacock previously described the flow profile in the aortic sinus as turbulent, comprising spinning vortices superimposed with random motion of fluid particles (Peacock, 1990). In contrast, the flow profile at aortic bifurcations distal to the aortic sinus has been described as recirculatory (Asakura & Karino, 1990) and is characterized by bidirectional blood flow with the resultant effect of a low time-averaged shear stress (Davies *et al.*, 1999). Additionally, Butcher et al demonstrated that the shear stress-induced transcriptional response of valvular endothelial cells is vastly different from aortic endothelial cells. Over 400 genes were differentially expressed, and overall, the inflammatory response of valvular endothelial cells were found to be less pronounced than that of aortic endothelial cells (Butcher *et al.*, 2006). Hence, Tie1 may regulate atherosclerosis progression in a novel shear stress specific manner, discriminating between different flow profiles characteristic of unique anatomical and physiological locations within the vascular system.

Epidemiological studies have suggested an atheroprotective effect afforded by estrogen replacement therapy (Mikkola & Clarkson, 2002). This cardiovascular benefit is likely to be a combination of lipoprotein profile modulation and regulation of inflammatory components. Postmenopausal women taking oral conjugated equine estrogen had decreased LDL and increased HDL both of which are associated with decreased risk for atherosclerosis (Walsh *et al.*, 1998). Reckless and co-authors (Reckless *et al.*, 1997) previously showed that 12 week-old ApoE null mice that were fed high-fat

diet containing tamoxifen for 3 months and promptly sacrificed had decreased total plasma cholesterol and LDL levels, increased HDL content and reduced atherosclerotic lesions as compared to untreated controls. We also observed that tamoxifen reduces atherosclerosis, even after short-term administration (2 weeks). However, in our studies, tamoxifen reduced lesions at the distal aorta 4 weeks post-treatment (12 week-old mice), but the effect was lost at 16 weeks (24 week-old mice). We also found a temporary reduction in cholesterol 4 weeks post tamoxifen treatment, however this effect was lost by 16 weeks. Although our results showed tamoxifen treatment reduced triglycerides up to 16 weeks post treatment, the deletion of Tie1 restored serum triglycerides to levels similar to untreated control animals while reducing average atherosclerotic lesions.

In summary, we showed that the attenuation of Tie1 reduced advanced stage atherosclerosis in a dose dependent manner. We also demonstrated that the reduction in atherosclerosis progression was localized to specific locations of the aorta, likely due to the different shear stress profiles experienced at each area. Hence, Tie1 may modulate atherosclerosis progression by mediating the effects of local hemodynamics on the endothelium.

CHAPTER IV

IMMORTALIZED MOUSE AORTIC ENDOTHELIAL CELLS ARE A UNIQUE PRIMARY CELL LINE FOR IN VITRO RESEARCH

Introduction

Our understanding in endothelial biology has significantly increased since the development of techniques for isolation and propagation of primary cells *in vitro*. Beginning in the 1970's, Jaffe et al (Jaffe *et al.*, 1973) were the first to culture human umbilical vein endothelial cells *in vitro* for up to five months, paving the way for a numerous studies in endothelial function. Together with the evolution of technology for genetically modified animals, the isolation of primary endothelial cells from transgenic and knockout mice has facilitated cellular and molecular investigations into notable phenotypic changes.

Endothelial cells have been isolated from murine lung (Gerritsen *et al.*, 1995; Dong *et al.*, 1997; Marelli-Berg *et al.*, 2000; Cha *et al.*, 2005), lymph nodes (Toyama-Sorimachi *et al.*, 1993) and brain (Marelli-Berg *et al.*, 2000). However, the isolation of primary endothelial cells from the mouse can be both time-consuming and costly, especially from certain organs, including the aorta, whereby endothelial cells can only be passaged for very few times before senescence occurs. Along with a plethora of publications reporting different techniques to isolate primary endothelial cells, there are also several formulations of proprietary (Endothelial Growth Media [EGM], Cambrex) and custom-made specialty media to promote endothelial growth.

Some of the early experiments in isolating macrovascular endothelial cells from mice (Merrilees & Scott, 1981; McGuire & Orkin, 1987) used the same enzymatic dissociation techniques previously employed for large animals. However, this method proved tumultuous as cultures were often contaminated with other cell types such as smooth muscle cells and fibroblasts, which are also released by the enzyme (Gimbrone *et al.*, 1974; Gumkowski *et al.*, 1987). Since then these obstacles have been addressed either by using endothelial selective media (Gumkowski *et al.*, 1987; Magee *et al.*, 1994; Geiger *et al.*, 1997) or selection by endothelial specific markers (Auerbach *et al.*, 1982; Gumkowski *et al.*, 1987; Kevil & Bullard, 2001).

Nishiyama et al (Nishiyama *et al.*, 2007), Chen et al (Chen *et al.*, 2004), Kobayahi et al (Kobayashi *et al.*, 2005) and Kevil et al (Kevil & Bullard, 2001) have all previously described enzymatic dissociation methods to isolate murine aortic endothelial cells. Nishiyama and co-workers digest aortic chunks with collagenase and primarily relies on a custom-made selective media to enrich the endothelial cell population. However, this method creates mixture of heterogeneous cells. Dr. Chen describes cannulating both ends of the thoracic aorta, and either tieing off or cauterizing open branches to create a sealed environment. Following which, trypsin is flushed into the aorta for two minutes and collected, and this process is repeated several times. They report that this method yields more than 95% endothelial purity by PECAM immunostaining and 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate-labeled acetylated low-density lipoprotein (di-I-acetylated-LDL / DiI-AcLDL) uptake. DiI-AcLDL is a synthetic compound that depends on the binding of LDL with its cognate receptor. Once sequestered into the endothelial cell, the DiI fragment is released and accumulates in the

cytosol as a fluorescent marker. However, this method poses technical difficulties in sealing off all openings of the aorta. More importantly, the number of endothelial cells obtained from this method is very low, thus limiting the degree of experiments performed. Along similar lines, Kobayashi et al isolates the aorta and seals off all the openings by cauterization except for one end. They fill the aorta with type II collagenase and tie the end shut while letting the aorta incubate at 37^{o}C. The primary principle of this protocol relies on the theory of differential sedimentation following transfer of the cell mixture onto tissue culture dishes. Dr. Kobayashi and co-workers report that disposing of the media 2 hours after seeding the cells removes smooth muscle cells, and the custom-made media will select for enrichment of the endothelial population. An alternate modification of the enzymatic dissociation method, Kevil et al incubates the aorta with dispase, and stains the cells with fluorescein isothiocyanate-*Bandeirea simplicifolia* lectin-I (FITC BS-I) to allow sorting by flow-assisted cytometry (FACS) (Kevil & Bullard, 2001). This method yields a relatively pure population of endothelial cells, although the return from FACs is consistently less than 1% (personal communication). Since these primary endothelial cells are limited in the number of passages, studies will still have to begin with a large number of animals and experiments have to be done with fewer cells.

In an alternate non-enzymatic approach Suh et al (Suh *et al.*, 1999), Lincoln et al (Lincoln *et al.*, 2003), and Huang et al (Huang *et al.*, 2003) report using an explant technique in combination with endothelial selection media to isolate murine aortic endothelial cells. Dr. Suh and co-workers cut the aorta longitudinally and placed 1 square millimeter pieces lumen-side down on matrigel-coated plates. Matrigel contains a rich

mixture of growth factors conducive for endothelial cell propagation. Improving on this technique, Dr. Huang and Dr. Lincoln relied on the uptake of DiI-AcLDL by endothelial cells. Placing a cloning cylinder around cells that fluoresced with DiI and dislodging them with trypsin, Lincoln et al employed a clonal expansion technique to isolate endothelial cells. Dr. Huang capitalized on the accuracy of FAC-sorting and retrieved endothelial cells that were positive for DiI-AcLDL uptake. While this variety of methods assures a relatively homogenous population comprising only of endothelial cells, the primary cultures can only propagate to very few passages hence making these techniques inefficient cost and time-wise.

Although these obstacles can be overcome by immortalizing endothelial cells *in vitro*, the process of immortalization may result in phenotypic changes such as loss of surface antigens (Harder *et al.*, 1991), and reduced responsiveness to cytokines (Lidington *et al.*, 1999). Moreover, primary endothelial cells are highly resistive to transfection efforts (Lidington *et al.*, 1999) and immortalized cell lines cannot be used for the studies in cell cycle progression.

The generation of H-2Kb-tsA58 transgenic mice has allowed investigators to circumnavigate the problems of immortalized cell lines. These mice express a themolabile strain (tsA58) of the simian virus (SV)40 large T antigen (tsA58 TAg) linked to an inducible major histocompatibility complex H-2K promoter (Jat *et al.*, 1991). In primary cells derived from this mice, the T antigen is only functionally evident at the reduced temperature of 33^{o}C and promoter activity can be upregulated by supplementing the media with interferon (IFN)-γ (Jat *et al.*, 1991). In this chapter, we describe a technique of isolating endothelial cells and selecting PECAM positive ones with FAC-

sorting after a period of cell expansion. We also report a novel custom-made media at a time-efficient and cost-effective rate.

Experimental Procedures

Genotyping

At three weeks of age, tail samples from offspring were digested in 100 mM Tris pH 8.5, 5 mM EDTA, 0.2% sodium dodecyl sulfate, 200 mM NaCl, 100 μg/ml of Proteinase K overnight at 55^{o}C. Mice were genotyped by polymerase chain reaction with REDExtract-N-Amp PCR Reaction Mix (Sigma) using the following primers,
tsA58: 5'- CCT CTG AGC TAT TCC AGA AGT AGT G -3' and 5'- TTA GAG CTT TAA ATC TCT GTA GGT AG -3'

Mouse Aortic Endothelial Cell Isolation (MAEC)

The Immorto mouse is a transgenic mouse generated by the introduction of thermolabile SV40 T Ag, tsA58 (Jat *et al.*, 1991). In this model, the simian virus 40 (SV40) large tumor antigen of a temperature-sensitive strain (tsA58) is fused with the major histocompatibility complex promoter H-2K^{b}. Expression of the promoter and the large T antigen protein is only evident when immorto mouse-derived cells are cultured at a permissive temperature (33^{o}C). The addition of IFN-γ was also used to further enhance promoter activity(Jat *et al.*, 1991). A 4 to 8 week-old immorto mouse was anesthetized with isoflurane and sacrificed by cervical dislocation. The lumbar aorta was punctured and 5ml of Hanks Balanced Salt Solution (HBSS) was perfused through the left ventricle

to flush out blood. The heart, lung and aorta bloc was excised and transferred to a dish containing HBSS and antibiotics. The heart and lungs were removed. The rest of the aorta was incubated at 37^{o}C for 15 minutes with filtered 10mg/ml collagenase Type II (Worthington Chemicals) and 1x antibiotics dissolved in dispase (Roche). The adventitia was gently removed and the remaining intima layer comprising the endothelium and internal elastic lamina was cut open longitudinally and incubated at 37^{o}C for 30 minutes in filtered 20 mg/ml collagenase Type II solution and 1x antibiotics dissolved in dispase. The aorta was dissociated by pipetting several times in the dissolving solution and filtered through a 100 μm sterile cell strainer (Fisher Scientific). Culture media (MCDB 131 [Gibco], 10% FBS [Hyclone], 10 U/ml heparin [Sigma], 2.75 nM hydrocortisone [Sigma] and 0.2% Bovine Brain Extract [Hammond Celltech], 10 U/ml recombinant murine IFN-γ [Peprotech], 1x antibiotics [Gibco]) was added to the cell suspension and centrifuged at 375 x g for 10 minutes. The cell mixture was then plated onto a fibronectin coated culture dish and propagated at 33^{o}C in a mixture of 5% carbon dioxide and 95% oxygen. MAECs were isolated from wild-type and Tie1$^{flox/flox}$:SCL-ER^{T}-Cre immorto mice.

Flow Assisted Cell Sorting

MAECs were released from the plate by incubating with Accutase (Invitrogen) for 10 minutes at 37^{o}C. Cells were collected by centrifugation at 375 x g for 10 minutes at 4^{o}C and resuspended in HBSS (supplemented with 1% FBS and 1x antibiotics). The cells were then incubated with APC conjugated CD31 (BD Biosciences 551262) at 1 mg/1x10^{6} cells for 30 minutes on ice with agitation every 10 minutes. MAECs were washed 3 times

in HBSS, filtered through a 40μm cell strainer (Fisher Scientific) and stored on ice before cell sorting. Propidium Iodide (Invitrogen) was added at 1:1000 dilution prior to sorting. CD31+ cells were collected in culture media, centrifuged and plated onto collagen (Rat tail collagen type I, BD Biosciences) coated dishes.

Mouse Aortic Endothelial Cell Characterization

MAECs were rinsed with phosphate buffered saline and briefly fixed with methanol at -20^{o}C and blocked with 10% normal goat serum (Jackson ImmunoResearch) for 1 hour at room temperature. Immunostaining was performed at 4^{o}C overnight with either CD31 (BD Biosciences, cat# 553370) or VECadherin (Cayman Chemical, cat#160840) and counterstained with goat anti-rat Alexa 488 (BD Biosciences) or goat anti-rabbit 488. MAECs were also cultured on matrigel (BD Biosciences) coated dishes overnight and observed for network formation. DiI-AcLDL (Biomedical Technologies) was added to the media at a 10μg/ml for up to 6 hours and washed to observe uptake in MAECs.

Shear Stress Experiments

Shear stress experiments were performed using a custom cone-and-plate shear stress viscometer design. An inverted servo motor (ElectroCraft, USA) is attached to a plexiglass cone with 0.5^{o} angle (Figure 4.1A, B). A Motomatic II motor controller (Figure 4.1C) (Reliance Electric, ElectroCraft, USA) regulates velocity in laminar flow, and a digital function generator (Instek, San Diego, CA, USA) is used to produce a sinusoidal waveform modulating oscillatory flow. A digital oscilloscope (Tektronix, USA) is used in parallel to monitor the output magnitude and waveform (Figure 4.1D).

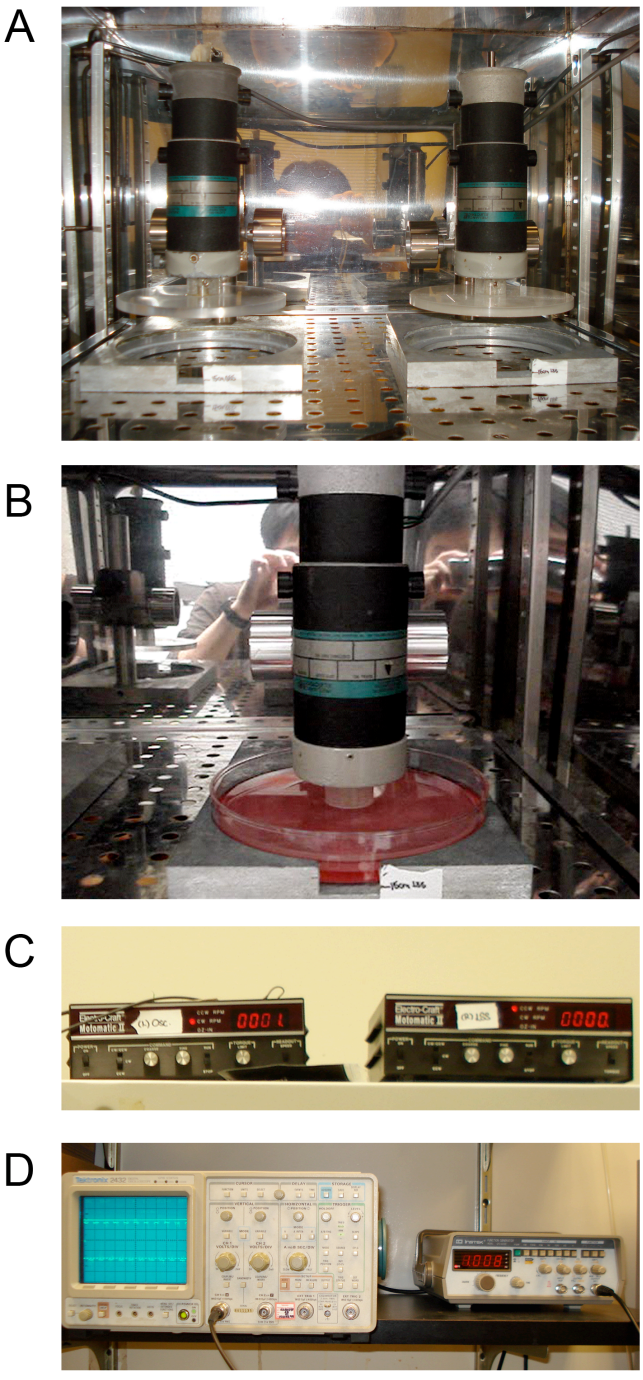

Figure 4.1 ***Cone-and-plate shear stress apparatus.*** **A, B.** A plexiglass inverted cone is attached to a step-motor. The motor height is controlled by a modified microscope stage. **C.** The speed of cone rotation is regulated by the Motomatic II motor controller. **D.** A

digital function generator produces sinusoidal waveforms to modulate oscillatory flow and a digital oscilloscope is used in parallel to monitor the output magnitude and waveform.

The servomotor and cone are lowered onto the culture dish by a step controlled base stand. Laminar shear is attained with unidirectional motion of the cone while oscillatory shear is achieved by bidirectional motion at ± 5 dynes/cm^2. Shear stress experiments are performed for 24 hours in complete media in a sterile, 5% CO_2 incubator at 37^{o}C.

Results

Characterization of murine aortic endothelial cells

To determine the effect of Tie1 deletion *in vitro*, we developed an immortalized murine aortic endothelial cell (MAEC) line immorto mice. Several passages after expanding the population, MAECs were characterized and tested for endothelial qualities. At passage 10, MAECs were immunostained with CD31 and FACs analysis reported 89.7% positive staining (Figure 4.2A). Immunostaining with PECAM showed uniform cytoplasmic staining in individual cells and localization of PECAM to the cell border when in apposition with adjacent cells (Figure 4.2B). Our MAECs displayed cobblestone morphology characteristic of endothelial cells (Figure 4.2C, D). We found robust cell border staining with VE-Cadherin (Figure 4.2E) also uptake of DiI-AcLDL (Figure 4.2F). MAECs cultured on matrigel showed robust network formation within 24 hours (Figure 4.2G, H). We also subjected the MAECs to further rigorous testing by placing them under flow conditions and we found that MAECs aligned to the direction of fluid flow after 24 hours of high shear laminar flow (Figure 4.2J) as compared to static control (Figure 4.2I). These results demonstrate that MAECs isolated from immortalized mice display the same characteristics of many primary endothelial cell lines.

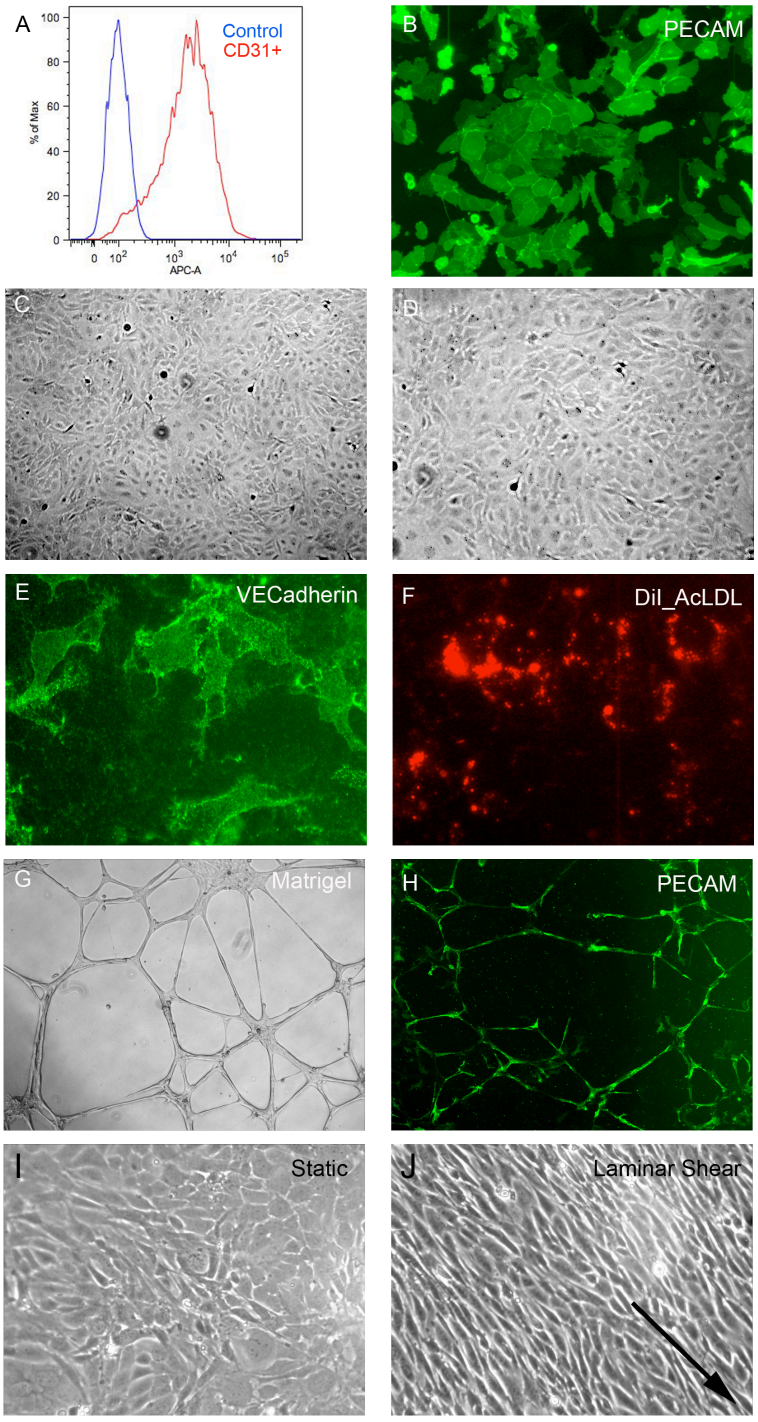

Figure 4.2 ***Characterization of murine aortic endothelial cells.*** **A.** Flow cytometry analysis of MAECs, 90% CD31+. **B.** PECAM immunostaining. **C, D.** low and high magnification phase contrast images displaying cobblestone morphology. **E.** VECadherin immunostaining. **F.** DiI-AcLDL uptake. Formation of networks following culture on matrigel, **G.** light microscopy, and **H.** PECAM immunofluorescence image. **J.** Alignment

of MAECs to direction of flow after 24hours of 20 dynes/cm^2 laminar flow compared to **I.** static culture.

Discussion

A variety of custom-made media has been used in endothelial cell culture. We report a media formulation that is not only permissive for endothelial cell growth but selects for endothelial enrichment. We used MCDB 131 as the basal media. This was originally developed by Knedler and Ham as a reduced serum-supplemented medium for the culture of human microvascular endothelial cells (Knedler & Ham, 1987), since then many reports have used this as a basal media for endothelial cell culture too (Kevil & Bullard, 2001; Lincoln *et al.*, 2003). We supplemented the media with 10% fetal bovine serum (FBS), a high FBS content provides a variety of growth factors for endothelial cell culture (Kobayashi *et al.*, 2005; Nishiyama *et al.*, 2007). Heparin and hydrocortisone is also added to facilitate presentation of growth factors to their cognate receptors on the cell surface and reduce endothelial cell inflammation (Kevil & Bullard, 2001) respectively. While proprietary endothelial growth media kits include growth factors such as VEGF, bFGF, IGF and EGF (EGM, Cambrex), many custom-made media formulations (Suh *et al.*, 1999; Kobayashi *et al.*, 2005) use endothelial cell growth supplements (ECGS) commercially available from biotechnology vendors (Invitrogen). We supplement our media with Bovine Brain Extract (Hammond Celltech) isolated from pituitary glands. This supplement provides a rich blend of growth factors that promote endothelial cell growth.

To further investigate the effect of shear stress on Tie1 expression *in vitro*, we devised a novel method of isolating genetically modified mouse aortic endothelial cells maintaining a high percentage of cells displaying endothelial characteristics. The culture of mouse aortic endothelial cells is a technically challenging process often resulting in

low yields with poor propagation properties. We have circumnavigated these problems through the generation of conditionally immortalized aortic endothelial cells from Tie1$^{flox/flox}$:SCL-ER^{T}-Cre immorto mice and selecting with PECAM by flow-assisted cell sorting. Jat et al (Jat *et al.*, 1991) first reported the derivation of conditionally immortalized cell lines from the H-2K^{b}-tsA58 transgenic mouse, following which several groups have successfully isolated a variety of cell lines from this mouse (Whitehead *et al.*, 1993; Lidington *et al.*, 2002; Langley *et al.*, 2003). In this model, the large tumor antigen (TAg) of a temperature-sensitive strain (tsA58) of the simian virus 40 (SV40) is fused with the major histocompatibility complex promoter H-2K^{b} that is active in a wide range of tissues and is induced by interferon (IFN). The presence of the inducible tsA58 TAg allows for the extended propagation of MAECs under permissive conditions (33^{o}C, IFN-γ). MAECs transferred to normal culture conditions (37^{o}C) were confirmed to be endothelial by their characteristic cobblestone morphology, formation of microtubules on Matrigel, expression of CD31 and uptake of DiI-Ac-LDL. Additionally, MAECs aligned to the direction of laminar flow after only twenty-four hours (Remuzzi *et al.*, 1984). Our results indicate that immortalized mouse aortic endothelial cells display the characteristics germane to many primary endothelial cell lines and is a useful cell line for the study of endothelial function *in vitro*.