UNIVERSITY OF CALIFORNIA, SAN DIEGO

Endothelial Nuclear Hormone Receptors in Atherosclerosis

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requirements for the degree of Doctor of Philosophy

in

Biology

by

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DEDICATION

God take and receive my liberty, my memory, my understanding and will.

All that I am and have He has given me.

God grant me the serenity to accept the things I cannot change,

Courage to change the things I can,

And wisdom to know the difference.

Living one day at a time,

Enjoying one moment at a time,

Accepting hardships as the pathway to peace,

Taking, as He did, this sinful world as it is, not as I would have it;

Trusting that He will make all things right if I surrender to his will,

That I may be reasonably happy in this life,

And supremely happy in the next.

Amen

EPIGRAPH

Believe those who are seeking the truth; doubt those who find it.

Andre Gide

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ABSTRACT OF THE DISSERTATION

Endothelial Nuclear Hormone Receptors in Atherosclerosis

by

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Doctor of Philosophy in Molecular Biology

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Atherosclerosis is a disease of dysregulated inflammation and metabolism. Many nuclear receptors have established roles in regulating metabolism and inflammation through various tissues and cell types, making them attractive therapeutic targets for cardiovascular disease. Thiazolidinediones (TZDs), pharmaceutical ligands for nuclear receptor peroxisome proliferator activated receptor gamma (PPARγ), are used to improve insulin sensitivity in diabetic

patients. Recently their effect on coronary heart disease has been called into question. PPARγ has been shown to be athero-protective in mice via the macrophage by enhancing cholesterol efflux and suppressing inflammation, but its role in lesion smooth muscle and endothelial cells is unknown. Some reports suggest PPARγ can suppress oxidation, hypertension and inflammation in the endothelium. Thus, endothelial PPARγ would be predicted to play a protective role in atherosclerosis. To test this, PPARγ-flox, Tie2-cre, LDLr-/- mice were irradiated and reconstituted with wildtype PPARγ bone marrow to generate an endothelial-specific PPARγ knockout atherogenic model. Endothelial PPARγ deficiency resulted in significantly increased lesion area and greater macrophage infiltration. Gene expression of pro-atherosclerotic chemokines and adhesion molecules was increased in primary endothelial cells from PPARγ-deficient mice relative to wildtype, both *in vivo* and with TLR ligand challenge *in vitro*. In agreement with this support for an anti-inflammatory role, PPARγ ligand could suppress monocyte adhesion to both human and mouse primary endothelial cells. While PPARγ ligand did not suppress classic endothelial adhesion molecules such as VCAM and ICAM, it did suppress the membrane-bound chemokine, fractalkine (CX3CL1). This molecule is strongly chemotactic and deficiency of its cognate receptor has been shown to improve atherosclerosis in LDLr-/- mice. Immunohistochemistry, luminex assay and neutralizing antibodies are being employed to determine the contribution of fractalkine to atherosclerosis in the endothelial PPARγ-deficient model. In an effort to identify additional nuclear receptors as potential targets for cardiovascular disease treatment, primary

endothelial gene expression was compared between wildtype mice and LDLr-/-

mice fed atherogenic diet. Expression of several nuclear receptors was

significantly altered in the atherogenic model and investigation of their function

in this context is underway.

INTRODUCTION

As people around the world become more sedentary and food readily available, the occurrence of obesity and associated health risks continue to grow. Atherosclerosis is a metabolic and inflammatory disease, often associated with obesity. It is often described as a hardening or thickening of the artery wall caused by accumulation of lipids or cholesterol. The development of the atherosclerotic plaque or lesion involves a complex interplay of leukocytes, smooth muscle cells and endothelial cells, and as a whole is affected by shear stress, vasomotor activity, modified forms of cholesterol and endotoxins. These cells may be involved in proliferation, apoptosis or inflammation, any of which may be pro- or anti- atherosclerotic depending on the extent of plaque progression.

Lining the lumen of the blood vessel, the endothelium is directly exposed to shear stress and modified lipids and cholesterol. In response to these stimuli, endothelial cells express chemokines and adhesion molecules. Blood-borne monocytes and T-cells adhere as a result, and subsequently migrate through endothelial junctions. These resident immune cells proliferate, secrete inflammatory cytokines, accumulate lipids and activate endothelial and smooth muscle cells, contributing to the thickness and instability of the plaque. Thus, the endothelium plays a critical role in plaque initiation and is a potential therapeutic target for the prevention of atherosclerosis.

The PPAR and LXR nuclear receptor sub-families have been implicated as regulators of atherogenesis due to their known roles in lipid and glucose homeostasis, as well as inflammation (review, Barish 2004). Indeed, a number of studies suggest that they are promising therapeutic targets for cardiovascular disease. PPARγ ligands (TZDs)

are used to treat diabetes, a disease which pre-disposes patients to atherosclerosis. TZDs effectively lower LDL and triglycerides while raising HDL and dramatically decreasing lesion size in mice (Li 2004). A number of studies specifically designed to look at cardiovascular outcomes or surrogate disease markers, suggest a potential benefit for TZDs in the treatment of human atherosclerosis (Mazzone 2006, Dormandy 2005). In addition to its prominent expression in adipose tissue, PPARγ is expressed in endothelial and smooth muscle cells, as well as foam cell macrophages. In the latter, it regulates cholesterol trafficking and inflammation, its deletion increasing lesion size in an atherosclerosis model (Chawla 2001). Recently, a postdoctoral fellow in our lab has made progress in determining the *in vivo* function of endothelial PPARγ in atherosclerosis using an endothelial specific knockout. Results indicate greater macrophage infiltration of aortic lesions and greater sinus valve lesion area in mice lacking endothelial PPARγ, suggesting a protective role for endothelial PPARγ in this disease.

The increased atherosclerosis in mice lacking endothelial PPARγ might be due to increased inflammatory activity, altered lipid management and impaired vasofunction. Anti-inflammatory activity, similar to that in the macrophage, may reduce monocyte adhesion and migration. Indeed, TZDs have been shown to reduce chemokines and adhesion molecules in human umbilical vein endothelial cells (HUVEC) (Murao 1999, Wang 2002), as well as inhibit macrophage homing to atherosclerotic lesions in mice (Pasceri 2000). However, these studies use supra-pharmalogical concentrations that are capable of off-target effects such as activation of PPARα and PPARδ. Interestingly, laminar flow, characteristic of aortic regions resistant to atherosclerosis, stimulates the

production of PPARγ ligands, epoxyeicosatrienoic acids (EETs), which repress LPS

stimulated NFκB activity in bovine aorta endotheial cells (BAEC) and HUVEC (Liu

2004). In the absence of shear stress, these EETs are modified and become PPARγ

antagonists. More recently, it has been shown that mice with an endothelial specific

deletion of PPARγ are dyslipidemic and have impaired vaso-reactivity, both

characteristics associated with atherosclerosis (Kanda 2009). While these reports are

suggestive of reasons for endothelial- PPARγ conferred athero-protection, they are not

conclusive.

In vitro endothelial cell studies present several challenges.   Murine and human

cell lines with some endothelial characteristics are quite different from primary cells and

not generally accepted by those that regularly study endothelial cell biology. The most

common sources of endothelial cells for *in vitro* studies are the bovine aorta and human

umbilical vein because of the quantity of cells obtained and their well-characterized

isolation and culture conditions. As a consequence, these cells are commercially

available. Primary murine endothelial cells are not, but there are several published

protocols for their isolation (van Beijnum 2008, Zhao 2002, Pozzi 2000). In addition,

results can vary widely with passage and thus, experiments are limited by the original

quantity of cells isolated.   The aim of my thesis work was to establish an *in vitro* system

to determine the role of endothelial -PPARγ in mediating protection against

atherosclerosis, as it is observed in our *in vivo* model. This effort expanded to identify *in*

vitro systems for the study of additional nuclear receptors in the endothelium and those

that may also play a role in atherosclerosis. Given the therapeutic role of nuclear receptors in

an array of disease contexts, we have characterized the nuclear receptor gene expression profiles

of human primary aorta, coronary artery and umbilical vein endothelial cells as well as and mouse primary heart and lung endothelial cells. In addition, we have revealed endothelial nuclear receptor expression changes in the context of an atherosclerotic mouse model which will lend to investigation of nuclear receptor-mediated improvements in vascular health.

Chapter 1. Defining the Role of Endothelial PPARγ in Atherosclerosis

1.1 PPARγ Effects on Gene Expression in Human EC

Initially, HUVEC gene expression was characterized in an effort to foster future studies with a commonly used endothelial *in vitro* model. In addition, resources for studies in human cells such as antibodies and siRNA were readily available. PPARγ RNA and protein expression was verified in HUVEC. Consistent with previously published data, PPARγ expression was low relative to adipocytes and macrophages. Genes activated in other cell types by TZD treatment, including LXRα, CD36 and ABCG1, were not significantly induced in these cells. Toll-like receptors and IFNγ receptor, both shown to be pro-atherogenic (review, Hansson 2006), were found to be expressed by qPCR. However, the observed rosiglitazone-mediated suppression of inflammatory genes induced by LPS and IFNγ in macrophages was not observed in these cells. It was determined that a candidate approach might not reveal PPARγ target genes in these models and a microarray experiment was designed in an attempt to find PPARγ target genes. Very few genes were up-regulated by rosiglitazone and the majority of genes that were significantly altered were repressed only in the presence of IFNγ (Table 1.1). A large number of these repressed genes encode proteins required for oxidative phosphorylation. Reactive oxygen species (ROS), resulting from oxidative phosphorylation, bind nitric oxide in the endothelium, thereby inhibiting optimal vasofunction. Thus, PPARγ may confer some protection by reducing ROS production. However, these results were not robust and could not be validated with qPCR in larger sample numbers. There are many reasons for discrepancies between microarray and qPCR results, including which genes are used for normalization, primer and probe

sensitivities, amplification bias, etc. QPCR normalization was attempted with several housekeeping genes, but results still failed to mimic those of microarray. The lack of ligand activation in these cells and the inability to validate microarray data prompted investigation in additional *in vitro* models.

In general, gene expression in a given cell type may depend on anatomical origin. Thus, PPARγ RNA expression was assayed in human aorta (HAEC) and coronary artery (HCAEC) endothelial cells. PPARγ RNA was found to be at least 10-fold greater in these cells. Pro-inflammatory genes implicated in atherosclerosis, like MCP-1 and VCAM, were more robustly induced by LPS and IFNγ in HCAEC (Fig1.1) and thus chosen for subsequent studies.

As observed with HUVEC, induction of classic PPARγ targets such as ABCG1, LXRα and SCD-1 was not observed in HCAEC, but are in fact slightly up-regulated with lenti-conferred PPARγ shRNA. The elevated CD36 scavenger receptor expression in the absence of PPARγ is particularly striking and consistent (Fig1.2). Thus, there is an apparent lack of PPARγ ligand transcriptional activation in all human endothelial cells assayed. Up-regulated scavenger receptor expression is a requisite of foam cell development and thus plays a key role in atherosclerotic plaque lipid accumulation and instability. Indeed, knockout of CD36, confers protection against atherosclerosis in mice (Binder 2003, Febbraio 2000, 2004). CD36 is a classic ligand-activated PPARγ target gene and so this result is unexpected. The de-repression of target genes seen in HCAEC with PPARγ knockdown suggests this nuclear receptor represses target genes in endothelial cells *in vitro* or in the absence of laminar flow. Perhaps in this context, PPARγ is predominantly repressive of its target genes, including CD36. Lesions and

plaques most commonly form in branched or curved regions of the aorta with increased oscillatory flow or turbulence whereas regions subjected to laminar flow are protected. Thus the lack of laminar flow *in vitro* may create a model for PPARγ activity in aortic regions prone to atherosclerosis.

There are several potential explanations for this lack of ligand activated PPARγ activity *in vitro*. First, a naturally occurring splice variant of PPARγ, termed PPARγ ORF4, has been reported in humans and mice. It is detected in tumors and healthy tissues, although at low levels. It lacks the ligand binding and AF2 domains, and has dominant-negative activity when over-expressed (Bouanchou 2007, Sabatino 2005). Its presence could be one reason for lack of PPARγ ligand transcriptional activation. Q-PCR with primers designed to span the unique exon-intron junction revealed the presence of this reported variant in human and mouse endothelial cells (Fig1.3). While this may be a reason for a lack of PPARγ activation by ligand in these cells, there may also be endogenous PPARγ antagonists. In the absence of laminar flow, epoxide hydrolase activity is increased, producing DHETs (dihydroxyeicosatrienoic acids), the hydrolyzed by-products of EETs, that can act as inhibitors of rosiglitazone (as shown by luciferase assay in BAECs), reducing rosiglitazone activity 50% (Liu 2005).

In addition to ligand activated transcription, PPARγ can also repress transcription in the presence of ligand by stabilizing repressor complexes on inflammatory gene promoters regulated by NF-kB, STAT1 and AP-1 (review, Ricote 2007). In an effort to determine if PPARγ ligand represses inflammation and adhesion in endothelial cells, we assayed ligand effects on gene expression induced by pro-inflammatory stimuli, LPS and IFNγ, in HCAECs. In general, experimental results varied greatly with exposure time, lot

(individual), passage number and PPARγ ligand co-treatment versus pre-treatment. Effects on control versus PPARγ knockdown HCAECs were also variable. Thus, it this cell type and context was deemed unsuitable for determining the role of PPARγ in endothelial inflammation.

Due to the challenge of controlling experimental variability in human cells, it was deemed necessary to isolate primary endothelial cells from mice. Although technically challenging due to their minute quantity in any given tissue, the use of primary mouse endothelial cells is increasing as appropriate tools and reagents become readily available and optimized protocols published (van Beijnum 2008, Zhao 2002, Pozzi 2000). This provides a source of endothelial cells of similar genetic background and environmental conditions limited only by the availability of the mouse strain. In addition, cells can be isolated from genetically modified mice and mice treated with various conditions such as drug and high cholesterol diet. In addition, inflammation studies are not potentially complicated by viral infection used to achieve knockdown. Finally, RNA may be extracted from freshly isolated endothelial cells to capture and compare *in vivo* gene expression.

1.2 Isolation & Validation of Mouse EC

Cells were isolated based on a protocol assembled from the collective experience of Bill Atkinson, Yaw-Chyn Lim, and Mandy Zervoglos of the Vascular Research Division, Department of Pathology, Brigham and Women's Hospital (http://vrd.bwh.harvard.edu/core_facilities/mlec.html). See Appendix (A.2) for a detailed description of the isolation protocol used for these studies. Endothelial cells were

isolated from heart and lung using immuno-magnetic selection.   Viable cells were never successfully obtained by FACS sorting in our hands. Cells were cultured in EGM®-2MV Microvascular Endothelial Cell Growth Medium-2, 5% serum (Lonza Inc) and passaged with Accutase (Innovative Cell Technologies). These reagents were selected for optimal cultured cell viability and growth.

Identification and purity of cultured EC were assessed by morphology, gene expression (QPCR), as well as immunofluorescence and FACS analysis with CD31 and VEGFR antibodies. They were deemed functional by their ability to take up acetylated LDL and form tubes on matrigel (Fig 1.4). Freshly isolated cell purity was assessed by comparing gene expression in WT cells to those with an endothelial specific deletion of PPARγ. All validation, gene expression and functional studies were performed with cells passaged less than five times.

1.3 *In Vivo* PPARγ WT vs KO EC Gene Expression Comparison

As mentioned previously, these studies were motivated by the results of an atherosclerotic study in an endothelial specific PPARγ knockout mouse model.   The model is generated by breeding Tie2-cre mice with PPARγ flox which generates a tissue-specific knock out of PPARγ in endothelium and hematopoietic stem cell lineages (Kisanuki 2001). To put this model in the context of atherosclerosis, these mice are further bred into the LDLR null background. This gives rise to an atherosclerosis model with an endothelial and hematopoeitic PPARγ deletion. These mice are sub-lethally irradiated and transplanted with wildtype PPARγ bone marrow with a GFP transgene marker which makes the deletion of PPARγ endothelial-specific. This provides a model for specifically determining the contribution of endothelial PPARg to atherosclerosis and

its effect on lesion monocyte infiltration (Fig 1.5). Confocal microscopy revealed increased atherosclerotic lesion macrophage infiltration after two weeks of atherogenic diet. This is relatively early in the development of the disease and it suggests a role for endothelial PPARγ in early stages and prevention. A microarray experiment was designed to identify endothelial gene expression changes due to the absence of PPARγ in the early stages of pathology. Endothelial cells were harvested from endothelial PPARγ wildtype and knockout, LDLr-/- mice at zero week and after two weeks of atherogenic diet. RNA was extracted immediately following isolation of cells. (These cells were never seeded and spent no time in culture). RNA from heart endothelial cells was analyzed, as lung-derived cells expressed relatively little PPARγ.

Only 40 genes were down-regulated in the absence of PPARγ at 0 week compared to control endothelial cells, whereas over 1000 genes were up-regulated (1.5-5-fold), including classic target genes CD36 and lipoprotein lipase and PPARγ endothelial target, GPIHBP. In agreement with the HUVEC microarray results, this suggests PPARγ acts predominantly as a transcriptional repressor in endothelial cells in the absence of an atherogenic diet. Contrastingly, CD36 expression doubled in control cells after 2 weeks of diet, while there was no change in knockout cells. Indeed, CD36 expression is suppressed ~40% in the knockout compared to control cells after 2 weeks diet. This may be evidence of the sought after PPARγ transcriptional activation triggered by the presence of increased cholesterol or modified forms of cholesterol in the wildtype cells, but not in the knockout.

Strikingly, there were no significant differences between PPARγ knockout at 0 week and PPARγ wildtype after 2 weeks of atherogenic diet. In addition, there were less

than 100 significant gene expression differences between PPARγ wildtype and knockout endothelial cells after 2 weeks atherogenic diet. These results imply that an endothelial PPARγ deletion plays a role in the initiation of atherosclerosis and, without any high cholesterol challenge, the absence of PPARγ mimics that of atherosclerotic condition in wildtype cells after two weeks.

Many of the genes most up-regulated (two to five-fold) in the absence of PPARγ under basal conditions (0 week) were those reportedly involved in endothelial cell migration and extracellular matrix remodeling. This activity is particularly relevant to plaque stability and increased risk of thrombosis. In addition, KEGG and GO analysis revealed numerous genes found in several cellular functions or structures that were also up-regulated under basal knockdown conditions, namely proteasomal degradation, gap junction, apoptosis and oxidative phosphorylation. All components were increased approximately two-fold.   Also of note, was the increase in several chemokines and adhesion molecules implicated in atherosclerosis. Q-PCR validated much of these data. See Table 1.2 for a summary of these results.

Gene expression suggests several pathways are up-regulated in the endothelial PPARγ knockout. Studies point to a correlation between an increase in these activities and advanced atherosclerosis. For example, the ubiquitin–proteasome system is the major pathway (up to 80% to 90%) of nonlysosomal degradation of intracellular and oxidized proteins. Targeting the proteasome with proteasome inhibitors has been shown to worsen atherosclerosis in pigs (Herrmann 2007). In addition, apoptosis in endothelial cells is generally considered atherogenic, decreasing plaque stability, creating molecules for adhesion, and impairing vasofunction (i.e. hardening artery). There is increased

endothelial apoptosis in plaques or regions of turbulence as opposed to shear stress. Furthermore, oxidative phosphorylation and its resultant reactive oxygen species produce oxidized lipoproteins capable of inducing endothelial cell apoptosis, promoting foam cell development and thereby plaque development. As mentioned previously, many genes encoding components of the oxidative phosphorylation pathway were down-regulated by PPARγ ligand in HUVEC as assayed by microarray. It is difficult to predict which changes are causes or effects of increased atherosclerosis. Further investigation of these results is ongoing.

1.4 *In Vitro* PPARγ Effects on Mouse Endothelial Cell Gene Expression

Unlike what was observed of human endothelial cells (HUVEC and HCAEC), classic PPARγ targets are robustly induced by ligand in mouse endothelial cells, more so in those derived from heart than lung (Fig 1.6). Reasons for this may be due to the nature of the source. Depending on the function of the tissue the blood vessel infiltrates, endothelial cells may play very different roles. Arterial and venous endothelial cells have distinct gene expression profiles as do endothelial cells of the micro- and macro-vasculature (Davies 2008). Retinol binding protein 7 (Rbp7), a mediator of lipid uptake, has been proven by EMSA and ChIP to be a direct PPARγ target gene and thus it's expression may be an indicator of PPARγ transcriptional activation (Zizola 2008). When Caprioli et al. made a transgenic mouse expressing a fused Rbp7-lacZ gene, they observed Rbp7 expression in endothelium of the heart, adipose and skeletal muscle, but not the brain, liver or lung. In addition, lacZ expression was much stronger in microvasculature than large vessels. Expression in heart endothelium but not in lung correlates with our observations of PPARγ expression. Thus, it is possible PPARγ

transcriptional activation may be restricted to the microvasculature primarily functioning to regulate lipid uptake and metabolism, while it acts predominantly as a repressor in large vessels to reduce inflammation as needed.

To query PPARγ ligand effects on inflammation in wildtype mouse endothelial cells, they were treated with LPS in the after DMSO (control) or 1μM rosiglitazone (TZD, PPARγ ligand) overnight treatment. Cells were harvested for RNA after 15h treatment and gene expression was assayed by QPCR. 15h was chosen to more closely model a chronic inflammatory condition. LPS induced cytokines implicated in atherosclerosis, such as MIP-2, CX3CL1/fractalkine and MCP-1 as well as adhesion molecules such as ICAM, VCAM and E-selectin as much as 800 fold over control. Notably, TLR2 (20 fold) and LDLR (4 fold) were also induced. Curiously, PPARγ isoform 2, but not isoform 1, was also induced 20 fold, an isoform is predominantly found in white adipose tissue with a key role in lipid uptake. Both isoforms are expressed in bone marrow derived macrophages and are dramatically down-regulated after 15h LPS treatment (Barish 2005), suggesting inflammatory signaling effects on PPARγ expression differs in macrophages and endothelial cells. All of these genes were slightly repressed by rosiglitazone treatment supporting an anti-inflammatory role for this ligand. However, we observed temporally dynamic LPS-induced changes in HCAEC RNA expression in an extensive time course experiment and a similar diligent time course experiment with protein analysis will be required to determine the overall effect of PPARγ ligand on inflammation.

More striking than PPARγ ligand effects on inflammation, was the absence of the nuclear receptor itself. Endothelial cells were simultaneously harvested from PPARγ-

flox, cre-negative and cre-positive mice and cultured. Cells were stimulated overnite

with DMSO or rostiglitazone, and subsequently for 8h with LPS, a time closer to when

many cytokines were observed to reach peak expression with LPS treatment in HCAECs.

While rosiglitazone suppression of cytokine and adhesion molecule gene expression was

not observed in PPARγ wildtype or knockout cells, the absence of PPARγ in most cases

enhanced the expression of these genes (Fig 1.7). These included TLR2, TLR4, CXCL16,

E-selectin, P-selectin, GM-CSF, GRO alpha, MCP-1, CX3CL1 and CXCR4. There is

increasing evidence for all of these factors to be pro-atherogenic, functioning to recruit

and aid the infiltration of pro-inflammatory monocytes, leukocytes and platelets. In

particular, the endothelium may play a specific role in pro-atherogenic TLR2 signaling.

While a total TLR2 knockout reduces lesion area in LDLr-/- mice, transplant of wildtype

bone marrow into these mice does not alter this protective effect, suggesting it is

occurring through a cell type other than hematopoietic derived cells, such as the

endothelium (Mullick 2005). Indeed laser confocal microscopy reveals increased

expression of TLR2 in areas disturbed blood flow and plaque-prone regions of the aorta,

such as the lesser curvature of the aortic arch, and this expression is exclusive to the

endothelium (Mullick 2008). These gene expression differences indicate that PPARγ

confers suppression of LPS-stimulated inflammation. Additional experiments with

TLR2-specific agonsits, IFNγ or TNFα will confirm how broad PPARγ's role is in

inflammation.

1.5 Endothelial PPARγ Inhibits Leukocyte Adhesion

As mentioned previously, there was increased macrophage infiltration in the

aortic lesions of endothelial-specific PPARγ knockout mice. This could be due to

increased proliferation, migration or adhesion of monocytes to the endothelium. There is evidence that PPARγ ligands can suppress adhesion molecule expression. However, the ligands or the concentrations used are not specific for PPARγ. (Pasceri 2000, Wang 2002). Thus, an assay was developed to determine if PPARγ ligand can suppress monocyte adhesion. Briefly, endothelial cells are treated overnite with PPARγ ligand (1uM rosiglitazone), washed, treated with LPS for 6h, washed again, and incubated with fluorescently labeled monocytes. After incubation, all non-adhering monocytes are washed away and fluorescence of remaining cells is quantified with a fluorescence plate reader. This method demonstrated that PPARγ ligand treatment, of either human or mouse endothelial cells, suppresses monocyte adhesion (Fig 1.8a).

In an effort to determine which adhesion molecule(s) may be responsible for this effect, endothelial cells were harvested for RNA after fluorescence readings. While no differences were observed in classic endothelial adhesion molecules such as VCAM, ICAM, and E-selectin between LPS and LPS with rosiglitazone treated cells, there was a significant inhibition of CX3CL1, otherwise known as fractalkine (Fig 1.8b). Fractalkine is expressed in smooth muscle cells and endothelial cells and is membrane bound in the latter (Bazan 1997). It is highly chemotactic to many leukocytes with CX3CR1. CX3CR1 knockout, ApoE-/- mice have reduced atherosclerotic lesions and the deletion of CX3CL1 further reduces suppressed atherosclerosis in CCR2-/- knockout mice (Saederup 2008), suggesting these two chemokines may be simultaneously targeted for synergistic therapeutic effects.

CONCLUSION

Work in our lab has demonstrated a protective role for endothlial PPARγ in atherosclerosis. Microarray reveals primary endothelial cells isolated from LDLr-/- mice with an endothelial specific deletion of PPARγ, compared to those expressing wildtype PPARγ, have increased expression of genes corresponding to oxidative phosphorylation, proteasome structure, apoptosis and gap junction proteins. When stimulated *in vitro* with TLR4 and TLR2 ligands, these cells also express a higher level of pro-inflammatory atherosclerotic markers. These markers include TLR4 and TLR2 themselves. In particular, TLR2 expression is increased under all conditions (basal or stimulated) in PPARγ knockout endothelial cells relative to wildtype. Xu et al have shown that PPARγ agonists repress TRL2, MyD88 and CD14 expression in primary mouse microglia, but this study uses non-specific ligands or concentrations. Dasu et al showed that pioglitazone reduces TLR2 & 4 expression in peritoneal macrophages of db/db mice and human monocytes. In addition, bacterial infection induction of PPARγ expression in macrophages has been shown to be TLR2 dependent. Simultaneous treatment with PPARγ antagonist proves this bacterial induction of PPARγ serves to produce lipid droplets (Almeida 2009). Interestingly, in our endothelial cells, TLR ligands LPS and Pam3CSK4 strongly induce PPARg isoform 2, but not 1 and the PPARg2 isoform is responsible for lipid uptake in adipocytes. The elevated TLR2 expression in PPARγ knockout endothelial cells suggests this nuclear receptor may suppress TLR2 expression in anti-inflammatory feedback inhibition loop. There is no presently no evidence for PPARγ transcriptional regulation of TLR2.

Reports of endogenous PPARγ ligands led to the investigation of ligand activity in these cells as well. The ability of PPARγ ligand to inhibit pro-inflammatory markers

varied with time and a monocyte adhesion assay was thus developed to determine if ligand treatment has a functional effect on pro-atherosclerotic activity. PPARγ ligand treatment proved to dramatically suppress adhesion of monocytes. QPCR revealed gene expression of classic adhesion molecules such as VCAM and ICAM were not altered. Instead, CX3CL1/fractalkine, a potent chemokine that is membrane bound in atherosclerotic lesion endothelium, was down-regulated.

A search for PPARγ ligand activated target genes revealed dramatic differences between the human and mouse endothelial cells assayed. Ligand did not induce CD36, LXRα or ABCG1 in human coronary artery endothelial cells as it is known to in adipocytes and macrophages. Conversely the knockdown of PPARγ in endothelial cells caused CD36 expression to increase more than 10-fold, with a similar trend in other classic targets. Contrary to the effects in human cells, ligand dramatically induced these targets in mouse heart and lung endothelial cells *in vitro*. This induction was not observed in PPARγ knockout endothelial cells confirming the dependence of ligand effects on PPARγ. Unlike the human PPARγ knockdown cells, there was no increased basal expression of these targets in the PPARγ knockout cells. This may be explained by species differences or off target effects of lentivirus in the human cells. Alternatively, this may be a due to differences between macro and micro vascular physiology. This is supported by the reported expression of PPARγ target gene Rbp7 in capillary beds of mice, but not in large vessels. The availability of human microvascular and/or mouse macrovascular endothelial cells would likely make it possible to resolve these differences.

Gene expression analysis is an ideal tool for screening and initial inquiry. However, one cannot assume gene expression changes have an effect on cellular

functions. Rigorous time course and dose response experiments revealed gene expression analysis in HCAECs due to inflammatory signaling is highly dependent on dose and duration, peaking and resuming basal levels within a couple of hours. Even rigorously assaying protein expression does not reveal if these expression changes have any functional significance. Therefore, it is important to develop functional assays to evaluate the physiological effects of nuclear receptor expression and corresponding ligand treatment. In accordance with this, assays are being developed and optimized to test the effects of PPARγ ligand and deletion in endothelial cells on oxidative phosphorylation and ROS production, as well as endothelial-leukocyte chemotaxis and transmigration.

In addition, while effects on lipid or cholesterol metabolism and inflammation in microvasculature due to PPARγ knockout may account for atheroprotective effects, there may be a different mechanism of protection in large vessels exposed to greater shear stress, turbulence and mechanical forces. To address this, methods of laser microdissection are being investigated and mouse aorta immunohistochemistry optimized. Based on the results of these techniques, neutralizing antibodies *in vivo* may be used to determine proteins responsible for the more extensive atherosclerosis and macrophage infiltration observed in endothelial PPARγ knockout mice.

The endothelium is a heterogeneous organ, its physiology and gene expression tailored to the role it plays in its surrounding tissue, be that lung, heart, brain or aorta. PPARγ proves to be no exception to these variations. Primary mouse endothelial cell gene expression and functional analysis supports an anti-inflammatory and lipid managing role for PPARγ and its ligand. However, determining its functions and

respective mechanisms in atherosclerotic lesion endothelial cells will most likely require immunohistochemistry, *in situ* hybridization or laser capture microdissection with subsequent genetic analysis.

Chapter 2. Characterizing Nuclear Receptor Expression in Endothelial Cells

Human and mouse endothelial cells were assessed for nuclear receptor expression using a high throughput Q-PCR platform. For human studies, RNA was isolated from coronary artery endothelial cells (HCAEC), aorta endothelial cells (HAEC) and umbilical vein endothelial cells (HUVEC). For mouse studies, endothelial cells were isolated from hearts (MHEC) and lungs (MLEC) of common strains, C57BL/6J and 129/SvJ. In an effort to identify potential roles for nuclear receptors in vascular disease, endothelial cells were similarly isolated from LDLr-/- mice fed normal chow or atherogenic diet. RNA was extracted at the time of harvest in an effort to capture *in vivo* gene expression. In addition nuclear receptor expression was assayed in cultured mouse lung and heart endothelial cells, passages 2-4. Several methods were used to validate cultured mouse endothelial cell identity and function, including gene expression by Q-PCR, immunofluorescence, matrigel tube forming assay and ac-LDL uptake (Fig 1.4).

2.1 The Atlas of Endothelial Nuclear Receptors

There are 50 known human nuclear receptors. Gene expression analysis revealed the presence of 28 in human endothelial cells (Fig 2.1A), including eight members of the endocrine receptor family, which are activated by high-affinity hormonal lipids, seven adopted heterodimeric orphan receptors, which are regulated by low-affinity dietary lipids, and 12 true orphan receptors: six constitutive activators and seven constitutive repressors. Expression levels vary modestly among HUVEC, HAEC and HCAEC.

Gene expression analysis revealed 35 of the 49 known mouse nuclear receptors were expressed in heart and lung endothelial cells. This includes twelve endocrine receptors,

eight adopted heterodimeric orphan receptors, eight constitutive orphan activators and seven constitutive orphan repressors (Fig 2.1B). Expression levels for several nuclear receptors can vary greatly between heart and lung derived cells. In general, they do not differ between strains.

Table 2.1 shows a complete tabulation of all expressed and non-expressed receptors in both species along with their classification and nomenclature.

The Endothelial Endocrine Receptors

Both mouse and human endothelial cells express androgen receptor (AR), estrogen receptor beta (ERβ), mineralocorticoid receptor (MR), glucocorticoid receptor (GR), Vitamin D receptor (VDR), thyroid hormone receptor alpha (TRα), and retinoic acid receptors alpha and gamma (RARα, RARγ). In addition mouse endothelial cells express progesterone receptor (PR), estrogen receptor alpha (ERα), thyroid hormone receptor (TRβ) and retinoic acid receptor beta (RARβ). In mice, ERβ and PR were only detected in mouse endothelial cells derived from lung tissue. While AR was detected in heart endothelial cells, it was 3-10 times higher in those from lung. Contrastingly, ERα was two to four-fold higher in mouse endothelial cells from heart versus lung. These differences in expression based on anatomical origin may indicate tissue specific functions.

The effect of estradiol treatment in cardiovascular health in post-menopausal women is controversial. While epidemiological evidence suggests hormone replacement therapy protects against coronary heart disease (CHD), large randomized controlled trials contest it can be detrimental (Hodis 2003, Cherry 2002, Hulley 1998). Interestingly, the

effects may largely be due to when therapy is initiated (Sherwood 2007). Billon-Gales et al. investigated endothelial ERα mediated effects of estradiol on atherosclerosis *in vivo* using an LDLr-/-, endothelial ERα knockout mouse model. The reduction of atherosclerotic lesions by estradiol is abolished in the absence of endothelial ERα. In contrast, protection conferred by estradiol is maintained in the absence of ERα in cells of hematopoietic lineage. This points to an exclusive role for endothelial ERα in estradiol-mediated protection against CHD. While evidence points to increased nitric oxide production and inhibition of platelet aggregation, the exact causes of ERα mediated protection in the endothelium are yet to be determined.

While PR was undetected in human cells, protein expression has been reported in human aortic endothelial cells (Welter 2003). Gene expression can change dramatically in primary cells under culture conditions. Indeed, expression of most nuclear receptors in primary mouse endothelial cells had dropped dramatically by P2 (data not shown).

The Endothelial Adopted Orphan Receptors

Adopted orphan receptors, peroxisome proliferator activated receptors (PPAR) α,δ, γ, liver x receptors (LXR)α,β, and retinoid xreceptors (RXR)α,β are expressed in both mouse and human endothelial cells. Farnesoid X receptor, FXRα, is expressed exclusively in mouse endothelial cells and expression unexectedly increases with passage number. Human and mouse PPARγ expression varied noticeably with anatomical source. MHEC PPARγ expression was approximately ten-fold higher than that of MLEC, and HAEC or HCAEC expression was as at least ten-fold higher than HUVEC. This was the

only significant difference observed in nuclear receptor expression among the various sources of human endothelial cells.

PPARγ ligands have been shown to repress inflammatory cytokines, adhesion molecules and reactive oxygen species in the endothelium both *in vitro* and *in vivo* as well as improve vascular tone and function through increased nitric oxide production (Murao 1999, Marx 2000, Pasceri 2000, Calabro 2005, Inoue 2001, Hwang 2007, Goya 2006, Calneck 2003). These effects have implications for treatment of diabetic retinopathy, macular degeneration and cardiovascular disease. PPARγ ligands also modulate proliferation and apoptosis of endothelial cells (Bishop-Bailey 1999, Lee 2006), potentially influencing atherosclerotic lesion or plaque stability *in vivo*. Many of these reports however use PPARγ ligands or ligand concentrations that with PPARγ - independent effects. The presence of an endogenous antagonist in the absence of shear stress may be the reason only high concentrations of PPARγ ligand activate this receptor *in vitro* (Liu 2005). Use of *in vitro* flow systems as well as genetic analysis could more definitively determine the endothelial role of PPARγ. Large clinical trials have established that PPARα ligands, fibrates, are cardio-protective, lowering lipid levels and improving endothelial function. However, as with PPARγ ligand, it is unclear where and how these effects are mediated.

The Endothelial Orphan Receptors

13 of 25 members of the orphan nuclear receptor family are expressed in both mouse and human endothelial cells. These include both constitutive activators [neuronal growth factor 1B (NGFIB), neuron-derived orphan A receptor (NOR) 1, nuclear receptor-

related (NURR) 1, estrogen-related receptor (ERR) 1, and RAR-related orphan receptors (ROR)α and γ] and constitutive repressors [liver receptor homolog 1 (LRH-1), Rev-Erb α and β, chicken ovalbumin upstream promoter-transcription factors(COUP-TF) 1 and 2, testicular receptors (TR)2 and 4]. These receptors can bind DNA as monomers, homodimers, or RXR heterodimers. Germ cell nuclear factor (GCNF) and RORβ were detected in mouse endothelial cells and tailless (TLX) in human cells, exclusively. The functions of most of these nuclear receptors are unknown in endothelium.

A recent survey of the diurnal expression profiles of all 49 mouse nuclear receptors in white and brown adipose tissue, liver, and skeletal muscle revealed that 25 of the 45 NRs are expressed in a rhythmic cycle (Yang 2006). Although such a rigorous temporal characterization was not carried out in this case, several nuclear receptors increased significantly from harvest time ZT6 to ZT10, most notably NGFI-B, NOR1, Nurr1, Rev-erbα and Rev-erbβ (Fig2.2). NGFI-B, NOR1, and NURR1 bind a common DNA element. They have been implicated in T cell apoptosis and dopaminergic neuron development and more recently, hepatic gluconeogenesis, β-adrenergic signaling in muscle and acute myeloid leukemia (Zetterstrom 1997, Masuyama 2001, Pearen 2006, Chao 2007, Pei 2006, Mullican 2007). Rev-erbs are constitutive repressors and closely related to the constitutively activating RORs. These bind a common DNA response element to mediate core clock genes such as Bmal. There is little known about the role of the circadian clock in vascular disease. Deaths from cardiovascular disease occur most often in the morning, when the endothelium is vulnerable and blood pressure surges. Recent investigation of Bmal-1 knockout and Clock mutant mice reveal increased vascular injury, and endothelial dysfunction, possibly due to reduced nitric oxide

production (Anea 2009). The evidence for temporal expression patterns of nuclear receptors in the endothelium suggests a new site whereby they may play a role in circadian regulation of energy metabolism and vascular disease.

2.2 Nuclear Receptors and Atherosclerosis

There was significantly increased expression of nine nuclear receptors in the MLECs of the atherosclerosis-induced model relative to wildtype (Fig 2.3). These included endocrine receptors PR and GR, adopted orphan receptors PPARγ, RARγ and LXRα, orphan receptors repressors TR2 and RORα, and orphan receptor activators COUPTF1 & 2. GR, PPARγ, LXRα and CoupTF1 were also elevated in MHECs approximately two-fold.

PR showed the greatest change, expression was 5 fold higher in the LDLR-/- mouse compared to wildtype, and further increased (resulting 6 fold) with two weeks of atherogenic diet. However, little is understood about the role of PR in CHD. Different reported PR ligands have been shown to oppose the beneficial effects of estrogen in animal models and clinical trials measuring disease markers in women, but not coronary incidents, suggest progestins may be harmful in humans as well (Lamon-Fava 2005, Wassman 2005).

Adopted orphan receptors PPARγ and LXRα expressions were increased 4 fold and 3 fold respectively with atherogenic diet. The PPAR and LXR nuclear receptor sub-families have been implicated as regulators of atherogenesis due to their known roles in lipid and glucose homeostasis, as well as inflammation (review Barish 2004). Indeed, a number of studies suggest that they are promising therapeutic targets for cardiovascular

disease (Li 2004, Mazzone 2006, Dormandy 2005). In the macrophage, PPARγ mediates cholesterol trafficking and inflammation. Consequently, its absence results in greater atherosclerotic lesion area (Chawla 2001). While there is *in vivo* and *in vitro* support for a protective role of endothelial PPARγ against atherosclerosis, an in vivo atherogenic challenge in a tissue specific knockout model will ultimately define the relevance of these studies.

2.3 Endothelial PPARδ and Atherosclerosis

While PPARδ expression did not significantly change in the atherogenic model, there was an increasing trend in lung endothelial cells. PPARδ has been shown to be athero-protective for mice via its anti-inflammatory activity in the macrophage (Lee 2003). To determine if endothelial PPARδ is also anti-inflammatory, endothelial cells were harvested from lungs of Tie2-Cre positive and negative, PPARd-flox mice, and stimulated with TLR ligands, LPS or Pam3CSK4 after overnite treatment with PPARδ ligand GW1516. Not only could ligand suppress induction of inflammatory genes considered pro-atherosclerotic, PPARδ deficiency conversely increased expression, with few exceptions (Fig 1.9). There was also a decrease in some proteins considered athero-protective, such as MCP-3, CXCL12 and IL-1R antagonist. This indicates an anti-inflammatory and thus protective role for this nuclear receptor in the endothelium.

There is less known about the role of orphan receptors in atherosclerosis. The activity of these receptors is regulated by expression level, post-translational modifications, and the presence of co-factors. *In vivo* studies of endothelial specific

deletion, mutation or over-expression may be required to establish the relevance of these receptors to this disease.

CONCLUSION

This study provides a comprehensive nuclear receptor profile of human and mouse primary endothelial cells in a context that is amenable to *in vitro* assays. Namely RNA was isolated from human cells including HUVEC, HAEC and HCAEC and mouse cells including MHEC and MLEC. All cells were harvested in early passage (P2-P4). In addition, RNA was isolated from freshly isolated MHEC and MLEC to capture *in vivo* expression. While RNA expression generally decreases *in vitro*, maybe due to a removal from mechanical, cellular or molecular stimuli, the nuclear receptor expression profile was maintained, arguing that these systems will support relevant mechanistic and functional studies. A significant number of nuclear receptors expressed in mouse endothelial cells were not observed in human, including endocrine receptors PR, ERα, RARβ and TRβ, adopted orphan receptor FXRα, and orphan receptors RORβ, GCNF, Reverbβ. TLX is the only nuclear receptor expressed in human cells, not expressed in mouse. Interestingly, none of the endothelial cells assayed express the xenobiotic receptors CAR or SXR/PXR. However, endothelial nuclear receptor expression was found to differ with anatomical origin. One cannot disregard the possibility that there are different nuclear receptor profiles in endothelial cells from other organs or in different physiological or diseased contexts. Instead, this data should be used as a guide in the consideration and selection of appropriate endothelial cell models for experimentation.

Coronary heart disease, including atherosclerosis, is the leading cause of death in western society. It is a close companion of obesity and insulin resistance, collectively termed the metabolic syndrome. The occurrence of the metabolic syndrome continues to increase in the western world as well as developing countries and the associated economic strain is alarming. Thus, it is vital to both our well-being and financial sustainability that we pursue cost-effective methods and therapies to prevent and treat this disease. Towards this goal, nine nuclear receptors were identified of which respective gene expression was elevated in freshly isolated endothelial cells from LDLr-/- mice compared to wild type mice fed normal chow. These included PR, GR, RARγ, PPARγ, LXRα, RORα, TR2, COUPTF1&2. In almost all cases they were further increased when LDLr-/- mice were fed atherogenic diet. These effects were more pronounced in MLEC than MHEC. These changes may be a response to increased circulating lipid levels or differences in vascular tone observed in this model, but determining the cause requires more in-depth studies. These data prompt further investigation of the roles of these nuclear receptors in the endothelium and particularly within the context of atherosclerosis. Confirmation of a protective or exacerbating role for these nuclear receptors in atherosclerosis, which may be dependent on the stage of lesion progression, is important for the development of therapies and pharmaceuticals to treat this epidemic.

Finally, this data hints at a role for nuclear receptors Reverbs and NGFI-B family members in diurnal or circadian rhythm in the endothelium. These nuclear receptor sub-families, as well as others, have been shown to have redundant functions in other tissues, and thus multiple endothelial specific deletions in the same mouse model may be required to determine if there is a systemic metabolic influence or a role in vascular

disease. Such challenges presented by the super family of nuclear receptors combined with the practice of primary endothelial cell isolation will take us on a difficult journey with many forks in the road. However, this study presents a map and tools for the exploration of nuclear receptors in the endothelium and signs with arrows pointing to roles in insulin signaling, cholesterol regulation, inflammation, circadian rhythmicity and beyond.
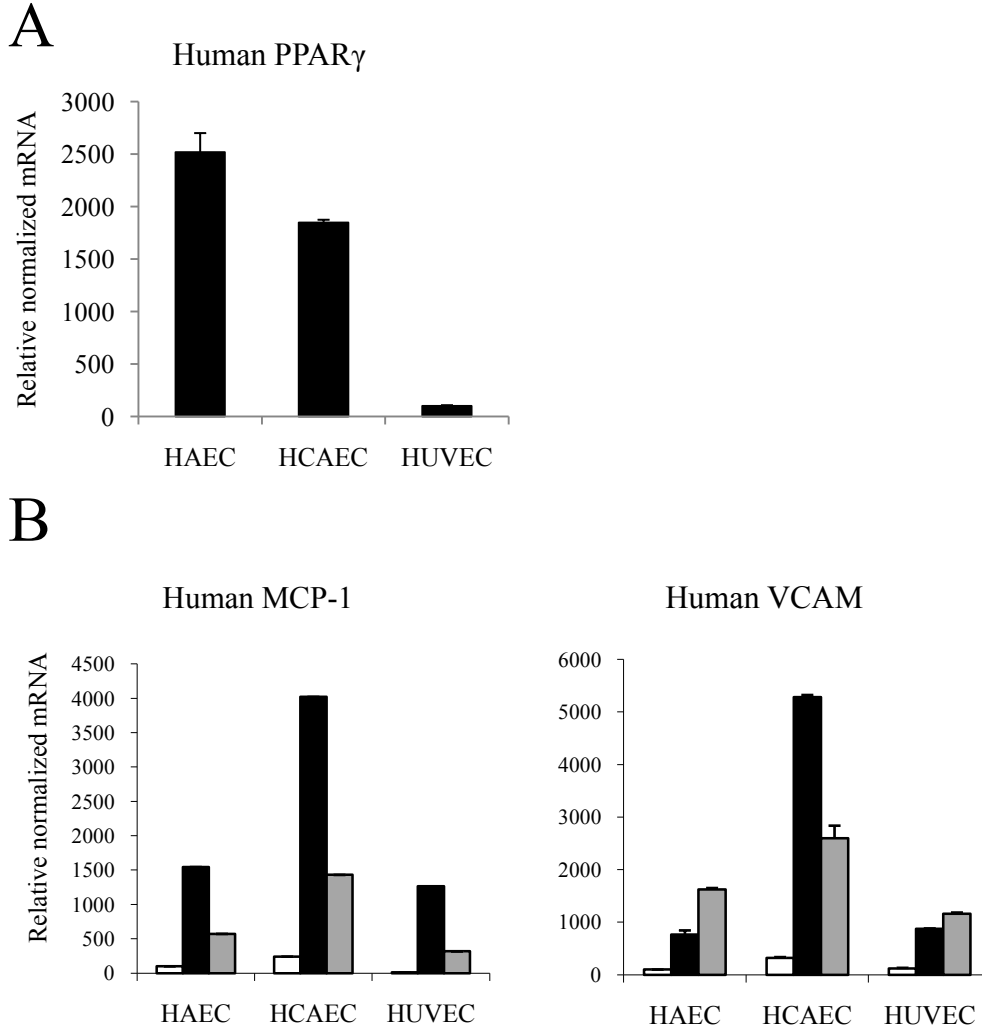
A.1   FIGURES

Figure 1.1 Human coronary artery endothelial cells express PPARγ and are responsive to inflammatory stimuli. RNA was isolated from (A) non-stimulated human aortic (HAEC), coronary artery (HCAEC) and human umbilical vein endothelial cells (HUVEC) and (B) HAEC, HCAEC and HUVEC stimulated with PBS (white bar),1μg/ml LPS (black bar) or 2ng/ml IFNγ (gray bar) for 12h. Gene expression was analyzed by qPCR.
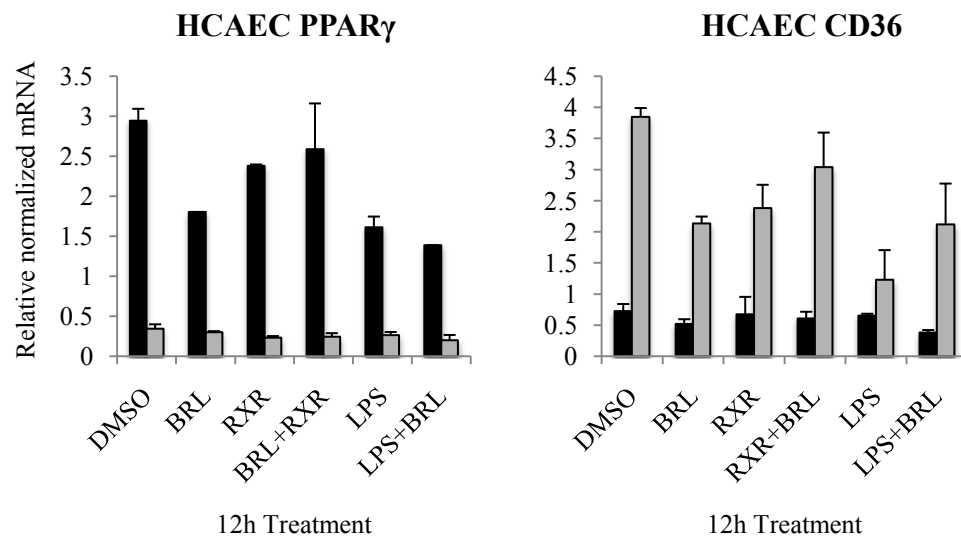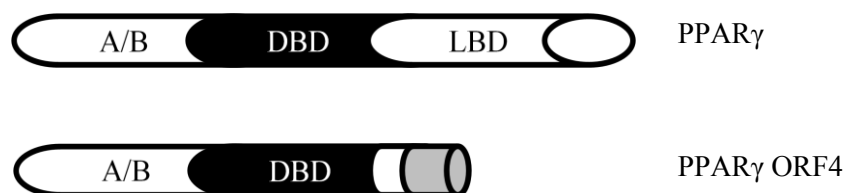
Figure 1.2 Scavenger receptor CD36 is up-regulated in the absence of PPARγ in human endothelial cells. HCAEC were transduced with lentivirus expressing control (black bars) or PPARγ (gray bars) shRNA. Cells were passages twice before treatments and harvested for RNA. Expression was measured by QPCR.
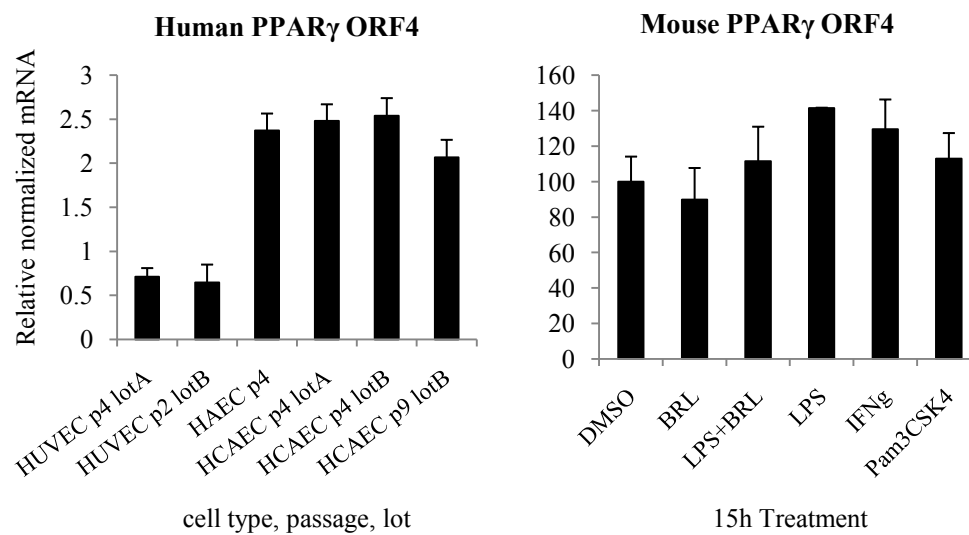
A

B

Figure 1.3 Human and mouse endothelial cells express a splice variant of PPARγ. (A) A schematic of PPARγ and splice variant (PPARγ ORF4) that includes part of intron 4 (gray region). (B) Primers spanning the unique exon-intron junction of PPARγ ORF4 were used to quantify relative levels in human endothelial cells and mouse lung-derived endothelial cells with different ligand treatments and inflammatory stimuli.
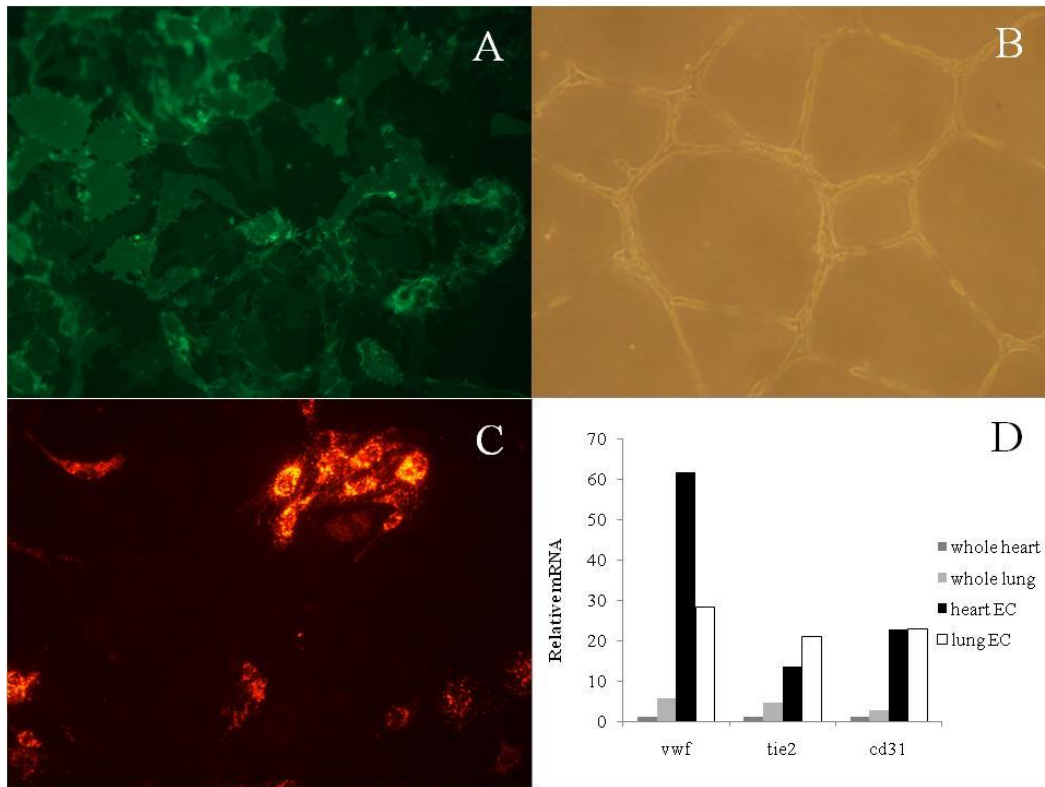
Figure 1.4 Validation of primary mouse endothelial cells. Endothelial cells were purified from homogenized and collagenase-digested mouse hearts and lungs using immunomagnetic beads. Endothelial cells were validated with CD31-FITC immunoreactivity (A), tube-forming assay (B), Ac-LDL uptake (C), and gene expression by QPCR (D).
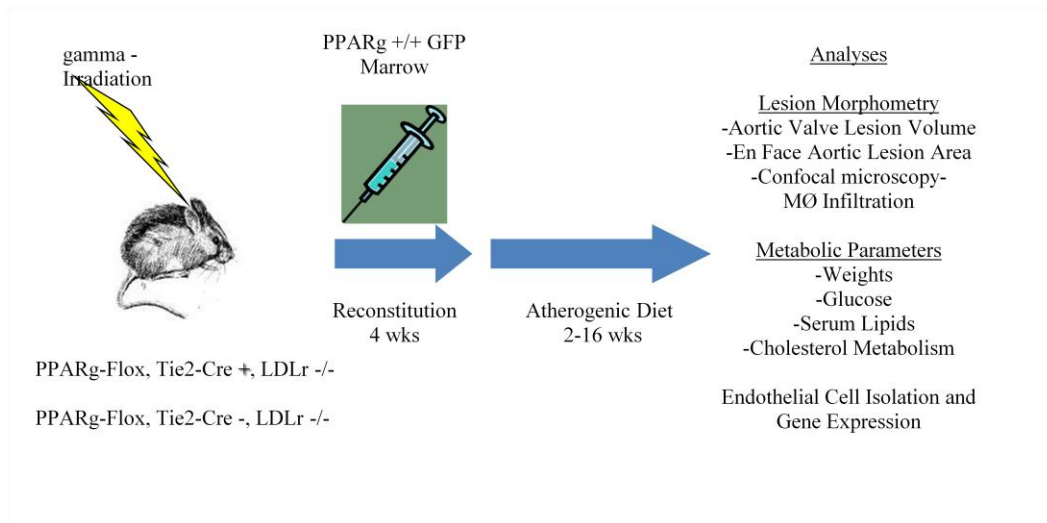
Figure 1.5 Development of endothelial PPARγ knockout atherogenic mouse model. PPARγ-Flox, Tie2-Cre negative and positive mice were sub-lethally irradiated and bone marrow re-constituted with PPARγ wildtype GFP+ bone marrow 24h later. After 4 weeks reconstitution, mice were fed atherogenic diet until harvests at various time points ranging from 2-16 weeks.
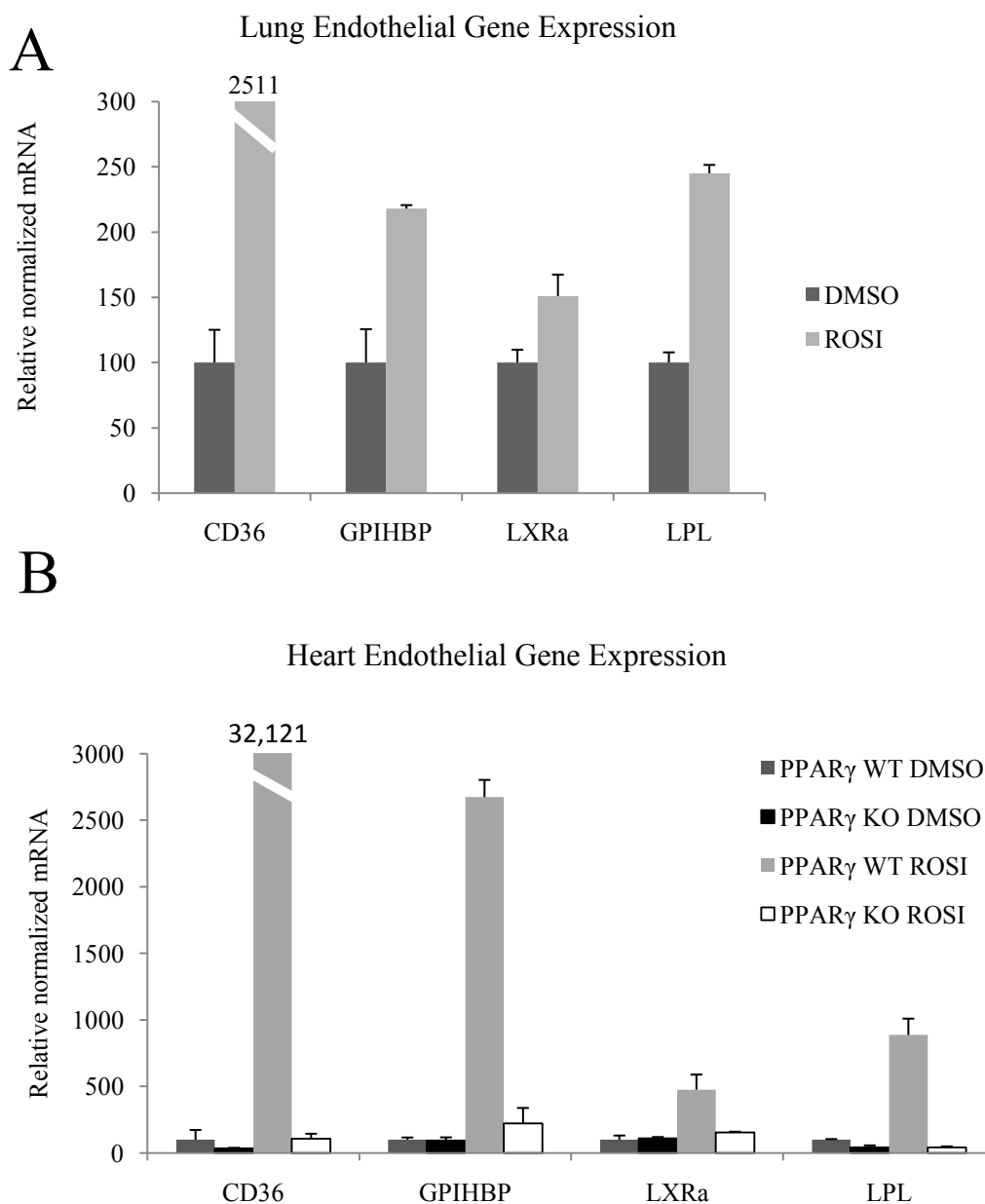
Figure 1.6 PPARγ ligand activation in mouse lung & heart endothelial cells.　　(A) Endothelial cells were isolated from lungs of C57BL/6 wildtype mice cultured to P4 and treated for 8h with 1μM rosiglitazone or DMSO. RNA was isolated and gene expression assayed by QPCR. (B) Endothelial cells were isolated from hearts of PPARγ- Flox,Tie2-Cre negative or positive mice, and similarly treated.
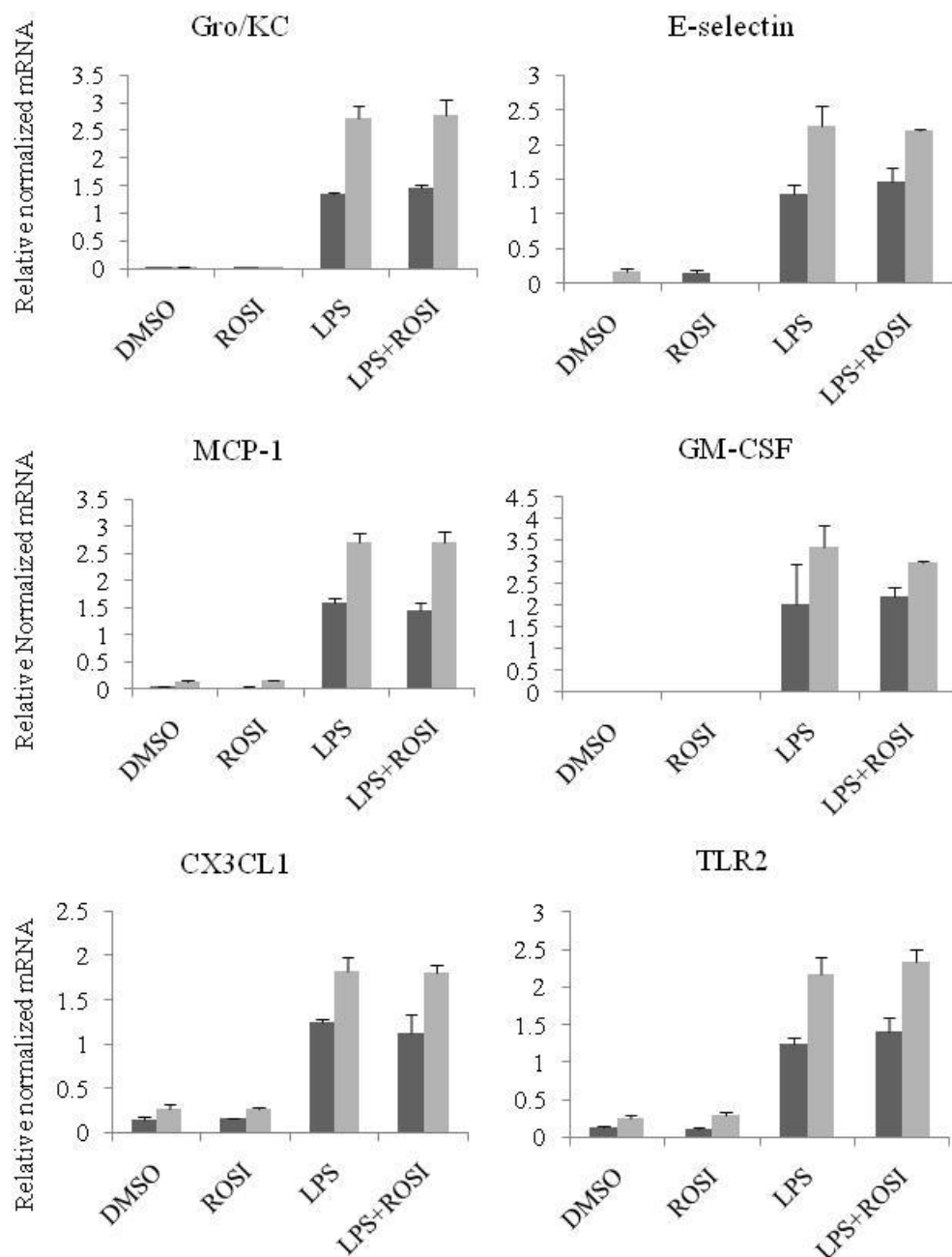
Figure 1.7 Pro-atherosclerotic gene expression is increased in PPARγ-deficient endothelial cells. Primary heart endothelial cells (P4) from PPARγ-flox, Tie2-cre positive and negative mice, were stimulated with DMSO or 1uM rosiglitazone overnite, followed by 8h treatment of 100ng/ml LPS. RNA was isolated and gene expression analyzed by QPCR.
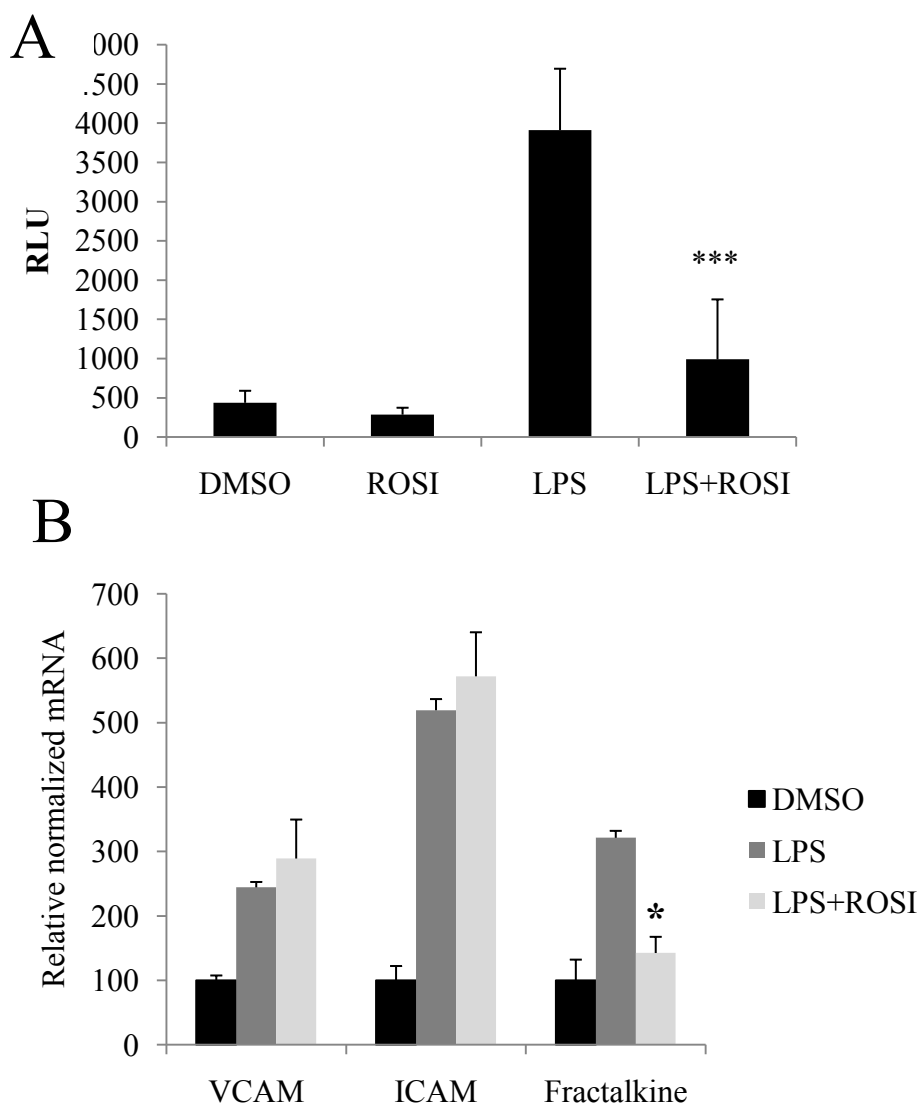
Figure 1.8   PPARγ ligand suppresses adhesion of monocytes to LPS-stimulated endothelial cells. (A) Primary mouse endothelial cells were stimulated overnite with DMSO or 1μM rosiglitazone and subsequently with 100ng/ml LPS for 6h. Endothelial cells were washed and co-incubated with calcein-AM-labeled human monocytes for 30m. Adherant cells were quantified with a fluorescence plate reader ex/em 485/535. (B) RNA was isolated from cells in adhesion assay and endothelial gene expression assayed by QPCR with primers specific to mouse.
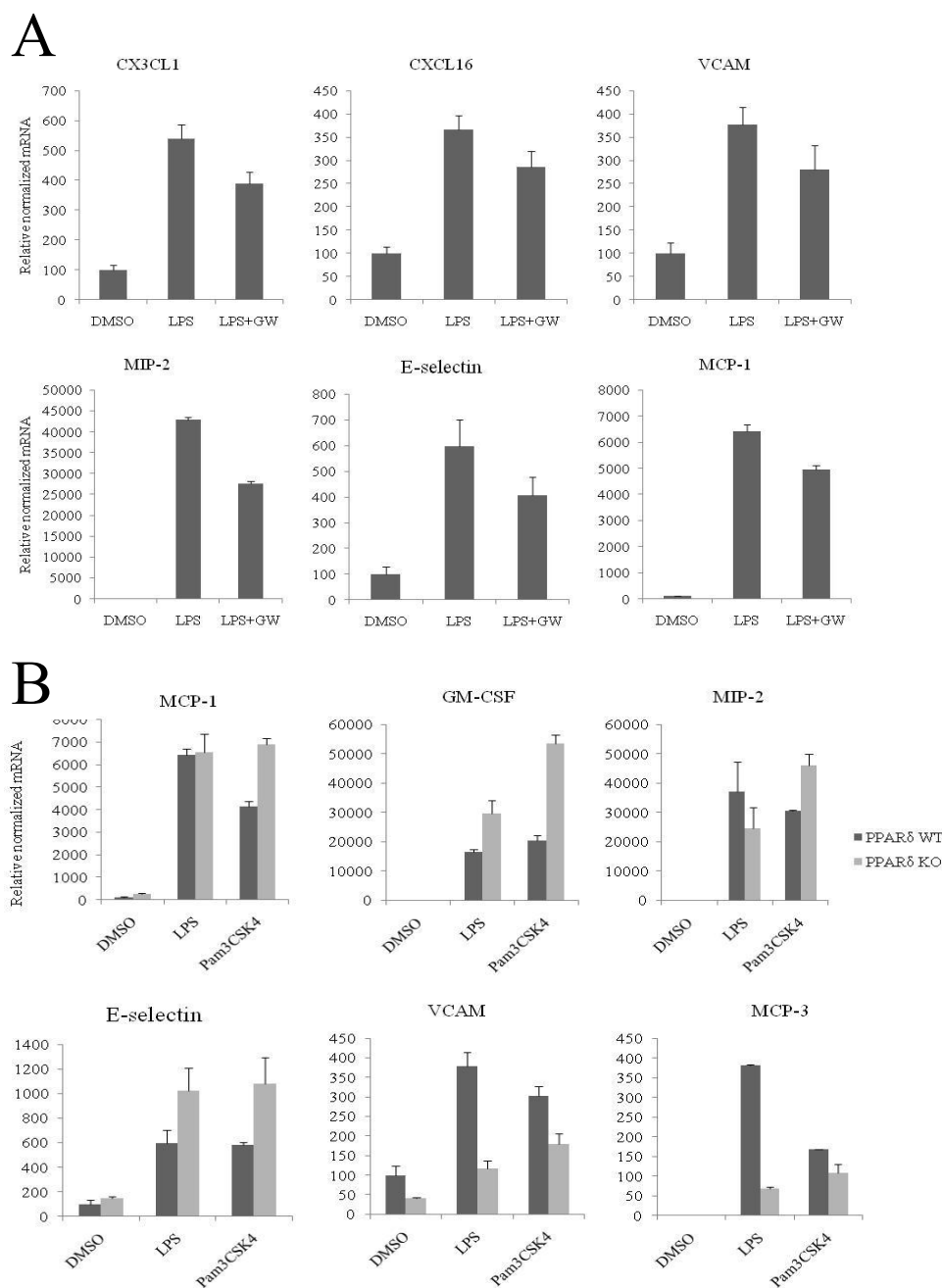
Figure 1.9 PPARδ Regulation of Inflammatory Markers of Atherosclerosis. (A) Endothelial cells were harvested from lungs of wildtype C57BL/6 mice. P4 cell were pre-treated overnite with 100nM PPARδ ligand GW1516 or DMSO and subsequently treated for 12h with 100ngl/ml LPS or PBS. (B) Cells were from lungs of PPARγ-Flox, Tie2-Cre positive or negative mice and treated with LPS or Pam3CSK4 for 12h.
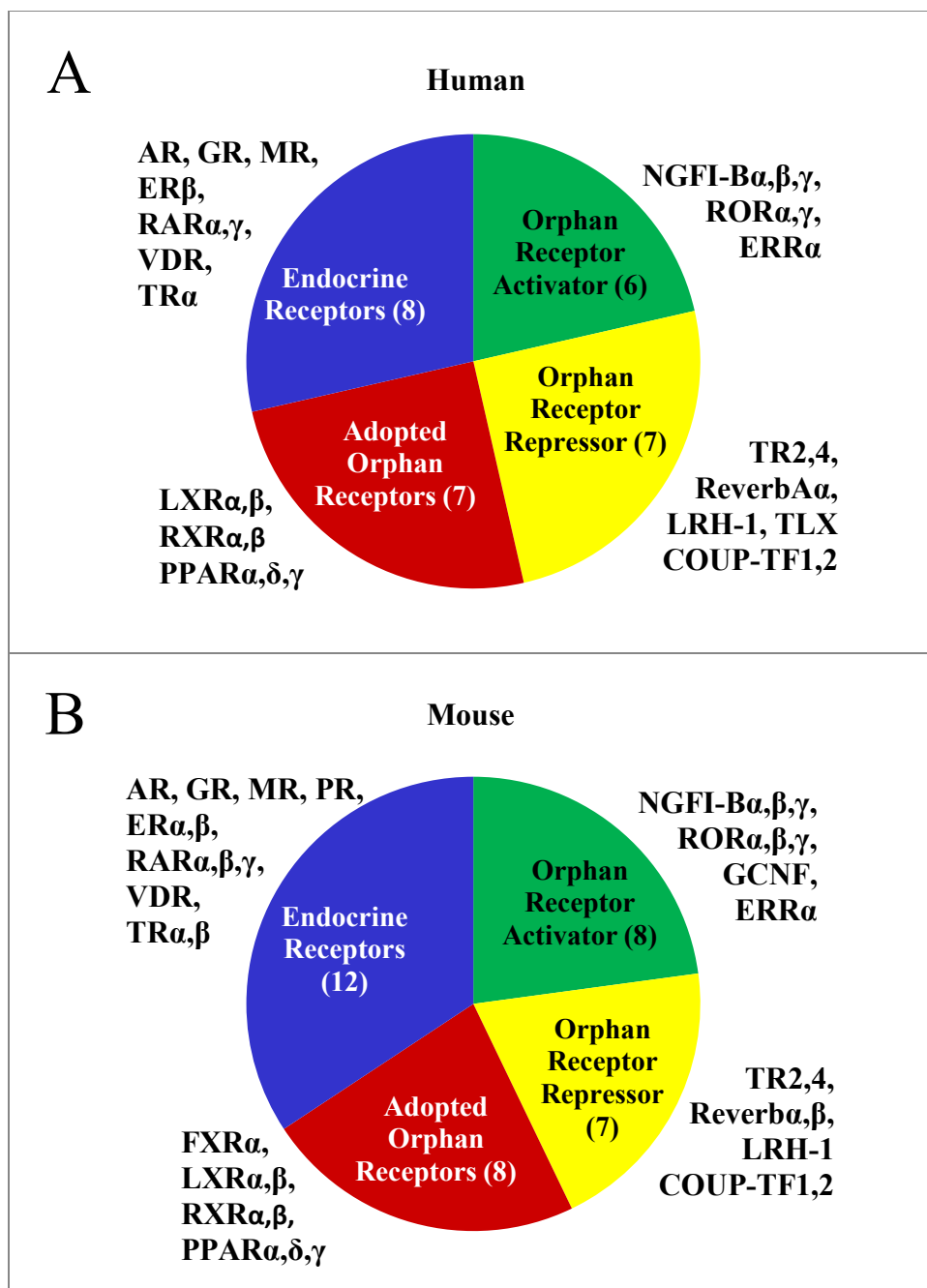
Figure 2.1 Human and mouse endothelial nuclear receptor gene expression profile. QPCR was used to analyze basal gene expression in (A) multiple lots of human endothelial cells: HUVEC, HCAEC and HAEC and (B) heart and lung primary endothelial cells from 12 week old C57BL/6J and 129/SvJ male mice. Primer sequences are available at www.nursa.org.
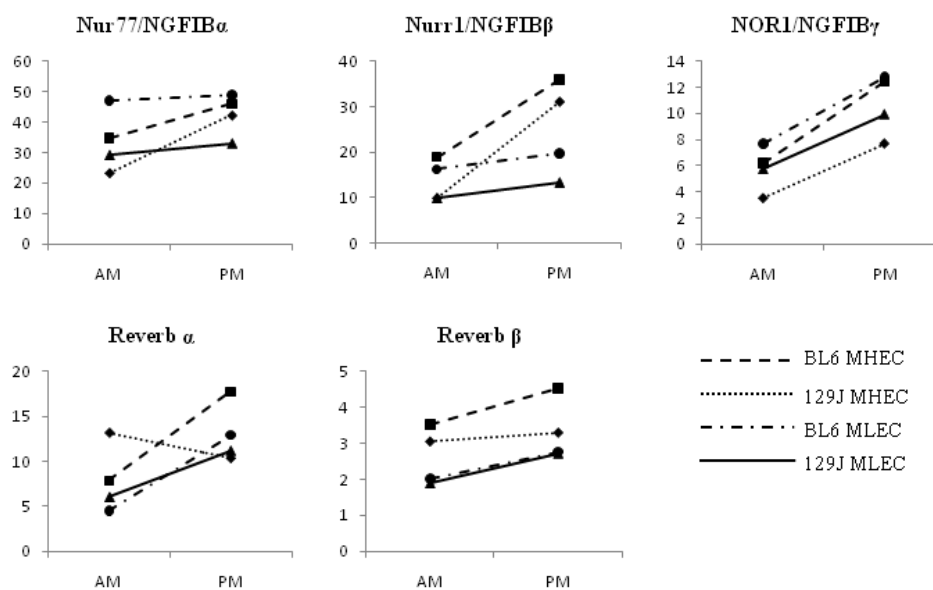
Figure 2.2   Endothelial nuclear receptor gene expression depends on time of day. Primary endothelial cells were harvested from heart (MHEC) and lung (MLEC) at ZT6 and ZT10 and nuclear receptor gene expression assayed by QPCR.
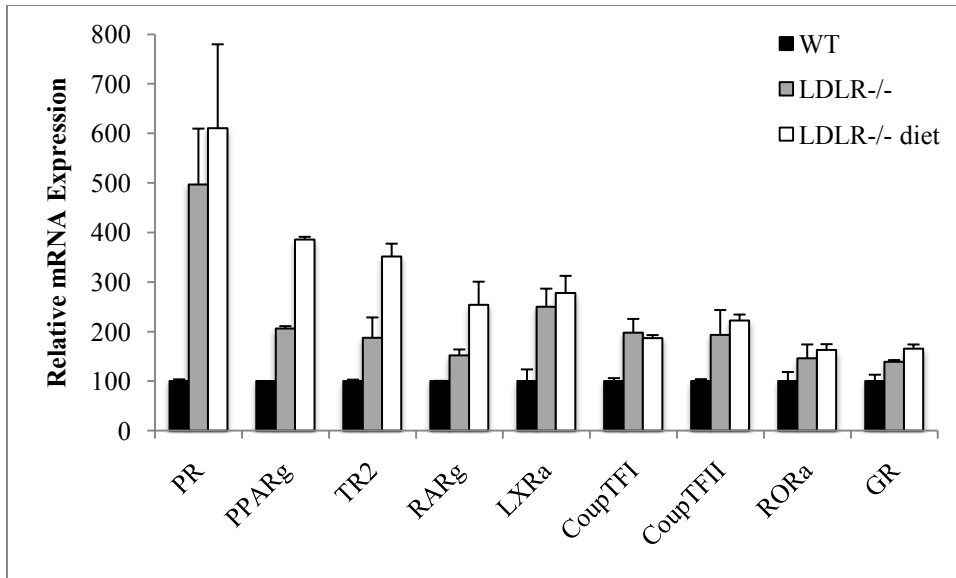
Figure 2.3 Primary mouse endothelial cell nuclear receptor expression increases with atherosclerosis. EC nuclear receptor expression of wild type mice fed normal chow were compared to those from LDLr-/- mice fed normal chow or atherogenic high cholesterol diet. All mice were in a C57BL/6J background. Mice were harvested in groups of five for lung and heart endothelial cells. RNA was extracted immediately following EC isolation. The data presented is an average of two cohorts of pooled lung cells. Error bars represent SD.

A2.   TABLES

Table 1.1   HUVEC Gene Expression: IFNγ or DMSO +/- PPARγ Ligand

Gene	Description	BRL+IFNg vs IFNg	BRL vs DMSO
Chemokine		-	-
CCL14	chemokine (C-C motif) ligand 14	0.674	-
CCL2	chemokine (C-C motif) ligand 2	0.544	-
CCL23	chemokine (C-C motif) ligand 23	0.711	-
CCRL1	chemokine (C-C motif) receptor-like 1	0.625	-
CXCL2	chemokine (C-X-C motif) ligand 2	0.493	-
CXCL6	chemokine (C-X-C motif) (granulocyte chemotactic protein 2)	0.699	-
CXCL9	chemokine (C-X-C motif) ligand 9	0.654	-
CXCR4	chemokine (C-X-C motif) receptor 4	0.474	-
Oxidative Phosphorylation			
NOX4	NADPH oxidase 4	0.592	-
NDUFA12	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 12	0.593	0.568
NDUFA4	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4, 9kDa	0.731	0.573
COX5B	cytochrome c oxidase subunit Vb	0.673	0.635
COX6A1	cytochrome c oxidase subunit VIa polypeptide 1	0.677	0.669
MRPL13	mitochondrial ribosomal protein L13	0.539	0.674
MRPL18	mitochondrial ribosomal protein L18	0.551	0.714
ATP5J	ATP synthase, H+ transporting, mitochondrial F0 complex	0.595	0.655
Alzheimer's Disease			
APP	amyloid beta (A4) precursor protein	0.256	-
APBB2	amyloid beta (A4) precursor protein-binding	0.497	-
APPBP1	amyloid beta precursor protein binding protein 1	0.663	-
SNCA	synuclein, alpha (non A4 component of amyloid precursor)	0.456	-
SNCB	synuclein, beta	0.604	-
BACE2	beta-site APP-cleaving enzyme 2	0.526	-
PSEN1	presenilin 1 (Alzheimer disease 3)	0.557	-
SERPINB1	serpin peptidase inhibitor, clade B (ovalbumin), member 1	0.692	-
Lipid Metabolism			
ABCA6	ATP-binding cassette, sub-family A (ABC1), member 6	0.715	
LDLRAD3	low density lipoprotein receptor class A domain containing 3	0.703	
LRP8	low density lipoprotein receptor-related protein 8, apo e receptor	0.703	
PTGES3	prostaglandin E synthase 3 (cytosolic)	0.709	
ACAT2	acetyl-Coenzyme A acetyltransferase 2	0.706	
LEPR	leptin receptor	0.681	

Table 1.2 Gene Expression Increased in the Absence of PPARγ in Mouse Endothelial Cells

Gene	Description
p53 signaling	
Bax	BCL2-associated X protein
Bbc3	BCL2 binding component 3
Casp8	caspase 8
Cdkn1a	cyclin-dependent kinase inhibitor 1A (P21)
Chek2	CHK2 checkpoint homolog (S. pombe)
Fas	Fas (TNF receptor superfamily member 6)
Gap junction	
Cdc2a	cell division cycle 2 homolog A (S. pombe)
Gnai2	guanine nucleotide binding protein (G protein), alpha inhibiting 2
Hras1	Harvey rat sarcoma virus oncogene 1
Kras	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
Map2k2	mitogen-activated protein kinase kinase 2
Pdgfa	platelet derived growth factor, alpha
Pdgfb	platelet derived growth factor, B polypeptide
Plcb4	phospholipase C, beta 4
Tuba1a	tubulin, alpha 1A
Oxidative phosphorylation	
Atp5o	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit
Atp6v0e	ATPase, H+ transporting, lysosomal V0 subunit E
Atp6v1b2	ATPase, H+ transporting, lysosomal V1 subunit B2
Cox4i2	cytochrome c oxidase subunit IV isoform 2
Cox5a	cytochrome c oxidase, subunit Va
Ndufa1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1
Ndufa12	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 12
Ndufb8	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8
Uqcrb	ubiquinol-cytochrome c reductase binding protein
Uqcrc2	ubiquinol cytochrome c reductase core protein 2
Chemokine	
Cx3cl1	chemokine (C-X3-C motif) ligand 1
Cxcl1	chemokine (C-X-C motif) ligand 1
Cxcl10	chemokine (C-X-C motif) ligand 10
Cxcl12	chemokine (C-X-C motif) ligand 12
Cxcl9	chemokine (C-X-C motif) ligand 9
Cxcr4	chemokine (C-X-C motif) receptor 4

Table 2.1   Endothelial Nuclear Receptor Expression

	Endocrine Receptors	**Adopted Orphan Receptors**	**Orphan Receptors**
Human & Mouse	**Expressed** AR (NR3C4) ERβ (NR3A2) GR (NR3C1) MR (NR3C2) RARα,γ(NR1B1,1B3) TRα (NR1A1) VDR (NR1I1)	**Expressed** LXRα,β (NR1H3,1H2) PPARα,δ,γ (NR1C1,1C2,1C3) RXRα,β (NR2B1,2B2)	**Expressed** COUP-TF1,2 (NR2F1,2F2) ERRα (NR3B1) LRH-1 (NR5A2) NGFIBα,β,γ (NR4A1-3) Reverbα (NR1D1) RORα,γ (NR1F1,1F3) TR2,4 (NR2C1,2C2)
Human Only			TLX (NR2E1)
Mouse Only	ERα (NR3A1) PR (NR3C3) RARβ (NR1B2) TRβ (NR1A2)	FXRα (NR1H4)	GCNF(NR6A1) Reverbβ (NR1D2) RORβ (NR1F2)
	Unexpressed	**Unexpressed** CAR (NR1I4) FXRβ (NR1H5) PXR (NR1I2) RXRγ (NR2B3)	**Unexpressed** COUPTF3 (NR2F3) DAX (NR0B1) ERRβ,γ (NR3B2,3B3) HNF4α,γ (NR2A1,2A3) PNR (NR2E3) SF1 (NR5A1) SHP (NR0B2)

A.3   Role of NR4A Family in Macrophage, An Atherosclerosis Study

A role in atherosclerosis has recently been suggested for another set of nuclear receptors belonging to the NR4A nuclear receptor subfamily [see (Maxwell 2006)] for review]. The NR4A receptors include Nur77, Nurr1 and NOR-1, which are classified as orphan receptors lacking a known endogenous ligand activator. Instead, activity is controlled by expression, covalent modifications and the presence of cofactors. Their DNA binding domains are >90% homologous and they bind identical response elements. They are rapidly induced and down-regulated in numerous cell types by a diverse range of stimuli and signaling pathways, and thus are commonly referred to as "immediate early genes." They have been implicated in T cell apoptosis and dopaminergic neuron development and more recently, hepatic gluconeogenesis, β-adrenergic signaling in muscle and acute myeloid leukemia (Zetterstrom 1997, Masuyama 2001, Pearen 2006, Chao 2007, Pei 2006, Mullican 2007). In addition, they are induced by LPS, IFNγ, oxidized cholesterol and TNFα in macrophages (Barish 2005, Pei 2005). Their over-expression reduces cholesterol uptake and scavenger receptor expression in human macrophage cell lines, while their knockdown causes the converse. Antibodies to Nur77, Nurr1 and NOR1 all co-localize with macrophage markers in human atherosclerotic lesions (Bonta 2006). They also modulate expression and directly bind the promoters of genes with known roles in atherosclerosis, such as matrix-metalloproteinases, plasminogen activator inhibitor, osteopontin, hypoxia inducible factor and IKKi (Mix 2007, Gruber 2003, Lammi 2004, Schmidt 2006, Pei 2005). They can also bind and repress NF-κB directly. Thus, like glucocorticoid receptor, PPARs and TLRs, these receptors are capable of modulating metabolism and inflammation.

Initially, *in vitro* experiments with the murine macrophage cell line RAW264.7 were performed to determine if these nuclear receptors enhanced or reduced the expression of inflammatory cytokines implicated in atherosclerosis. Macrophages were transfected with GFP (control), Nur77 or a dominant negative (DN-NR4A) construct that blocks activity of all three receptors (Woronicz 1994). This dominant negative activity was confirmed by individual co-transfections with vectors carrying genes for each of the three NR4As and an NR4A luciferase reporter. Resulting cells were stimulated with LPS or IFNγ 24 hours later. Macrophages stimulated with LPS for 8h and over-expressing Nur77 expressed less MMP-9, MCP-1 and TNFα (reductions of 50 to 90%) than control as assayed by qPCR. In addition, when stimulated with IFNγ for 8h, Nur77 overexpression completely suppressed IL-6 induction and suppressed MCP-1, MMP-9 and TNFα as much as 90%. Conversely, those cells tranfected with DN-NR4A expressed 10-fold IL-6, 7-fold iNOS, 3-fold MIP-2 and 2-fold TNFα over control cells when stimulated with LPS for 8h. A similar comparison after 2h stimulation revealed 100-fold induction of IL-6 and a 5-fold induction of MCP-1 and MMP-9 in DN-NR4A transfected macrophages over control. This data supports a protective role for the NR4A family by suppressing pro-atherosclerotic cytokine expression.

To examine the cardiovascular impacts of macrophage NR4A receptors *in vivo*, Ldlr-/- mice were irradiated and injected with fetal liver cells from Nur77-/- mice or NOR-1-/- mice to reconstitute the bone marrow. After 4 weeks of reconstitution, mice were fed atherogenic (high cholesterol) diet for 16 weeks. Weight and fasting glucose were measured at 16 weeks and serum cholesterol and triglycerides were measured at the initiation of diet, after 8 weeks of diet and at termination. These values did not differ

significantly between cohorts. Because the loss of NR4A family members in hematopoietic stem cells can cause acute myeloid leukemia in mice, white blood cell count was measured after 8 weeks of atherogenic diet and at termination. Counts were normal and didn't differ from control transplants.

Mice were sacrificed after 16 weeks of atherogenic diet. After formaldehyde perfusion, aortae were dissected and cleaned of adventitial tissues, stained with Sudan IV, and pinned for *en face* analysis. Image analysis software was used to count pixels corresponding to stained lesions and total aorta area. Lesion area was calculated as a percentage of total aorta area. There was no difference in lesion areas between mice reconstituted with wild-type bone marrow versus Nur77 knockout versus NOR-1 knockout bone marrow.

It is possible that the NR4As play redundant roles in atherogenesis by targeting similar genes, potentially explaining the apparent absence of a phenotype. Functional redundancy of these receptors has been previously reported (Cheng 1997). Heart valve lesions from these animals have yet to be analyzed, but based on the results from the aortas, are unlikely to reveal a difference in valve lesions. Indeed, it may be difficult to dissect the functions of these nuclear receptors or determine if they affect atherosclerosis without a combination knockout or knockdown approach. Double knockout mice, unfortunately, develop leukemia at a very young age, which would confound such studies. Thus, determining the role of macrophage NR4As in atherosclerosis may require the use of a conditional knockout, a dominant negative mutant that blocks all three receptors or over-expression.

A.4   Isolation of Primary Mouse Endothelial Cells

This protocol is designed for the isolation of endothelial cells from 5 mice. This can provide the equivalent of nine (lung-derived) and three (heart-derived) confluent 10cm cell culture plates (or equivalent) of endothelial cells at P4. It is not recommended to use cells after P4. Scaling down is possible if necessary. If extracting RNA immediately following harvest (no culture), approximately 2 μg of RNA can be obtained from heart EC and 8μg from lung EC.

Day 1:

- Wash 120μl anti-rat IgG coated dynabeads (Invitrogen Cat#110-35) in 5ml Buffer 1 (PBS/0.1%BSA) 3 times using Dynal magnet
- Re-suspend in 120μl Buffer 1 and add 15ul rat anti-mouse CD31 antibody (BD Pharmingen Cat#557355)
- Incubate overnite on rotator at 4°C

Day 2:

- Sacrifice mice with CO_2
- Before opening chest cavity, exsanguinate via the heart left ventricle with 1ml syringe and 22G needle
- Remove lungs and hearts and place in separate dishes of cold isolation buffer (high glucose DMEM, 20%FBS). Keep on ice.
- Transfer all to TC hood
- Cut hearts in half and put in 40ml cold isolation media, gently agitate/rotate in cold room.
- Mince half of lung tissue in a dry dish for one minute with curved scissors. Re-suspend in 25ml collagenase solution (2mg/ml Type I collagenase/ PBS with Ca/Mg). Rotate at 37° for 30m. Repeat with second half of lung tissue, followed by heart (also halved).
- While incubating, wash beads with 5ml Buffer 1 three times and re-suspend in 120μl Buffer 1.
- Use a 20cc syringe and 18 gauge needle to triturate each suspension 8 times, go slow, avoid frothing.
- Pipette suspensions through 40uM disposable cell strainers into 50ml conical tubes.
- Wash with 10ml cold isolation medium.
- Spin down at 400g for 8min (at 4 degrees if possible)
- Carefully aspirate collagenase solution.
- Re-suspend lung and heart pellets with 7 ml and 3ml cold Buffer 1, respectively.

- Transfer to 5ml Falcon round-bottom tubes (split lung between 2 tubes) and add 45μl prepared beads to each lung sample and 30μl to heart sample.
- Incubate on rotator in cold room 30-40min
- Divide heart sample between two round bottom tubes and the lung sample between 3 tubes. Bring total volume to 4ml with Buffer 1.
- Mount on magnet, invert for 2m, aspirate suspension. Remove magnet and triturate cell bound beads in 4ml fresh Buffer 1 **vigorously** 5 times, but avoid creating bubbles. Repeat this entire washing procedure 5 times.
- Mount on magnet, invert for 2m and aspirate Buffer 1. Remove magnet and re-suspend beads and cells in 2ml and 3ml EC Growth Media (Lonza EGM-2MV recommended) for heart and lung respectively. Plate on 0.2% gelatin coated plates (3.5cm, heart and 6cm, lung).

Day 3:

- Wash dishes 3 times with PBS and change media every other day therafter.
- Cells usually can be passaged 5-10d later.

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