DEVELOPMENT OF VASCULAR SMOOTH MUSCLE CELL MODELS FOR INVESTIGATING THE ROLE OF CMKLR1/CHEMERIN SIGNALLING IN ATHEROSCLEROSIS

by

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Abstract

Chemerin is a fat tissue-secreted protein that activates cell proliferation and migration though chemokine-like receptor 1 (CMKLR1) signalling. Serum chemerin is positively correlated to atherosclerosis severity, however the functional role of chemerin in atherosclerosis remains unknown. The study goal was to assess the role of chemerin/CMKLR1 signalling in human and mouse vascular smooth muscle cell (VSMC) migration and proliferation (important stages of atherosclerotic plaque formation). A protocol for differentiating human adipose-derived stem cells into SMCs was investigated. Morphological VSMC-characteristics were observed, as was chemerin and CMKLR1 expression, however further VSMC phenotype confirmation is required. Therefore, a second approach used primary aortic mouse VSMCs from CMKLR1-expressing and CMKLR1-deficient mice. Mouse VSMCs expressed chemerin and CMKLR1, and secreted low levels of chemerin. Low chemerin concentrations enhanced VSMC migration via CMKLR1 signalling and high concentrations inhibited migration via a CMKLR1-independent mechanism. This data suggests the involvement of chemerin/CMKLR1 signalling in atherogenic VSMC processes.

List of Abbreviations Used

Abs- absorbance

Akt- protein kinase B

ApoE- apolipoprotein E

ASC- adipose derived stem cell

baPWV- brachial ankle pulse wave velocity

BMI- body mass index

BrdU- bromodeoxyuridine

BSA- bovine serum albumin

CAD- coronary artery disease

cAMP- cyclic adenosine monophosphate

CCRL2- C-C chemokine-like receptor 2

CMKLR1- chemokine-like receptor 1

Cnn1- calponin 1

Ct- cycle threshold

CVD- cardiovascular disease

CYC-A-cyclophilin-A

Da- dalton

DMEM- Dulbecco's modified eagle medium

DNA- deoxyribonucleic acid

EC- endothelial cell

EdU- 5'ethynyl-2'- deoxyuridine

ELISA- enzyme-linked immunosorbent assay

eNos- endothelial nitric oxide synthase 3

ERK- extracellular signal-regulated kinases

ESAM- endothelial cell-specific adhesion molecule

E-SEL- endothelial selectin

FBS- fetal bovine serum

G1- growth phase 1

GAPDH- glyceraldehyde 3-phosphate dehydrogenase

GPCR- G-protein coupled receptor

GPR1- G-protein coupled receptor 1

HDL- high density lipoprotein

HRP- horseradish peroxidase

hs-CRP- high sensitivity C-reactive protein

ICAM- intercellular adhesion molecule

IL- interleukin

KO- knockout

LDL- low density lipoprotein

MMP- matrix metalloproteinase

MTT- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Myl9- myosin light chain 9, regulatory

NF-κB- nuclear factor kappa-light-chain-enhancer of activated B cells

PBS- phosphate buffered saline

PDGF- platelet-derived growth factor

PECAM- platelet endothelial cell adhesion molecule-1

qPCR- quantitative polymerized chain reaction

RARRES2- retinoic acid receptor responder 2

RNA- ribonucleic acid

ROS- reactive oxygen species

RT- room temperature

SMA- smooth muscle actin

SMC- smooth muscle cell

SM-MHC- smooth muscle myosin heavy chain

TIG2- tazarotene-induced gene 2

TNF- tumour necrosis factor

VCAM- vascular cell adhesion protein 1

VEGF- vascular endothelial growth factor

VSMC- vascular smooth muscle cell

WT- wildtype

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Chapter I. Introduction

1.1 Atherosclerosis

1.1.1 Definition of atherosclerosis

Atherosclerosis is a disease where an artery wall thickens due to accumulation of calcium, cholesterol, and triglycerides, forming plaques. Arterial plaque reduces the space available for blood to flow through the artery causing increased blood pressure, or hypertension. When plaque has formed in an artery there is an increased risk of heart attack and stroke, where blood flow is completely stopped preventing it from reaching the heart and the brain, respectively. The term cardiovascular disease (CVD) describes a number of cardiac events and conditions (including heart attack and stroke) most of which involve atherosclerotic plaque formation.

1.1.2 Prevalence and ethnic differences

Over 20 million deaths worldwide each year are due to CVD. CVD makes up 30% of all deaths, making it leading cause of death globally[1]. In Canada, someone dies from heart attack or stroke due to atherosclerotic plaque rupture every 7 minutes [2]. In Nova Scotia, cardiovascular disease is the largest killer, claiming 2800 citizens each year [3]. CVD is a leading cause of death in both males and females, however it develops 7-10 years later in women than in men such that men over the age of 45 and women over 55

are at an increased risk [4]. There are a number of additional risk factors which may influence the likelihood for an individual to develop atherosclerosis. Ethnically, it has been found that the burden of atherosclerosis is higher for symptomatic white and Asian-American individuals compared to black and Hispanic individuals, as determined by angiography and electron beam tomography [5]. Despite this, there are no ethnic groups which have not been affected by atherosclerosis.

1.1.3 Atherosclerosis mechanism

Arteries are composed of three layers. The outermost layer being the *tunica externa* which is composed of connective tissue made of collagen fibers. The middle layer is the *tunica media* (or media) which is the largest layer and is composed of vascular smooth muscle cells (VMSCs). The innermost layer, which is in direct contact with blood flow is the *tunica intima* (commonly called intima), which is composed of a thin layer of endothelial cells. The mechanism of how endothelial cells are involved in atherosclerosis development is largely unknown, however it is suggested that smoking, high levels of cholesterol in the blood, or high blood pressure could cause damage to the endothelium which allows for fat and cholesterol to accumulate (Figure 1). The presence of cholesterol in the intima causes macrophages to produce increased amounts of reactive oxygen species (ROS) which oxidize the cholesterol and initiate an inflammatory response. In this response, nitric oxide production is increased and macrophages consume the oxidized cholesterol, forming foam cells. Foam cells secrete inflammatory cytokines and encourage monocyte recruitment to the area. The foam cells migrate to the interior

of the vessel, and begin forming a plaque [6]. It is at this stage, that the smooth muscle cells begin to proliferate then migrate to the interior of the artery, contributing to a cap which forms over the plaque.

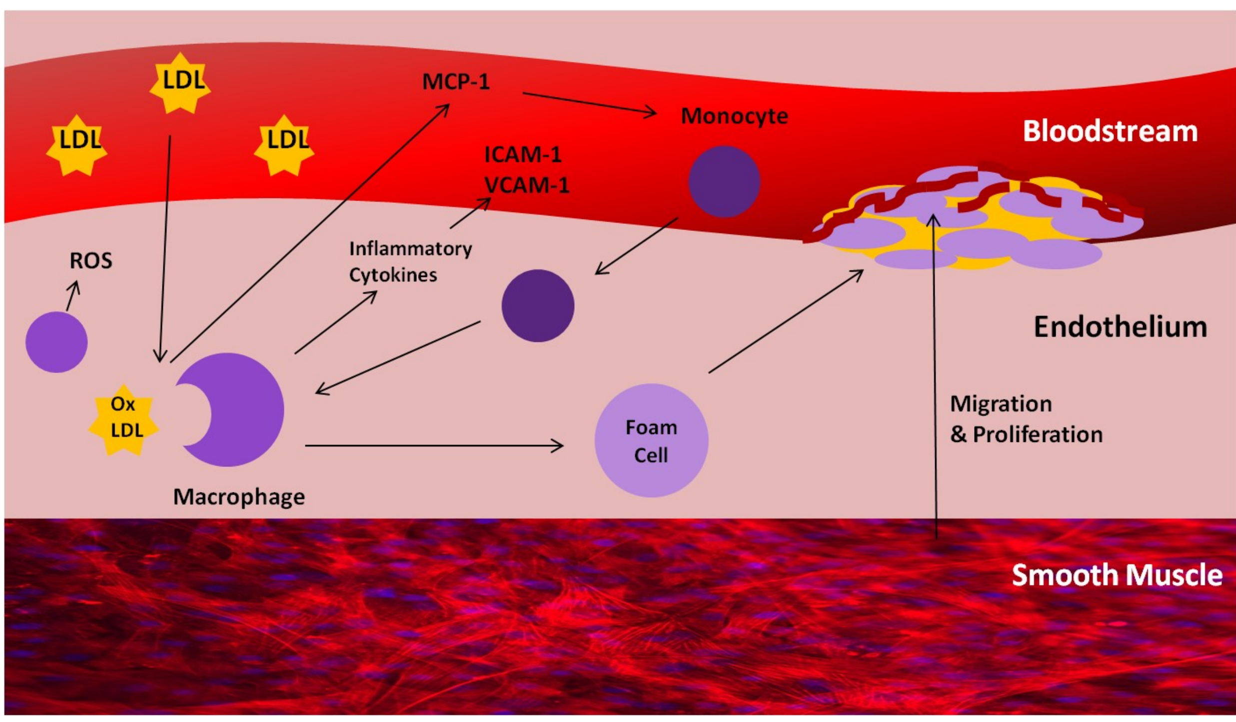
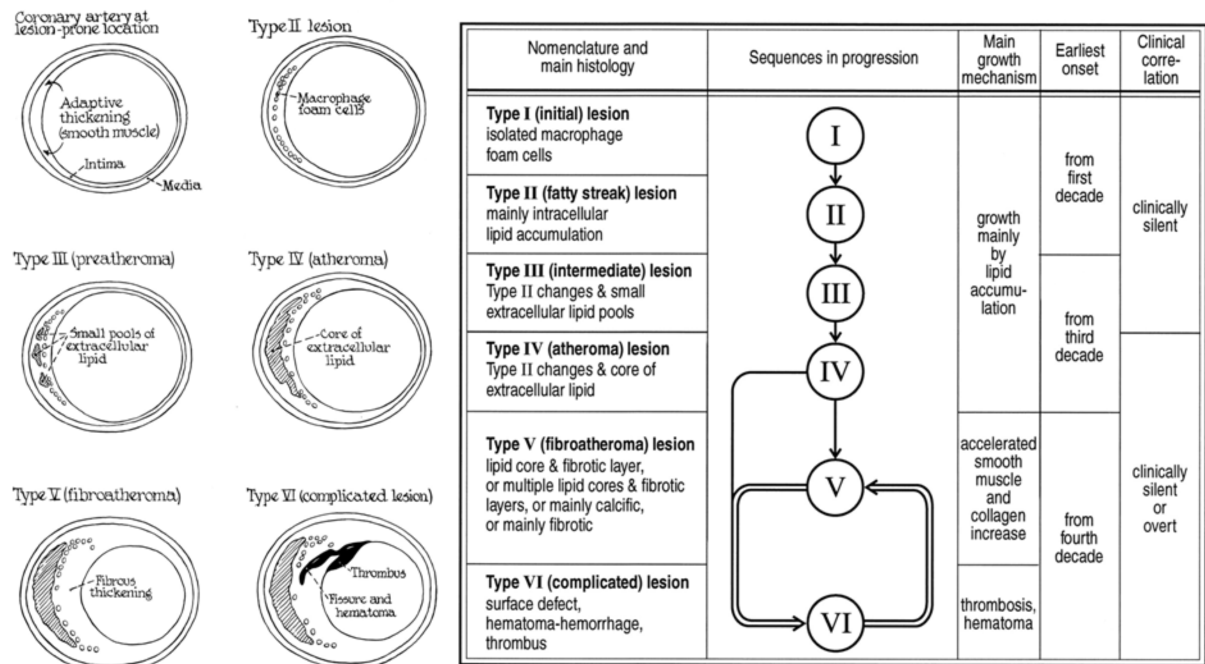


Figure 1: Mechanism of atherosclerotic plaque development in a blood vessel.

Upon understanding the mechanism of atherosclerotic plaque development, it is then important to understand the clinical classification of atherosclerotic lesions. Lesions are classified into six groups with increasing severity (Figure 2). Not all lesions produce symptoms in inflicted individuals and types I-III are often asymptomatic or "clinically silent", while types IV-VI are usually symptomatic. It is the more severe plaques which are often referred to as unstable plaques which are at a greater risk for rupture causing acute cardiac events.



Nomenclature and main histology	Sequences in progression	Main growth mechanism	Earliest onset	Clinical correlation
Type I (initial) lesion isolated macrophage foam cells	I	growth mainly by lipid accumulation	from first decade	clinically silent
Type II (fatty streak) lesion mainly intracellular lipid accumulation	II			
Type III (intermediate) lesion Type II changes & small extracellular lipid pools	III		from third decade	
Type IV (atheroma) lesion Type II changes & core of extracellular lipid	IV			clinically silent or overt
Type V (fibroatheroma) lesion lipid core & fibrotic layer, or multiple lipid cores & fibrotic layers, or mainly calcific, or mainly fibrotic	V	accelerated smooth muscle and collagen increase	from fourth decade	
Type VI (complicated) lesion surface defect, hematoma-hemorrhage, thrombus	VI	thrombosis, hematoma		

Figure 2: The American Heart Association's histological classification of atherosclerotic lesions (Stary et al., 1995 -Used with permission from Wolters Kluwer Health Inc. License # 3673670939896) [7].

1.1.4 Co-morbidities and atherosclerosis risk

There are a number of major risk factors which have been linked to the development of atherosclerosis. One major risk factor is having high blood concentrations of low-density lipoprotein (LDL) cholesterol (the “bad” cholesterol), or having low blood concentrations of high-density lipoprotein (HDL) cholesterol (the “good” cholesterol). Another major risk factor is high blood pressure which tends to increase with age. Smoking increases the risk for atherosclerosis by damaging and tightening blood vessels. It also limits the amount of oxygen available to the body’s tissues. Additional major risk factors are insulin resistance, diabetes, being overweight or obese, physical inactivity, old age, family history of heart disease, and a diet high in trans fats, cholesterol, sodium and sugar. Atherosclerosis develops over time, and by the time an individual is middle-aged enough plaque may have accumulated to increase their risk of heart attack or stroke. The risk associated with this process increases for men after the age of 45 and women after the age of 55. Minor risk factors that may affect atherosclerosis are sleep apnea (may increase blood pressure and promote heart attack or stroke), stress (emotionally upsetting events are often associated with a heart attack or stroke in prone individuals), and high alcohol consumption (may cause damage to heart muscle) [8].

The above major and minor risk factors which have been associated with atherosclerosis also explain linkages to co-morbidities often observed in patients with atherosclerosis. Some of the most common co-morbidities which have been associated

with atherosclerosis are obesity, diabetes, alcoholism, chronic tobacco use, hypertension, polycystic ovary syndrome, and some inflammatory diseases.

1.1.4.1 Obesity and atherosclerosis

The relationship between obesity and atherosclerosis is a subject which has been disputed for many years. Early research using human autopsy subjects found weak to no correlation between body weight and CVD. Despite this early work, subsequent publications have confirmed that specific risk factors (such as dyslipidemia, insulin resistance, and pro-inflammatory as well as pro-thrombosis characteristics) associated with obesity have led obesity to be considered a cause of atherosclerosis as opposed to an individual risk factor. The pro-inflammatory state in obesity is characterized by increased serum concentrations of C-reactive protein (hs-CRP), which is thought to play a role in the stability of atherosclerotic plaques. Many fat-secreted cytokines (collectively termed "adipokines") also play a role in the pro-inflammatory state of obesity and may influence the production and stability of atherosclerotic plaque. The pro-thrombosis state in obesity is characterized by increased amounts of plasminogen activator inhibitor-1 which promotes blood clotting and may enhance the size of coronary thrombosis accompanying coronary plaque rupture [9].

The link between obesity and atherosclerosis has been researched in a number of human studies. One study included autopsies of over 3000 individuals aged 15 to 34 years who had died of unrelated external causes. Atherosclerotic lesions in the right

coronary artery and left anterior coronary artery were graded in these individuals and were compared to the individuals' body mass index (BMI). The severity of lesions (also sometimes termed "fatty streaks") was found to positively correlate with BMI in men, while in women correlation between lesion severity and BMI was not observed. This indicates that because women and men have different fat distribution, not all fat tissue may be equally harmful and location may play a role in its function [10]. This study rationalizes the lack of correlation between lesions and BMI in women in two ways. Firstly, they note that premenopausal women generally have a delay in the progression of atherosclerosis. Secondly, they explain that abdominal adipose tissue carries a higher risk for atherosclerotic plaque in men than it does for women, and men are more likely to have an "apple" shape (increased abdominal adiposity) as opposed to a "pear" shape (increased adiposity in the hip region) which is more common in women. In this sense, correlations made to an individual's waist-to-hip ratio may be more accurate than BMI.

The factors influencing the link between weight and atherosclerosis extend beyond gender differences. Research has discovered that ethnicity can promote increased susceptibility for some populations over others. For example, South Asian populations may gain only a moderate amount of weight however the corresponding risk of CVD is very large [11]. It is also more common for South Asian populations to develop insulin resistance following moderate weight gain. In general white populations of European origin have been found to have an increased predisposition for atherogenic dyslipidemia compared to other populations. Meanwhile, black individuals of African origin are more prone to developing hypertension and type 2 diabetes following weight gain. Black

populations develop less atherogenic dyslipidemia compared to whites with the same degree of weight gain. Native Americans and Hispanics are especially susceptible to developing type 2 diabetes, but are less likely to develop hypertension than blacks [12]. Clearly, understanding the influence of atherosclerotic risk factors associated with weight gain is a complex subject, however the link between atherosclerosis and obesity is well established.

1.1.4.2 Diabetes and atherosclerosis

Given the inter-connectedness of the symptoms and risk factors associated with diabetes and atherosclerosis, it is not surprising that the two diseases are linked. Many people with type 2 diabetes have metabolic syndrome (defined as having three out of five possible symptoms which include increased waist circumference, raised triglycerides, reduced HDL cholesterol, elevated blood pressure, and high blood glucose due to insulin resistance), which likely accounts for the increased risk of CVD [9, 13]. However, the presence of diabetes increases the risk of CVD beyond what has been observed with metabolic syndrome alone. Additionally, an increased risk of CVD in type 1 diabetics has also been observed [13, 14]. This is interesting because type 1 diabetes (caused by a lack of insulin production rather than insulin resistance) is rarely associated with metabolic syndrome, suggesting that the conditions specific to diabetics such as hyperglycemia (high blood glucose) may play a role in CVD. In investigating the mechanistic connection between diabetes and atherosclerosis, numerous studies have focused on the role of high glucose conditions on endothelial cells, smooth muscle cells, and macrophages. It has

been proposed that glucose may act directly or indirectly on plaque formation by generating advanced glycation end-products or reactive oxygen species. High glucose concentrations have also been associated with protein kinase C activation in vascular cells through the aldose reductase pathway. This pathway has been associated with increased inflammation and increased nuclear factor kappa B (NF-κB) activation, a protein which has been found in high levels in atherosclerotic lesions. The role of NF-κB in atherosclerotic lesions however, has not been confirmed. One possible explanation for the involvement of glucose-activated increase in NF-κB in atherosclerosis is by the increase in adhesion molecule expression promoting monocyte adhesion to endothelial cells, a key stage in the formation of plaque. The effect of high glucose in atherosclerosis is just one avenue of which the two diseases could be related. The influence of insulin resistance, impaired insulin production, and hyperlipidemia on atherosclerosis, are additional areas which may provide insight into the relationship between diabetes and atherosclerotic plaque formation.

1.1.4.3 Tobacco & alcohol in atherosclerosis

The consumption of alcohol and intake of tobacco in any form (including second-hand smoking) have both been associated with atherosclerosis. The effect of alcohol on the formation of atherosclerotic plaque has been debated in scientific literature. Low amounts of alcohol when taken on a regular basis have been shown to protect against cardiovascular disease [15-17], while heavy drinking promotes a severe risk condition. One study examined the effect of alcohol on the incidence and progression of

atherosclerosis over a 5-year period. They discovered that subjects who consumed small amounts of alcohol daily had a lower risk for atherosclerosis compared to heavy drinkers and abstainers. One factor which affects this balance negatively is alcohol-induced hypertension, while alcohol-increased HDL cholesterol levels may play a role in the optimistic results found in the light-drinking group. The low-alcohol intake group had half the risk of carotid stenosis, likely from counteracting plaque thrombosis. Alcohol reduces the ability for blood to coagulate by inhibiting various coagulation factors and enhancing thrombocyte survival time. Another study failed to find any beneficial effects of limited occasional alcohol intake on intima-media thickening (representing early stages of atherosclerosis), suggesting that beneficial results are seen only when alcohol is consumed in very low amounts on a daily basis [18]. Interestingly, the non-alcoholic components of red wine have been found to inhibit the oxidation of LDL cholesterol, preventing plaque formation [19, 20]. This process is mediated by free radical generators and metal ions. An early study by Frankel et al. (1993) determined that red wine inhibited Cu^{2+}-catalyzed oxidation of LDL [19]. A later study comparing the effects of red wine compared to grape juice showed that both inhibited LDL oxidation, however flavonoids from the red wine were absorbed more efficiently than those in grape juice [21]. Other phenol sources have been examined such as the high phenol-containing Chilean berry and polyphenols from cacao, in both cases LDL oxidation was inhibited [22, 23]. The cacao polyphenols also reduced the number of atherosclerotic lesions and the plaque area in atherosclerotic apolipoprotein E deficient (apoE $^{-/-}$) mice [23]. These examples suggest that the beneficial effects of alcohol consumption on reduced risk of heart disease are

somewhat disputed, but that phenolic-related benefits may be gained from non-alcoholic dietary sources.

Tobacco smoke contains over 4000 different compounds including reactive oxygen and nitrogen species, and has been associated with endothelial cell injury and altered cardiac cellular metabolism [24-28]. It is suggested that tobacco smoke exposure increases vascular inflammation, oxidative stress, and mitochondrial damage [29-32]. It is also hypothesized that smoking cigarettes could modify the lipid profile, promoting atherosclerosis [33]. Smoking reduces arterial oxygen carrying capacity through increased serum carboxyhemoglobin levels, and causes oxidative phosphorylation dysfunction in cardiac cells [24-26]. The activity of the myocardial mitochondrial cytochrome oxidase (final enzyme in the respiratory electron transport chain responsible for conversion of oxygen to water and ATP synthesis) falls 25% after a single 30-min exposure to second-hand smoke in rats and the activity continues to decline with prolonged exposure [24]. Tobacco smoke also has been found to inhibit mitochondrial oxidative phosphorylation in platelets, resulting in increased generation of mitochondrial ROS [26].

While heavy drinking alone has been found to increase risk of incidence and progression of atherosclerosis, the risk is increased when this is paired with smoking. The combined increase in atherosclerosis risk from exposing the body to alcohol and tobacco is evident even when the tobacco intake is low due to inhalation of second hand smoke (environmental tobacco smoke or ETS) [28]. A study which examined the effects of

ethanol and tobacco smoke exposure in hypercholesterolemic mice found that the combined exposure increased atherosclerosis. They further suggest that the protective role of low doses of ethanol on cardiovasculature does not exist when paired with tobacco smoke. They also note that mitochondrial oxidative stress was correlated with the size of the atherosclerotic plaque and mitochondrial oxidative stress may play an important role in plaque development [28].

1.1.5 The role of smooth muscle cells in atherosclerosis

It is well established that smooth muscle cells play an important role in the final stage of atherosclerosis where they proliferate and migrate over the endothelial layer, on top of the plaque, forming a cap. In understanding the factors which affect VSMC migration and proliferation it is helpful to first understand the embryonic derivation of smooth muscle cells. Smooth muscle cells have different embryonic origins, depending on the segment of the arterial system involved. In some vertebrates, smooth muscle cells in the upper portion of the thoracic aorta are derived from a neuroectodermal source while those in the abdominal aorta are derived from a mesenchymal source [34, 35]. While this has not been confirmed in humans, it is likely. Human smooth muscle cells of the coronary arteries appear to originate from a third precursor population in the intracardiac mesenchyme. The existence of these different lineages suggests that smooth muscle cells in different parts of the arterial tree may respond differently to the stimuli that generate atherosclerotic plaque in these areas. To further complicate matters, the smooth muscle cells in large arteries may be heterogeneous with different proliferative

and matrix-producing capabilities [36]. Because VSMCs may have different lineages, they may respond differently to different cytokines, mitogens, chemotactic factors, or extracellular matrixes [37, 38].

The various lineages of smooth muscle cells play a role in the differing phenotypes they display [39-42]. The smooth muscle cells in normal mature blood vessels have the most predominant phenotype which is known as the contractile phenotype. VSMCs with this phenotype are myofilament-rich which allows the cells to regulate the diameter of the blood vessel (vasodilation and vasoconstriction) and the blood flow [7, 43-45]. In the event of injury to the vessel (such as in atherosclerosis) these cells change from a contractile phenotype to a synthetic, migratory and proliferative phenotype. In addition to increased migration and proliferation, cells in this phenotype m can secrete more extracellular matrix proteins to aid in vascular reconstruction and may therefore be referred to as having a secretory phenotype [39]. The VSMCs in this proliferative/ migratory/ secretory phenotype are myofilament-poor but are relatively rich in rough-surfaced endoplasmic reticulum [7]. Intimal VSMCs which are in close proximity to the thrombotic deposits of type V and VI lesions may contain even higher amounts of rough-surfaced endoplasmic reticulum. Basement-membrane rich or pancake-shaped VSMCs are a variant found in type IV, V, and VI lesions, which commonly occur near a lipid core in the lesion [46, 47]. Basement membrane thickening may represent an attempt by the cells to restore the integrity of the tissue and provide improved anchorage for the cells in the injury repair process [7, 48]. Regardless of VSMCs being basement membrane-rich or not, the phenotypic change from the contractile state to a proliferative

and migratory state is necessary for the reconstruction and healing of the vessel. Following tissue injury, cells produce growth factors such as epidermal growth factor, fibroblast growth factor, insulin-like growth factor, platelet-derived growth factor (PDGF), transforming growth factor, vascular endothelial growth factor (VEGF), and angiotensin II. These cells act in a paracrine manner to activate the healing response in neighboring cells. The phenotypic change of VSMCs to a proliferative/ migrating phenotype is an example of one such response. If they fail to return to a contractile phenotype, they then induce pathogenic vascular remodelling and generate vascular lesions [39, 43, 44, 49].

Angiotensin II is an octapeptide hormone which is involved in many cellular processes such as proliferation, differentiation, regeneration, and apoptosis [39]. In VMSCs, the duration of exposure to angiotensin II is crucial. Acute exposure is necessary for normal cell physiology including tissue repair after injury, however chronic exposure causes VSMCs to proliferate, migrate, and secrete extracellular matrix proteins via angiotensin II receptors. This process occurs in blood vessel scarring or thickening causing narrowing of the blood vessel, a component of atherosclerosis.

VEGF is expressed in VSMCs under normal physiological conditions, however upon vascular injury it is over-expressed [39]. Some triggers of overexpression include hypoxia, ultraviolet light, ROS and mechanical injury [50]. VEGF receptors are known to be expressed in both endothelial and VSMCs [51]. Endothelial cells can produce VEGF upon exposure to hypoxia, ROS and fibroblast growth factor 2, which can then act upon

VSMCs [52, 53]. It is suggested that VEGF assists with vascular injury repair by stimulating matrix metalloproteinase (MMP) production by VSMCs which facilitates migration and intimal thickening [54]. VEGF has not been shown to mediate VSMC proliferation.

The PDGF family of growth factors are produced by platelets, macrophages, endothelial cells, fibroblasts and keratinocytes [39, 55]. The PDGFs bind to two different transmembrane tyrosine kinase receptors (α and β). PDGF stimulates VSMC migration via binding with the PDGF β-receptor. This is demonstrated by the increased expression of this receptor in migrating VSMCs. When the PDGF β-receptor is not down-regulated following wound repair the result is continued neointimal thickening [56, 57]. PDGF has also been found to play a role in proliferation in addition to migration of VSMCs.

1.1.5.1 Relationship between proliferation and migration in VSMCs

The signalling pathways which are activated when exposed to mitogens, growth factors or peptides often trigger pathways that are known to stimulate both proliferation and migration. However, most research suggests that this is not always the case, and that migration and proliferation are not interdependent. One study examined the relationship between migration and proliferation in response to PDGF. They found that proliferation occurred only at high concentrations of PDGF, while migration was activated only at low concentrations, suggesting that the response is dose-dependent [39, 58]. Another possible mechanism which may affect whether a cell migrates or proliferates is the cell cycle

arrest in a specific phase. VSMCs can migrate during the G1 phase (first growth phase during the interphase) of the cell cycle, but not in other phases [59, 60].

1.1.5.2 Factors affecting VSMC proliferation

The three different lineages and two phenotypes of smooth muscle cells are not the only factors which may influence their ability to proliferate and migrate. In addition to the growth factors described above which affect proliferation, the composition of the connective tissue surrounding the artery may also play an important role. In a healthy artery, the outer layer is primarily composed of type I and III fibrillar collagen, while in an artery with atherosclerotic plaque, this layer is largely proteoglycan mixed with collagen fibrils. When human arterial smooth muscle cells are plated on collagen, the collagen inhibits cell proliferation by up-regulating specific inhibitors of the cell cycle [38]. If the collagen is degraded by collagenase, smooth muscle cells may be more likely to replicate, as they do when they are cultured without collagen. It is possible that other matrix molecules such as fibronectin and heparin sulfate may be involved in this process, as they can also inhibit the cell-cycle, which could promote chemokine expression in macrophages. These processes suggest that the environment surrounding smooth muscle cells is an important influence on the inflammatory and proliferative properties of the cells [34].

1.1.5.3 Factors affecting VSMC migration

There are a number of growth factors and inflammatory cytokines which may cause an environment to become a pro-migratory for VSMCs. Table 1 provides a summary of agents known to promote VSMC migration

Table 1: Summary of agents promoting VSMC migration

Growth Factors and Cytokines	**Extracellular Matrix Components**	**Other Molecules**
Angiotensin II	Collagen I, IV	ATP, UTP
Basic fibroblast growth factor (bFBF)	Collagen VIII	Noepinephrine
Heparin-binding epidermal growth factor	Fibronectin	High glucose
Insulin-like growth factor-1 (IGF-1)	Hyaluronan	Histamine
Interleukin-1β (IL-1β)	Laminin	Serotonin
Interleukin-6 (IL-6)	Osteopontin	Sphingosine-1 phosphate
Platelet-derived growth factor (PDGF)	Thrombospondin	
Transforming growth factor- β1 (TGFβ1)	Vitronectin	
Tumor necrosis factor (TNF)		
Thrombin		
Urokinase plasminogen activator		
Vascular endothelial growth factor (VEGF)		

In addition to the growth factors and cytokines listed above, there are other key players which may have a more direct role in VSMC migration. These molecules include

integrins, integrin-linked kinase, focal adhesion kinase, cortactin-cofilin, and metalloproteinases.

Integrins are a family of transmembrane glycoproteins that mediate cell-cell and cell-matrix interactions [39, 61]. The integrin αvβ3 is one of the most highly expressed integrins, and is expressed on endothelial and VSMCs. It is known to mediate migration of VSMCs through binding with a number of ligands, including, fibronectin, and vitronectin. The expression of αvβ3 is increased when VSMCs are treated with thrombin, as well as other growth factors and mitogens which represent an atherogenic state [62]. Interestingly, the location of αvβ3 on VSMCs can change depending on the bioavailability of PDGF such that αvβ3 are more densely distributed on the side of the cell which is migrating toward PDGF. In the absence of PDGF, αvβ3 is uniformly distributed on the cell. In a healthy artery, αvβ3 expression is minimal.

Integrin-linked kinase is a serine-threonine protein kinase which is an important component of the focal adhesion complex. It acts by anchoring actin filaments to integrin receptors and the cell membrane, an important process in cell migration. Its function however, is not well understood. It is suggested that integrin-linked kinase may have two functions in VSMCs. First, it may function as a scaffold protein at focal adhesion sites. Second, integrin-linked kinase can activate signal transduction via its kinase domain [63].

Focal adhesion kinase is a 125 kDa protein tyrosine kinase which plays a prominent role in integrin signalling, an important aspect of cell migration. It is

maintained as an inactive state where its catalytic domain is blocked inhibiting ATP and substrate binding. Focal adhesion kinase becomes activated in the presence of certain integrins and later serves as a docking site for SH2 domain proteins [64]. Enhanced phosphorylation of focal adhesion kinase in VSMCs has been observed following vascular injury or with vascular stimulation with growth factors or angiotensin II. In VSMCs activation of focal adhesion kinase has been linked to migration and to the transmission of survival signals [65]. Interestingly, when a protein called focal adhesion kinase-related non-kinase (containing the same C-terminal domain as focal adhesion kinase) is over-expressed it reduces phosphorylation of focal adhesion kinase, and reduces Ang-II-stimulated VSMC migration [66].

The actin-severing protein cofilin is essential for directed cell migration and chemotaxis. It acts by increasing the number of free barbed ends capable of initiating actin polymerization. This process is crucial in actin-based cell migration. The protein cortactin can interact directly with colfilin to inhibit its actin-severing activity, providing regulation of actin-based cell migration.

Another group of important factors in VSMC migration are MMPs, which facilitate the removal of the basement membrane around VSMCs and allowing them to migrate. The presence of inflammatory cytokines and growth factors in atherosclerotic blood vessels can increase the production of MMPs, therefore promoting cell migration [39, 67].

1.1.5.4 VSMC apoptosis and plaque rupture

The migration and proliferation of VSMCs is an important part of atherosclerotic plaque development as it allows for the formation of a cap which forms over the plaque, reducing the vessel diameter for blood flow but also preventing the plaque from rupture. Apoptosis of VSMCs is a phenomenon which like migration and proliferation is significantly reduced in healthy cells but increased in atherosclerosis. This is reflected in the low apoptotic and mitotic indices for VSMCs in healthy arteries compared to those in a diseased atherosclerotic state [68]. One major difference between apoptosis, and proliferation and migration, is that while the latter protects the plaque, the former causes the VSMC cap to thin, making the plaque at a greater risk for rupture. There are two pathways that regulate VSMC apoptosis, which are via the membrane death receptors of the TNF family, and also via mitochondrial amplification. Both pathways involve a downstream caspase cascade which signals for apoptosis.

1.1.5.5 Therapies targeting VSMC proliferation and migration

While the migration and proliferation of VSMCs has a protective role in improving plaque stability, too much proliferation and migration will lead to over-narrowing of an artery in a process called restenosis. Patients with restenosis may be given a drug-eluting stent to maintain proper blood flow throughout the artery. Stents are tubes which are surgically placed in an artery, and a drug-eluting stent will slowly release a drug to the area. There are two drugs available clinically as drug-eluting stents which

inhibit VSMC proliferation and migration to prevent further narrowing of the artery, rapamycin (Cypher™) and paclitaxel (Taxus™) [69, 70]. Rapamycin (also called sirolimus) is a macrocyclic triene produced by the bacterium *Streptomyces hygroscopius*. It has been found to reduce restenosis, but has been shown to reduce the proliferation and migration of endothelial cells as well, preventing the important re-endothelialization process involved in vascular healing [71, 72]. Paclitaxel was isolated from the bark of the Western yew tree in 1971 [73], and induces cell arrest in VSMCs, inhibiting proliferation and migration both in vitro and in vivo [74, 75], as well as inhibits neointimal formation [76]. In both drug-eluting stents, there is a risk of vascular thrombosis occurring. These therapies validate the rationale for targeting the inhibition of VSMC proliferation and migration, however the risks associated with them describe the need for improved therapeutic options.

1.1.6 Cytokines in atherosclerosis

There are a large number of cytokines which are suspected to play a role in atherosclerosis, some of which have been listed in Table 1. Two inflammatory cytokines which are well known to be involved in atherosclerotic plaque development are IL-6 and TNF.

TNF is an inflammatory cytokine which is produced by macrophages, mast cells, endothelial cells, cardiomyocytes, fibroblasts, nerve cells and adipocytes [77, 78]. TNF was one of the first proteins to be identified as a part of the adipose tissue inflammatory

pathways [79, 80]. It is suspected to directly and indirectly influence insulin resistance is humans. In this context, TNF is primarily produced by adipose-infiltrating macrophages [81], causing low-grade inflammation which induces insulin resistance and endothelial dysfunction, leading to diabetes and cardiovascular disease [82]. Its function in promoting cell apoptosis is well documented, especially for VSMCs and endothelial cells in atherosclerosis [77, 83, 84]. TNF has also been shown to promote atherosclerotic processes by up-regulating the expression of vascular cell adhesion molecule-1 (VCAM-1), E-selectin, and intercellular adhesion molecule-1 (ICAM-1) in human aortic endothelial cells and VSMCs [85]. Interestingly, when TNF expression is down-regulated in endothelial cells, their expression of VCAM-1 and ICAM-1 is also reduced [86]. Elevated circulating TNF levels have been associated with an increased incidence of cardiac events including angina, myocardial infarctions, and ischemic strokes [79, 87, 88]. TNF along with hsCRP have been shown to be predictive of cardiac events, alongside usual cardiovascular risk factors. However, additional research is required before it can be used as a clinical biomarker for CVD [89].

IL-6 is a pro-inflammatory cytokine produced by many cell types including fibroblasts, endothelial cells, and lymphocytes. The pro-inflammatory nature of IL-6 has been described in atherosclerotic apoE-/- mouse studies. For example, treatment of apoE-expressing (apoE $^{+/+}$) mice with IL-6 resulted in a 5 fold increase in fatty streak size, whereas apoE$^{-/-}$ mice on low- and high-fat diets showed a 2 fold increase [90, 91]. Alternatively, IL-6 deficient apoE$^{-/-}$ mice showed enhanced plaque formation with reduced collagen content and reduced production of IL-10 as well as reduced

inflammatory cell recruitment [92]. In healthy humans, IL-6 circulates at low blood concentrations but is increased in obese individuals. IL-6 is an important cytokine for stimulating secretion of hs-CRP (which has been linked to cardiovascular disease) [79]. In atherosclerosis, macrophages are responsible for the majority of IL-6 secretion, however its exact mechanistic role is unclear. IL-6 interacts with an IL-6 receptor as well as a signal-transducing protein which is also used by other members of the IL-6 family. IL-6 levels have been found to be elevated in patients with myocardial infarction, and also correlate with blood pressure in healthy adults, and are increased in patients with atherosclerosis [93, 94]. IL-6 has been found to exhibit some anti-inflammatory properties as well, where it suppresses the release of TNF [79], however additional research is required in understanding its role in atherosclerosis.

1.1.7 Adipokines in atherosclerosis

Adipokines are cytokines which are secreted from fat tissue, and several have been linked to atherosclerosis. This connection is not surprising given the close relationship between obesity and atherosclerosis, and that individuals who have increased amounts of visceral white adipose tissue are at a greater risk for CVD. Two adipokines which have been studied in relation to atherosclerosis are adiponectin and leptin.

Adiponectin is a polypeptide produced by adipose tissue which is secreted as several different isoforms with varying molecular weights [79, 95]. Adiponectin has a strong inverse association with insulin resistance and type 2 diabetes, such that patients

with type 2 diabetes or impaired glucose tolerance have low adiponectin levels [96]. Several studies have found that adiponectin levels are also lower in patients with CVD compared to healthy controls, and the incidence of myocardial infarction has been associated with low plasma adiponectin levels [97, 98]. There have been some studies which have found no significant association between adiponectin and CVD, and so its role in CVD is still being debated [99]. Despite this, it has been discovered that adiponectin does have direct anti-atherosclerotic effects. For example, adiponectin has been found to strongly inhibit the production of the adhesion molecules ICAM-1, VCAM-1 and E-selectin in endothelial cells which play an important role in monocyte recruitment during plaque formation [100]. Adiponectin has also been shown to inhibit the inflammatory cytokines TNF-induced NF-κB activation in endothelial cells (10). Interestingly, injection of globular adiponectin in mice reduced the neointimal formation in the endothelial layer and also the differentiation of macrophages into foam cells [100-103]. This suggests a protective role for adiponectin in atherosclerosis, however these results have not yet been confirmed in humans. There is a need for increased research regarding the role of adiponectin in CVD, especially with respect to the roles of the various adiponectin isoforms.

Leptin is a 16 kDa hormone secreted by adipocytes and its circulating levels are correlated with white adipose tissue mass [95]. It is suggested that the primary role of leptin is in weight regulation. In this way, leptin functions to decrease food intake and increase energy consumption by acting on specific hypothalamic nuclei, where leptin induces anorexigenic factors as such as cocaine amphetamine-related transcript and

suppresses orexigenic neuropeptides such as neuropeptide Y [104]. Interestingly, genetic deficiency of the gene encoding for leptin or its receptors provokes severe obesity and diabetes mellitus [95]. Other functions of leptin which have been determined are in immune system modulation, inflammatory response, and blood pressure regulation [79, 105-107]. Because leptin was found to influence risk factors for CVD, it was suggested that leptin may play a more direct role in this disease process, independent of increased adiposity. To further assess this, some studies focused on direct correlations between leptin and CVD. It was discovered that serum leptin concentrations have been associated with risk of myocardial infarction and stroke [108]. Additionally, there are a number of studies which have found proatherogenic roles involving leptin. For example, leptin has been shown to have a role in platelet aggregation [109] and to have proatherogenic properties acting on both endothelial and VSMCs [110, 111]. Leptin has also been positively correlated with hs-CRP as well as IL-6 [95, 112] and found to induce proliferation and migration of VSMCs by stimulating phosphorylation and activation of mitogen activated protein kinases and upregulating phosphatidylinostidol 3-kinase activity [113]. Other studies have found that leptin induces the production of matrix metalloproteinase-2 (MMP-2) which plays a key role in plaque rupture [111, 114, 115]. Additionally, leptin has been found to stimulate vascular remodeling by promoting profibrotic cytokine production [116], increase secretion of the proatherogenic enzyme lipoprotein lipase in cultured human and mouse macrophages [117], and induce hs-CRP expression in human coronary endothelial cells [118]. Leptin may also play a role in inducing caveolin-1 in endothelial cells, which is involved in regulating the movement of LDL in the endothelial layer during plaque formation, as well as by inhibiting eNOS

function [119, 120] and by playing a role in NO-mediated vasodilation of blood vessels [121]. Lastly, leptin also has been reported to increase the expression of plasminogen activator inhibitor-1 in coronary endothelial cells, which is known to play a role in the progression of atherosclerosis [122].

While leptin and adiponectin have been studied in relation to atherosclerosis, there are some more recently discovered adipokines whose possible roles have not been well examined. These include visfatin, resistin and chemerin. Resistin was discovered in 2001 and is secreted by adipose tissue, macrophages, neutrophils, and other cell types [95]. Resistin production is restricted to adipocytes in mice, however in humans its primary source is from circulating monocytes and macrophages [123]. It has been suggested that resistin may play a regulatory role in insulin resistance but that it also may be involved in inflammation, endothelial dysfunction, thrombosis, angiogenesis and VSMC dysfunction in CVD [124, 125]. One study found that there was an increase in plasma resistin levels in patients with unstable angina (chest pain caused by lack of oxygen to the heart) compared to those with the less severe stable angina [126]. Resistin levels were also elevated in patients with acute coronary syndrome and it was hypothesized that the release of resistin occurs during plaque rupture [127]. It was also suggested that macrophage-secreted resistin interferes with endothelial and VSMC functions. One study found that resistin exerts a proliferative effect on human aortic SMCs through both extracellular signal-related kinases (ERK) 1/2 and protein kinase B (Akt) signalling pathways [77, 128]. In ERK signalling proteins in a cell communicate a signal from a surface receptor to DNA in the nucleus, while in Akt signalling a growth

factor activates phosphatidylinositol 3-kinase, which in both pathways promotes cell growth and survival. More research regarding the mechanistic role of resistin in atherosclerosis is required to determine if these hypotheses are correct. The adipokine visfatin was first discovered in 2005 in liver, bone marrow and muscle but has since been found to be secreted by visceral fat [95, 129]. These regions represent those with the highest visfatin expression, however it is expressed at some level in all tissues. Interestingly, visfatin expression is very low in subcutaneous fat and it is predominately secreted from macrophages rather than adipocytes in visceral adipose tissue [79, 129]. Visfatin has been associated with insulin resistance and impaired glucose metabolism, and is secreted under pro-inflammatory conditions by macrophages in visceral fat making it logical that visfatin may also play a role in atherosclerosis [130, 131]. To date there has been debate about whether a correlation exists between visfatin and atherosclerosis. Most studies show increased levels of visfatin in patients with diabetes, obesity, hypertension, and renal and cardiovascular diseases [132], however, others report decreased visfatin levels in these diseases. One study found that visfatin expression was increased in human symptomatic plaques compared to asymptomatic plaques [95, 133]. Recently, it was discovered that visfatin expression in pericoronary fat was positively correlated with coronary atherosclerosis [134], and also that individuals with increased LDL and hs-CRP also had increased visfatin blood levels [135]. These studies suggest that visfatin likely acts as an inflammatory mediator in the development of atherosclerosis. It was also determined that visfatin induces monocyte chemotactic protein-1 in human endothelial cells [136], and that foam cells secrete visfatin leading to an increase in IL-8, TNF and MMP-9. Further research is required to determine the exact role of visfatin in

atherosclerosis. One of the most newly discovered adipokines is chemerin. It was determined to act as an adipokine in 2007, and its role in cardiovascular disease is a topic which is beginning to be explored and is the focus of this research. Figure 3 shows a summary of what is known about the roles of adipokines in atherosclerosis.

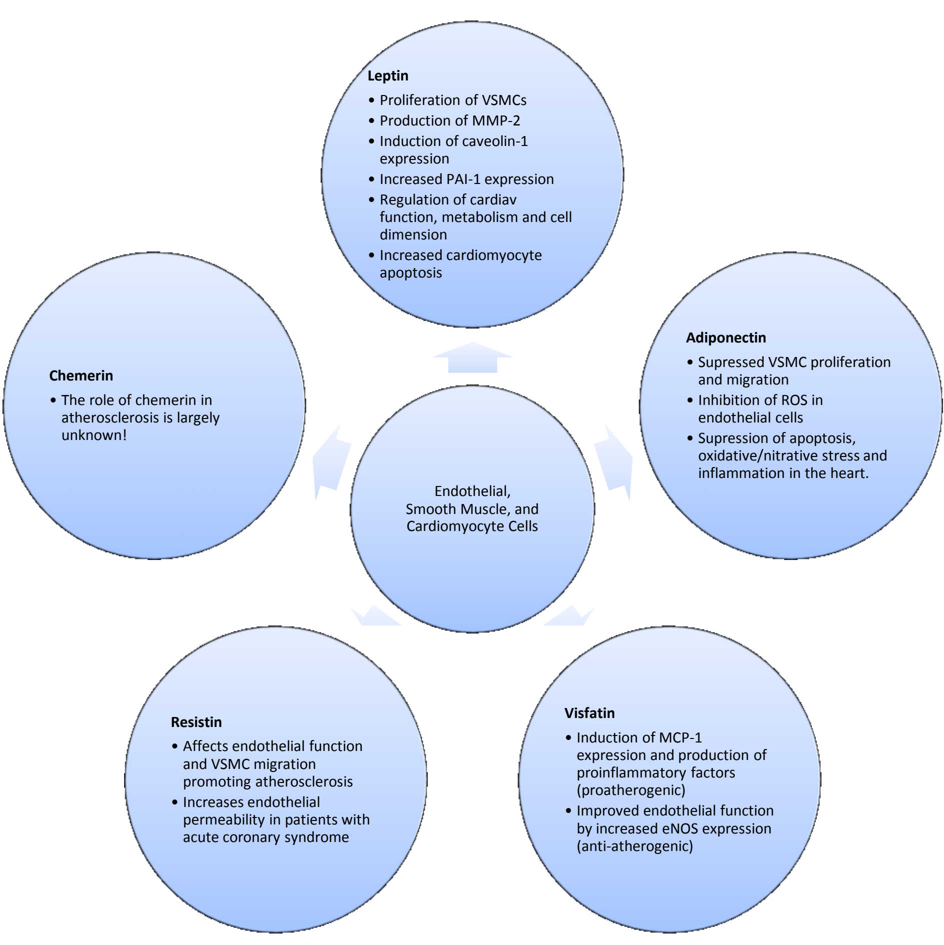


Figure 3: Schematic representation of the role of adipokines in atherosclerosis (Adapted from Scotece, 2012) [95].

1.2 Chemerin & CMKLR1

1.2.1 Chemerin discovery, expression and receptors

Chemerin was initially discovered in 1997 as a retinoid responsive gene in psoriatic skin lesions [137, 138]. It was originally called tazarotene-induced gene 2 (TIG2) or retinoic acid receptor responder 2 (RARRES2) [138]. Despite this early discovery, evidence of its biological function did not occur until 2003 when chemerin was detected in human inflammatory fluids including ascites from ovarian cancer patients and synovial fluid from rheumatoid arthritis patients [139]. It was then determined that chemerin was a ligand for the previously discovered G-protein coupled receptor (GPCR) called chemokine-like receptor 1 (CMKLR1), and that chemerin acted as a chemoattractant for cells expressing CMKLR1 [138-141]. Chemerin was found to be highly expressed in the liver and white adipose tissue, moderately expressed in the lungs and brown adipose tissue, and minimally expressed in tissues such as the heart ovary and kidney [142]. Interestingly, CMKLR1 was found to be highly expressed in macrophages, immature dendritic cells, and in white adipose tissue, and at lower levels in bone, lung, brain, heart and placenta [139, 143]. Because both chemerin and CMKLR1 were highly expressed in white adipose tissue, it prompted the hypothesis that chemerin could be acting as a secreted adipokine. This was confirmed by Goralski et al. in 2007, and chemerin was classified as a novel adipokine. It was determined that chemerin regulated adipogenesis and adipocyte metabolism which was evidenced by data showing that a loss in either chemerin or CMKLR1 prohibited adipocyte differentiation and modified the

expression of genes important in glucose and lipid metabolism [143]. In addition to CMKLR1, chemerin also can bind with the receptors G-protein coupled receptor 1 (GPR1), and chemokine receptor-like 2 (CCRL2). GPR1 is highly expressed in adipose tissue, but its expression is limited in leukocytes [144], while CCRL2 is not highly expressed in adipose tissue, but is detected in lung, heart, spleen and leukocytes [145]. CCRL2 lacks an intracellular signalling domain and CCRL2-bound chemerin is not internalized. Therefore, rather than activation of intracellular signalling, it is believed that CCRL2 binding serves to increase local chemerin concentrations, possibly facilitating chemerin binding and activation of CMKLR1 and GPR1 [145, 146]. Subsequent research of chemerin and its receptors has determined that it also plays a role in osteoclastogenesis [146, 147], angiogenesis [148], proliferation, migration, differentiation, renal function, energy metabolism [137, 146, 149, 150], and inflammatory processes in skin [151] and adipose tissue [152].

The mechanisms and pathways in which chemerin and its receptors are involved are largely unknown. Early research has suggested that CMKLR1 activation resulted in intracellular calcium release and a reduction in cAMP accumulation [141]. These effects were inhibited by treatment with pertussis toxin (an endotoxin produced by the bacterium *Bordetella pertussis*), which inactivate G-proteins, suggesting that a GPCR was likely involved [139]. Additional research regarding the cell signalling of CMKLR1 and GPR1 are required to fully understand the pathways through which they are functioning.

1.2.2 Chemerin structure and processing

Chemerin exists in several isoforms but is secreted as an inactive 163 amino acid protein referred to as pre-prochemerin which is enzymatically cleaved by various serine and cysteine proteases (Figure 4) [143, 147] into active and inactive isoforms ranging from 152- 158 amino acids in length. Initially pre-prochemerin undergoes N-terminal processing by a yet to be determined mechanism into the 18 kDa (162 amino acid) inactive protein prochemerin [139]. Prochemerin undergoes cleavage at the C-terminal by extracellular proteases of the coagulation, fibrinolytic, or inflammatory cascade to produce the active 16 kDa forms of chemerin [137, 139, 143, 145]. Each of the active chemerin isoforms have varying lengths and degrees of activity toward CMKLR1. In vitro recombinant human chemerin studies have determined that there are six chemerin isoforms, including chemerinG^{152}, chemerinF^{154}, chemerinA^{155}, chemerinF^{156}, chemerinS^{157}, and chemerinK^{158} [137, 147]. The enzyme elastase can activate prochemerin by cleaving 6, 8 or 11 C-terminal amino acids, while cathespin G can remove 7 amino acids. ChemerinK^{158} is formed when prochemerin is cleaved by plasmin or tryptase [137, 147]. ChemerinS^{157} can be formed either by cleavage of prochemerin with elastase followed by staphopain B, cathespin K, or cathepsin L, or from the interaction of ChemerinK^{158} with carboxypeptidase N or B. Meanwhile, chemerinF^{156} can be formed by cleavage of prochemerin by cathepsin G or the newly reported kallikrein 7 [151]. ChemerinA^{155} is created when proteinase 3, tryptase or elastase interact with prochemerin, while chemerinF^{154} is formed following cleaving of either chemerinF^{156} or chemerinS^{157} by chymase. Lastly, chemerinG^{152} can be formed by the interaction of

prochemerin with elastase. Of these isoforms, chemerinG[152], chemerinF[154], and chemerinA[155] are all inactive or with very low biological activity, chemerinK[158] has low activity, chemerinF[156] has moderately high activity and chemerinS[157] has the highest biological activity.

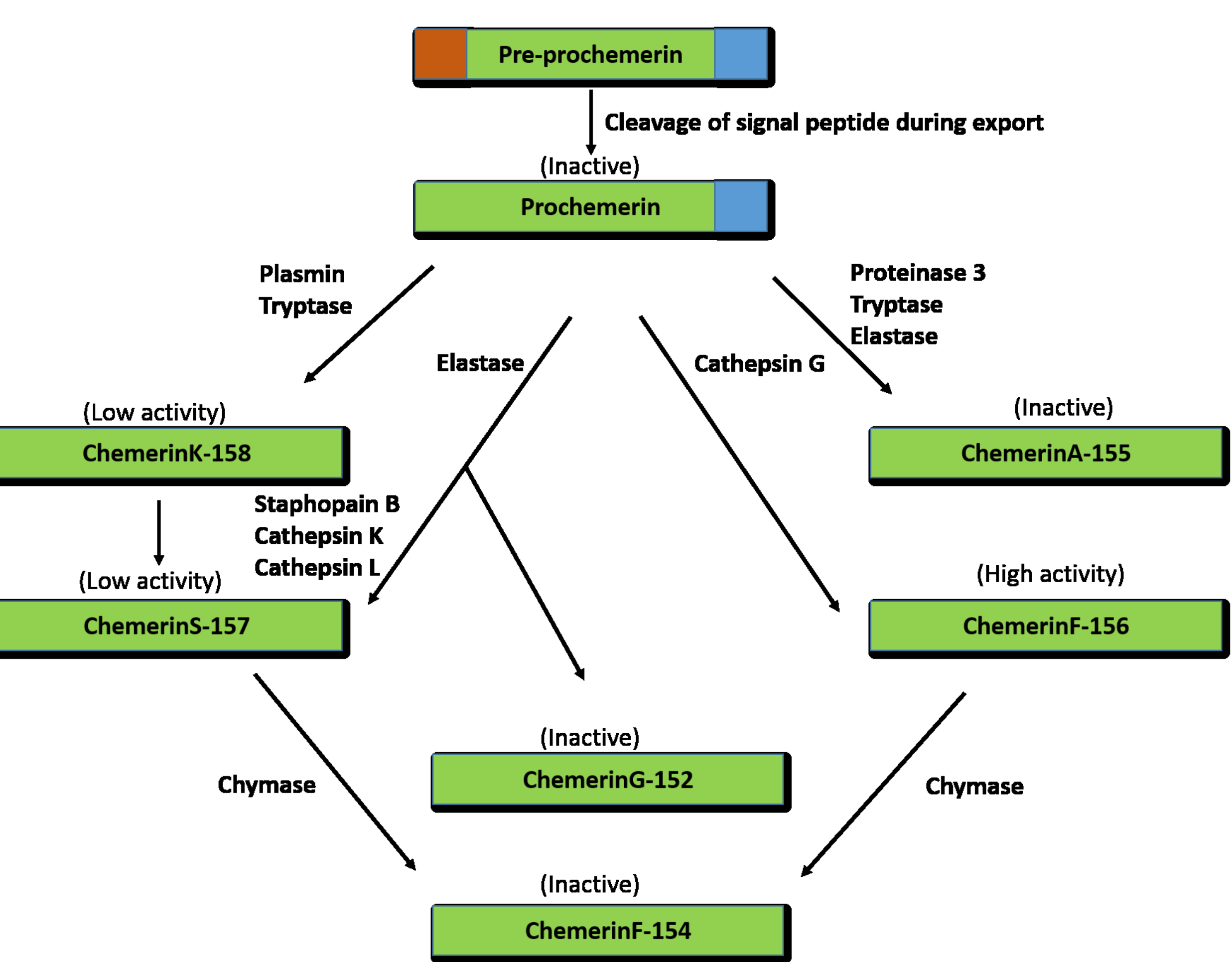


Figure 4: Chemerin processing into various isoforms.

1.2.3 Chemerin isoform distribution in humans

The locations of the chemerin isoforms differ in distribution among bodily fluids. The distribution of chemerin isoforms has been explored in various body fluids using specific antibodies for chemerinK^{158} and chemerinS^{157} [147, 153]. Prochemerin was found to be the dominant isoform in plasma from healthy subjects, constituting 80% of the total chemerin (combined prochemerin and active and inactive chemerin isoforms), while chemerinS^{157} was almost undetectable. This was the opposite in synovial fluid from arthritis patients which had 2-fold higher total chemerin compared to plasma, however it was composed of only 25% prochemerin meaning it was 75% chemerinK^{158} and chemerinS^{157}. To date, the presence of chemerinK^{158}, S^{157}, and A^{155} have been verified in human blood, chemerinF^{154} in hemofiltrate, chemerinS^{157} in ascites, chemerinK^{158} in synovial fluid and chemerinK^{158} in cerebrospinal fluid [140, 153-156]. These findings indicate that while total chemerin differs in different regions of the body, so do the concentrations of biologically active chemerin isoforms. This variability is most likely attributable to the relative expression and activity of proteases with the ability to activate or deactivate chemerin at any given anatomical site.

1.2.4 Chemerin in disease

Circulating chemerin levels have been correlated to a number of diseases, many of which are associated with chronic inflammation. Given that chemerin is largely secreted by adipocytes, the most obvious disease with which it is associated is obesity.

Serum chemerin concentrations were found to be positively correlated with BMI [157, 158] as well as waist-to-hip ratio and accumulation of abdominal visceral fat [157, 159]. Increased chemerin has also been associated with diabetes [137], polycystic ovary syndrome [160], Crohn's disease, ulcerative colitis [161], chronic kidney disease [162-164], chronic pancreatitis [165], arthritis [166, 167], pre-eclampsia [168], liver disease [169-171], psoriasis [138], and atherosclerosis [172, 173]. Increased chemerin in blood has been positively correlated with a number of inflammatory markers, such as hsCRP [174-177], IL-6, TNF, as well as the adipokines resistin and leptin [161, 163, 170, 175, 177]. The exact mechanisms of how chemerin relates to these inflammatory cytokines remains unknown. Some studies have treated cultured human chondrocytes with chemerin and discovered that the secretion of inflammatory mediators, such as IL-6, IL-8, TNF, CCL2, and IL-1β were increased [166, 167]. Also, when human intestinal epithelial cells and adipocytes were treated with TNF, chemerin secretion was increased [178-180]. These studies show that chemerin may contribute to acute inflammation generation and resolution, and may also be a contributing factor in chronic inflammation.

1.2.5 Chemerin in cell migration and proliferation processes

Migration and proliferation are cellular processes which are prevalent in inflammatory healing mechanisms following tissue injury. For this reason, it makes sense that chemerin may be playing a role in cell migration and proliferation. While chemerin has not been researched in relation to VSMC proliferation and migration as they relate to atherosclerosis, it has been researched in various other cell types. For example, chemerin

has been found to regulate the proliferation and differentiation of mouse C2C12 myoblasts which are precursors to skeletal muscle cells [181]. In this study it was determined that chemerin promotes proliferation but inhibits differentiation of the C2C12 cells. A second study looked at migration of natural killer cells and dendritic cells in human oral lichen planus lesions and found that chemerin and CMKLR1 played an important role in their migration [152]. These studies suggest that chemerin and CMKLR1 may also play a role on VSMC migration and proliferation.

1.3 Chemerin in atherosclerosis

1.3.1 Correlative studies

The majority of current literature examining the relationship between chemerin and atherosclerosis is correlative in nature. For example, Xiaotao et al. (2012) examined the correlation between serum chemerin and the presence and extent of coronary artery disease (CAD) [173]. A total of 132 patients with CAD, and 56 patients without CAD were included in the study where they underwent coronary angiography to evaluate atherosclerotic plaque severity and also provided blood samples. Serum chemerin was significantly elevated in CAD patients compared to healthy patients, and was also correlated with BMI, triglycerides, and LDL. A similar study involving more participants examined serum chemerin levels in Chinese adults with and without CAD. In this study 239 patients had CAD and 191 did not. As was the case in the previous study, serum chemerin was found to be an independent risk factor for coronary atherosclerosis [182].

A study by Yoo et al. (2012), examined the correlation between serum chemerin with atherosclerosis as measured by arterial stiffness and carotid intima-media thickness in obese and non-obese subjects [183]. Arterial stiffness was measured by recording the brachial ankle pulse wave velocity (baPWV), which measures the volume of blood between the ankle and wrist over time, where a higher baPWV reading correlates to high arterial stiffness. Intima-media thickness is a measure of the increasing thickness of an artery as a result of inflammation or plaque formation which limits blood flow. Having a high intima-media thickness and baPWV reading would represent the worst case scenario. There were 58 obese and 62 non-obese individuals who participated in this study and it was discovered that serum chemerin was increased in obese individuals compared to lean controls and was correlated with BMI, waist circumference, LDL, triglycerides, and hs-CRP. Serum chemerin was significantly associated with the baPWV, but not carotid intima-media thickness. Therefore, circulating chemerin was found to be an independent risk factor for arterial stiffness.

1.3.2 Chemerin as a predictor of atherosclerosis

The majority of correlative studies describe similar findings where chemerin has been correlated to the presence and extent of atherosclerosis. There are a few studies however which have not found a correlation between chemerin and atherosclerosis. One such study by Lehrke et al. (2009) aimed to examine the relationship between chemerin and 1) markers of inflammation, 2) components of metabolic syndrome, and 3) coronary atherosclerotic plaque burden and morphology[177]. The study involved 303 patients

with stable typical or atypical chest pain who did not have coronary artery stenosis (abnormal narrowing of the vessel). The atherosclerotic plaques in these individuals were determined by CT-angiography and were classified as calcified, mixed, or non-calcified. In this study, chemerin was highly correlated with hs-CRP, IL-6,TNF, resistin, and leptin, as well as, hypertension among other metabolic syndrome characteristics. Chemerin was weakly correlated with coronary plaque burden and the number of non-calcified plaques, however when these values were corrected to eliminate the bias of other cardiovascular risk factors, this correlation was lost and chemerin was not found to be an independent predictor of coronary atherosclerosis. Another study by Becker et al. (2010) found that expression of human chemerin induced insulin resistance in skeletal muscle but did not affect weight, lipid levels, or the extent of atherosclerosis in LDL receptor KO mice on a high-fat diet [172]. In this study, mice were injected with chemerin, and having the presence of additional chemerin did not influence the severity of atherosclerotic lesions. One possible explanation for this negative result is this is that the high-fat diet was already inducing high chemerin production in both the chemerin-injected and control groups, such that additional chemerin only increased blood glucose and did not alter atherosclerotic lesions. Although both of the described studies describe a lack in correlation between chemerin and atherosclerotic lesion size and morphology, they do not completely rule out its involvement in the overall atherosclerosis process.

1.3.3 Advancements in the role of chemerin and CMKLR1 in atherosclerosis in humans

More recent research has focused on understanding possible mechanisms of chemerin and CMKLR1 signalling in atherosclerosis by looking at locations of protein expression. It has been discovered that periadventitial fat (fat surrounding arteries and organs) has a great impact on the formation of atherosclerotic plaque, due to its direct proximity to arteries. Adipokines secreted by periadventitial fat are especially important in atherosclerosis for this reason. One study looked at protein expression of the adipokines adiponectin, visfatin, leptin, vaspin and chemerin, in periaortic and epicardial adipose tissue from 41 autopsy cases. They found that adiponectin, visfatin, and chemerin protein expression was associated with both aortic and coronary atherosclerosis, while vaspin and leptin were only correlated with aortic atherosclerosis [134]. Another study focused on chemerin and CMKLR1 expression exclusively. In this study they examined 40 autopsy cases aged 14-81 who died of acute coronary occlusion. Only 34/ 40 of aorta samples had atherosclerotic lesions. Anti-chemerin and anti-CMKLR1 primary antibodies were used to determine the protein expression of chemerin and CMKLR1 in periaortic fat, aortic VSMCs, and aortic foam cells. Chemerin was detected in 37/40 periaortic samples, 40/40 VSMCs, and 27/34 foam cells in atherosclerotic lesions. CMKLR1 was not expressed in periaortic tissue, but it was detected in VSMCs (10/40) and foam cells (30/34). Overall, a correlation between protein expression and atherosclerotic lesion severity was observed [184]. These studies support the hypothesis

that locally produced adipokines, such as chemerin, may be involved in atherogenesis and that different adipokines may play a role in different areas of the vasculature.

1.4 Project rationale & research objectives

1.4.1 Project rationale

As has been described, research regarding the role of chemerin in atherosclerosis has been largely correlative in nature. That being said, it is known that chemerin regulates migration and proliferation processes, such as in the migration of dendritic and natural killer cells and proliferation of mouse C2C12 myoblast and preadipocyte cells. It is also known that chemerin and CMKLR1 are expressed at a protein level in VSMCs and foam cells, but that CMKLR1 was not detected in periaortic fat. Based on this prior knowledge, it is reasonable to predict that chemerin may play a role in regulating VSMC migration and proliferation processes in atherosclerosis.

1.4.2 Research objectives

1. To develop a human SMC model by differentiating adipose-derived stem cells (ASCs) into SMCs.

2. To develop a method for isolation and culturing of VSMCs from the aortas of $CMKLR1^{+/+}$ and $CMKLR1^{-/-}$ mice.

3. To determine the effect of chemerin/CMKLR1 signalling on human SMC and mouse VSMC function as it relates to atherosclerosis.

1.4.3 Hypothesis

Chemerin and CMKLR1 play a role in the proliferation and migration of VSMCs as it relates to atherosclerosis.

Chapter II. Experimental

2.1 Materials and methods

2.1.1 Human ASC to SMC differentiation model

2.1.1.1 Human cell culture methods & differentiation experiments

Human adipose-derived stem cells (Zenbio Cat # ASC-F, Lot #ASC0053) were maintained in subcutaneous preadipocyte maintenance medium (Zenbio, Cat # PM-1) and those which were differentiated to smooth muscle cells were cultured in smooth muscle basal medium (Lonza, Cat # CC-3181). The cell media compositions are listed in Table 2. Cells were maintained at 40-90% confluence and plated in 12-well plates (40 000 cells/well) and cultured for 0, 7 and 14 days. At these time points cells were harvested for RNA isolation and gene expression analysis and also for treatment with actin and nuclei fluorescent stains and fluorescent imaging.

Table 2: Composition of medium used for human ASC differentiation experiments.

Subcutaneous Preadipocyte Medium (PM-1) Zenbio	Smooth Muscle Basal Medium (SmBM) Lonza
DMEM/ Hams F-12 (1:1, v/v) HEPES pH 7.4 Fetal bovine serum Penicillin Streptomycin Amphotericin B D-glucose (3.13 g/L)	Human epidermal growth factor (hEGF) Insulin Human fibroblastic growth factor (hFGF-B) Fetal bovine serum Gentamicin Amphotericin-B

2.1.1.2 Human cell staining & fluorescent imaging

On days 0, 7 and 14, differentiated and undifferentiated cells in 12-well plates were stained with the actin stain fluorescein isothiocyanate labeled phalloidin (Sigma, Cat # P5282) and nuclei stain Hoescht 33258 (Biotium, Cat #40045). In doing this, cells were first washed 3X with phosphate-buffered saline (PBS) (500 µL/ well) and fixed with filter sterilized paraformaldehyde (4% in PBS) for 15 minutes at room temperature (RT) on plate shaker (300 rpm). Paraformaldehyde was removed and cells were washed 3X with PBS (500 µL/ well), then permeablized in 0.3% Triton X-100 in PBS (500 µL/ well) for 5 minutes. Cells were washed 3X with PBS (500 µL/ well) for 5 minutes each wash. Non-specific binding was blocked using 1% bovine serum albumin (BSA) in PBS (500 µL/ well) for 30 minutes. Blocking solution was removed and a 1:500 solution of

phalloidin containing 0.1% BSA, and 0.05% Tween-20 in PBS (500 µL/ well) was added and incubated for 1 hour with gentle shaking. Cells were rinsed 3X with PBS (500 µL/ well) followed by staining with Hoescht 33258 (0.3 µg/ mL in PBS) for 3 minutes and rinsing 2X with PBS (500 µL/ well). Cells were then imaged using a Zeiss Axiovert 200 microscope with a Hamamatsu Orca R2 camera.

2.1.1.3 RNA isolation, and quantitative PCR

Cells were lysed in RLTplus buffer and RNA was isolated using the RNeasy mini plus kit (Qiagen; Germantown, MD, USA, Cat #74134) according to the manufacturer's instructions. Reverse transcription was used to generate copy DNA (cDNA) from 0.2 µg of isolated RNA using RNA to cDNA EcoDry premix (Clontech; Mountain View, CA, Cat# 639549). Exon- spanning quantitative real-time PCR (qPCR) primers were designed using NCBI primer BLAST (Table 3). Gene expression was measured using the Roche FastStart SYBR green Master (Roche; Laval, QC, Cat# 04673484001) on a Light Cycler 96 instrument according to the manufacturer's instructions. Relative gene expression was calculated using the ΔΔCt method [185] with glyceraldehyde 3-phosphate dehydrogenase *(GAPDH)* and cyclophilin A *(CYC-A)* as the reference genes.

<u>Table 3:</u> Human qPCR Primers

Gene	Accession	Sequence 5' to 3'
Calponin 1 (*Cnn1*)	NM_001299	F: AACCATACACAGGTGCAGTC R: GATGTTCCGCCCTTCTCTTAG
Myosin Light Chain 9, Regulatory (*Myl9*)	NM_006097	F: GTGATTCGCAACGCCTTTG R: TTCTTATCAATGGGTGCCTCC
Leptin	NM_000230	F: TTCACACACGCAGTCAGTCT R: GGAGGTTCTCCAGGTCGTTG
Adiponectin	NM_001177800	F: ATGGCCCCTGCACTACTCTA R: CAGGGATGAGTTCGGCACTT
Osterix	NM_001300837	F: ATGGCTCGTGGTACAAGGC R: GCAAAGTCAGATGGGTAAGTAGG
Osteocalcin	NM_199173	F: TTTCTGCTCACTCTGCTGACCC R: CTGTTCACTACCTTATTGCCCTCC
Chemerin	NM_002889	F: TGGAAGAAACCCGAGTGCAAA R: AGAACTTGGGTCTCTATGGGG
CMKLR1	NM_002046	F: AAGGGGAGGAGAAATAGAGTCCAC R: TGGCTTCCAAGGGGGATAAGTC
Glyceraldehyde 3-phosphate dehydrogenase *(GAPDH)*	NM_001289746	F: GAGTCAACGGATTTGGTCGT R: TTGATTTTGGAGGGATCTCG
Cyclophilin-A *(CYC-A)*	NM_001300981	F: TTCATCTGCACTGCCAAGAC R: TCGAGTTGTCCACAGTCAGC

2.114 ELISA measurement of human secreted total chemerin

The concentration of total chemerin secreted into cell culture media was determined using a DuoSet enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Cat # DY2324). Mouse anti-human chemerin capture antibody (100 µL/well of 4 µg/ mL) was added to a 96-well plate. The plate was sealed and placed on an orbital shaker (400 revolutions/ minute at RT) for 16 hours to allow for antibody-binding to the well surface. The solution was aspirated and washed 3X with wash buffer (300 µL/well). A blocking reagent diluent solution (300 µL/well of 1% bovine serum albumin in PBS) was added and incubated for 1 hour with shaking at RT. Wells were aspirated and washed 3X with wash buffer as described previously. The standards were prepared by serially diluting a human chemerin-157 stock solution (160 ng/mL) in reagent diluent to generate a 7-point standard curve with chemerin concentrations of 0, 62.5, 125, 250, 500, 1000, and 2000 pg/mL. The diluted media samples (1:20) and standards were added (100 µL/well) and incubated with shaking for 2 hours at RT, followed by aspiration and 3X wash. Next, biotinylated goat anti-human chemerin detection antibody (100 µL/ well of 200 ng/mL) was added and incubated with shaking for 2 hours at RT, followed by aspiration and 3X wash. Streptavidin-HRP (100 µL / well) was added and the plate was covered in aluminum foil and incubated for 20 minutes with shaking, followed by aspiration and a 3X wash. Substrate solution (100 µL/ well) containing a 1:1 mixture of hydrogen peroxide and tetramethylbenzidine was added to each well, the plate was covered and incubated for 20 minutes without shaking. Stop solution (50 µL/ well of 2M sulphuric acid) was added and absorbance (450 nm for detection of the coloured reagent

and 540 nm to correct for volume) was immediately read and recorded. The specific absorbance (Abs) of each sample was calculated using the following equation:

$$Abs_{Sample} = (Abs_{450nm} - Abs_{540nm}) - Abs_{Blank} \quad \text{(Eq. 1)}$$

GraphPad prism software was used to create a 4-phase logarithmic standard curve and the total chemerin concentration of each sample was interpolated.

2. 1.2 Mouse vascular smooth muscle cell model

2.1.2.1 Animal protocol and housing

All protocols were conducted in accordance with the Canadian Council on Animal Care guidelines and approved by the Dalhousie University Committee on Laboratory Animals. Animals were maintained at 25 °C on a 12h light: 12h darkness cycle with access to food and water and allowed to feed *ad libitum*. CMKLR1$^{-/-}$ mice were purchased from Deltagen, and the coding region of the CMKLR1 gene which was replaced is located in the 3rd exon. The genotype of breeding CMKLR1$^{+/+}$ and CMKLR1$^{-/-}$ mice was confirmed by genotyping and offspring were used for experiments.

2.1.2.2 Murine aortic harvest, VSMC isolation & characterization

CMKLR1$^{+/+}$ and CMKLR1$^{-/-}$ mice were euthanized by intraperitoneal injection with a syringe (1 mL with 26 ½ gauge needle) containing sodium pentobarbital (90 mg/kg). The mouse was placed in supine position and pinned on a styrofoam board. The body was sprayed with ethanol (70%) and skin was removed using sharp sterile scissors and toothed forceps. A second set of sterile scissors and forceps were used to open the thorax. The lungs and liver were removed to access the aorta, and the bottom abdominal region of the aorta was cut, followed by PBS perfusion of the aorta via injection of the left ventricle. Sterile scissors and angled forceps were used to harvest the aorta, which was then placed on a dissecting dish in Fungizone (2-3 drops of filter sterilized solution containing 10 µL of 0.25 mg/mL [Cat # 15290018, Life Technologies] in 10 mL DMEM) and pinned using micro dissecting needles. The remainder of the dissection was completed using a dissecting microscope and fine angled forceps and microdissecting scissors. The ragged periadventitial fat was removed leaving the smooth aorta. The aorta was placed in a second dissecting petri dish with DMEM (2-3 drops). The aorta was cut into square pieces (1-2 mm in width) and the pieces were placed in a tissue culture tube (1.5 mL) containing freshly prepared type II collagenase enzyme solution (100µL of filter sterilized 3.75 mg type II collagenase in 2.75 mL DMEM). The tube was loosely capped and placed in a standard 37°C, 5% CO_2 incubator for 6 hours. Cells were agitated via pipetting at the 5 hour point. Following enzymatic digestion the cells were placed in a polypropylene tube (15mL), diluted with DMEM (3 mL), and centrifuged for 5 min (300 x g at RT). Medium was aspirated, and cells were re-suspended in DMEM (1 mL) and

placed in a T25 flask containing DMEM (5 mL). Upon achieving 80-90% confluence, cells were moved to a T75 flask. Initial gene expression characterization was completed using RNA isolation and qPCR techniques (See sections 2.113) using primers listed in Table 4.

Table 4: Mouse qPCR Primers

Gene	Accession	Sequence 5' to 3'
Actin, alpha 2, smooth muscle, aorta	NM_007392	F: GTGAAGAGGAAGACAGCACAG R: GCCCATTCCAACCATTACTCC
Calponin1 (*Cnn1*)	NM_009922	F: CAGAGAAACAAGAGCGGAGAT R: GTTTGGGATCATAGAGGTGACG
Myosin light chain 9, regulatory (*Myl9*)	NM_172118	F: AGGCAAGATGTCGAGCAAGAG R: GTTCTTCCCCAGAGAGGCCAG
Von Willebrand factor homolog (*VWF*)	NM_011708	F: GGGTGACCAAAGCATCTCCA R: CATCGATTCTGGCCGCAAAG
Intercellular adhesion molecule 2 (*ICAM2*)	NM_010494	F: CACGTTCAACAGCACAGCTC R: TCCTGCATCGGCTCATAGAC
Endothelial cell- selective adhesion molecule (*ESAM*)	NM_027102	F: GACTGAGTACCCTTGCTGCC R: TCTCCCTCTACCGCTTCCAA
Platelet/ endothelial cell adhesion molecule (*PECAM*)	NM_008816	F: CTCCCTTGAGCCTCACCAAG R: GGAGCCTTCCGTTCTTAGGG
Endothelial cell selectin (*ESEL*)	NM_011345	F: GCTGGAGAACTTGCGTTTAAG R: AGATAAGGCTTCACACTGGAC
Endothelial cell nitric oxide synthase 3 (*eNos*)	NM_008713	F: CTGCCACCTGATCCTAACTTG R: CAGCCAAACACCAAAGTCATG
Chemerin	NM_027852	F: TACAGGTGGCTCTGGAGGAGTTC R: CTTCTCCCGTTTGGTTTGATTG
CMKLR1	NM_008153	F: GCTTTGGCTACTTTGTGGACTT R: CAGTGTTCACGGTCTTCTTCATCTTG
Cyclophilin-A *(CYCA)*	NM_008907	F: GAGCTGTTTGCAGACAAAGTTC R: CCCTGGCACATGAATCCTGG

2.1.2.3 ELISA measurement of mouse secreted total chemerin

Total secreted chemerin was measured using a Quantikine ELISA Mouse Chemerin Immunoassay (R&D Systems, Cat # MCHM00). Assay diluent RD-1 was added (50 µL/ well) to the provided monoclonal mouse chemerin antibody-coated 96-well microplate, followed by the addition of standard, control or sample (50 µL/ well). Plate was covered with adhesive strips and incubated for 2 hours at RT on a horizontal orbital microplate shaker (500 +/- 50 rpm). Wells were aspirated and washed 5X with wash buffer (400 µL/ well), ensuring complete removal of liquid after each wash. Following final wash, plate was inverted and blotted against clean paper towel. Mouse chemerin conjugate (100 µL/ well) was added, and the plate was covered with a new adhesive strip then incubated at RT for 2 hours. The plate was washed 5X with wash buffer (400 µL/ well as previously described, and substrate solution (100 µL/ well) was added, followed by a 30 minute RT incubation on the benchtop while wrapped in tinfoil. Finally, stop solution (100 µL/ well) was added and absorbance of plate was read within 30 minutes using a microplate reader at 450 and 540 nm as is described in Section 2.114.

2.1.2.4 Bromodeoxyuridine (BrdU) proliferation assay

Mouse VSMCs were plated in a 96 well plate (2000 cells/ well) and allowed to adhere for 24 hours followed by serum starvation for 24 hours to synchronize the cell cycle. Cells were then treated with chemerin (0.1- 30 nM) (R&D Systems, Cat # 2325-CM-025) alone or in combination with TNF (0.1- 10 nM) (Sigma-Aldrich, Cat # T7539)

for 24 hours. Cell proliferation was measured using a BrdU assay kit (Cell Signaling Technology Cat #5492), beginning with a 1 hour incubation with 1X BrdU (100 µL/well) in a 37°C, 5% CO_2 incubator. Medium was removed and fixing/denaturing solution (60 µL/ well) was added and incubated at RT for 30 min. Fixing/ denaturing solution was removed and cells were treated with 1X BrdU detection antibody solution (50 µL/ well) for 1 hour at RT. The detection antibody solution was removed, and cells were washed 3X with wash buffer (50 µL/ well). A solution of 1X HRP-conjugated secondary antibody solution (50 µL/ well) was added and cells were incubated at RT for 30 min, followed by a 3X wash with wash buffer. A working solution of 1:1 luminol/enhancer solution and stable peroxide buffer (50 µL/ well) was added and plate was read within 10 min using a plate-based luminometer to measure luminescence (in relative light units, RLU) at 425 nM. The assay principle and process are summarized diagrammatically in Figure 5.

2.1.2.5 Methyltetrazolium (MTT) cell viability assay

Cells were plated in a 96 well plate (2000 cells/ well) and treated with chemerin alone or in combination with TNF as described in Section 2.1.2.4. Filter sterilized (0.45 micron filter) MTT solution was added (5 mg/ mL MTT in PBS) to each well (20 µL/ well) except for the "blank" wells and the plate was wrapped in aluminum foil and incubated at 37°C for 2 hours. Media was removed, and DMSO (50 µL/ well) was added and plate was shaken (500 revolutions/min) for 5 minutes. Absorbance (550 nm) was read using a plate reader. Cell viability is reported as the fold-change in viability

compared to the blank. The assay principle and process are summarized diagrammatically in Figure 6.

2.1.2.6 Transwell cell migration assay

Harvested CMKLR1$^{+/+}$ and CMKLR1$^{-/-}$ aortic VSMCs were maintained in logarithmic growth at 40 to 80% confluence with complete medium (DMEM + 10% FBS) with cell splitting every 3-4 days. For the assay, cells were split and allowed to grow in complete medium (DMEM + 10% FBS) for 24 hours then incubated in serum-free medium (DMEM) for an additional 24 hours. Cells were trypsinized, collected by centrifugation and re-suspended in serum-free DMEM medium. Cells (1.0 x 10^5 per well) were added to the upper chamber of 8 μm pore ThinCert tissue culture inserts (Greiner Bio One; Monroe, NC, USA, cat# 662638).
Treatments (600 μL) in serum-free medium were placed in the lower chamber as indicated and cells were allowed to migrate for 6 hours. Non-adherent cells were removed from the upper chamber prior to adherent cell fixation in 100% methanol at -20° C for 10 minutes. Cells adhered to the upper side of the membrane were removed using a cotton swab and the insert was washed 2X in PBS. Migrated cells adhered to the lower side of the insert were labeled for 20 minutes in 1 mg/ml Hoescht 33258 (Biotium, Cat #40045). Inserts were washed 1x in PBS, carefully removed and mounted on glass slides using aqueous mounting medium (Sigma Aldrich; Oakville, Ontario, Canada, cat# F4680). Five random-field images per insert were obtained at 40X magnification using a Zeiss Axiovert 200 with a Hamamatsu Orca R2 camera. Stained cells on the underside of

each membrane were counted visually and expressed as fold change over vehicle control (Figure 7).

2.1.3 Statistics

All data are expressed as mean +/- SEM. Comparisons between two groups utilized an unpaired t-test and comparisons between three or more treatments were performed using 1-way ANOVA analyses with GraphPad Prism software. A p-value of less than or equal to 0.05 was considered significant.

Chapter III. Results

3.1 Human ASC to SMC differentiation study results

3.1.1 Differentiated ASCs show SMC morphology

The differentiation of human ASCs into SMCs has not been well documented in the literature. Because of this, it was important to come up with an approach to encourage differentiation and also prove that the differentiation was successful. One such approach was through examining cell morphology using phase contrast microscopy and also by imaging cells labeled with fluorescent nuclei and actin stains (Figures 8 & 9). What is most important to note is that the differentiated cells displayed three primary characteristics of vascular smooth muscle cells including (1) directional uniformity, (2) cell elongation and (3) formation of "hills and valleys". The final characteristic can especially be observed in the fluorescent images (Figure 9).

3.1.2 Effect of the ASC to SMC differentiation protocol on gene expression

In continuing to determine the phenotype of differentiated ASCs, an analysis of the expression of SMC-positive genes and non-SMC negative control genes was conducted. In the first approach several SMC genes were chosen including calponin 1 and myosin light chain 9, regulatory. These genes were expressed both in the ASCs and

differentiated SMCs and were actually higher in the undifferentiated cells with increasing confluence and were reduced by 50% in the differentiated SMCs with increasing confluence (Figures 10a & b). Because these markers were also expressed in the ASCs at a similar level it was difficult to make a conclusion about the phenotype of the differentiated cells. ASCs have been well documented to differentiate into adipocytes, osteoblasts and chondrocytes. In the second approach markers for adipocytes (adiponectin and leptin) and osteoblasts (osterix and osteocalcin) were tested and were confirmed to be absent in the differentiated cells (data not shown).

3.1.3 Evaluation of *CMKLR1* expression and chemerin expression and secretion in undifferentiated ASCs and differentiated cells.

The gene expression and presence of chemerin protein in ASCs and SMCs has not been well documented. Most literature studies have focused on the correlation between chemerin serum concentrations and heart disease, but not at its role mechanistically within atherosclerosis [182, 186]. It was therefore important to characterise the model to determine if chemerin and CMKLR1 were detectable in ASCs in the undifferentiated state and following completion of the SMC differentiation protocol. It was determined that both chemerin and *CMKLR1* were expressed and that *CMKLR1* expression remained consistent in the differentiated cells, while the ASCs showed increased *CMKLR1* expression during the course of the differentiation period (Figure 11a). The expression of chemerin in the ASCs did not change throughout the 28-day differentiation period, while the differentiated cells showed a 50% reduction in chemerin expression at days 14 and 28 (Figure 11b). The ELISA results described the total chemerin secretion in media exposed

to the cells for 48 hours and collected at various time points during the differentiation period. The ELISA studies resulted in secretion of chemerin in the undifferentiated and differentiated cells in the 1-2 nM range (Figure 12).

3.2 Mouse VSMC study results

3.2.1 SMC and EC-specific gene expression profiles in aortic CMKLR1$^{+/+}$ and CMKLR1$^{-/-}$ VSMCs

While the human differentiated ASCs appeared morphologically like SMCs, it was difficult to determine their level of functionality through gene expression analysis because the ASCs also expressed several SMC-specific genes. For this reason, the focus of this project was turned to the isolation of primary mouse VSMCs. When the aorta is harvested from a mouse, both the thick smooth muscle cell and thin interior endothelial cell layers are collected and time in culture allows for the smooth muscle cells to take over, producing an enriched smooth muscle cell population. The morphology of the VSMCs varies depending on whether they are in a contractile (long stretched cells) or proliferative/ migratory (cobble-stone or pancake-like cells) phenotype. In CMKLR1$^{+/+}$ VSMCs, the expression of the SMC-specific gene calponin 1 was significantly increased by 3-fold by days 7 and 14. The SMC-specific gene myosin light chain 9, regulatory was significantly increased 2-fold by day 14 (Figure 13a). In contrast, several EC-specific genes including von willebrand factor homolog, intercellular adhesion molecule 2, endothelial cell-selective adhesion molecule, platelet/endothelial cell adhesion molecule,

endothelial cell selectin, and endothelial cell nitric oxide synthase 3*,* were all significantly decreased by 40-50% after 7 days and 60% after 14 days in culture (Figure 13b). These results were also confirmed in CMKLR1$^{-/-}$ VSMCs where it was demonstrated that *Cnn1* and *Myl9* expression increased with confluence and *E-SEL* and *ESAM* expression decreased with cellular confluence (Figure 14)

3.2.2 Chemerin and *CMKLR1* gene expression in aortic CMKLR1$^{+/+}$ and CMKLR1$^{-/-}$ VSMCs

As is the case in the human VSMCs, chemerin and *CMKLR1* gene expression in mouse VSMCs has not been studied. In CMKLR1$^{+/+}$ VSMCs, it was discovered that both genes are expressed (with more *CMKLR1* expression being observed compared to chemerin, according to Ct value) and that the expression of *CMKLR1* is consistent with increasing confluence while chemerin expression increases, and is significantly increased by day 14 (Figure 15). CMKLR1$^{-/-}$ VSMCs express similar amounts of chemerin at day 0 and 7, but expression is 50% lower at day 14. As is expected, the CMKLR1$^{-/-}$ VSMCs express little to no *CMKLR1* compared to CMKLR1$^{+/+}$ VSMCs.

3.2.3 Secretion of total chemerin by CMKLR1$^{+/+}$ and CMKLR1$^{-/-}$ VSMCs

The secretion of chemerin in cell media exposed to cells for 24 hours has been detected to reach 8 pM in the CMKLR1$^{-/-}$ VSMCs and over 4 pM in the CMKLR1$^{+/+}$ VSMCs by day 14 in culture (Figure 16). Both the CMKLR1$^{+/+}$ and CMKLR1$^{-/-}$ Day 14 detected chemerin concentrations are significantly different than the CMKLR1$^{+/+}$ and CMKLR1$^{-/-}$ Day 0 and Day 7 results.

3.2.4 Cell-culture promoted phenotypic changes in VSMCs and FBS was verified as a positive control for VSMC proliferation and migration

Before beginning proliferation and migration studies, mouse VSMCs were passaged at least five times such that the cells noticeably proliferated faster and were more uniform morphologically (Figure 17). It was determined using preliminary transwell migration assays and BrdU assays that mouse VSMC migration and proliferation can be induced using FBS and that it acts as an ideal positive control. The BrdU results show that 5% and 10% serum produce a maximal proliferation response and so the intermediate 1% serum concentration was chosen to examine the positive or negative interactive effect of chemerin with serum in proliferation and cell viability assays (Figure 18). For the migration assays, 20% FBS was chosen to act as a positive control based upon preliminary studies.

3.2.5 Chemerin does not significantly affect proliferation of CMKLR1$^{+/+}$ VSMCs in the BrdU assay.

It is thought that FBS may contain some amount of chemerin naturally, however interestingly when chemerin was added at 0.1, 1, 10 and 30 nM concentrations to CMKLR1$^{+/+}$ VSMCs a trend where a slight increase occurred at 0.1 nM chemerin, followed by a steady decrease to 10 nM then a slight increase once again at 30 nM in both 0% and 1 % serum (Figures 19a and b). Because these results are not statistically significant and the changes are minimal it can be concluded that chemerin does not affect CMKLR1$^{+/+}$ VSMC proliferation to an observable extent when using the BrdU assay.

3.2.6 Effect of TNF and chemerin co-treatment on the proliferation of CMKLR1$^{+/+}$ VSMCs

Some adipokines work in combination with inflammatory cytokines to influence cell migration and proliferation. Because TNF is a common inflammatory cytokine in atherosclerosis, and induces chemerin secretion from adipocytes [180], it could function in combination with chemerin to influence VSMC proliferation. A TNF dose-dependent decrease in the amount of BrdU-incorporated DNA was detected in CMKLR1$^{+/+}$ VSMCs however there was no additional effect in combination with increasing chemerin

concentration. The decrease in proliferation was statistically significant for the 1 nM and 10 nM TNF concentrations and was approximately a 50% compared to the zero control (Figure 20a). Despite the observed decrease in proliferation, MTT assay results confirm that there was no loss in cell viability at these TNF and chemerin concentrations (Figure 20b).

3.2.7 Chemerin does not significantly affect proliferation of CMKLR1$^{-/-}$ VSMCs using the BrdU assay.

There is a large amount of variability in the 0% serum CMKLR1$^{-/-}$ BrdU assay, however no significant effect of chemerin concentration on proliferation was observed (Figure 21a). The 1% serum data has very little variability, and again does not show any effect of chemerin on VSMC proliferation (Figure 21b). MTT assays were done in both 0% and 1% serum and both showed no reduction in cell viability, further suggesting that chemerin does not influence mouse VSMC proliferation in the CMKLR1$^{-/-}$ cells (Figure 21c).

3.2.8 Chemerin has differential dose-dependent effects on the migration of CMKLR1$^{+/+}$ and CMKLR1$^{-/-}$ VSMCs

Both the CMKLR1$^{+/+}$ and CMKLR1$^{-/-}$ transwell migration studies were highly reproducible and produced consistent results. While the difference in fold migration compared to vehicle between CMKLR1$^{+/+}$ and CMKLR1$^{-/-}$ VSMCs is not statistically significant, the CMKLR1$^{+/+}$ cells consistently show a slight increase in migration at 0.01 and 0.1 nM chemerin concentrations, while the CMKLR1$^{-/-}$ cells show a decrease at these concentrations, compared to the vehicle. Both the CMKLR1$^{+/+}$ and CMKLR1$^{-/-}$ cells are decreased at 10 nM chemerin, and also show almost entirely inhibited migration at 100 nM chemerin, which is statistically significant compared to that at 0 nM chemerin (Figure 22). Subsequent t-tests determined that migration approaches statistical significance between CMKLR1$^{+/+}$ and CMKLR1$^{-/-}$ cell migration at 0.1 nM chemerin ($P= 0.0745$), however when 0.01 and 0.1 nM data were combined to increase statistical power, a significant difference in migration is observed between the CMKLR1$^{+/+}$ and CMKLR1$^{-/-}$ VSMCs using t-test analyses ($P= 0.0106$).

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Chapter IV. Discussion

4.1 Human ASC to SMC differentiation study

4.1.1 Factors affecting ASC to SMC differentiation and characterization

The biggest challenge in using an ASC to SMC differentiation model is confirming that the differentiated cells are functional SMCs. In this study, smooth muscle basal medium containing human epidermal growth factor (hEGF) and human fibroblastic growth factor (hFGF) was used to stimulate differentiation of ASCs into SMCs. A study by Wang (2010) treated ASCs for 7 days with either DMEM supplemented with recombinant human transforming growth factor beta 1 (TGF-β1) or recombinant human bone morphogenetic protein 4 (BMP4) to stimulate the differentiation of ASCs to SMCs. They found increased gene expression of *α-SMA* (10-fold), calponin (4-fold), *SM22 α* (12-fold) and *SM-MHC* (4-fold) in differentiated cells compared to undifferentiated control cells. They also found that when ASCs were embedded in a collagen lattice they displayed contraction which could be further strengthened in response to cabachol (cholinergic agonist) stimulation. In the current study, only morphological and gene expression analyses were used for characterization of the ASCs that were subjected to the SMC differentiation protocol. Both calponin 1 and myosin light chain 9, regulatory were suppressed in the differentiated cells compared to the undifferentiated cells at days 14 and 28. It is possible that the growth factors used in this experiment (while effective in maintaining primary VSMCs) may not have been sufficient to promote differentiation

into functional SMCs. Another possibility is that because relative mRNA expression is being examined, it could be that the ASCs in PM-1 medium were differentiating in to a cell type which also expressed these markers to a greater extent compared to the differentiated cells. It is well known that ASCs can differentiate into osteoblasts and adipocytes and a negative control approach was also examined. It was confirmed that the adipocyte markers leptin and adiponectin and the osteoblast markers osteocalcin and osterix were negative in both the undifferentiated and differentiated cells suggesting that cells in both treatment groups are not differentiating into these cell types. This provides evidence that despite the SMC-specific gene expression results, the cells in the differentiation treatment group which display morphological SMC characteristics and lack expression of adipocyte and osteoblast markers may be differentiating into SMCs. To confirm this, a final gene expression approach would be to examine the disappearance of ASC-specific genes such as CD10 and CD200 which are consistently expressed in both mouse and human ASCs [187]. Lastly, perhaps one of the most functional approaches would be to study the ability of the differentiated cells to display a contractile phenotype. Combined these experiments would be sufficient to confirm the morphologically observed SMC phenotype.

4.1.2 Chemerin & CMKLR1 in ASCs and differentiated cells

Most studies investigating chemerin in atherosclerosis are correlative in nature and the expression of chemerin and *CMKLR1* have not yet been documented in subcutaneous ASCs or SMCS [173, 182, 183]. Recently, a study by Kostopoulos (2014)

examined the protein expression of chemerin and CMKLR1 in various human vascular tissues from autopsy patients, 25% of whom had died of acute coronary occlusion. They found chemerin was detected in the periaortic adipose tissue, aortic VSMCs and in foam cells of atherosclerotic lesions. CMKLR1 was not present in the periaortic adipose tissue (fat-tissue surrounding the aorta), but was detected in the VSMCs and foam cells. They noted a positive correlation between chemerin protein expression and atherosclerosis severity in the autopsy patients. They also state that the presence of chemerin and CMKLR1 in these tissues suggests that chemerin and CMKLR1 signalling contributes to plaque progression. They do not rule out the possible involvement of other CMKLR1 ligands such as lipid mediator resolvin E1, or the existence of other chemerin receptors such as GPR1 and CCRL2 [184]. This study was the first of its kind as it examined the presence of chemerin and CMKLR1 protein in various atherosclerotic tissues in humans, however gene expression analyses and chemerin secretion analyses were not possible due to the use of formaldehyde-preserved tissues. The current study advances research in the area of chemerin and vascular function by showing that the cells in the undifferentiated and differentiated treatment groups both expressed chemerin and *CMKLR1*. Because both chemerin and *CMKLR1* are expressed, functional signalling between the two is possible. Also, *CMKLR1* gene expression remained consistent in the cells of the SMC differentiation treatment group, while the undifferentiated ASCs showed increased *CMKLR1* expression. This could be suggestive of a change occurring in the ASCs, such as differentiation into a preadipocyte-like cell type. Interestingly, the expression of chemerin in the ASCs did not change throughout the 28-day differentiation period, while the cells in the SMC differentiation treatment group showed a 50% inhibition in chemerin

expression at days 14 and 28. Meanwhile, the ELISA results which examined chemerin secretion in media samples collected during the differentiation period showed equivalent increasing secretion of chemerin from cells of the undifferentiated and differentiated treatment groups in the 1-2 nM range. These values are considered to be in the CMKLR1-activating range but are lower than the 10-20 nM total chemerin concentrations detected in the plasma and serum of obese individuals [157]. These results note the importance of recognizing that protein secretion does not always follow a similar trend as gene expression. The gene expression of chemerin and CMKLR1 agrees with the previous literature in suggesting their possible involvement in atherogenic plaque formation [184]. The added finding that human ASCs also express chemerin and *CMKLR1* and secrete chemerin is one which may have implications in adipogenesis, and is something which should be investigated further.

4.2 Mouse Cell Study Discussion

4.2.1 Characterization of CMKLR1$^{+/+}$ and CMKLR1$^{-/-}$ VSMCs

Because harvested cells from a mouse aorta contain primarily VSMCs but also a small number of endothelial cells, it is important to show the development of the mixed cell population into a primarily pure VSMC population. In doing this, the gene expression of SMC-specific and EC-specific genes were assessed in CMKLR1$^{+/+}$ VSMCs. The fold expression of the SMC-specific gene *mCnn1* was significantly increase by 3-fold by days 7 and 14, while the SMC-specific gene *mMyl9* was significantly increased 2-fold by day 14. These results reflect the increasing number of VSMCs in the

population. In contrast, several EC-specific genes including *VWF, ICAM2, ESAM, PECAM, E-SEL*, and *eNOS*, were all significantly decreased by 50% after 7 days in culture. These decreased genes also reflect the increasing VSMC population, something which is also visually evident as the cells become more uniform. These data suggest that after 7 days in culture a primarily pure VSMC population has been produced and the cells can then be used for experiments. This experiment was repeated for the CMKLR1$^{-/-}$ VSMCs and produced the same results.

4.2.2 Possible role for chemerin in atherosclerosis given gene expression of chemerin and CMKLR1 and secretion of chemerin in mouse VSMCs

As was the case in humans, the role of chemerin and CMKLR1 in mouse VSMCs has not been studied. It was discovered that both chemerin and *CMKLR1* are expressed in CMKLR1$^{+/+}$ VSMCs and that chemerin is expressed in CMKLR1$^{-/-}$ VSMCs. This data suggests that because chemerin is expressed in both CMKLR1$^{+/+}$ and CMKLR1$^{-/-}$ VSMCs it may be involved in atherogenic VSMC functions. Also, because *CMKLR1* is expressed in CMKLR1$^{+/+}$ VSMCs chemerin/CMKLR1 signalling may be a prominent pathway which regulates these functions. In addition to gene expression, chemerin was found to be increasingly secreted from both CMKLR1$^{+/+}$ and CMKLR1$^{-/-}$ VSMCs with confluence, reaching concentrations of 4-8 pM. There are a number of possible areas in the pathology of atherosclerosis where chemerin may be involved, including in pro-inflammatory or anti-inflammatory activity on immune cells such as by encouraging the recruitment of

macrophages in atherosclerosis. This is plausible given the chemoattractant properties of chemerin on murine macrophages [188]. Another area of interest would be in alteration of insulin sensitivity of VSMCs, or the alteration of endothelial cell function especially in relation to the production of nitric oxide and inflammatory cytokines. Finally, a possible role for chemerin/CMKLR1 may be in VSMC apoptosis, migration and proliferation.

The *CMKLR1* and chemerin gene expression profiles were similar between the human ASC to SMC differentiation protocol and with increasing confluence of cultured CMKLR1$^{+/+}$ mouse VSMCs. In the differentiation protocol, cells become confluent over a 28-day period suggesting that observed increase in chemerin expression and secretion in both the human and mouse models are confluence-driven processes. The mouse VSMCs secreted chemerin at concentrations of 4-8 pM, which is 125-250 fold lower than that detected in human ASCs and differentiated SMCs (Figure 16). It is important to keep in mind that media from mouse VSMCs was collected after 24 hours while the media from human ASCs and SMCs were collected after 48 hours, so more than double the secreted chemerin could be expected for the latter model. Therefore, it can be estimated that the mouse VSMCs secreted 62.5- 125 fold less total chemerin than the human ASCs/ SMCs. The increased secreted chemerin in the human ASC/SMC model suggests that chemerin could act in an autocrine manner where it binds with local CMKLR1 to promote or inhibit VSMC proliferation or migration, which in turn will alter the thickness of the VSMC cap over the plaque. Alternatively, VSMC-secreted chemerin could behave in a paracrine manner such that it acts as a chemoattractant for macrophages in

atherosclerosis, or acts upon endothelial cells altering their ability to produce inflammatory cytokines or nitric oxide. The chemerin secretion in mouse VSMCs is likely more similar to primary human VSMCs, as the SMC-differentiation treatment group cells may have increased chemerin secretion given their ASC descent. The reduced chemerin detected in media from primary mouse VSMCs may suggest that they are not a primary source of chemerin, but rather are acted upon by an alternative source such as periaortic fat (which secretes chemerin but does not express *CMKLR1*) [184]. This finding opens up a number of avenues which can be explored, however this study aims to focus on VSMC proliferation and migration.

4.2.3 Examining proliferation & migration in mouse VSMCs

In atherosclerosis VSMCs transfer from a contractile phenotype to a proliferative/ migratory phenotype (also called a synthetic phenotype) in response to the forming plaque. Before examining proliferation and migration properties in CMKLR1$^{+/+}$ and CMKLR1$^{-/-}$ VSMCs it was important to wait until the cells in culture morphologically displayed a proliferative/ migratory phenotype. A possible explanation for this phenotypic change is that it occurs in response to increased uptake of glucose in cell media. It is suggested that cultured VSMCs do not experience the glucose fluctuations that they would in vivo, altering their glucose uptake patterns, and promoting the change to a proliferative/ migratory phenotype [13]. Cells that have adopted the proliferative/ migratory phenotype appear less elongated and have a more cobblestone morphology which is sometimes referred to as epitheliod or rhomboid [189]. The cells also divide

faster, requiring cell splitting bi-weekly. Another method which was not used for confirming the proliferative/ migratory phenotype was to assess the expression of marker genes. Typically, it is the loss of contractile markers which is most associated with the phenotypical change to the synthetic phenotype. For example, alpha smooth muscle actin and calponin are associated with the contractile phenotype and their expression is reduced in the synthetic phenotype. Conversely, expression of collagen, PDGF, and MMP isoforms may be high in the synthetic phenotype and reduced in the contractile phenotype. This being said, cells may not always portray one phenotype or the other but rather an intermediate phenotype. For this reason, the visual morphological differences combined with gene expression data and observations on changes in the rate of cell division is best for determining the phenotype of the VSMCs. This approach was employed with the exception of gene expression analyses before performing proliferation or migration studies.

Once cells had adopted a migratory/proliferative phenotype, FBS was tested as a positive control for the proliferation/ migration assays. FBS contains several components including growth factors such as insulin-like growth factor 1, transforming growth factor beta 1, and fibroblast growth factor-2. These growth factors likely played a role in encouraging increased VSMC migration and proliferation, which was observed upon treatment with FBS. The FBS results confirmed that the proliferation and migration assays were working properly, such that the effect of chemerin/CMKLR1 on VSMC proliferation and migration could be investigated.

In the present study, it was discovered that chemerin did not significantly alter the proliferation of CMKLR1$^{+/+}$ and CMKLR1$^{-/-}$ VSMCs using the BrdU assay. The assay was performed in both serum-free and 1% serum environments. Similar trends were observed with increasing chemerin concentration however statistical significance was not present. This was also the case in the CMKLR1$^{-/-}$ VSMCs however the serum-free trial using these cells contained significant variability which could be due to greater biological variability between the CMKLR1$^{-/-}$ mice used or between the cultured VSMCs, or also error in the assay procedure for these trials. It is possible that additional trials or an alternative approach for assessing proliferation could provide stronger evidence. For the CMKLR1$^{-/-}$ VSMCs, MTT assays completed for the serum-free and 1% serum conditions showed no reduction in cell viability, suggesting that while a dose-dependent effect of chemerin on the rate of BrdU-incorporated DNA synthesis was not observed, the cells were also maintaining metabolic activity at these chemerin concentrations (reduction in cell viability was not observed). Therefore, cell toxicity was not a factor influencing the proliferation results. This finding is interesting as it is different from other adipokines such as leptin and adiponectin. Leptin is known to promote VSMC proliferation and migration, while adiponectin has been found to inhibit VSMC proliferation and migration [113, 190-192]. The mechanisms of the effect of these adipokines on proliferation are largely still under investigation.

Another consideration was that chemerin may work in combination with other molecules such as inflammatory cytokines to influence VSMC proliferation. One inflammatory cytokine largely associated with atherosclerosis is TNF. One study in mice

found that administration of adiponectin decreased serum TNF levels [193] which may be related to the decreased VSMC proliferation which was observed. With this in mind, VSMCs were treated with increasing concentrations of chemerin in combination with increasing concentrations of TNF in the BrdU assay. A TNF dose-dependent decrease in the amount of BrdU-incorporated DNA was detected in CMKLR1$^{+/+}$ VSMCs however there was no additional effect in combination with increasing chemerin concentration. Therefore, chemerin does not act in combination with TNF to alter mouse VSMC proliferation. Despite the observed decrease in cell proliferation, MTT assay results confirm that there was no loss in cell viability at these TNF concentrations. Unlike the observed TNF-mediated reduction in cell proliferation, most literature sources note that TNF induces VSMC proliferation [194]. The unchanged cell viability which was observed despite TNF-reduced proliferation could be due to arrest in the Growth 1 (G1) phase (first of four phases in the cell cycle during eukaryotic cell division) such that while the cells are metabolically active, the cells are not undergoing new DNA synthesis and completing the mitosis cycle. TNF has been found to arrest some types of cancer cell such as mouse fibrosarcoma cells in the G1 phase [195].

The final finding was that while chemerin may not influence mouse VSMC proliferation, it does influence migration. VSMCs isolated from CMKLR1$^{+/+}$ mice migrated to a higher degree than those which lacked CMKLR1 when exposed to low chemerin concentrations (0.01- 0.1 nM). This suggests that chemerin/CMKLR1 signalling is involved in the promotion of VSMC migration at low chemerin concentrations using cells that display a phenotype similar to those present in

atherosclerosis. The partial inhibition of CMKLR1$^{-/-}$ VSMC migration was visible upon exposure to low chemerin concentrations and the migration of both CMKLR1$^{+/+}$ and CMKLR1$^{-/-}$ VSMCs was entirely inhibited upon exposure to 100 nM chemerin, suggesting that chemerin can influence the migration of VSMCs independently of CMKLR1, especially at higher chemerin concentrations. It is possibly that this inhibitory response could be mediated through the GPR1 receptor. Further investigation of chemerin/GPR1 signalling is required to determine if this pathway is responsible for the observed CMKLR1-independent inhibition of VSMC migration. . These results suggest that chemerin/CMKLR1 may be important for regulating VSMC migration which if chemerin/CMKLR1 signalling is acting to increase cell migration at low chemerin concentrations, it may lead to limited area available for blood flow creating high blood pressure, and more VSMCs which are susceptible of plaque-disrupting apoptosis. Conversely, if chemerin concentrations are too high and are inhibiting cell migration than the cap which forms over the plaque will be thin making it unstable, and increasing the likelihood of rupture. It is important to note that 100 nM chemerin is a 4-5 fold higher concentration than that which has been detected in humans and is therefore a non-physiological concentration, but was important for characterizing the degree to which dose-dependent observations were evident. Interestingly, chemerin is behaving similarly to the adipokine adiponectin, which also inhibits VSMC migration. While the mechanism of adiponectin-inhibited migration is largely unknown, a study by Motobayashi (2009) found that adiponectin inhibits insulin-like growth factor-1 induced cell migration by suppressing ERK 1/2 activation in VSMCs [192]. Additional research is required to determine whether or not chemerin/CMKLR1 activates VSMC migration at low

chemerin concentrations via the ERK 1/2 pathway or through a separate signalling pathway. It was determined that chemerin activates ERK 1/2 signalling in mouse adipocytes exposed to low chemerin concentrations [143], which is further evidence that it may be acting through this pathway to inhibit CMKLR1$^{-/-}$ VSMC migration at low chemerin concentrations.

Chapter V. Limitations & Future Directions

5.1 Human ASC to SMC differentiation study

The biggest challenge with the human ASC to SMC differentiation model was the lack of existing protocols. There are many protocols available for differentiating ASCs into adipocytes, chondrocytes and osteoblasts, but not for VSMCs. Recently, the potential for differentiating ASCs into SMCs has begun to be of interest in the tissue regeneration field for making contractile bladder SMCs, however distinct protocols are not widely reported [196, 197]. While there is little information available it is important to note that there are also various kinds of SMCs and even if a functional VSMC could be differentiated, the properties of VSMCs often differ based on location (near the top or bottom of the aorta for example) as was discussed in Section 1.15, and so not all differentiated VSMCs may represent the various VSMCs which exist naturally in vivo. Wang (2010) suggested using two different growth factors (TGF-β1 and BMP4) to promote differentiation of ASCs into SMCs, and in this study smooth muscle basal medium containing two alternative growth factors were used (hEGF and hFGF-B) [198]. It is possible that using the previously reported growth factors could have yielded more convincing gene expression data, suggesting a functional SMC phenotype. Despite this, the morphological change in the cells was suggestive that the experiment was successful, however to be sure additional gene expression or protein expression data would be required. Two SMC-specific genes which could be assessed are *SM22 α* and *SM-MHC* [198]. The SM-actin, *Cnn1* and *Myl9* genes which were investigated were detected in the

differentiated cells however they were also prevalent in the ASCs making them difficult markers for assessing the phenotypic change. It would also be very important to investigate the loss of ASC-specific genes such as *CD10* and *CD200*, and confirm the absence of negative control genes such as those specific to chondrocytes. If the cells differentiated using this model can be confirmed to be SMCs then the next step would be to investigate the effect of chemerin/CMKLR1 on their ability to migrate and proliferate. It would also be important to compare these results to those of primary human VSMCs to see if they are consistent. The benefit of using an ASC to VSMC differentiation protocol to obtain human VSMCs compared to purchasing primary human VSMC is that ASCs have a reduced cost and are readily available. It is also very interesting that subcutaneous ASCs express chemerin and *CMKLR1* and also secrete chemerin, and so there are a number of experiments which could further investigate what role chemerin may be playing in the ASCs and if it is involved in determining what kind of cell they differentiate into such a preadipocytes, adipocytes or an alternative cell type.

5.2 Mouse VSMC study

Unlike the human ASC differentiation model, the mouse VSMC isolation model is a very consistent method for obtaining a VSMC population. The cell population becomes a primarily pure VSMC population within 7 days in culture. The mouse VSMCs can be cryostocked and passaged up to 35 times making them a resilient cell line to work with. The biggest challenge in working with primary mouse VSMCs is that they can exist in either a contractile or proliferative/ migratory phenotype or an intermediate phenotype

which may involve aspects of both [189]. Confirming which phenotype the cells are displaying is complex, and the cells can change between phenotypes which can introduce significant variability when assessing proliferation and migration. One benefit of cell culture is that it can act as an unfamiliar environment for the cells promoting them to switch to the proliferative/migratory phenotype. Therefore, cell culture is a potentially very useful method for achieving the proliferative/ migratory phenotype and could also be used to further study the variables involved during phenotypic change. However, because the degree to which a phenotype has been adopted cannot be determined visually, research on the use of precise biomarkers for each state is required. Also, because the cells are isolated from different animals there is significant biological variability, which can also affect the observed results. The MTT and transwell migration assays produced consistent results, however the BrdU assay contained a number of antibody treatment and washing steps which added to the variability of the assay. It is possible that trying an alternative approach such as injecting ethynyl deoxyuridine (EdU) or BrdU into mice and histologically assessing VSMC proliferation in an atherosclerotic mouse model could be a useful way to confirm if the BrdU in vivo assay results reflect what may be happening in vitro. Also, while the transwell migration assay investigates cell migration through a membrane toward a higher chemerin concentration, the assay could be adapted to better represent the physiological environment in atherosclerosis by first allowing endothelial cells to adhere to the membrane, and then assessing how VSMCs might move through this layer. Additionally, a scratch assay involving removable inserts could be used to see if chemerin influences VSMC migration across a surface when entirely surrounded by a chemerin-containing solution. The observation

that chemerin inhibits migration can be further assessed by beginning to determine what kind of signalling pathway may be involved. In doing this, additional studies using both $GPR1^{-/-}$ mice and GPRI/CMKLR1 DBL KO mice would be useful to determine if signalling through CMKLR1 and GPR1 have opposing effects on the proliferation of VSMCs. A final consideration is that while only dose-dependent effects of chemerin were assessed, it may instead be acting in a time-dependent manner such that the length of time VSMCs are exposed to chemerin may influence their proliferation and migration.

5.3 Mouse & human VSMC studies

Moving forward it would be interesting to also assess the effect of chemerin/CMKLR1 signalling on VSMC apoptosis, as it greatly influences atherosclerotic plaque stability and may promote rupture. Proliferation and migration are important in restenosis, limiting the area available for blood flow, and while their regulation is important in long-term prevention they do not have the same ability to cause an acute heart attack or stroke as VSMC apoptosis can. Future studies in this area could involve caspase 3/7 ELISA or flow cytometry analyses of propidium iodide or annexin 5 stained cells. A second consideration would be to use shRNA to knockout either or both chemerin and CMKLR1 or GPR1 in human and mouse VSMCs, to further assess their involvement on migration, proliferation and apoptosis functions.

Chapter VI. Conclusion

The human ASC to SMC differentiation model is one which requires additional research to be considered a reliable protocol. Despite the additional work required, it appears to be a promising model based upon the morphological and negative-control gene expression results. The expression of chemerin and *CMKLR1* in the cells of the SMC-differentiation treatment and undifferentiated groups, and secretion of chemerin from both cell types are novel findings and research exploring the role of chemerin and CMKLR1 in these cells is required. Additionally, primary mouse aortic CMKLR1$^{+/+}$ and CMKLR1$^{-/-}$ VSMCs were successfully isolated and characterized. They were also found to express chemerin and *CMKLR1* and secrete chemerin, which has not been previously reported. The primary mouse VSMCs secreted chemerin to a lesser extent than the human cells, despite similar gene expression results which is likely due to the adipose-derived lineage of the differentiated human cells. The expression of chemerin/*CMKLR1* in mouse VSMCs make them a more accurate model for studying their effect on VSMC processes. The mouse chemerin secretion data suggests that chemerin may be supplied in a paracrine fashion by alternative tissues and act upon local CMKLR1 to regulate VSMC function. Chemerin did not influence the proliferation of CMKLR1$^{+/+}$ and CMKLR1$^{-/-}$ VSMCs but did inhibit migration of both cell types at high concentrations. At low chemerin concentrations, CMKLR1$^{-/-}$ VSMC migration was reduced suggesting that chemerin/CMKLR1 signalling promotes VSMC migration. This may suggest that chemerin/GPR1 signalling has an inhibitory effect on migration at low and high chemerin concentrations which is independent of CMKLR1. Therefore, the regulation of

chemerin/CMKLR1 is important in VSMC migration as it relates to plaque stability atherosclerosis. Further research is required to determine the extent to which chemerin is involved in VSMC proliferation and migration, the involvement of other chemerin receptors such as GPR1, what signalling pathway is activated during the observed inhibition of migration, and whether targeting chemerin signalling through pharmacological intervention could be beneficial in preventing restenosis.

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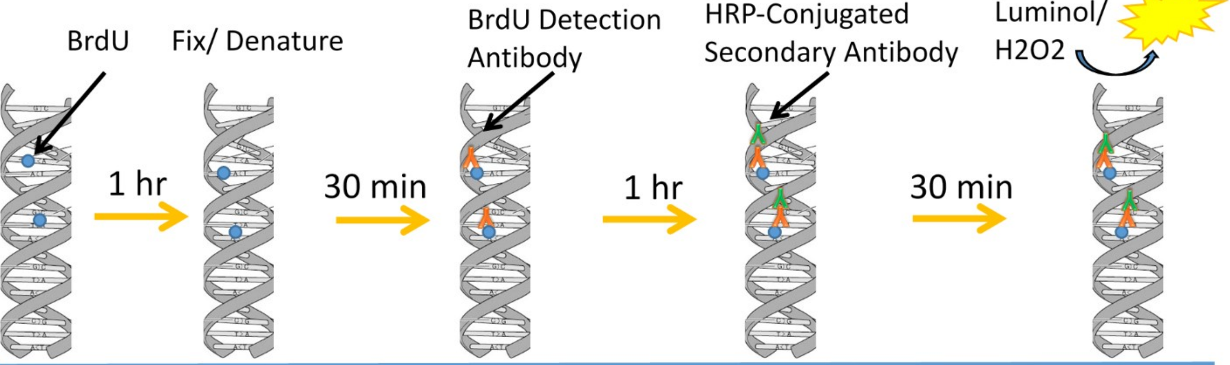
. APPENDIX



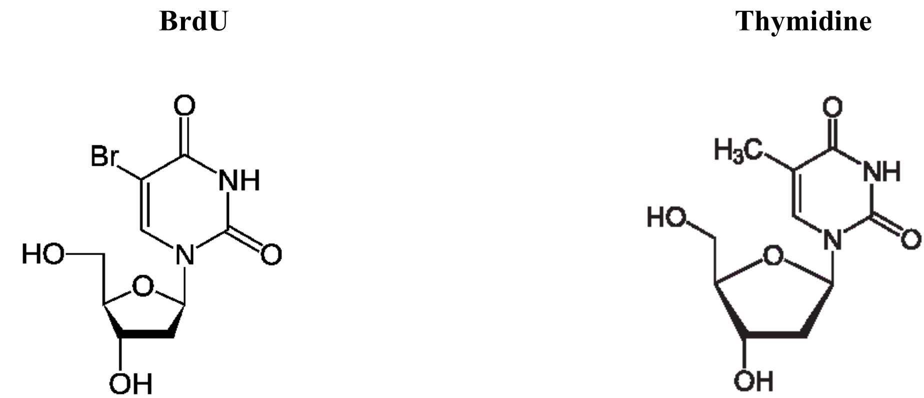


Figure 5: BrdU assay. The thymidine analogue bromodeoxyuridine (BrdU) can be incorporated into DNA as it is synthesized, and then labeled with a primary and HRP-liked secondary antibody, which reacts with luminol and peroxide to produce luminescence. The luminescence is quantified as relative light units (RLU).

Figure 6: MTT assay. MTT (yellow) is converted to formazan (purple) by the mitochondrial enzyme succinate dehydrogenase in metabolically active cells, which can be quantified by absorbance.

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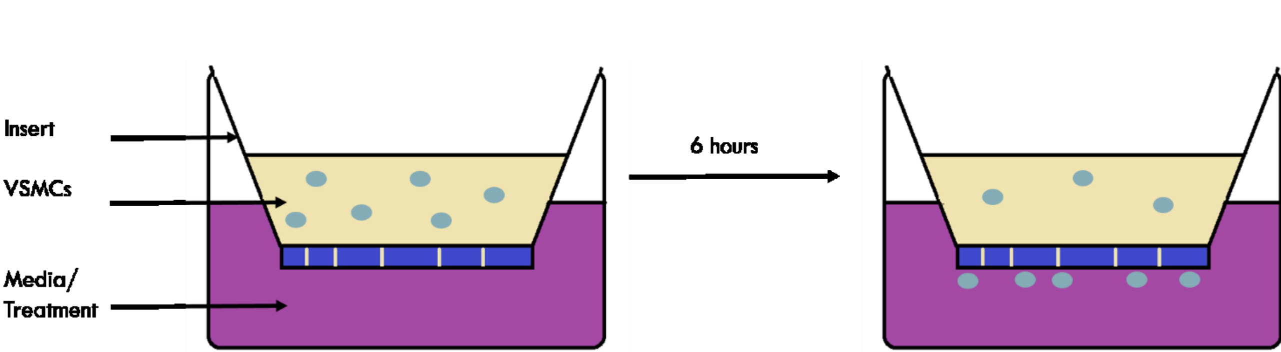


Figure 7: Transwell cell migration assay. The VSMCs are placed in the upper chamber of the insert and chemerin-containing media is placed in the bottom of the wells. The VSMCs can pass through the membrane and adhere on the opposite side which can then be removed and stained for fluorescent imaging.

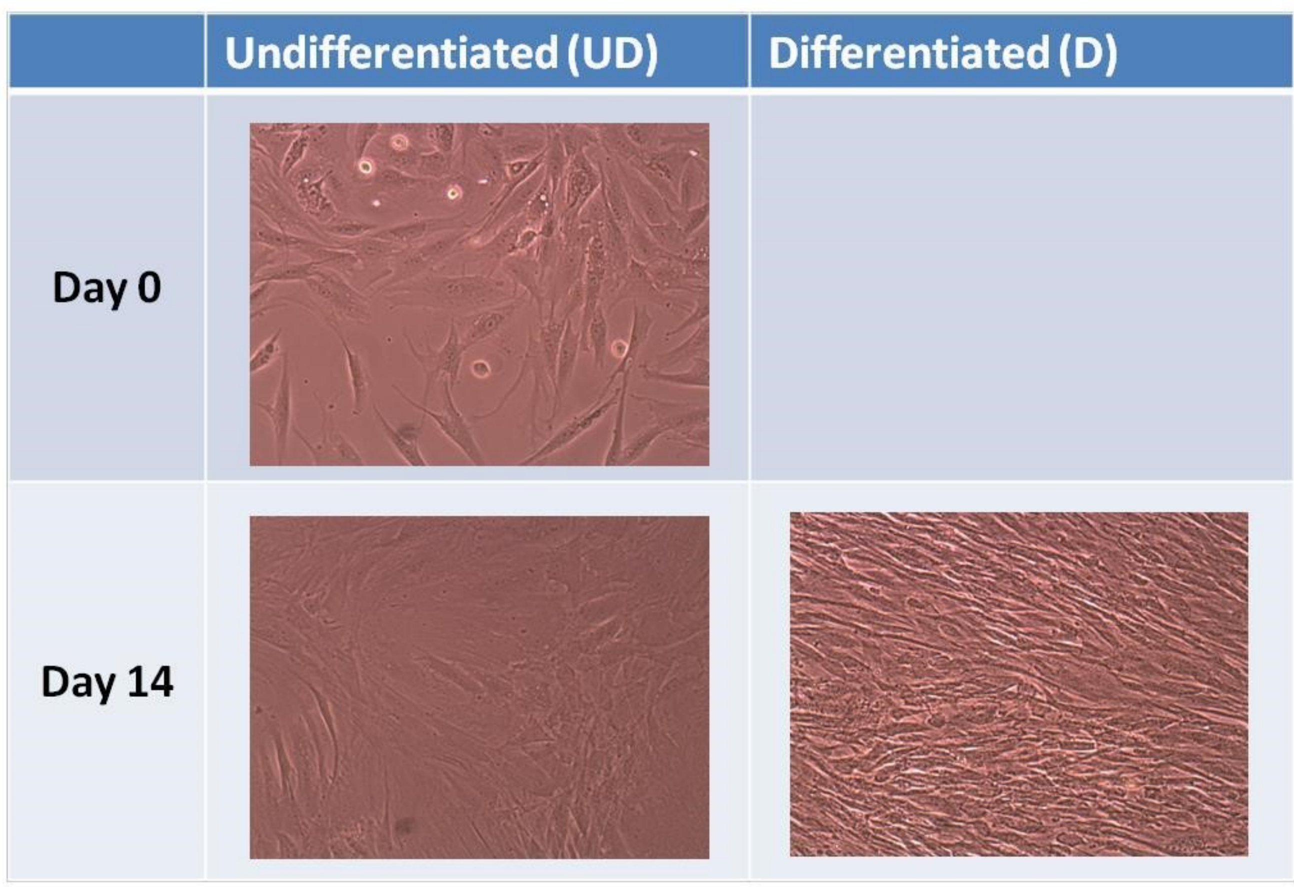


Figure 8: Phase contrast image of differentiated and undifferentiated ASCs. Day 14 differentiated cells are elongated and directionally uniform. Images taken with Nikon compound microscope at 10X magnification.

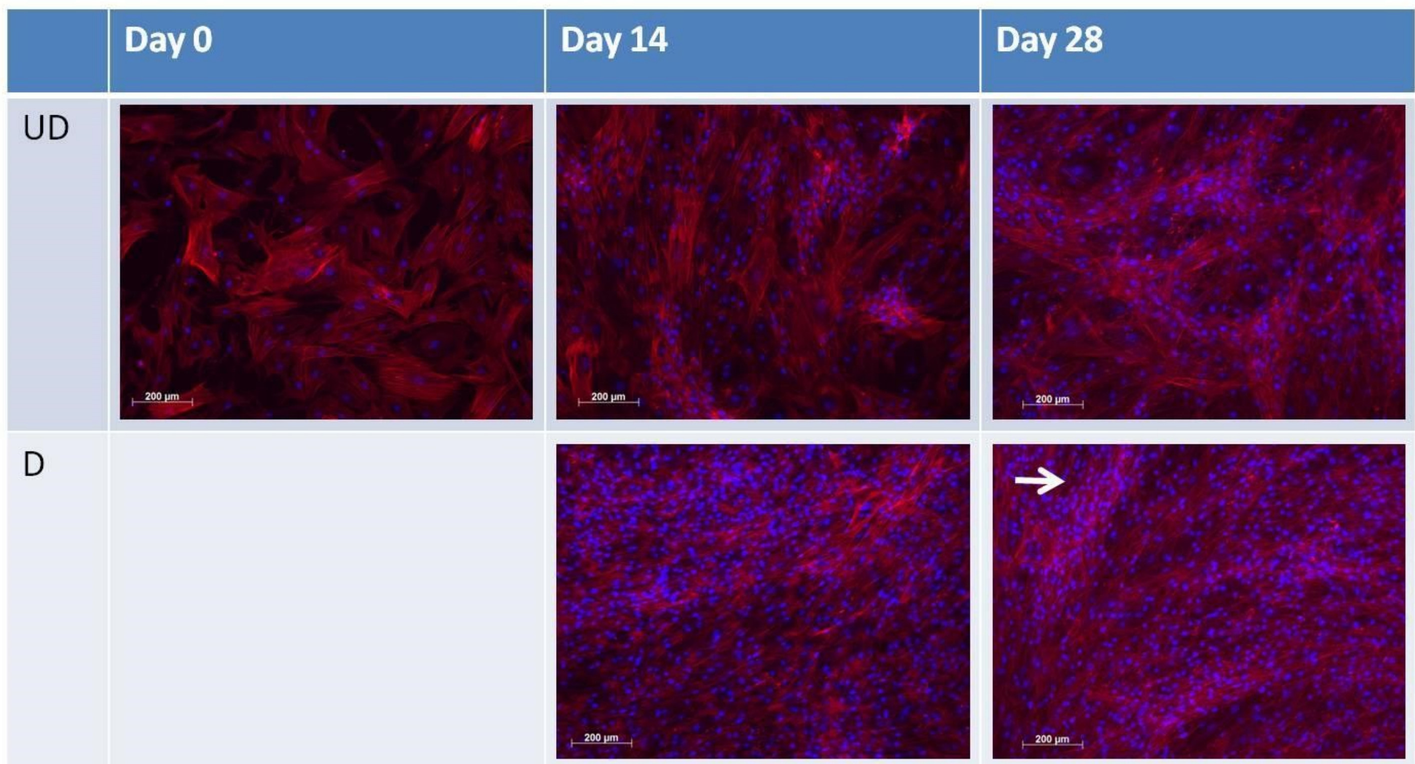
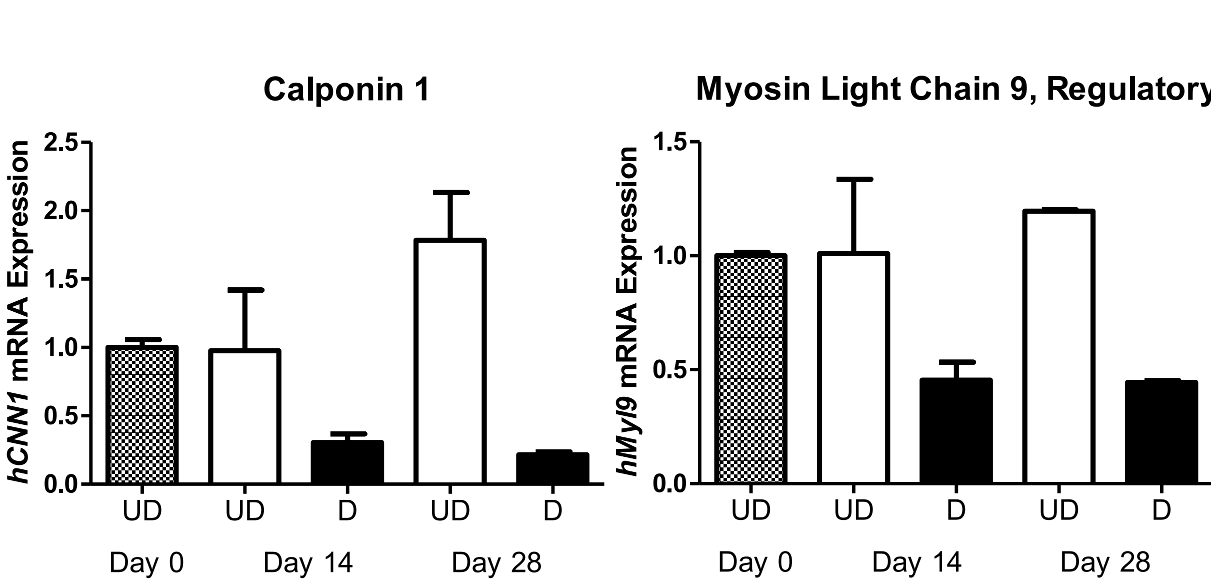


Figure 9: Fluorescent image of differentiated and undifferentiated ASCs. Cells were stained with phalloidin (actin stain, red) and HOESCHT (nuclei stain, blue) and imaged using a Zeiss Axiovert 200 microscope with a Hamamatsu Orca R2 camera at 10X magnification. The white arrow is noting an example of the SMC phenotype which is that they form hills and valleys.



<u>Figure 10 A & B:</u> The SMC genes calponin 1 and myosin light chain 9, regulatory are not increased compared to undifferentiated ASCs. For each experiment, cells in each treatment were plated in duplicate, and each duplicate was quantified by qPCR in duplicate. The above data represents two independent experiments (N=2).

A

B

CMKLR1

CMKLR1 mRNA Expression

15
10
5
0

UD UD D UD D

Day 0 Day 14 Day 28

Chemerin

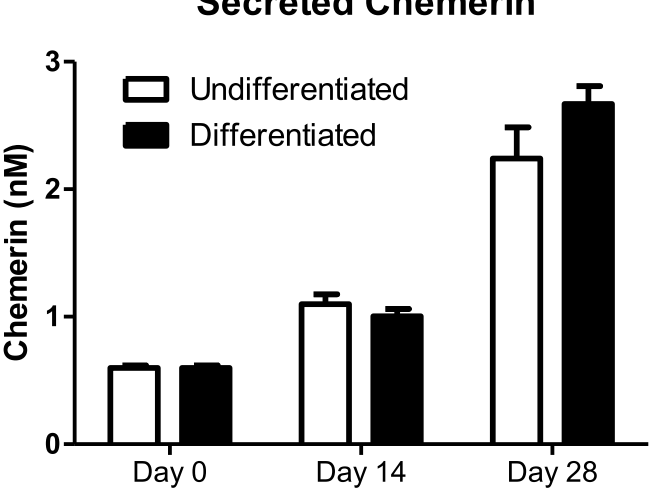
Chemerin mRNA Expression

40
30
20
10
0

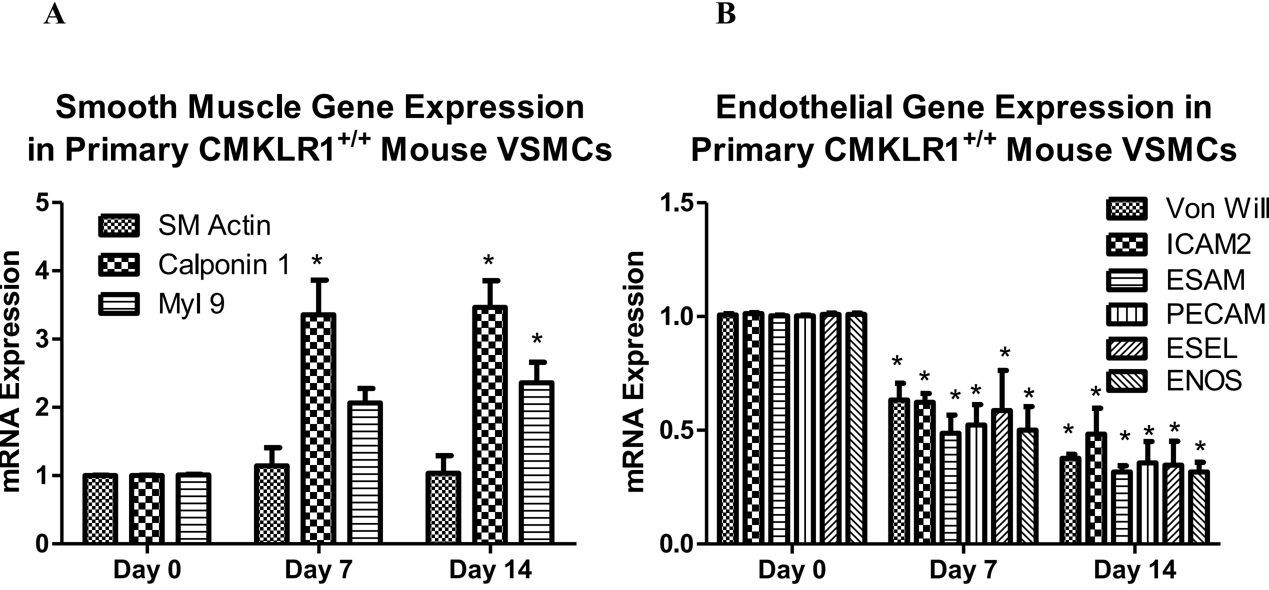
UD D UD D UD

Day 0 Day 14 Day 28

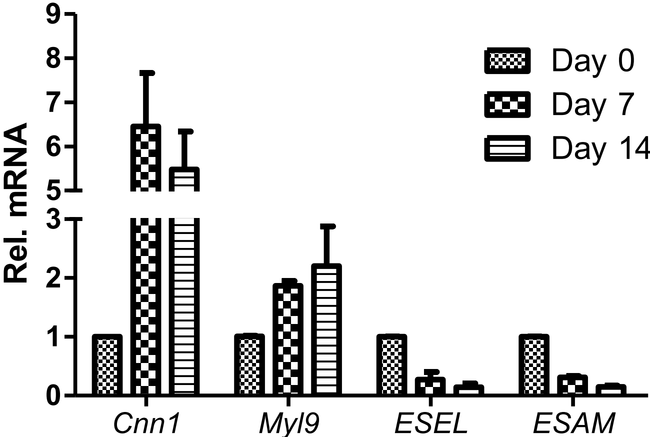
<u>Figure 11 A & B:</u> *CMKLR1* and chemerin gene expression in differentiated and undifferentiated ASCs. A) CMKLR1was consistently expressed in differentiated cells throughout the differentiation period but increased in undifferentiated cells. B) Chemerin was consistently expressed in undifferentiated cells but decreased in differentiated cells throughout the differentiation period. For each experiment, cells in each treatment were plated in duplicate, and each duplicate was quantified by qPCR in duplicate. The above data represents two independent experiments (N=2).



<u>Figure 12</u>: Total chemerin in cell media of differentiated and undifferentiated ASCs. Chemerin was detected in the cell media of differentiated SMCs and undifferentiated ASCs in the 1-2 nM concentration range, and increased throughout the differentiation period. Data was collected from one experiment containing three independent replicates for each condition, which were measured in duplicate using the ELISA assay (N=1).



<u>Figure 13 A & B:</u> CMKLR1$^{+/+}$ VSMCs show increased SMC-specific genes and decreased EC-specific genes by day 7 in culture. This data reflects the morphological observations that the harvested cell population is becoming one containing purely VSMCs. For each experiment, each condition was repeated in a minimum of triplicate, and read using qPCR in duplicate. Three experiments were averaged (N=3). * Statistical significance compared to Day 0 control using 1-way ANOVA with a Bonferroni multiple comparison post-test.



<u>Figure 14:</u> Confirmatory increase in SMC-specific genes (*Cnn1* and *Myl9*) and decrease in EC-specific genes (*ESEL* and *ESAM*) in CMKLR1 KO VSMCs. For each experiment, each condition was repeated in a minimum of triplicate, and read using qPCR in duplicate. Two experiments were averaged (N=2).

A

Relative Chemerin mRNA Expression

20
15
10
5
0
Rel. mRNA
CMKLR1$^{+/+}$
CMKLR1$^{-/-}$
Day 0
Day 7
Day 14

B

Relative *CMKLR1* mRNA Expression

1.5
1.0
0.5
0.0
Rel. mRNA
CMKLR1$^{+/+}$
CMKLR1$^{-/-}$
Day 0
Day 7
Day 14

Figure 15 A & B: Chemerin and *CMKLR1* expression in CMKLR1$^{-/-}$ and CMKLR1$^{+/+}$ VSMCS. Chemerin expression increases in CMKLR1$^{-/-}$ and CMKLR1$^{+/+}$ VSMCs with increased confluence while CMKLR1 expression remains constant. The CMKLR1$^{-/-}$ phenotype is demonstrated by the lack of CMKLR1 gene expression**.** For each experiment, each condition was repeated in a minimum of triplicate, and read using qPCR in duplicate. For the CMKLR1$^{+/+}$ data two experiments were averaged (N=2) and for the CMKLR1$^{-/-}$ data three experiments were averaged (N=3).

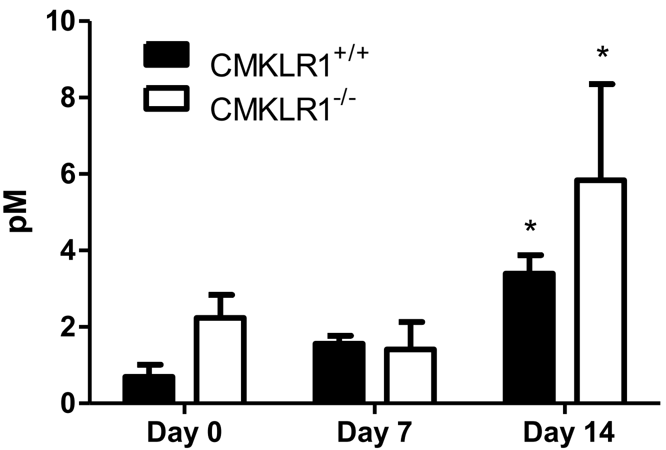
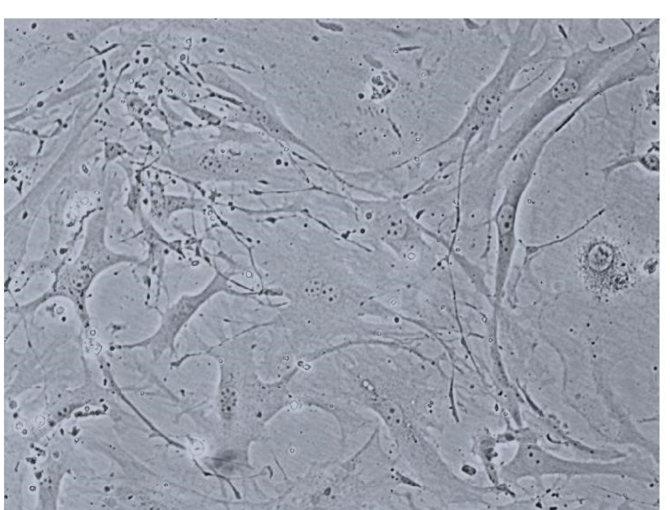


Figure 16: Chemerin was detected in cell media from both CMKLR1$^{-/-}$ and CMKLR1$^{+/+}$ VSMCs at concentrations of up to 8 pM. VSMC media from three CMKLR1$^{-/-}$ and three CMKLR1$^{+/+}$ mice was analyzed, and each sample was tested in duplicate (N=3). * Both the CMKLR1$^{-/-}$ and CMKLR1$^{+/+}$ Day 14 detected chemerin are significantly different than both CMKLR1$^{-/-}$ and CMKLR1$^{+/+}$ Day 0 and Day 7 results using a 1-way ANOVA with a Bonferroni multiple comparison post-test.



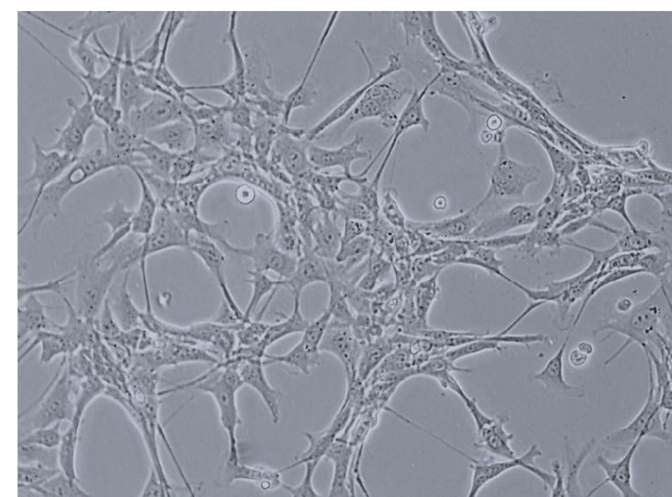
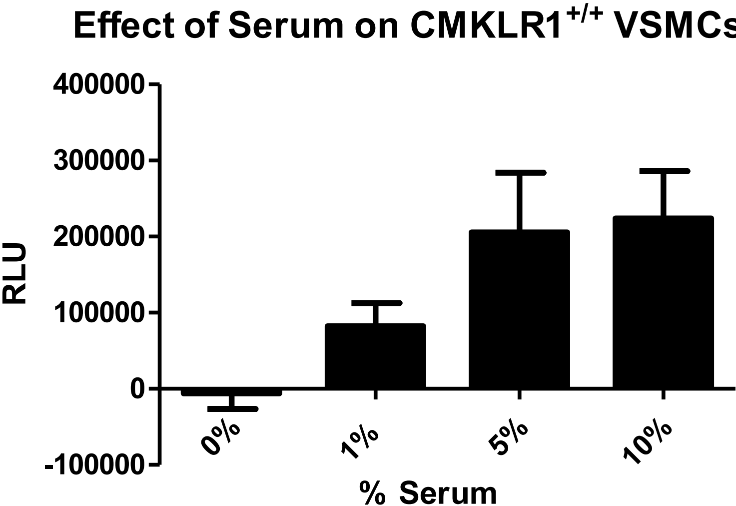
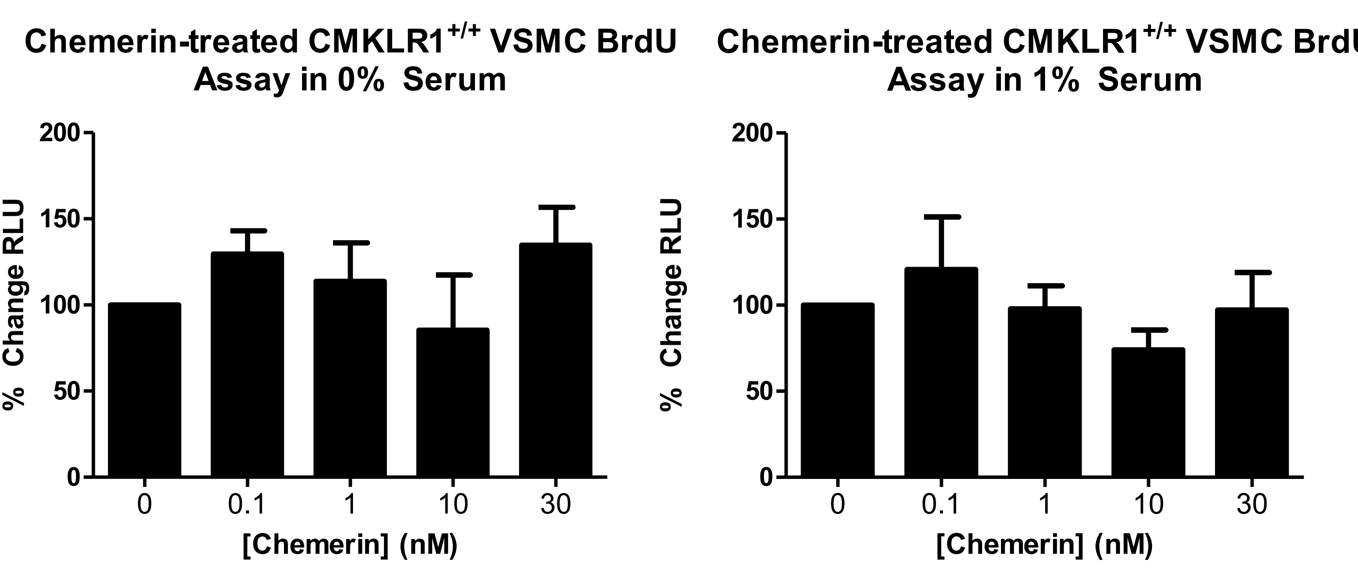


Figure 17: Primary mouse VSMCs change from a contractile (left) to a migratory/proliferative (right) phenotype in cell culture. The cells on the left are longer and more stretched, while on the right the cells are shorter, more uniform and require cell splitting more frequently. Images were taken using a Nikon compound microscope at 10X magnification.



<u>Figure 18</u>: FBS acts as a positive control for VSMC proliferation as was quantified by the BrdU assay. The maximal response was detected with 5% serum and so a lower FBS concentration is sufficient for demonstrating the effect of FBS on proliferation while not exceeding the detection level. Each treatment was measured in triplicate and averaged (N=1).



<u>Figure 19:</u> **Chemerin does not significantly affect CMKLR1$^{+/+}$ VSMC proliferation using the BrdU assay.** A similar trend was observed in both 1% and 0 % FBS, suggesting chemerin may play a role, however the differences are not statistically significant as was determined using a 1-way ANOVA. Each treatment of each experiment was tested in triplicate and averaged. The above data is representative of six experiments using cells from different mice (N=6).

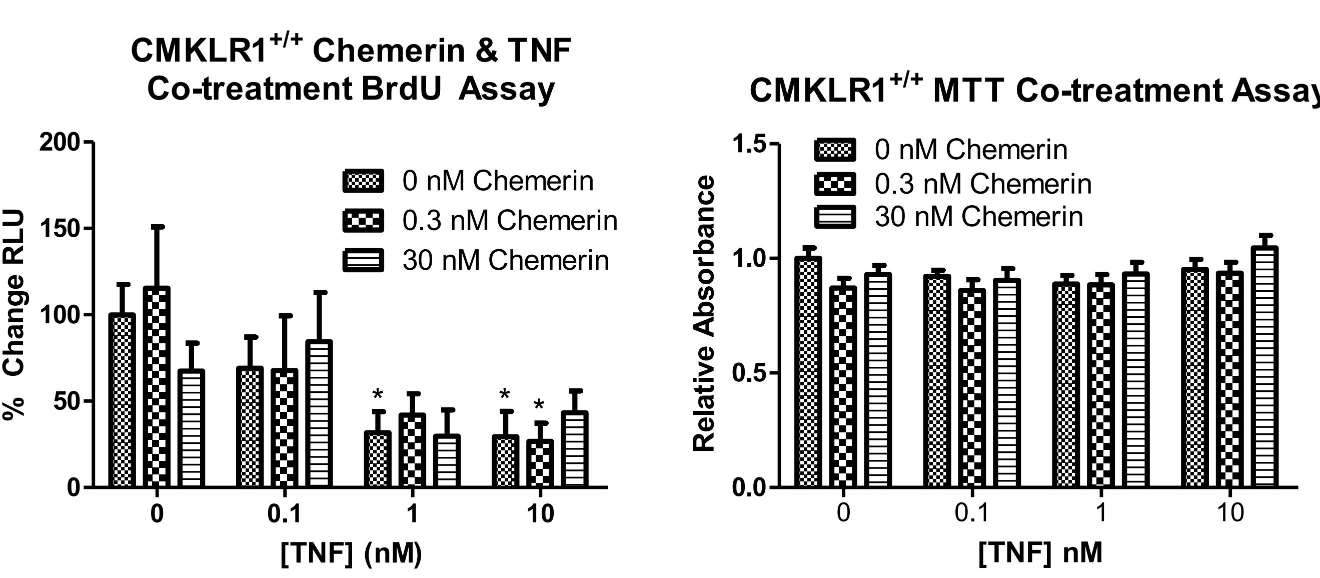
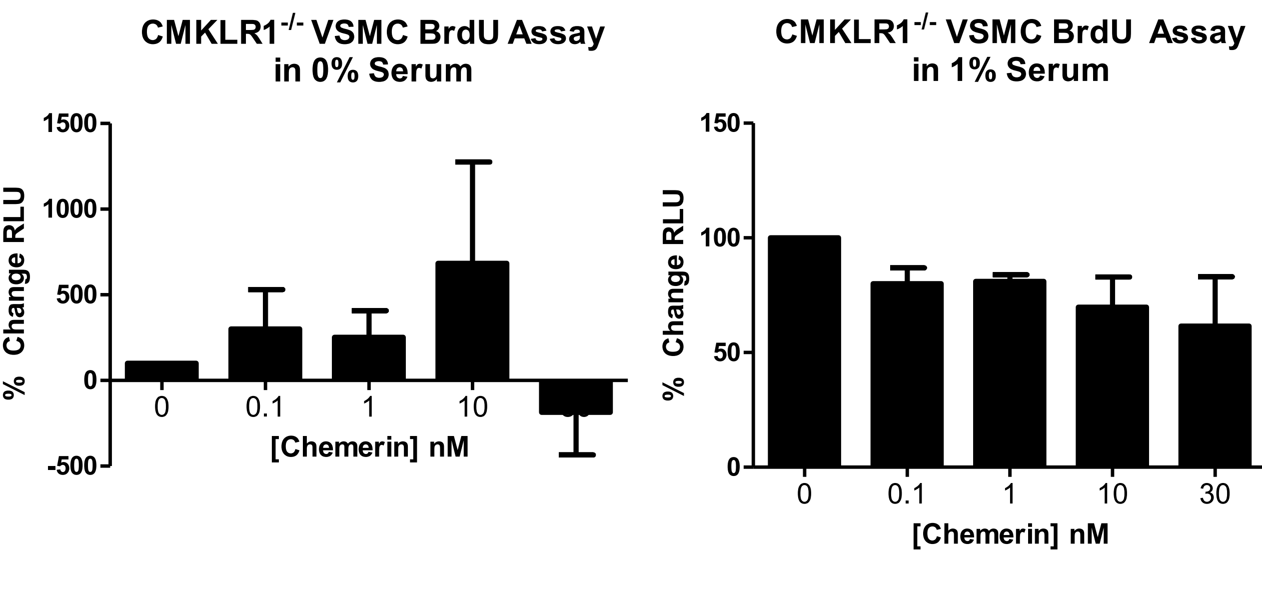
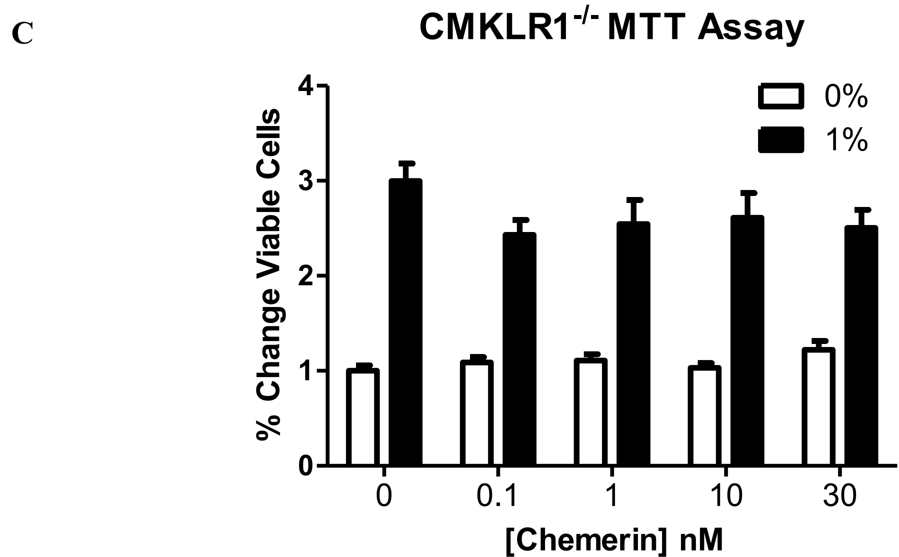


Figure 20: A) TNF inhibits DNA synthesis in CMKLR1$^{+/+}$ VSMCs. For each BrdU assay different treatment was tested in triplicate and averaged. Three independent experiments using VSMCs from different mice were used for the BrdU assay (N=3). **B) Metabolic activity of chemerin and TNF co-treated VSMCs is not reduced with increasing concentration.** For each MTT assay, treatments were tested in a minimum of quadruplicate, and averaged. VSMCs from three biologically different mice were used in each of the three assays included in the above graph (N=3). * Statistical significance was determined between 1 and 10 nM TNF compared to 0 nM TNF using a 1-way ANOVA and Bonferroni multiple comparisons post-test.

A **B**



C



<u>Figure 21:</u> **A & B) Chemerin does not affect the proliferation of CMKLR1$^{-/-}$ VSMCs with or without serum.** In the absence of serum proliferation results are highly variable, however with serum the results are consistently unchanged (with a slight and non-significant decreasing trend). Treatments were measured in triplicate and averaged for each experiment. Three experiments were combined in the above data (N=3). **C) MTT assay results show no change in CMKLR1$^{-/-}$ VSMC viability with increasing chemerin concentration**. Each treatment was measured in quintuplicate and averaged for each experiment. Three experiments were combined in the above data (N=3).

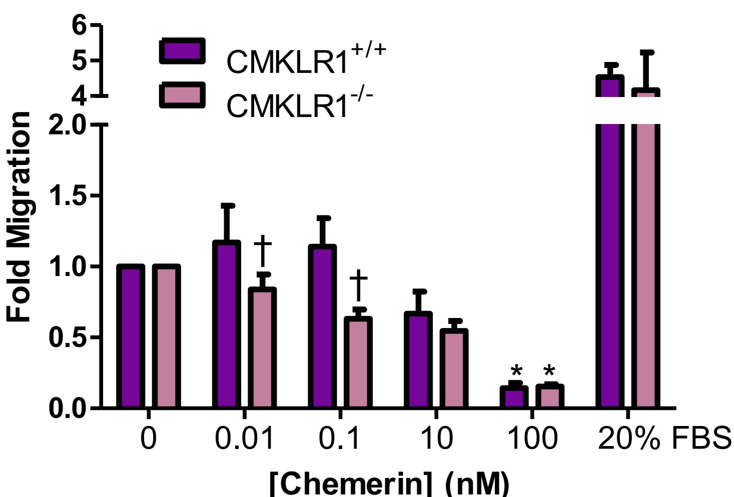


Figure 22: Effect of increasing chemerin concentrations on CMKLR1$^{+/+}$ and CMKLR1$^{-/-}$ VSMC migration using the transwell migration assay. Chemerin inhibits mouse VSMC migration at 10 nM and 100 nM concentrations in CMKLR1$^{+/+}$ and CMKLR1$^{-/-}$ VSMCs, and also at 0.01 nM and 0.1 nM concentrations in only CMKLR1$^{-/-}$ VSMCs. Each treatment was tested in duplicate for each experiment. Three CMKLR1$^{+/+}$ and CMKLR1$^{-/-}$ experiments were performed and combined in the above data (N=3). * A 1-way ANOVA confirmed statistical significance ($P < 0.05$) between the 100 nM and the 0 nM groups in both CMKLR1$^{+/+}$ and CMKLR1$^{-/-}$ VSMCs. † Subsequent t-tests determined that migration approaches statistical significance between CMKLR1$^{+/+}$ and CMKLR1$^{-/-}$ VSMC migration at 0.1 nM chemerin ($P= 0.0745$). When 0.01 and 0.1 nM CMKLR1$^{+/+}$ and CMKLR1$^{-/-}$ data are combined a significant difference is observed using t-test analyses ($P= 0.0106$).