

Investigating the initial molecular interactions  
between hepatocyte- like cells with the  
extracellular matrix and hepatic sinusoidal  
endothelial cells

and

The loss of LAR inhibits the activation of the  
PDGF receptor by NHERF2 via the PKC  $\delta$   
pathway



UNIVERSITY OF  
BIRMINGHAM

Daniel Blakeway  
MRes Molecular and Cellular  
Biology

School of Biosciences  
2012

UNIVERSITY OF  
BIRMINGHAM

**University of Birmingham Research Archive**

**e-theses repository**

This unpublished thesis/dissertation is copyright of the author and/or third parties. The intellectual property rights of the author or third parties in respect of this work are as defined by The Copyright Designs and Patents Act 1988 or as modified by any successor legislation.

Any use made of information contained in this thesis/dissertation must be in accordance with that legislation and must be properly acknowledged. Further distribution or reproduction in any format is prohibited without the permission of the copyright holder.

The first research project investigated the initial interactions between embryonic stem cell-derived hepatocyte-like cells (ES-HLCs) with the extracellular matrix and hepatic sinusoidal endothelial cells. Liver disease is a major cause of mortality worldwide; present treatments aim to slow down the progression to prevent further damage however this is only effective if the disease is controlled before irreversible damage has occurred. Developments in the understanding of hepatocyte proliferation have led to cell transplantation as a promising alternative. Current research aims to identify an alternative sustainable source, ES-HLCs offer a great potential for a renewable source of functional hepatocytes; however transplanted cells express low engraftment levels. The project investigated the interactions between ES-HLCs with the extracellular matrix and hepatic sinusoidal endothelial cells (HSEC). Fluorescence activated cell sorting (FACS) was carried out on various types of HSEC to quantify the level of the integrins being expressed. Static adhesion assays with ES-HLCs were performed on a range of extra cellular matrix (ECM) proteins to give a measure of cell interaction and adhesion. FACS analysis showed integrin- $\beta$ 1 and basal cell adhesion molecule (BCAM), were highly expressed. ES-HLCs showed preferential binding to the ECM protein Laminin, this adherence was significantly reduced when the cells were treated with  $\beta$ 1 and BCAM blocking antibodies; furthermore ES-HLC adherence was reduced when laminin was pre-treated with a recombinant Human BCAM/Fs. Both BCAM and integrin-  $\beta$ 1 are crucial in regulating adhesion in hepatocyte-like cells; suggesting potential targets to manipulate to improve ES-HLC engraftment in vivo.

The second research project investigated the role of leukocyte common antigen-related receptor like PTP (LAR-RPTP), in the regulation of the platelet derived growth factor receptor (PDGFR). The PDGFR is a cell surface tyrosine kinase receptor specific for members of the platelet derived growth factor (PDGF). PDGFs have been associated to have a pivotal role in a range of cellular processes; from cell growth, motility, differentiation and survival in connective tissues. PDGF is also associated with diseases such as tumour formation. The aim of the project was to further investigate the role of LAR in the regulation of the PDGF receptor and its effect upon downstream signal transduction molecules. The project aims to further examine the hypothesis that the PDGFR is regulated though LAR, which helps to promote ligand induced activation of the PDGF  $\beta$ -receptor through its mechanism of down regulating c-Abl activity.

The effects of Protein kinase C delta (PKC  $\delta$ ) were examined; PKC  $\delta$  is an enzyme protein that is linked to abelson murine leukemia viral oncogene homolog 1 (c-Abl) and is linked with the regulation of the PDGF receptor; through inhibiting the phosphorylation of the tyrosine residues. The structural proteins Na<sup>+</sup>/H<sup>+</sup> Exchanger Regulatory Factor (NHERF1) and sodium-hydrogen exchange regulatory cofactor NHE-RF2 (NHERF2) were also investigated; these proteins are known to bind to the C-terminal of the PDGF receptor, crucial for the receptor to become dimerized. siRNA knockdown of PKC  $\delta$ , NHERF1 and NHERF2 proteins was performed in the absence of LAR (LAR  $\Delta$  P mouse embryonic fibroblasts (MEF) cells), the knockdown samples were then analysed through western blot technique. The data obtained showed that in the absence of PKC  $\delta$  and NHERF-2, PDGFR phosphorylation and activity increased; and in the absence of NHERF1 the activity was inhibited. The important regulatory role of these proteins was further determined when the down signalling molecule extracellular signal-regulated kinases (ERK) was investigated. The results facilitate a novel mechanism that LAR regulates PDGFR signalling through ligand induced activation of the PDGF  $\beta$ -receptor, through its mechanism of down regulating c-Abl activity; reducing the activation of PKC  $\delta$  which inhibits the PDGF  $\beta$ - receptor activation and as well as dissociating NHERF1 while promoting NHERF2 binding affinity to the PDGFR diminishing receptor dimerization.

## **Contents**

<b>1.0. <u>Introduction</u>:</b>	<b>Page 6</b>
<b>1.1. Background:</b>	<b>Page 6</b>
<b>1.2. Clinical approaches and treatments:</b>	<b>Page 8</b>
<b>1.3. Hepatocyte Transplantation and clinical developments:</b>	<b>Page 9</b>
<b>1.4. Stem cell isolation and transplantation:</b>	<b>Page 10</b>
<b>1.5. Hepatocyte transplantation and initial interactions:</b>	<b>Page 12</b>
<b>1.6. Aims of research project:</b>	<b>Page 15</b>
<b>2.0. <u>Materials and Methods</u>:</b>	<b>Page 15</b>
<b>2.1. Cell Culture and Preparation:</b>	<b>Page 15</b>
<b>2.2. Isolation of Binary Epithelial and Hepatic Sinusoidal Cells:</b>	<b>Page 16</b>
<b>2.3. Flow cytometry: Fluorescence-activated cell sorting (FACS):</b>	<b>Page 18</b>
<b>2.4. ECM Static Adhesion Assay:</b>	<b>Page 19</b>
<b>2.5. HUH Static Adhesion on HSEC:</b>	<b>Page 22</b>
<b>3.0. <u>Results</u>:</b>	<b>Page 23</b>
<b>3.1. FACS Analysis:</b>	<b>Page 23</b>
<b>3.2. The interaction between embryonic stem cells and the extracellular matrix:</b>	<b>Page 28</b>
<b>3.3. The interaction between HUH 7.5 cells and the extracellular matrix:</b>	<b>Page 31</b>
<b>3.4. The interaction between HUH 7.5 cells and a hepatic sinusoidal endothelial cell monolayer:</b>	<b>Page 34</b>
<b>4.0. <u>Discussion</u>:</b>	<b>Page 36</b>
<b>4.1. Conclusion:</b>	<b>Page 41</b>
<b>5.0. <u>References</u>:</b>	<b>Page 42</b>

## **Acknowledgements**

I would like to thank

Dr N. Davies, H. Dawes, Dr P. Newsome and the Newsome Research Group  
for their guidance and support.

## Abstract

Liver disease is a major cause of mortality worldwide; in the UK it is the only major cause of death that is still increasing on a yearly basis. Present treatments for liver diseases aim to slow down the progression to prevent further damage, this is only effective if the disease is controlled before irreversible damage has occurred; in many cases a liver transplant is the only option. Developments in the understanding of hepatocyte proliferation have led to cell transplantation as a promising alternative. Research aims to identify an alternative sustainable source, embryonic stem cell-derived hepatocyte-like cells (ES-HLCs) are a great potential renewable source of functional hepatocytes; however transplanted cells express low engraftment levels. The research project investigated the initial interactions between ES-HLCs with the extracellular matrix and hepatic sinusoidal endothelial cells. FACS was carried out on various types of HSEC to quantify the level of the integrins being expressed. Static adhesion assays with HLCs were performed on a range of ECM proteins to give a measure of cell interaction and adhesion. FACS analysis showed integrin- $\beta$ 1 and basal cell adhesion molecule (BCAM), were highly expressed. HLCs showed preferential binding to the ECM protein Laminin. HLC adherence to laminin was significantly reduced when the cells were treated with  $\beta$ 1 and BCAM blocking antibodies; furthermore HLC adherence was reduced when laminin was pre-treated with a recombinant Human BCAM/Fs. Both BCAM and integrin-  $\beta$ 1 are crucial in regulating adhesion in hepatocyte-like cells; suggesting potential targets to manipulate to improve HLC engraftment in vivo.

## **1. Introduction**

### **1.1. Background**

Liver disease is a major cause of mortality worldwide; in the UK it is the only major cause of death that is still increasing on a yearly basis and is now the fifth biggest 'killer' (after heart, cancer, respiratory disease and strokes) (Office for National Statistics, 2008). Statistics show that there has been a progressive rise in liver-disease related deaths in the UK since 1997, with an increase of 12% from 2005 to 2008 (British Liver Trust, 2009); where a total of 46,244 lives were lost. Figures show that in 2008 twice as many people died from liver disease compared to that in 1991 (Office for National Statistics, 2008).

Although these figures show an accurate representation of the increase in deaths related to liver disease; the British Liver Trust state that the actual numbers are higher than those being represented. The reasons for this are believed to be due to the stigma associated to liver disease and the attribution of liver-related deaths to other causes (patients can survive with up to 70% liver damage, which often leads to multiple organ dysfunction or failure) (British Liver Trust, 2009).

There are many types of liver disease which can be categorised into different groups. Liver disease can be caused by infection, for example hepatitis A, B, C, D and E virus.

Hepatitis B (HBV) is one of the most common infectious diseases in the world; and the most prevalent serious liver infection; affecting two billion people worldwide, of which 350 million suffer from chronic HBV infections (World Health Organization, 2008). Figures show that between 500,000 and 700,000 people die each year from liver cancer or cirrhosis caused by HBV infections. In the UK alone there was an estimated 326,000 people suffering from HBV infections (Hepatitis B Foundation UK, 2007). HBV can cause both an acute and a chronic illness. Generally the acute illness causes short term liver inflammation, jaundice and can rarely develop into acute liver failure. Chronic HBV illness is caused by long term infection (greater than six months), constant inflammation and scarring leads to cirrhosis and in some cases the development of liver cancer known as hepatocellular carcinoma (Chang, M. (2007)).

Another major-occurring form of liver disease is caused by chemical damage due to poisoning and/or substance abuse of drugs or alcohol. In the UK over the last 30 years there has been a massive increase in deaths related to alcoholic liver disease (ALD), figures show

that mortality rates have risen to over 450% (BMA, 2009). The drastic increase in ALD has meant that hospital admissions have more than doubled from 1995 to 2007, with a 71% increase (800,000 a year) (LAPE, 2012); it has also caused a financial impact; figures show that alcohol misuse costs the NHS roughly £2.7 billion each year (Department of Health, 2012). Early stages of ALD may not seem to serious, and in most cases the process is silent until later stages of liver disease have developed; and has become an acute illness with a high risk of immediate mortality (25-50%) (Department of Health, 2012). Early stages of ALD result in a fatty liver which is due to storage of fat broken down from excessive alcohol intake. This can be reversed with a reduced alcohol intake however continuation of excessive abuse can lead to inflammation and scarring of the liver leading to fibrosis and eventually cirrhosis; leading to liver failure and/or increased risks of liver cancer. Alcohol abuse with a fatty liver can also lead to alcoholic hepatitis, where the liver becomes swollen; this can happen suddenly and can result in liver failure (British Liver Trust 2007).

Liver disease can also be caused by genetic, autoimmune, vascular, congenital and metabolic damage (non-alcoholic fatty liver) (British Liver Trust 2007).

Haemochromatosis (HFE) is an example of a genetic disorder which can lead to liver disease. People suffering with this disorder absorb at least twice the amount of iron as normal; when the iron intake reaches a certain level (around 5 grams) it is then deposited in the body, often affecting the liver and heart. The extra iron is toxic and can be potentially lethal (Andrews et al., 1999).

Primary biliary cirrhosis (PBC) is a chronic autoimmune disease that leads to the progressive destruction of the small bile ducts (Henryk, 2010). When these ducts are damaged the bile can no longer flow through, instead the bile builds up in the liver causing organ dysfunction and inflammation which leads to cholestasis (British Liver Trust 2007). Over time this leads to scarring of the liver, known as fibrosis; the liver then becomes less efficient at repairing itself and eventually leads to liver cirrhosis (Henryk, 2010).



## **1.2. Clinical approaches and treatments**

The majority of present treatments for liver diseases aim to slow down the progression of the disease in order to prevent further damage. For instance anti-viral medications can be used to treat infections, for example with hepatitis B and C, to control or clear the virus. This type of treatment however is still undergoing active research and drug development.

In the case of ALD, and non-fatty liver disease the most effective treatments include cutting out alcohol, exercise and improving diet; in order to reverse the amount of fatty deposits leading to inflammation and fibrosis. This can however only be achieved in earlier stages of liver disease, before excessive fibrosis and cirrhosis has occurred. In later stages of liver disease cirrhosis can cause the development of hepatocellular carcinoma (HCC), if detected early the cancer can be removed or targeted with chemicals or radiation; however a liver transplant may be the only effective treatment option.

Other drug treatments include steroid-based drugs to help alleviate symptoms in autoimmune diseases; and for cholestatic liver diseases, ursodeoxycholic acid (naturally occurring bile acid) can be administered to help protect the liver from a build up of chemicals from the bile.

In Haemochromatosis (HFE) patients can attempt to keep iron levels under control, by the process of venesection; whereby a quantity of blood is regularly removed in order to prevent iron levels becoming excessive.

As stated above, present treatments are focused on slowing down the progression of liver disease in a hope to prevent further damage or complications occurring. Treatments are only effective if the disease is controlled from an early stage, and in most cases long term illness is caused due to irreversible damage, such as scarring and cirrhosis. When treatments stop being effective, in later stages of a disease, a liver transplant is the only option. Although usually successful, a transplant is a major operation with risks; the donor liver is also seen as a foreign object so immunosuppressant medication is crucial for the rest of the patient's life, which in itself can lead to further complications (Cleveland Clinic, 2009). The number of liver transplants that can be implemented is restricted by the number of donors available; figures show that in 2007-2008 a total of 1,121 patients were registered for a liver transplant and only 58% of these actually received the operation (NHS, 2012). Figures also show that the ALD, hepatitis C and PBC related cirrhosis were the main causes for liver transplantations (NHS, 2012).

### 1.3. Hepatocyte Transplantation and clinical developments

Recent developments in the understanding of hepatocyte proliferation potential have led to liver cell transplantation as a promising alternative to orthotopic liver transplantation (OLT). Hepatocyte transplantation (HT) offers many advantages in comparison, most notably that the procedure is less intrusive and that the patient's liver 'architecture' remains intact after the engrafted hepatocytes are transplanted (Fox *et al.*, 1997). This means that the transplantation is less stressful (metabolically) reducing the risks to the patient. Hepatocyte transplantation can also be repeated meaning that the consequence of potential graft loss or rejection is less severe compared to organ rejection (Fox *et al.*, 1997). Hepatocytes have been shown to survive and function once transplanted in the liver through widespread animal models; they have also been seen to participate in a regenerative process (Fabrega *et al.*, 1995).

Hepatocyte transplantation has been performed in clinical trials with patients suffering from both acute liver failure and liver-based metabolic diseases; where the aim was to replace deficient cells with fully functioning hepatocytes (Fox *et al.*, 2004). The results so far for the clinical trials that have been conducted involving liver-based metabolic diseases have been promising; there was a clear improvement in the disease phenotype, however cell functionality often declines after 9 months possibly due to immunosuppression (Puppi *et al.*, 2008). Clinical trials involving acute liver failure have shown improvements in hepatic activity and a reduction in ammonia and bilirubin levels (Strom *et al.*, 1999); however at present no significant clinical outcome. Hepatocyte transplantation has also been used with patients suffering with chronic liver disease, however further trials need to confirm the efficiency of cell engraftment; variable results are seen, which may be in relation to fibrosis impairing cell engraftment (Hughes *et al.*, 2005).

At present human hepatocytes are obtained from donor liver tissue that is unused in orthotopic liver transplantations. Hepatocytes are isolated using a collagenase perfusion technique, which digests the donor tissue; the cells are then purified through centrifugation (Fabrega *et al.*, 1995). Protocol procedures follow strict conditions (good manufacturing practice) for clinical use, ensuring the final cell product is free from contamination of microorganisms (Mitry *et al.*, 2003). Primary human hepatocytes are therefore limited by the

availability of unused donor liver-tissue; there have been reports that good quality hepatocytes could be isolated from diseased livers (removed at transplantation). These hepatocytes would be an additional source; however the safety aspect of using these cells for transplantation has yet to be confirmed (Lehec *et al.*, 2009). There are also storage issues with primary human hepatocytes, cells are ideally transplanted immediately after isolation, where possible however this can be hard to achieve in most cases and is impractical. Hepatocytes can also be cryopreserved for future use or in case of emergency treatment (Bhogal *et al.*, 2011). At present however cryopreservation of hepatocytes seems to have detrimental effects on the cell viability and function; and often thawed cells are not suitable for transplantation (Terry *et al.*, 2006). A study investigating the mechanism of cryopreservation –induced nonattachment in primary hepatocytes (Terry *et al.*, 2007), showed a loss in hepatocyte attachment. The study focused on beta1-integrin, which is a cell matrix adhesion molecule. The results showed conclusively that cryopreserved hepatocytes had significant lower levels of beta-1 expression compared to fresh hepatocytes and that there was a correlation between the amount of beta-1 expressed and hepatocyte engraftment (Terry *et al.*, 2007). Current research aims to optimize a freeze/thaw protocol carried out in good manufacturing practice conditions in order to obtain a significant improvement in hepatocyte quality for clinical use.

#### **1.4. Stem cell isolation and transplantation**

As stated above primary hepatocytes are isolated from donor livers, this is a major limitation for the clinical application of hepatocyte transplantation as a primary therapy for liver disease, due to a lack of availability. A big emphasis in current research is to find and identify an alternative sustainable source of hepatocyte-like cells and to develop cell-based hepatocyte treatments as novel therapies for liver disease. Recent studies have demonstrated that there has been an advancement in the isolation of stem cells; and a progression in the characterisation of the cells potential ability to differentiate into hepatic cell types (Sancho-Bru1 *et al.*, 2009). Completed studies have shown, in a preclinical application, the potential regeneration that can be achieved using stem cell therapy, although at an early stage the results obtained from stem cell-hepatocyte transplantation suggest an increase in liver function and improved hepatic activity in chronic liver disease (Stutchfield *et al.*, 2010). It has been suggested that hepatocyte-like cells can be obtained from multiple sources; these

include adult stem cells, human embryonic stem cells and induced pluripotent cells. Isolated mesenchymal- like stem cells have been shown to engraft and differentiate into HLC's once transplanted in vivo (Najimi et *al.*, 2007).

Human embryonic stem cell-derived hepatocyte-like cells (ES-HLCs) are a great potential renewable source of functional hepatocytes, they have the potential to differentiate towards cells of a hepatic lineage with many characteristics of primary human hepatocytes. Studies completed (Yamamoto et *al.*, 2003) have confirmed that ES-HLCs are able to grow and exhibit a morphology similar to native mature hepatocytes and also have ability to express hepatocyte –specific genes. Yamamoto et *al.*, 2003 also state that transplanted ES-HLCs showed both engraftment and regenerative capabilities in carbon tetrachloride – injured mouse liver models (Yamamoto et *al.*, 2003).

There are fundamental issues at present with successful transplantation of hepatocyte- like cells; research completed by (Gupta et *at.*, 1998) state that transplanted cells express low engraftment levels into host liver parenchyma. The research shows that the majority of transplanted cells are lost early; after 24 hours post transplantation only a 30% survival of transplanted hepatocytes. Further research into understanding the mechanisms regulating cell adherence and engraftment within the liver is therefore crucial for hepatocyte-like cell transplantation, as a therapeutic application. Refining routes of transplant delivery to increase engraft efficiency is also critical (Basma, et al., 2009).

As stated above human embryonic stem cell-derived hepatocyte-like cells (ES-HLCs) are a great potential renewable source of functional hepatocytes, they have the potential to differentiate towards cells of a hepatic lineage with many characteristics of primary human hepatocytes; however at present transplanted cells have low engraftment levels within host livers. Increasing engraftment efficiency and sustained survival of transplanted ES-HLCs need to be addressed before this source of derived hepatocyte-like cells can be confirmed as a potential therapy. Mechanisms in which ES-HLCs interact with host liver upon transplantation are still unclear; this is therefore a major research area to investigate.

The research project aims to investigate the initial molecular interactions between hepatocyte-like cells with the extracellular matrix and hepatic sinusoidal endothelial cells (HSEC).

### **1.5. Hepatocyte transplantation and initial interactions**

It has been shown in previous studies that once transplanted hepatocyte-like cells initially interact with HSEC; the cells then disrupt the endothelium and adhere to the underlying ECM before crossing into the liver parenchyma (Basma, et al., 2009). Gupta et al., established that transplanted cells had a greater success of engraftment if deposited within hepatic sinusoids; it was found that hepatocytes transplanted into portal areas were cleared. It was also stated that for transplanted hepatocytes to enter into the liver parenchyma the cells must first integrate through the HSEC barrier and through the underlying extracellular matrix (Gupta et al., 1998). The increased survival of transplanted hepatocytes in sinusoids was further shown in studies, MacFee et al., state superior hepatocyte survival in sinusoids in the spleen and liver is seen; when hepatocytes are transplanted into a rat, cells have a higher engraftment compared to those seen in a mouse, where the spleen lacks sinusoids (MacFee et al., 1988). Studies have suggested that hepatocytes disrupt and integrate through the hepatic sinusoidal endothelium through many possible ways. Cytokines may be one such example; it is known that they are capable of having an effect on the endothelium; examples such as tumour necrosis factor and interleukins which may be released by macrophages as a direct immune response to the transplanted hepatocytes, which may cause disruption to the HSEC (Lindner et al., 1997).

It has also been suggested that cytokines could be released by Kupffer cells and or by the endothelial cells (Nakagawa et al., 1996).

Studies state that upon hepatocyte transplantation into sinusoids there is an over expression of hepatocyte growth factor, these growth factors have been shown to target endothelial cells in the proximate areas to the transplanted cells; the factors are released during cell adherence and engraftment and could be responsible for changes in HSEC morphology (Yazigi et al., 1995).

The research project will investigate the initial interactions between hepatic sinusoidal endothelium cells and hepatocyte like cells; these interactions are crucial to further understanding the mechanism of cellular adherence.

Studies have shown that hepatic sinusoids have a unique structural organisation in that they lack basement membranes. It has also been shown that once adhered to HSEC, hepatocyte engraftment is most probably due to association with the extracellular matrix and its components (Darby *et al.*, 1986).

The research project will also investigate the interaction of hepatocyte- like cells with a range of extracellular matrix components; to determine the importance of the of the ECM components with binding to hepatocytes and to give a quantitative idea of the levels of adherence.

Research completed (Gupta *et al.*, 1998) suggest that hepatocyte-like cell engraftment is increased when ECM-like components are present; the study shows more efficient engraftment levels when ECM-like components were initially injected into the host liver before cell transplantation. The role integrins contribute in the adhesion of hepatocyte- like cells to both HSEC and ECM components will therefore also be investigated. Integrin-  $\beta$ 1 and basal cell adhesion molecule (BCAM) in particular will be explored.

Integrin-  $\beta$ 1 has been shown in previous studies to play an important role in regulating adhesion; it is a well established adhesion molecule. Integrin-  $\beta$ 1 has been shown to be a receptor for the extra cellular matrix protein laminin (Carloni *et al.*, 1998).

Studies investigating the effects of integrin-  $\beta$ 1 have shown that it is crucial for survival of transplanted hepatocytes in the host liver paranchyma. (Pinkse *et al.*, 2004) studied the effect of the presence and absence of the ECM component integrin-  $\beta$ 1 upon hepatocyte function and survival; they state that hepatocytes in the presence of integrin-  $\beta$ 1 (in native ECM conditions) survive and maintain function. In the absence of integrin-  $\beta$ 1 hepatocytes go into apoptosis (Pinkse *et al.*, 2004). Other studies also show a loss in cell adhesion to ECM components and to endothelial cells when integrin-  $\beta$ 1 is blocked (Pinkse *et al.*, 2004; Potocnik *et al.*, 2000).

Basal cell adhesion molecule (BCAM) (CD239) is a cell surface glycoprotein, studies have shown that it interacts and functions as a receptor for laminin (Udani *et al.*, 1998). BCAM is also believed to be involved with cell-cell adhesion and migration; and is highly expressed in

carcinomas (Schon *et al.*, 2001). The interactions with BCAM and hepatocytes-like cells are at present unclear, as stated it is known that BCAM is a receptor for laminin; an important ECM protein which regulates attachment, migration and organisation of cells. BCAM is therefore a potential target to manipulate to increase hepatocyte adherence to laminin/ extracellular matrix.

The investigation will use two types of cells, embryonic stem cell – hepatocyte like cells (ES-HLC's) and HUH 7.5 cells.

HUH 7.5 cells are a liver carcinoma cell line. These cells express high levels of BCAM and alpha-1-fetoprotein (AFP); AFP is produced in the liver during foetal development, a precursor to serum albumin. These cells will be used as a surrogate hepatocyte, presenting similar characteristics seen with foetal hepatocytes.

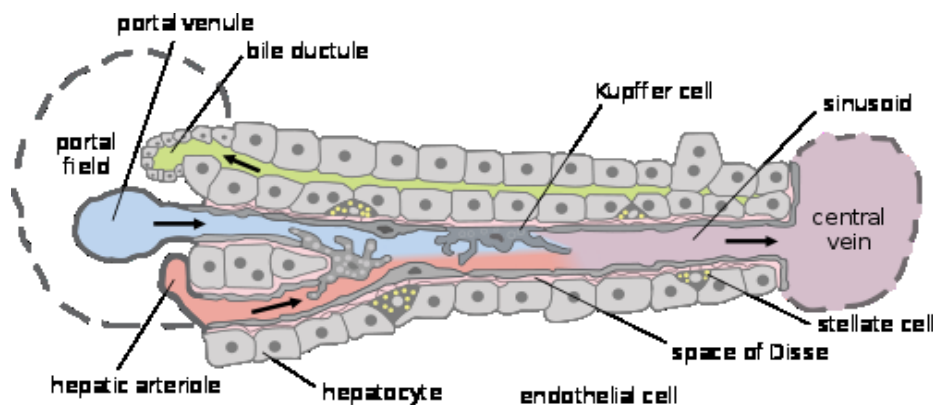


Figure 1: Diagram showing the liver cellular structure; including the flow of blood through a sinusoid into a central hepatic vein (Tissupath, 2011).

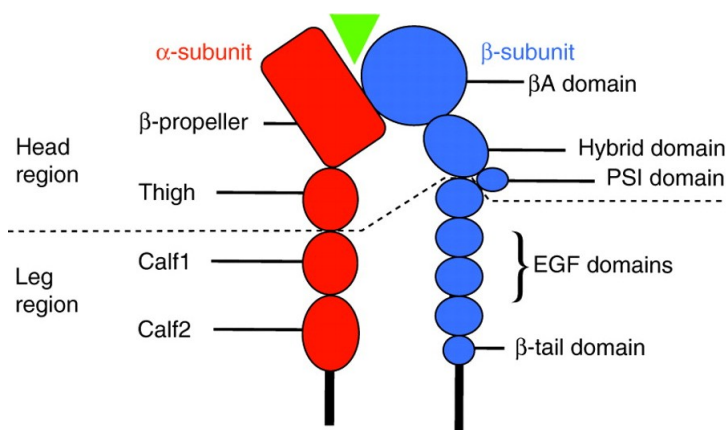


Figure 2: Diagram showing the structure of an integrin. A schematic showing the structure of the head region (both the  $\alpha$  and  $\beta$  subunits of the propeller and thigh domains; with support from the leg region. Ligand binding takes place between the  $\beta$ A domain and the propeller domain (Janet *et al.*, 2009)

## **1.6. Aims of research project**

- To investigate the initial molecular interactions between hepatocyte- like cells with the extracellular matrix and hepatic sinusoidal endothelial cells (HSEC). The investigation will involve finding the integrins being expressed in both HSEC and hepatocyte –like cells, and to understand the role they expressed in cell adhesion.
- To investigate the initial interactions between hepatic sinusoidal endothelium cells and hepatocyte like cells; these interactions are crucial to further understanding the mechanism of cellular adherence. If the regulating factors involved can be discovered then the potential to increase cellular adherence to HSEC would be an essential first step in increasing the overall engraftment of transplanted ES-HLCs (or other hepatocyte like cells). Investigating the interactions of hepatocyte like cells with components of the extra cellular matrix is also critical in understanding the mechanism of cell adherence and engraftment.
- The research project will also investigate the interaction of hepatocyte- like cells with a range of extracellular matrix components; to determine the importance of the of the ECM components with binding to hepatocytes and to give a quantitative idea of the levels of adherence.

## **2. Materials and Methods**

### **2.1. Cell Culture and Preparation**

Cell culture of HUH 7.5 cells

Under sterile conditions:



HUH 7.5 cells (human hepatoma cell line) frozen in 10% Dimethyl sulfoxide (DMSO), for cell membrane stability and survival, were resurrected at 37 °C in a water bath (rapidly thawed).

The cells were grown in culture under sterile conditions in Dulbecco's Modified Eagle Media (DMEM) + 10% foetal calf serum (FCS). The DMEM also contained 1% Glutamine-Penicillin-Streptomycin (GPS), a one-step supplementation to help reduce the risk of contamination.

After several days of growth the cells were passaged using the cell dissociation reagent TrypLE™ Express. The cells were grown in 5mls of DMEM + 10% FCS to confluence in 25cm<sup>2</sup> flasks; roughly 2-3 x10<sup>6</sup> cells per flask. The HUH 7.5 cells were used in ECM static adhesion and flow cytometry assays.

## **2.2. Isolation of Binary Epithelial and Hepatic Sinusoidal Cells**

Under sterile conditions:

A slice of human liver was placed in a sterile Petri dish, under aseptic conditions within a laminar flow cabinet. Using two sterile scalpels the liver was finely chopped, and the diced tissue was placed into a sterile 200ml beaker. Sterile PBS (20ml) was added to the beaker along with a 5ml aliquot of collagenase. The beaker was then swirled to mix, before being incubated at 37 °C for 25-45 minutes; depending on the texture of the tissue. The tissue 'digest' was then strained through a sterile fine mesh into a fresh beaker (sterile); and washed through several times with PBS. The volume was made up to a total of 200ml with PBS and the digest was separated into 8 aliquots into universal containers; and centrifuged at 2000xg for 5minutes. The 8 pellets were then resuspended into 4 universal containers; the spare tubes were washed to ensure no loss of cells before being centrifuged again at 2000xg. This was repeated until the pellets were combined into one universal container; the total volume was made up to 24ml with PBS.

Preparation of percoll gradients were done according to protocol:

Stock percoll – 99ml percoll +11ml 10x PBS

33% percoll – 33ml of stock + 67ml 1x PBS

77% percoll – 77ml of stock + 23ml 1x PBS

Percoll consists of polyvinylpyrrolidone (PVP) coated colloidal silica particles; it possesses low osmolarity and viscosity and is non toxic. (Pertoft, et *al.*, 1978)

It is an efficient tool in density separation; used in the isolation of cells and organelles by density centrifugation.

Percoll-33% (3 ml) was added to 8 15 ml conical bottomed tubes. Percoll -77% (3 ml) was then allotted underneath the 33% percoll gradient. Finally 3 ml of cell suspension was layered onto the percoll gradient; the 8 tubes where then centrifuged for 20 minutes at 2000 xg (note the break speed set to 0). The top layer was then discarded and the band of cells at the percoll gradient was removed; 2 aliquots were put into 1 universal (a total of 4 universals). PBS (25 ml) was added and mixed to prevent a further gradient formation, before being centrifuged again at 2000 xg. This was repeated until all the cell pellets had been combined into one tube; the pellet was then resuspended with 500 µl of PBS, 50 µl of HEA 125 and + 10 µl FCS was also added. The cells were left to incubate for 30 minutes at 37 °C. HEA 125 is an epithelium- specific glycoprotein (Ep-CAM).

The pellet was then washed (14ml PBS) and centrifuged at 2000xg for 5minutes, before being resuspended in 1ml of ice cold PBS. 12 µl of cold Dynabeads (+ 10 µl FCS) was then added and left to incubate at 4 °C for 30 minutes; with constant agitation. Dynabeads are super-paramagnetic spherical polymer particles (mono sized). They have a defined surface for the coupling of specific cells (Axl, et *al.*, 2007).

After incubation 5 ml of ice cold PBS was added to the tube, which was then placed in a magnet for 5 minutes. Whilst in the magnet the supernatant was removed carefully with a pipette and transferred to a second 15 ml conical bottom tube – Tube 2.

BEC isolation: 5 ml of cold PBS was added to the first tube, it was mixed well before being left for 5 minutes in the magnet. As above, the supernatant was removed and added to tube 2. Finally the beads (tube 1) were resuspended in cold PBS for a further minute in the magnet;

the PBS was then discarded and the beads were suspended in 5 ml of BEC medium and transferred into a 25 cm collagen coated flask and incubated at 37 °C.

HSEC isolation: Tube 2 was placed in the magnet for 2 minutes; to remove any residual beads. While in the magnet the supernatant was removed and placed into a fresh tube; the volume was made up to 14 ml with PBS and centrifuged at 2000 xg for 5 minutes. The pellet was resuspended in 1ml of PBS + 10 µl FCS and 10 µl of CD31 coated magnetic beads; and was incubated for 30 minutes at 4 °C with continual agitation.

CD31, also known as platelet endothelial cell adhesion molecule (PECAM-1) is a protein that is responsible for a large portion of endothelial cell intercellular junctions (NCBI et *al.*, 2012).

Cold PBS (5 ml) was added and mixed, and the tube was placed back into the magnet (approx 2minutes). Whilst in the magnet the supernatant was removed and discarded; this step was repeated three times. The beads were then resuspended in 5 ml of HSEC media and transferred to a 25 cm<sup>2</sup> collagen coated flask and incubated at 37 °C.

### **2.3. Flow cytometry: Fluorescence-activated cell sorting (FACS)**

Under sterile conditions:

FACS was carried out on various types of HSEC cells (normal or diseased) and HUH 7.5 cells.

The cells to be used were taken out of the incubator, the media was removed (the media was kept in a 15ml universal tube) from the flask and the cells were washed in 5 ml of PBS. TrypLE™ Express (GIBCO®) (5 ml) was then added to the flask, and left to incubate at 37 °C for 5 minutes. Once the cells had become dissociated (checked under a microscope), the media was re-added to deactivate the TrypLE™ Express and the cell solution was transferred into a 15 ml universal tube. The solution was then filtered using 50 µm membrane filters, and washed through with PBS before being centrifuged at 2000xg for 5 minutes. The supernatant was removed and the pellet was resuspended in 2 ml of PBS. The cells were counted by adding 10 µl of Trypan Blue mixed with 10 µl of the cell solution (1:2 ratio) on a haemocytometer glass slip. Sterile FCS (1/500) (2 µl) was added to the cell solution; from

which a 100  $\mu$ l of cells was then distributed into 18 FACS tubes. A specific antibody (2  $\mu$ l) was then added to each of the FACS tubes; as follows:

Tube	Antibody	Tube	Antibody
1	Cells only	10	$\beta$ 3 (IgG2a)
2	IgG1 isotype control (PE)	11	IgG2b isotype control (PE)
3	$\alpha$ 1 (IgG1)	12	$\beta$ 4 (IgG2b)*
4	$\alpha$ 3 (IgG1)	13	$\alpha$ v $\beta$ 3 (IgG2b)*
5	$\alpha$ 4 (IgG1)	14	$\alpha$ v $\beta$ 5 (IgG2b)*
6	$\beta$ 1 (IgG1)	15	$\alpha$ v $\beta$ 6 (IgG2b)*
7	$\beta$ 2 (IgG1)	16	BCAM (IgG2b)
8	IgG2a isotype control (PE)	17	IgG1 (FITC)
9	$\alpha$ 2 (IgG2a)	18	$\alpha$ 5 (FITC)

Table 1: The antibodies used for FACS analysis. \* denotes unconjugated antibodies.

Note: BCAM antibody was only used with HUH 7.5 cells

The 4 unconjugated antibodies were added first to the respective FACS tubes, and left on a plate mover at 4 °C for 30 minutes. PBS (1 ml) was then added to the 4 tubes, and centrifuged at 2000xg for 5 minutes. The excess liquid was poured off and secondary antibody (IgG2b) was added to the tubes; the primary antibodies (see table above) were also added to the respective tubes and again left at 4 °C for 20 minutes. PBS (1 ml) was added to each of the FACS tubes and centrifuged again for 5 minutes at 2000xg. The samples were then run on a FACS machine.

## 2.4. ECM Static Adhesion Assay

### Reagents

#### Cells

24- Well plate

PBS

DMEM containing 0.1% BSA

ECM proteins- Laminin, Fibronectin, Collagen, Osteopontin,

Methanol

Hematoxylin

Recombinant ECM proteins – rhBCAM (B-CAM/Fc chimeric molecule), vascular cell adhesion molecule (VCAM)

Blocking antibodies –  $\beta 1$ , IgG1,  $\alpha V\beta 5$ , BCAM, IgG2b (goat),  $\alpha V$

ECM Protein	Concentration (1mg/ml)	Dilution with PBS (200 $\mu$ l per well)
Laminin	4 $\mu$ l	196 $\mu$ l
Fibronectin	10 $\mu$ l	190 $\mu$ l
OPN	20 $\mu$ l	180 $\mu$ l
rhVCAM	2 $\mu$ l	198 $\mu$ l
rhBCAM	50 $\mu$ l	450 $\mu$ l

Table 2: Shows relevant ECM protein concentration.

### Plate Preparation

Under sterile conditions:

The wells of the 24-well plate were washed with sterile PBS, before 200  $\mu$ l of the recombinant ECM proteins were added (to the specific wells) at the desired concentration (see table 2 below). The plate was then sealed with Parafilm and left for 30 minutes at 37 °C. The wells were then washed twice with sterile PBS; 200  $\mu$ l of 0.1% BSA in PBS was then added to the wells and left for 10 minutes at room temperature. The wells were again washed twice with PBS before 200  $\mu$ l of the recombinant ECM proteins (e.g. rBCAM, rVCAM) were added to the specific wells. The plate was left to incubate for 30 minutes at room temperature, and the wells were washed twice with PBS; and also kept in 1 ml of PBS until ready to use.

### Assay

Under sterile conditions:

Static adhesion assays were completed with both embryonic stem cells and HUH 7.5 cells. Depending on the assay the cells were dissociated from the flask using either Trypsin EDTA (1x) (Sigma) for ES cells; or TrypLE™ Express for HUH 7.5 cells. For both cases the media was firstly removed from a 25 cm<sup>2</sup> flask, and the cells were washed with PBS before 2.5 ml of the dissociation solution was added. The flask was left to incubate at 37 °C until the cells had become dissociated (checked under a microscope). The media was then re-added (5 ml) to the flask to neutralize the dissociation solution; L-15 media containing 10% FCS was used for the ES cells while DMEM containing 10% FCS was used for the HUH 7.5 cells. The cell solution was then transferred to a 15 ml universal tube and any remaining cells were washed with sterile PBS and scraped using a cell scraper before being added to the universal tube. The cell solution was then filtered through a 100 µ sterile filter, and washed through with PBS. The cell solution was then centrifuged at 2000xg for 5 minutes, the supernatant was poured off and the pellet was resuspended in 2 ml of PBS; the cells were then counted (as above) and re-centrifuged. The pellet was resuspended in 3 ml of the appropriate media and left to rest for an hour at 37 °C.

In the assays where the cells were treated with blocking antibodies (e.g. β1 and BCAM blocking assays) the cells were left to rest for 30 minutes before being treated with the antibody. For these assays 5 µl of a specific antibody was added to 500 µl of the cell solution and left for a further 30 minutes.

After the cells had been rested, they were then washed with PBS and centrifuged at 2000xg for 5 minutes and resuspended in DMEM media containing 0.1% BSA to give a cell concentration/ density of 1x10<sup>6</sup> cells per ml. The cells were then added to the pre-treated well plate; the PBS was removed from the wells and 200 µl of cells (in DMEM + 0.1% BSA) were added per well. The plate was left undisturbed for one hour at room temperature to allow for the cell adhesion. The wells were then washed twice in PBS, and the remaining adhered cells were fixed using 200 µl of MeOH for 5 minutes. The wells were then washed twice more with PBS.

In non-sterile conditions:

The adhered cells were stained using Leica Mayers 30 second Hematoxylin. Hematoxylin is a long lasting stable nuclear stain. The cells/wells were then washed with 3 times with water

and left in 1ml PBS until ready for cell counting. Cells were counted on 20 x magnification with 10 fields of view per well.

## **2.5. HUH Static Adhesion on HSEC**

Cells

24- Well plate

PBS

DMEM containing 10% FCS

HSEC media

Methanol

Recombinant ECM proteins – rhBCAM (B-CAM/Fc chimeric molecule), vascular cell adhesion molecule (VCAM)

Blocking antibodies –  $\beta$ 1, IgG1, BCAM, IgG2b (goat)

### HSEC Monolayer Preparation

Under sterile conditions

HSEC cells were isolated and grown on collagen (Rat Tail) coated 6-well plates to a confluent monolayer. The HSEC cells were seeded into 7 wells in total, at a density of  $2 \times 10^6$  cells/ml. Once confluent the HSEC cells (monolayer) were then stimulated with Interferon –  $\gamma$ ; this causes up-regulation of DNA mimicking conditions seen in-vivo in the liver. The HSEC cells were then left to rest for 24 hours.

### Static Adhesion Assay

Under sterile conditions

HUH 7.5 cells were dissociated from a 25 cm<sup>2</sup> flask(s) using 2.5 ml of TrypLE™ Express (see above). The cells were washed with sterile PBS and centrifuged at 2000xg for 5 minutes; and the pellet was resuspended in 1 ml of PBS + 0.1% BSA. Carboxyfluorescein succinimidyl ester (CFSE) fluorescent dye (5mM) (1 µl) added to the cell solution and left to incubate in the dark at 37 °C for 10 minutes; the cell solution was agitated at 5 minutes.

Cold FCS (3 ml) was added to the cell solution, which was then washed and centrifuged at 2000xg for 5 minutes. The supernatant was removed and the pellet was washed resuspended in PBS and re-centrifuged. The cell pellet was then resuspended in DMEM + 10% FCS; the HUH cells were then split into universal tubes and treated with the antibodies –  $\beta$ 1, IgG1, BCAM, IgG2b (see above). The cells were rested for 30 minutes at 37 °C; then centrifuged for 5 minutes at 2000xg and resuspended in PBS + 0.1% BSA at a density of 1 x10<sup>6</sup> cells/ml (e.g. 1 ml per well).

The HSEC media was removed from the well plate; 2 wells of the HSEC mono-layer were treated with 200 µl of rhBCAM and rhVCAM and left to incubate for 30 minutes at 37 °C. The wells were then washed twice in sterile PBS.

HUH 7.5 (2 ml) cell suspension was then gently added to each specific well and left at room temperature for 1 hour in the dark (covered with foil). The cell solution was then removed and 1 ml of cold methanol was added to fix the adhered cells. The methanol was aspirated off and the wells were washed twice in sterile PBS.

The adhered HUH 7.5 cells were then counted under an inverted fluorescence microscope under a FITC laser. 20 fields of view at x20 magnification per well covering the total areas were counted. The quality of HSEC was also checked, making sure it was still present.

### **3. Results**

#### **3.1. Integrin expression profile for HSEC and HUH 7.5 cells**

Fluorescence-activated cell sorting (FACS) analysis was performed on both HSEC and HUH 7.5 cells. Both HSEC and HUH 7.5 cells were treated with specific antibodies that were tagged with a relevant fluorophore. The fluorophore R-phycoerythrin (PE) was used; it has an



absorbance wavelength of 480 nm and an emission wavelength of 578 nm, its visible colour is yellow. The fluorophore Fluorescein FITC was also used; it has an absorbance wavelength of 495 nm and an emission wavelength of 518 nm, visible colour is green. FACS analysis is an accurate quantitative technique, it was used to show the amount of integrins that was being expressed in both cell types.

A range of HSEC liver cells were tested, these included normal and diseased samples. Various types of diseased samples were tested, comprising of: Primary Biliary cirrhosis (PBC), Haemochromatosis (HFE), Alcoholic Liver disease (ALD), Porphyria and Sero-negative.

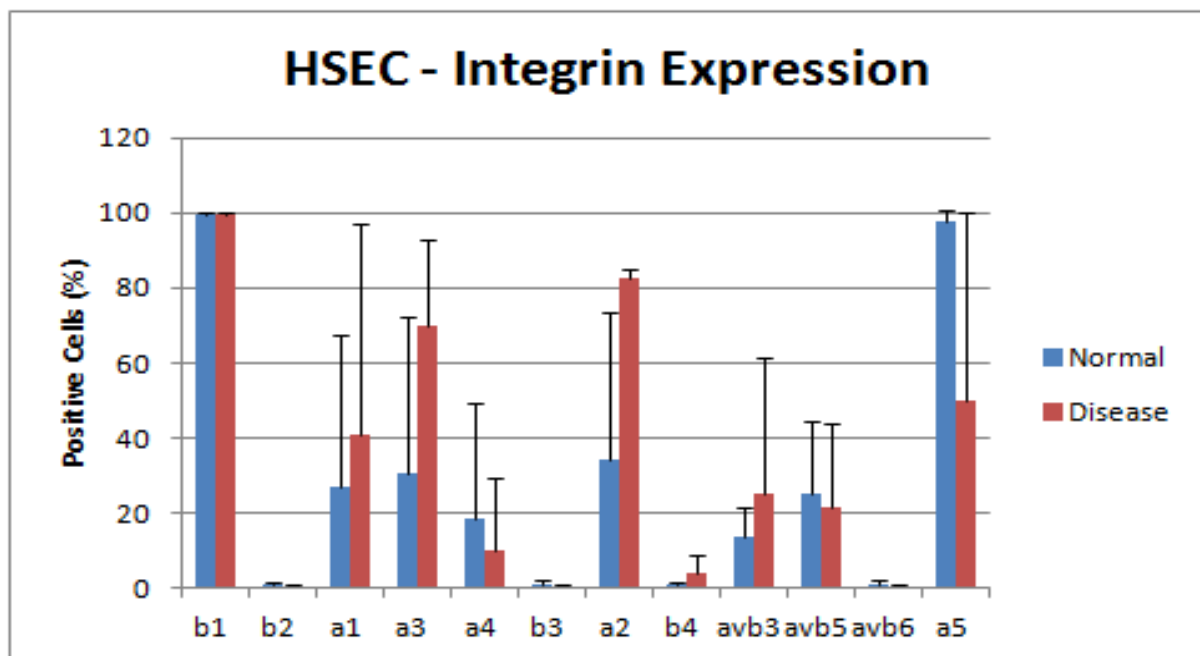


Figure 3: Shows integrin expression in both normal and diseased HSEC liver cells, obtained by fluorescence-activated cell sorting flow cytometry. Diseased HSEC samples include: primary biliary cirrhosis (PBC), hemochromatosis (HFE), alcohol liver disease (ALD), sero-negative and porphyria.

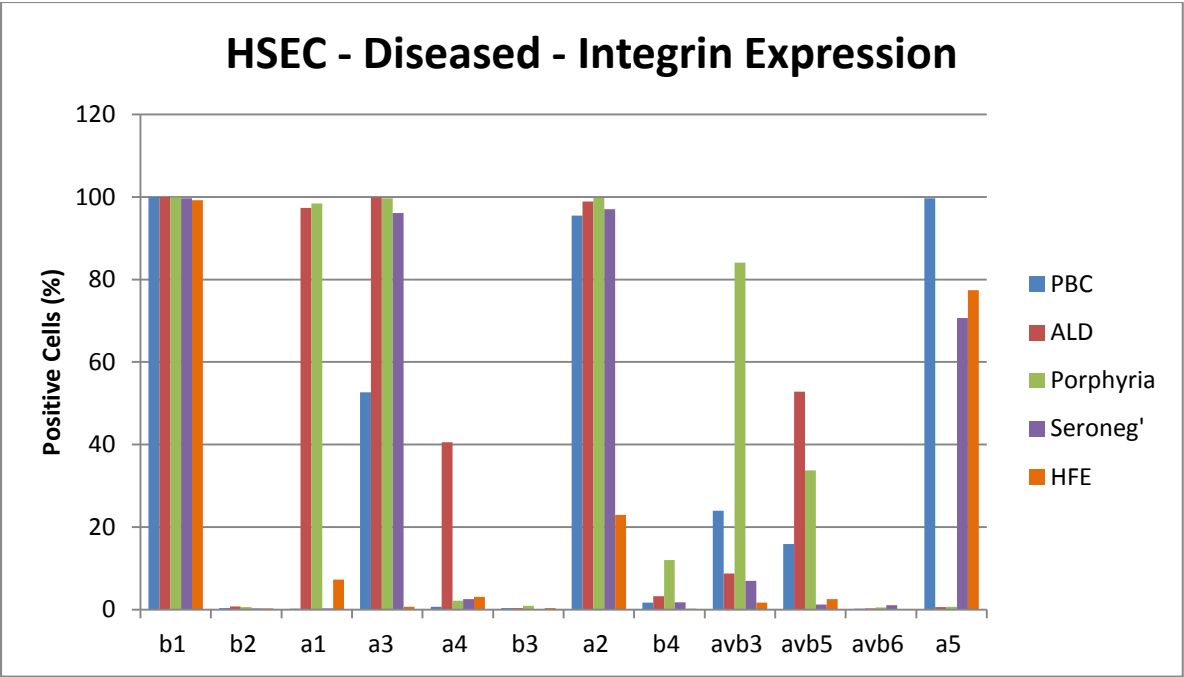


Figure 4: Shows integrin expression in diseased HSEC liver cells, obtained by fluorescence-activated cell sorting flow cytometry. Diseased HSEC samples include: primary biliary cirrhosis (PBC), hemochromatosis (HFE), alcohol liver disease (ALD), sero-negative and porphyria.

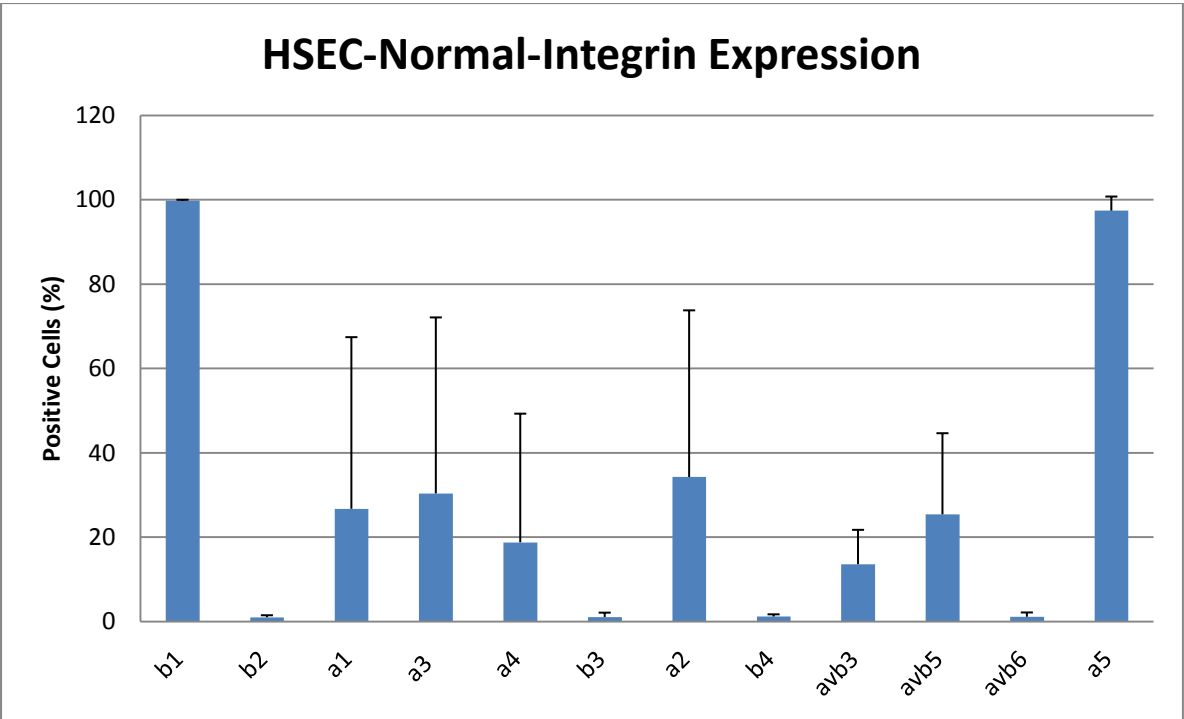


Figure 5: Shows integrin expression in normal HSEC liver cells, obtained by fluorescence-activated cell sorting flow cytometry.

In normal HSEC both  $\beta 1$  and  $\alpha 5$  integrins were expressed in high quantities (Fig.3) (Fig.5). In normal HSEC  $\beta 1$  integrin was at 99.76% positive cell expression; and  $\alpha 5$  was at 97.4% positive cell expression. In comparison  $\beta 1$  integrin was also highly expressed in all the HSEC disease types (Fig.3) (Fig.4); (positive cell expression: PBC: 99.95%, ALD: 99.96%, Porphyria: 99.88%, Sero-negative: 99.68%, HFE: 99.2%).  $\alpha 5$  integrin had variable expression between the several disease types; a high expression in PBC, Sero-negative and HFE disease however very little expression in ALD (0.6%) and Porphyria (0.68%). In most HSEC disease types (excluding HFE)  $\alpha 3$  and  $\alpha 2$  integrins are highly expressed.  $\alpha 2$  integrin in particular is expressed in excessive levels (positive cell expression: PBC: 95.48%, ALD: 98.89%, Porphyria: 99.75%, Sero-negative: 97.04%) in comparison to that seen in normal HSEC expression (positive cell expression 34.25%).

In both normal and diseased HSEC  $\beta 1$  integrin is highly expressed with little variation, likewise  $\alpha 5$  integrin is highly expressed in normal HSEC but is variable between different types of diseased HSEC. In the majority of the diseased HSEC that was analysed (with the exception on HFE)  $\alpha 3$  and  $\alpha 2$  integrins were also highly expressed; the 'over-expression' of these integrins is not present in normal HSEC.

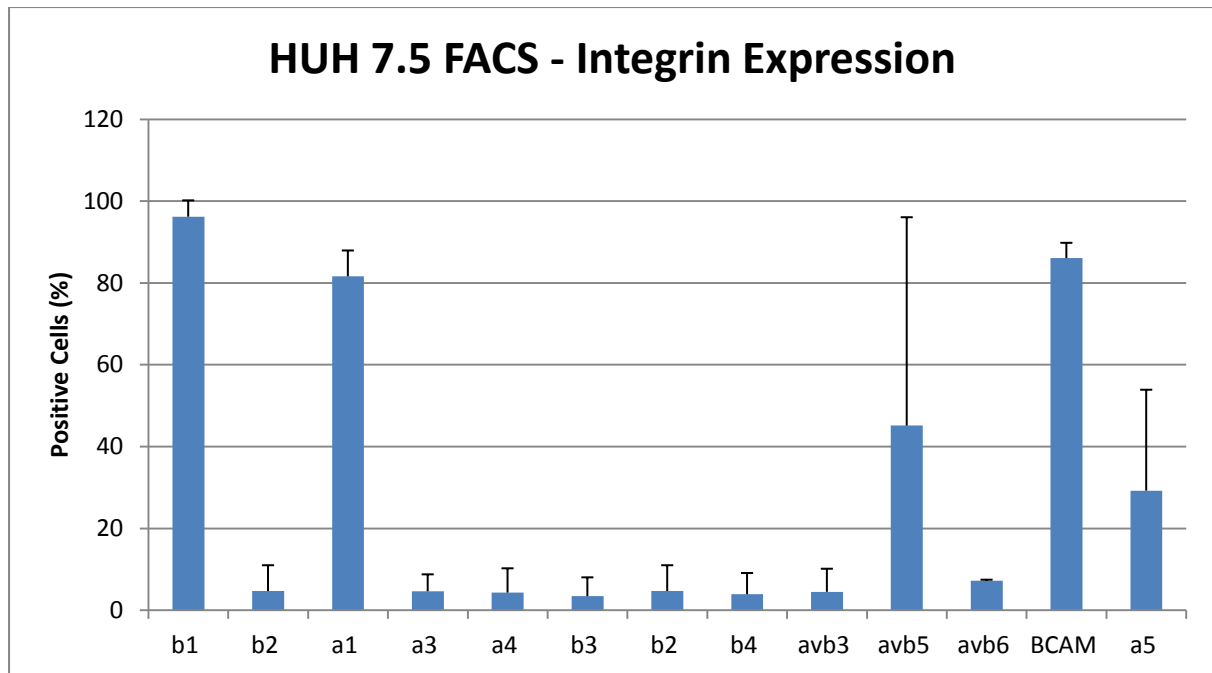


Figure 6: Shows integrin expression in HUH 7.5 cells, obtained by fluorescence-activated cell sorting flow cytometry.

HUH 7.5 FACS analyses showed that the integrin  $\beta 1$  was expressed in high quantities (Fig.4); its positive cell expression was 96.25%. This closely resembles the figures seen with HSEC analysis. The basal cell adhesion molecule (BCAM) is also highly expressed in HUH 7.5 cells, its positive cell expression 86.07%;  $\alpha 1$  also showed significant expression (81.62%). The high expression of both integrin- $\beta 1$  and BCAM suggests their relative importance in HUH 7.5 cell (hepatocyte-like cell) adherence.

### Cell Adhesion expression

Static adhesion assays were performed using both ES and HUH 7.5 cells. A range of extra cellular matrix (ECM) proteins were used and various types of blocking antibodies. The static assays gave a quantitative measure of cell adhesion to the ECM.

### 3.2. The interaction between embryonic stem cells and the extracellular matrix

The interactions and adherence of embryonic stem cell hepatocyte-like cells were tested with the ECM components laminin, fibronectin, osteopontin, collagen and vascular cell adhesion molecule (VCAM). ES cell binding to the ECM components was compared to the control – BSA; cell binding to BSA represented non-specific binding.

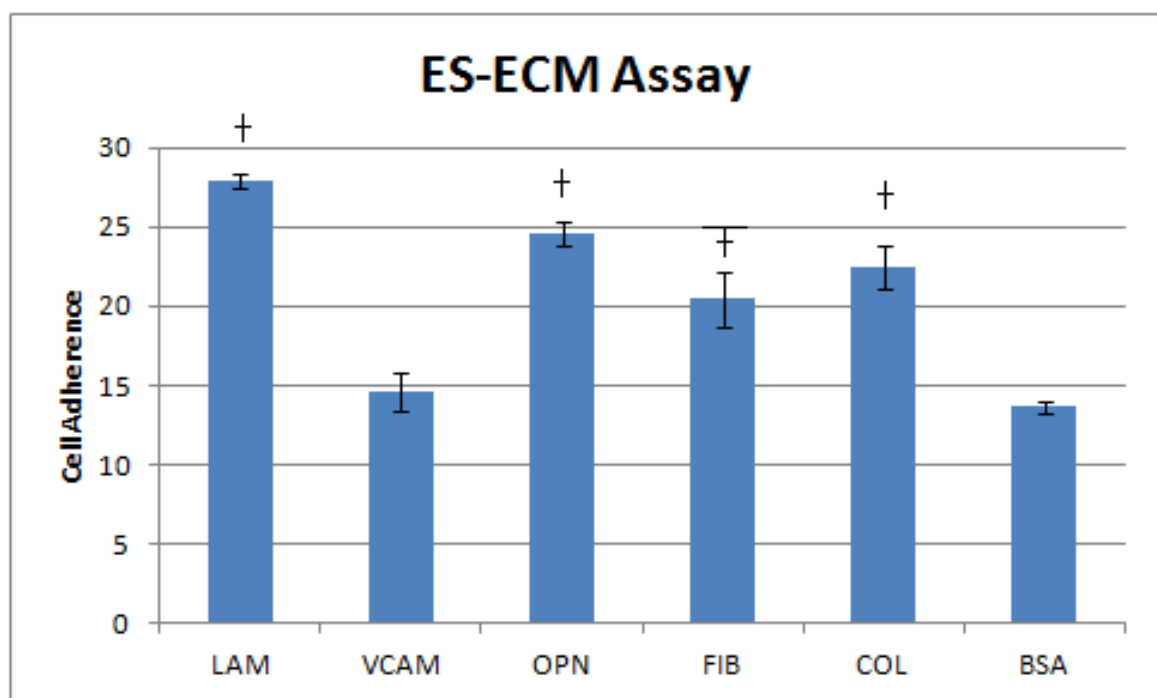


Figure 7: Static adhesion assay showing ES-HLCs bound to ECM proteins. + denotes statistically significant relative to untreated control ( $P < 0.05$ ).

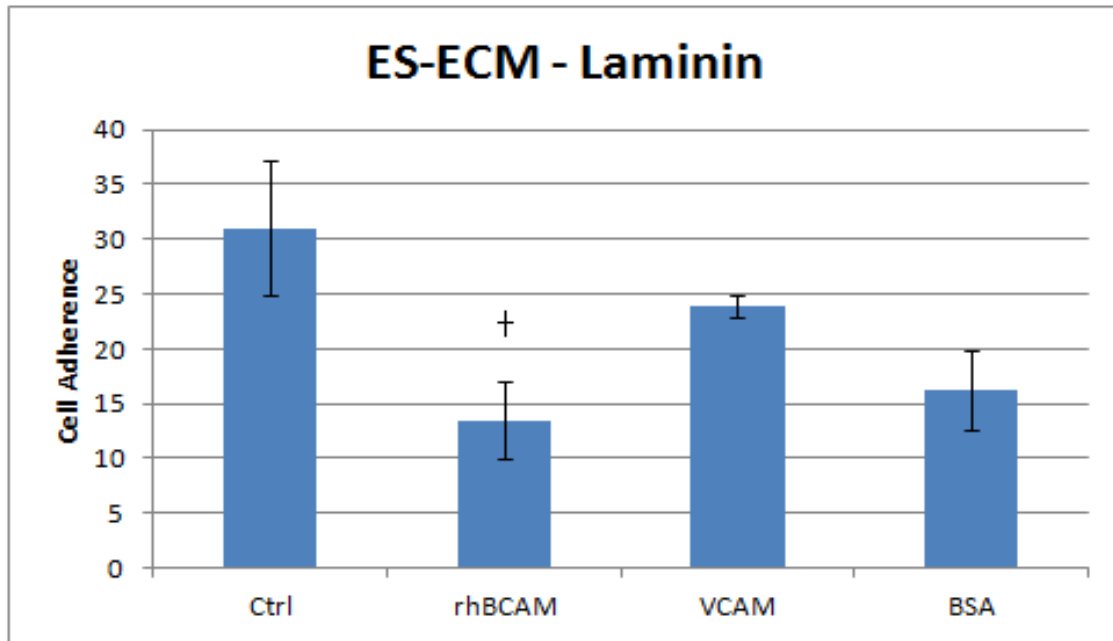


Figure 8: Static adhesion assay showing ES-HLCs bound to laminin; pre-treated with rhBCAM and VCAM. + denotes statistically significant relative to untreated control ( $P < 0.05$ ).

ES cell static adherence to laminin, osteopontin, collagen and fibronectin was statistically significant ( $P < 0.05$ ) compared to the non-specific binding seen with BSA; however there was no significant change in cell adherence to VCAM (Fig.7). ES cells bound preferably to both laminin and osteopontin; figures show that twice the amount of cells bound to laminin in comparison to values seen with non-specific binding (Fig.7).

ES cell adherence to laminin was further analysed; laminin was pre-treated with recombinant Human BCAM/Fs (rhBCAM), and also with VCAM. Cell binding to laminin was greatly reduced when the laminin had been treated with rhBCAM, in comparison to the binding seen with the control (Fig.8); the cell adherence was statistically significant ( $P < 0.05$ ). There was no significant change in cell adherence when the laminin was treated with VCAM.

This suggests the rhBCAM is competitively binding to the BCAM binding site on laminin; this further confirms the molecular interactions between laminin and BCAM.

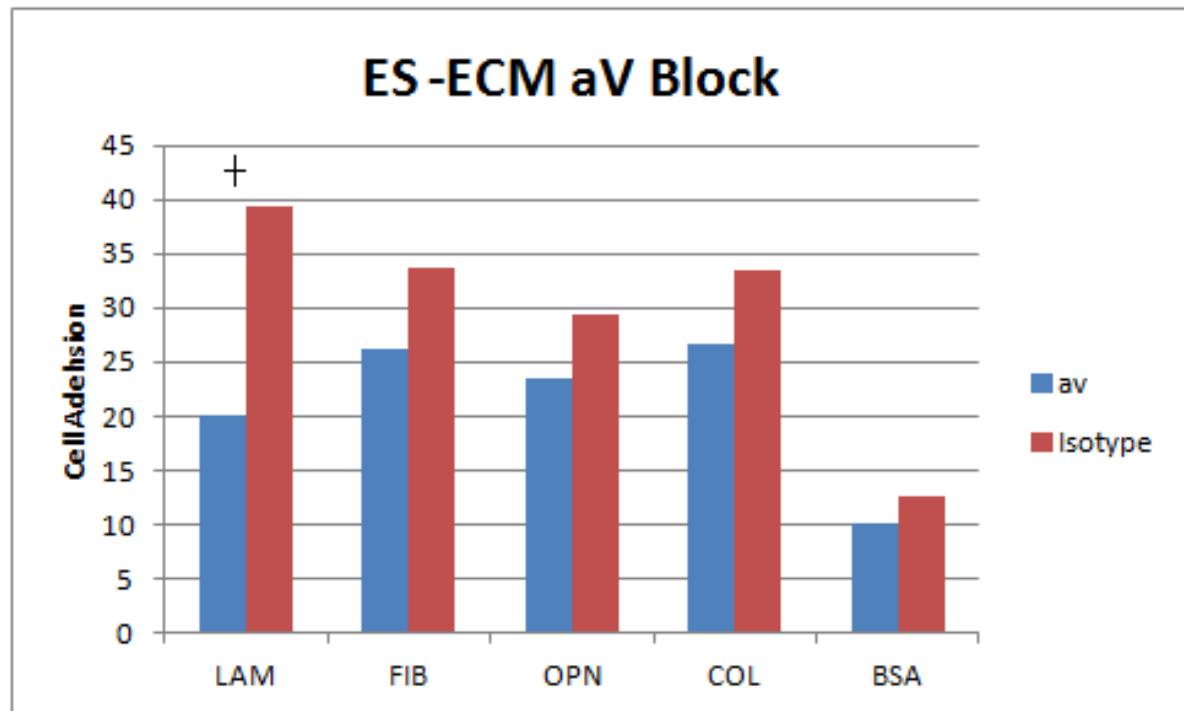


Figure 9: Static adhesion assay showing ES cells bound to ECM components. Cells have been treated with  $\alpha V$  blocking antibody. + denotes statistically significant relative to untreated control ( $P < 0.05$ ).

ES cell static adherence with the ECM components was further analysed when the cells were treated with a blocking antibody. ES cells were treated with  $\alpha V$  blocking antibody and IgG1 isotype. There was an overall reduction in adherence with ES cells that had been treated with  $\alpha V$  antibody; and a statistically significant ( $P < 0.05$ ) reduction in laminin binding (Fig.9).

### 3.3. The interaction between HUH 7.5 cells and the extracellular matrix

The interactions and adherence of HUH 7.5 cells were tested with the ECM components laminin, fibronectin, osteopontin, collagen and vascular cell adhesion molecule (VCAM). HUH 7.5 cell binding to the ECM components was compared to the control – BSA; cell binding to BSA represented non-specific binding.

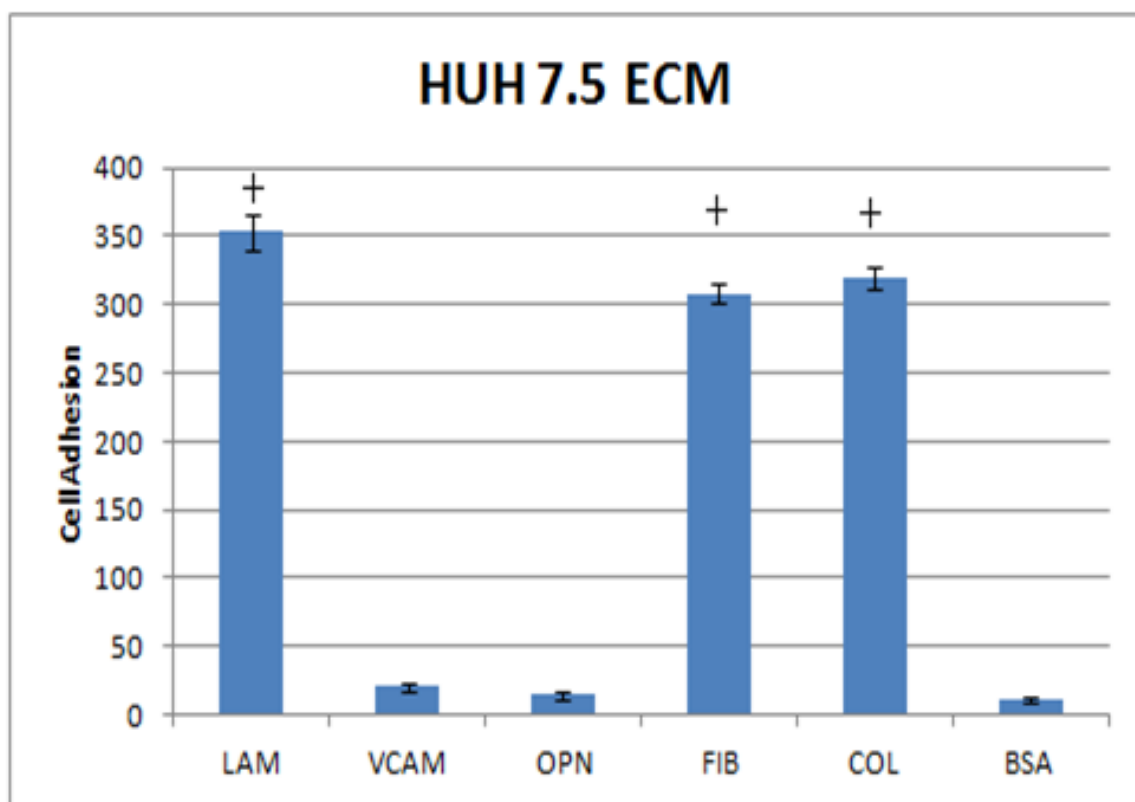


Figure 10: Static adhesion assay showing HUH 7.5 cells bound to ECM components. + - denotes statistically significant relative to untreated control ( $P < 0.05$ ).



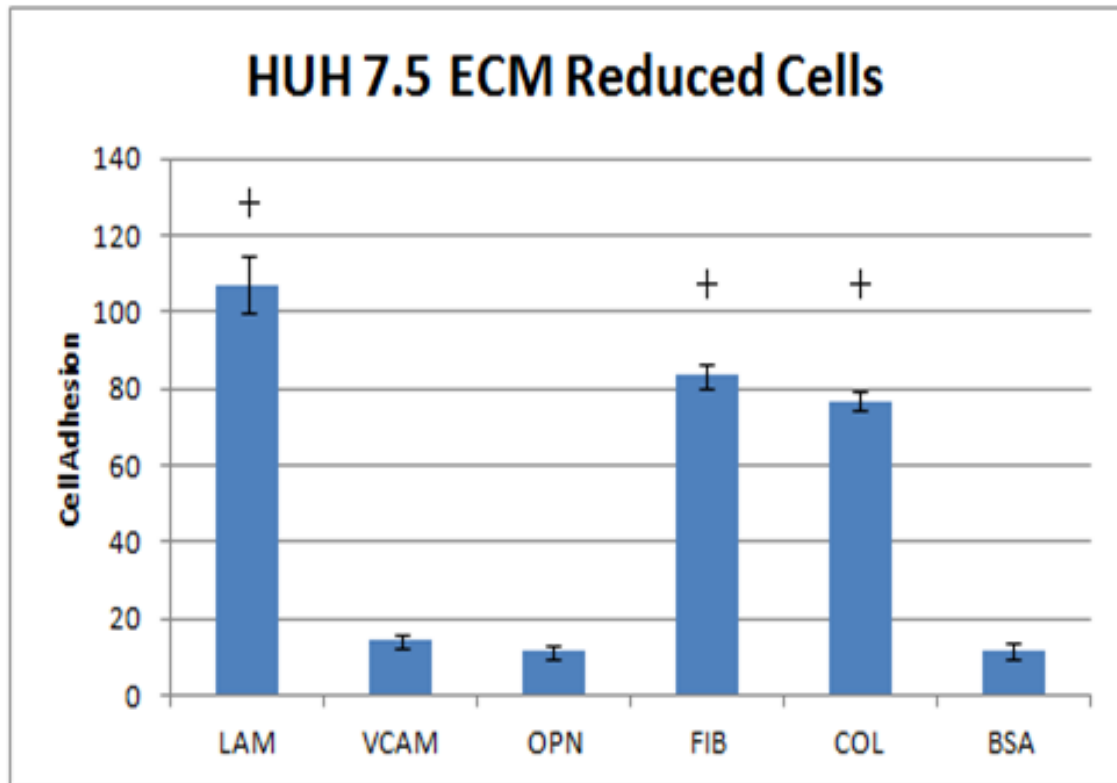


Figure 11: Static adhesion assay showing HUH 7.5 cells bound to ECM components. Reduced amount of cells used (1/10). + denotes statistically significant relative to untreated control ( $P < 0.05$ ).

HUH 7.5 cells bound preferably to laminin, fibronectin and collagen in comparison to the control BSA (non-specific binding) (Fig.10) (Fig.11). There was also very little cell binding to VCAM and osteopontin. HUH 7.5 cell adherence to laminin, collagen and fibronectin was statistically significant ( $P < 0.05$ ) compared to the non-specific binding seen with BSA; however there was no significant change in VCAM or OPN (Fig.10) (Fig.11).

The HUH 7.5 cell binding to laminin, fibronectin and collagen was significantly high. The assay was repeated with a reduced amount of cells (1/10) (Fig.11). HUH 7.5 cell binding to laminin, fibronectin and collagen was again shown to be statistically significant ( $P < 0.05$ ) in comparison to BSA.

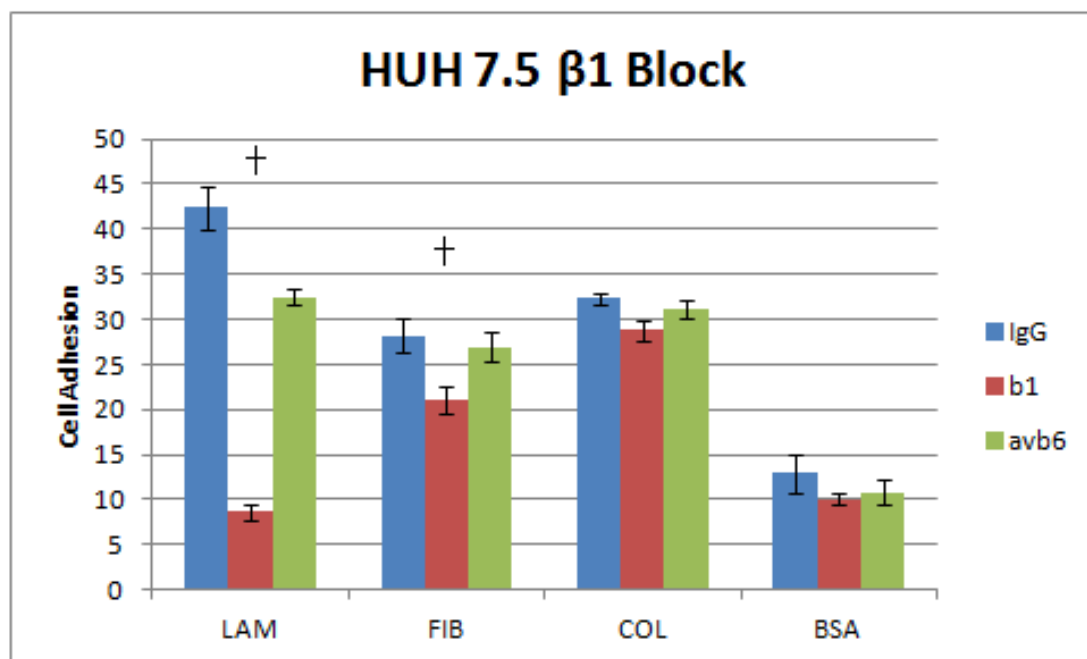


Figure 12: Static adhesion assay showing HUH 7.5 cells bound to ECM components. Cells treated with IgG 1,  $\beta 1$  and  $\alpha V\beta 6$  blocking antibodies. + - denotes statistically significant relative to untreated control ( $P < 0.05$ ).

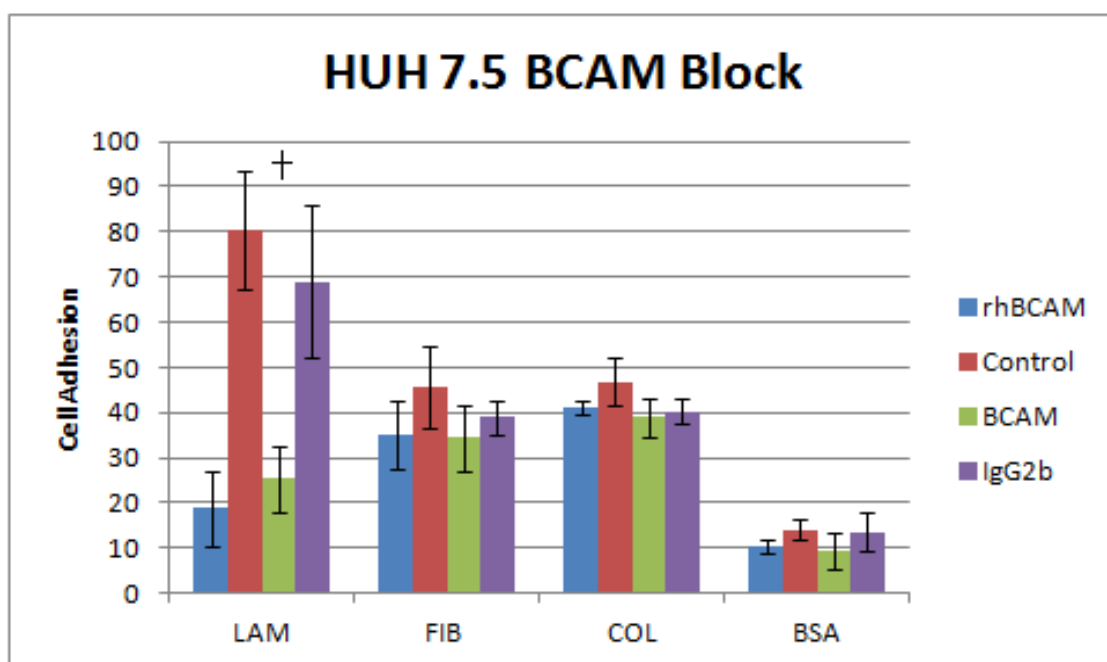


Figure 13: Static adhesion assay showing HUH 7.5 cells bound to ECM components. Cells were treated with BCAM and IgG2b blocking antibodies. ECM components were also treated with rhBCAM. + - denotes statistically significant relative to untreated control ( $P < 0.05$ ).

HUH 7.5 cell adherence to the ECM components: laminin, fibronectin and collagen was further analysed; the cells were treated with a blocking antibody. HUH 7.5 cells were treated with IgG1-isotype,  $\beta 1$  and  $\alpha V\beta 6$  blocking antibodies. Cells that were treated with  $\beta 1$  blocking antibody showed a decrease in adherence to fibronectin, and a substantial decrease in adherence to laminin compared to that of the control isotype (Fig.12); the reduction was statistically significant ( $P<0.05$ ). HUH 7.5 cells treated with  $\alpha V\beta 6$  antibody showed a small reduction in adherence to laminin, but not significant change.

In a separate static assay HUH 7.5 cells were treated with BCAM and IgG2b blocking antibodies; ECM components were also treated with rhBCAM. Cells that were treated with BCAM blocking antibody show a massive reduction in cell adherence to laminin; the large reduction in cell binding was also seen when the laminin had been pre-treated with rhBCAM (Fig.13). Both decreases in HUH 7.5 cell binding was statistically significant ( $P<0.05$ ) in comparison to the cells bound to the control. Adherence of cells to fibronectin and collagen were not significantly affected by BCAM blocking antibody; also cell binding was not affected by the treatment of these ECM components with rhBCAM (Fig.13).

### **3.4. The interaction between HUH 7.5 cells and a hepatic sinusoidal endothelial cell monolayer**

The interactions and adherence of embryonic HUH 7.5 cells were tested with HSEC static adhesion assay. A HSEC monolayer was grown, to which a range of treated HUH 7.5 cells were then added. The interactions between the HSEC monolayer and the HUH 7.5 cells gave a quantitative measure of adherence; between hepatocyte-like cells and liver endothelium.

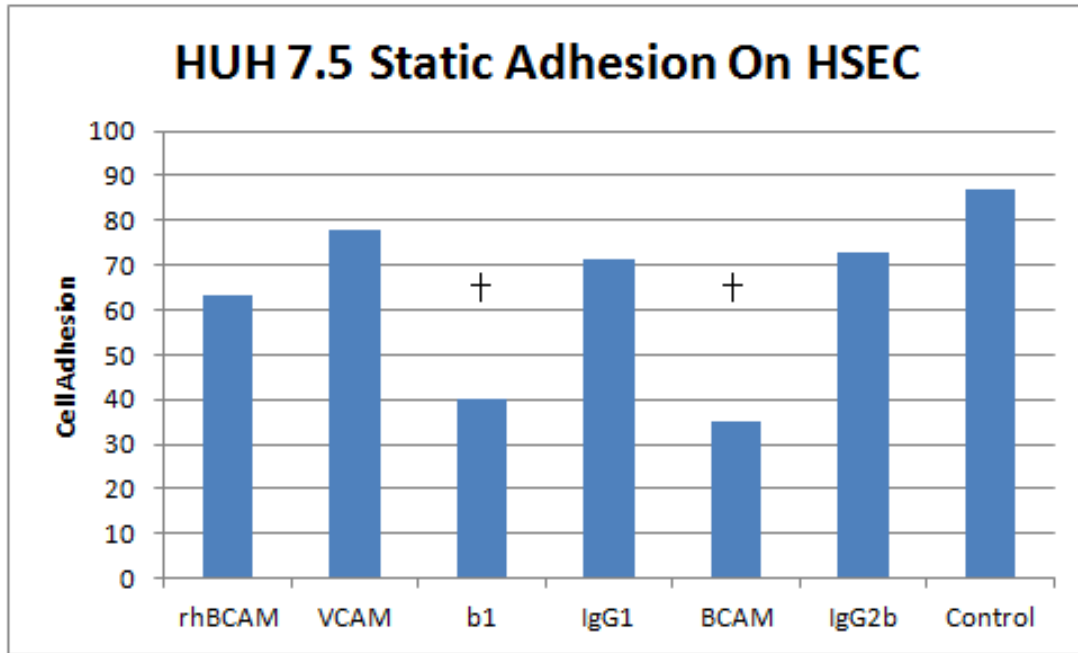


Figure 14: Static adhesion assay showing HUH 7.5 cells bound to HSEC monolayer. Cells were treated with  $\beta$ 1, IgG1, IgG2b and BCAM blocking antibodies. HSEC was also treated with rhBCAM and VCAM. + - denotes statistically significant relative to untreated control ( $P < 0.05$ ).

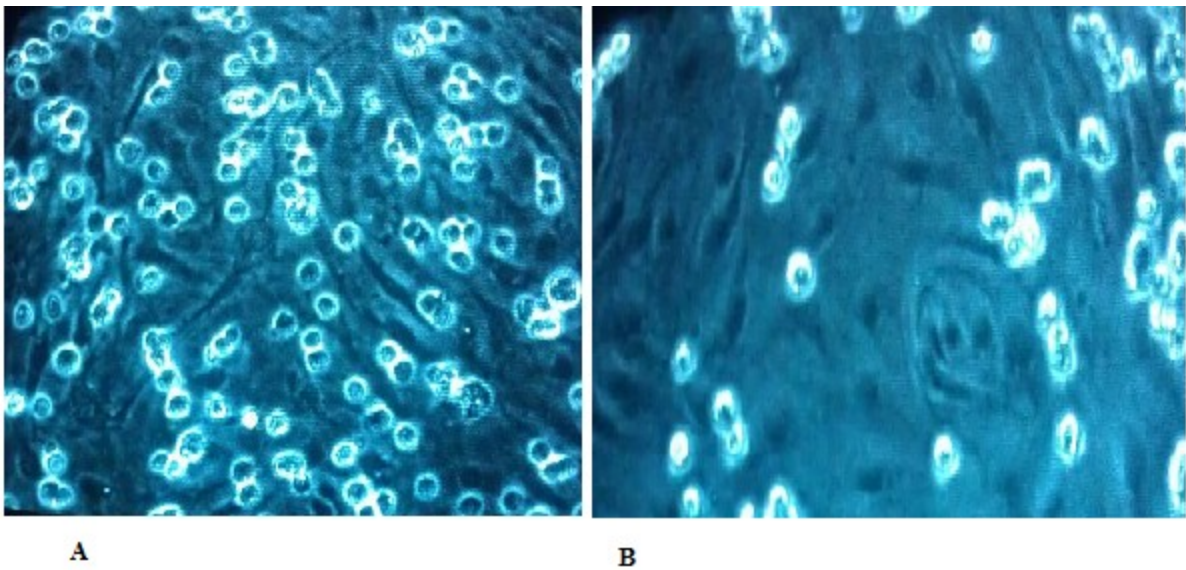


Figure 15: Fluorescent microscopy showing HUH 7.5 cells bound to a HSEC monolayer. A – shows Control cells. B – shows HUH 7.5 treated with BCAM blocking antibody.

HUH 7.5 cell adherence to the HSEC monolayer varied depending on what blocking antibody the cells were treated with. HUH 7.5 cells that were treated with  $\beta 1$  blocking antibody showed a reduction in cell binding to the HSEC. This trend was also seen when the cells were treated with BCAM blocking antibody (Fig.12) (Fig.15). In both cases the reduction in cell adherence was statistically significant ( $P < 0.05$ ) in comparison to the cells bound to the control. Both antibody control isotypes – IgG1 and IgG2b showed little change in cell adherence. HSEC that had been treated with rhBCAM and VCAM also showed little reduction in cell adherence; rhBCAM although a reduction in cell binding showed no statistical significance ( $P > 0.05$ ).

#### **4. Discussion**

Fluorescence-activated cell sorting (FACS) analysis was performed on both HSEC and HUH 7.5 cells. FACS analysis was performed to show the level of integrins being expressed in both hepatic sinusoidal endothelial cells and HUH 7.5 cells, which were used as a surrogate hepatocyte. Integrins with high expression may have a potential role with hepatocyte-like cell adhesion both initially to HSEC and to the underlying ECM components.

To gain an accurate insight into the specific integrins that were being expressed, a range of primary HSEC liver cells were tested. The HSEC that was tested included normal and diseased samples. An array of diseased liver type were tested to show the significant difference, if any in the integrins being expression between the disease types and also if the integrin expression varied in comparison to the values seen in a normal liver.

The types of diseased samples that were tested, comprised of: Primary Biliary cirrhosis (PBC), Haemochromatosis (HFE), Alcoholic Liver disease (ALD), Porphyria and Sero-negative.

The results obtained from FACS analysis clearly show that in all samples of normal HSEC (n=6) there is high expression of both  $\beta 1$  and  $\alpha 5$  integrins (Fig.2) (Fig.5). Integrin -  $\beta 1$  had an average of 99.76% positive cell expression in NRL HSEC and integrin-  $\alpha 5$  had an average positive cell expression at 97.4%. The high expression of Integrin -  $\beta 1$  was also seen in all variants of the diseased HSEC, with average positive cell expression all greater than 99% (positive cell expression: PBC: 99.95%, ALD: 99.96%, Porphyria: 99.88%, Sero-negative: 99.68%, HFE: 99.2%). Integrin -  $\beta 1$  is a well establish adhesion molecule, studies have shown its role with the regulation of cell adherence (Carloni *et al.*, 1998); whilst other studies have demonstrated its importance for hepatocyte survival (Pinkse *et al.*, 2004). The FACS analysis performed has further confirmed these suggestions; the high expression of integrin -  $\beta 1$  across all the types of HSEC illustrates its importance. Integrin -  $\beta 1$  could well be a potential target for manipulation to increase cell adherence.

All other integrin expression was very variable, with the exception of integrin-  $\alpha 2$ , across the different types of diseased HSEC samples. Integrin-  $\alpha 2$  was highly expressed in all disease types (excluding HFE) (positive cell expression: PBC: 95.48%, ALD: 98.89%, Porphyria: 99.75%, Sero-negative: 97.04%); although this specific integrin was only expressed in low levels of NRL HSEC (34.25%). The results suggest that integrin-  $\alpha 2$  is therefore being expressed by HSEC cells in relation to disease. Studies have shown that integrin-  $\alpha 2$  mediates binding to collagen type IV, which is seen in high quantities in liver sinusoids; it has also been shown to be involved with cancer metastasis and cell migration within the liver (Yoshimura *et al.*, 2009). Further research would need to be carried out to investigate the differences in integrin-  $\alpha 2$  expression (NRL compared to diseased), and its potential as a target to increase hepatocyte cell adherence in diseased livers. Furthermore the unique differences in integrin expression with the varied types of diseased HSEC could help identify certain characteristics of each disease; and possibly help with future research to find a more effective therapeutic treatment.

The results obtained from FACS analysis on HUH 7.5 cells also showed integrin  $\beta 1$  to be highly expressed (Fig.6). The average (n=3) positive cell expression was 96.25%; which closely resembles the FACS data seen with HSEC analysis. As stated above HUH 7.5 cells were used as a surrogate hepatocyte as they have similar characteristics to those seen with

foetal hepatocytes. HUH 7.5 cells express high levels of BCAM; this was confirmed with the FACS data. BCAM was positively expressed at an average of 86.07%. Integrin  $\alpha 1$  was also highly expressed (positive cell expression 81.62%) this could be a potential adhesion molecule of interest.

The high expression of integrin- $\beta 1$  in both HSEC and HUH 7.5 FACS data suggests the important role this molecule has with hepatocyte adhesion. High levels of BCAM expression in HUH 7.5 cells also suggest the relative importance in cell adhesion.

Both integrin- $\beta 1$  and BCAM were further studied through static adhesion assays with a range of extra cellular matrix (ECM) components and HSEC. Static adhesion assays were performed using both ES (ES-HLCs) and HUH 7.5 cells.

The interactions and adherence of embryonic stem cell hepatocyte-like cells were tested with the ECM components laminin, fibronectin, osteopontin, collagen and vascular cell adhesion molecule (VCAM). Static adhesion data showed that ES-HLCs had a high binding affinity to laminin, osteopontin, collagen and fibronectin (Fig.7). In all cases binding was statistically significantly higher ( $P < 0.05$ ). ES-HLCs adherence to laminin in particular was almost double that in comparison to non-specific binding seen with BSA (control). ES-HLCs however had a low level of attachment to VCAM molecule. The preferential binding of ES-HLCs to laminin is significant; laminin is an important ECM protein which regulates attachment, migration and organisation of cells. The interactions between ES-HLCs with osteopontin and fibronectin should be further researched to gain a better insight into the mechanisms of hepatocyte-ECM adherence.

ES-HLCs adherence with laminin was further investigated. Laminin was pre-treated with a recombinant Human BCAM/Fs (chimeric molecule) (rhBCAM), and also with VCAM.

Static adhesion data showed that the pre-treatment of laminin with recombinant Human BCAM/Fs (rhBCAM) significantly reduced ES-HLCs adherence in comparison to binding seen with the control (Fig.8). Cell adherence was statistically significant ( $P < 0.05$ ) with a total loss in adherence of  $56\% \pm 2.5$ . The data (Fig.8) also showed that there was little change with cell adherence when laminin was treated with VCAM ( $22.8\% \pm 1.4$ ).

The static adhesion data suggests that recombinant Human BCAM/Fs (chimeric molecule), is competitively bound to the BCAM binding site on laminin, resulting in a saturation of the binding site. This in turn is blocking the BCAM (being expressed by the ES-HLCs) interaction with the laminin; therefore a loss in cell adherence. This also further confirms the molecular interactions between laminin and BCAM.

Further analysis of ES-HLCs static adherence to the ECM components: laminin, osteopontin, collagen and fibronectin was completed. As determined from previous results ES-HLCs preferentially bound to these ECM proteins. The effects of a blocking antibody on cell adhesion were tested. ES-HLCs were treated with IgG1 isotype along and  $\alpha V$  blocking antibody. The data shows that ES-HLCs that were treated with  $\alpha V$  antibody showed an overall reduction in adherence to each of the ECM components, (laminin  $49\% \pm 1.8$ , fibronectin  $22\% \pm 2.6$ , osteopontin  $20\% \pm 0.8$  and collagen  $21\% \pm 2.8$ ) however only the reduction in laminin was statistically significant ( $P < 0.05$ ) (Fig.9).

The data suggests that the  $\alpha V$  antibody is blocking integrin expression on the ES-HLCs.  $\alpha V$  integrins are well known receptors that recognise a wide array of ligands;  $\alpha V$  integrins are receptors for laminin, osteopontin, and fibronectin. The significant reduction in cell adherence to laminin binding is very interesting, further research in this area is needed to confirm these findings.

The interactions and adherence of HUH 7.5 cells were tested with the ECM components laminin, fibronectin, osteopontin, collagen and vascular cell adhesion molecule (VCAM). The data shows that HUH 7.5 cells bound preferably to laminin, fibronectin and collagen; there was a considerable difference in cell adherence with all three of these ECM proteins in comparison to the control BSA (non-specific binding) (Fig.9) (Fig.11). HUH 7.5 cell binding to laminin, fibronectin and collagen was statistically significant ( $P < 0.05$ ).

HUH 7.5 cell binding to laminin, fibronectin and collagen was extremely high; because of this as an accurate representation of the amount of cells bound was immeasurable (human error).



The assay was repeated with a reduced amount of cells (1/10) to gain a more precise measurement of cell adhesion. (Fig.11) HUH 7.5 cell binding to laminin, fibronectin and collagen was again shown to be statistically significant ( $P<0.05$ ); data shows that almost 10 times the amount of cells adhered to laminin in comparison to BSA (non-specific binding). The data obtained compliments that seen with ES-HLC interaction with ECM components; in both cases significant binding to laminin, fibronectin and collagen with very little binding to VCAM. Both sets of data show the importance of these ECM proteins, in the regulation of cell adherence.

Further analysis of HUH 7.5 static adherence to the ECM components: laminin, collagen and fibronectin was completed. As determined from previous results HUH 7.5 preferentially bound to these ECM proteins. The effects of a blocking antibody on cell adhesion were tested. HUH 7.5 cells were treated with IgG1-isotype,  $\beta 1$  and  $\alpha V\beta 6$  blocking antibodies. The data showed that HUH 7.5 cells treated with  $\beta 1$  blocking antibody showed a substantially significant decrease in adherence to laminin; a 79.6% ( $\pm 3.1$ ,  $P<0.05$ ) reduction of cells bound to laminin when compared to the cells treated with IgG1. Data shows that  $\beta 1$  blocking antibody also affected cellular adhesion with fibronectin (26.2%  $\pm 2.4$ ).

The effect of  $\beta 1$  blocking antibody on cell adherence shows the importance of the integrin- $\beta 1$  in the regulation of hepatocyte-like cell adherence. When the integrin- $\beta 1$  is blocked there is a loss of hepatocyte-like cell adhesion in the extracellular matrix; the loss of cell adherence when integrin- $\beta 1$  is blocked is also seen in endothelial cells. This is shown in the data obtained from HUH 7.5 cell static adhesion assay with HSEC (Fig.14). The data showed that HUH 7.5 cells that had been treated with  $\beta 1$  blocking antibody had a significantly lower binding affinity to the HSEC monolayer in comparison to the control ( $P<0.05$ ). The results obtained are conclusive and compliment studies previously completed (Pinkse *et al.*, 2004; Potocnik *et al.*, 2000).

In a separate static assay HUH 7.5 cells were treated with BCAM and IgG2b blocking antibodies; the ECM components: laminin, collagen and fibronectin were also treated with recombinant Human BCAM/Fs (rhBCAM). The data showed that cells treated with BCAM

blocking antibody had a significant reduction in cell adherence to laminin ( $68.7\% \pm 2.6$ ,  $P < 0.05$ ) (Fig.13). A significant reduction was also seen in cell adherence when laminin had been pre-treated with rhBCAM ( $77.4\% \pm 1.0$ ,  $P < 0.05$ ) (Fig.13). HUH 7.5 cell adherence to the other ECM proteins was not significantly affected by either the BCAM blocking antibody or by recombinant Human BCAM/Fs (rhBCAM).

The results further conclude the interactions and functions of BCAM as a receptor for laminin (Udani et al., 1998). The effect of BCAM blocking antibody on cell adherence shows the importance of the BCAM in the regulation of hepatocyte-like cell adherence. A significant loss in hepatocyte-like cell adhesion to laminin occurs when BCAM is blocked; furthermore a loss of cell adherence is also seen in endothelial cells when hepatocyte-like cells are treated with BCAM blocking antibody. Data obtained from HUH 7.5 static adhesion assay with HSEC (Fig.14) (Fig.15), showed a significantly lower binding affinity of HUH 7.5 cells treated with BCAM antibody to the HSEC monolayer ( $P < 0.05$ ). Recombinant Human BCAM/Fs however did not seem to have any significant effect on HUH 7.5 – HSEC adherence.

The data suggests the involvement of BCAM with cell-cell adhesion and migration as previously suggested (Schon et al., 2001).

#### **4.1. Conclusion**

Data obtained from the research completed suggests that integrin- $\beta 1$  and BCAM both play critical roles in the regulation of hepatocyte-like cell adhesion. The data suggest that both adhesion molecules play a crucial role in the interactions of hepatocyte-like cells between both the extracellular matrix and the hepatic sinusoidal endothelium cells. In conclusion both integrin- $\beta 1$  and basal cell adhesion molecule are potential targets in which to manipulate to improve the engraftment efficiency of transplanted ES-HLCs and hepatocytes. Future research studying the interactions of ES-HLCs and hepatocytes with ECM components and HSEC may be useful in identifying molecular interactions to manipulate to improve engraftment in vivo.

## 5.0. References

1. Andrews, N. (1999) Disorders of Iron Metabolism. **New England Journal of Medicine** 341 (26).  
Neurauter, A., Bonyhadi, M., Lien, E. et al. (2007) T. Cell Isolation and Expansion Using Dynabeads. **Advances in Biochemical Engineering/Biotechnology**. Volume 106
2. Basma, H., Soto-Gutiérrez, A., Rao, G. et al. (2009) Differentiation and Transplantation of Human Embryonic Stem Cell-Derived Hepatocytes. **Gastroenterology**. Volume 136, Issue 3, Pages 990–999.
3. Bhogal, RH., Hodson, J., Bartlett D., et al. (2011) Isolation of primary human hepatocytes from normal and diseased liver tissue: A one hundred liver experience.
4. BMA (2009) The nation's drinking as a major health issue, The human cost of alcohol misuse [online], Available from [http://www.bma.org.uk/images/humancostalcohol\\_tcm41-184494.pdf](http://www.bma.org.uk/images/humancostalcohol_tcm41-184494.pdf) [Accessed 27 Feb, 2012]
5. British Liver Trust (2009) Analysis of official mortality statistics covering all deaths related to liver dysfunction covering ICD K70-76 and other codes including C22-24 (liver cancer), and B15-B19 (viral hepatitis), Available from <http://www.britishlivertrust.org.uk/home/the-liver.aspx> [Accessed 2 March, 2012]
6. British Liver Trust Hepatitis B Foundation UK (2007). Rising Curve: Hepatitis B Infection in the UK.[online], Available from <http://www.britishlivertrust.org.uk/home/the-liver/liver-diseases.aspx> [Accessed 27 Feb, 2012]
7. Carloni, V., Romanelli, RG., Mercurio, AM., et al. (1998) Knockout of alpha6 beta1-integrin expression reverses the transformed phenotype of hepatocarcinoma cells. **Gastroenterology**. 82 (6): 380-388
8. Chang, M. (2007) Hepatitis B virus infection. *Seminars in fetal & neonatal medicine* 12 (3): 160– 167. doi:10.1016/j.siny.2007.01.013. PMID 17336170 [accessed March 3 2012]
9. Cleveland Clinic (2009) [online], Available from [http://my.clevelandclinic.org/services/liver\\_transplantation/hic\\_liver\\_transplantation.aspx](http://my.clevelandclinic.org/services/liver_transplantation/hic_liver_transplantation.aspx), [Accessed March 3 2012]
10. Dancygier, H. (2012) *Clinical Hepatology Principles and Practice*. **Springer**. pp. 895–. ISBN 9783642045097.
11. Darby, H., Gupta, S., Johnstone, R., et.al. (1986) Observations on rat spleen reticulum during the development of syngeneic hepatocellular implants. **J Exp Pathology**; 67:329-339.
12. Department of Health (2008) Safe, Sensible, Social - Consultation on further action Impact Assessments[Accessed Feb 27, 2012]
13. Dhawan, A., Mitry, RR., Hughes, RD., et al (2004). Hepatocyte transplantation for inherited factor VII deficiency. **Transplantation**; 78: 1812.
14. Fabrega, AJ., Bommineni, VR., Blanchard, J., et al (1995) Amelioration of analbuminemia by transplantation of allogeneic hepatocytes in tolerized rats. **Transplantation**; 59:1362.
15. Fitzpatrick, E., Mitry, RR., Dhawan, A (2009) Human hepatocyte transplantation: State of the art. **Intern Med**; 266: 339.

16. Fox, J., Roy-Chowdhury, N (1997) Hepatocyte transplantation in liver failure and inherited metabolic disorders. **J Hepatol**; 40: 878.
17. Fox, J., Roy-Chowdhury, N (2004) Hepatocyte transplantation in Acute Liver Failure **J Hepatol**; 20: 624.
18. Gupta, S., Rajvanshi, P., Sokhi, R., (1999) Entry and Integration of Transplanted Hepatocytes in Rat Liver Plates Occur by Disruption of Hepatic Sinusoidal Endothelium, , **Heptatology**, Vol.29.
19. Hughes, R., Mitry R., Dhawan A (2005) Hepatocyte transplantation for metabolic liver disease: UK experience. **J Soc Med**; 50: 88
20. Janet, A., Askari, P., Buckley, A., et al (2009) Linking integrin conformation to function, **Journal of Cell Science**; 30: 169-171
21. LAPE (n.d) [online]. Available from <http://www.lape.org.uk/data.html> [accessed 27 Feb, 2012]
22. Lehec, S., Hughes, R., Mitry, R. et al (2009) Experience of microbiological screening of human hepatocytes for clinical transplantation. **Cell Transplant**; 24: 177-183
23. Lindner, H., Holler, E., Ertl, B. et al. (1997) Peripheral blood mononuclear cells induced programmed cell death in human endothelial cells and may prevent repair: role of cytokines. **Blood**;89:1931-1938.
24. MacPhee, P., Schmidt, E., Keown, P., (1998) Microcirculatory changes in livers of mice infected with murine hepatitis virus. Evidence from microcorrosion casts and measurements of red cell velocity. **Microvasc Res**; 66:120-128
25. Mitry, R., Hughes R., et al (2003) Human hepatocyte isolation and relationship of cell viability to early graft function. **Cell Transplant**
26. Najimi, M., Khuu, D., Lysy, P., et al (2007) Adult-derived human liver mesenchymal-like cells as a potential progenitor reservoir of hepatocytes? **Cell Transplant**; 16: 717.
27. Nakagawa, K., Miller, F., Simms, D., et al (1996) Mechanisms of interleukin-2–induced hepatic toxicity. **Cancer Res** ;56:507-510
28. NCBI (n.d) [online] PECAM1 platelet/endothelial cell adhesion molecule 1. Available from <http://www.ncbi.nlm.nih.gov/sites/entrez?Db=gene&Cmd=ShowDetailView&TermToSearch=5175> [accessed Feb 27, 2012]
29. NHS (2012) [online] Organ transplant. Available from <http://www.uktransplant.org.uk/ukt/default.jsp>, [accessed March 3 2012]
30. Office for National Statistics (2008) (online) Health Service Quarterly, No. 40 p59-60, [ accessed Feb 27, 2012]
31. Pertoft, T., Laurent, T., and L.Kagedal (1998) The Efficiency of Percoll and Ficoll Density Gradient Media in the Isolation of Marrow Derived Human Mesenchymal Stem Cells with Osteogenic Potential. **Biochem**. Available from [http://dx.doi.org/10.1016/0003-2697\(78\)90419-0](http://dx.doi.org/10.1016/0003-2697(78)90419-0), 197
32. Pinkse, G., Voorhoeve, M., Noteborn, M. et al (2004) Hepatocyte survival depends on beta1-integrin-mediated attachment of hepatocytes to hepatic extracellular matrix. **Liver Int**; 22: 120-127
33. Potocnik, A. (2000) Fetal and Adult Hematopoietic Stem Cells Require  $\beta$ 1 Integrin Function for Colonizing Fetal Liver, Spleen, and Bone Marrow. **Immunity**. Volume 12, Issue 6, 653-663,

34. Puppi, J., Tan, N., Mitry, R. et al (2008) Hepatocyte transplantation followed by auxiliary liver transplantation —A novel treatment for ornithine transcarbamylase deficiency. **J Transpl** 2008; 8: 452.
35. Sancho-Bru I, M., Najimi, M., Caruso et al (2009) Recent advances in clinical practice Stem and progenitor cells for liver repopulation: can we standardise the process from bench to bedside?, **Gut**; 20: 66-70
36. Schon, M. (2001) Basal-cell adhesion molecule (B-CAM) is induced in epithelial skin tumors and inflammatory epidermis, and is expressed at cell-cell and cell-substrate contact sites. **Journal of Investigative Dermatology** 117(4): 995-997
37. Strom, S., Chowdhury, J., Fox, I. (1999) Hepatocyte transplantation for the treatment of human disease. **Liver Dis**; 10: 20-23
38. Stutchfield, B., Forbes, S., Wigmore, S. (2010) Prospects for stem cell transplantation in the treatment of hepatic disease, **Liver Transpl**;16(7):827-36.
39. Terry, C., Dhawan, A., Mitry, R. et al. (2006) Cryopreservation of isolated human hepatocytes for transplantation: State of the art. **Cryobiology**; 08: 111-117
40. Terry, C., Hughes, R., Mitry, R. et al (2007) Cryopreservation-induced nonattachment of human hepatocytes: Role of adhesion molecules. **Cell Transplant**; 20: 110-115
41. Tissupath (2011) [online]. Available from <http://tissupath.com.au/education-medical-student-liver> [accessed 3 March 2012]
42. Udani, M. (1998) Basal cell adhesion molecule/lutheran protein. The receptor critical for sickle cell adhesion to laminin. **Journal of Clinical Investigation** 101(11): 2550-2558
43. World Health Organization (n.d) [online]. Available from <http://www.who.int/mediacentre/factsheets/fs204/en/index.html> [accessed Feb 29, 2012]
44. Yamamoto, H., Quinn, G., Asari, A. et al (2003) Differentiation of embryonic stem cells into hepatocytes: Biological functions and therapeutic application, **Hepatology** Volume 37, Issue 5, pages 983–993.
45. Yazigi, N., Carrick, T., Bucuvalas, J. (1995) Increased liver engraftment of transplanted hepatocytes following selective hepatocyte injury. **Hepatology**;22:211A.
46. Yoshimura, A., Kristen, F., Lindsay, S. et al (2009) Schulick Integrin A2 Mediates Selective Metastasis to the Liver, **Cancer Res**;69:7320-7328

## **Project 2**

### **Contents**

**1.0. Introduction: Page 49**

**1.1. PDGF receptor: Page 49**

**1.2. 1.2 Autophosphorylation and Dimerization of PDGF receptor: Page 50**

**1.3. PI3-kinase pathway + PLC- $\gamma$  pathway: Page 51**

**1.4. The mitogen activated protein kinase (MAP kinase) pathway: Page 52**

**1.5. Src kinase pathway: Page 53**

**1.6. Protein tyrosine phosphatases: Page 54**

**1.7. LAR-RPTP and its role in PDGF receptor signalling: Page 55**

**1.8.1. c-ABL and G protein-coupled receptor kinases (GRKs): Page 56**

**1.8.2. Protein Kinase C, NHERF-1 and NHERF-2: Page 57**

**1.9. Aim of the research project: Page 57**

**2.0. Materials and Methods: Page 59**

**2.1. Cell Culture and Preparation: Page 59**

**2.2. Western Blot and Immunoprecipitation: Page 60**

**2.3. Phorbol 12-myristate 13-acetate (PMA): Page 63**

**2.4. siRNA Knockdown optimisation: Page 64**

**2.5. Knockdown of PKC  $\delta$ , NHERF-1 and NHERF-2: Page 66**

**2.6. Down signalling pathway: ERK: Page 67**

**3.0. Results: Page 67**

**3.1. Testing Immunoprecipitate's: Page 67**

**3.2. PMA: Page 69**

**3.3. siRNA Optimisation: Page 71**

**3.4. siRNA knockdown: Page 74**

**3.5. Downsignalling pathway: Page 80**

**4.0. Discussion: Page 83**

**4.1. Conclusion: Page 88**

**5.0. References: Page 91**

## **Acknowledgements**

I would like to thank

W. Zheng, Dr C. Hellberg and the Hellberg Research Group  
for their guidance and support.



## Abstract

The platelet derived growth factor receptor (PDGFR) is a cell surface tyrosine kinase receptor specific for members of the platelet derived growth factor (PDGF). PDGFs have been associated to have a pivotal role in a range of cellular processes; from cell growth, motility, differentiation and survival in connective tissues. PDGF is also associated with diseases such as cancer. The aim of the research project was to investigate the role of leukocyte common antigen-related receptor like PTP (LAR-RPTP), in the regulation of the PDGF receptor. siRNA optimisation for the knockdown of PKC  $\delta$ , NHERF1 and NHERF2 proteins was performed in the absence of LAR (LAR  $\Delta$  P MEF cells), was utilized and analysed through western blot technique. The data obtained showed that in the absence of PKC  $\delta$  and NHERF-2, PDGFR phosphorylation and activity increased; and in the absence of NHERF1 the activity was inhibited. The important regulatory role of these proteins was further determined when the downstream signalling molecule ERK was investigated; an increase in ERK activity ensued with successful knockdowns of PKC  $\delta$  and NHERF2. The results obtained facilitate a novel mechanism that LAR regulates PDGFR signalling through ligand induced activation of the PDGF  $\beta$ -receptor, through its mechanism of down regulating c-Abl activity. Thus reducing the activation of PKC  $\delta$  and in turn GRK2, which desensitises and inhibits PDGF  $\beta$ -receptor activation and downstream signalling pathways; as well as dissociating NHERF1 while promoting NHERF2 binding affinity to the PDGFR, further diminishing receptor dimerization.

## 1.0. Introduction

### 1.1. PDGF receptor

Platelet derived growth factor receptors (PDGFR) are cell surface tyrosine kinase receptors specific for members of the platelet derived growth factor (PDGF) (Williams, *et.al*, 1989). Members of the Platelet derived growth factor (PDGF) have been associated to have a pivotal role in a range of cellular processes; the subunits A and B of the PDGF in particular have been shown to have an important function with cell growth, motility, differentiation and survival in connective tissues (Heldin, et al, 1996). PDGF is also associated with diseases such as tumour formation and growth (Williams, et.al, 1989). There are two structurally different forms of the PDGFR, known as  $\alpha$  and  $\beta$ ; which are separately encoded by different genes. Upon binding of the PDGF to the PDGFR it causes the receptor to undergo change leading to the autophosphorylation between the two receptors,  $\alpha$  and  $\beta$ . There are two types of dimerization that can take place, PDGF-R homo- or heterodimerization, which is dependent upon the growth factor that is bound (Heldin, et al, 1992).

The PDGF molecule is dimeric and consists of a variety of disulfide bonded homodimers, these are known as A, B, C and D, which are polypeptide chains. There is also the heterodimer polypeptide chain know as AB. Examples include PDGF –AA, AB, BB, CC and DD (Heldin, et al, 1992). The PDGF molecule, in all its isoforms binds to and activates the two structurally different PDGF protein tyrosine kinase receptors; PDGF  $\alpha$  and PDGF  $\beta$ ; which in turn leads to an effect with their target cells. As stated the PDGF molecule is dimeric, in this case each of its isoforms have two different receptor binding epitopes and can therefore bind to both the  $\alpha$  and  $\beta$  receptor at the same time which leads to PDGF receptor dimerization. (Chen, et al., 2011).

These isoforms are native in their monomeric forms, but once bound to the PDGFR- $\alpha$  and PDGFR- $\beta$  it leads to receptor dimerization causing conformational change which can result in three possible receptor combinations,  $\alpha\alpha$ ,  $\beta\beta$  and  $\alpha\beta$ .

The PDGF subunits bind specifically to the ligand binding sites on the receptor; for example PDGF-AA only bind to PDGFR- $\alpha\alpha$ , PDGF-AB and CC bind to  $\alpha\alpha$  and  $\alpha\beta$ , whereas PDGF-

DD binds with high affinity to  $\beta\beta$  and partly to  $\alpha\beta$ . PDGF-BB however is unique and is able to bind with a high affinity to all three types of the receptor combinations (Heldin, et al, 1992).

## **1.2. Autophosphorylation and Dimerization of PDGF receptor**

PDGF receptor dimerization causes the juxtaposition of the intracellular domains which in turn leads to the activation of the kinase through the autophosphorylation of the tyrosine residues between the two receptors,  $\alpha$  and  $\beta$ ; the receptor molecules also undergo a conformational change allowing for full enzymatic activity to be obtained (Kelly *et al.*, 1991). The majority of the phosphorylation sites have been shown to be localised outside the kinase domain on both the  $\alpha$  and  $\beta$  receptors. Studies have shown that both receptors are significantly auto phosphorylated when they have undergone dimerization, (Claesson-Welsh *et al.*, 1994) have shown that 11 of the 15 tyrosine residues of the  $\beta$  receptor are autophosphorylation sites (in the intracellular non-catalytic areas).

The phosphorylated tyrosine residue along with C-terminal amino acid residues together form a specific binding site which is designed for the binding of signal transduction molecules; the autophosphorylation of the receptor permits for the recruitment of the site specific molecules enabling for the signal to be propagated downstream (Chen, et al., 2011).

Studies have shown (Pawson *et al.*, 1997) that the large majority of the transduction molecules that bind to the phosphorylated PDGF receptors when there is an increased activity and a conformational change have Src homology 2 (SH2) cytoplasmic domains present; these domains are able to specifically recognise phosphorylated tyrosine residues on the PDGF (any many other) receptors (Heldin *et al.*, 1996). The binding in these domains have been shown to have a high specificity, the signal transduction molecules themselves vary; some are equipped with adaptor molecules which have been shown can contain sub units which can possess catalytic ability while other signal transduction molecules have a range of different enzymatic activities. Once bound to the activated PDGF receptor, the interaction results in the catalytic action becoming up regulated by the receptor through tyrosine phosphorylation which in turn generates a signal that is unique for each type of transduction molecule.

This leads to the initiation of different signalling pathways which can lead to an array of effects such as the modulation of gene transcription and cytoskeletal rearrangement along with other physiological effects (Zheng, *et al.*, 2011).

There are numerous signalling pathways that can be activated which are dependent upon the enzyme activity of the SH2 domain containing the signal transduction molecule; there are several binding sites to the phosphorylated PDGF receptor that have been identified for the transduction molecules. These include the Src family of protein tyrosine kinases, phosphatidylinositol 3' kinase (PI3 kinase), protein tyrosine phosphatase SHP-2, GTPase activating protein (GAP), phospholipase C- $\gamma$  (PLC $\gamma$ ) and also certain adaptor molecules such as GRB2 and 7, Shc, Crk (Songyang *et al.*, 1993).

After binding of the PDGF ligand, the active PDGF receptor will eventually become internalised and degraded, as part of a negative feedback system. The rate at which this internalisation and degradation of the PDGF receptor takes place is dependent on the activity of the kinase and the autophosphorylation of the receptor (Sorkin *et al.*, 1991),(Huang, et al., 2011); the degradation leading to PDGF receptor inactivation usually takes place between 30 to 60 minutes after the receptor has begun to be internalised. The ligand receptor complex is internalised through endosomes which then fuse with lysosomes which lead to the degradation of the receptor complex causing the inactivation of the receptor (Sorkin *et al.*, 1991).

### **1.3. PI3-kinase pathway + PLC- $\gamma$ pathway**

The phospholipid kinase PI-3 kinase has been shown to bind to the phosphorylated sites on the PDGF receptor (Kaslauskas *et al.*, 1989); the binding of the PI-3 kinase to the PDGF receptor causes a conformational change of the kinase which leads to an increased enzymatic activity (Backer *et al.*, 1992). The PI-3 kinase is activated by a large range of receptor tyrosine kinases (RTKs); RTKs are high affinity cell surface receptors which are seen in polypeptide growth factors, hormones and cytokines (Robinson, et al., 2000). RTK's are key regulators in cellular processes and have also been associated with the development of an array of cancers (Moonhee, et al., 2009).

The activation of PI-3 kinase is the major contributing factor in the activation of Phosphatidylinositol (3,4,5)-trisphosphate (PI(3,4,5)P<sub>3</sub>) or Phosphatidylinositol (3,4)-bisphosphate (PI(3,4)P<sub>2</sub>). This occurs by the recruitment of PLC $\gamma$ , due to the activation of a RTK; PLC $\gamma$  once activated hydrolyses its substrate PtdIns(4,5)P<sub>2</sub>. This action leads to the formation of two secondary messengers, inositol trisphosphate (IP<sub>3</sub>) and diacylglycerol (aka PIP<sub>2</sub>). IP<sub>3</sub> governs the release of calcium (Ca<sup>2+</sup>) from intracellular supplies, which in turn bind to calmodulin leading to the activation of protein kinases (Walter, et al., 2003; Zheng, *et al.*, 2011).

The phosphorylation of PIP<sub>2</sub> by the PI-3 kinase leads to the generation of PIP<sub>3</sub>; PIP<sub>3</sub> functions as a secondary messenger which in turn activates downstream signal transduction molecules. These include tyrosine kinases Btk and Itk and serine/threonine kinase Akt (Burgering *et al.*, 1995).

Akt activation plays a critical role in major biological functions including the survival, proliferation and cell growth; and is associated with many cancers (Williams, *et al.*, 1989). The generation of PIP<sub>3</sub> also leads to the activation of the protein kinase C family (Toker *et al.*, 1994; Akimoto *et al.*, 1996), along with p70 S6 kinase (Cheatham *et al.*, 1994), the c-Jun Nterminal kinase and GTPases (Lopez-Illasaca *et al.*, 1997).

For example PIP<sub>3</sub> has been shown to bind to the PH domain of the Akt kinase which leads to conformation change in the kinase; in turn this allows for Akt phosphorylation to occur resulting in the activation of the Akt kinase (Klippel *et al.*, 1997). The resultant activation of Akt leads to many cellular processes occurring, as stated Akt kinase effects cell survival, cycle, metabolism and angiogenesis; Akt has been shown to inhibit the Bcl-2-associated death promoter (BAD) molecule, BAD is a pro apoptotic protein which is involved in initiating apoptosis (Heldin *et al.*, 1998).

#### **1.4. The mitogen activated protein kinase (MAP kinase) pathway**

The adaptor protein known as growth factor receptor bound protein 2 (Grb2) bind to the PDGF receptor in its phosphorylated state. When the PDGF receptor is stimulated the Grb2 protein forms a complex with the son of sevenless homolog 1 (Sos1); the complex is recruited by the SH3 domain on the Grb2 protein. The complex that is formed functions as a

nucleotide exchange factor, the complex is recruited to the membrane by the SH2 domain on the Grb2 protein; which is bound to the activated PDGF receptor (Keshet, et al., 2010).

This allows for the interaction with Ras, which leads to the conversion exchange of the GDP inactive form to the GTP bound active form (Schlessinger, 1994). The active GTP-bound Ras interacts with and activates a range of signalling proteins; in particular the downstream Raf-1 molecule. Raf-1 molecule has a stimulatory effect, activating the mitogen activated protein kinase kinase (MAPKK, MEK); Raf-1 is the first kinase within the MAP kinase cascade, it activates MAPKK, MEK through the phosphorylation of a Ser residue (Heldin *et al.*, 1996).

The activation of MAPKK, MEK in turn leads to the phosphorylation and activation of the MAP kinases Erk 1 and Erk 2; once active these kinases are translocated into the nucleus leading to the phosphorylation of a range of cytoplasmic substrates (Keshet, et al., 2010). Erk 1 and Erk 2 are also responsible for the activity of many transcription factors contributing to the regulation of a variety of cellular processes including cell growth, proliferation, differentiation, migration and apoptosis (Satoh *et al.*, 1993). MAP kinases have also been found to be involved with cellular immunoresponses (Nanberg *et al.*, 1993).

### **1.5. Src kinase pathway**

Src is a proto-oncogene encoding a tyrosine kinase; members of this family are associated with having a SH2 domain, SH3 domain and a kinase domain which have been characterised (Erpel *et al.*, 1995). The kinase activity of the Src molecule is controlled by a tyrosine residue known as T 527, which resides within the C-terminal of the molecule. The tyrosine 527 residue plays a crucial role when the Src molecule is phosphorylated; the tyrosine residue controls molecular interactions within the SH2 domain keeping the Src kinase in its in-active state (Kypta *et al.*, 1990). However the mechanism involved in the binding to the PDGF receptor causes the displacement of the tyrosine 527 residue; this results in other tyrosine residues that are present in both the SH2 and SH3 domain becoming phosphorylated which induces the activation of the Src kinase. Studies have shown the Src kinase has a positive effect on the induction on stimulating cellular growth. (Alonso *et al.*, 1995; Stover *et al.*, 1996).

## 1.6. Protein tyrosine phosphatases

Protein tyrosine phosphatases are a type of diverse but highly specific enzymes that remove phosphate groups from tyrosine residues on phosphorylated proteins. The action of PTPs are crucial for regulating signalling pathways by reversing tyrosine phosphorylation, which is a common mechanism used for the activation of protein kinases to interact and alter transcription factors leading to cell proliferation, migration and adhesion. (Ostman *et al.*, 2006). The maintenance of an appropriate level of protein tyrosine phosphorylation is therefore critical for the function of many cellular activities. PTP enzymes are tyrosine specific protein phosphatases (PTPase) that catalyse and remove the phosphate group which is bound to the tyrosine residue, achieved through the use of a cysteinyl-phosphate enzyme intermediate.

PTPase are crucial in regulating many signal transduction pathways including the MAP kinase cascade; and in turn are key in determining the activity of cell growth, proliferation and cycle.

PTPs can be categorised into 2 types, including receptor like forms and non-receptor forms. Receptor like PTPs (RPTP) comprise of a transmembrane domain and various variable extracellular domains; along with intracellular components which in most cases contain two PTP domains that are the active catalytic section of the protein.

There is a large diversity in the structure of non-receptor PTPs; studies have shown that they exhibit many sequences which allow them to bind to specific proteins with high affinity (Mauro *et al.*, 1994).

There are many examples of such PTPs that regulate the critical signalling pathways, such as SHP-2 and TC-PTP which have been shown to interact and dephosphorylate the PDGF receptor tyrosine residues which are phosphorylated during ligand binding (Persson *et al.*, 2003). Studies have also shown that the activity of these phosphatases could potentially adjust specific signalling pathways that are connected to the active form of the PDGF receptor; by altering the protein tyrosine phosphatases downstream signalling could be adjusted in accordance of a cellular response to an external stimulus (Persson *et al.*, 2003).

### 1.7. LAR-RPTP and its role in PDGF receptor signalling

The leukocyte common antigen-related (LAR) are a subfamily of PTPs that belong to the group of receptor like protein tyrosine phosphatases. Studies have shown that the leukocyte common antigen-related subfamily is generally comprised of three homologs known as RPTP- $\sigma$ , RPTP- $\delta$  and LAR-RPTP (Chagnon *et al.*, 2004).

LAR-RPTP has a receptor like structure which has the ability to monitor the extracellular environment, and in turn has the ability to modulate signals through cytosolic PTP domains into the cell.

LAR-RPTP has two intracellular domains, it also contains a membrane distal domain which has weak catalytic activity; the intracellular domain also comprises of a membrane proximal domain (D1) which possess high catalytic activity. The intracellular domains are bound to a hydrophobic transmembrane (stretch) which in turn connects to the extracellular domains of the LAR-RPTP. The extracellular domains comprise of eight fibronectin type III domains and three immunoglobulin-like domains (Pulido *et al.*, 1995).

LAR is known to be expressed in many various cell types; LAR-RPTP has been documented to be present in fibroblast, cardiac, epithelial and neuronal cells (Streuli *et al.*, 1992). Studies (Chagnon *et al.*, 2004) have shown that LAR-RPTP plays an important role in signal transduction; LAR had previously been known to be involved with nervous system development, studies investigating the role LAR exhibits have now shown that it is involved with the regulating of nerve regeneration as well as with axonal growth and development (Zheng, *et al.*, 2011). LAR has also been shown to have a role with integrin focal adhesions and also it has been suggested that LAR dephosphorylates beta-catenin therefore playing a regulatory role with cadherin adhesions (Mooney, *et al.*, 2003).

More recently LAR has been determined to play important roles in the regulation of tyrosine kinase signal transduction; in that LAR exhibits a dual function with the regulation of these kinases. Studies have shown that LAR can directly dephosphorylate receptor tyrosine kinases which lead to receptor inactivation and the modulation of downstream signalling. A recent study (Niu *et al.*, 2007) involving LAR was shown to interact with the insulin like growth factor 1 receptor (IGF-1R); which lead to the inhibition of IGF-1 signalling in smooth muscle cells. The inhibition of IGF-1 was then associated with loss of cell proliferation and migration due to down regulation. It has also been suggested that LAR regulates the activity



of the cytoplasmic tyrosine kinase c-Abl, keeping the kinase in its inactive form (Zheng, *et al.*, 2011).

LAR has also been seen to enhance signal transduction through the act of dephosphorylating tyrosine residues that have a negative regulatory effect upon tyrosine kinases. It has been shown that certain protein tyrosine kinases are linked with the substrate of LAR-RPTP, examples of such kinases are the Src, Fyn and Lck kinases; LAR has also been shown to activate these kinases (Tsujikawa *et al.*, 2002).

Another study (Zheng, *et al.*, 2011) previously carried out by the Hellberg research group demonstrated that LAR positively regulated PDGF  $\beta$  receptor with respect to receptor activation and signal transduction. The study showed that in the absence of LAR (in cells where phosphatase domains of LAR had been deleted) PDGF  $\beta$  receptor activation was impaired. As a knock on effect there was also a decrease in phosphorylation of crucial downstream signalling molecules connected to the PDGF  $\beta$  receptor. The study also showed that with the inhibition of c-Abl, PDGF  $\beta$  receptor phosphorylation was reverted, in LAR $\Delta$ P MEF cells; indicating that LAR is critical for maintain the inactive form of c-Abl, which in turn allows for PDGF  $\beta$  receptor phosphorylation (Zheng, *et al.*, 2011).

The studies that have been conducted give evidence to suggest that LAR-RPTP has the ability to either up regulate or down regulate cell functions dependent upon the cell type and signalling pathway (Zheng, *et al.*, 2011).

### **1.8.1 c-ABL and G protein-coupled receptor kinases (GRKs)**

c-Abl is a proto-oncogene which encodes a cytoplasmic protein tyrosine kinase which has been shown to lead to cellular processes including differentiation, division and adhesion (Szczylik, *et al.*, 1991). The activity of c-Abl is regulated negatively by its SH3 domain; and CDC2 mediates the DNA-binding activity of the c-Abl tyrosine kinase through phosphorylation, potentially showing that it has a cell cycle function (Szczylik, *et al.*, 1991).

GRKs are a type of protein kinases which regulate G protein- coupled receptors through the phosphorylation of their intracellular domains, after the association of G-proteins have been activated.

The phosphorylation of the serine and threonine residues prevents reactivation of the signalling pathway; due to the recruitment of arrestin proteins to the phosphorylated residues preventing the reassociation of G proteins (Evron *et al.*, 2012).

### **1.8.2 Protein Kinase C, NHERF-1 and NHERF-2**

Protein Kinase C (PKC) is part of the serine and threonine-specific protein kinase family; it is activated by calcium and diacylglycerol (Huppi, *et al.*, 1999). They are responsible for a range of cellular signalling pathways and are known to phosphorylate many proteins depending on their expression. Studies have shown that PKC is involved in a range of cellular processes including B cell signalling as well as the regulation of growth, differentiation and apoptosis (Blake, *et al.*, 1999).

Sodium-hydrogen exchange regulatory cofactor (NHERF-2) is a scaffold protein connecting membrane proteins linking them to the actin cytoskeleton, they also help in the regulation of the surface expression of these proteins; NHERF-2 has been shown to be vital for cAMP phosphorylation (Hall, *et al.*, 1998).

Sodium-hydrogen antiporter 3 regulator 1 (NHERF-1) is a scaffold protein which has been shown to regulate the sodium-hydrogen antiporter 3; current studies have also shown that NHERF-1 plays an important role in both enzyme and protein regulation through the binding of an effector molecule to the target allosteric site (Farago, *et al.*, 2010).

## **1.9. Aim of the research project**

- To investigate the role of LAR in the regulation of the PDGF receptor and its effect upon downstream signal transduction molecules, in particular the ERK1/2 pathway. As previously stated the Hellberg research group have determined a mechanism for PDGF signalling whereby the receptor is regulated through LAR, which helps to promote ligand induced activation of the PDGF  $\beta$ -receptor through its mechanism of down regulating c-Abl activity.
- To investigate the effects of c-Abl and the kinases associated with respects to the activation of the PDGF  $\beta$ -receptor. The serine/threonine-specific protein kinase PKC  $\delta$  and its effect will be examined; PKC  $\delta$  is an enzyme protein that is linked to c-Abl and the PDGF receptor, situated in-between. The hypothesis, that PKC  $\delta$  regulates

PDGF receptor activation by inhibiting phosphorylation of the tyrosine residues will be tested. PKC  $\delta$  will be knocked down using specific small interfering RNA to determine the effect it has upon the PDGF receptor; this will be conducted in LAR $\Delta$ P mouse embryonic fibroblast (MEF) cells, in which the phosphatase domains of LAR have been deleted, thus c-Abl will not be suppressed.

- The structural proteins NHERF1 and NHERF2 will also be investigated; these proteins are known to bind to the C-terminal of the PDGF receptor which is crucial for the receptor to become dimerized. As with PKC  $\delta$ , both NHERF1 and 2 will be knockdown using siRNA in order to determine the effect upon the PDGF receptor in the absence of LAR.
- The effects of the knockdown of PKC  $\delta$ , NHERF1 and NHERF2 will also be further assessed by examining the downstream signal transduction molecules, the ERK1/2 and the Akt pathways; to determine if the effect upon the PDGF receptor is reflected further down the signalling pathway.

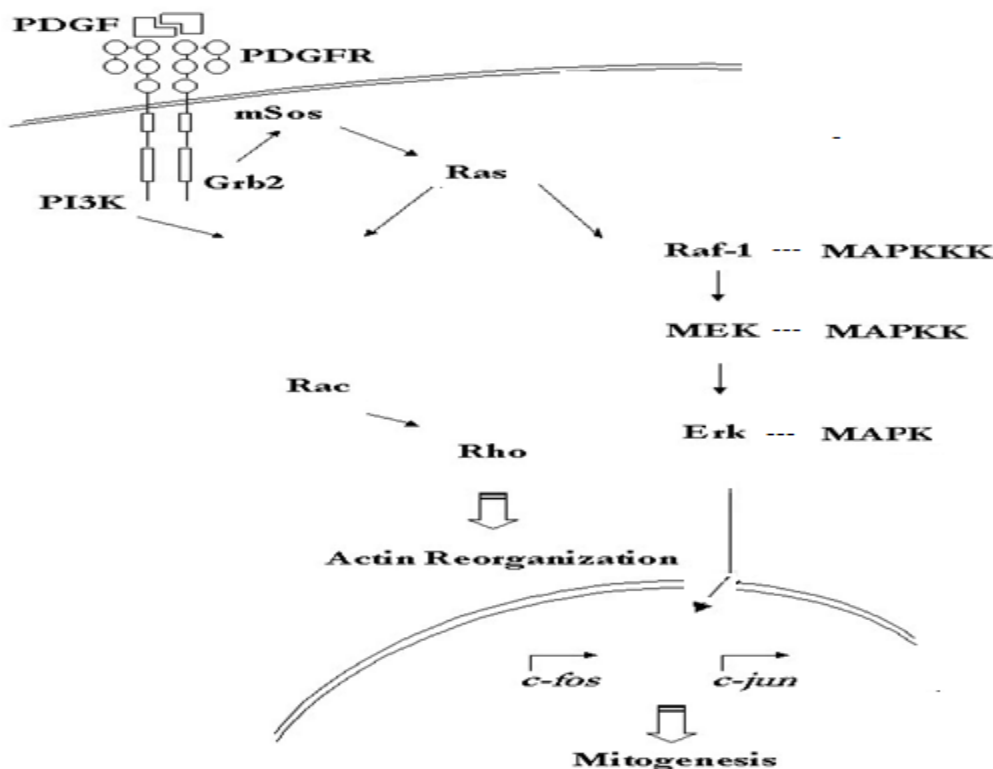


Figure 1: The mechanism of PDGF  $\beta$  receptor signalling, showing the Ras/mitogen-activated protein kinase cascade. The direct interaction from Ras activates Raf causing the phosphorylation and activation of MEK which in turn activates ERK 1 and 2 leading to the activation of transcription factors (Ligaya, *et al.*, 1999) (Villari, 2009)

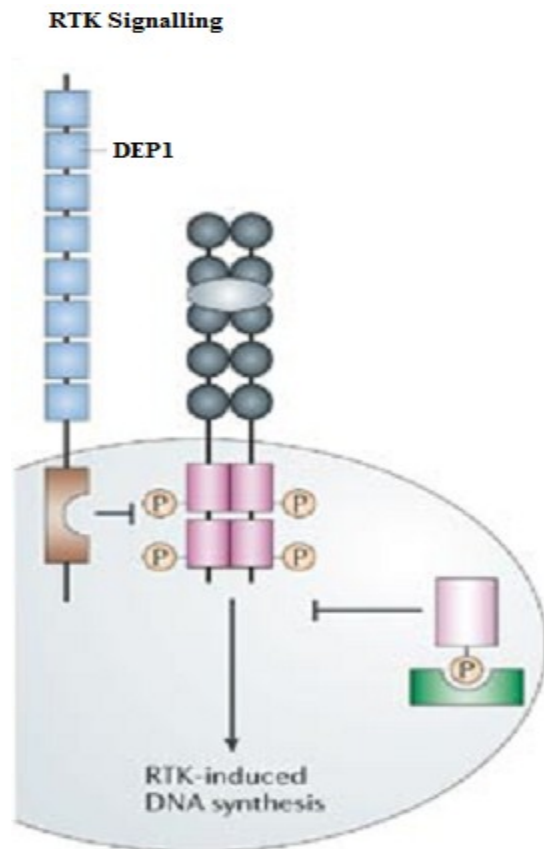


Figure 2: Schematic illustration showing the structure of protein-tyrosine phosphatases (PTPs) (e.g. the leukocyte common antigen-related (LAR)). PTPs have the ability to antagonize receptor-tyrosine kinase (RTK) signalling by either direct dephosphorylation or through density-enhanced Phosphatase-1 (DEP-1) mediated dephosphorylation as seen with platelet-derived growth factor (PDGF) receptors. PTPs have also been shown to promote RTK signalling through the dephosphorylation and inactivation of inhibitory components (Östman *et al.*, 2006).

## 2.0. Materials and Methods

### 2.1. Cell Culture and Preparation

Cell culture of MEF LAR WT and MEF LAR  $\Delta$ P cells

Under sterile conditions:

Mouse embryonic fibroblast (MEF) cells frozen in 10% Dimethyl sulfoxide (DMSO), for cell membrane stability and survival, were resurrected at 37 °C in a water bath (rapidly thawed). Two cells types were used to investigate the effect of the receptor tyrosine phosphatase (RPTP) LAR upon the platelet-derived growth factor (PDGF) receptor signalling. The MEF

cells that were used were isolated from mice which had had the LAR phosphatase domains deleted; wild type MEF cells were also isolated.

The cells were grown in culture under sterile conditions in Dulbecco's Modified Eagle Media (DMEM) + 10% foetal bovine serum (FBS). The DMEM also contained 1% Glutamine-Penicillin-Streptomycin (GPS), a one-step supplementation to help reduce the risk of contamination.

After several days of growth the cells were passaged using the cell dissociation reagent Trypsin (0.25% Trypsin-EDTA 1x). The cells were grown in 15ml of DMEM + 10% FBS to confluence in 162cm<sup>2</sup> flasks; roughly  $6 \times 10^6$  cells per flask.

## **2.2. Western Blot and Immunoprecipitation**

Testing for phosphorylation of PDGF receptor

Under sterile conditions confluent cells in 25cm<sup>2</sup> flasks were starved in DMEM (starvation media, containing no FBS or growth factors) for 16 hours. The cells were starved in order for them to up-regulate and express the PDGF receptor. The cells were then stimulated using a recombinant human PDGF ligand (homodimer PDGF- BB); this was used to stimulate the PDGF receptor for a set time. 2µl of the PDGF ligand was added to 5ml of starvation media to give an overall concentration of 20ng/ml (The stock concentration of the ligand was 500µg/ml, and was diluted by 1/10 using PDGF-BB buffer (NaCl)). This concentration was determined to be optimal for receptor stimulation, for a set time of 10 minutes at 37 °C. After 10 minutes of stimulation the flask of cells were put on ice, and the cells were washed 3 times with cold PBS, to remove the stimulation media. Note the cells were washed gently as to not disrupt adherence. Lysis buffer (0.5% TX-100, 0.5% Deoxycholic acid, 150mM NaCl, 20mM Tris pH 7.5, 10 mM EDTA) at pH7.5 was then added to the cells. 10 µl of aprotinin and 10 µl sodium orthovanadate was added to 10 ml of lysis buffer. Aprotinin is a protease inhibitor; when the cells are lysed proteases are released which lead to protein degradation, Aprotinin was therefore used to inhibit this process from occurring. Sodium orthovanadate, the chemical compound Na<sub>3</sub>VO<sub>4</sub>, acts as protein tyrosine phosphatase inhibitor; it binds to the active sites on the protein tyrosine phosphatase. The inhibition of protein tyrosine

phosphatase's helps preserve the phosphorylation of the protein under investigation as phosphatases that are present within the cell lysate are inhibited. 300µl of lysis buffer was then added to each 25 cm<sup>2</sup> flask of cells, and was left for 15 minutes in order for the cells to be completely lysed. The cells were scraped from the flask and then placed into eppendorfs, and left on ice for a further 15 minutes. The eppendorf's were then centrifuged at 1300xg for 15 minutes, the supernatant was then removed and aliquoted into a new eppendorf. The supernatant contained the proteins of interest and was removed with care not to disrupt the pellet which contained non-soluble membrane proteins.

The protein concentration of each sample was then tested using UV spectroscopy; using absorbance set at 595nm. 5µl of each sample was added to a well (96 –well plate), including a control using lysis buffer. 200µl of Coomassie brilliant blue was then added to each of the sample wells, and left for 10 minutes for the changes to take place, the plate was then read using a spectrophotometer to calculate the protein concentration.

The data obtained from the UV spectroscopy was then used to determine how much lysate buffer to add to each protein sample in order to make the concentrations the same, to be used in the Immunoprecipitation assay. 10µl of protein sample was also removed and aliquoted into new eppendorfs to test for total cell lysate, to compare to the IP. 10µl of 2X sample buffer (125mM Tris HCl pH 6.8, 20 % glycerol, 4% sodium dodecyl sulphate (SDS), 0.02% bromphenol blue) was added to each of the 10µl of total cell lysate samples, which were then boiled for 5 minutes at 95 °C.

A selected antibody was added to the immunoprecipitation samples; CTβ antibody, CTβ serum were used. Both CT β antibody and serum detect and bind specifically to the PDGF receptor, using these antibodies with a high binding affinity and specificity resulted in an efficient precipitation of unwanted proteins from the sample.

2µl of CTβ antibody (rabbit antibody concentration 1.89µg/ml) was added to each sample and was left overnight to mix (at 5 °C). CTβ serum was also tested for the use in IP, 20µl was added to each sample, and was also left overnight to mix (at 5 °C). After leaving overnight 30µl of magnetic protease beads was added to each sample and left to mix for one hour at 5 °C. The tubes were then placed in a magnet and left for 20 seconds before the supernatant was removed. The pellet was then washed with 1ml of lysis buffer and was resuspended before being placed back into the magnet. This was repeated for a total of 3 washes.

Alternatively WGA beads were used; Wheat germ agarose beads have a high binding affinity to glycosylated proteins; it is well known that the PDGF receptor is highly glycosylated, WGA beads therefore offered a good alternative for precipitation of sample proteins.

30µl was added to each sample and left to mix for one hour at 5 °C. The beads were then centrifuged at 1300xg for 1 minute; the supernatant was removed and the pellet was washed with 1ml of lysis buffer. The pellet was resuspended and centrifuged again at 1300xg for 1 minute. This was repeated for a total of 3 washes.

In both cases 2X sample buffer was added after the third wash in the same ratio as with the total cell lysate (30µl was added to the 30µl sample). The samples were then briefly centrifuged and boiled for 5 minutes at 95 °C.

The samples were then run on either 7% or 10% gels (separation gel containing 17.4% of 40% acrylamide, 19.8% of 2 M Tris-Cl pH6.8, 0,5% of 20% SDS, 61.8% distilled water, 0,1% of TEMED (N, N, N',N'-tetramethylethylenediamine, MERCK) and 0,4% APS and with the staking gel containing 10% of the 40% acrylamide, 13.8% of 0,5 M Tris-Cl pH6.8, 5% of 20% SDS solution, 13.1% of glycerol, 62.2% distilled water, 0.1% TEMED and 5% of 10% APS)

IP samples were loaded in equal amounts (15µl per well), and the volume of the total cell lysate samples loaded depended upon the concentration (taken from UV spectroscopy data). Also 5µl of pageruler pre-stained protein ladder marker was also loaded in the gel. The gels were run at 175volts for roughly 55 minutes, after which the gel were transferred to nitrocellulose membranes by electrical transferring machine in a Trans blot turbo machine and blotted for 7 minutes.

The membrane was then taken out and the gel discarded; the membrane was washed in PBS-T and then left for 1 hour in antibody blocking buffer. After 1 hour the membrane was washed in PBS-T and PY99 antibody was added to the membrane, which was left over night at 5 °C (excluding SiRNA optimisation see below). PY99, also known as p-Tyr is a mouse monoclonal IgG<sub>2</sub> antibody that specifically detects and binds to phosphotyrosine containing proteins; it was used to show the amount of phosphorylated PDGF receptor present in each sample (in this case the antibody will only bind to the PDGF receptor as other protein kinases have been precipitated from the sample).

After leaving over night the membrane was then washed for 5 minutes in PBS-T (0.05% Tween-20) 6 times. A secondary antibody linked to a peroxide marker (IgG – HRP goat-anti mouse) was then added to the membrane (2µl of antibody in 20ml of PBS-T, 1/1000 dilution). The membrane was incubated for one hour with the antibody at room temperature, after which the membrane was washed for another 6 times with PBS-T.

The membrane was then subjected to a developing solution, a pro-chemiluminescent reagent (600µl of both developing solution A and B) (ECL western blotting detection reagents from GE healthcare) for 1 minute before being analysed using Gene Snap software (CCD camera detects the light signal produced from the reaction between the pro-chemiluminescent reagent and the peroxide marker; shows the amount of protein of interest present in sample. Gene Snap setting that were used included a photo set at 5 micro-seconds with upper white light and no filter, the membranes were also exposed for 10 minutes with no light or filter. After exposure the membrane was then stripped using sodium hydroxide (NaOH) for 10 minutes, before being rewashed in PBS-T 6 times for 5 minutes. The membrane was re-blocked using antibody blocking buffer and washed once with PBS-T before CTβ antibody was added (excluding SiRNA optimisation see below). CTβ is an anti PDGF receptor antibody; it was used to show the amount of PDGF receptor present within the sample. The antibody was again left over night at 5 °C, and the protocol was repeated as with the PY99 antibody; however the secondary antibody used was a IgG – HRP goat-anti rabbit.

### **2.3. Phorbol 12-myristate 13-acetate (PMA)**

PMA is a diester of phorbol which is regularly used in the activation of the signal transduction enzyme protein kinase C (PKC). PMA has an effect on PKC due to its parallel resemblance of diacylglycerol which is a natural activator of classic PKC isoforms (Castagna et al, 1982). PKC would be activated in cells that had been treated with PMA; left incubated over night would lead to the degradation and eventual depletion of PKC within the cells. The depletion of PKC would in turn show the effect that the ‘knock out’ of the PKC signalling



pathway has upon the PDGF receptor; with respect to receptor phosphorylation and expression.

As above, cells were seeded and grown to confluence in 25cm<sup>2</sup> flasks with DMEM complete media until confluent. 8 flasks in total were used as follows:

	PMA	DMSO
LAR Wild type	Un-stimulated	Un-stimulated
	10min-20ng/ml PDGF ligand	10min-20ng/ml PDGF ligand
LAR ΔP	Un-stimulated	Un-stimulated
	10min-20ng/ml PDGF ligand	10min-20ng/ml PDGF ligand

The cells were starved overnight in media containing either 1μM of PMA or DMSO, in order for them to up-regulate and express the PDGF receptor. DMSO was used as a control, as PMA is dissolved in DMSO; therefore any change in PDGF receptor phosphorylation could be noted. 4 flasks (as seen above) were then stimulated using a recombinant human PDGF ligand (homodimer PDGF- BB). 2μl of the PDGF ligand was added to 5ml of starvation media to give an overall concentration of 20ng/ml.

After 10 minutes of stimulation all 8 flasks were put on ice and lysis buffer was added as above. Protein concentration was analysed using UV spectroscopy; the data was then used to determine equally concentrations of the samples for the use in IP. CTβ antibody (2 μl) was used and all samples including total cell lysate was run on 7% gels for 55 minutes at 175 volts. Membranes containing IP samples were then blotted initially with PY99 antibody and then re-blotted with CTβ; and membranes containing the total cell lysate samples were initially blotted using PKC Δ (IgG mouse antibody) antibody and re-blotted with CTβ

## 2.4. siRNA Knockdown optimisation

### Knockdown optimisation of PKC delta, NHERF-1 and NHERF-2.

siRNA optimisation for the knockdown of PKC δ, NHERF-1 and NHERF-2 in LAR Δ P MEF cells was analysed in order to find the optimal concentration of siRNA and length of

time of transfection to be used in order to achieve a successful knockdown with respect to the PDGF receptor for the MEF cell line. siRNA stock amount of PKC  $\delta$ , NHERF-1 and NHERF-2 was 10 nmol; the tubes were briefly centrifuged and the siRNA was resuspended with 500  $\mu$ l of sterile distilled water (dH<sub>2</sub>O) making the final concentration of siRNA to 20  $\mu$ M. The solution in each tube was gently mixed to avoid the introduction of bubbles; the solution was then further mixed for 30 minutes using an orbital mixer/shaker at room temperature. This additional mixing step allows for a more reliable resuspension (shown by UV spectroscopy). The tubes were then briefly centrifuged before the siRNA was aliquoted into small volumes (50  $\mu$ l into 7 eppendorfs, and 30  $\mu$ l into 5 eppendorfs) and stored at -20 degrees C. This was to ensure that the freeze- thaw events were limited to no more than 5 per tube.

Cells were seeded in 12 well plates, at  $6 \times 10^4$  per well; roughly 30% confluent per well. Cells were counted using a cell counter; where 5  $\mu$ l of trypan blue was mixed with 5  $\mu$ l of cell sample. Each siRNA knockdown optimisation used 4 plates (hours), in which 5 wells (concentrations) were used on each plate; as follows.

Hours	Conc' nM	Conc' nM	Conc' nM	Conc' nM	Conc' nM
24	0	1	10	50	100
48	0	1	10	50	100
72	0	1	10	50	100
96	0	1	10	50	100

siRNA concentrations for PKC  $\Delta$ , NHERF-1 and NHERF-2 were made up as follows

Concentration (nM)	Starvation media (DMEM) ( $\mu$ l)	siRNA duplex ( $\mu$ l)	Interferon ( $\mu$ l)
0	200	0	0
1	200	1.2	4
10	200	12	8
50	200	3 from stock	8
100	200	6 from stock	8

Note: At 1 and 10 nM concentrations siRNA duplexes were first diluted using sterile dH<sub>2</sub>O; whereby 1 µl of siRNA duplex from stock was diluted with 19 µl of dH<sub>2</sub>O.

The transfected cells were left for the set amount of time (as above); after which they were lysed and their protein concentrations calculated using UV spectroscopy as previously stated above (follow protocol). The total cell lysate samples were run on a 10% gel for 50 minutes at 175 Volts. The membrane was then blotted initially with either PKC  $\Delta$ , NHERF-1 or NHERF-2 to show the amount of protein present and to measure the effect of the siRNA knockdown present for at concentration at the different time points. The membrane was stripped and re-blotted with Alix antibody which was used to show the volume of siRNA sample that had been loaded into each well.

## 2.5. Knockdown of PKC $\delta$ , NHERF-1 and NHERF-2

Data obtained from the siRNA optimisation assays for PKC  $\delta$ , NHERF-1 and NHERF-2 was analysed; the conditions which favoured the optimum knockdown for each was then further tested. The data showed that 100nM concentration of siRNA duplex at 48 hours had the biggest knockdown effect for all three siRNA's.

These optimisation conditions were then used in further knockdown experiments to show the effects of siRNA knockdown of PKC  $\delta$ , NHERF-1 and NHERF-2 on the phosphorylation of the PDGF receptor.

As above LAR  $\Delta$  P MEF cells were seeded in 6 well plates, at  $6 \times 10^4$  per well; roughly 40% confluent per well. Cells were counted using a cell counter; where 5 µl of trypan blue was mixed with 5 µl of cell sample. For each siRNA knockdown 4 samples were tested:

1	LAR $\Delta$ P cells + ctrl siRNA un-stimulated by PDGF-BB ligand
2	LAR $\Delta$ P cells + ctrl siRNA stimulated by PDGF –BB ligand (20ng/ml) - 10 minutes
3	LAR $\Delta$ P cells + siRNA un-stimulated by PDGF-BB ligand
4	LAR $\Delta$ P cells + siRNA stimulated by PDGF –BB ligand (20ng/ml) - 10 minutes

The transfected cells were left for 48 hours; after which they were lysed and their protein concentrations calculated using UV spectroscopy as previously stated above (follow

protocol). 30 µl of WGA beads were used for each sample for the siRNA immunoprecipitates. The samples were run on a 5% gel for 55 minutes at 175 Volts. The membrane was then blotted initially with PY99 to show the amount of phosphorylated PDGF receptor present in each sample. The membrane was stripped and re-blotted with CTβ to measure the amount of PDGF receptor present in each sample (not phosphorylation).

## **2.6. Down signalling pathway: ERK**

Successful siRNA knockdowns of PKC δ, NHERF-1 and NHERF-2 were further analysed; ERK a downstream signalling protein was analysed to see if the siRNA knockdown on the PDGF receptor contributed to further changes. Successful knockdowns in theory should lead to a difference in downstream signalling; by knocking out PKC δ, NHERF-1 and NHERF-2 the ERK pathways could potentially be affected. To investigate this total cell lysate from samples that had had a successful knockdown were loaded into a 10% gel and run at 175 Volts for 55 minutes. The membrane was initially blotted with anti- phosphatase ERK ½ (p-ERK) antibody (rabbit ab). This antibody detects phosphorylated ERK molecules present within the sample. The membrane was stripped and re-blotted with anti ERK ½ protein (rabbit) which detects any form of ERK proteins present.

## **3.0. Results**

### **3.1. Protein Immunoprecipitate profile**

Immunoprecipitates and western blot analysis was performed on both MEF wild type and LAR ΔP cells. As stated above, CTβ antibody, CTβ serum and WGA beads were tested in order to determine which method gave the best results for showing the amount of protein of interest (PDGF receptor) that was present within the sample. 2µl of CTβ antibody and 20µl CTβ serum was added to each sample which was also left over night to mix (at 5 °C). After leaving overnight 30µl of magnetic protease beads was added to each sample and left to mix for one hour at 5 °C. 30µl of WGA beads was added to each sample and left to mix for one hour at 5 °C. The beads were then centrifuged at 1300 rpm for 1 minute (3 times, as above).

All samples (CT $\beta$  antibody, CT $\beta$  serum and WGA beads) were run on 7% gels for 55 minutes at 175 volts; the membranes were then initially treated with PY99 antibody to test for the amount of PDGF receptor phosphorylation present per sample. The samples were also treated with CT $\beta$  antibody to test for the amount of PDGF receptor present per sample.

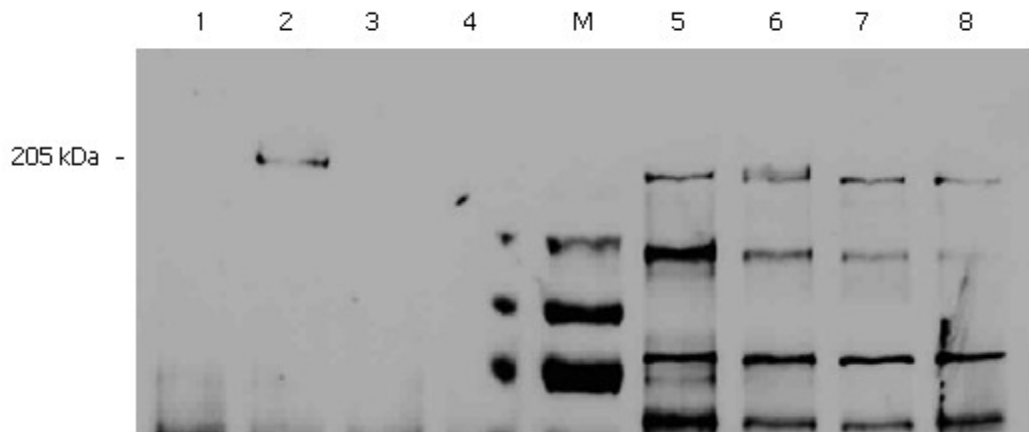


Figure 3. PY99 antibody western blot showing PDGF phosphorylation in MEF LAR WT and LAR  $\Delta$ P cells. Lanes 1-4 CT $\beta$  antibody IP where lane 1 is WT, lane 2 is WT + PDGF-BB stimulated, lane 3 is LAR  $\Delta$ P and lane 4 LAR  $\Delta$ P + PDGF-BB stimulated. Lanes 5-8 show the total cell lysate respectively.

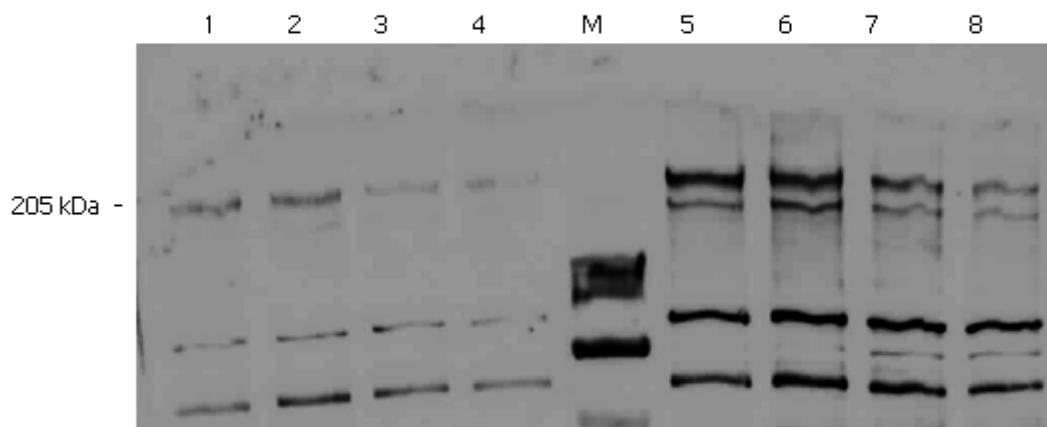


Figure 4. CT $\beta$  antibody western blot showing PDGF receptor present in MEF LAR WT and LAR  $\Delta$ P cells. Lanes 1-4 CT $\beta$  antibody IP where lane 1 is WT, lane 2 is WT + PDGF-BB stimulated, lane 3 is LAR  $\Delta$ P and lane 4 LAR  $\Delta$ P + PDGF-BB stimulated. Lanes 5-8 show the total cell lysate respectively.

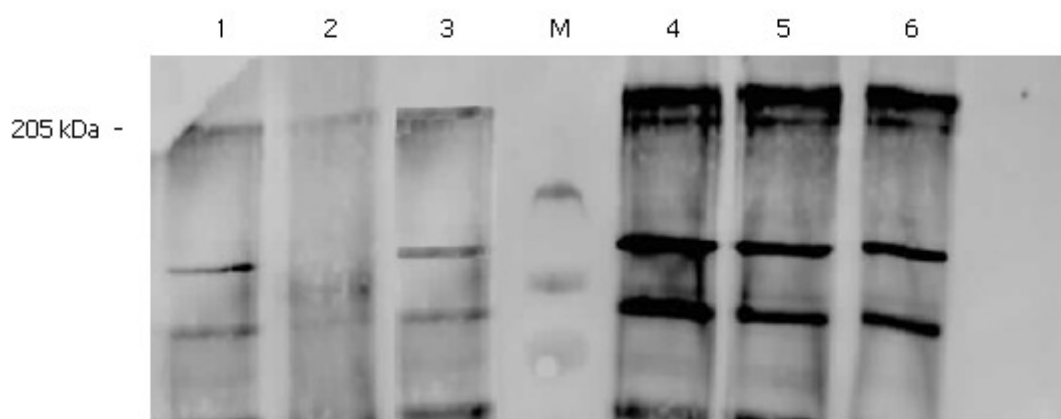


Figure 5. PY99 antibody western blot showing the phosphorylation of PDGF receptor in LAR  $\Delta$ P cells for a range of Immunoprecipitates; including CT $\beta$  ab, CT $\beta$  serum and WGA beads (from lanes 1-3 respectively). Lanes 4-6 show the corresponding results for total cell lysate.

Western blot analysis showed that CT $\beta$  antibody and the WGA beads gave a stronger immunoprecipitate signal than that obtained using CT $\beta$  serum. This result can be clearly seen in figure 3, when the signal from the 3 different IP's are compared. From this further analysis of CT $\beta$  antibody and WGA beads were carried out as can be seen in figures 3 and 4. The use of CT $\beta$  antibody gave a more precise IP, as the antibody detects and binds to the PDGF receptor, meaning the separation or precipitation from other proteins is highly specific; however a great signal was found to be achieved using WGA beads which bind to highly glycosylated proteins. It was therefore decided that for future experiments that either CT $\beta$  antibody or WGA beads should be used with regards to Immunoprecipitation, depending upon the resulting signal intensity.

### 3.2. The effects of PMA upon PKC

Both MEF wild type and LAR  $\Delta$ P cells were treated either with PMA or DMSO (control). As stated above PMA activates PKC, leading to its degradation. Western blot analysis was performed on the samples to determine the effect that an effectively depleted 'knockdown' PKC pathway had upon the PDGF receptor expression in both cell types. Immunoprecipitates were attained using CT $\beta$  antibody, samples were then run on 7% gels; membranes were blotted initially with PY99 antibody to give a measurement of the amount of PDGF receptor

phosphorylation per sample to measure the effect of PKC. The membrane was stripped and re-blotted with CT $\beta$  antibody to give a measurement of the amount of PDGF receptor present.

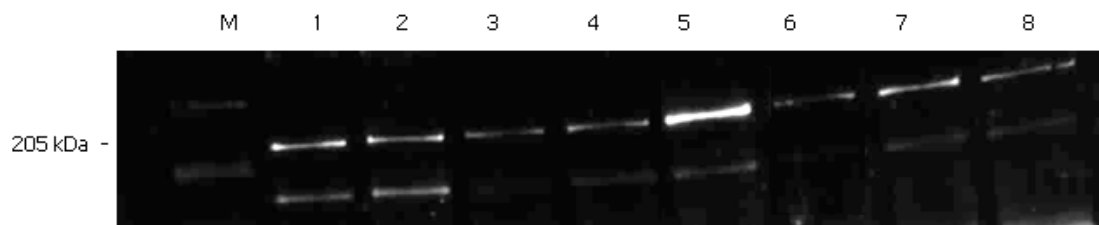


Figure 6. PY99 antibody western blot showing PDGF receptor phosphorylation in PMA/DMSO treated samples. Lanes 1-4 PMA treated samples including: LAR  $\Delta$ P stimulation (PDGF-BB ligand) and un-stimulated, WT stimulation (PDGF-BB ligand) and un-stimulated. Lanes 5-8 show corresponding DMSO treated samples.

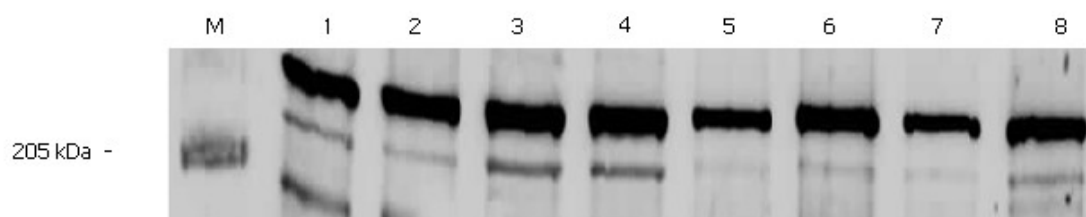


Figure 7. CT $\beta$  antibody western blot showing the amount of PDGF receptor present in PMA/DMSO treated samples. Lanes 1-4 PMA treated samples including: wild type cells un-stimulated and 10 min stimulation (PDGF-BB ligand) and LAR  $\Delta$ P cells unstimulated and 10 min stimulation (PDGF-BB ligand). Lanes 5-8 show corresponding DMSO treated samples.

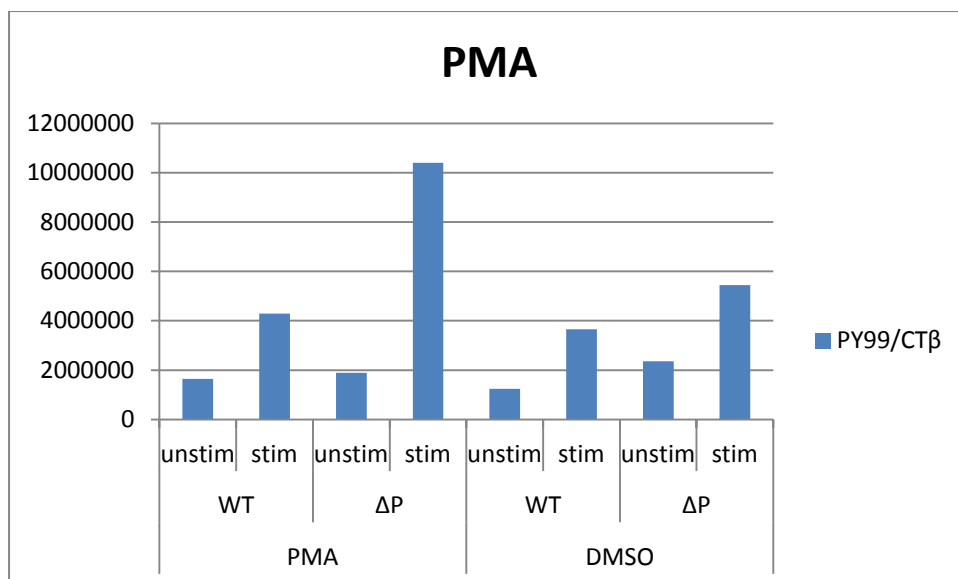


Figure 8. Western blot data showing the amount of PDGF receptor expression in PMA and DMSO IP samples; from left to right: wild type MEF un-stimulated and PDGF-BB stimulated; and LAR  $\Delta P$  un-stimulated and PDGF-BB stimulated. Error bars: standard deviation

The data obtained from western blot analysis clearly shows that PMA has a significant effect upon the PDGF receptor. The result suggests that with prolonged exposure to PMA, which leads to the degradation of PKC, that it has a positive effect on PDGF receptor expression and phosphorylation in LAR  $\Delta P$  cells; thus suggesting PKC has an inhibitory role in regulating PDGFR activity.

This result can be further determined with band quantification, figure 8 clearly shows the effect that PMA has upon the PDGF receptor; whereby the PDGF receptor expression is almost doubled (roughly 47%) in PMA samples when stimulated in comparison to those treated with DMSO control.

### 3.3. siRNA Knockdown Optimisation

siRNA optimisation was performed on LAR  $\Delta P$  MEF cells in order to analyse optimal conditions for the knockdown of PKC  $\delta$ , NHERF-1 and NHERF-2 proteins. A range of conditions were tested, these included a variety of siRNA concentrations set over a range of transfection times (as mentioned above). All samples were run on 10% gels; membranes were then blotted initially with either PKC  $\delta$ , NHERF-1 or NHERF-2 to show the amount of



protein present and to measure the effect of the siRNA knockdown. The membrane was stripped and re-blotted with Alix antibody which was used to show the volume of siRNA sample that had been loaded into each well.

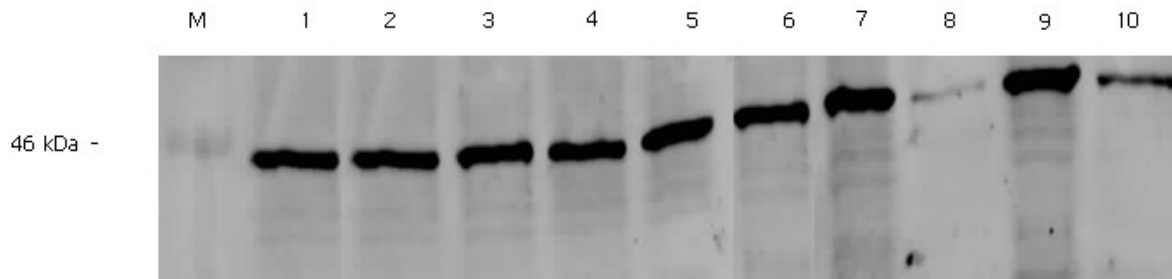


Figure 9. NHERF-2 antibody western blot showing the amount of NHERF-2 phosphorylated within the samples. From left to right: Protein marker, lane 1-5 24 hours – control, 1nM, 10nM, 50nM and 100nM siRNA concentrations. Lanes 6-10 show siRNA concentrations at 48 hours.

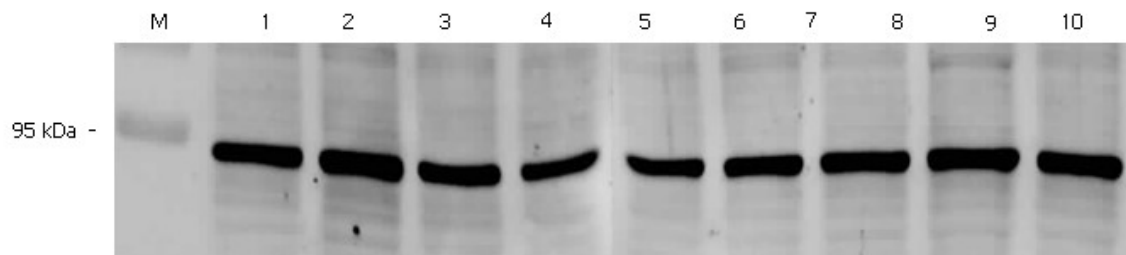


Figure 10. ALIX antibody western blot showing the amount of NHERF-2 present within the samples. From left to right: Protein marker, lane 1-5 24 hours – control, 1nM, 10nM, 50nM and 100nM siRNA concentrations. Lanes 6-10 show siRNA concentrations at 48 hours.

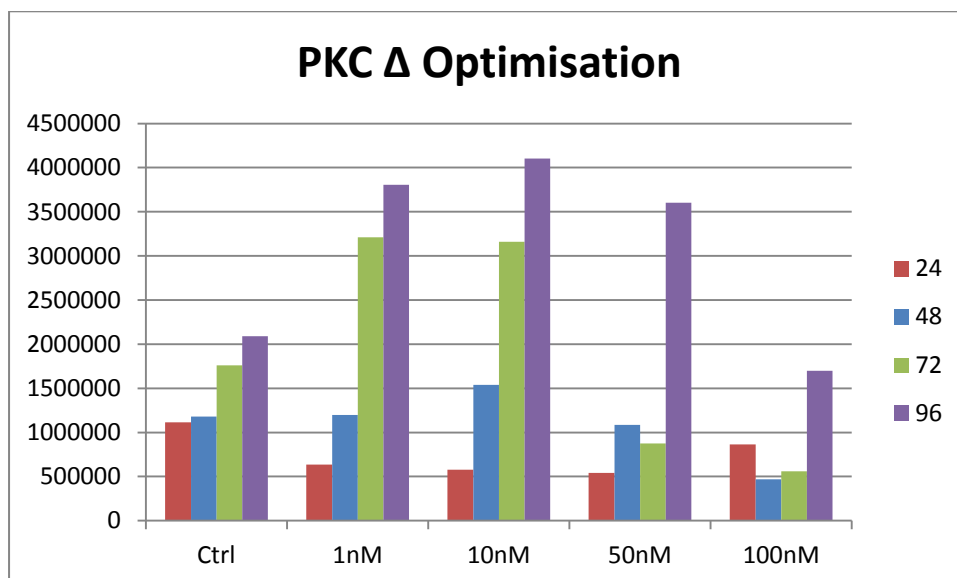


Figure 11. Graph of western blot data showing PKC  $\delta$  optimisation at 24, 48, 72 and 96 hours for a range of siRNA concentrations.

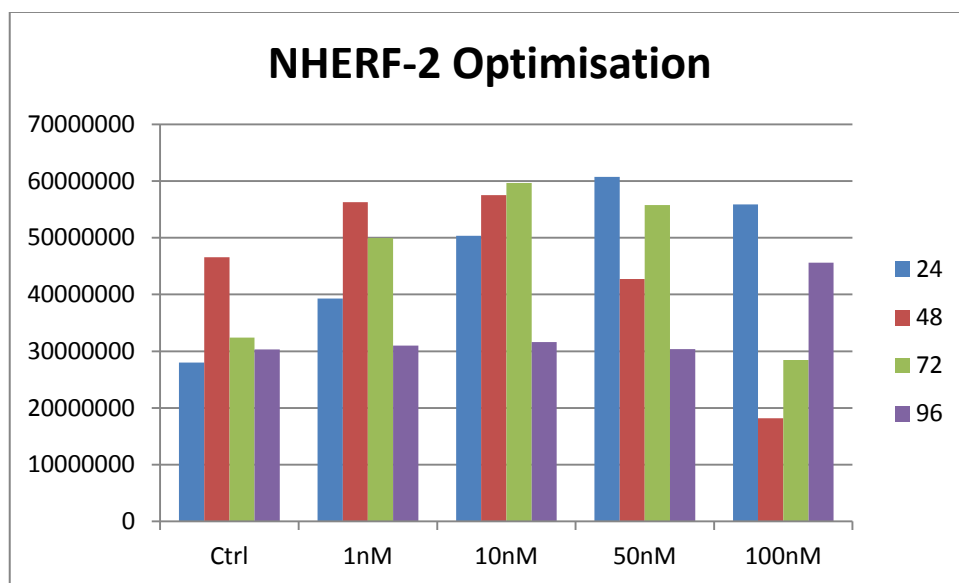


Figure 12. Graph of western blot data showing NHERF-2 optimisation at 24, 48, 72 and 96 hours for a range of siRNA concentrations.

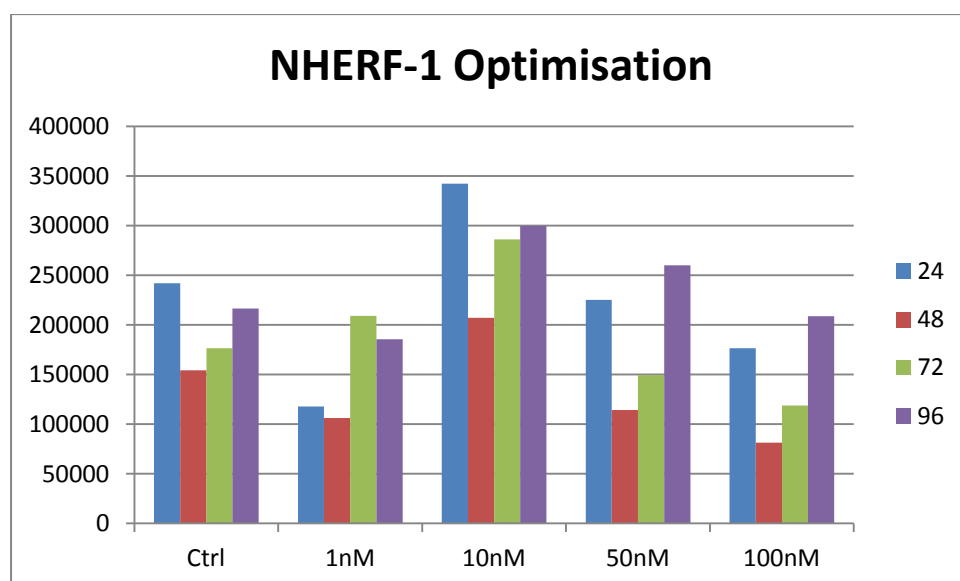


Figure 13. Graph of western blot data showing NHERF-1 optimisation at 24, 48, 72 and 96 hours for a range of siRNA concentrations.

Western blot analysis showed that the optimal knockdown condition for PKC  $\delta$  was at the concentration of 100nM (siRNA) at 48 hours. This was further shown through the use of band quantification (phosphorylated data/ amount of protein present); figure 10 shows the amount of knockdown per sample. The data clearly shows that at 100nM at 48 hours there is roughly a 60% reduction in PKC  $\delta$ , in comparison to the control siRNA. At this concentration

the reduction of PKC  $\delta$  is at its greatest, the large reduction indicates there is a successful siRNA knockdown taking place. The siRNA knockdown is most effective at this condition; the data also shows that the other siRNA knockdowns (at different conditions) are not as effective, this can be clearly seen in figure 10 where there are no other significant reduction in protein amounts (greater than 40%). This trend can also be seen with both NHERF2 and NHERF1 siRNA knockdowns; in both cases the greatest reduction in protein amount per sample occurs at 100nM siRNA concentration at 48 hours. This is shown in figure 11 and 12 where the reduction in NHERF2 and NHERF1 is again significantly different, 61% and 47% reduction respectively. As with PKC  $\Delta$  there are no other significant protein reductions; which indicates less effective siRNA knockdowns.

### **3.4. siRNA expression profile**

Data obtained from the siRNA optimisation assays for PKC  $\delta$ , NHERF-1 and NHERF-2 was employed for the siRNA knockdown experiments; the conditions which gave the most significant knockdown were used (100nM concentration of siRNA at 48 hours).

siRNA knockdown was performed on LAR  $\Delta$ P MEF cells, the knockdown of PKC  $\delta$ , NHERF-1 and NHERF-2 proteins were all analysed to investigate and measure any effect upon the PDGF receptor amount and phosphorylation. Immunoprecipitates were attained using WGA beads and samples were then run on 7% gels; membranes were blotted initially with PY99 antibody to give a measurement of the amount of PDGF receptor phosphorylation per sample, to measure the effect of the siRNA knockdown. The membrane was stripped and re-blotted with CT $\beta$  antibody to give a measurement of the amount of PDGF receptor present.

## PKC $\delta$

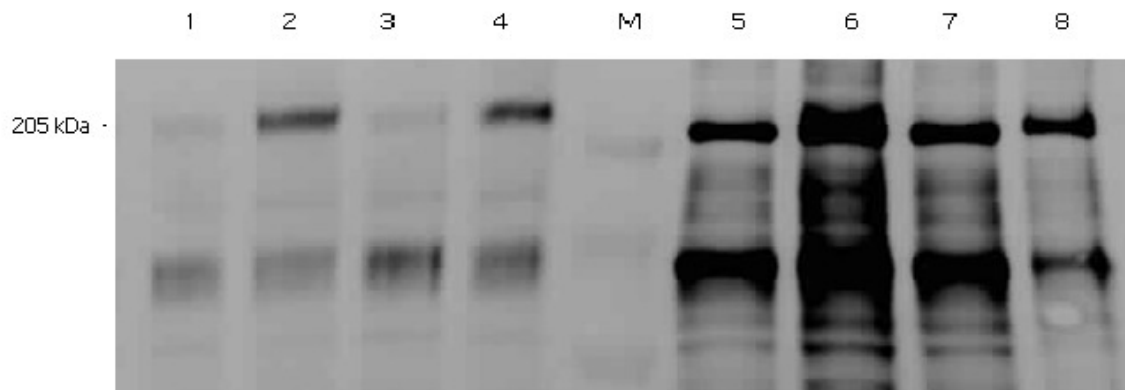


Figure 14. PY99 antibody western blot , showing the amount of PDGF phosphorylation in the samples after PKC  $\delta$  siRNA knockdown. Where lane 1 and 2 are control siRNA, un-stimulated and PDGF-BB ligand stimulation (10 minutes) respectively. Lane 3 and 4 are PKC  $\delta$  siRNA un-stimulated and PDGF-BB ligand stimulation (10 minutes) respectively. Lanes 5-8 contain the total cell lysate equivalent.

←205kDa

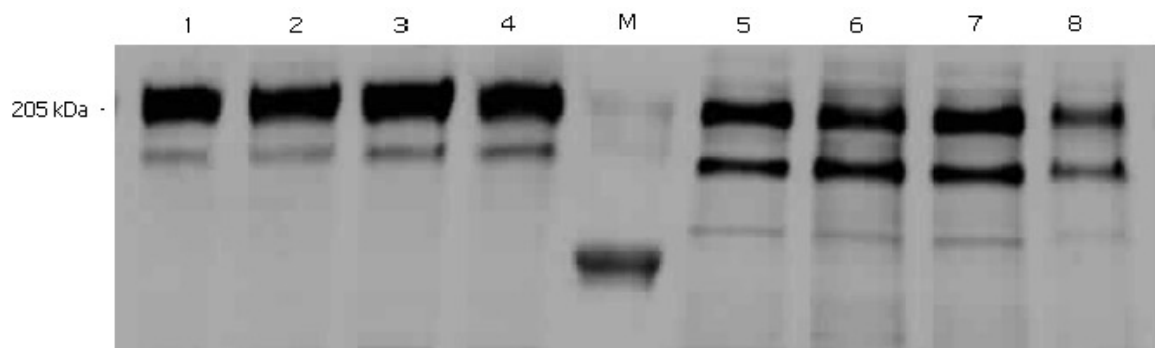


Figure 15. CTB antibody western blot, showing the amount of PDGF present in the samples after PKC  $\delta$  siRNA knockdown. Where lane 1 and 2 are control siRNA, un-stimulated and PDGF-BB ligand stimulation (10 minutes) respectively. Lane 3 and 4 are PKC  $\delta$  siRNA un-stimulated and PDGF-BB ligand stimulation (10 minutes) respectively. Lanes 5-8 contain the total cell lysate equivalent.

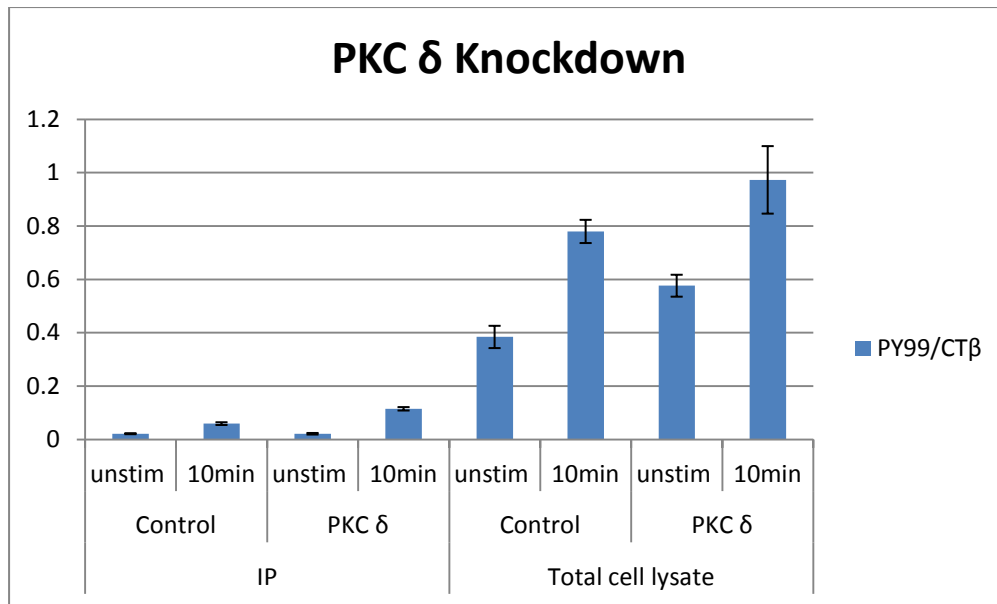


Figure 16. Western blot data showing the amount of PDGF receptor expression; from left to right, IP samples: control siRNA and PKC  $\delta$  siRNA and total cell lysate sample of the control and PKC  $\delta$  knockdown. Error bars: standard deviation.

## NHERF-2

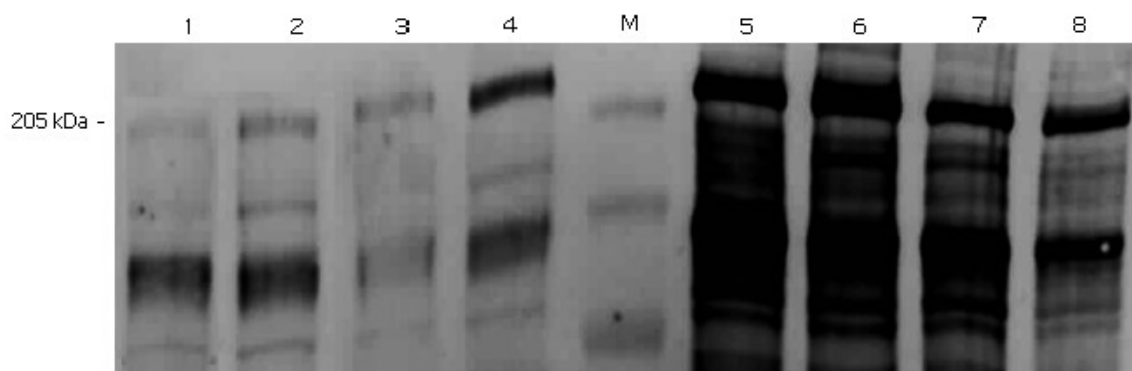


Figure 17. PY99 antibody western blot, showing the amount of PDGF phosphorylation in the samples after NHERF-2 siRNA knockdown. Where lane 1 and 2 are control siRNA, un-stimulated and PDGF-BB ligand stimulation (10 minutes) respectively. Lane 3 and 4 are NHERF-2 siRNA un-stimulated and PDGF-BB ligand stimulation (10 minutes) respectively. Lanes 5-8 contain the total cell lysate equivalent.

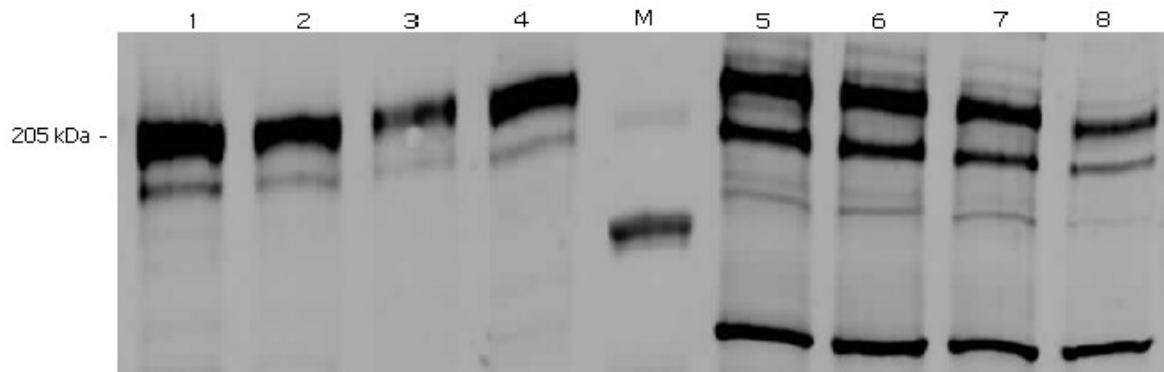


Figure 18. CT $\beta$  antibody western blot, showing the amount of PDGF present in the samples after NHERF-2 siRNA knockdown. Where lane 1 and 2 are control siRNA, un-stimulated and PDGF-BB ligand stimulation (10 minutes) respectively. Lane 3 and 4 are NHERF-2 siRNA un-stimulated and PDGF-BB ligand stimulation (10 minutes) respectively. Lanes 5-8 contain the total cell lysate equivalent.

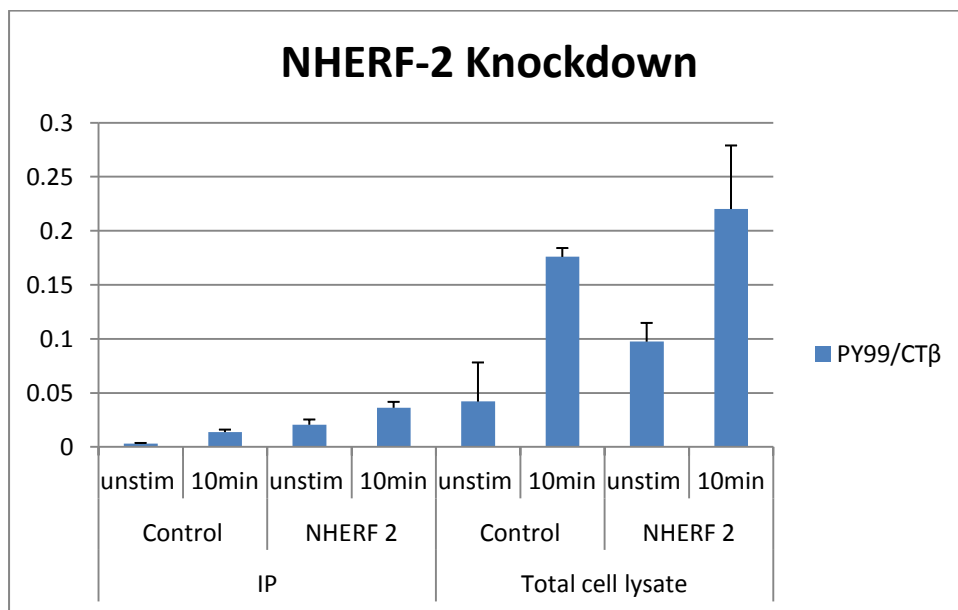


Figure 19. Western blot data showing the amount of PDGF receptor expression; from left to right, IP samples: control siRNA and NHERF-2 siRNA and total cell lysate sample of the control and NHERF-2 knockdown. Error bars: standard deviation

## NHERF-1

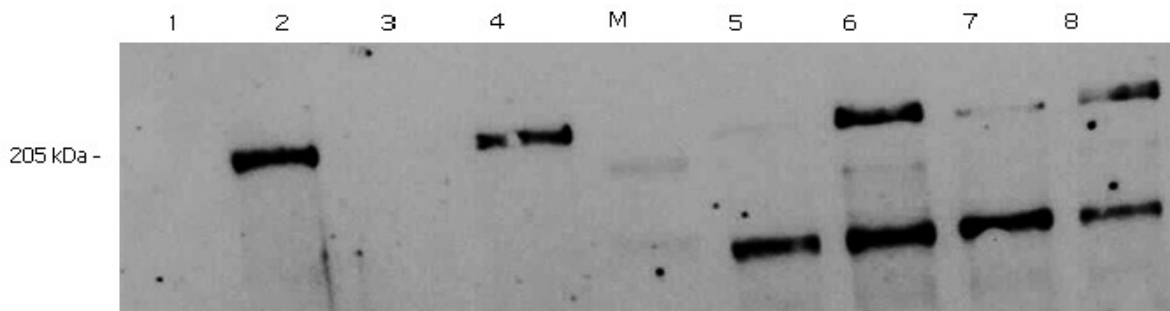


Figure 20. PY99 antibody western blot, showing the amount of PDGF phosphorylation in the samples after NHERF-1 siRNA knockdown. Where lane 1 and 2 are control siRNA, un-stimulated and PDGF-BB ligand stimulation (10 minutes) respectively. Lane 3 and 4 are NHERF-1 siRNA un-stimulated and PDGF-BB ligand stimulation (10 minutes) respectively. Lanes 5-8 contain the total cell lysate equivalent

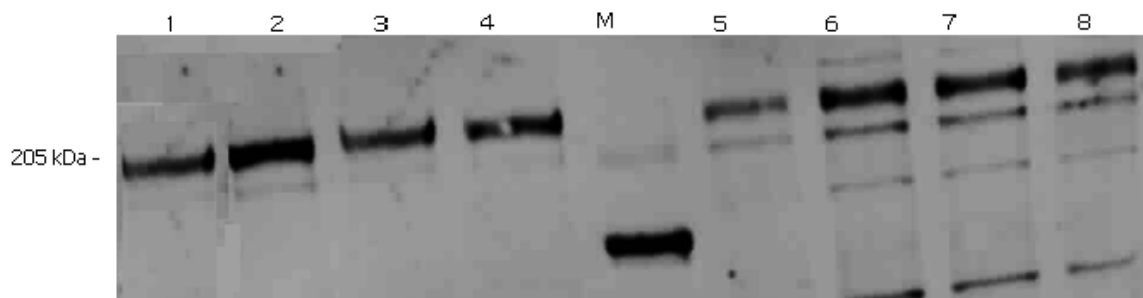


Figure 21. CT $\beta$  antibody western blot, showing the amount of PDGF present in the samples after NHERF-1 siRNA knockdown. Where lane 1 and 2 are control siRNA, un-stimulated and PDGF-BB ligand stimulation (10 minutes) respectively. Lane 3 and 4 are NHERF-1 siRNA un-stimulated and PDGF-BB ligand stimulation (10 minutes) respectively. Lanes 5-8 contain the total cell lysate equivalent.

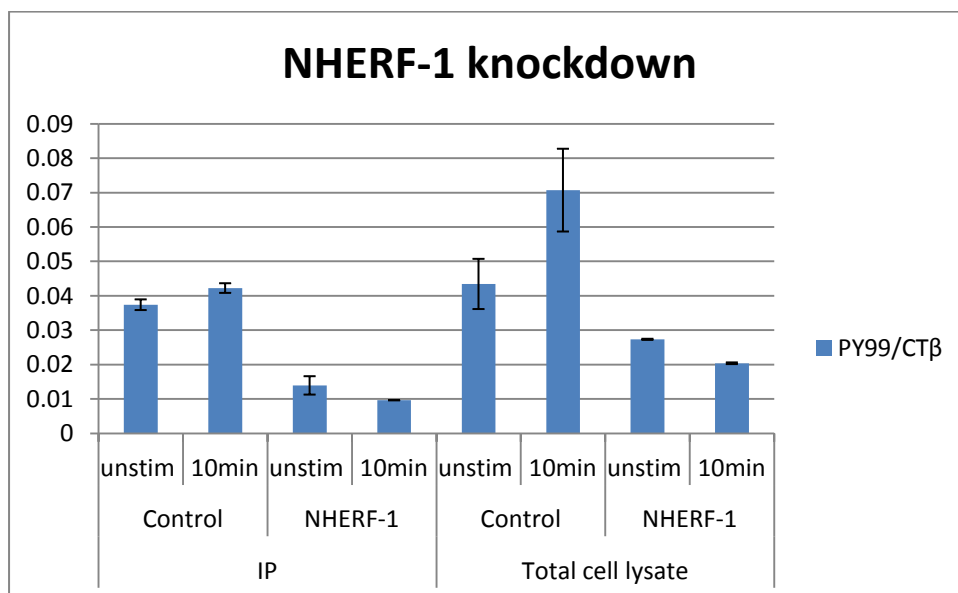


Figure 22. Western blot data showing the amount of PDGF receptor expression; from left to right, IP samples: control siRNA and NHERF-1 siRNA and total cell lysate sample of the control and NHERF-1 knockdown. Error bars: standard deviation.

The data obtained from western blot analysis clearly shows that the siRNA knockdown of PKC  $\delta$  has a significant effect upon the PDGF receptor. The results suggest that when PKC  $\delta$  is inhibited (a successful knockdown) it has a positive effect on PDGF receptor expression and phosphorylation in LAR  $\Delta$ P cells; figure 14 clearly shows a higher amount of PDGF receptor phosphorylation in the PDGF-BB stimulated sample (lane 4) in comparison to the stimulated control siRNA sample (lane 2). This result can be further determined with band quantification (whereby phosphorylated PDGF receptor (PY99) data is divided by the amount of receptor present (CT $\beta$ )). Figure 16 clearly shows the effect that the knockdown of PKC  $\delta$  has upon the PDGF receptor; whereby the PDGF receptor expression is more than doubled (roughly 52%) in IP samples where PKC  $\delta$  (stimulated) has been inhibited; in comparison to the control siRNA (stimulated). This pattern is also closely resembled in the total cell lysate samples where there is a 20% increase, as seen in figure 16.

Western blot analysis also showed that the siRNA knockdown of NHERF-2 equally had a significant effect upon the PDGF receptor. As with the PKC  $\delta$  knockdown, results suggest that when the NHERF-2 structural protein is inhibited it leads to a positive effect on PDGF receptor phosphorylation and expression in LAR  $\Delta$ P cells. This is shown in figure 17 where there is a distinctive increase in PDGF phosphorylation in stimulated samples treated with NHERF-2 knockdown siRNA (lane 4), in comparison to that seen in the control samples (lane 2). This result can be further determined with band quantification (whereby phosphorylated PDGF receptor (PY99) data is divided by the amount of receptor present (CT $\beta$ )). Figure 19 clearly shows the effect that the knockdown of NHERF-2 has upon the PDGF receptor; whereby the expression of the PDGF is more than doubled (roughly 62%) in IP samples where NHERF-2 (stimulated) has been inhibited; in comparison to the control siRNA (stimulated). This can also be seen with the total cell lysate samples where there is a 20% increase, as seen in figure 19.

Western blot analysis for NHERF-1 siRNA knockdown also shows that the protein plays a major role relating to PDGF receptor expression in LAR  $\Delta$ P cells. The data suggests that when NHERF-1 is inhibited it leads to a negative effect upon PDGF receptor phosphorylation and expression. Figure 20 shows that difference in PDGF receptor phosphorylation in control and NHERF-1 knockdown samples; the amount of phosphorylation in NHERF-1 siRNA



samples is visibly lower in comparison to that of the control (lanes 4 and 2 respectively). This result can be further determined with band quantification (whereby phosphorylated PDGF receptor (PY99) data is divided by the amount of receptor present (CT $\beta$ )). Figure 22 shows conclusively the effect of the inhibition of NHERF-1 has upon the PDGF receptor expression; whereby the expression is significantly reduced, roughly 77% in IP samples where NHERF-1 is inhibited compared to control siRNA. This pattern is repeated with total cell lysate samples where there is a reduction of 71% as seen in figure 22.

### 3.5. Downstream protein signalling pathway expression

Successful siRNA knockdowns of PKC  $\delta$ , NHERF-1 and NHERF-2 were further analysed; ERK a downstream signalling protein was analysed to see if the siRNA knockdown on the PDGF receptor contributed to further changes.

Total cell lysate from samples that had achieved a successful knockdown were loaded into a 10% gel. The membrane was initially blotted with anti- phosphatase ERK  $\frac{1}{2}$  (p-ERK) antibody (rabbit ab). This antibody detects phosphorylated ERK molecules present within the sample. The membrane was stripped and re-blotted with anti ERK  $\frac{1}{2}$  Protein (rabbit) which detects the total amount of ERK proteins present.

#### pERK

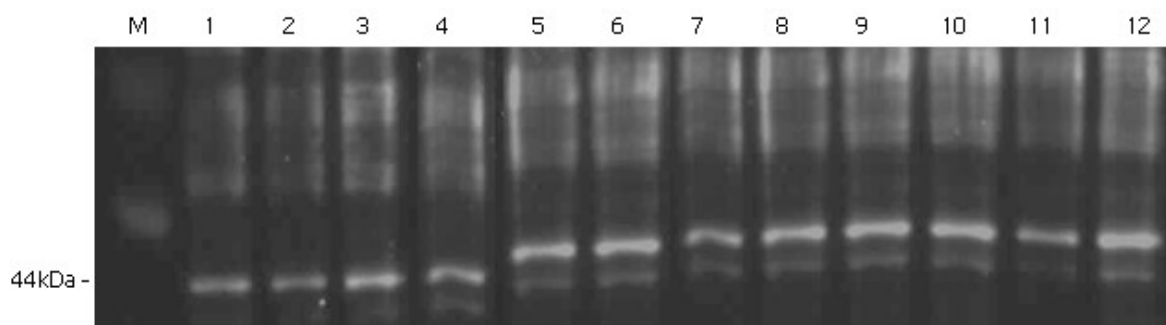


Figure 23. pERK antibody western blot shows the amount of downstream receptor present within the total cell lysate samples (double band). Lanes 1-4 show PKC  $\delta$ , 5-8 NHERF-1 and 9-12 NHERF-2. For each set of total cell lysates from left to right: 1-control siRNA, 2- control siRNA + stimulation, 3- active siRNA, 4- active siRNA + stimulation.

## ERK

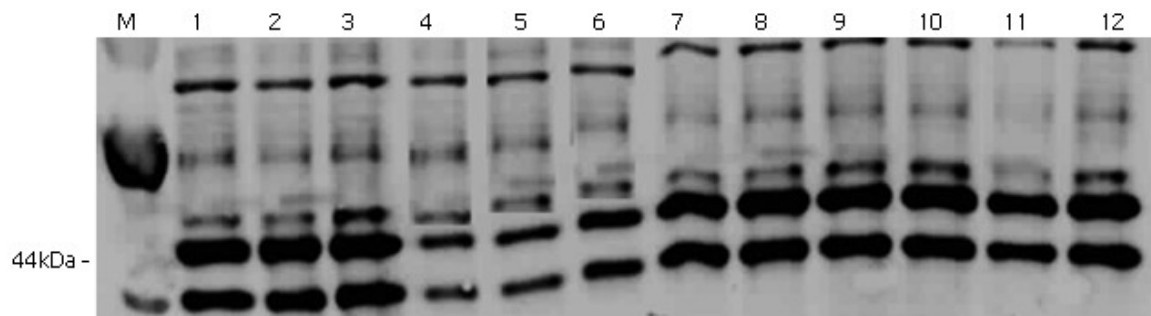


Figure 24. ERK antibody western blot shows the amount of downstream receptor present within the total cell lysate samples (double band). Lanes 1-4 show PKC  $\delta$ , 5-8 NHERF-1 and 9-12 NHERF-2. For each set of total cell lysates from left to right: 1-control siRNA, 2- control siRNA + stimulation, 3- active siRNA, 4- active siRNA + stimulation.

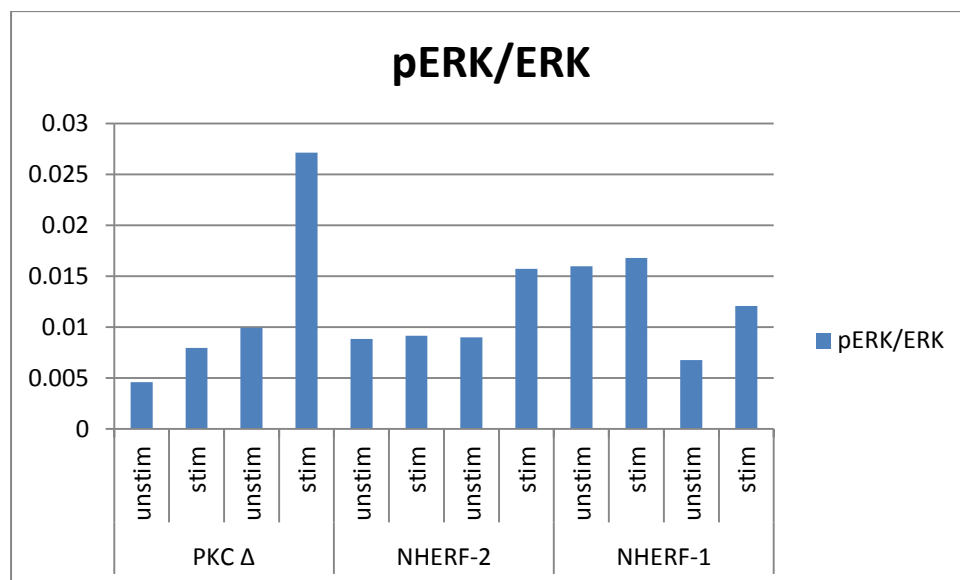


Figure 25. Western blot data showing the amount of ERK protein expression in successful siRNA knockdowns of PKC $\delta$  NHERF-2 and NHERF-1. From left to right, total cell lysate samples: control –(un-stimulated and stimulated), and siRNA (un-stimulated and stimulated).

Western blot analysis confirms there are differences with ERK expression when PKC  $\delta$ , NHERF-2 and NHERF-1 proteins are inhibited. Figure 23 and 24 show the amount of ERK phosphorylation and the total amount of ERK present in each sample; further analysis through band quantification clearly shows that there is a significant difference when PKC  $\delta$  and NHERF-2 are knocked down. This can be seen in figure 25, which shows the amount of

ERK being expressed in each sample; when PKC  $\delta$  is inhibited there is a significant increase in ERK expression; data shows a 70% increase in the stimulated sample when compared to that of the control siRNA stimulated sample. The same pattern is seen too with NHERF-2 knockdown, whereby there is an increase of roughly 42%, figure 25 shows this data; however the change in ERK expression is not as significant as with PKC  $\delta$ . NHERF-1 knockdown however follows the same pattern as seen with PDGF expression; after a successful knockdown of NHERF-1 there is a decrease in ERK expression. Data shows that there is a 28% decrease in ERK expression when compared to the stimulated control siRNA sample; this can be clearly seen in figure 25.

#### **4.0. Discussion**

Immunoprecipitates were obtained from both mouse embryonic fibroblast (MEF) wild type cells and LAR  $\Delta P$  cells; the PDGF protein was isolated while other proteins present were precipitated from the sample. The samples were subjected to western blot analysis to determine which IP method gave the best PDGF receptor signal. CT $\beta$  antibody, CT $\beta$  serum and WGA beads were investigated according to a set protocol (as stated above).

The results obtained from western blot analysis show that CT $\beta$  antibody and WGA beads gave the highest PDGF receptor signal; figure 5 shows PDGF receptor phosphorylation in IP samples that have been treated with PY99 antibody, an anti-phosphotyrosine antibody. It can be clearly seen that PDGF receptor phosphorylation is greater in both CT $\beta$  antibody and WGA IP samples in comparison to the CT $\beta$  serum IP. This pattern was further repeated when IP samples were treated again with CT $\beta$  antibody (an anti PDGFR antibody), whereby there was a greater amount of PDGF receptor present in CT $\beta$  antibody and WGA IP samples. These two methods of collecting IPs were then utilized in later assays.

The effect of PMA upon the PDGF receptor was investigated to determine the outcome of the signal transduction enzyme protein kinase C (PKC), has upon the expression of the PDGF receptor. PMA is a diester of phorbol which is regularly used in the activation of PKC.

It is hypothesised that PKC ( $\delta$ ) regulates PDGF receptor activation through inhibiting phosphorylation of the tyrosine residues.

MEF wild type and LAR  $\Delta$ P cells were treated with PMA (and DMSO as a control); samples treated with PMA overnight resulted in degraded PKC thus no active kinases. Western blot analysis was performed on the samples to determine the effect that the active PKC signalling pathway had upon the PDGF receptor expression in both cell types.

The results obtained from western blot analysis show that PDGF receptor phosphorylation is increased in both MEF WT and LAR  $\Delta$ P cells when treated with PMA, in comparison to samples treated with DMSO. Figure 6 shows PDGF receptor phosphorylation, it can be clearly seen that there is a increase in receptor phosphorylation in WT cells when treated with PMA; more significant however is the increase in the receptor phosphorylation in LAR  $\Delta$ P cells when treated with PMA compared to that of DMSO treated samples. This pattern is repeated when samples are stimulated with PDGF-BB ligand; an increase in PDGF phosphorylation is still present in both cell types. Figure 7 shows samples treated with CT $\beta$  antibody; the amount of PDGF receptor present in samples treated with PMA appears less affected when compared to the DMSO samples. This suggests that the PMA does not have an effect upon amount of receptor expressed in each sample; however PMA is seen to activate the phosphorylation and thus activation of the PDGF receptor.

This somewhat suggests that active PKC, regulates the PDGF  $\beta$ -receptor through inhibiting phosphorylation of the tyrosine residues due to serine phosphorylation thus inhibiting full  $\beta$ -receptor activation. Moreover the significant difference seen with LAR  $\Delta$ P cells in comparison to wild type cells (when treated with PMA) suggests that LAR plays an inhibitory role with blocking PKC activation; possibly through the mechanism of down regulating c-Abl activity which has a 'knock on' effect with PKC activation, thus allowing for PDGF  $\beta$ -receptor activation. The PMA experiment should be repeated to determine the results obtained.

siRNA optimisation was performed on LAR  $\Delta$ P MEF cells in order to analyse optimal conditions for the knockdown of PKC  $\delta$ , NHERF-1 and NHERF-2 proteins.

Once optimal conditions had been determined (stated above) siRNA knockdown was performed on LAR  $\Delta$ P MEF cells, the knockdown of PKC  $\delta$ , NHERF-1 and NHERF-2 proteins were all analysed to investigate and measure any effect upon the PDGF receptor with respects to expression and phosphorylation. Immunoprecipitates from samples were collected and subjected to western blot analysis.

The results obtain from the analysis showed that PDGF receptor phosphorylation is increased with the knockdown of PKC  $\delta$ ; this can be seen in figure 14; which shows the knockdown of samples treated with PCK  $\delta$ , compared to samples treated with control siRNA. Western blot analysis illustrated that when samples were stimulated with PDGF –BB ligand that PDGF receptor phosphorylation was greater when PCK  $\delta$  had been knocked down (figure 14). This pattern was not seen however when the total amount of PDGF receptor was investigated, using CT $\beta$  antibody. Figure 15 shows samples from both PCK  $\delta$  and control siRNA, it can be clearly seen that in both IP and total cell lysate samples that the amount of PDGF receptor present has no significant differences between PCK  $\delta$  and control siRNA samples.

Figure 16 additionally highlights this concept whereby the result of the knockdown of PKC  $\delta$  upon the PDGF receptor can be clearly seen. The PDGF receptor expression is more than doubled (roughly 52%) in IP samples when PKC  $\delta$  (stimulated) has been inhibited; in comparison to the control siRNA. This pattern is also closely resembled in the total cell lysate samples where there is a 20% increase (figure 16).

The results further suggest that inhibiting the activation of PKC  $\delta$  increase the phosphorylation of the PDGF  $\beta$ -receptor; and that once active, PKC regulates the PDGF  $\beta$ -receptor through inhibiting phosphorylation of the receptor which inhibits full activation.

It has been proposed (Yuan *et al.*, 1998; Krasel *et al.*, 2001; Wu *et al.*, 2005) that c-Abl activates the signal transduction enzyme protein kinase C (PKC), once active PKC then activates the G protein-coupled receptor kinase-2 (GRK2), which has been shown to be a substrate for PKC (Krasel *et al.*, 2001). PKC phosphorylation of the serine residues of GRK2 increases its activity; which has been suggested due to the relief of tonic inhibition by calmodulin (Krasel *et al.*, 2001). GRK2 has been shown to phosphorylate the serine residues

on the PDGF  $\beta$ -receptor which in turn diminishes the signalling by the receptor (Wu *et al.*, 2005).

The results obtain from the PKC  $\delta$  knockdown further conclude that shown with the PMA assays, in that the inhibition of PKC  $\delta$  in turn leads to an increase in PDGF  $\beta$ - receptor phosphohrylation in LAR  $\Delta$ P cells; this suggests LAR plays an inhibitory role blocking PKC activation through the mechanism of down regulating c-Abl activity allowing for PDGF  $\beta$ -receptor activation. siRNA knockdown of PKC  $\delta$  should therefore be further tested on wild type MEF cells to confirm this hypothesised mechanism.

The results attained from the analysis also showed that the PDGF receptor phosphorylation is increased with the knockdown of NHERF2; this can be seen in figure 17; which shows the knockdown of samples treated with NHERF2, compared to those treated with control siRNA. Western blot analysis illustrated that when samples were stimulated with PDGF –BB ligand the PDGF receptor phosphorylation was significantly greater when NHERF2 had been knocked down (figure 17). This pattern was not seen however when the total amount of PDGF receptor was investigated. Figure 18 shows samples from both NHERF2 and control siRNA, it can be clearly seen that in both IP and total cell lysate samples that the amount of PDGF receptor present has no significant differences between samples treated with NHERF2 siRNA and control siRNA.

PDGF receptor phosphorylation was more than doubled (roughly 62%) in IP samples that had been stimulated by PDGF-BB ligand where NHERF2 had been knocked down (figure 19); compared to that of the control. A 20% increase in PDGF expression was also seen in total cell lysate samples. The data suggests that NHERF2 has an inhibitory effect upon PDGF receptor activation when expressed in cells. It has been suggested that NHERF2 binds directly to the PDGF receptor via the carboxyl terminus (Maudsley *et al.*, 2000).

The results obtained from Western blot analysis for NHERF-1 siRNA knockdown also show that the protein plays a major role relating to PDGF receptor expression in LAR  $\Delta$ P cells. The data shows that samples treated with NHERF-1 knockdown siRNA had less PDGF  $\beta$ -receptor phosphorylation compared to control siRNA (figure 20). Again there is little difference in the total amount of PDGF receptor present, as seen with CT $\beta$  antibody (figure 21). The results obtained are illustrated in figure 20, where the effect of the inhibition of NHERF-1 upon the PDGF receptor is further determined; roughly a 77% reduction in PDGF receptor expression

in IP samples with the knockdown of NHERF1 in comparison to control siRNA. A reduction of 71% is also seen in total cell lysate samples.

This data suggests that NHERF1 has a crucial role in PDGF receptor activation. The results obtained can be further determined as it has been previously suggested (Maudsley *et al.*, 2000) that the Na<sup>+</sup>/H<sup>+</sup> exchanger regulatory factor (NHERF1) protein interacts with the PDGF receptor through the binding via the carboxyl terminus; it has been shown to have a high binding affinity with the PDZ domain (Lau *et al.*, 2001). The binding of NHERF1 to the PDGF receptor potentiates the autophosphorylation of the PDGF receptor which leads to the dimerization and full activation of the receptor; which in turn leads to the activation of downstream signal regulated kinase's such as the MAPK pathway. It has been suggested that the potential ability for NHERF1 to increase PDGF receptor activity and signalling is dependant upon the ability for NHERF1 to oligomerize; this has been shown to occur when NHERF1 is bound via the carboxyl terminus (PDGF-CT) (Maudsley *et al.*, 2000). It has also been suggested that NHERF2 also actively binds to the PDGF-CT; this results in no stimulatory signal for the autophosphorylation of the PDGF receptor thus reducing receptor tyrosine kinase activity, when NHERF2 is over expressed in cells.

The findings reveal that NHERF1 and NHERF2 have the ability to bind directly to the PDGF receptor, thus leading to the increase or decrease respectively in receptor autophosphorylation and activity; presenting a novel mechanism in which the activity of the PDGF receptor is regulated.

As stated above the G protein-coupled receptor kinase-2 (GRK2) has the ability to phosphorylate serine residues on the PDGF  $\beta$ -receptor leading to the desensitisation of the receptor. It has been stated (Hildreth *et al.*, 2004) that GRK 2 over expression can also significantly reduce the binding association between PDGF  $\beta$ -receptor and NHERF1 due to the serine residue phosphorylation. This suggests that GRK2 not only desensitises the PDGF  $\beta$ -receptor it also plays a crucial role in dissociating NHERF1 from the receptor thus diminishing autophosphorylation and receptor activation. GRK2 could also be associated with increased NHERF2 expression which further inhibits PDGF receptor dimerization and activation (Zheng, *et al.*, 2011).

siRNA knockdown of both NHERF1 and NHERF2 should be carried on in wild type cells to determine the effect LAR has upon the signalling mechanism.

Successful siRNA knockdown of PKC  $\delta$ , NHERF1 and NHERF 2 was further investigated by analysing the downstream signalling protein ERK. Western blot analysis shows that there are differences with ERK expression when PKC  $\delta$ , NHERF-2 and NHERF-1 proteins are inhibited. When PKC  $\delta$  is inhibited there is a significant increase in ERK expression; data shows a 70% increase in the stimulated sample when compared to that of the control siRNA stimulated sample (figure 25). An increase in ERK expression is also seen with successful NHERF-2 knockdown, whereby there is an increase of roughly 42% (figure 25); however the change in ERK expression is not as significant as with PKC  $\delta$ . NHERF-1 knockdown showed there is a decrease in ERK expression. Data shows that there is a 28% decrease in ERK expression when compared to the stimulated control siRNA sample (figure 25); suggesting the potential ability for NHERF1 to increase PDGF receptor activity is also crucial for downstream signalling.

By inhibiting PKC  $\delta$  through siRNA knockdown it in turn leads to the diminishing of GRK2 activation. The data suggests that a loss of GRK2 activity, by which the PDGF  $\beta$ -receptor becomes desensitised due to serine phosphorylation, results in an increase in MAP kinase pathway. The results therefore show that GRK2 plays a critical regulatory role not only in reducing the activity of the PDGF  $\beta$ -receptor but also in key downstream signalling pathways, including the MAP kinase pathway. As expected NHERF2 also regulates downstream signalling by inhibiting PDGF receptor dimerization reducing its full activation; however the due to the significant effect seen by the reduction in GRK 2, could suggest that GRK2 also regulate down downstream molecules through the inhibition of other signals. It has been suggested (Carmen, *et al.*, 2006) that GRK2 regulates the activity of ERK through either a direct or coordinate interaction with the mitogen-activated protein kinase kinase (MEK). Therefore GRK2 also has a crucial role in controlling ERK activation as well as regulating the PDGF  $\beta$ -receptor.



## 4.1. Conclusion

The data obtained from the research study has shown that the knockdown of PKC  $\delta$ , NHERF1 and NHERF2 affects the phosphorylation and activity of the PDGF receptor in cells where the LAR phosphatase domains have been deleted. The knockdown of PKC  $\delta$  and NHERF2 was shown to increase PDGF receptor phosphorylation, suggesting that PKC  $\delta$  plays an inhibitory role in the activation of the receptor; whereas the knockdown of NHERF1 had a detrimental effect upon receptor activation suggesting NHERF1 is crucial for PDGF receptor dimerization.

The results further determine the hypothesis that LAR regulates the cytoplasmic tyrosine kinase c-Abl; with the deletion of LAR there is no down regulation of c-Abl, which can therefore lead to activation of the signal transduction enzyme protein kinase C (PKC). PKC activates the G protein-coupled receptor kinase-2 (GRK2), a substrate for PKC; which then phosphorylates the serine residues on the PDGF  $\beta$ -receptor, in turn desensitises the receptor and diminishes signalling. GRK2 over expression has also been associated with the significant reduction in the binding association between PDGF  $\beta$ -receptor and NHERF1; dissociating NHERF1 from the receptor thus diminishing autophosphorylation and receptor activation. The results also show that when PKC  $\delta$  is knocked down there is a significant increase in activity in the downstream signalling protein ERK; a greater increase seen than in NHERF2 knockdown. This further gives evidence to the suggestion that GRK2 regulates the activity of ERK through either a direct or coordinate interaction with the mitogen-activated protein kinase kinase (MEK). Therefore GRK2 also has a crucial role in controlling ERK activation as well as negatively regulating the PDGF  $\beta$ -receptor.

The data obtained facilitates the novel mechanism that LAR helps to regulate PDGF receptor signalling by ligand induced activation of the PDGF  $\beta$ -receptor, through its mechanism of down regulating c-Abl activity. This inhibits the activation of PKC  $\delta$  and in turn GRK2, which desensitises PDGF  $\beta$  receptor activation and downstream signalling pathways; as well as dissociating NHERF1 and promoting NHERF2 binding to the PDGF receptor further diminishing receptor dimerization.

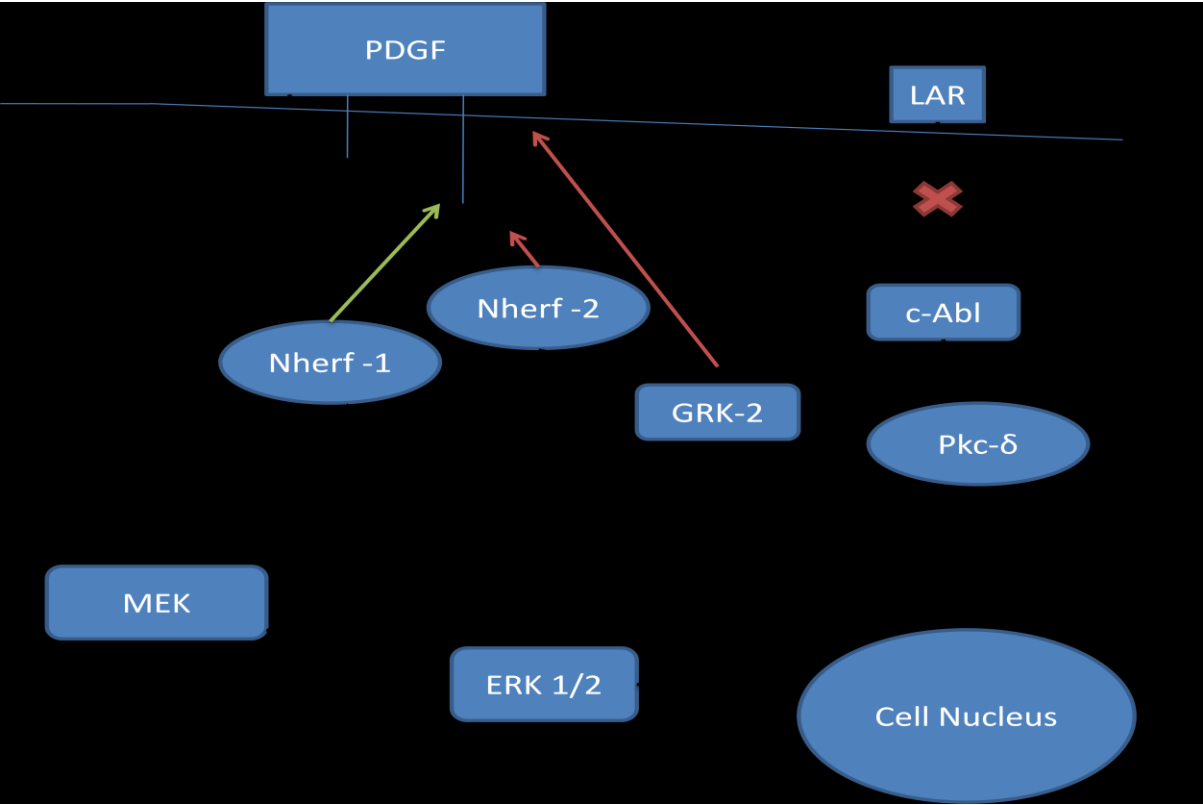


Figure 26: The mechanism of LAR upon PDGF receptor signalling

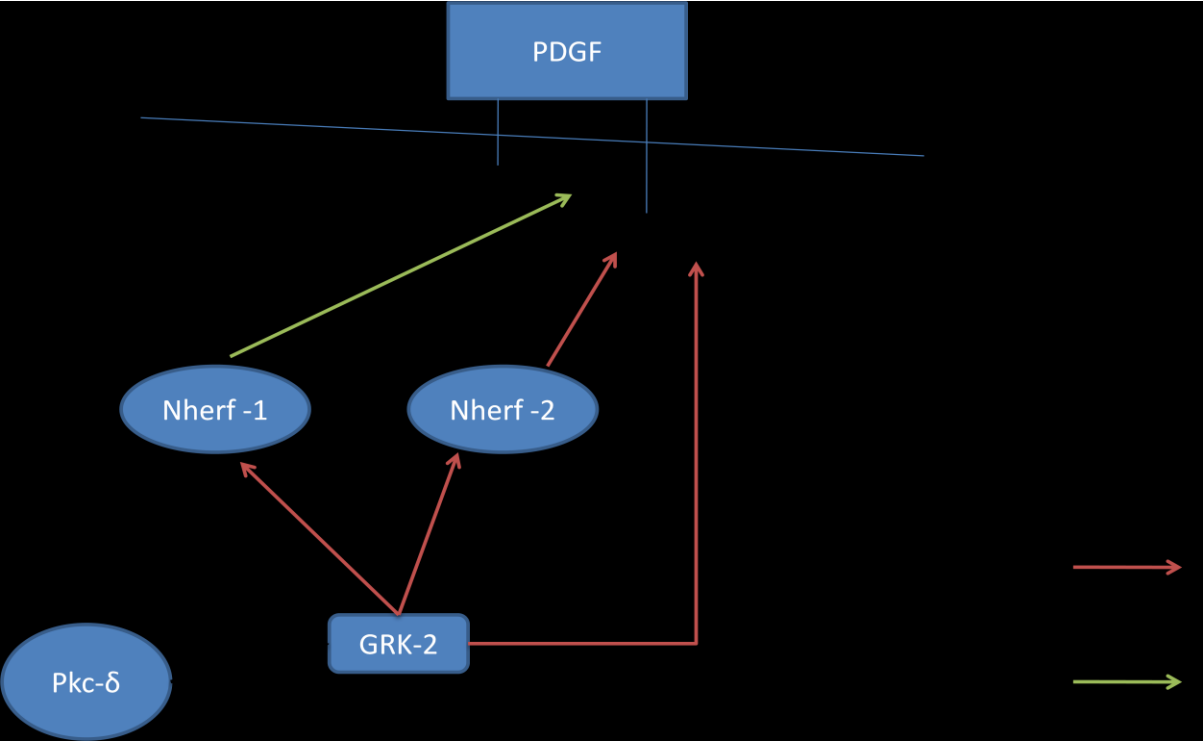


Figure 27: The mechanism of PKC-δ and GRK2 upon PDGF-β receptor activation and signalling

## 5.0. References

1. Adachi, M., Imai, K., (2002) The proapoptotic BH3-only protein BAD transduces cell death signals independently of its interaction with Bcl-2. **Cell death and differentiation** 9 (11): 1240–1247
2. Akimoto, K., Takahashi, R., Moriya, S. et al (1996) EGF or PDGF receptors activate atypical PKC lambda through phosphatidylinositol 3-kinase. **EMBO J**; 15: 788-798
3. Alonso, G., Koegl, M., Mazurenko, N. et al (1995) Sequence requirements for binding of Src family tyrosine kinases to activated growth factor receptors, **J. Biol. Chem.** 270
4. Backer, J., Myers Jr. M., Shoelson, S. et al (1992) Phosphatidylinositol 3P-kinase is activated by association with IRS-1 during insulin stimulation, **EMBO J.** 11 3469-3479.
5. Blake, R., Garcia-Paramio, P., Parker, P. et al (April 1999). Src promotes PKCdelta degradation. **Cell Growth Differ.** 10 (4): 231–41
6. Burgering, B., Coffey, P., (1995)Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction, **Nature**; 376: 599-602.
7. Carmen, J., Cristina, M., Annemieke, K. et al (2006) G Protein-coupled Receptor Kinase 2 Negatively Regulates Chemokine Signaling at a Level Downstream from G Protein Subunits. **Molecular Biology of the Cell** Vol. 17, 25–31
8. Castagna, M. et al (1982). **Journal of Biological Chemistry** 257 (13): 7847–7851.
9. Chagnon, M., Uetani, N., Tremblay, M., (2004) Functional significance of the LAR receptor protein tyrosine phosphatase family in development and diseases. **Biochem. Cell Biol.** 82
10. Cheatham, B., Vlahos, C., Cheatham, L., et al (1994) Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation, **Mol. Cell. Biol.** 14 4902-4911
11. Chen, H., Gu X, Liu Y., Wang, J., et al (2011) PDGF signalling controls age-dependent proliferation in pancreatic  $\beta$ -cells, **Nature**. 12;478(7369):349-55. doi: 10.1038/nature10502.
12. Claesson-Welsh, L. (1994) Platelet-derived growth factor receptor signals, **J. Biol. Chem**; 269: 32023-32026.
13. Erpel, T., Courtneidge, S.A. (1995) Src family protein tyrosine kinases and cellular signal transduction pathways. **Curr. Opin. Cell Biol.** 7; 176-182
14. Evron, T., Daigle, T., Caron, M. (2012). GRK2: multiple roles beyond G protein-coupled receptor desensitization. **Trends Pharmacol. Sci**; 33 (3): 154-64. doi:10.1016/j.tips.2011.12.003
15. Farago, B., Li, J., Cornilescu, G. et al (2010) Activation of Nanoscale Allosteric Protein Domain Motion Revealed by Neutron Spin Echo Spectroscopy. **Biophysical Journal** 99 (10): 3473–3482. Bibcode:2010BpJ....99.3473F.doi:10.1016/j.bpj.2010.09.058

16. Hall, R., Ostedgaard, L., Premont, R. et al (1998) C-terminal motif found in the beta2-adrenergic receptor, P2Y1 receptor and cystic fibrosis transmembrane conductance regulator determines binding to the Na<sup>+</sup>/H<sup>+</sup> exchanger regulatory factor family of PDZ proteins. **Natl. Acad. Sci.** 95 (15): 8496–501. doi:10.1073/pnas.95.15.8496.
17. Heldin, C., Ostman, A., Eriksson, A. et al (1992) Platelet-derived growth factor: isoform-specific signalling via heterodimeric or homodimeric receptor complexes. **Kidney Int.** 41 (3): 571–4.
18. Heldin, C., Ostman, A., Ronnstrand, L. (1998) Signal transduction via platelet-derived growth factor receptors. **Biochim. Biophys. Acta** 1378
19. Heldin, C., Westermark, B., (1996) Role of platelet-derived growth factor in vivo. **The Molecular and Cellular Biology of Wound Repair**, 2nd edn, pp. 249-273.
20. Hildreth, K., Wu, J., et al (2004) Phosphorylation of the platelet-derived growth factor receptor-beta by G protein-coupled receptor kinase-2 reduces receptor signaling and interaction with the Na(+)/H(+) exchanger regulatory factor. **J Biol Chem.** 1;279(40):41775-82..
21. Huppi, K., Siwarski, D., Goodnight, J. (June 1994). Assignment of the protein kinase C delta polypeptide gene (PRKCD) to human chromosome 3 and mouse chromosome 14. **Genomics** 19 (1): 161–2. doi:10.1006/geno.1994.1028
22. Kazlauskas, A., Cooper, J.A. (1989) Autophosphorylation of the PDGF receptor in the kinase insert region regulates interactions with cell proteins. **Cell** 58; 1121-1133
23. Keshet, Y., Seger, R. (2010) The MAP Kinase Signaling Cascades: A System of Hundreds of Components Regulates a Diverse Array of Physiological Functions. **Methods in Molecular Biology** Volume 661, pp 3-38
24. Klippel, A., Kavanaugh, W., Pot, D. et al (1997) A specific product of phosphatidylinositol 3-kinase directly activates the protein kinase Akt through its pleckstrin homology domain. **Mol. Cell. Biol.** 17 338-344.
25. Krasel, C., Dammeier, S., Winstel, R., et al (2001) Phosphorylation of GRK2 by protein kinase C abolishes its inhibition by calmodulin J Biol Chem. 19;276(3):1911-5.
26. Kypta, R., Goldberg, Y., Ulug, E. et al (1990) Association between the PDGF receptor and members of the src family of tyrosine kinases, *Cell* 62 481-492
27. Lau, A., Hall, R. (2001) Oligomerization of NHERF-1 and NHERF-2 PDZ Domains: Differential Regulation by Association with Receptor Carboxyl-Termini and by Phosphorylation, **Biochemistry.** 40 (29), pp 8572–8580 DOI: 10.1021/bi0103516
28. Ligaya, L., Stice, C., Douglas, V. (1999) Regulation of Platelet-Derived Growth Factor Signalling by Activated p21. **Frontiers in Bioscience** 4, d72-86
29. Lopez-Ilasaca, M., Li, W., Uren, A. (1997) Requirement of phosphatidylinositol- 3 kinase for activation of JNK/SAPKs by PDGF. **Biochem. Biophys.** 232 273-277.
30. Maudsley, S., Musa, A., Blitzer, L. et al (2000) Platelet-Derived Growth Factor Receptor Association with Na<sup>+</sup>/H<sup>+</sup> Exchanger Regulatory Factor Potentiates Receptor Activity, **Mol. Cell. Biol.** vol. 20 no. 22 8352-8363
31. Mauro, L., Dixon, J. (1994) Zip codes' direct intracellular protein tyrosine phosphatases to the correct cellular address. **Trends Biochem. Sci.** 19, 151–155

32. Minzhou Huang, L., Satchell, J., DuHadaway, G. (2011) RhoB links PDGF signaling to cell migration by coordinating activation and localization of Cdc42 and Rac. Volume 112, Issue 6, p 1572–1584
33. Mooney, C. LeVea, C. (2003) **Med. Chem.** 3 (7) 809–819
34. Mooney, R., Kulas, D., Bley, L. et al (1997). The Protein Tyrosine Phosphatase LAR Has a Major Impact on Insulin Receptor Dephosphorylation. **Biochem. Biophys. Research** 235,709–712
35. Moonhee, K., Jaehoon, J., Kyunglim, L (2009) Roles of ERK, PI3 kinase, and PLC- $\gamma$  pathways induced by overexpression of translationally controlled tumor protein in HeLa cells. **Biochemistry and Biophysics** Volume 485, Issue 1, Pages 82–87
36. Nanberg, E., Westermark, B. (1993) Platelet-derived growth factor increases the turnover of GTP/GDP on Ras in permeabilized fibroblasts, **J. Biol. Chem.** 268 18187-18194
37. Niu, J. Li, Z. Hakim, M. (2007) **J. Biol. Chem.** 282 (27) 19808–19819
38. Ostman, A., Hellberg, C., Bohmer, (2006) F. D., Protein-tyrosine phosphatases and cancer, **Nature** vol. 6
39. Östman, A., Hellberg, C., Böhmer, F (2006) Protein-tyrosine phosphatases and cancer. **Nature Reviews Cancer** 6, 307-320 doi:10.1038/nrc1837
40. Pawson, T., Scott, J.D. (1997) Signalling through scaffold, anchoring, and adaptor proteins. **Science** 278 2075-2080.
41. Persson, C., Savenhed, C., Bourdeau, A., et al (2004) Site-Selective Regulation of Platelet-Derived Growth Factor  $\beta$  Receptor Tyrosine Phosphorylation by T-Cell Protein Tyrosine Phosphatases **Mol. Cell. Biol.** 24 2190-2201
42. Pulido, R., Serra-Pages, C., Tang, M. et al (1995) The LAR/PTP delta? PTP sigma subfamily of transmembrane protein tyrosine-phosphatases: multiple human LAR, PTP delta, and PTP sigma isoforms are expressed in tissue-specific association with the LAR-interacting protein LIP-1. **Proc. Natl. Acad. Sci.**, 92 11686-11690
43. Robinson, D., Wu, Y., Lin, F. (2000). The protein tyrosine kinase family of the human genome. **Oncogene** 19 (49): 5548–5557.
44. Satoh, T., Fantl, W., Escobedo, J. et al (1993) Platelet-derived growth factor receptor mediates activation of Ras through different signaling pathways in different cell types, **Mol. Cell. Biol.** 13 3706-3713
45. Schlessinger, J. (1993) How receptor tyrosine kinases activate Ras. **Trends Biol. Sci.** 18 273-275
46. Schlessinger, J. (1994) SH2/SH3 Signaling Proteins. **Curr. Opin. Gen. Dev.** 4: 25-30
47. Sorkin, A., Westermark, B., Heldin, C (1991) Effect of receptor kinase inactivation on the rate of internalization and degradation of PDGF and the PDGF L-receptor, **J. Cell Biol.** 112 469-478.
48. Stover, D.R., Furet, P., Lydon, N (1996) Modulation of the SH2 binding specificity and kinase activity of Src by tyrosine phosphorylation within its SH2 domain, **J. Biol. Chem.** 271 (1996) 12481-12487.
49. Streuli, M (1996) **Curr. Opin. Cell Biol.** 8 182–188
50. Streuli, M., Krueger, N., Ariniello, P. (1992) **EMBO J.** 11 897-907
51. Szczylik, C., Skorski, T., Nicolaides, N. et al (1991). Selective inhibition of leukemia cell proliferation by BCR-ABL antisense oligodeoxynucleotides. **Science**; 253 (5019): 562-5. doi:10.1126/science.1857987

52. Toker, A., Meyer, M., Reddy, K.. et al (1994) Activation of protein kinase C family members by the novel polyphosphoinositides PtdIns-3,4-P2 and PtdIns-3,4,5-P3. **J. Biol. Chem.** 269
53. Tsujilawa, K., Ichijo, T., Moriyama, K. et al (2002) Regulation of Lck and Fyn tyrosine kinase activities by transmembrane protein tyrosine phosphatase leukocyte common antigen related molecule. **Mol Can** 155-163
54. Villari, G. (2009) Ras-Raf-MEK-ERK-MAPK pathway, <http://flipper.diff.org/app/pathways/1874>
55. Walter, F., Boron, P. (2003) Medical Physiology: A Cellular And Molecular Approach. Elsevier/Saunders. pp. 1300. ISBN 1-4160-2328-3.
56. Williams, L. (1989) Signal transduction by the platelet-derived growth factor receptor. **Science** 243 (4898): 1564–70.
57. Wills, J. Bateman, C. Korey, A. et al (1999) **Neuron** 22 (2) 301–312
58. Wu, J., Goswami, R., Kim, L. et al (2005) The platelet-derived growth factor receptor-beta phosphorylates and activates G protein-coupled receptor kinase-2. A mechanism for feedback inhibition. **J Biol Chem.** 280(35):31027-35.
59. Zheng, W., Lennartsson, J., Hendriks, W. et al (2011) The LAR protein tyrosine phosphatase enables PDGF  $\beta$ -receptor activation through attenuation of the c-Abl kinase activity **Cell Signal.** 23(6):1050-6.

