



# **Changes in protein expression and activation in rat arteriovenous fistula *in vitro***

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**HÁSKÓLI ÍSLANDS**

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- May 2016



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## Abstract

### Changes in protein expression and activation in rat arteriovenous fistula *in vitro*

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**Introduction:** Over 400,000 people were being treated with hemodialysis because of end stage renal disease in 2013. Of these 62.5% had arteriovenous fistula as their vascular access. Arteriovenous fistula are the preferred vascular access for hemodialysis as they are associated with lower infection rates and lower all-cause mortality than other methods of establishing vascular access. The drawback to using arteriovenous fistula is that they need to mature before being used. This maturation involves vessel wall thickening and vessel dilation. Studies today show that up to 60% of arteriovenous fistula do not mature enough to be used. Recent studies have shown that several proteins, including Eph-B4, caveolin-1, Akt-1 and eNOS can affect fistula maturation. The aim of this study was to assess the changes in protein expression and activation in a rat arteriovenous fistula using a novel bioreactor flow chamber.

**Methods and Materials:** A bioreactor flow chamber that can subject an arteriovenous fistula to arterial levels of flow and shear stress was created. A rat fistula made from the jugular vein and carotid artery was placed in the bioreactor and connected to needles. These needles lead to tubing that runs through a peristaltic pump. A media of endothelial basal media and xanthan gum was prepared so that the viscosity of the media is similar to that of blood and used as intra- and extraluminal fluid. Each sample was ran in the bioreactor for an hour. As a control the other jugular vein was also harvested and kept in static conditions while the sample was in the bioreactor. One sample was analyzed with an immunofluorescence assay to assess the state of the endothelium. A western blot assay was then performed where total amounts of Eph-B4, caveolin-1, Akt-1 and eNOS were assessed. Phosphorylated versions of caveolin-1, Akt-1 and eNOS were also assessed. GAPDH was used as loading control.

**Results:** The immunofluorescence assay showed that the endothelium was not destroyed in the bioreactor. Only two proteins assessed showed bands in the western blot, total caveolin-1 and total Akt-1. They showed no trends towards changes. A band was also observed for GAPDH which showed that the loading for the samples was uneven.

**Discussion:** These results from the immunofluorescence assay show that arteriovenous fistula can be put under arterial conditions in the bioreactor without their endothelium being destroyed. The results from the western blot are unreliable but the fact that there do not seem to be changes in the total amount of caveolin-1 and Akt-1 also suggest that the endothelium is preserved. No phosphorylated proteins showed bands so no conclusions about protein activation can be drawn. We conclude that the bioreactor flow chamber model is viable for further, exciting research into fistula maturation.

## Acknowledgements

I want to thank Dr. Alan Dardik MD/PhD for allowing me to work in his lab and for his guidance throughout my stay in his lab at Yale University. I would like to thank all his group for their help, especially Dr. Hualong Bai MD for making the fistula and Jeans M. Santana for guiding me through immunofluorescence and western blotting protocols. I would also like to thank Mehmet Kural for his help with the technical aspects of the bioreactor. Lastly I would like to thank my family for their support throughout this process.

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## Abbreviations

AVF .....	Arteriovenous fistula
AVG .....	Arteriovenous graft
CA-Akt .....	Constitutively active Akt-1
Cav-1 .....	Caveolin-1
COUP-TFII .....	COUP Transcription Factor II
DAPI .....	4',6 – diamidino-2-phenylindole
DN-Akt .....	Dominant Negative Akt
DOPPS .....	Dialysis Outcomes and Practice Patterns Study
EBM .....	Endothelial Basal Media
eNOS .....	Endothelial Nitric Oxide Synthase
ESRD .....	End Stage Renal Disease
GAPDH .....	Glyceraldehyde 3-Phosphate Dehydrogenase
iNOS .....	Inducible Nitric Oxide Synthase
IVC .....	Inferior Vena Cava
KO .....	Knock Out
nNOS .....	Neuronal Nitric Oxide Synthase
PECAM .....	Platelet Endothelial Cell Adhesion Molecule
PH Domain .....	Pleckstrin Homology Domaim
VEGF .....	Vascular Endothelial Growth Factor
WT .....	Wild Type



# 1. Introduction

## 1.1 Anatomy of blood vessels

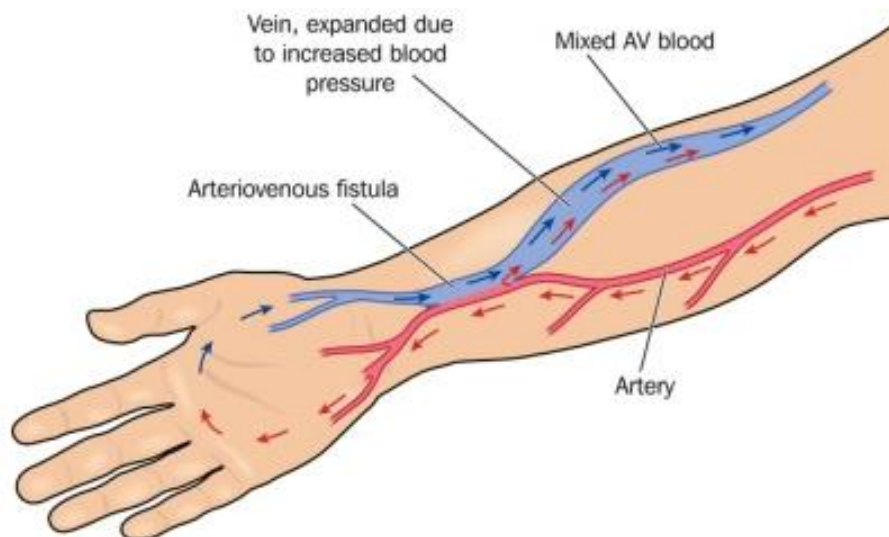
Although they share a common role of transporting blood, veins and arteries have different histological structures which help them perform their role in different environments. They share a three-layered structure with the adventitia being the outermost, then the media and the innermost being the intima. These layers have different compositions in the different types of vessels. In the arteries the intima consists of a single layer of endothelial cells on a basement membrane. The lamina elastica interna, made of elastin, separates the intima from the media. The media consists of smooth muscle cells with varying amounts of connective tissue, in general the larger an artery is the more layers of smooth muscle cells. The lamina elastic externa, also made of elastin, separates the media from the adventitia. The adventitia is made of loose connective tissue and is where the vasa vasorum lay in larger vessels. This connective tissue is mainly made of fibroblasts and collagen. Arteries are generally more rigid than veins and have a smaller lumen than comparable veins. These structural elements help arteries transport blood under high pressure and flow rates.(1)

Veins have a similar but different structure. The intima in both kinds of vessels is largely the same. The media of veins however is much less muscular and may contain more fibroblasts and collagen. The adventitia of veins is comparatively thicker than the adventitia of arteries and is made of longitudinal bundles of collagen and elastin. It is the thickest layer of veins. In addition to all of this, some veins have valves in their lumens to prevent backflow, this is not seen in arteries. This structure allows veins to serve as a reservoir of blood under low pressure, unlike the high pressure, high flow environment of arteries.(1)

When veins are surgically put into the arterial part of the vascular system they are subjected to shear stress, flow rates and pressure their walls are not used to. In response to these changes the vein undergoes changes in its wall structure, thickness and lumen diameter.(2) In addition to these changes, turbulent flow can arise in veins in arteriovenous fistula and affect its structure.(3)

## 1.2 Arteriovenous fistula use

Arteriovenous fistulas are created by connecting a vein to an artery. They can be done in a few different locations. One of the most common is in the wrist by connecting the radial artery to the cephalic vein thus creating what is known as a radiocephalic fistula like the one in figure 1. (4, 5) Connecting the brachial artery to the cephalic vein creating a brachiocephalic fistula at the elbow is also common. (5, 6) When selecting a site for arteriovenous fistula placement one should always consider the most distal placement first for several reasons. One reason is to try to minimize potential steal syndrome which is more severe when more proximal. Another is that if the most distal arteriovenous fistula fails more proximal one can be put in.(5)



**Figure 1:** An arteriovenous fistula. From (7)

At the end of 2013 there were 661,648 cases of end stage renal disease (ESRD) in the United States. While incidence has plateaued after rising from the eighties to the early 2000s prevalence is still rising by about 21,000 cases per year. At the end of 2013 63.7% of cases were being treated with hemodialysis, which equals around 421,470 patients on hemodialysis. Of those around 62.5% were using arteriovenous fistula as vascular access, giving a total of about 263,419 patients on hemodialysis using an arteriovenous fistula.(8)

Arteriovenous fistula use in recent years has been much higher in select European countries when compared to the United States. THE DOPPS study published in 2002 showed that arteriovenous fistula were used in 80% of patients in a 101 dialysis units France, Germany, Italy, Spain and the United Kingdom but only 24% of patients in 145 dialysis units in the United States. The study also reports that arteriovenous fistula survival in the European countries was longer than is the United States with the relative risk being 0.49. (9) Since this study was published the use of arteriovenous fistula at dialysis initiation and at one year of dialysis has risen in the United States.(8)

Arteriovenous fistulas are the preferred vascular access for hemodialysis.(5) They are associated with lower fatal infection rates, lower all-cause mortality and lower risk for cardiovascular events than central venous catheters and lower fatal infection rates and all-cause mortality than arteriovenous grafts.(10, 11) Arteriovenous fistula are also associated with lesser hospitalization.(5) Care related costs for arteriovenous grafts have been estimated to be fivefold those of arteriovenous fistula.(12) A potential drawback of using an arteriovenous fistula is that they need to mature before being cannulated. This maturation involves dilation of the venous arm and wall thickening. The time needed to allow this maturation to take place is not agreed upon, but guidelines currently recommend at least 1 month.(5) Failure to mature is also a major problem with using arteriovenous fistula. Studies report that 31-60% of fistulas fail to mature.(13-16) Fistula maturation success seems to be connected to the age of the patient with younger patients showing higher rates of successful maturation.(8) An understanding on the mechanisms behind the maturation of arteriovenous fistula could have great

clinical relevance in increasing the proportion of arteriovenous fistula that mature and remain patent and decreasing the amount of complications that follow.

### 1.3 Current research

Research has been done with the goal of understanding the adaptation of veins in the arterial environment. For example, one such experiment performed by Juncos et al showed that a genetic deficiency in MCP-1 proved to be protective in the murine model and increased arteriovenous fistula patency at 6 weeks after formation with increased luminal area and decreased wall thickness.(17) However, most research has mainly focused on vein grafts and not arteriovenous fistula. From this research we know that veins adapt to the arterial environment, both through dilation and wall thickening. This adaptation is necessary because the arterial environment has different hemodynamic factors and oxygen tension than venous environment. There are important differences in the environment and response between vein grafts and arteriovenous fistula. These differences include higher flow rates, lower pressure and higher shear stress in arteriovenous fistula which lead to more dilation and less wall thickening of arteriovenous fistula compared to vein grafts. (2)

A previous experiment with a linear bioreactor flow chamber simulating arterial shear stress in human saphenous vein grafts was performed by Model et al. In that experiment flow was adjusted for each sample to achieve arterial or venous shear stress while pressure was held near constant to minimize confounding effects. Vein grafts were also held in static conditions as a control. The results showed that the vein graft loses Eph-B4 expression without gaining Ephrin-B2 expression after 24 hours in arterial flow conditions. These changes did not occur when the vein graft was exposed to venous flow for the same amount of time nor when kept in static conditions. The general architecture of the veins was preserved at all points as shown by analysis of PECAM. This experiment shows that even without the influence of hormones and the immune system shear stress can affect venous identity. (18)

### 1.4 Eph receptors

Eph receptors are the largest family of tyrosine kinases in mammals. Their ligands are the Ephrins which are divided into two groups, Ephrin-A ligands which are bound to the membrane through a glycosylphosphatidylinositol (GPI) link and Ephrin-B ligands which are transmembrane proteins. Eph receptors are divided into two groups, A and B, based on their binding preference. It was thought that Eph-A receptors only bound to Ephrin-A ligands and vice versa but it has been discovered that Ephrin binding preference spans classes. Because of the membrane spanning region of Ephrin-Bs there is a possibility for bidirectional signaling when they bind to Eph receptors.(1)

Eph receptors and ephrins are expressed in most, if not all cells in the body. In some cases the receptor and ligand are both expressed on the same cell but in others they are expressed in a mutually exclusive pattern.(19) In the vascular system Eph-B4 is expressed on vein endothelial cells and ephrin-B2 is expressed in arterial endothelial cells. Other Eph receptors are expressed throughout the vascular system. In ephrin-B2 knockout mice angiogenesis is greatly disturbed. This provides the information that ephrin-B2 and Eph-B4 are important for embryological development of the circulatory

system. (1, 20) Findings show that even in ephrin-B2 only knockout mice venous development is disturbed. A recent study showed that in mesenchymal tissue involved in angiogenesis selective lack of ephrin-B2 in endothelial tissue is lethal despite the expression of ephrin-B2 in other tissues. This underlines the important role of ephrins and Eph receptors in vasculogenesis. (21) This might suggest that interaction between ephrin-B2 and Eph-B4 is necessary for normal development. (22)

Much work has been put into understanding the regulatory pathways of ephrin signaling. It is now known that Delta/Notch pathway can increase ephrin-B2 expression and inhibit Eph-B4 expression. In turn, vascular endothelial growth factor (VEGF) induces the Delta/Notch pathway and is itself induced by sonic hedgehog. This cascade is inhibited in venous endothelial cells by COUP-TFII which inhibits VEGF and thus decreases the inhibitory effect of Delta/Notch on Eph-B4 and decreases the stimulating effect on Ephrin-B2. (23)

Eph-B4 and ephrin-B2 persist into adulthood as markers of identity and their expression has been proven to be plastic under certain circumstances, such as when veins are put in the arterial environment. Kudo et al showed that when vein grafts are subjected to the arterial environment Eph-B4 expression is downregulated while Ephrin-B2 expression is not upregulated. (24) Surprisingly, when an arteriovenous fistula is made in a mouse model the expression of Eph-B4 goes up in the venous arm of the arteriovenous fistula. (25)

Eph-B4 has a role in regulating wall thickening in veins in the arterial environment in adults. Muto et al showed that stimulating Eph-B4 inhibits venous wall thickening and conserves vein identity in vein grafts surgically placed in the arterial environment in a mouse model. When Eph-B4 was inhibited increased wall thickening was observed. Furthermore, this study showed that Eph-B4 was associated with caveolin-1 and loss of caveolin-1 also inhibited wall thickening in the vein graft. (26)

## 1.5 Caveolin

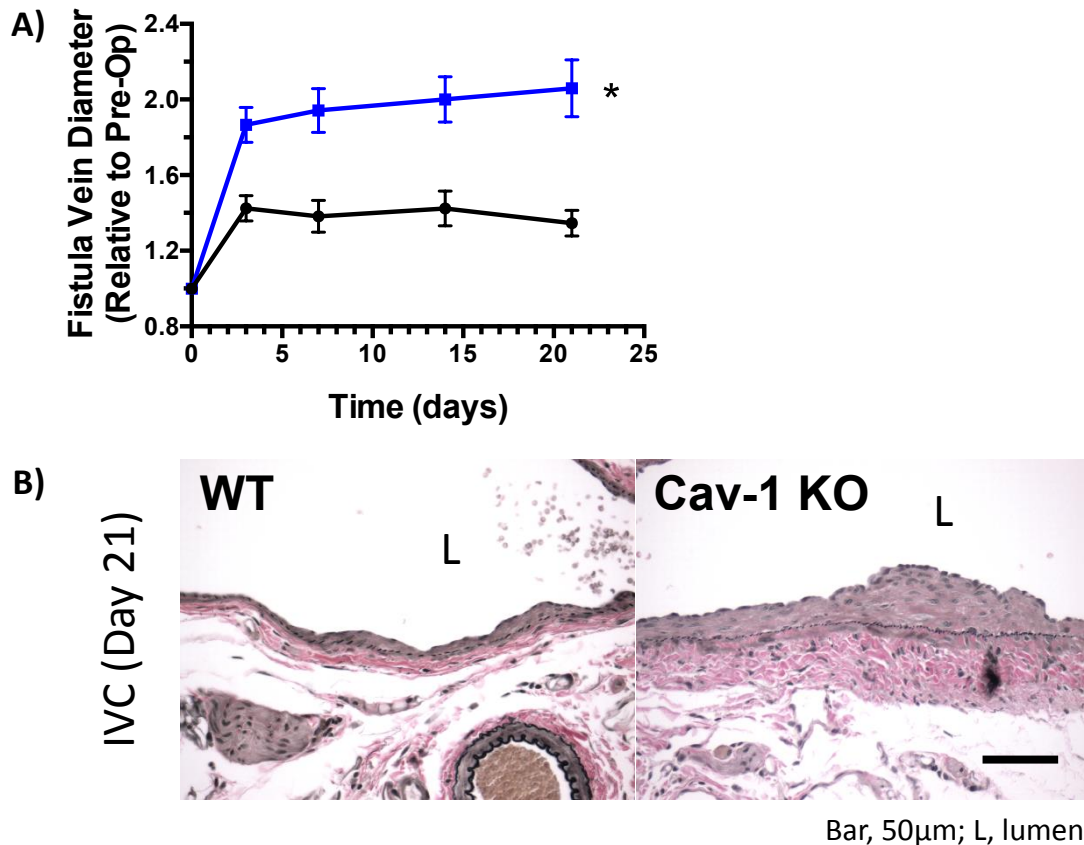
The caveolin family consists of three different proteins, caveolin-1 (also known as VIP21), caveolin-2 and caveolin-3. (27-29) Caveolin-1 also has two different isoforms,  $\alpha$  and  $\beta$ , where the N-terminus of  $\beta$  is shortened by 21 amino acids when compared with the  $\alpha$  form. These isoforms have been shown to differ in their distribution within a single cell. (30) Caveolins are associated with caveolae, antibodies against caveolin-1 have been shown to coat the cytoplasmic protein component of caveolae. (27) Studies have shown that without caveolin-1 caveolae formation is inhibited. (31) Caveolin-1 has also been found in the trans-Golgi network. (32) While caveolins are small proteins they seem to form large complexes with each other. Caveolin-1 forms oligomeric complexes with itself as does caveolin-3. (29, 33) However, caveolin-2 needs caveolin-1 to migrate to the plasma membrane and form oligomers. (34, 35)

The caveolins are a major component of the protein coat of caveolae. (29, 34-36) Caveolae are flask shaped indentations in the plasma membrane of cells. They are often considered to be a subtype of lipid rafts which are highly organized regions of the plasma membrane with a high concentration of certain lipids such as cholesterol and sphingolipids. (37) Caveolae especially have a high concentration of cholesterol when compared to other types of lipid rafts. Caveolin-1 has been shown to

bind tightly to cholesterol.(38) Caveolae serve to concentrate signaling molecules to certain areas on the plasma membrane. Several signaling molecules, such as G-proteins and eNOS, have been found to bind to caveolin-1 and group together in caveolae.(39, 40) In particular, Eph-B1 has been shown to localize to caveolae and caveolin-1 seems to affect its signaling. This kind of interaction also happens with the other class of Eph receptors as Eph-A2 has also been shown to interact with caveolin-1.(41) eNOs in particular has been shown to bind to different forms of caveolins in different cells, caveolin-1 in endothelial cells and caveolin-3 in cardiac myocytes. The same study showed that nearly all eNOs in endothelial cells can be found in association with caveolin. (42) Several studies have shown that caveolins can affect downstream signaling of proteins.(43, 44) All of this suggests that caveolin-1 could bind to and effect Eph-B4 and eNOS, proteins that play a major role in fistula maturation.

Caveolin-1 in endothelial cells in blood vessels is also known to be a mechanosensor. It has a role in sensing the shear stress that endothelial cells in blood vessels are subject to and relaying signals in response to changes. A study by Yu et al showed that caveolin-1 knockout mice showed impaired flow-dependent arterial remodeling. (45)

Research by the Dardik lab has shown that caveolin-1 is very important in arteriovenous fistula maturation. Muto et al showed that with the loss of caveolin-1 in caveolin-1 knock out mice the inhibitory effects of Eph-B4 on vein wall thickening in vein grafts is lost.(26) In mice with an infrarenal aorto-caval arteriovenous fistula caveolin-1 is increased 21 days after the creation of fistula. Caveolin-1 knock out mice show a larger diameter and thicker vessel walls in the venous arm of fistula compared to wild type mice as can be seen in figure 2. (46)



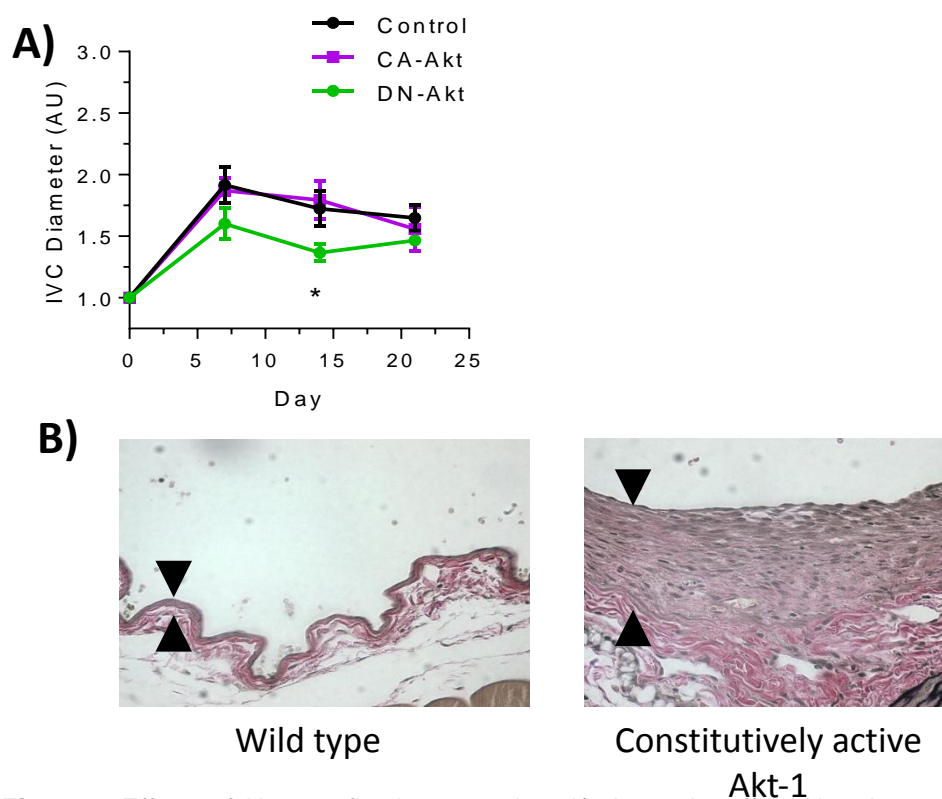
**Figure 2:** Effects of caveolin on fistula maturation. A) shows the vein dilation. B) shows vessel wall thickening. IVC: Inferior Vena Cava, KO: Knock Out, WT: Wild Type. Adapted from (46)

## 1.6 Akt

Akt, also known as Protein kinase B or RAC, is a serine-threonine kinase. (47) Akt is mostly cytosolic. (48) Orthologs of Akt have been cloned from both *Drosophila melanogaster* and *Caenorhabditis elegans* showing that this protein is widely conserved, suggesting that it is important for normal cell function. (49, 50) Akt has three isoforms, Akt-1/PKB $\alpha$ , Akt-2/PKB $\beta$  and Akt-3/PKB- $\gamma$  which share about 80% of their sequence identity. (51-53) They all contain an active kinase site with specificity to serine and threonine in substrate proteins. (47, 54) On the N-terminus of these proteins is a pleckstrin homology domain through which protein-lipid interaction or protein-protein interaction can be achieved. (55, 56) This domain is important for the activation of Akt. It has been shown in experiments that mutations that inhibit the phospholipid binding ability of the PH domain result in a reduced ability of Akt to be activated by growth factors. (57) Conversely, mutations that increase the phospholipid binding activity of the PH domain increase the activation of Akt. (58) However other studies have shown that the PH domain is not necessary for Akt activation but rather plays a role in translocating Akt to the plasma membrane where can then be activated. Another important player in this translocation is phosphoinositide kinase-3 (PI-3-K). (48) This kinase can be activated through growth factor receptors like platelet derived growth factor (PDGF). When activated it phosphorylates phosphoinositides (PI) to produce PI3P, PI(3,4)P2 and PI(3,4,5)P3. These products bind to the PH domain and cause Akt to form protein complexes. (56) When PI 3-K is blocked by its inhibitor wortmannin decreases the activation of Akt by growth factors. (57) For the full activation of Akt two

amino acids are most important, threonine 308 and serine 473. When the kinase is mutated so that these to amino acid residues cannot be phosphorylated Akt cannot be activated.(59) The activation of Akt is thus complex and has many regulatory features, perhaps underlining its important role in normal cell function.

Eph-B4 has been shown to regulate Akt-1 in venous remodeling to the arterial environment. Foster et al have shown that Akt-1 expression and phosphorylation is increased in arteriovenous fistula in a mouse model. When an constitutively active Akt-1 was delivered to the fistula with an adenovirus it caused an increase in both arteriovenous dilation and vein wall thickness. Akt-1 knockout model showed the opposite effect. This can be seen from figure 3. When stimulating Eph-B4 in wild type mice the vein diameter and wall thickness was decreased compared to unstimulated mice. This effect was abolished when the Eph-B4 stimulation was performed in Akt-1 knockout mice. They suggest that Eph-B4 has a negative regulating effect on Akt-1. (25)



**Figure 3:** Effects of Akt-1 on fistula maturation. A) shows the effect Akt-1 has on vessel diameter. B) shows the effect Akt-1 has on vessel wall thickness. Arrowheads indicate the endothelial layer. CA: Constitutively active, DN: Dominant negative, IVC: Inferior Vena Cava. Adapted from reference (25)

## 1.7 eNOS

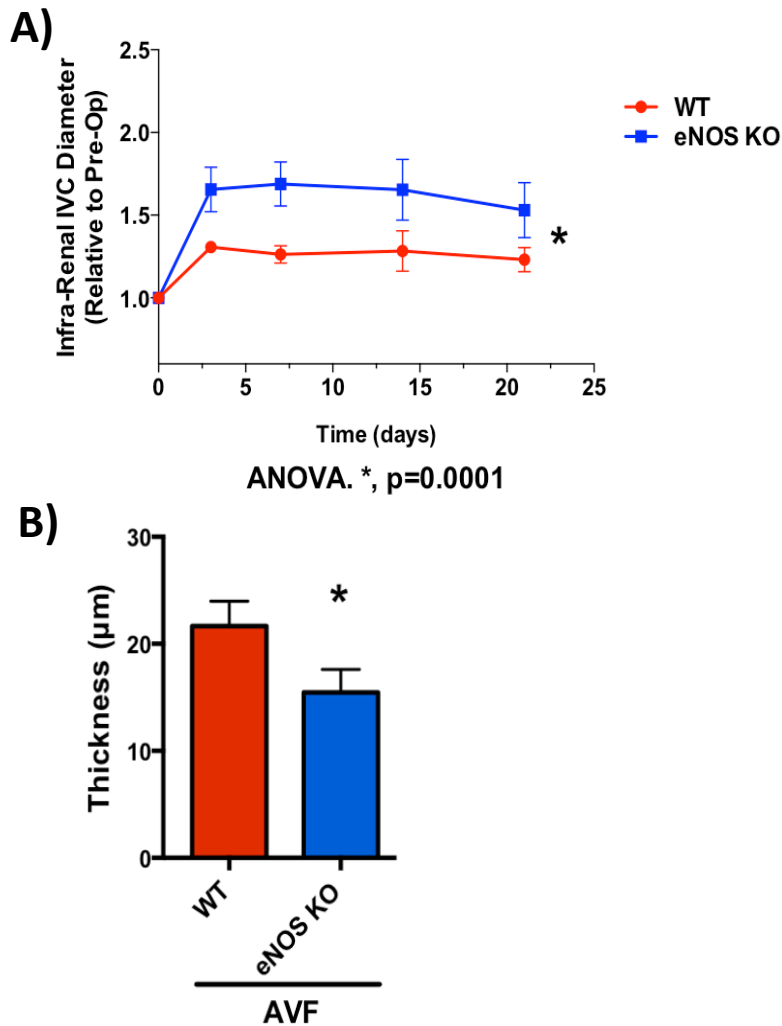
It is well established that endothelial nitric oxide synthase (eNOS, also known as NOS3) plays an important role in the vascular system. Its product, nitric oxide, is a vasodilator. It is found in the endothelial layer of blood vessels and studies have shown that decreased NO formation is connected to cardiovascular disease. Other forms of this enzyme include neuronal NOS (NOS1) and inducible

NOS (NOS2). iNOS produces larger amounts of NO than eNOS. Both iNOS and nNOS are typically involved in other processes than eNOS such as host defense, inflammation and neuronal signaling.(60)

Although best known for its vasodilatory effects eNOS also influences other processes that are important to arteriovenous fistula maturation, e.g. cellular proliferation. Yang et al. showed that increased NO formation inhibited cell proliferation in bovine fetal vascular endothelial cells.(61) This demonstrates that eNOS could have a direct effect on vessel wall thickness.

A study from Wang et al shows that the activities of Eph-B4 and eNOS are linked in vein graft adaptation to the arterial environment. Their data suggest that Eph-B4 is a negative regulator of eNOS and that eNOS downregulates Eph-B4. They showed that in knock-out eNOS mice expression of Eph-B4 was increased when compared to wild type mice. The vein grafts in the knock out eNOS mice showed less remodeling and less wall thickening than the wild type mice. (62) Ongoing research at the Dardik lab has shed some light on the role of eNOS in arteriovenous fistula maturation using a infrarenal aorto-caval fistula in mice. Following the creation of fistula phosphorylation of eNOS is increased for the first 7 days and then returns to baseline. The fistula in eNOS knockout mice have a larger diameter when compared to wild type mice. The veins in the eNOS knockout mice also show less neointimal thickening than the wild type mice as can be seen from figure 4. The Dardik lab has also shown that chronic Eph-B4 stimulation results in less eNOS phosphorylation while inactivating Eph-B4 shows the opposite results. This suggest that Eph-B4 mediates venous remodeling to the arterial environment through eNOS.(63)

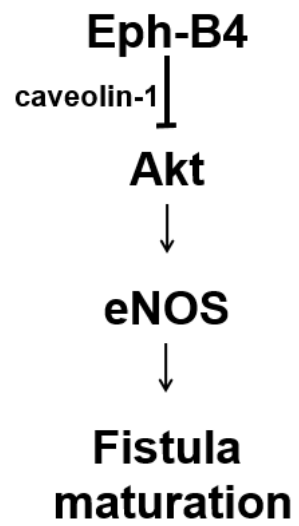




**Figure 4:** Effects of eNOS on fistula maturation. A) shows the effect eNOS has on dilation. B) shows the effect eNOS has on vessel endothelial thickening. IVC: Inferior Vena Cava, KO: Knock Out, WT: Wild Type. Adapted from (63)

## 1.8 Arteriovenous fistula maturation signaling pathway

Research done at the Dardik lab has shed light on the pathway in which the aforementioned proteins lay. Eph-B4 downregulates fistula maturation. This effect is abolished in caveolin-1 knockout mice suggesting they lay in the same pathway.(46) Akt-1 has an opposite effect on fistula maturation to Eph-B4 and caveolin-1, that is it increases dilation and vessel wall thickening. The effects of stimulating Eph-B4 are abolished in Akt-1 knockout mice, again suggesting that Akt-1 lies downstream to Eph-B4 and is inhibited by it.(25) It has been demonstrated that Akt-1 stimulation leads to increased eNOS phosphorylation and that Eph-B4 decreases it.(63) This further solidifies that Akt-1 and Eph-B4 have opposite effects and leads us to believe that the pathway these proteins make up looks like the one in figure 5.



**Figure 5:** Fistula maturation pathway

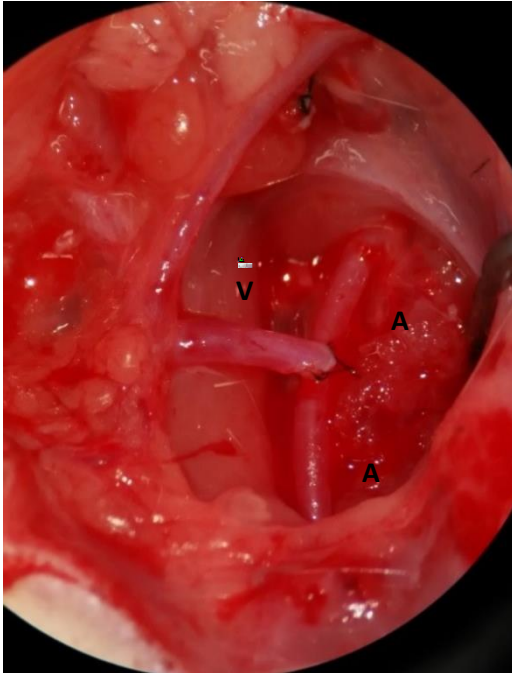
## 2 Aims

The aims of this study were to study the changes in protein expression and activation in the venous arm of rat arteriovenous fistula that were subjected to arterial levels of shear stress for an hour in vitro. The model used is a one of a kind branched bioreactor flow chamber which gives us the opportunity to study the response of the vessels themselves without influence from external factors such as the immune system.

### 3 Methods and materials

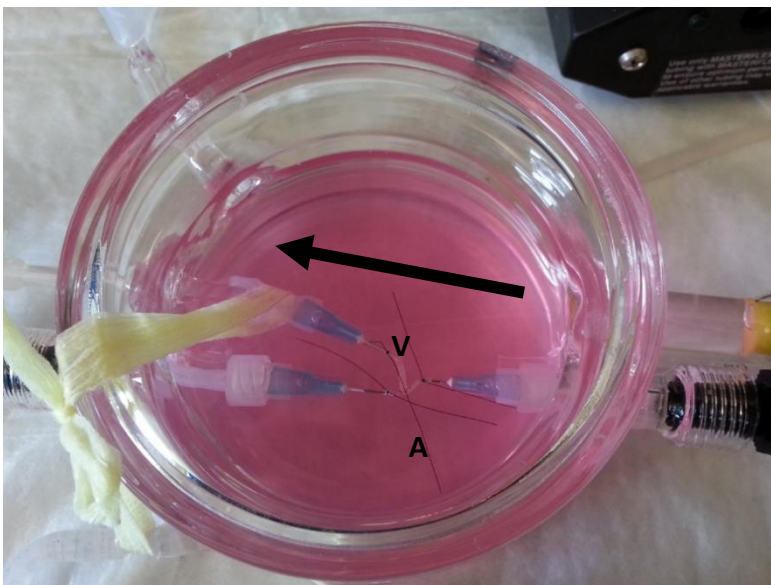
#### 3.1 Bioreactor flow chamber and fistula

An arteriovenous fistula was made by connecting the internal jugular vein to the carotid artery in a rat. This can be seen in figure 6. The arteriovenous fistula was then harvested and connected to the needles in the bioreactor with silk sutures like can be seen in figure 7.



**Figure 6:** Arteriovenous fistula in vivo. The venous arm is labelled with V and the artery is labelled with A.

The samples were generated by placing the arteriovenous fistula under arterial shear stress conditions in a bioreactor flow chamber. Each sample was in the bioreactor for 1 hour. Controls were prepared by placing rat internal jugular veins in static conditions in the same media while the samples were in the bioreactor.

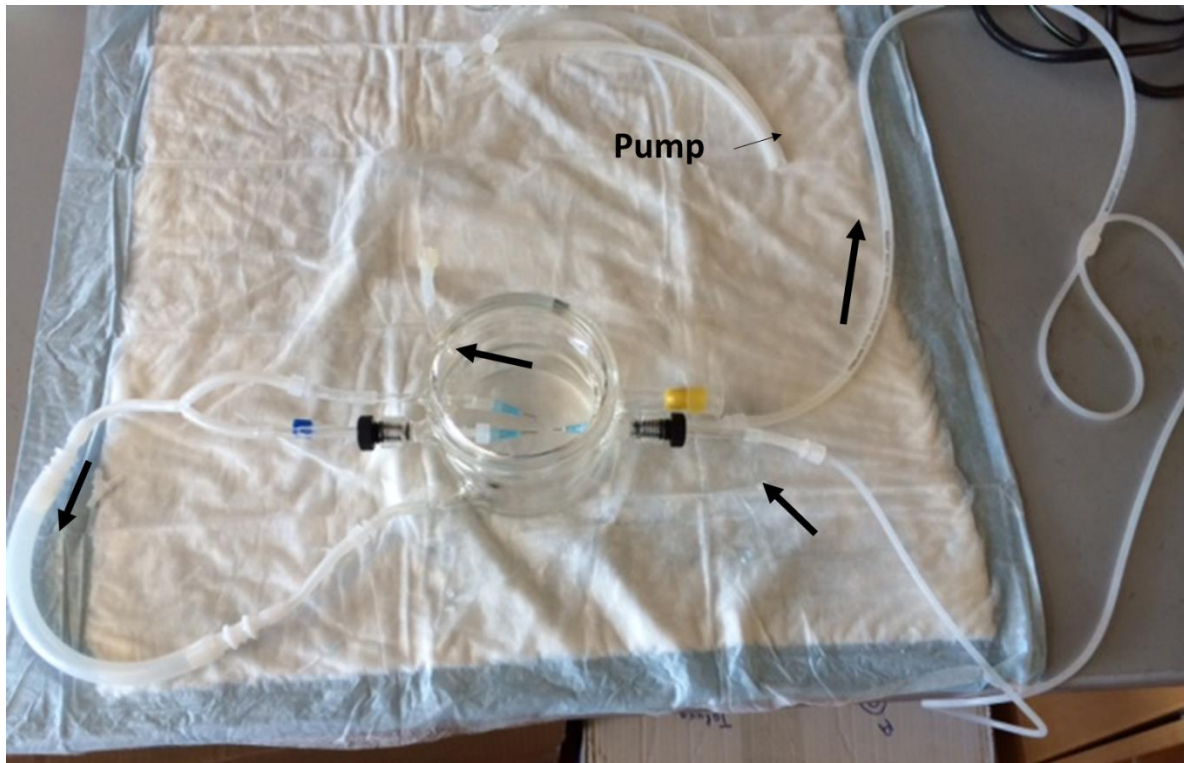


**Figure 7:** Set up of the arteriovenous fistula in the bioreactor. The arrow indicates the direction of flow. The venous arm is labelled with V and the artery is labelled with A.

A flow chamber bioreactor was used to simulate arterial flow through the arteriovenous fistula. The bioreactor is set up like shown in figures 8 and 9. The arteriovenous fistula lies in the reservoir. A 13 mm tube connects the reservoir to the inflow end of the arteriovenous fistula artery through a 25G needle. This tube lies through a digital roller pump (Masterflex L/S peristaltic pump with Easy Load II head) which pumps at 0.6 ml/min to achieve a shear stress of 20 dyne/cm<sup>2</sup>. This flow rate was calculated according to the Hagen-Poiseuille equation,  $\tau_{\text{mean}} = \frac{4\mu Q}{\pi R^3}$ , where the inner radius of the tube R is 0.3mm (the needle gauges were 0.51mm so the fistulas were estimated at 0.6mm), the fluid viscosity  $\mu$  is 4 centipoise, the flow rate is Q and  $\tau$  is the mean shear stress. The arterial and venous outflow ends are connected to tubing through two 25G needles. The outflow tubing of the arterial and venous arms join and connect to tubing that leads back into the reservoir.



**Figure 8:** Set up of the bioreactor during a run



**Figure 9:** Flow direction in the bioreactor flow chamber. The direction of flow is indicated with arrows. The placement of the pump is labelled.

The media used in the flow chamber was Endothelial Basal Medium (EBM) (Lonza CC-3156). To imitate the viscosity of blood the media was mixed with 0.7 g of xanthan gum (Sigma G-1253) per liter of media. The media was left to stir on a stir plate at 37°C overnight. With the xanthan gum a viscosity of 4 centipoise was reached which is similar to viscosity of blood. To inhibit bacterial growth an antibiotic-antimycotic solution containing streptomycin, penicillin and amphotericin B (Gibco, catalogue no. 15240062) was added to the media and it was kept at 4°C before use.

After the samples had been run in the bioreactor for 1 hour the venous arm of the fistula was cut off and placed in an Eppendorf tube. The tube was then submerged in liquid nitrogen to freeze the sample. Then the same process was followed for the control. The samples were then stored at -80°C until analysis.

### 3.2 Immunofluorescence

Immunofluorescence assay was performed on one sample to assess the state of the endothelium. This sample had been run in the bioreactor with a media of EBM without xanthan gum. The sample was sent to pathology to be fixed in paraffin wax and put on slides. An immunofluorescence protocol from Giovanni Zito at the Greco lab was then followed (see appendix 1). The pictures were taken using a microscope and Volocity 6.3 software. The antibodies used can be seen in table 1.

**Table 1:** Antibodies used in immunofluorescence assay

Antibody against	Origin	Order	Catalogue number	Concentration	Producer
Total eNOS	Rabbit	Primary	sc-654	1:100	Santa Cruz Biotechnology
Phosphorylated eNOS	Rabbit	Primary	ab87750	1:100	Abcam
Von Willebrand's	Sheep	Primary	Ab11713	1:100	Abcam
Anti-sheep	Donkey	Secondary	A-11015 488	1:200	Invitrogen
Anti-Rabbit	Donkey	Secondary	A-10042 568	1:200	Invitrogen

### 3.3 Western blot

To analyze the samples a western blot assay was performed to isolate proteins and compare their amounts. The samples were prepared by first being submerged in liquid nitrogen and then crushed. 300  $\mu$ L of lysis buffer (see appendix 2) was then added to each sample and left for 30 minutes. A sonic dismembrator (Model 60, Fischer Scientific) was used to further disrupt the cells. The samples were then spun down using a centrifuge (Hermle Z216 MK). The supernatant was then harvested and the pellet discarded. Protein quantification was performed on the samples using a plate reader (BioTek Synergy HT) and Gen5.11 software for analysis. Then a western blot was performed following a protocol from Fang Yi (see appendix 3). Antibodies used for the western blot can be seen in table 2. The films were developed in a medical film processor (Parker X-ray, model SRX-101A).

**Table 2:** Antibodies used for western blotting

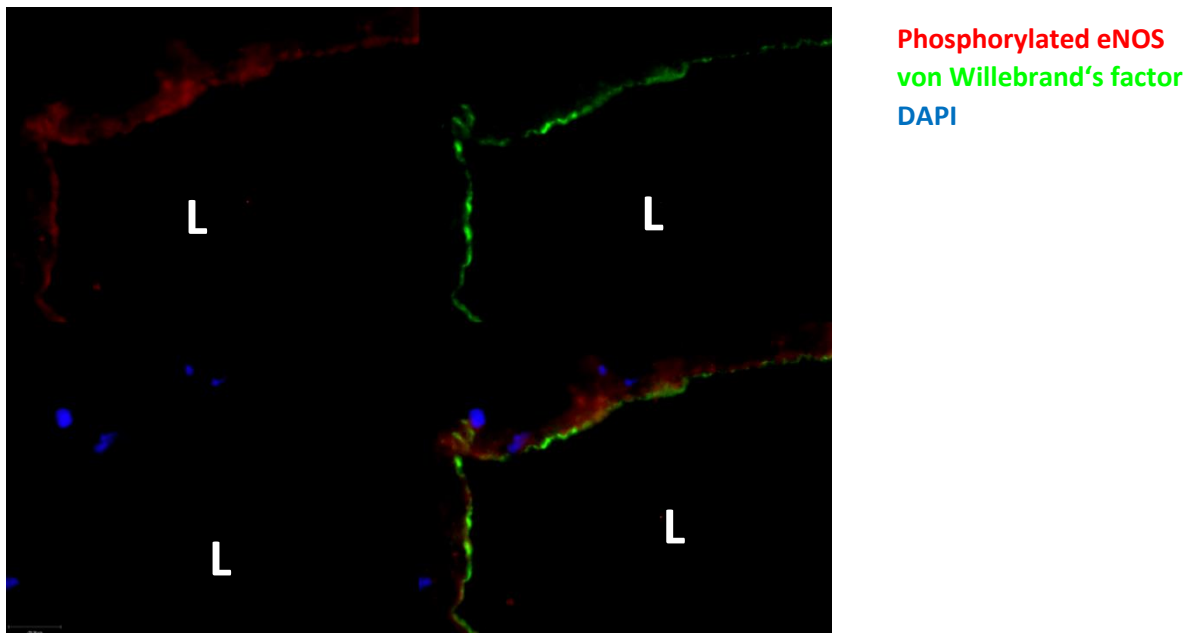
Antibody against	Origin	Order	Catalogue number	Concentration	Producer
Eph-B4	Rabbit	Primary	sc-5536	1:1000	Santa Cruz Biotechnology
Phosphorylated Akt-1	Rabbit	Primary	9018S	1:1000	Cell Signaling Technology
Total Akt-1	Rabbit	Primary	2938S	1:1000	Cell Signaling Technology
Phosphorylated eNOS	Rabbit	Primary	9571S	1:1000	Cell Signaling Technology
Total eNOS	Rabbit	Primary	sc-654	1:1000	Santa Cruz Biotechnology
Phosphorylated caveolin-1	Rabbit	Primary	3251S	1:1000	Cell Signaling Technology
Total caveolin-1	Mouse	Primary	610057	1:1000	BD Biosciences
GAPDH	Rabbit	Primary	2118S	1:1000	Cell Signaling Technology
Anti-rabbit	Goat	Secondary	7074S	1:5000	Cell Signaling Technology
Anti-mouse	Horse	Secondary	7076S	1:5000	Cell Signaling Technology



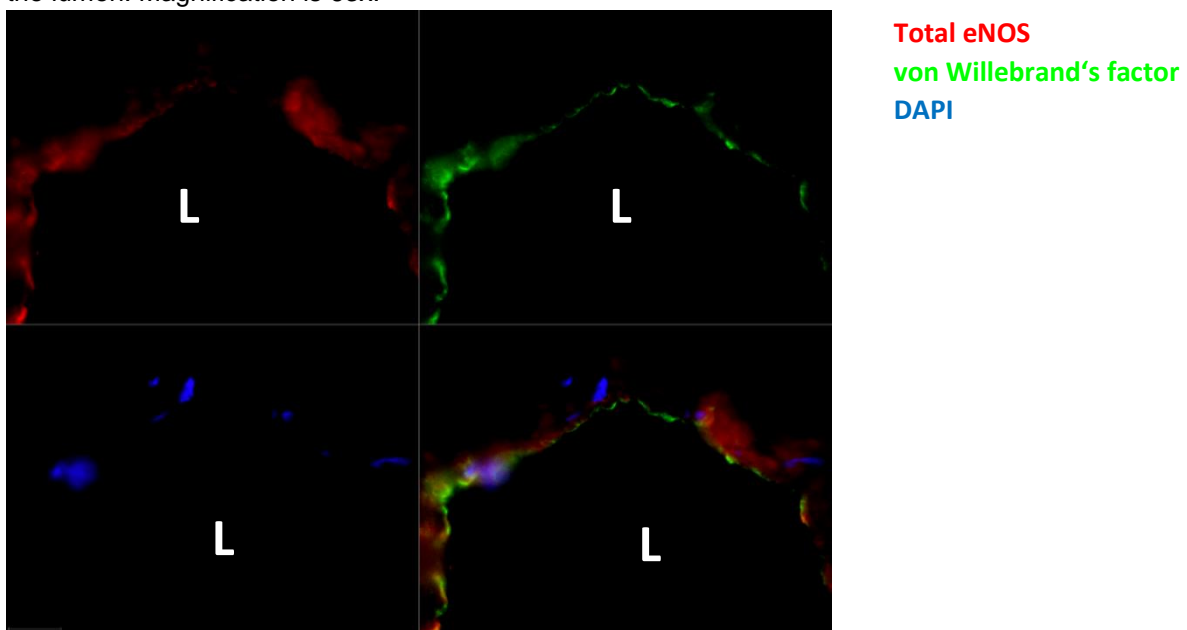
## 4 Results

### 4.1 Immunofluorescence

The results of the immunofluorescence assay can be seen in figures 10 and 11. The results show the location of total eNOS and phosphorylated eNOS along with von Willebrand's factor and DAPI in a vein from an arteriovenous fistula run in the bioreactor with EBM as media. Both total and phosphorylated eNOS are found in the same place in the sample and seem to line the lumen of the vein. This indicates that the endothelium is intact.



**Figure 10:** Immunifluorescence results. Immunofluorescence photos to evaluate the state of the endothelium. Phosphorylated eNOS is red, von Willebrand's factor is green and DAPI is blue. L marks the lumen. Magnification is 63x.

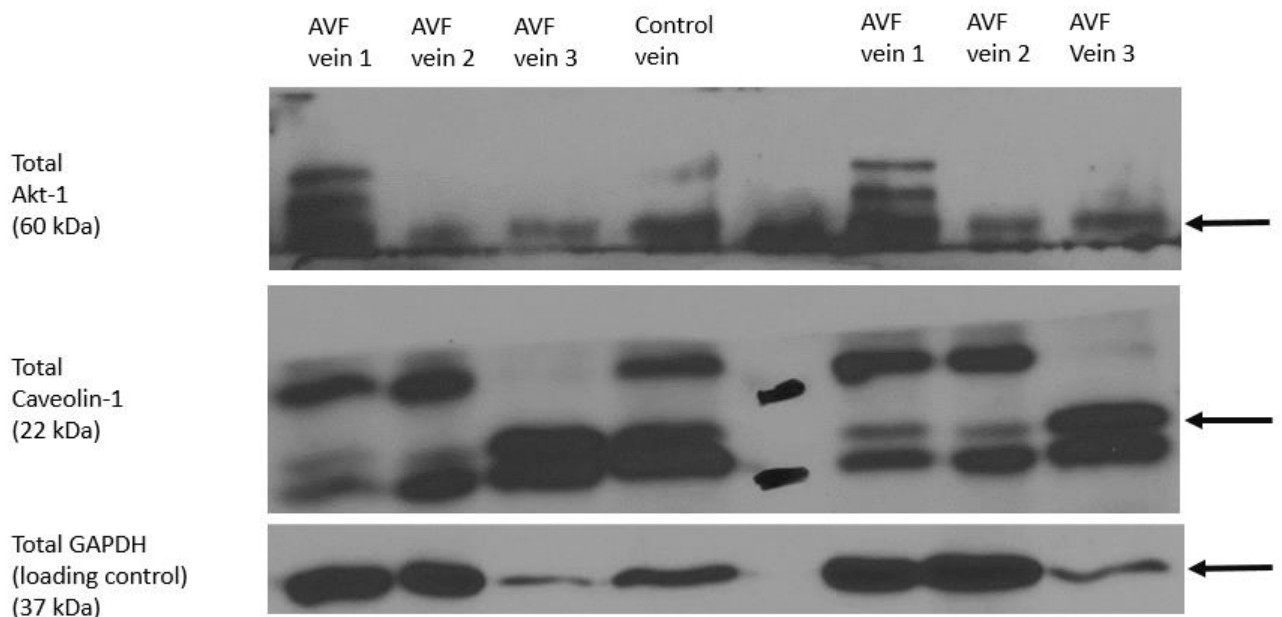


**Figure 11:** Immunofluorescence results. Immunofluorescence photos to evaluate the state of the endothelium. Total eNOS is red, von Willebrand's factor is green and DAPI is blue. L marks the lumen. Magnification is 63x.



## 4.2 Western blot

Results from the western blot can be seen in figure 12. These results show the isolation of proteins from the samples. Three samples of veins from arteriovenous fistula run through the bioreactor were compared to one control vein. Only two proteins in addition to the loading control showed bands. These proteins were total Akt-1 and total caveolin-1. Multiple bands were observed for both proteins indicating unspecific binding of the antibodies. The loading control shows that the loading of the samples was uneven.



**Figure 12:** Results of western blotting. Bands for Akt-1, caveolin-1 and GAPDH can be seen with arrows indicating the appropriate bands.

## 5 Discussion

### 5.1 Results

The immunofluorescence data shows that proteins found in the endothelium, eNOS and von Willebrand's factor, are found in the same place in the sample. We conclude that they are found in the endothelium of the sample and that the endothelium of this sample was not destroyed in the bioreactor flow chamber.

The results of the western blot showed no changes in protein expression for total Akt-1 and total caveolin-1. However, it is hard to draw conclusions as these proteins showed multiple bands suggesting unspecific binding. Other proteins assessed showed no bands, except for the loading control. As the bands for GAPDH show, the protein loading was uneven and therefore the results are unreliable. A densitometry was not performed because of this and other reasons such as the fact that the GAPDH membrane seems to be oversaturated and there is a lot of background and unspecific binding shown on the film.

While it is hard to draw conclusions from this western blot the fact that the total protein amount does not seem to change suggests that the endothelium is not destroyed in the samples. Thus the western blot results strengthen our findings from the immunofluorescence assay. We conclude that the endothelium is not destroyed in the bioreactor.

In the end only two proteins showed bands after western blot analysis. This could be because of several reasons. Blood vessels in rats are small and results in small amounts of tissue, and thus proteins, in samples. This is especially true for veins which are much harder to analyze than e.g. arteries which have much thicker vessel walls. The samples used in this study were very small and therefore the total amount of protein was not very high. This could result in proteins that are not present in high concentration in the tissue, such as the phosphorylated versions of the proteins assessed, to not show up in bands. What could also lower protein amounts is partial loss of the endothelium. The vessels are subjected to surgical manipulation and after that the venous arm is put under higher shear stress than it has previously been under. This could result in the loss of cells from the endothelium in the samples. The proteins assessed are mostly found in the endothelium so the loss of it or parts of it would result in lower amounts detected or no detection of the proteins at all. The state of the endothelium was assessed in a sample that had been run for an hour in the bioreactor. In that sample the endothelium was intact. However, only EBM was used in that experiment as media. The addition of xanthan gum raises the viscosity which could affect the endothelium and result in a higher level of destruction. This seems unlikely as the total amounts of Akt-1 and caveolin-1 did not seem to change. However if this experiment would be repeated a sample

with run with media of the right viscosity would have to be analyzed with regards to the endothelium, preferably with immunofluorescence.

Another problem that could have affected the western blotting is the lysis of the sample. If the cells are not lysed properly the proteins would not be released and be harder to detect. If the experiment is repeated another approach to the lysis of the samples might give better results.

## **5.2 Bioreactor flow chamber**

This experiment was one of the first steps in using this model to investigate arteriovenous fistula. Bioreactors have been used to put veins under arterial levels of shear stress in a linear set up to imitate vein grafts.(18) However, this type of bioreactor flow chamber that can be used to subject a branched vessel structure such as a arteriovenous fistula to different flow rates and levels of shear stress has not been used or developed anywhere else. It is therefore one of a kind and presents us with a unique opportunity to study arteriovenous fistula maturation. In addition to being able to control the flow rate through the fistula with the pump the bioreactor enables us to increase the resistance in each outflow arm independently and thus control the flow through each arm. This is achieved by using a clamp on the outflow tubing. The angle at which the venous arm connects to the artery can also easily be manipulated which is much harder and more unreliable in *in vivo* models.

While we did not detect any trends in protein expression or activation the results from the immunofluorescence are promising. They show that arteriovenous fistula can withstand the surgical manipulation and shear stress of the run in the bioreactor without losing its structure. This was a necessary first step in using the bioreactor flow chamber as a model.

This model was designed to assess the response of the fistula without external factors. While that gives us an opportunity to clear up the role of the fistula itself in its maturation the results of experiments using this model cannot be directly transferred to *in vivo* models where external factors such as the immune system play a role in the maturation process. The results of experiments conducted in the bioreactor are therefore limited but could lead to further experiments *in vivo*.

## **5.3 Future steps**

The first step following the results presented in this thesis would be to redo the experiment to get more accurate results. Some steps that could be taken have already been mentioned such as to assess the state of the endothelium after running a sample in the bioreactor with the media used in the experiment. An hour run time might be too short to see any significant change in the expression of proteins and if the experiment is repeated a greater run time would be better to assess the changes in total amount of protein.

It would be interesting to add phosphorylated Eph-B4 on the list of proteins assessed in the western blot. However this would have to be done through an immunoprecipitation western blot as there is currently no antibody for phosphorylated Eph-B4.

The media used for the bioreactor flow chamber is difficult to mix as the xanthan gum does not dissolve easily in the EBM. Therefore the media had to be left on a stir plate overnight at 37°C. This makes an antibiotic-antimycotic solution necessary as bacterial or fungal growth could affect the arteriovenous fistula itself or the viscosity of the media. If the viscosity of the media were to be increased it would no longer simulate that of blood and affect the shear stress the vessel walls are under as can be seen by the Hagen-Poiseuille equation. If this experiment were to be repeated ideally a better method of mixing the media would be devised so that the antibiotic-antimycotic solution is no longer needed as it could potentially affect the fistula and its response.

If we would repeat the experiment and get results we would expect to see some trends in the expression and activation of proteins similar to those detected in *in vivo* models. It has previously been shown that caveolin-1 can act as a mechanosensor and therefore we suggest that it could detect the increased shear stress which could lead to different levels of the protein or different levels of activation. (45) It has also been shown that caveolin-1 interacts with Eph-B4, Akt-1 and eNOS. (26, 39, 40) It could affect the total levels and activation of these proteins in response to increased shear stress. In line with recent results from the Dardik lab we would expect Eph-B4 levels to rise as happens in arteriovenous fistula *in vivo*. (25)

The bioreactor model can be used in many exciting projects in the future. It could be used for longer runs where the samples could be analyzed for mRNA expression. This would require the runs to take place in an aseptic environment which would require modifications to the bioreactor. Stimulants and inhibitors could be added to the media and protein expression and interaction be analyzed in the samples. Signaling molecules from the sample could be isolated and analyzed from the media. For this experiment to be realistic the amount of media used in the bioreactor would have to be reduced to raise the concentration levels of the signaling molecules. This model also has the possibility of changing the angle that the vein connects to the artery. This angle can affect the amount of turbulent flow within the fistula. The bioreactor could be used to assess whether this angle affects protein expression or activation and thus maturation. Results from these or similar experiments could then be applied to *in vivo* models which could lead to increased understanding of arteriovenous fistula maturation. That knowledge could then in turn lead to novel clinical targets or therapies which could impact the lives of the hundreds of thousands of people whose end stage renal disease is being treated with hemodialysis.

## 6 References

1. Fancher TT, Muto A, Fitzgerald TN, Magri D, Gortler D, Nishibe T, et al. Control of blood vessel identity: from embryo to adult. *Annals of vascular diseases*. 2008;1(1):28-34.
2. Lu DY, Chen EY, Wong DJ, Yamamoto K, Protack CD, Williams WT, et al. Vein graft adaptation and fistula maturation in the arterial environment. *The Journal of surgical research*. 2014;188(1):162-73.
3. Marie Y, Guy A, Tullett K, Krishnan H, Jones RG, Inston NG. Patterns of blood flow as a predictor of maturation of arteriovenous fistula for haemodialysis. *The journal of vascular access*. 2014;15(3):169-74.
4. Brescia MJ, Cimino JE, Appel K, Hurwicz BJ. Chronic hemodialysis using venipuncture and a surgically created arteriovenous fistula. *The New England journal of medicine*. 1966;275(20):1089-92.
5. National Kidney Foundation. KDOQI Clinical Practice Guidelines and Clinical Practice Recommendations for 2006 Updates: Hemodialysis Adequacy, Peritoneal Dialysis Adequacy and Vascular Access. *American journal of kidney diseases : the official journal of the National Kidney Foundation*. 2006;48:S1-S322 (suppl 1).
6. Polo JR, Romero A. Brachiocephalic fistulas for vascular access. *Nephron*. 1989;52(1):105-6.
7. Kaing G. Taking Care of Arteriovenous Fistula. 2014.
8. United States Renal Data System. 2015 USRDS annual data report: Epidemiology of kidney disease in the United States. Bethesda, MD: National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, 2015.
9. Pisoni RL, Young EW, Dykstra DM, Greenwood RN, Hecking E, Gillespie B, et al. Vascular access use in Europe and the United States: results from the DOPPS. *Kidney international*. 2002;61(1):305-16.
10. Dhingra RK, Young EW, Hulbert-Shearon TE, Leavey SF, Port FK. Type of vascular access and mortality in US hemodialysis patients. *Kidney international*. 2001;60(4):1443-51.
11. Ravani P, Palmer SC, Oliver MJ, Quinn RR, MacRae JM, Tai DJ, et al. Associations between hemodialysis access type and clinical outcomes: a systematic review. *Journal of the American Society of Nephrology : JASN*. 2013;24(3):465-73.
12. Lee H, Manns B, Taub K, Ghali WA, Dean S, Johnson D, et al. Cost analysis of ongoing care of patients with end-stage renal disease: the impact of dialysis modality and dialysis access. *American journal of kidney diseases : the official journal of the National Kidney Foundation*. 2002;40(3):611-22.
13. Vazquez MA. Vascular access for dialysis: recent lessons and new insights. *Current opinion in nephrology and hypertension*. 2009;18(2):116-21.
14. Dember LM, Beck GJ, Allon M, et al. Effect of clopidogrel on early failure of arteriovenous fistulas for hemodialysis: A randomized controlled trial. *JAMA*. 2008;299(18):2164-71.
15. Schild AF, Prieto J, Glenn M, Livingstone J, Alfieri K, Raines J. Maturation and failure rates in a large series of arteriovenous dialysis access fistulas. *Vascular and endovascular surgery*. 2004;38(5):449-53.
16. Biuckians A, Scott EC, Meier GH, Panneton JM, Glickman MH. The natural history of autologous fistulas as first-time dialysis access in the KDOQI era. *Journal of Vascular Surgery*. 2008;47(2):415-21.
17. Juncos JP, Grande JP, Kang L, Ackerman AW, Croatt AJ, Katusic ZS, et al. MCP-1 contributes to arteriovenous fistula failure. *Journal of the American Society of Nephrology : JASN*. 2011;22(1):43-8.
18. Model LS, Hall MR, Wong DJ, Muto A, Kondo Y, Ziegler KR, et al. Arterial shear stress reduces eph-b4 expression in adult human veins. *The Yale journal of biology and medicine*. 2014;87(3):359-71.
19. Lisabeth EM, Falivelli G, Pasquale EB. Eph Receptor Signaling and Ephrins. *Cold Spring Harbor Perspectives in Biology*. 2013;5(9):20.

20. Wang HU, Chen ZF, Anderson DJ. Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. *Cell*. 1998;93(5):741-53.
21. Gerety SS, Anderson DJ. Cardiovascular ephrinB2 function is essential for embryonic angiogenesis. *Development (Cambridge, England)*. 2002;129(6):1397-410.
22. Rivera M, Muto A, Feigel A, Kondo Y, Dardik A. Venous and arterial identity: a role for caveolae? *Vascular*. 2009;17 Suppl 1:S10-4.
23. Muto A, Model L, Ziegler K, Eghbalieh SD, Dardik A. Mechanisms of vein graft adaptation to the arterial circulation: insights into the neointimal algorithm and management strategies. *Circulation journal : official journal of the Japanese Circulation Society*. 2010;74(8):1501-12.
24. Kudo FA, Muto A, Maloney SP, Pimiento JM, Bergaya S, Fitzgerald TN, et al. Venous identity is lost but arterial identity is not gained during vein graft adaptation. *Arteriosclerosis, thrombosis, and vascular biology*. 2007;27(7):1562-71.
25. Foster TR, editor Eph-B4 Mediated AVF Remodeling via Akt-1. Annual Meeting of VA Surgeons; 2016; Virginia Beach.
26. Muto A, Yi T, Harrison KD, Davalos A, Fancher TT, Ziegler KR, et al. Eph-B4 prevents venous adaptive remodeling in the adult arterial environment. *The Journal of experimental medicine*. 2011;208(3):561-75.
27. Rothberg KG, Heuser JE, Donzell WC, Ying YS, Glenney JR, Anderson RG. Caveolin, a protein component of caveolae membrane coats. *Cell*. 1992;68(4):673-82.
28. Scherer PE, Okamoto T, Chun M, Nishimoto I, Lodish HF, Lisanti MP. Identification, sequence, and expression of caveolin-2 defines a caveolin gene family. *Proceedings of the National Academy of Sciences of the United States of America*. 1996;93(1):131-5.
29. Tang Z, Scherer PE, Okamoto T, Song K, Chu C, Kohtz DS, et al. Molecular cloning of caveolin-3, a novel member of the caveolin gene family expressed predominantly in muscle. *The Journal of biological chemistry*. 1996;271(4):2255-61.
30. Scherer PE, Tang Z, Chun M, Sargiacomo M, Lodish HF, Lisanti MP. Caveolin isoforms differ in their N-terminal protein sequence and subcellular distribution. Identification and epitope mapping of an isoform-specific monoclonal antibody probe. *The Journal of biological chemistry*. 1995;270(27):16395-401.
31. Lisanti MP, Tang ZL, Sargiacomo M. Caveolin forms a hetero-oligomeric protein complex that interacts with an apical GPI-linked protein: implications for the biogenesis of caveolae. *The Journal of cell biology*. 1993;123(3):595-604.
32. Kurzchalia TV, Dupree P, Parton RG, Kellner R, Virta H, Lehnert M, et al. VIP21, a 21-kD membrane protein is an integral component of trans-Golgi-network-derived transport vesicles. *The Journal of cell biology*. 1992;118(5):1003-14.
33. Sargiacomo M, Scherer PE, Tang Z, Kubler E, Song KS, Sanders MC, et al. Oligomeric structure of caveolin: implications for caveolae membrane organization. *Proceedings of the National Academy of Sciences of the United States of America*. 1995;92(20):9407-11.
34. Parolini I, Sargiacomo M, Galbiati F, Rizzo G, Grignani F, Engelman JA, et al. Expression of caveolin-1 is required for the transport of caveolin-2 to the plasma membrane. Retention of caveolin-2 at the level of the golgi complex. *The Journal of biological chemistry*. 1999;274(36):25718-25.
35. Scherer PE, Lewis RY, Volonte D, Engelman JA, Galbiati F, Couet J, et al. Cell-type and tissue-specific expression of caveolin-2. Caveolins 1 and 2 co-localize and form a stable hetero-oligomeric complex in vivo. *The Journal of biological chemistry*. 1997;272(46):29337-46.
36. Glenney JR, Jr., Soppet D. Sequence and expression of caveolin, a protein component of caveolae plasma membrane domains phosphorylated on tyrosine in Rous sarcoma virus-transformed fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America*. 1992;89(21):10517-21.
37. Galbiati F, Razani B, Lisanti MP. Emerging Themes in Lipid Rafts and Caveolae. *Cell*. 2001;106(4):403-11.

38. Murata M, Peranen J, Schreiner R, Wieland F, Kurzchalia TV, Simons K. VIP21/caveolin is a cholesterol-binding protein. *Proceedings of the National Academy of Sciences of the United States of America*. 1995;92(22):10339-43.
39. Lisanti MP, Scherer PE, Tang Z, Sargiacomo M. Caveolae, caveolin and caveolin-rich membrane domains: a signalling hypothesis. *Trends in cell biology*. 1994;4(7):231-5.
40. Razani B, Woodman SE, Lisanti MP. Caveolae: from cell biology to animal physiology. *Pharmacological reviews*. 2002;54(3):431-67.
41. Vihanto MM, Vindis C, Djonov V, Cerretti DP, Huynh-Do U. Caveolin-1 is required for signaling and membrane targeting of EphB1 receptor tyrosine kinase. *Journal of cell science*. 2006;119(Pt 11):2299-309.
42. Feron O, Belhassen L, Kobzik L, Smith TW, Kelly RA, Michel T. Endothelial nitric oxide synthase targeting to caveolae. Specific interactions with caveolin isoforms in cardiac myocytes and endothelial cells. *The Journal of biological chemistry*. 1996;271(37):22810-4.
43. Li S, Couet J, Lisanti MP. Src tyrosine kinases, Galpha subunits, and H-Ras share a common membrane-anchored scaffolding protein, caveolin. Caveolin binding negatively regulates the auto-activation of Src tyrosine kinases. *The Journal of biological chemistry*. 1996;271(46):29182-90.
44. Couet J, Sargiacomo M, Lisanti MP. Interaction of a receptor tyrosine kinase, EGF-R, with caveolins. Caveolin binding negatively regulates tyrosine and serine/threonine kinase activities. *The Journal of biological chemistry*. 1997;272(48):30429-38.
45. Yu J, Bergaya S, Murata T, Alp IF, Bauer MP, Lin MI, et al. Direct evidence for the role of caveolin-1 and caveolae in mechanotransduction and remodeling of blood vessels. *The Journal of clinical investigation*. 2006;116(5):1284-91.
46. Hashimoto T, editor Arteriovenous fistula adaptation requires caveolin-1 signaling. *Vascular Research Initiatives Conference*; 2016; Nashville, Tennessee.
47. Coffey PJ, Woodgett JR. Molecular-Cloning and Characterization of a Novel Putative Protein-Serine Kinase Related to the cAMP-Dependent and Protein-Kinase-C Families. *European Journal of Biochemistry*. 1991;201(2):475-81.
48. Andjelković M, Alessi DR, Meier R, Fernandez A, Lamb NJC, Frech M, et al. Role of translocation in the activation and function of protein kinase B. *Journal of Biological Chemistry*. 1997;272(50):31515-24.
49. Franke TF, Tartof KD, Tsichlis PN. The SH2-Like Akt Homology (AH) Domain of c-Akt is Present in Multiple Copies in the Genome of Vertebrate and Invertebrate Eukaryotes - Cloning and Characterization of the *Drosophila melanogaster* c-Akt Homolog DAKT1. *Oncogene*. 1994;9(1):141-8.
50. Paradis S, Ruvkun G. *Caenorhabditis elegans* Akt/PKB transduces insulin receptor-like signals from AGE-1 PI3 kinase to the DAF-16 transcription factor. *Genes & development*. 1998;12(16):2488-98.
51. Jones PF, Jakubowicz T, Hemmings BA. Molecular cloning of a second form of rac protein kinase. *Cell regulation*. 1991;2(12):1001-9.
52. Nakatani K, Sakaue H, Thompson DA, Weigel RJ, Roth RA. Identification of a human Akt3 (Protein kinase B  $\gamma$ ) which contains the regulatory serine phosphorylation site. *Biochemical and Biophysical Research Communications*. 1999;257(3):906-10.
53. Kandel ES, Hay N. The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. *Experimental Cell Research*. 1999;253(1):210-29.
54. Bellacosa A, Testa JR, Staal SP, Tsichlis PN. A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science (New York, NY)*. 1991;254(5029):274-7.
55. Musacchio A, Gibson T, Rice P, Thompson J, Saraste M. The PH Domain - A Common Piece in the Structural Patchwork of Signaling Proteins. *Trends in Biochemical Sciences*. 1993;18(9):343-8.
56. Datta K, Franke TF, Chan TO, Makris A, Yang SI, Kaplan DR, et al. AH/PH domain-mediated interaction between Akt molecules and its potential role in Akt regulation. *Molecular and cellular biology*. 1995;15(4):2304-10.

57. Franke TF, Yang SI, Chan TO, Datta K, Kazlauskas A, Morrison DK, et al. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell*. 1995;81(5):727-36.
58. Bellacosa A, Chan TO, Ahmed NN, Datta K, Malstrom S, Stokoe D, et al. Akt activation by growth factors is a multiple-step process: The role of the PH domain. *Oncogene*. 1998;17(3):313-25.
59. Alessi DR, Caudwell FB, Andjelkovic M, Hemmings BA, Cohen P. Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase. *FEBS Letters*. 1996;399(3):333-8.
60. Oliveira-Paula GH, Lacchini R, Tanus-Santos JE. Endothelial nitric oxide synthase: From biochemistry and gene structure to clinical implications of NOS3 polymorphisms. *Gene*. 2016;575(2 Pt 3):584-99.
61. Yang WD, Ando J, Korenaga R, Toyooka T, Kamiya A. Exogenous Nitric Oxide Inhibits Proliferation of Cultured Vascular Endothelial Cells. *Biochemical and Biophysical Research Communications*. 1994;203(2):1160-7.
62. Wang M, Collins MJ, Foster TR, Bai H, Hashimoto T, Santana JM, et al. Eph-B4 mediates vein graft adaptation by regulation of endothelial nitric oxide synthase. *J Vasc Surg*. 2016.
63. Santana JM, editor eNOS is a mediator of arteriovenous fistula maturation. Sarnoff Conference; 2016; Boston, Massachusetts.



## 7 Appendix 1

### **Immunofluorescence Protocol on paraffin sections**

Giovanni Zito – Greco Lab

1. Heat the slides at 60 degrees for 1h
2. Clear the slides with Xilene (2x10')
3. Rehydrate the sections with serial ethanol passages:  
100% (2x5' each)  
90% (1x5')  
80% (1x5')  
70% (1x5')  
50% (1x5')  
30% (1x5')
4. Wash with PBS (2x 3')
5. Antigen retrieval: Nacitrate 10mM pH 6.0. Use the retriever filled out with Nacitrate (1L). Let cool the slides down at room temperature for 5' by adding.
6. Permeabilize with PBS/0.2% TritonX, 10' room temperature
7. Wash with PBS (1x5')
8. Blocking solution (the same that we use for frozen sections) at least 1h at room temperature.
9. Incubation with primary antibody diluted in blocking solution, over night at 4 degrees.
10. Wash the slides with PBS/0.2% TritonX (2x20')
11. Wash with PBS (1x20')
12. Incubate with secondary antibody diluted in blocking solution, 1h at room temperature. Keep the slides in a dark environment
13. Wash the slides with PBS/0.2% TritonX (2x20'). Keep the slides in a dark environment
14. Wash with PBS (1x20'). Keep the slides in a dark environment
15. Mount the slides with mounting solution (with DAPI)

Downloaded from

[[http://www.yale.edu/grecolab/Lab\\_Protocols\\_files/Immunofluorescence%20Protocol%20on%20paraffin%20sections.pdf](http://www.yale.edu/grecolab/Lab_Protocols_files/Immunofluorescence%20Protocol%20on%20paraffin%20sections.pdf)] 03.14.2016

## 8 Appendix 2

### *Lysis buffer*

To make 5 mL:

500 uL 10xRIPA,

500uL 10% SDS,

50uL Phosphatase I inhibitor,

50uL Phosphatase II inhibitor,

50 uLPMSF (100mM),

750 uL Protease inhibitor,

3.1 mL distilled water.

## 9 Appendix 3

### General protocol for Western Blotting (AP and HRP conjugates)

(last updated 3-23-07)

By : Fang Yi

#### BUFFERs and Solutions:

Transfer buffer (1.5L, always prepare fresh before transfer):

- 50 mM Tris, 39 mM Glycine, 20% methanol (v/v), pH 8.0
- Methanol slows down the transfer, add SDS to larger protein to speed up the transfer

20X TBS stock (no Tween 20) (200 ml)

- 200 mM Tris, 3M NaCl, pH 8.0

1X TBS<sup>T</sup> (prepare right before the analysis) (1L)

- 10 mM Tris, 150 mM NaCl, pH 8.0, 0.1% Tween

Blocking buffer:

- 1X TBST with 5% non-fat milk

**For AP conjugates:**

TSM

- 100 mM Tris, 100 mM NaCl, 50 mM MgCl<sub>2</sub>, pH 9.5

For development:

- BciP, NTB, TSM

**For HRP conjugate (colorimetric only):**

10X Tris-saline:

- 9% (w/v) NaCl in 1 M Tris-HCl, pH 8.0

Staining solution for HRP:

- 18 mg 4-chloro-1-naphthol (sigma cat#: C8890) in 6 ml methanol. Add 24 ml 1X Tris-saline followed by 600 ul 3% hydrogen peroxide (final 0.2% H<sub>2</sub>O<sub>2</sub>)

#### Procedures:

##### 1. SDS-page Gel:

- Run a protein gel to separate the proteins, runs it until the bromophenol blue front reaches the end of gel.
- also load a protein marker to use as a control for protein transfer.

##### 2. During gel running, prepare:

- PVDF membrane:

Cut appropriate size membrane, and activate it by:

- Soak in 100% methanol for 10 sec. (PVDF is hydrophobic)
- Soak in ddH<sub>2</sub>O for 30 sec

- Soak in transfer buffer for >10 minutes

#### Whatman paper filter & sponges

- Soak cut filters (whatman paper) and sponges in transfer buffer for 10 minutes (push bubbles out)
- (don't soak the gel in transfer buffer if you are transferring small proteins); but soaking in transfer buffer for short time (eg. 3 minutes) can wash salt off gel to reduce current during transfer.

3. Assemble the transfer apparatus in the order of  
Black(negative) –Sponge---Filter---Gel---PVDF memberane—filter---sponge—**RED end**
4. Ensure the packing is tight and the chamber does not leak. ( if you press the sponges to the inner side, you should see the solution levels)
5. Transfer at RT (ice in apparatus) for 120 minutes at constant current (350 mA)
  - This setting may need to be optimized for diff. size of proteins. Small proteins (<20 kD) tends to transfer faster, larger protein might need longer transfer time
  - The setting that has worked for me (working with proteins with diff. sizes from 37kD to 180kD) is: total work ( W ) =  $I \times V \times \text{time} \sim 15$  . For example, if using a constant voltage of 40 volts, current is 0.2 A, then a time setting of 2 hrs ( $40 \times 0.2 \times 2 = 16$ ) usually is enough for complete transfer as judged by the marker.
6. Disassemble the chamber. To ensure your protein is transferred before all the following steps, commassie blue stain the SDS-gel to estimate the transfer efficiency
7. Block the membrane for 1 hr in blocking buffer: 5% (w/v) non-fat milk in TBST.
8. Wash membrane for 3\* 5 min w/ TBST;
9. Incubate membrane in Primary antibodies diluted in blocking buffer (TBST) at 4 deg overnight and 1 hr at room temperature.
10. Wash for 3\*5 min w/ TBST;
11. Incubate with 2<sup>nd</sup> Ab for 1 hr at RT in TBST
12. wash for 2\*5 min w/ TBST
13. wash 1\*5 min w/ TBS
14. wash 2\*10 min/TSM (if using AP conjugated 2<sup>nd</sup> Antibody)
15. Development:
  - a) Colorimetric: Stain with AP or HRP staining solution until the signal is clearly visible
    - For AP-conjugated 2<sup>nd</sup> Ab, add 33 ul BciP , 42 ul NBT in 10 ml TSM, pH 9.5 (5-15 minutes)
    - For HRP: Staining solution for HRP: 18 mg 4-chloro-1-naphthol (sigma cat#: C8890) in 6 ml methanol. Add 24 ml 1X Tris-saline followed by 600 ul 3% hydrogen peroxide (final 0.2% H<sub>2</sub>O<sub>2</sub>) (1-5 minutes)
    - at RT for 5~15 minutes until the band shows up; (Don't shake blots during color development)
  - b) ECL solution followed by Film Exposure:

1. Prepare ECL reagents: mix the two reagents 1:1 (~1ml per blot) and pipette enough vol on a piece of Saran wrap taped to the bench.
2. Drag the blot along the edge of the tray, drain excess TBST, then place the blot with protein side down on the ECL solution.
3. Incubate for 1 min then drain excess reagent and transfer the blot to a plastic report cover.
4. Expose immediately (few seconds — 1 hr).

**16. Stop solution: 20 mM Tris, pH 7.4, 5 mM EDTA OR rinse twice in water.**

**17. Dry the membrane and expose ASAP.**

**TIPs from the Millipore Protein Blotting Handbook (5<sup>th</sup> version):**

**To reduce persistent background:**

- **Use High salt wash after the 2<sup>nd</sup> Ab incubation (PBS or TBS with 0.5 M NaCl and 0.2% SDS)**
  - ◆ **Incubate the membrane in high salt buffer for 30 minutes w/ gentle shaking**
  - ◆ **Rinse blot with Milli-Q water and proceed as usual**

**To reduce overall high background:**

**Use higher dilution of the 2<sup>nd</sup> Ab**

**To reduce high non-specific signal:**

**Use higher dilution of the 1<sup>st</sup> Ab and lower protein yield.**

**To view the protein on the membrane:**

**Soak in 20% methanol, watch on the light box**

**Role of Tween:**

**0.05% -0.1% Tween can help renature the antigen, thus increasing improved recognition of specific antibodies; but too high % of Tween can wash away blotted proteins**