

**THE REGULATION OF PLEXIN B1 EXPRESSION IN PROSTATE CANCER
METASTASIS**

By

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DECLARATION

This thesis is as a result of my own work except where otherwise stated.

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Abstract

Prostate cancer is a significant disease affecting most men as they age. Although the overall lifetime mortality from the disease is about 3% the disease has a high prevalence and the treatment for the disease carries with it unwanted morbidity. Our group has shown that plexin B1 protein expression is elevated in prostate cancer and have suggested a link with metastatic prostate cancer.

The mechanism for the increased expression of plexin B1 in prostate cancer is unknown, the aim of this study was to elucidate the cause for this upregulation. I hypothesized that microRNAs are linked with the increased expression of plexin B1, either through loss of miRNA binding sites in the 3'UTR of plexinB1 mRNA or through loss of specific miRNAs that regulate plexinB1 expression. I found no evidence for plexinB1 mRNA variants with truncated 3'UTR resistant to miRNA down regulation. Two splice variants were identified in prostate cancer cells that are predicted to alter plexinB1 function, one being variant R hitherto unproven in literature. I performed an insilico and an in vitro functional screen to identify candidate miRNAs that regulate plexinB1 expression. The candidate miRNAs were then assessed to establish if they directly bound to the 3'UTR of plexinB1 and regulated expression of a reporter gene and to determine if they had any effect on endogenous plexinB1 mRNA and protein levels in prostate cancer cell lines. MiRNAs 199a and miRNA 214-3p reduced expression of the reporter gene and endogenous plexinB1, suggesting that these miRNAs negatively regulate plexinB1 expression directly by binding to the 3'UTR of plexinB1. MiRNA 1 was found to down regulate endogenous plexinB1 expression

probably indirectly. I conclude that there is a role for specific microRNAs in regulating plexin B1 expression with potential for prostate cancer therapy but further studies are needed to validate my findings.

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Publications

Function of mutant and wild-type plexin B1 in prostate cancer cells.

Damola A, Legendre A, Ball S, Masters JR, Williamson M.

Prostate. 2013 Sep;73(12):1326-35. doi: 10.1002/pros.22678. Epub 2013 Jun 15

The prostate cancer tumour suppressor micro RNA 1, regulates plexin B1 expression

Damola A, Williamson M.

The Journal of Urology, 2013 April Vol. 189, Issue 4, Supplement, Page e540

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Chapter 1

Introduction

Plexins and Semaphorins Overview

The Semaphorin-Plexin signalling pathway is responsible for many normal functions in the body such as the embryological development of the nervous, cardiovascular and skeletal systems (Perala et al., 2012). This pathway is involved in the immune system as well as in tumour progression. This section aims to summarise what is known about the structure of Semaphorins and Plexins and also their normal functions.

Semaphorins

Semaphorins (Sema) belong to a super family of over 30 proteins found mainly in multicellular organisms (Potiron et al., 2009). There are 8 classes within the semaphorin family and they consist of 21 vertebrate genes and 8 invertebrates genes (Neufeld and Kessler, 2008). The 8 classes are shown in figure 1 below.

Classes 1 and 2 are invertebrate Semaphorins, while 3 to 7 represent the vertebrates and class 5 comprises both invertebrate and vertebrate Semaphorins, finally class V are the viral semaphorins (Perala et al., 2012). The Semaphorins are mainly known for their role in the nervous system, but they are expressed in various other tissues and organs including renal, cardiac, endocrine, gastrointestinal and seem to be expressed more in the developmental stages in humans and wane with age (Yazdani and Terman, 2006).

[Fig 1 modified from Neufeld and Kessler](#) (Neufeld, G. and O. Kessler 2008)

Diagram of the Semaphorins, with their receptors Plexins and Neuropilins.

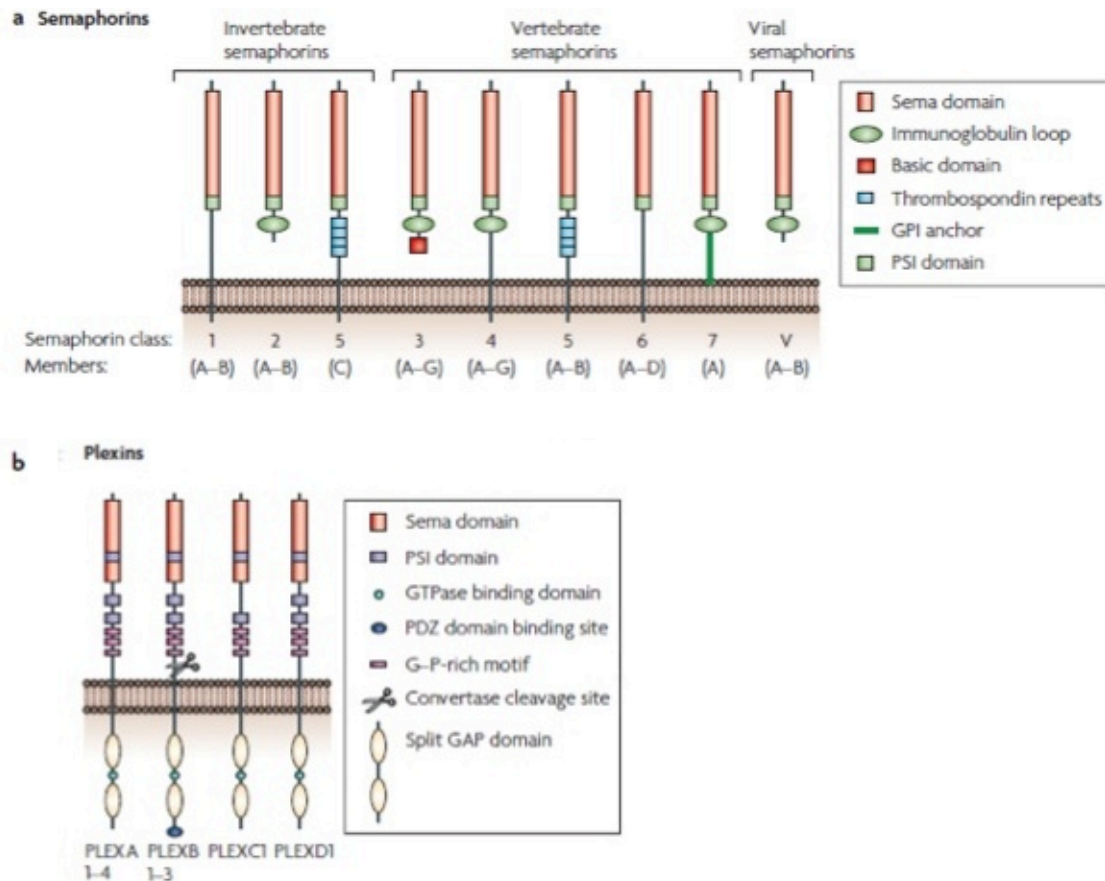


Fig 1a: The 8 classes of semaphorin showing their subunits and domains and relationship with the cell wall.

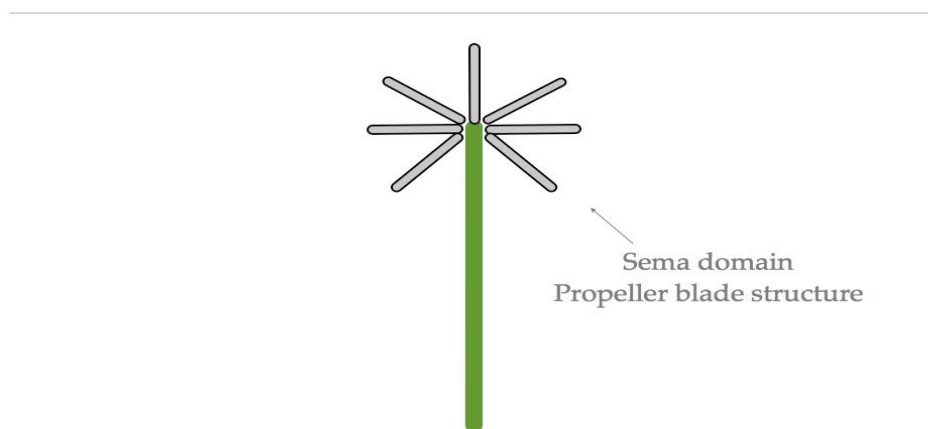
Fig 1b: Plexins, showing their subunits and domains.

[Structure and functions of the domains of Semaphorins](#)

Semaphorins are homodimers composed of paired identical polypeptide chains. They have a characteristic feature, the Semaphorin domain that contains approximately 500

amino-acids. This domain also occurs in the Plexins and Met families (Potiron et al., 2009). This Sema domain is essential for semaphorin signaling and it is through the analysis of the X-ray structure of the Sema domains of Semaphorin 3D and Semaphorin 4D that it is known that this segment is a conserved seven blade beta propeller structure (Neufeld and Kessler, 2008, Yazdani and Terman, 2006). The seven bladed structure of the Sema domain, which is seen in other proteins, is the largest of its kind due to the number of amino acids present (Gherardi et al., 2004). It is given the name due to the similarity to a propeller with its multi-stranded beta sheets that surround a central axis, see figure 2.

[Fig 2 Schematic diagram of the propellar blade structure of the SEMA domain](#)



The Sema domain is the region where most of the interactions occur between semaphorin and other proteins that lead to signaling effects. For instance the sema-

sema interaction of the blades of the propeller structure of semaphorin and that of the plexins (Yazdani and Terman, 2006, Gherardi et al., 2004, Sakurai et al., 2012)

There is a conserved stretch of 54 amino-acids that occurs after the carboxy-terminal of the sema domain that bears homology to the N-terminal of the same region of beta integrins and is designated the PSI (Plexin-Semaphorin-Integrin) domain. Its role is to correctly orient semaphorin with its receptor, but it may also be involved in signaling as the PSI domain of β 1 integrins binds to antibodies (Hota and Buck, 2012, Kozlov et al., 2004). Only the viral Semaphorin V lacks the PSI domain. Another recurrent domain in the Semaphorins is the immunoglobulin-type domain, whose function is to enhance the effect of the Sema domain (Yazdani and Terman, 2006), see figure 1.

Semaphorins diverge in their C-termini and can be either transmembrane, anchored into the plasma membrane or secreted. Class 3 Semaphorins are the only secreted vertebrate Semaphorins and are distinguished by the presence of a conserved basic domain at their C- termini. Classes 4 to 7 are all cell membrane anchored proteins. There are structures that differentiate each class, such as thrombospondin repeats which are peculiar to class 5. The thrombospondin repeat domain in Sema 5A has been shown to regulate Sema5A axon guidance cues by forming links with proteoglycans (Kantor et al., 2004). Class 7 have a GPI anchor that holds them to the cell wall (Neufeld and Kessler, 2008).

Plexins

Plexins are the primary receptors of Semaphorins. They are a large family of trans-membrane glycoproteins. The first Plexin molecule was discovered in the African clawed frog *xenopus laevis* using an antibody produced from plexiform layers of its optic tectum, hence the name Plexin (Takagi et al., 1987) . Like Semaphorins, Plexins are widely expressed in nervous and other tissues in the body.

Structure

The nine vertebrate Plexins are divided into 4 subfamilies as follows;

4 in type A (A1, A2, A3 and A4)

3 in type B (B1, B2 and B3)

1 in type C (C1)

1 in type D (D1)

Plexins have a characteristic split GTPase-activation protein (GAP) cytoplasmic domain and are the only known membrane spanning receptors that interact directly with small intracellular GTPases (Neufeld and Kessler, 2008). Their role will be discussed later.

Diagrams of the Plexins are shown in figure 1.

The extracellular domain of all Plexins is distinguished by the Sema domain, the presence of PSI and glycine-proline (G-P) rich motifs that the plexins share with tyrosine kinase receptors belonging to the Met family. The Plexin B family contains in addition a C-terminal binding site for the PDZ domain containing GEF (PDZ-RHOGEF) and LARG

and in their extracellular domain a conserved cleavage site for furin-like proprotein convertases that is not found in other Plexins. Plexin B1 is of a particular interest because of the link with prostate cancer as highlighted by Wong et al (Wong et al., 2007), this will be discussed in detail later.

The variants of Plexin B1

The human Plexin B1 gene is found on Chromosome 3 p21 and it has 37 exons and composed of over 26,000 nucleotide bases. One of the key steps in the process that leads to protein production is the transcription of genes in DNA to RNA. The DNA is first transcribed to precursor messenger RNA (pre-mRNA) in the nucleus following which splicing occurs to remove the intervening sequences (introns) (Black, 2003). This results in mature mRNA containing only exons. This is termed constitutive splicing and is the conventional form of splicing. Splicing is catalysed by Spliceosomes which are macromolecular complexes. Other events that occur post transcription within the nucleus include 5' capping and 3' polyadenylation (Hui, 2009). There are two main forms of the Plexin B1 mRNA produced by constitutive splicing as listed below (NCBI, 2011);

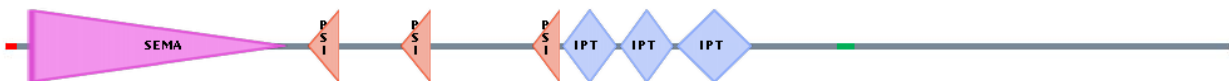
Variant 1 (NM002673)

Variant 2 (NM001130082)

The variants 1 and 2 mRNA are similar but differ in the 5' UTR region of the mRNA, they code for the exactly the same full length plexin B1 protein with 2135 amino acids. This full length is a single pass transmembrane receptor. A breakdown of the 2135 amino acid is as follows: The extracellular domain (ECD) is composed of type 1 transmembrane glycoprotein that contains 1471 amino acids. This ECD contains the

semaphoring domain, three PSI domains, and three IPT repeats. The cytoplasmic region or domain (CD) contains 612 aminoacids. The schematic diagram of the protein is shown in figure 3 below.

[Fig 3: Schematic diagram of protein for variant 1 and 2 courtesy of plasma proteome database](#)



(SEMA- Semaphorin domain; PSI- Plexins, semaphorins, integrins domain; IPT- Immunoglobulin-like Plexin Transcription factors domain)

There is another form of splicing termed alternative splicing that leads to the formation of other variants of mRNA. Alternative splicing allows multiple proteins to be produced from a single gene. This occurs at precursor mRNA (pre-mRNA) level(Hui, 2009).

Alternative splicing accounts for the hundreds of thousands of proteins produced by humans, despite an estimated 20,000 to 25,000 coding genes (Hui, 2009, 2004).

Alternative splicing occurs due to the following four main methods; a) Cassetted exon skipping or inclusion; b) alternative 5' splice sites; c) alternative 3' splice sites; d) intron inclusion(Nilsen and Graveley, 2010). This means that one gene may produce a variety of different mature mRNAs and therefore proteins termed alternatively spliced variant proteins. This phenomenon of alternative splicing was first discovered by Berget et al with their work on the viral hexon mRNA in 1977(Berget et al., 1977). There is a

correlation between the complexity of the organism and the occurrence of alternative splicing, with more complex organisms having a greater proportion of their genes undergoing alternative splicing (McManus and Graveley, 2011).

In the case of Plexin B1 gene, alternative splicing online human databases have shown up to 21 possible different alternatively spliced mRNA variants out of which only 5 produce a form of the Plexin B1 protein (Project, 2011, NCBI, 2011, Ensembl, 2011). The two most common forms of alternatively spliced variants of Plexin B1 mRNA according to the online databases are:

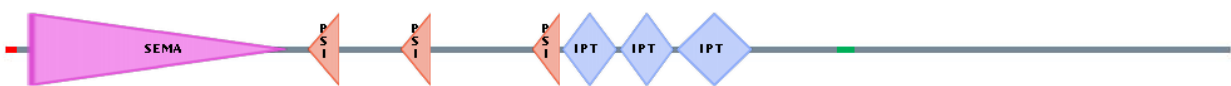
Truncated variant 'AJ011414'

Variant R 'AJ011415'

The truncated variant has a significantly shorter 3' UTR end, it is the shorter of the two presumably because it has a different polyadenylation signal site closer to the 5' end of the mRNA. Its mechanism of alternative splicing includes an exon skipping, it doesn't have the exon 11. The splicing out of exon 11 in this variant results in a shift in the reading frame. Translation of the mRNA is terminated prematurely due to the presence of a stop codon in the new frame. The protein product of this mRNA does not contain the inter-membranous or cytoplasmic component unlike the full protein. The protein of the truncated variant contains only 729 amino acids (NCBI, 2011, Tamagnone et al., 1999). The truncated variant as shown in the figure 4 below only has two PSI domains and lacks the transmembranous and cytoplasmic domains therefore it is a secreted form of the protein. Variant R is the longer of the two alternatively spliced variants and one of its modes of formation is also exon 11 skipping. Variant R also appears to have a

shortened 3' UTR end but not to the same extent as the truncated variant. It is similar to the full length mRNA of Plexin B1 and its protein contains 1952 amino acids(NCBI, 2011, Tamagnone et al., 1999). A summary of the common variants of plexin B1 are shown in table 1 below.

[Fig 4: Schematic diagram of protein for variant R and Truncated variant courtesy of plasma proteome database](#)



Protein- variant R (AJ011415)



Protein- truncated Variant (AJ011414)

(SEMA- Semaphorin domain; PSI- Plexins, semaphorins, integrins domain; IPT- Immunoglobulin-like Plexin Transcription factors domain)

[Table 1: The common variants of plexin B1](#)

The common variants of Plexin B1	
Constitutively spliced	Alternatively spliced
Variant 1 'NM002673' (7325bp)	Variant R 'AJ011515' (5859bp)
Variant 2 'NM001130082.1' (7158bp)	Truncated variant 'AJ011414'

The Semaphorin signalling pathway

Semaphorin binds to Plexin to activate a signalling pathway. Upon activating the signalling pathway changes occur within the cytoskeleton which causes cellular movement and changes in morphology, such as axonal retraction (Kruger et al., 2005).

The semaphorin signaling pathway involves the interaction of various proteins with the extracellular and intracellular regions of Plexins. The extracellular interactions are the start of the signalling pathway that continues downstream into the cell. For example neuropilins, which are essential co-receptors to Sema 3s which do not bind directly to Plexin, true for all SEMA 3 ligands the exception being SEMA3E (Capparuccia and Tamagnone, 2009); Offtrack (OTK) a receptor tyrosine kinase which combines with Sema 1a and Plexin A leading to axonal repulsion; CD72 and Tim 2 which are receptors to the Sema 4s involved in immune response; Also Sema 4 interacts with Met and ErbB2 through Plexin B1(Zhou et al., 2008).

The various Semaphorins require specific Plexin receptors to function. Sometimes the same Plexin acts as a receptor for different Semaphorins. Examples of these are listed in table 2 below;

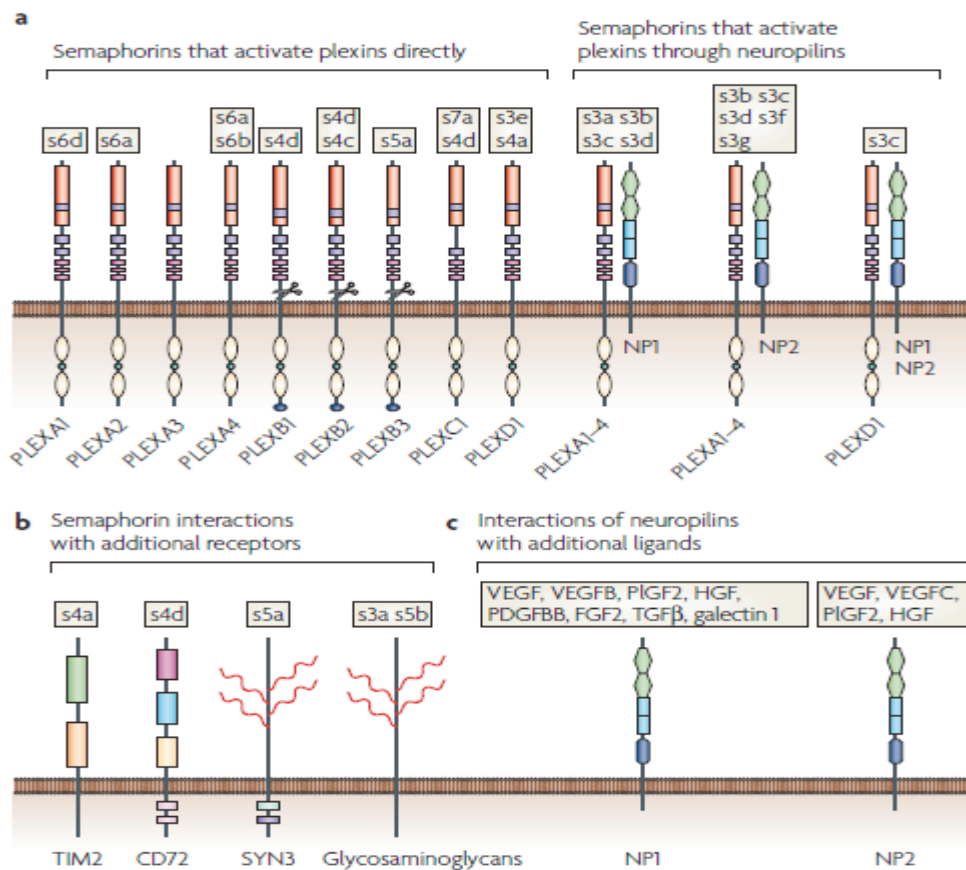
Table 2: The interactions of the Semaphorins and their receptors

Semaphorin	Receptor –Plexins (others)
1A,1B	A
2A	B
3A,3B,C,D,F	A (with neuropilin co-receptor)
3E	D
4A	(Tim-2)
4D	B1
5A	B3
6A,6B	A4
7	C1
V	C1

Figure 5 shows a broader example of the Semaphorins and their receptors involved in signal transduction.

Fig 5 courtesy of Neufeld and Kessler (Neufeld, G. and O. Kessler 2008)

The Interactions of semaphorins with its receptors



The signalling pathway also involves a variety of intracellular components. Some examples of the signaling pathways are listed below, focusing on Sema4D and its main receptor Plexin B1;

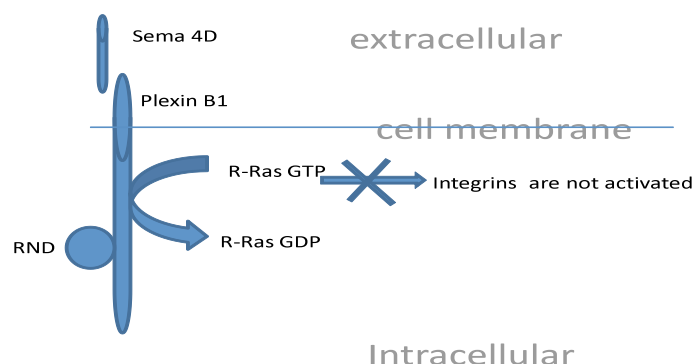
- a) The GTPases
- b) The receptor tyrosine kinases.

A) The GTPases-

1. The R-Ras

Sema 4D binds to Plexin B1 to activate the R-Ras GTPase activation protein (GAP) domain activity of Plexin B1, see figure 6. The stimulation of the GAP domain is also dependent on RND1 binding to the intracellular region of Plexin B1 which then allows Plexin B1 to act on R-Ras, converting it to R-Ras GDP from R-Ras GTP (Pasterkamp, 2005, Kruger et al., 2005). R-Ras regulates integrin activity, causing integrin mediated cell adhesion to the extracellular matrix. When Sema 4D binds to Plexin B1 the effect is to decrease activated R-Ras leading to reduced cellular adhesion to the extracellular matrix and detachment of the cells. This was proven by experiments involving the mutated forms of Plexin B1 that were unable to interact with R-Ras or RND1 resulting in unchanged level of GTP bound R-Ras. Integrins are involved in intracellular pathways which lead to important functions like cell death and movement. Thus there is inhibition of integrin activities, such as directional migration of cells leading instead to retraction.

[Figure 6: The Sema4D-Plexin B1 pathway involving R-Ras](#)

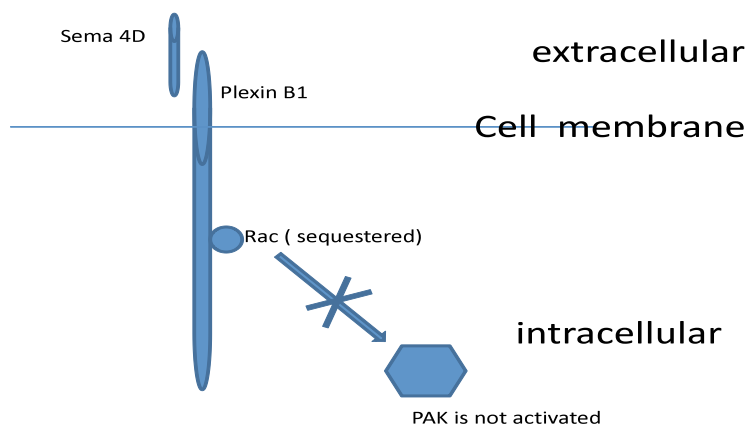


2. Rac

Another GTPase involved in the signaling pathway is Rac. Rac binds to an intracellular region of Plexin B1 (figure 7). Binding leads to the sequestering of Rac, which in turn reduces the amount of free active Rac in the cytoplasm. Rac activates p21-activated kinase, which polymerises actin. So there is inhibition of p21- activated kinase (PAK). The final result is to inhibit cellular actin polymerization and lamellipodia therefore causing repulsion (Wannemacher et al., 2011, Kruger et al., 2005).

Figure 7

The Sema4D-Plexin B1 pathway involving Rac



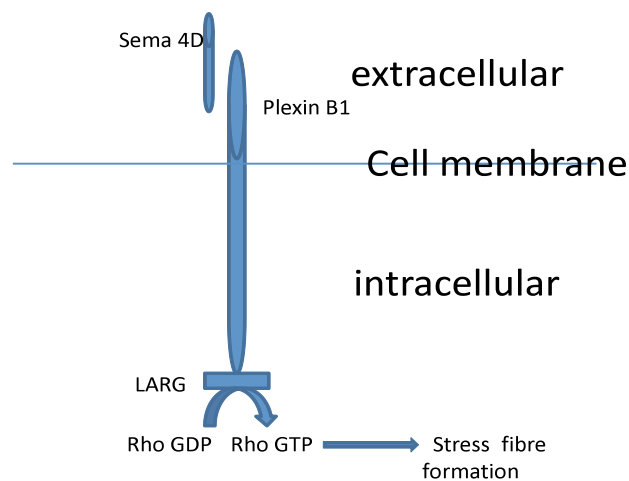
3. RhoA

The intracellular C terminal PDZ-binding region of Plexin B1 binds to the Rho specific guanine nucleotide exchange factors (RhoGEFs) (Kruger et al., 2005, Ch'ng and Kumanogoh, 2010). RhoA activation leads to repulsive growth of nerve cells due to the formation of stress fibres that cause contraction within the cytoplasm. The activation of

RhoA occurs through the Sema 4D-Plexin B1 signalling pathway. After Sema 4D binds to Plexin B1 the PDZ domain of Plexin B1 stimulates the RhoAGEFs LARG (Leukaemia associated RhoGEF) and PDZ-RhoGEF to cause the conversion of RhoA-GDP to activated RhoA-GTP, see figure 8.

Figure 8

The Sema4D-Plexin B1 pathway involving Rho



4Rnd

Rnd1 is another member of the Rho family. It has been shown to cause contraction or shrinkage of cells by Oinuma et al (Oinuma et al., 2003) interacting directly with plexin B1 in the presence of Sema 4D

5Rap

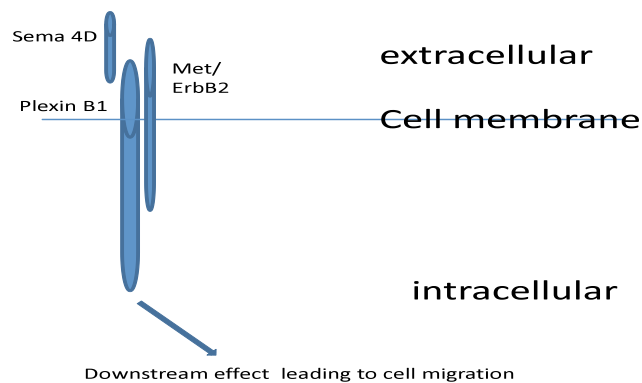
Plexins also act as GAPs for Rap (Wang et al., 2012)

B) The Receptor Tyrosine Kinases (RTK)

The binding of Sema 4D to Plexin B1 also stimulates the tyrosine kinase activity of Met and ErbB2 leading to cell migration (Zhou et al., 2008). Met or ErbB2 binds to the extracellular region of Plexin B1 in the presence of bound Sema 4D which causes the phosphorylation of Met or ErbB2 and Plexin B1.

Figure 9

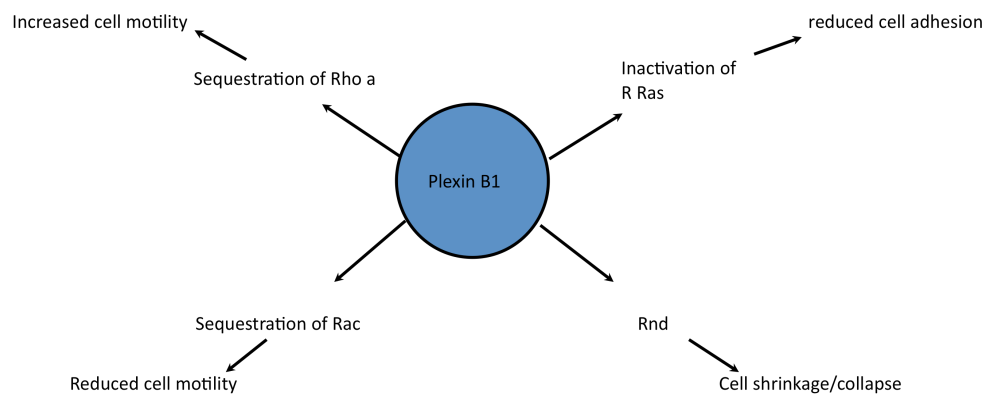
The Sema4D-Plexin B1 pathway involving Met or ErbB2



To summarise the different interactions with plexin B1 causes a number of action within the cell that is shown in the schematic diagram in figure 10 below.

Figure 10

A Schematic diagram of Plexin B1 function



Functions of the Semaphorin-Plexin pathway

The Semaphorins are present in many tissues of the body. The semaphorin-plexin pathway is involved in organogenesis, angiogenesis, Immunity and in tumour progression.

Semaphorin 4D

Immunity: SEMA4D was the first semaphorin identified in the immune system. SEMA4D (also called CD100) activates B cells in the immune system, increasing the quantity of B cells and antibody production. SEMA4D is expressed at high levels in tissues such as those of the spleen and the thymus. It is expressed in high levels on T cells, and in lower levels in B cells and macrophages. Its receptors include Plexin B1 and CD72 (Ch'ng and Kumanogoh, 2010, Nkyimbeng-Takwi and Chapoval, 2011). Plexin B1 is mainly found in epithelial and endothelial cells such as in the kidney, prostate and digestive tract among others. In the immune system Plexin B1 is expressed on the surface of dendritic cells (DC) and activated T cells. Several studies have shown the effect of SEMA4D through Plexin B1 is to increase B cell and DC activity, for instance B cell proliferation in mice (Nkyimbeng-Takwi and Chapoval, 2011). CD72 on the other hand is found mainly on the surface of B cells and in lower quantity on macrophages. It has a negative signalling effect on these cells, these signals are switched off by SEMA4D after binding. The intracellular region of the CD72 contains a tyrosine based domain ITIM that binds to tyrosine phosphatase SHP1, this SHP1 is released once SEMA4D binds to CD72 allowing the activation of B cells.

Angiogenesis: Angiogenesis is the creation of new blood vessels and is promoted by the Semaphorin-Plexin pathway. For instance in-vitro studies have shown the proangiogenic effects of the Semaphorin 4D and Plexin B1 interaction (Perala et al., 2012). Plexin B1 is found in endothelial cells and SEMA 4D signalling through Plexin B1 induces this angiogenic effect. Studies on porcine aortic endothelial cells showed

this effect occurs through the binding of the Rho GEFs, PDZ-RhoGEF and leukaemia-associated RhoGEF (LARG) to the PDZ region of plexin B1. In human umbilical vein endothelial cells the signalling pathway required the activation of Met tyrosine kinase (Roth et al., 2009). Both the Met and RhoGEF mechanism cause endothelial cell migration.

Neuronal guidance: The Sema 4D- Plexin B1 signalling pathway is also involved in neuronal development where they act as guidance cues. For instance experiments carried out on mice revealed that during development GnRH-1 secreting nerve cells require the presence of Semaphorin4D and Plexin B1 in order to migrate to the hypothalamus (Giacobini et al., 2008). It was shown that the signaling pathway also involved the phosphorylation of Met and that Plexin B1 deficient mice showed reduced migration to the hypothalamus from their olfactory origin.

Other Semaphorins

Immunity:

Other Semaphorins have been shown to be involved in the immune system (Roth et al., 2009). Including SEMA 4A, SEMA 7D and SEMA 3A. SEMA 4A is expressed on the surfaces of B cells and DC and its receptor in immune cells is Tim 2, which stands for T cell Ig and mucin domain 2. Tim 2 is found in activated T cells. SEMA 4A increases T cell activity by binding to its receptor rather than stimulating B cell activity. SEMA 7A is found in activated lymphocytes and its main receptor is Plexin C1. It may play a role as a negative effector on T cells helping to regulate their response (Roth et al., 2009).

SEMA 3A and its co receptor neuropilin 1 are found in the thymus and their levels increase during the maturation of the thymocytes. SEMA 3A is found on the surfaces of activated T cells and DC. Neuropilin 1 and SEMA 3A are involved in the negative feedback control of T cells production as shown when antibodies against SEMA 3A in DC and T cell co cultures caused an increase in T cell production(Lepelletier et al., 2006).

Angiogenesis:

There are other Semaphorins, such as SEMA 3E and SEMA 3A that are also involved angiogenesis. For instance SEMA 3E, which is the only class of SEMA 3 that does not require neuropilins to function, is expressed in developing endothelial cells along with its receptor Plexin D1(Neufeld et al., 2012). The presence SEMA 3E causes the organised growth of blood vessels within the boundary of the developing somite through its interaction with the receptor Plexin D1. In the heart, SEMA 6D and its receptor Plexin A1 are involved in embryonic development (Tran et al., 2007). This has been shown both in chicken and mice studies where the developing ventricular chambers were found to be influenced by the repulsive effect of SEMA 6D. It was found that chicken hearts are thinner and smaller in the absence of SEMA 6D or the receptor Plexin A1.

Development:

In lung development the Semaphorins also have an important role to play. They are involved in the development of blood vessels required for gaseous exchange. In terms of lung tissue specifically studies on mice lung tissue have revealed the presence of

SEMA 3A, 3C and 3F in invitro mouse lung tissue (Potiron et al., 2009). It appears SEMA 3A is located in the mesenchyme involved in inhibiting branching of the lung tree whereas the other two are involved in promoting growth through branching. Neuropilin 1 and 2 are also involved, as is Plexin A1 the receptor. It appears the SEMA 3A is involved early on the regulation of the growth of the main branches and later on its expression falls. SEMA 3C and 3F are involved in the growth of the terminal segments. Both the SEMA3F mRNA and protein have been shown to be expressed in adult human lung tissue.

There are a number of semaphorins and plexins that are involved in the body's skeletal development. The first stage of bone development is the transformation of cartilage that originates from chondrocytes to bone. Two important bone cell types are involved, osteoblasts that produce new bone material and the osteoclasts that reabsorb bone ensuring normal bone density is maintained as their roles balance out. The Plexin A1 and A2, SEMA 6D, SEMA 7A, SEMA 3A, and neuropilin 1 are involved in the skeletal development. Studies have shown the expression of SEMA 6D in osteoclasts; Plexin A1, A2 in chondrocytes and osteoblasts (Perala et al., 2012). In Plexin A1 knockout mice there is a defect in the regulation of osteoblastic function with resultant increase in bone and skeletal deformities. Plexin A1 is the receptor for SEMA 6D. Polymorphism of Plexin A2 is linked with osteoporosis in women and Plexin A2 may be used as marker for the disease (Roth et al., 2009). SEMA 7A is expressed by osteoblasts and may be essential for the production of new bone. A recent mouse study involving SEMA 3A seems to offer hope as a plausible therapy for osteoporosis where mice with deficient

bone states improved following injection of SEMA 3A into their blood stream (Hayashi et al., 2012). It is expressed by osteoblasts, osteoclasts and chondrocytes and seems to be a crucial semaphorin in conjunction with neuropilin 1 in bone homeostasis (Roth et al., 2009). It appears to be osteoprotective by increasing osteoblastic activity while reducing osteoclastic effects. Plexin D1 is also important when it comes to the development of the vertebrae with Plexin D1 knock out being fatal in mice, as they were incompatible with life, having split or fused vertebrae.

In the kidney Semaphorins 3A and 3F and their receptors neuropilin 1 and 2 are expressed and have been shown to have a role in the morphogenesis of the kidney. SEMA 3A is important in the development of the glomerular filtration barrier through the negative control on the development of the podocytes. It also has an inhibitory effect on the branching of the ureteric bud as discovered with knockdown Plexin 3A experiments. There is also a negative effect on the migration of the endothelium during development of the kidney (Reidy and Tufro, 2011, Roth et al., 2009). The SEMA 4D-Plexin B1 signalling pathway also seems to have an inhibitory effect on the ureteric branching by activating Rho A but without the influence of Met.

Semaphorin, Plexin and Cancer

The Semaphorins and their receptors play a role in disease processes in the human body. One such disease is cancer. There are several studies that show this link with

cancer ranging from the involvement of semaphorins in angiogenesis to the direct link with invasion and metastasis of tumour cells (Tamagnone, 2012).

The Semaphorin family has been shown to be involved in preventing tumour angiogenesis and metastasis like the Semaphorin 3 group while other semaphorins have been shown to promote angiogenesis and metastasis like Semaphorin 4D (Sakurai et al., 2012).

Angiogenesis is the process of new blood vessel formation and it is an essential part of the normal functioning of the body. Angiogenesis is also vitally important for major diseases like cancer both in terms of survival and progression and this has led to the development of anti-angiogenic drugs for the treatment of cancer.

There are a number of factors involved in angiogenesis, one such key factor being the vascular endothelial growth factor VEGF. Not only is VEGF involved in normal angiogenesis but also in pathological angiogenesis such as in cancer as will be discussed below. Examples of other factors are platelet derived growth factor (PDGF) that activate smooth muscle cell growth and multiplication which is important in new vessel formation. Other factors include the fibroblast growth factor (FGF), that are responsible for the proliferation of blood vessels, smooth muscles and fibroblasts, Angiopoietin-Tie family that stabilize blood vessels and EphrinB2-EphB4 pathway that are involved in the differentiation of larger vessels of veins and arteries (Shibuya, 2008). The VEGF group in humans are made up of at least four members; VEGF-A, VEGF-B, VEGF-C, VEGF-D. VEGF-A is the most important of the group and contains the subgroup VEGF-A 121, VEGF-A165 and VEGF-A189, the differences occurring due to

alternative splicing (Neufeld et al., 2012). Type 165 and 189 have the basic stretches in the carboxyl terminal region, while the type 121 does not. VEGF-A according to mice studies appears essential for embryonic survival and is involved in development for new blood vessels from scratch and angiogenesis (Shibuya, 2008). VEGF-A has the neuropilins as a receptor and this is significant in relation to the class 3 Semaphorins. It is only Sema 3E that combines directly with plexin-D1, and does not interact with a neuropilin.

It was initially discovered that neuropilin 1 then neuropilin 2 are the receptors for VEGF-A165, and this led to the link between semaphorin 3s and angiogenesis. As the semaphorin 3s shared the same receptors as these VEGFs, like Sema3A that has neuropilin 1 as a receptor, inferences were made that they would be anti-angiogenic by competitively inhibiting the VEGFs through the receptor. These inferences were confirmed by trials (Neufeld et al., 2012, Maione et al., 2012). The Sema 3 group are important in tumour regulation as by being anti-angiogenic it can be inferred that they may play a role in diminishing the growth of tumours and potentially they may be found in low levels in these cancer tissues. These inferences have been backed up by different studies, reviewed below, that show this link and importantly show how this group of semaphorins can be used as treatment of cancer especially in combination with other anti-angiogenic agents.

Maione et al in their study highlighted the issue of the current pitfalls of anti-angiogenic treatment such as sunitinib, a tyrosine inhibitor (Maione et al., 2012). These drugs

though initially effective in treating solid tumours, causing shrinkage and hypoxia eventually the tumour develop resistance and continue invading and eventually metastasize. They hypothesize that this resistance is due to the hypoxia that the tumours face with anti-angiogenic treatment which leads to increased invasiveness in search of more blood supply. The increased invasiveness may either be due to the increased in hepatocyte growth factor, increasing HIF 1 alpha expression or reduced expression of E-cadherin. In order to test their hypothesis the study was devised with two different types of mouse models of cancer; cervical and neuroendocrine pancreatic cancer. With each one they tested the effect of sunitinib only, Sema 3A only and combined effect of Sema 3A and sunitinib. It was shown that Sema 3A on its own was more effective in preventing metastatic spread compared to sunitinib and control but the effect of combined therapy had the greater effect with prolonged survival and remarkably some of the RIP-Tag mice (the pancreatic tumour mouse model) were found to have no tumours. The findings were similar in the cervical tumour mouse model (HPV16E2), with the sunitinib actually causing more liver metastasis compared to control and Sema 3A only, and the combined therapy being much more effective causing a clear drop in the number of metastases.

The effect of Sema 3A was attributed to a decrease in hypoxia within the tumour by normalising the vasculature as shown through pimonidazole immunostaining. There was also a reduced expression of HIF 1 alpha expression in the combined therapy group. Therefore it was proposed that this improvement in outcome was due to reduced

tumour hypoxia, which prevents invasiveness as well as increasing the uptake of chemotherapeutic agents within the tumour.

The other members of the Sema 3 group have also been shown to have a detrimental effect on tumours as in seen in the work of Neufeld et al (Neufeld et al., 2012). This group worked both on glioblastoma cell lines and mouse models to show the effects of the Sema 3 group on this form of brain tumour that is known to be highly vascularized. The first step was to transfect the glioblastoma cell line U87MG with six of the Sema3 group (A,B,D,E,F and G) excluding Sema3C. They showed differing morphological changes in the cell line depending on the type of Sema 3. There was contraction of the cells transfected with Sema 3A and Sema 3B and Sema 3F but not with the others. They also had reduced rates of proliferation in anchorage free growth in agar. These transfected cells were then implanted subcutaneously into nude mice and it was found that they all, except Sema 3G, inhibited tumour formation on these mice compared to control. The strongest effect being with sema 3D and Sema 3E. To further verify their findings they injected U87MG expressing luciferase into the cortex of CD-1 immune deficient mice and found that again besides Sema 3G and control, there was no luminescence seen in the other mice with transfected Sema 3s. Another glioblastoma cell line was also used, U373MG, with very similar results. This study showed greater effect within the brain of the Sema 3s compared to subcutaneous implantation which in turn had a more potent effect than the in- vitro work. The results of this study has been supported by papers with the general consensus being that the Sema 3 group are have a negative effect on tumour progression and may be used as a form of treatment for

patient with cancer (Neufeld et al., 2012, Sakurai et al., 2012). The exact mechanism of action for this group of semaphorins, are still being clarified but seem to suggest mainly an anti-angiogenic effect on these cancers.

When it comes to lung cancer so far the bulk of the evidence points again to the Sema 3s (Potiron et al., 2009). It appears that there is a lack of or relative reduction in some cases of lung cancer. Both types of lung cancer, small cell carcinoma (the more aggressive form) and non-small cell carcinoma commonly start off with a mutation, such as a deletion in chromosome 3p. This is of interest as it is the same chromosome that codes for Semaphorins. Sema 3F and Sema 3B are the two most linked with lung cancer with a reduced expression in a certain form of non-small cell carcinoma. It was shown through a transfection study that Sema 3B reduces the proliferation of lung cancer and Sema 3B was also found to cause the death of the cells.

There are other semaphorins that along with their receptors are known to have the opposite effect that is, aid the progression of cancer. However in the case of Sema 4D and Plexin B1 which shall be discussed later it may either promote or work against cancer progression depending on the interactions with its co receptors (Tamagnone, 2012).

Pancreatic cancer often presents late and has a poor prognosis, usually an average of 6 months. A study by Sandanandam et al has revealed the link between pancreatic cancer and Sema 5A in partnership with its receptor plexin 3B (Sadanandam et al., 2012). This group have shown using western blotting technique that there is an increase in the expression of Sema 5A protein in pancreatic cancer cell line Panc1. It was in this

same line they had earlier identified the expression of plexin B3. They were also able to show through proliferation assays that Sema 5A is pro-angiogenic being able to induce endothelial cell proliferation. They went onto inject nude mice with pancreatic cancer lines transfected with Sema 5A and showed that although there was a lower tumour load, there was an increase in micrometastases.

Semaphorin 4D, Plexin B1 and Cancer

Semaphorin 4D and its plexin B1 have been linked to breast cancer (Worzfeld et al., 2012). Worzfeld et al in their recent paper showed that in ErbB2 positive breast cancer Plexin B1 is able to be activated without its ligand Semaphorin, in the environment of over expression of ErbB2. They also showed that there is a correlation with increased Plexin B1 expression and poor prognosis of breast cancer positive for ErbB2. In contrast low levels of plexin B1 are correlated with poor prognosis in ER positive breast cancer (Okada et al., 2015). An increased expression of semaphorin 4D is seen in head and neck squamous carcinoma (Basile et al., 2006). Basile et al showed through tissue array studies that sema 4D expression was enhanced in prostate, breast, colon and lung cancer compared to normal tissue.

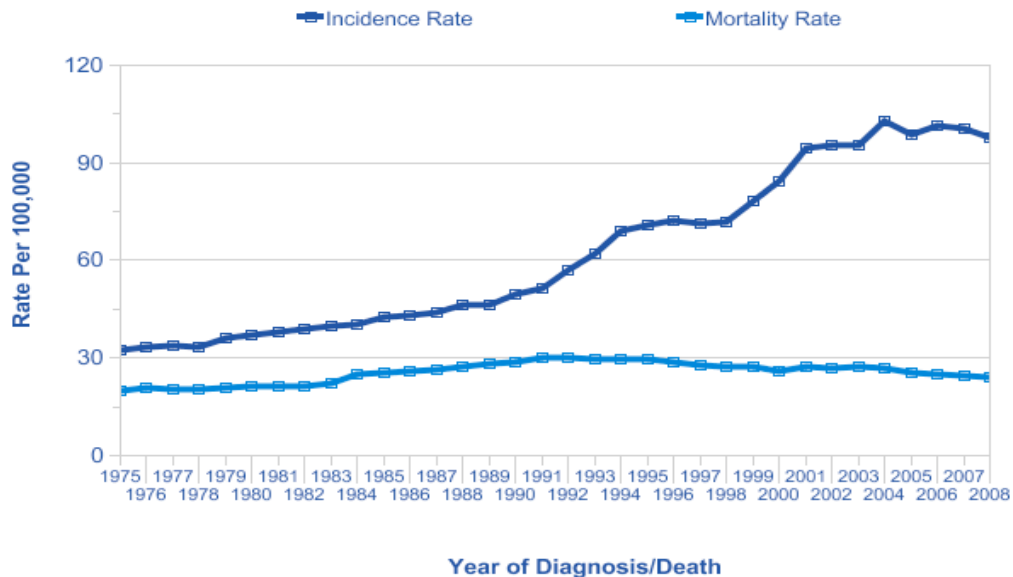
Prostate cancer a review

Background:

Prostate cancer is a significant disease. It is the most frequently occurring cancer in men in the UK with an incidence of 37,051 in 2008, accounting for 24% of newly diagnosed cancers in men. The incidence of the disease has increased steadily over the past 30 years and may be due in part to PSA testing and incidental findings after TURP operations. On the other hand mortality from the disease has remained steady over the same period. In men with the disease the overall mortality is approximately 3%

(2012, Quinn and Babb, 2002, Kirby and Fitzpatrick, 2008). This trend is shown in graph 1 below.

Graph 1: Courtesy of Cancer Research UK(2012)



There were 10,168 deaths from the disease in 2008, accounting for 12% of deaths in males due to cancer. It is the second most common cause of deaths from cancer after lung cancer. It is a disease that affects older men, with $\frac{3}{4}$ of the men affected being aged above 65 (Crawford, 2009, 2012). The largest number of cases diagnosed is between the ages of 70 to 74 years. Interestingly in the UK there is a socio economic correlation with the disease with the incidence being higher in the more affluent (Quinn and Babb, 2002).

Aetiology; Pathophysiology; Diagnosis and Natural history of Prostate cancer:

The aetiology of prostate cancer remains mainly idiopathic (Grover and Martin, 2002). There are a variety of risk factors associated with prostate cancer, the main being age as alluded to earlier (Crawford, 2009). Other risk factors include diet, race, obesity, family history and genetics. The association with diet is best shown in studies on migrants moving from places of low risk such as China and Japan to western countries (Grover and Martin, 2002). The migrant population's risk of prostate cancer increases especially in second generations with the adoption of western style diet. There is a strong racial association in prostate cancer. For instance in the USA between 2001 and 2005, black men were twice as likely to be affected compared to white men with an incidence rate of 259 per 100,000 in black men compared to 157 per 100,000 in white men (Ries, 2005). In terms of genetics and family history in prostate cancer it has been shown that males with first degree relatives with prostate cancer have a 2.0 relative risk of developing the disease and a relative risk of 8.8 if he has both a first-degree and second degree relative with the disease (Steinberg et al., 1990). Another important factor in the pathology of prostate cancer is the hormonal link. The link between hormones and the prostate has been documented as early as the 18th century when John Hunter described the effects of castration on the prostate of bulls when he observed castrated bulls had smaller prostate glands and reduced secretions as compared to their non-castrated counterparts (Marks, 2004). Although testosterone is the primary circulating androgen it is dihydrotestosterone (DHT) that is the primary intraprostatic androgen. DHT is produced by the metabolism of testosterone by the intracellular enzyme 5AR. Studies such as those done by Imperato-Mcginley and

colleagues in 2002 on individuals with congenital 5AR2 deficiency have been important in highlighting the role of testosterone and DHT in male sexual differentiation (Zhu and Imperato-McGinley, 2009, Imperato-McGinley and Zhu, 2002). These individuals have normal testosterone levels. They have been found not to develop BPH or prostate cancer, hence the hypothesis of DHT being one of the initiating factor in the development of prostate cancer.

The majority of prostate cancer is of the adenocarcinoma variety accounting for 95% of cases(Humphrey, 2012, Dunn and Kazer, 2011). The other histological variations include transitional cell morphology originating from the lining of the prostatic urethra. Other varieties include neuroendocrine, small cell and basal cell varieties (Crawford, 2009, Humphrey, 2012). The prostate is divided into 3 main zones of which 75% is the peripheral zone, 20% is the central zone and 5% the transitional zone. Prostate cancer mainly develops in the peripheral zone accounting for about 70% with another 20% arising from the central zone. Prostate cancer can be localised or multifocal in nature most being multifocal(Crawford, 2009).

The disease is diagnosed by histological analysis of prostate biopsies. Although the disease mainly is asymptomatic, patients can present with some lower urinary symptoms like increased frequency of micturition that sets up as cascade of examinations and investigations culminating in the histological confirmation(Dunn and Kazer, 2011).

The natural history of the disease is dependent on the stage at which it is diagnosed. The more aggressive the disease, as measured by Gleason score, the worse the prognosis and vice versa. The natural history of untreated low scoring prostate cancer was studied and it showed that not until 15 years did the progression free survival rate drop substantially. The prostate cancer mortality rate increased from 15 per 1000 person-years during the first 15 years to 44 per 1000 person-years beyond 15 years of follow-up (Johansson et al., 2004).

Plexin B1, Semaphorin 4D and Prostate cancer:

Semaphorins as stated before are best known for their role in the nervous tissue but they have also been shown to be present in other tissues involved in angiogenesis, control of the immune system and formation of new organs (Eickholt et al., 1999, Wong et al., 2007). It is this Sema4D-Plexin B1 signalling pathway that may be harnessed by the cancer cells during invasion and metastasis. It has been shown by Wong et al in 2007, that in prostate cancer there is an increased expression of Plexin B1 relative to non-cancerous prostate tissue(Wong et al., 2007). They found there was a significant difference between the levels of cores staining positive for Plexin B1 in 85 samples of primary prostate cancer tissue when compared to 85 samples of non-cancerous prostate tissue using a tissue array of radical prostatectomy samples. This significant increase in Plexin B1 was also replicated when quantitative reverse transcription PCR was performed. This study showed an increased level of expression of plexin B1 RNA in

cancer tissue compared to non-cancer. A high level of somatic missense mutations were found in prostate cancer tissue as well as metastatic lymph nodes compared to non-cancerous tissue. These mutations were shown to cause altered function in the cells such as increased cell motility which is linked to progression of cancer. This higher level of expression and mutation of Plexin B1 evident in prostate tumours suggests that Plexin B1 has a role in prostate cancer and is a potential target for therapy(Wong et al., 2007).

MicroRNAs overview

MicroRNAs are small RNAs that are approximately 19 to 25 nucleotides in length that do not code for proteins but play an important role regulating gene expression (Medina and Slack, 2008)They perform this regulatory function by mainly inhibiting translation or causing the degradation of mRNA(Almeida et al., 2011). MicroRNAs are important in physiological and pathological processes in the human body as it is estimated that up to 60% of all coding RNA are regulated by microRNAs(Friedman et al., 2009). This means that they directly affect the majority of cellular activities, one microRNA has many mRNA targets. MicroRNAs are highly conserved across species with humans having the highest number and according to the latest version of mirbase, the microRNA database, there are 2,588 mature human microRNAs(Mirbase).

Discovery and history of microRNAs

The study of microRNAs is still relatively recent as their existence was first reported in 1993 by Lee et al, when researching the lin-4 gene in the C Elegans (Lee et al., 1993).

It was found that lin-4 was essential for the sequential development of the larval stage of C Elegans as mutations that caused loss of function of the lin 4 gene led to developmental abnormalities. The abnormalities included the retention of features typical of early stages in the adult animal including extra larval molts, abnormal cuticles and the inability to lay eggs. It was the discovery of the link between lin 4 and another gene lin 14 in the C Elegans, that led to what we now know as microRNAs. C Elegans with an increased expression of lin 14 due to a mutation of the lin 14 gene region were found to have the same effect as the loss of function lin 4 mutation. This led to the investigation of the lin 4 gene as a negative regulator of the lin 14 gene. Firstly lin 4 was found not to code for any protein. It was also found to encode for two mRNA transcripts one of which was 22 nucleotides in length. On further investigation part of the sequence of these small RNA was complementary to the 3'UTR section of the lin 14 RNA. They showed that this 3'UTR section of mRNA was mutated in the C Elegans with lin 14 gain of function mutation, proving that the negative regulation of lin 14 by lin 4 is through the 3'UTR end of lin 14 (Lee et al., 1993). It was this discovery that has led to the microRNA era and we now understand that microRNAs are small non coding RNAs that function mainly by binding to the 3'UTR end of their target mRNA and thereby regulating their expression. The mechanism of action may also include binding to the coding region and the 5'UTR ends (Pang et al., 2010).

Biogenesis of MicroRNAs

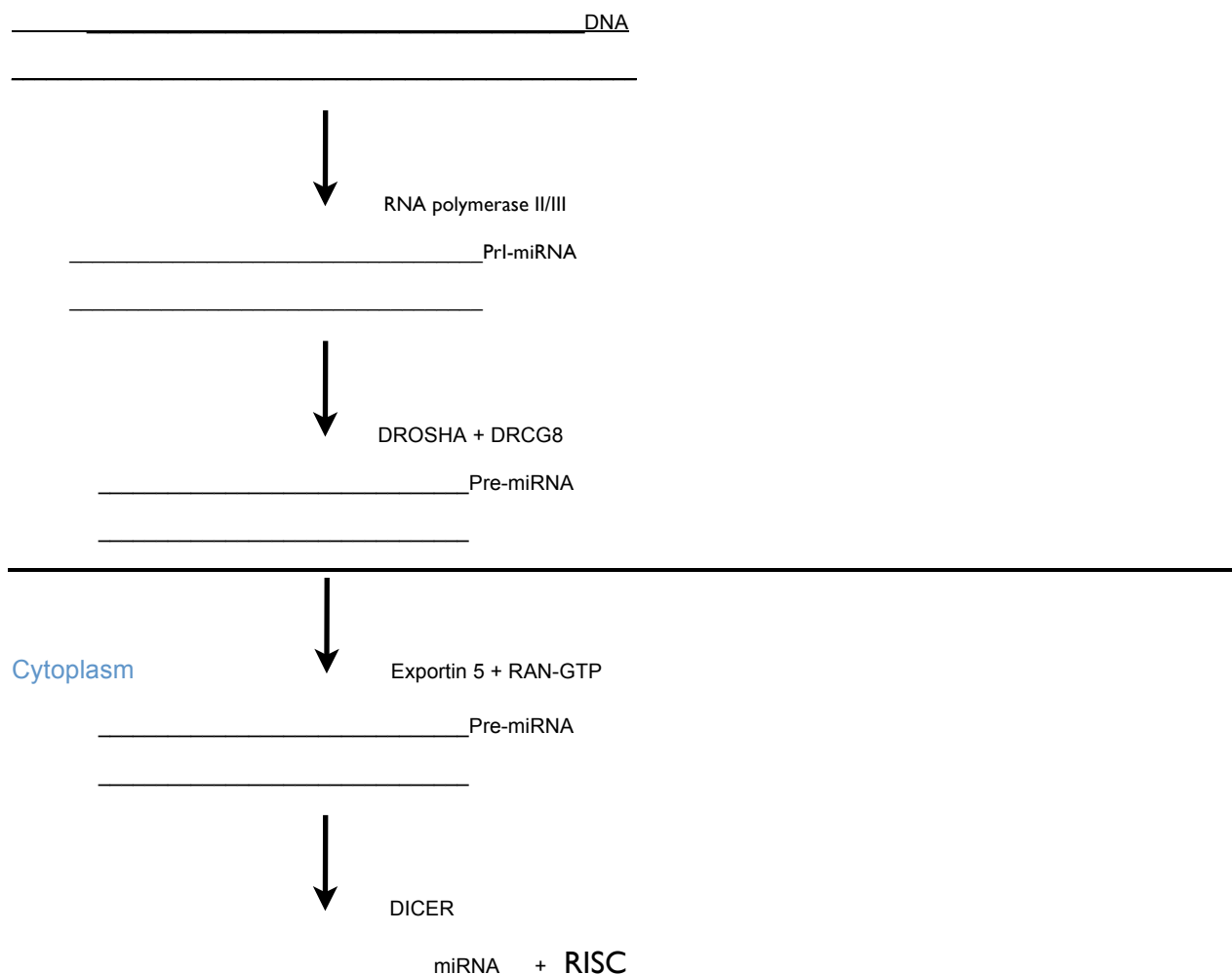
MicroRNAs start off by being transcribed in the nucleus and are then transported to the cytoplasm where they mature to effect change on mRNAs. MicroRNAs are initially

transcribed by the enzyme RNA polymerase II, into the primary microRNA transcripts termed pri-miRNA. There are also a smaller number of microRNAs that are transcribed by RNA polymerase III (Etheridge et al., 2011). The pri-miRNAs are double stranded long structures with stem loops comprising of tens to hundreds of nucleotides (Kim et al., 2009, Casanova-Salas et al., 2012). These pri-miRNAs do eventually produce multiple mature miRNAs. Pri-miRNAs are broken down within the nucleus into precursor microRNAs (pre-miRNAs) by a protein complex called the Microprocessor. This microprocessor has two main components; Drosha an RNA polymerase III, and the co factor called DiGeorge syndrome critical region 8 (DRCG8) a double stranded RNA binding protein. The microprocessor complex breaks down the pri-miRNA at the stem region of the stem loop, into 60 to 110 nucleotide pre-miRNAs that resemble hair-pin like structures. The pre-miRNA are then exported from the nucleus into the cytoplasm through the nuclear pore by Exportin-5, a double stranded RNA binding nuclear transport receptor, that also transports transfer RNA (Winter and Diederichs, 2011) . Exportin-5 transports the pre-miRNA by combining with RAN-GTP, and on the hydrolysis of the GTP in the cytoplasm the pre-miRNA is unbound(Ha and Kim, 2014). Once in the cytoplasm the pre-miRNA is broken down further into mature miRNA by the RNA polymerase III called Dicer. The Dicer does this by cleaving off the loop section of the pre-miRNA leaving a duplex miRNA. One of the two strands becomes the mature miRNA, the other, the complementary sequence of the duplex, is eventually degraded. The mature miRNA combines with the Argonaute protein to form the RISC (RNA induced silencing complex)(Lujambio and Lowe, 2012, Russo and Giordano, 2009). The RISC gives the miRNA stability with which it interacts with mRNAs

through its “seed” region, which is a 6-8 nucleotides region at the start of the 5’ end of the miRNA (Ameres and Zamore, 2013) . The figure 11 below is a schematic representation of miRNA biogenesis. In the biogenesis process sometimes both strands become active miRNAs because none get degraded(Ha and Kim, 2014). One of the strands is named 5p as it originates from the 5’ strand of the pre-miRNA, while the complementary sequence is termed 3p originating from the 3’ strand, an example being mir532-3p(Ha and Kim, 2014).

[Figure 11: A Schematic diagram of miRNA biogenesis](#)

Nucleus



The physiological role of microRNAs

The physiological role of microRNAs is to regulate RNA expression and therefore ultimately gene function. The regulatory role of microRNAs on mRNA is due to complementary binding of base pairs. The 6-8 nucleotide “seed” region of the microRNA, at the start its the 5'UTR end complements the 3' region of a particular mRNA and the level of pairing leads to the complete degradation of the mRNA or a halt to its translation(Shukla et al., 2011). This occurs mainly within the confines of the cell wall.

Although microRNAs are produced within the cell they have been shown to exist in varying quantities in different extracellular compartments (Weber et al., 2010). It appears they are ubiquitous as the study by Wang et al showed that microRNAs exist in all the 12 different body fluids they tested from serum to saliva (Wang et al., 2010). MicroRNAs, unlike other RNAs, are stable within the extracellular environment and even within fixed formalin tissue. This has been suggested to occur because they are found to be either within protective secretive vesicles and exosomes or bound to proteins such as nucleophosmin 1 (NPM1) or the Argonaut 2 complex, part of the RISC complex(Wang et al., 2010, Selth et al., 2012, Etheridge et al., 2011). Wang et al, for example, showed in their study that miRNAs such as mir-122 were bound to the NPM1 protein and that this protein provided protection against ribonuclease activity in the extracellular environment(Wang et al., 2010). Others have shown that miRNAs are able to exist for a substantial amount of time without decay in serum and even so after subjecting them to repeated freeze-thaw cycles (Chen et al., 2008).

Due to their existence in the extracellular compartment, there is a debate about the function of microRNAs within this area of the body. It is thought that they may exist in circulation due to the lysis of dead cells or perhaps they are actually a means of cell to cell communication (Wang et al., 2010, Selth et al., 2012). It has been suggested that microRNAs may be released specifically into the circulation depending on the bodies requirements and just like hormones set off a cascade of events by communicating to distant sites within the body(Kelly et al., 2013).

MiRNA and cancer

The possibility that microRNAs could be linked to cancer was first highlighted by the work of Calin et al (Calin et al., 2002). There is a common deletion in chromosome 13q14.3 found in patients with chronic lymphocytic leukaemia (CLL). The group investigated the genes found within the deleted region of the chromosome 13q14.3 to discover if any of them could be linked to the CLL. They identified two particular genes in the deleted region that coded for microRNAs 15 and 16 as the most likely candidates linked to CLL. The deletion at chromosome 13 has also been associated with other forms of cancer such as prostate cancer and multiple myeloma. On further investigation they also found a reduced expression of these microRNAs. They showed in their study that levels of microRNA 16 and MicroRNA 15 were reduced in at least 60% of patients

with CLL. The exact role of these microRNAs in leukaemia is still being investigated. It appears that microRNA are a significant factor in carcinogenesis as this group showed they often reside in weak areas of chromosomes (fragile sites), or missing sections of chromosomes liable to predispose to cancer(Calin et al., 2004). A possible mechanism of action in CLL was highlighted by a subsequent study by the group (Cimmino et al., 2005). They showed that the reduced expression of both microRNAs 15 and 16 in CLL are linked to the increased expression of B cell lymphoma 2(BCL2) protein, the protein linked to preventing apoptosis in B cell lymphoma. They studied the nucleotide sequences, and noted the 3'UTR end of the BCL2 mRNA is complementary to the first nine nucleotides of the 5' end of both microRNAs 15 and 16. Therefore they suggest the low levels of the microRNAs that occur in cases of CLL described earlier allowed for the increased expression of BCL2 protein and thus the link to CLL pathology (Cimmino et al., 2005). Trials of a drug ABT-199 that selectively blocks BCL2 protein have shown promising results in both animal models and an initial trial with patients with CLL, thus further suggesting the link between miRNAs 15 and 16 and CLL(Souers et al., 2013).

There have now been a number of studies linking miRNAs to various forms of cancer either as oncogenes or tumour suppressors. Other studies have investigated the important roles the RNA polymerase III enzymes drosha and dicer, key factors in the biogenesis of miRNA, may play in carcinogenesis(Lujambio and Lowe, 2012).

The prevailing consensus is that the miRNA biogenesis factors drosha and dicer have roles as tumour suppressors(Merritt et al., 2008, Khoshnaw et al., 2012, Zhu et al.,

2012). Karube et al looked into the influence dicer and drosha had on non-small cell lung cancer patients to investigate any relationship to the prognosis of the disease(Karube et al., 2005). Their hypothesis was linked to previous findings of low levels of the miRNA let 7 in non-small cell lung cancer (NSCLC), and they indeed found significant low levels of dicer was linked to poor prognosis in these patients(Karube et al., 2005). The investigation involved analyzing the samples of patients that underwent potentially curative surgery and following up their survival, using the quantitative real time polymerase chain reaction qPCR to quantify dicer levels. Other studies have found similar results in different cancers such as ovarian in the case of Merritt et al (Merritt et al., 2008), and also in breast cancer (Khoshnaw et al., 2012). These studies have not only looked into the levels of the RNA through qPCR, as in the case of Karube et al, but also compared the protein levels in tissue samples as well as standardized their results by comparing to normal tissue samples(Merritt et al., 2008). However more recent studies appear to suggest relationship to cancer is not as straightforward as shown in initial studies. For instance the link with prognosis and dicer levels in NSCLC appears to be only in certain sub groups and not in all patients when it was studied recently by Lonvik et al(Lonvik et al., 2014). Other groups have shown that in some other cancers low levels of dicer are linked with better prognosis as in the case of colorectal cancer(Vincenzi et al., 2013). The exact relationship is likely to be complex as shown in a recent study by Adams et al (Adams and Eischen, 2014), and may depend on cancer type, but there appears to be potential in this area for cancer therapeutics and future studies will no doubt shed more light on this.

With respect to miRNAs acting as oncogenes, there are a number of miRNAs that have been identified with this property such as the microRNAs mir 21 and mir 155(Si et al., 2007, Medina et al., 2010, Costinean et al., 2006). Mir 21 for instance has been shown to be upregulated in a variety of cancers such as breast cancer as shown by Si et al(Si et al., 2007). Their study compared the levels of mir21 in breast cancer tissue with matched normal samples using qPCR on an miRNA array and found the levels of mir21 to be elevated in the cancer tissue. They also showed that breast cancer cells transfected with anti-mir21 had a dose dependent decrease in growth and confirmed this in mouse models (Si et al., 2007). A further study this time on mice engineered to express mir21, showed that an increased mir21 expression caused lymphoma like effects on the mice in those mice that had the mir21 expression switched on which remarkably regressed once the mir21 was blocked to further suggest mir21 role as an oncogene(Medina et al., 2010). Another study looked at the involvement of mir21 in prostate cancer and found it was overexpressed in the disease and particularly linked with recurrent prostate cancer(Leite et al., 2015). Mir 155 is another miRNA that has also been shown to act as an oncogene. This was exemplified by Costinean et al (Costinean et al., 2006)when the transgenic mice they created to overexpress mir155 showed acute lymphoblastic lymphoma like disease suggesting a role of mir 155 in the initiation or progression of the disease.

MiRNAs have been shown to have tumour suppressor capabilities for instance as mentioned earlier in the first paragraph of this section in the case of the mir15 and mir16.

The mir34 family is another example that has been studied extensively for its tumour suppressive properties(Lodygin et al., 2008, Kasinski et al., 2014, Asmar et al., 2014). This family of miRNA is important and is part of the p53 tumour suppressor cascade, as shown by He et al(He et al., 2007). Their study found p53 controls the transcription of the mir 34 family. A study by Asmer et al on B cell lymphoma patients showed that in certain subgroups a combined defect in p53 and mir34a had a more negative prognostic effect than defects in p53 alone(Asmar et al., 2014). Thus suggesting mir34 has an important tumour suppressive role in its own right. Lodygin et al found the levels of mir 34a were reduced in a variety of cancer cells such as breast, prostate, bladder and melanoma (Lodygin et al., 2008). It is also reduced in B-cell lymphoma and it appears to follow a progressive pattern, significantly lower in high grade compared to low-grade B-cell lymphoma (Craig et al., 2011). These findings have led to studies into the potential therapeutic use of mir34 in cancer. For instance in a recent study by Kasinski et al, mir34a was transfected into non-small cell lung cancer cell lines and also injected into mouse models of the disease(Kasinski et al., 2014). The study found that the animals injected with the lipid based nanoparticle containing the mir34a had a reduction in tumour size compared to the control. To ascertain the clinical utility of this technique a phase one study is currently in progress using this nanoparticle in human subjects with liver metastases(Bouchie, 2013). Among other tumour suppressor miRNAs, we also have let 7 miRNA, as also shown in the Kasinski et al study where their effects were similar to mir34a on the cell and animal models of lung cancer(Kasinski et al., 2014). An interesting study by Trang and colleagues showed the gradual reduction in the levels of

let 7 as the lung tumour became more aggressive(Trang et al., 2010). This inverse relationship further strengthens the suggestion that let 7 is a tumour suppressor.

Another important tumour suppressor miRNA is mir1. This miRNA has been shown by several studies to act as a tumour suppressor in several cancers such as prostate, lung, mesothelioma and bladder (Letelier et al., 2014, Matakı et al., 2015, Xu et al., 2013, Hudson et al., 2012, Yoshino et al., 2012). In the study by Letelier et al, using microarray analysis of bladder cancer tissue and validating their data by qRT-PCR, mir 1 was shown to have significantly reduced expression compared to normal tissue(Letelier et al., 2014). Mir 1 was also shown to induce apoptosis and reduce cell growth when transfected into the bladder cancer cell line NOZ(Letelier et al., 2014). In terms of discovering the mechanism of action of mir 1 in bladder cancer they highlighted the protein VEGF-A, a protein that is essential in many instances for angiogenesis and found in high levels in bladder tumour with poor prognosis(Letelier et al., 2014). VEGF-A was repressed by mir1 in this study by Letelier. They suggest the reduced level of mir 1 in bladder tumour is a possible cause for the level and effect of VEGF-A in this tumour(Letelier et al., 2014). Other studies such as that by Matakı et al(Matakı et al., 2015), have also shown significant reduction in cancer characteristics such as proliferation, migration upon transfection of mir1 this time into lung cancer cell lines.

MiRNA and prostate cancer

In the case of prostate cancer, studies have not only shown a link between mir1 and the disease but also suggested that it is linked to prognosis of the disease as exemplified by further down regulation of mir1 levels in metastatic disease and by mir 1 being independently associated with the recurrence of prostate cancer(Hudson et al., 2012).

The study by Hudson et al in 2012 showed that the effect was due to repression of targets such as BRCA1 and that mir 1 also inhibited invasion and filopodia formation in the prostate cancer cell line LNCaP(Hudson et al., 2012). Interestingly this study showed that the complementary sequence of BRCA1 to which the seed region of mir1 binds to was found among the coding sequence of BRCA1 and not in its 3'UTR.

Various studies have been done and others are ongoing to elucidate the relationship between miRNAs and prostate cancer. This as stated earlier is due the unique properties of miRNAs such as their ability to withstand degradation by ribonuclease or to remain intact in plasma despite repeated freeze-thaw cycles. This makes them ideal candidates as biomarkers for both the diagnosis and prognosis of the disease(Cannistraci et al., 2014). A few candidate miRNAs have been suggested as diagnostic markers for prostate cancer, one such is the tumour suppressor let 7 that is down regulated in prostate cancer(Wagner et al., 2014). A study by Nadiminty et al in 2012 investigated let 7c, a member of the let 7 family, looking at its link to prostate cancer(Nadiminty et al., 2012). They found through their work on LNCaP cell lines and nude mice xenografted with human prostate cancer tumours that let 7c is a negative regulator of the androgen receptor (AR). Let 7c negatively regulates AR which is the key

factor in normal prostate development and also prostate cancer through direct inhibition of c-Myc. C-Myc contains complimentary sequence in its 3'UTR to the seed region of Let 7c(Nadiminty et al., 2012). Nadiminty et al also showed in their work that Let 7c reduced the proliferative characteristics in prostate cancer cells.

In terms of a marker for disease prognosis, among the many miRNAs that have been linked with prostate cancer, mir 221 has been shown in several studies to be down regulated in metastatic disease(Cannistraci et al., 2014, Goto et al., 2015). One such study by Kneitz et al(Kneitz et al., 2014) has shown that mir221 is decreased in metastatic prostate cancer and correlates with treatment failure of the disease as well as prostate cancer related death in this advanced prostate cancer group. To verify the prognostic value of mir221 they studied a cohort of patients that had high risk disease, these patients were followed up and the outcome correlated with the levels of mir221. They also transfected the prostate cancer cell lines DU145,PC3 and LNCaP with mir221 and found a decrease in growth and invasiveness in these cell lines. The clinical utility of mir221 is especially relevant in differentiating high risk prostate disease from low risk disease(Kneitz et al., 2014). Mir154 and mir379 are among others that have been linked with the prognosis of the disease for instance in metastatic bone disease as shown by Gururajan et al(Gururajan et al., 2014).

In summary this is an evolving area of study that may help in the clarification of prostate cancer diagnosis, prognosis and ultimately treatment of prostate cancer.

HYPOTHESIS, AIMS, AND OBJECTIVES

It has been shown that plexin B1 levels are increased in prostate cancer and results suggest a link with prostate cancer metastasis(Wong et al., 2007). It is also known that the expression levels of genes and thus their effect are regulated by microRNAs. There have been numerous studies that have shown the link between microRNAs and cancer, so it is possible that the expression levels of plexin B1 in prostate cancer may be as a result of aberrant regulation by microRNAs. A recent study by Qiang et al has suggested that a certain microRNA, miR 214 directly binds to the 3'UTR of plexin B1 and so suppresses the invasive and migratory effects of plexin B1 on cervical cancer cells(Qiang et al., 2011).

I therefore hypothesized that the elevation in plexin B1 in prostate cancer was as a result of one of the following two scenarios involving microRNAs;

1. The lack of microRNA binding sites in the sequence of plexin B1 as occurs in alternatively spliced variants of plexin B1.
2. The reduction in levels of certain miRNAs that normally act as tumour suppressors, i.e normally down-regulate the expression of plexin B1.

The aim of this research project was to identify the putative alternatively spliced variants of plexin B1 that lack microRNA binding sites that may account for the altered expression levels of plexin B1. I also aimed to discover which miRNAs may affect the expression of wild type plexin B1

My objectives included: (a) characterising the plexin B1 variants. (b) Identifying the microRNAs that may down regulate the expression of plexin B1 (c) Confirming the downward regulation of plexin B1 by the identified microRNAs i.e. reduced expression of plexin B1 by these microRNA, both in the plexin B1 mRNA and protein levels.

Chapter 2

Materials and Methods

Cell lines

The first step in this project was to confirm the existence of Plexin B1 mRNA in the chosen 9 cell lines. The 9 cell lines were as follows; PC3, DU145, LNCaP, 1542CP, 1542NP, YUSIK, GCT27, T24 and 22RV1. PC3 was first isolated by Kaighn et al from a lumbar vertebrae bony metastasis of a patient with prostate cancer(Kaighn et al., 1979). DU145 are epithelial cells derived from a tumour mass excised from the brain of a 69 year old man with prostate cancer and lymphocytic leukaemia(Stone et al., 1978). LNCaP was derived from a lymph node metastasis of a patient with prostate cancer(Horoszewicz et al., 1980). 1542 NP and 1542CP were derived from resected benign and malignant prostate tissue respectively from a patient with prostate cancer(Bright et al., 1997). 22RV1 are primary prostate carcinoma cells with bony metastasis derived from xenografts(Sramkoski et al., 1999). YUSIK is a cell line derived from lymph node metastasis of melanoma patient(Aziz et al., 2010). GCT27 is derived from a human germ cell testicular tumour cell line(Pera et al., 1987). T24 cell line is derived from a primary transitional cell carcinoma of the bladder(Bubenik et al., 1973).

YUSIK (melanoma) was used because PlexinB1 is thought to act as a tumour suppressor gene in melanoma. Prostate cancer cell lines (PC3, DU145, 1542C, 22RV1, LNCaP) and normal prostate cell line (1542NP) were used to determine if the potential Plexin B1 variants identified were cancer specific. I used the GCT27 (testicular germ cell) cell line and T24 (bladder) cell lines to determine if the potential Plexin B1 variants identified were also expressed in other urological cancer cell lines.

Cell culture

All cell lines were cultured in RPMI 1640 media (Invitrogen) supplemented with L-glutamine 5% and 8% foetal bovine serum. All cell lines were grown in T25 flasks at 37°C, in 5% CO₂ and passaged at 80-90% confluency.

Each passage was made by first aspirating the medium, and the cells then washed with PBS. The PBS was aspirated out of the T25 flask, and then 0.5mls of trypsin solution was added for a period of 5 minutes to detach the cells from the base of the flask. To make a passage of 1:10 ratio, a volume of 9.5mls of RPMI solution was then added to the T25 flask making a total volume of 10mls. Finally I made up 3 new T25 flasks by adding 1 ml from the cell suspension and 4mls of fresh RPMI solution to each flask.

RNA extraction

RNA was extracted using the Qiagen RNeasy mini kit following the Qiagen RNeasy Mini Handbook protocol. Once the cells were ~80-90% confluent they were washed with PBS, a physiological buffer, to remove serum to enhance the extraction process. The PBS was then aspirated and 0.1- 0.25% trypsin in PBS added to the flask. After the cells detached from the base of the flask, 8mls of medium was added and the content transferred to a polypropylene centrifuge tube and centrifuged at 300 x g for 5 min. The supernatant was completely aspirated and the cell pellet loosened thoroughly by flicking the tube. The cells were disrupted by adding the appropriate volume (600 µl, for 5×10^6 pelleted cells) of buffer RLT (a buffer from QIAGEN used in cell lysis) and mixed using a pipette. The lysate was then homogenized. This was done by pipetting the

lysate directly into the QIA shredder spin column placed in a 2ml collection tube, and centrifuging it for 2 minutes at full speed. An equal volume of 70% ethanol was added to the homogenized lysate, and mixed well using pipetting. 700 μ l of the sample was transferred, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube. This was centrifuged for 15 seconds at $\leq 8000 \times g$ ($\leq 10,000$ rpm) and the flow-through discarded. 700 μ l of Buffer RW1 was then added to the RNeasy spin column and centrifuged for 15s at $\leq 8000 \times g$ ($\leq 10,000$ rpm) to wash the spin column membrane and the flow-through was discarded. 500 μ l of Buffer RPE was used to wash the spin column membrane twice, firstly centrifuged for 15s at $\leq 8000 \times g$ ($\leq 10,000$ rpm) then centrifuged for 2s at $\leq 8000 \times g$ ($\leq 10,000$ rpm). To dispose of all possible Buffer RPE the RNeasy spin column was placed in a new 2ml collection tube, and centrifuge at full speed for 1 min. The RNA in the RNeasy spin column was then eluted using 30-50 μ l RNase free water added directly to the spin column membrane and centrifuged for 1 min at $\leq 8000 \times g$ ($\leq 10,000$ rpm). The RNA is stored at -80°C .

Experiments to identify splice variants in prostate cancer cells

In order to identify the splice variants of Plexin B1 mRNA, an online search was made of the following sites; National Center for Biotechnology Information (NCBI) Reference sequence database, the alternative splicing in human structural genomics project at the website <http://www.as3d.org>, and the Ensembl database[6-8]. The common variants attributed to Plexin B1 were found as follows;

Variant 1: NM_002673

Variant 2: NM001130082.1

Variant R: AJ011515

Truncated variant: AJ011414

To identify the above variants bespoke primers were designed by aligning the full length variant NM_002673 to the other variants R and truncated variant. Sense and Antisense primers were chosen in areas where variants R and the truncated variant had missing bases (at exon 11) in comparison to variants 1 and 2 (fig 1). Multiple areas of same bases were avoided, as were regions that could produce secondary structures e.g.- GGGCCCC. These primers were confirmed to be specific for PlexinB1 using the 'blast' nucleotide programme on the National Centre for Biotechnology Information at the website <http://www.ncbi.nlm.nih.gov> [7]. The 3'RACE PCR was used to identify both Variant R and truncated variant by amplifying their 3'ends.

The following primers were used;

Primer	Sequence (5'-3')
Plexin B 1 415 1 S	CATCAACAAGTACTATGACCAG (gene specific primer)
Plexin B 1 415 2 S	TGCAGCTGGGCTATCGGCTC (gene specific primer)
Plexin B 1 414 1 S	TGCCTGAGTTCACGGTGCAG (gene specific primer)
Plexin B 1 414 2 S	GTGCAGTATGACGGCGAGAG (gene specific primer)

Amplification of cDNA ends using 3'RACE (Rapid Amplification of cDNA Ends) system

The above primers are used to perform 3'RACE (rapid amplification of cDNA ends) PCR using a oligo dT tagged primer and a gene specific primer complementary to a sequence close to the stop codon.

The 3'RACE system is a method of producing multiple copies of a defined area within a 3' (untranslated region) UTR end of a given cDNA. I followed the Invitrogen 3'RACE protocol using the bespoke primers for the 3'UTR of plexin B1 I made earlier. The first stage is to synthesize the cDNA. I first mixed and centrifuged the components then added 1µg of RNA extracted from the cell lines to DEPC treated water to make a final volume of 11µl in a microcentrifuge tube. I then added 1µl of 10µM AP solution, mixed gently and briefly centrifuged the reaction. The mixture was then heated to 70°C for 10 minutes and chilled on ice for at least 1 minute. The content of the tube were collected by centrifugation and I then added the following; 2 µl of 10X PCR buffer, 2 µl of 25mM MgCl₂, 1µl 10mM dNTPmix, and 2 µl of 0.1 DTT. The content in the tube was then mixed and centrifuged briefly equilibrating the mixture to 42°C for 5 minutes. I then added 1 µl of SuperScript™ II RT and Incubated the tube at 42°C in a heat block for 50 minutes. The reaction was terminated by incubating it at 70°C for 15 minutes. I then chilled the tube on ice and collected the reaction by brief centrifugation. I added 1 µl of RNase H to the tube, mixed it, and incubated for 20 min at 37°C.

Then I followed the second stage of the protocol which is to amplify the target cDNA. I added the following to a fresh 0.5ml microcentrifuge tube; 5 µl of 10X PCR buffer, 3 µl of 25mM MgCl₂, 36.5 µl of distilled water, 1 µl of the primer, 1 µl of 10 µM AUAP, 0.5 µl of Taq DNA polymerase. I added 2 µl from the cDNA synthesis reaction to the tube. I then mixed gently and collected the reaction by brief centrifugation. I then incubated the reaction at 94°C for 3 min. I then carried out 20 to 35 cycles of PCR, according to the TaqDNA polymerase protocol. The sample was analysed with agarose gel electrophoresis and after extraction was sent for cDNA sequencing.

Exon 11 experiments

In the plexin B1 variant experiments I also used primers for exon 11 to differentiate Variant 1 and 2 (they have all their exon's present in the mRNA) from variants R and the truncated variant (both have missing exons 11). The primers are as below;

Plexin B1 Exon 11splice S TATGACTGTGTGGCGGTCAC (exon specific primer)

Plexin B1 Exon 11 splice AS CTGGTAGTCGAGGCTGGACG (exon specific primer)

RT-PCR

For the reverse transcriptase polymerase chain reaction (RT-PCR), RNase free eppendorfs tubes and tips were used for the procedure. I followed the Invitrogen

superscript III first strand synthesis system for RT-PCR protocol. The RNA extract was added to oligo dT primer, dNTP mix and DEPC- treated water to make 10 µl of mixture. This mixture was incubated for 5 minutes at 65°C and then placed in ice for at least one minute. A cDNA synthesis mixture was created using the following; 2µl of 10x RT buffer, 4 µl of 25mM MgCl₂, 2µl 0.1 M DTT, 1µl of RNase OUT and 1µl of Superscript III RT. This cDNA synthesis mix was added to the RNA mix and after brief centrifugation the resultant mixture was incubated at 50°C for 50 minutes and the reaction was terminated at 85°C for 5 minutes and then placed in ice. The reaction was then briefly centrifuged and 1µl of RNase H added to each tube and incubated for 20 minutes at 37°C. The cDNA synthesis reaction was stored at -20°C or used directly for the PCR reaction.

Two sets of mega PCR mixes were made, one with the particular plexin primers being investigated and the other mega mix with GAPDH primers with the following reagents 10x buffer, MgCl₂, dNTP, primer sense and antisense, Taq polymerase and completed with sterilized distilled water.

The reaction was as follows;

The first stage was heating the cDNA product to 95°C for 3 minutes. The second stage was 38 cycles of the following steps for plexin primers: A period of 1 minute of heating to 95°C to denature, 60°C for 1 minute to anneal, 72°C for 1 minute to elongate and finally the third stage is 1 cycle of heating the product to 72°C for 10 minutes. The same PCR process was done for GAPDH primer except in the second stage when it was 30 cycles as opposed to 38 cycles.

Agarose gel for electrophoresis

The cDNA PCR reaction products were analysed using a 1% agarose electrophoresis gel. This gel was made by first dissolving 2grams of agarose powder into 200mls of 1x TAE (Tris-base, acetic acid and EDTA) buffer. For the splice variant experiments to enhance the visualization of the individual bands of the cDNA product 3% agarose was used involving 6g in 200mls of 1x TAE buffer. The solution is heated up to boiling point to allow adequate dissolving of the agarose and once adequately cooled before it sets, 14µl of ethidium bromide which binds to DNA was added to the mixture to aid detection of DNA. The agarose gel is poured into a gel cast to set and then it is placed in an electrophoresis tank with 1x TAE buffer solution.

DNA gel extraction

To extract DNA from the agarose gel, I followed the QIAquick gel extraction kit protocol using a microcentrifuge. Firstly I excised the DNA from the agarose gel, weighed the gel slice in a colourless tube and added 3 volumes of buffer QG for 1 volume of gel (100mg=100µl). I then incubated for 10 minutes at 50°C when the gel dissolved completely, checking that the colour of the mixture was yellow similar to the QG buffer. I then placed the sample in the QIAquick spin column and put the column into a 2ml collection tube and centrifuged for 1 minute at 10,000 X g to bind the DNA. I then

discarded the flow through and then added another 0.5ml of buffer QG to the QIAquick column and centrifuged for 1 minute and discard the flow through. Then to wash the product I added 0.75mls of PE buffer to the QIAquick column and centrifuged for another 1 minute. After discarding the flow through I centrifuged for another 1 minute. The QIAquick column was then placed into a clear 1.5ml microcentrifuge tube and to elute the DNA I added 50µl of EB buffer to the centre of the QIAquick membrane and centrifuged the column for 1 minute at maximum speed.

Cloning of the pmir GLO Vector

The objective for this set of procedures was to make plasmid vector construct containing plexin B1 3'UTR that would be transfected into selected cell lines. The plasmid vector construct was made by the combination (ligation) of the plexin B1 3'UTR onto the pmirGLO vector. The pmirGLO vector contains the luciferase gene. I first performed PCR of the 3'UTR end of plexin B1 using primers designed to tag the ends of the PCR product to enable ligation with the cut vector end. I then performed gel electrophoresis to confirm the existence of the product and extracted the cDNA following the QIAquick gel extraction kit protocol using a microcentrifuge. The pmirGLO vector was digested, i.e. cut and linearized, using restriction enzymes to create compatible ends for cloning. I then performed ligation of the cut vector and the plexin B1 3'UTR to form a vector containing the 3'UTR of plexin B1 downstream of the luciferase gene (Luc-3'UTR). This ligation reaction was performed following the ligation protocol

with T4 DNA ligase from New England Biolabs. The reaction mixture, composed of the vector, the plexin B1 3'UTR, nuclease free water and T4 DNA ligase was mixed, incubated at 16°C overnight to ligate the sticky ends generated by the restriction enzyme digestion, heat inactivated at 65°C for 10 min and then chilled on ice. I then proceeded to transform the ligated pmirGLO vector- construct (Luc-3'UTR) into E. coli bacteria and selected them out on ampicillin-containing agar plates. The agar plates were made by autoclaving 500ml agar solution (made by dissolving 18.5g of agar into 500mls of deionized water) and on cooling adding 500ml of 10mg/ml of filtered sterilized ampicillin, this resultant mixture was poured into petri dish making the agar plate. The transformation was performed using the NEB 5 alpha competent E. coli high efficiency transformation protocol, from New England Biolabs. The resultant transformed colony of bacteria was greatly multiplied by picking each colony using a sterile pipette tip from the agar plate and adding it to a mixture of sterilized ampicillin and LB solution and then incubating the mixture at 37°C in an incubator shaker. The Luc-3'UTR vector was extracted by following the QIAprep miniprep kit protocol. I then performed PCR of Luc-3'UTR to amplify the product and then analysed the nucleotide sequence, to check the 3'UTR end was correctly inserted into the vector. With this confirmation I proceeded to make more LUC-3'UTR by transforming them into more E. coli and then this time purifying them using Qiagen maxiprep purification protocol.

Transformation of pmirGLO vector-construct into competent E. coli

The transformation was performed using the NEB 5 alpha competent E. coli high efficiency transformation protocol, from New England Biolabs. This involved thawing a tube of the NEB 5 alpha competent E. coli cells on ice for 10 minutes. Then mixing gently and carefully pipetting 50µl of cells into a transformation tube on ice. I then added 1µl of the Luc-3'UTR (vector construct) to the cell mixture and carefully flicked the tube to mix the cell and the DNA. I then placed the mixture on ice for 30 minutes. The mixture was then heat shocked at exactly 42°C for exactly 30 seconds and then placed on ice for 5 minutes and then added 950µl of SOC using a pipette. The mixture was then placed in an incubator shaker and shaken vigorously at 37°C for 60 minutes. I then mixed the cells thoroughly by flicking the tube and inverting, then performed several 10-fold serial dilutions in SOC and spread 50-100µl of each dilution onto a selection plate (ampicillin agar plate) and incubated overnight at 37°C.

pmirGLO vector-construct purification

The Luc-3'UTR vector was purified by following the QIAprep Miniprep kit protocol and also then on confirmation of the sequence more were produced using the E.coli bacteria transformation and then purified using the Qiagen maxiprep purification protocol.

For the first purification process using the QIAprep Miniprep kit protocol I centrifuged 5ml bacterial overnight culture at 6800 x g for 3 minutes at room temperature to obtain a pellet. I then resuspended the pelleted cells in 250µl P1 buffer and transferred it to a microcentrifuge tube. I then added 250µl P2 buffer and mixed thoroughly by inverting

the tube 4-6 times until the solution became clear. I then added 350 μ l of N3 buffer and mixed immediately by inverting the tube 4-6 times. I then centrifuged for 10 minutes at 17,900 x g in a table-top microcentrifuge. The supernatant was added to the QIAprep column and centrifuged for 60 seconds and the flow through decanted. I then washed the QIAprep spin column by adding 750 μ l PE buffer and centrifuged for 60 seconds and again discarded the flow through. This was followed by centrifuging the spin column for 1 minute to remove residual wash buffer. I then placed the column in a clean 1.5ml microcentrifuge tube and I eluted the LUC-3'UTR by adding 50 μ l of EB buffer to the centre of the QIAprep spin column and let it stand for 1 minute and centrifuged it for 1 minute.

On confirming the authenticity of the LUC-3'UTR by examining their sequence, I purified them from the bacteria using Qiagen maxiprep purification protocol. This involved harvesting the bacteria cells by centrifugation at 6000 x g for 15 minutes at 4°C. The bacteria pellet was then resuspended in 10mls of P1 buffer to allow complete mixing of lysis buffer by vortexing. I then added 10mls of P2 buffer and mixed gently by inverting 4 times and incubating at room temperature for 5 minutes. I then added 10mls of chilled (to enhance precipitation) P3 buffer and mixed immediately by inverting 4 times and incubated on ice for 20 minutes. I then centrifuged at 20,000 x g for 30 minutes at 4°C and removed the supernatant containing plasmid DNA promptly. I centrifuged the supernatant again at 20,000 X g for another 15 minutes at 4°C again removing the supernatant. I equilibrated a Qiagen tip 500 by applying 10mls QBT buffer and allowed the column to empty by gravity flow. I then applied the supernatant to the tip and

allowed it to enter the resin by gravity flow. I then washed the Qiagen tip with 2 X 30mls QC buffer to remove all contaminants and eluted the Luc-3'UTR vector with 25mls of QF buffer. The vector was precipitated by adding 10.5 ml isopropanol and mixed and centrifuged at 20,000 x g for 30minutes at 4°C, carefully decanting the supernatant. I washed the DNA pellet with 5mls of 70% ethanol and centrifuged at 20,000 x g for 10 minutes carefully decanting the supernatant without disturbing the pellet. The pellet was air dried and redissolved in 400µl of T.E. buffer and stored at 4°C. The concentrations of the vectors were measured by spectrometry using the Spectrophotometer Ultrospec 3100 Pro.

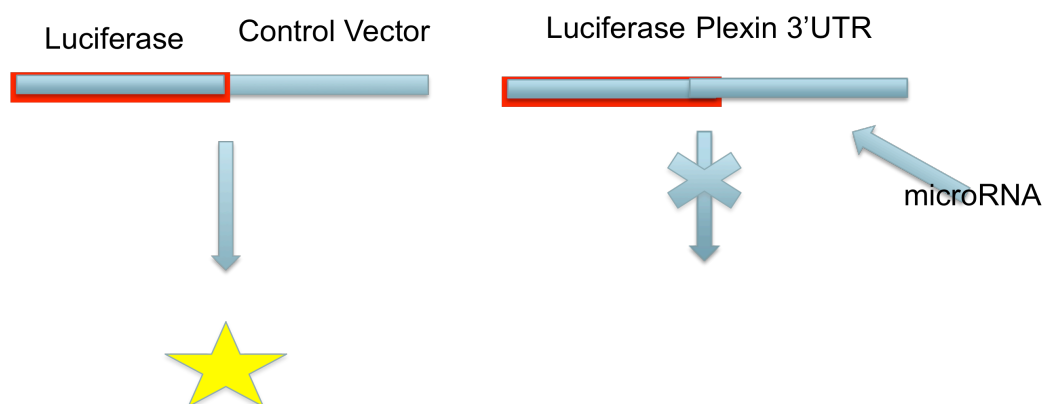
Transfection of pmir GLO vector and assessment by Luciferase reporter assay

The cells lines were transfected with both the Luc-3'UTR (vector construct) and Luc-control (pmirGLO vector only) following the invitrogen transfection protocol. Firstly the cells were plated using a specific number of cells (counted with the aid of a haemocytometer) per well of a 96 well plate and were incubated overnight with medium. On the second day I made a mixture of 200ng of vector in 25µl of opti-mem and 0.5µl of lipofectamine in 25µl of opti-mem and left to stand for 20 minutes. I then removed the medium from the well and added vector lipofectamine mixture to each well plus another 25µl of opti-mem to each well. This was left for 6 hours in the incubator and then replaced the opti-mem mixture with 75 µl of medium and kept in an incubator for 48hours.

I then assessed the transfection by measuring the expression of the luciferase after the addition of Dual Glo luciferase reagents according the promega Dual Glo luciferase assay system protocol as follows; I removed the 96 well plates from the incubator and added 75µl of Dual Glo luciferase reagent (an equal volume to the medium) to each well and mixed. I waited for 10 minutes then measured the firefly luminescence. To measure the Renilla luciferase activity I then added 75µl of Dual Glo Stop and Glo reagent (ratio 1:100) to each well and mixed. I waited at least 10 minutes and then measured the luminescence. I then normalized the reaction by the ratio of the luciferase firefly to renilla activity. I measured the luminescence using the Wallac 1420 Luminometer. The effect of the presence of the 3'UTR on luciferase expression was determined by comparing the luciferase activity of cells transfected with the Luc-3'UTR and control Luc constructs. A schematic diagram of the effect of differing interactions with and without the plexin B1 3'UTR is shown below in figure 12 below.

Figure 12

Effect of microRNA on luciferase activity



QPCR and Transcriptome array experiments

Quantitative polymerase reactions (qPCR) were carried out to verify and quantify the levels of expression of Plexin B1 in the transcriptome array experiments, in cell lines on their own as well as in cell lines transfected with mimics. In each case these reactions were done in triplicates to obtain a more accurate value. I used the eppendorph Mastercycler realplex machine to perform these reactions. The parameters for the programme were set as shown in table 4 below;

Table 3: The PCR parameters

Programme	Step	Temperature (°c)	Time (mm:ss)	Cycles
Heat Activation	1	95.0	03:00	1
PCR cycle	2	95.0	00:05	
	3	57.0	00:08	
Melt Curve	4	95.0	00:03	1
	5	60.0	00:15	
	6		20:00	
	7	95.0	00:15	

QPCR transcriptome array was performed to detect the microRNAs (miRNAs) that regulate Plexin B1 expression. Prior to performing the transcriptome arrays the QPCR reactions were optimized. Optimisation experiments included performing different QPCR reactions to ascertain which dye would be the most suitable for the transcriptome array from either Taqman or SYBR green dyes. SYBR green was chosen as it worked well with good CT values and was recommended by Qiagen the makers of the

transcriptome PCR array. The optimisation process also included performing qPCR using cDNA initially from cancer cell lines, then later cDNA from HeLa cells to closely resemble the QPCR of the transcriptome array (this array uses HeLa cells). Another part of the optimization process was to perform qPCR using different annealing temperatures to find which temperature best suited the primers and probes. After optimization qPCR was performed in the Mastercycler realplex machine with SYBR green dye using Qiagen Sure FIND transcriptome PCR arrays to identify potential miRNA that regulate Plexin B1. Primers for Plexin B1, and GAPDH were used for the qPCR process. The array contains HeLa cells transfected with miRNA mimics and also controls consisting of HeLa cells without miRNA mimics. The relative levels of expression of Plexin B1 in HeLa cells with the mimics relative to those without were quantified and normalised using the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as control.

In the other qPCR experiments (not involving the transcriptome array), I also performed optimisation experiments finding again SYBR green to be the dye of choice. I used the cDNA samples obtained from the first strand synthesis for these reactions, after they were diluted to a ratio of 1 in 5. For each reaction to make a total of volume 20 μ l per well, I used 10 μ l of Brilliant III Ultra-Fast SYBR green qPCR master mix, 6 μ l of nuclease free water, 1 μ l of both sense and antisense primers and 2 μ l of the diluted cDNA sample. I used three housekeeping genes GAPDH, B2M and Succinate Dehydrogenase (SDHA) for controls in these experiments. These samples were placed in a 96 well plate and the qPCR reaction done in the eppendorph Mastercycler realplex machine.

Co transfection of miRNAs and pmir GLO Vector experiments

The Candidate miRNAs were transfected into prostate cancer cell lines to assess their effects on the plexin B1 3'UTR found on the vector construct. The miRNAs were transfected into the cells along with LUC- 3'UTR (pmir GLO vector construct), or with LUC-Cont (pmirGLO vector alone). The co-transfection was done using the Qiagen transfection protocol database for cotransfection of Plasmid and miRNA(Qiagen). Using the fastforward cotransfection of the cell line protocol. For each well I seeded 4×10^6 cells into the 96 well plate in 100 μ l in culture medium and then incubated them for a few minutes at 37°C at 5% CO₂. I then diluted 0.2 μ g of vector (either Luc-control or Luc-3'UTR) in 50 μ l of opti-mem. I then added 1.6 μ mol of miRNA mimic to the diluted vector and mixed. I added 0.75 μ l attractene transfection reagent to the diluted vector and mixed. The mixture was then incubated for 15 minutes at room temperature to allow formation of tranfection complexes. I then added the complexes drop wise onto the cells in the 96 well plates and gently swirled the plate to allow uniform distribution. I incubated the cells with the transfection complexes for 48 hours. Then I analysed the results using the Dual GLO luciferase reporter assay system.

Transfection control experiments and transfection of miRNAs into the cell lines

I then proceeded to assess the effect of microRNAs on the expression levels of plexin B1 in the cell lines by transfecting the candidate miRNAs into the cell. In order to confirm transfection I conducted transfection control experiments by transfecting siRNA (All Star HS Cell Death Control siRNA) into cell lines following the Qiagen transfection

protocol database for the specific cell line(Qiagen) using PBS and water as control. After following the transfection steps as explained below in the miRNA transfection paragraph, I monitored the effects of transfection in the cells by placing the 6 well plate in an Incucyte imaging system taking images every 4 hours up to 56 hours to assess the changes in each well.

To transfect the miRNAs into the cells I followed the Qiagen transfection protocol database for the specific cell line (Qiagen). The steps were as follows; I seeded 2×10^5 cells per well of a 6-well plate in 2300 μ l of culture medium and then incubated them for a few minutes at 37°C at 5% CO₂. I then diluted 150ng miRNA in 100 μ l Opti-mem and added 24 μ l of HiPerfect transfection reagent and mixed the resultant mixture. The mixture was then incubated for 15 minutes at room temperature to allow formation of transfection complexes. I then added the complexes drop wise onto the cells in the 96 well plate and gently swirled the plate to allow uniform distribution. I incubated the cells with the transfection complexes for 48 hours. The total RNA in the transfected cells are then extracted using the Qiagen RNeasy mini handbook protocol. I then analysed the levels of plexin B1 RNA in these transfected cells by performing qRT-PCR.

Analysis of plexin B1 protein levels in cells transfected with miRNA

In order to assess the plexin B1 protein levels in the cell lines transfected with miRNA I followed the following step; I first extracted the protein, then measured the concentration of the protein, I performed SDS-PAGE and protein blotting and finally western blotting and the films were developed with aid of developer and fixer.

Protein extraction

To extract the protein I made use of Oinuma lysis buffer containing added inhibitors. A 100mls of lysis buffer consisted of 100mls of nuclease free water containing 2mls of 20mM Tris pH 8, 15ml of 150mM NaCl, 0.4mls of 4mM MgCl₂, 1ml of 1% NP40 and 10mls of 10% glycerol. To 1ml of the lysis buffer I added the following inhibitors; 10µl of 1mM PMSF (phenylmethanesulphonyl fluoride), 10µl of 10µg/ml Leupeptin, 20µl of Na Vanadate, 20µl of Na Fluoride and 10µl of Pepstatin. I first suctioned off the medium from the wells of the 6 well plates. I washed the cells with 2mls of ice cold PBS and after suctioning off the PBS I added 100µl of lysis buffer to each well and left on ice for 5 minutes. The cells were then scraped off using a cell scraper and the cell lysate was put into a 1.5ml eppendorf tube on ice for 15 minutes. I then centrifuged the tube at 13,000 rpm for 10 minutes at 4°C. I then collected the supernatant in a pre-chilled eppendorf tube.

Measurement of protein concentration

The protein concentration was calculated using the lowry method. The protein concentrations were normalised using the lowry protein assay kit. Serial dilutions of Bovine Serum Albumin (BSA) were made from the BSA standard solution. A standard of water 200mls volume in a tube was used as baseline control (the blank). I made a serial dilution of varying concentrations of BSA in 5 tubes as follows in the table 5 below;

[Table 4: BSA dilutions](#)

Tube	Content	Concentration
1	33.3% of BSA in 200 μ l	0.5mg/ml
2	16.7% of BSA in 200 μ l	0.25mg/ml
3	8.3% of BSA in 200 μ l	0.125mg/ml
4	4.2% of BSA in 200 μ l	0.0625mg/ml
5	2.1% of BSA in 200 μ l	0.03125mg/ml

The process of dilution involved adding 125 μ l of BSA into 375 μ l of water in a 10ml tube then decanting 200 μ l of the resultant mix into another 10ml tube (tube 2) containing 200 μ l of water and mixed by vortexing. 200 μ l of the resultant mix from tube 2 was decanted into tube 3, which contains 200 μ l of water. This process was repeated till tube 5 was made and then 200 μ l from tube 5 is discarded. I also discarded 100 μ l from tube 1 to make a final volume of 200 μ l for each tube. I then diluted 10 μ l of each lysate (protein extract) sample 20 folds with 190 μ l of water. This was followed by preparing the Folin-Ciocalteu reagent by diluting 2N Folin-Ciocalteu reagent with distilled water to make a 1N solution. With the aid of a timer I added 1ml of modified lowry protein assay reagent to each diluted BSA sample and standard and mixed by vortexing gently at 20 second intervals per reaction. The mixture was incubated for exactly 10 minutes at room temperature from the start of the first reaction in tube 1. I initiated the stop reaction at the end of the 10minutes by adding 100 μ l of 1N Folin-Ciocalteu reagent and mixed gently by vortexing. The samples were then incubated for 30 minutes at room temperature and

the concentration were measured using the UV/ visible spectrophotometer Ultrospec 3100 Pro. The standard curve was plotted automatically by the machine based on absorbance reading against the concentration of BSA standards. I multiplied the reading of each sample by 20 to get the protein concentration (as the lysate was diluted 20 folds).

SDS-PAGE and protein blotting

An equal amount of total protein from the lysate samples were normalised to the same volume using lysis buffer. I heated the mixture at 95°C for 5 minutes and the denatured protein samples were loaded onto the sodium dodecyl-sulphate (SDS)- polyacrylamide gel with appropriate concentration of acrylamide. I made the 8% resolving gel by mixing the following; 3.75mls of 1M Tris pH 8, 2.67ml of 30% acrylamide, 3.53mls of water, 50µl of 20% SDS, 100µl of ammonium persulfate (APS), 6µl of TEMED. The solution was poured onto plates on the assembled chamber and 70% ethanol was added onto the top and left to set for an hour. To make the stacking gel I made the following; 375µl of 1M Tris pH 6.8, 374µl of 30% acrylamide, 2.24ml of water, 15µl of 20% SDS, 30µl of 10% APS, and 5µl of TEMED. I poured off the ethanol from the chamber and added the stacking gel solution, placed the comb and left to set for 20 minutes. After the gel had set I loaded an equal amount of protein per well and I also loaded to one well a rainbow molecular marker to a well as a size reference. The chamber was filled with running buffer then I proceeded with electrophoresis firstly with 66V till the junction of the stacking and resolving gel was reached and 94V thereafter until the dye reached the end of the gel.

Western blotting

I then proceeded to perform western blotting. This was done by first separating the plates to expose the gel and the stacking gel section was discarded. The sponges are soaked in transfer buffer and the gel was rinsed in transfer buffer. The polyvinylidene fluoride (PVDF) membrane was prewashed in 100% methanol and then rinsed in transfer buffer and placed straight onto the gel removing any bubbles that may have formed on the surface. Filter paper was then placed on the PVDF membrane again removing any bubbles. A second filter paper was then placed on the initial filter paper and I then flipped this set up to reveal the gel onto a presoaked sponge. To complete the sandwich I added onto the gel 2 filter papers and another soaked sponge and secured the sandwich in a cartridge. The cartridge was placed into the mini trans-blot cell of Bio-Rad filled with transfer buffer and electrotransfer was performed overnight at 36V.

Figure 13: A Schematic diagram of a Western blott transfer sandwich



After the electrotransfer I rinsed the membrane briefly in PBS-Tween 20 (PBS-T), 0.1% Tween-20 in 1 X PBS. I then blocked the PVDF membrane with 5% non-fat milk (5g in 100mls of PBS Tween FOR \geq 1 hour at room temperature. After blocking, the membrane was probed with 10mls of 1% non-fat milk containing diluted primary antibody (3 μ l of plexin B1, 1 μ l of actin antibody as appropriate). The membrane was then washed four times with PBS-T for 4 minutes each time. After washing the membrane was incubated with 1 μ l of appropriate horseradish peroxide conjugated secondary antibody diluted in 10mls of 1% non-fat milk for 1 hour. The membrane was then washed in again in PBS-T four times for 4 minutes each time and was then ready for enhanced chemiluminescent development. The chemiluminescent signals were developed by Pierce ECL western blotting substrate. An appropriate volume of mixture of detection reagent 1 and 2 at ratio 1:1 was added to the membrane and incubated for 5 minutes at room temperature. The membrane was then placed between 2 sheets of film and set into Kodak biomas cassette. The membrane was exposed to a film and the films were developed with Kodak Developer and Fixer solution.

Statistical analysis

All cell line experiments were performed in triplicates to improve the validity of the results. Statistical analysis and significance were assessed with aid of SPSS 20, Origin and Microsoft Excel.

Chapter 3

Plexin B1 full length and variants expression in cell lines

Introduction

The receptor plexin B1 has been found in various organs of the body such as in blood cells, blood vessels and brain where it plays an important part in the early development of these areas. It also has a role in the normal functions of the body for instance in the immune system(Tamagnone et al., 1999, Nkyimbeng-Takwi and Chapoval, 2011). It has been shown that plexin B1 is present and expressed in varying amounts in different prostate cancer cell lines by Wong et al(Wong et al., 2007). What is not known is if all prostate cancer cell lines express plexin B1 and what the levels of expression of plexin B1 levels are in the cell lines.

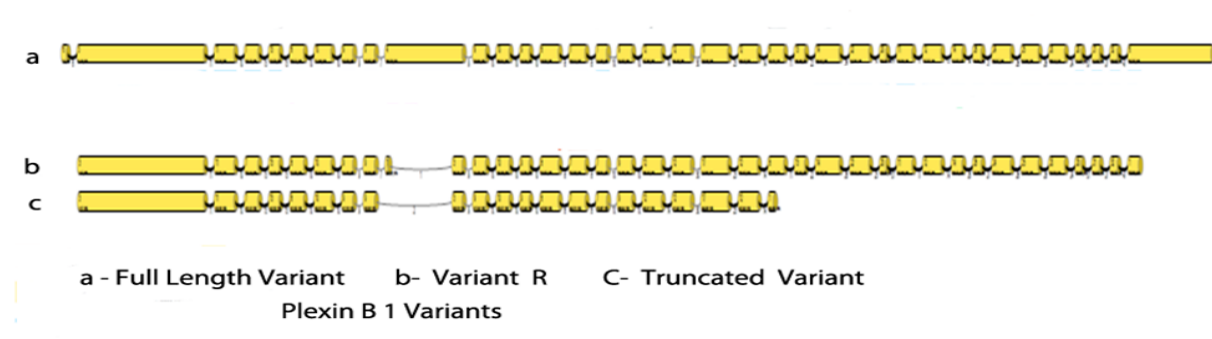
Plexin B1 has been shown to exist in different forms known as splice variants as shown by Tamagnone et al(Tamagnone et al., 1999). There have been more variants predicted to exist by online genome databases that have yet to be verified in the literature(NCBI, 2011). These variants are likely to act differently to the known plexin B1 variants, for instance interact differently with local miRNAs because of their different nucleotide sequence composition, and as such are worth discovering because of the potential for different cellular effect. It is not known if any of the cell lines express the alternative variants; variant R and the truncated variant.

The aim of this section of the investigation was to establish if prostate cancer cell lines express the full length variant version of plexin B1 mRNA. The next step was to investigate if these cell lines also expressed the two common alternative spliced variants in question, variant R and truncated variant. I started to find proof of the

existence of a variant of Plexin B1 by first seeking a plexinB 1 mRNA that lacked exon 11 a common trend in both alternatively spliced variants(NCBI, 2011), the absent exon 11 is highlighted in figure 14 below. I then followed this up by looking for transcripts with a shortened 3'UTR end equivalent to variant R and the Truncated variant.

Fig 14: Schematic of variants of plexin B1 mRNA

The diagram shows the variants of plexin B1, a the full length variant, b the variant R and c the truncated variant of plexin B1 courtesy of <http://www.as3d.org/human2006/genePLXNB1.html> and NCBI database



Results

The results are divided into the following sections

1. The use of RT-PCR to measure plexin B1 mRNA expression in cell lines.
2. The use of qRT-PCR to measure plexin B1 mRNA expression.
3. Alternative splicing of of plexin B1 exon 11 experiments.
4. The results of 3'RACE experiments for plexin B1 truncated variant and plexin B1 variant R.

1.The use of RT-PCR to measure plexin B1 mRNA expression in cell lines

The first step in this project is to confirm the existence of Plexin B1 mRNA in the chosen 9 cell lines. These cell lines are as follows; PC3, DU145, LNCaP, 1542CP, 1542NP, YUSIK, GCT27, T24 and 22RV1. The background and rationale for the use of these cell lines are covered in the second chapter.

To achieve the first step of the investigation, to confirm the expression of Plexin B1, RNA was extracted using the Qiagen RNA extraction kit protocol from the following cell lines; PC3/27, DU145/71, Yusik10, GCT27/75, 22RV1/5, 1542NP/51 and LNCaP/73. RTPCR was then performed using sense and antisense primers for Plexin B1 and the PCR products were run in gel electrophoresis. No bands were seen in the control of no cDNA (not shown).The cDNA products were then cut out of the gel and sequenced. The products were confirmed to be Plexin B1 mRNA for the 7 cell lines by comparing the sequence of the RT-PCR products extracted from the gel with the reference sequence of Plexin B1 on the NCBI database using the BLAST computer program. This is as shown in the image of agarose gel electrophoresis shown in figure 15 below. See also the image of an electropherogram in figure 16 below;

Fig 15: Electrophoresis gel showing presence of plexin B1 mRNA in cell lines

RNA from each cell line indicated was reversed transcribed and amplified by PCR and the products separated on an agarose gel for plexin B1 and the control GAPDH.

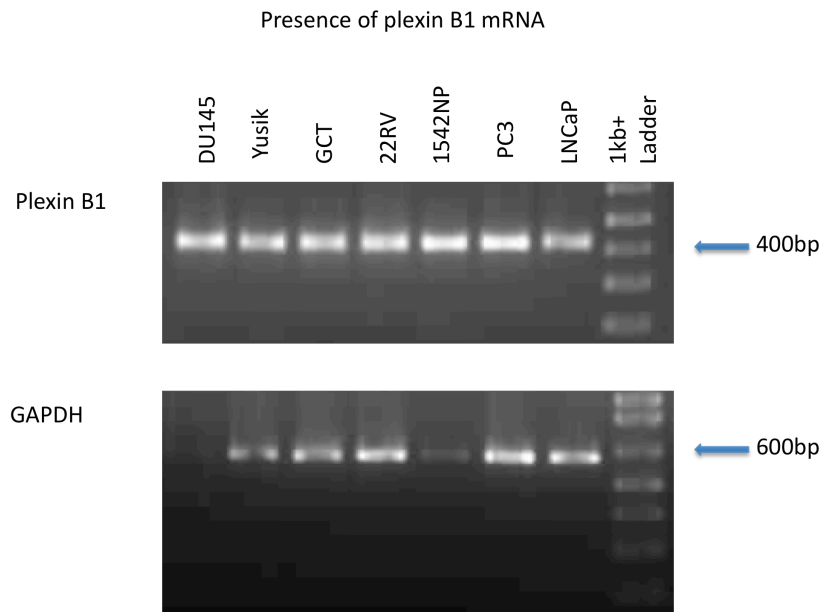
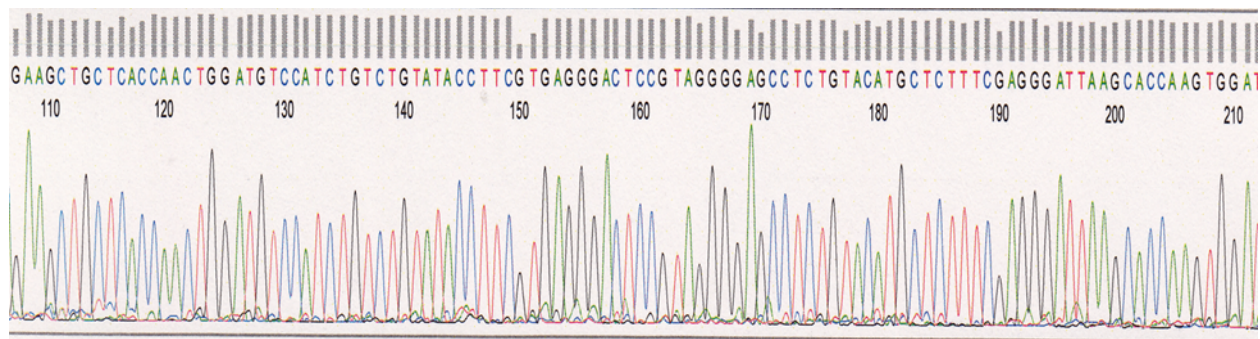


Fig 16: section of electropherogram showing presence of Plexin B1 in a cell line

DNA from the amplified products in Fig 14 was extracted from the gel and was sequenced by Sanger sequencing method (representative electropherogram)



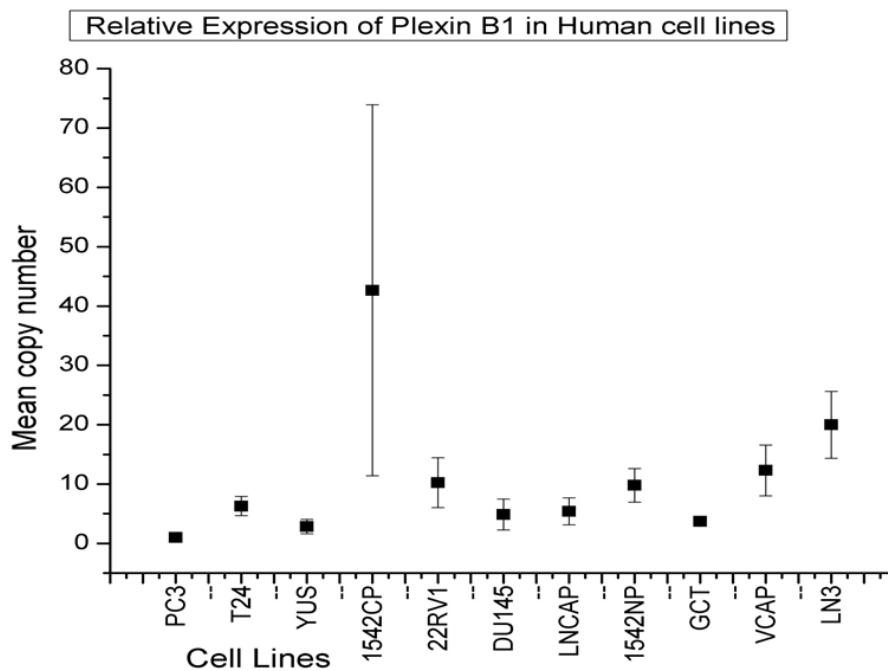
2.The use of qPCR to measure plexin B1 mRNA expression plexin B1 levels

In order to confirm the presence of plexin B1 in the cell lines, and to quantify the levels of expression of plexin B1, I performed a series of qRT-PCR experiments.

To obtain actual and relative levels of expression of Plexin B1 mRNA in the following cell lines; T24, Yusik, 1542C, PC3, 22RV1, DU145, LNCaP, 1542NP, GCT, VCAP, LNCaPLN3, I used Quantitative PCR with SYBR Green dye. The extracted RNA from two different passages of the above cell lines, were converted to cDNA using the protocol of the first stage of Invitrogen superscript III first strand synthesis for RTPCR. The cDNAs were then used to perform QPCR using the SYBR Green dye with a set QPCR protocol. The experiment was performed 3 times, and the results show all cell lines express Plexin B1 and at different levels. The transcript copy number was calculated using standard of known copy number. It was shown that all cell lines expressed Plexin B1 with 1542CP expressing the highest amount as shown in the graph 2 below. LNCaP LN3 had a higher expression level than LNCaP and the other cell lines except 1542CP.

Graph 2: QPCR quantification of the level of expression of plexin B1

The copy number of plexin B1 mRNA was measured by qPCR of cDNA using housekeeping controls GAPDH, B2M and SDHA. **The mean of the 3 experiments calculated and the error bars represent the SEM.**



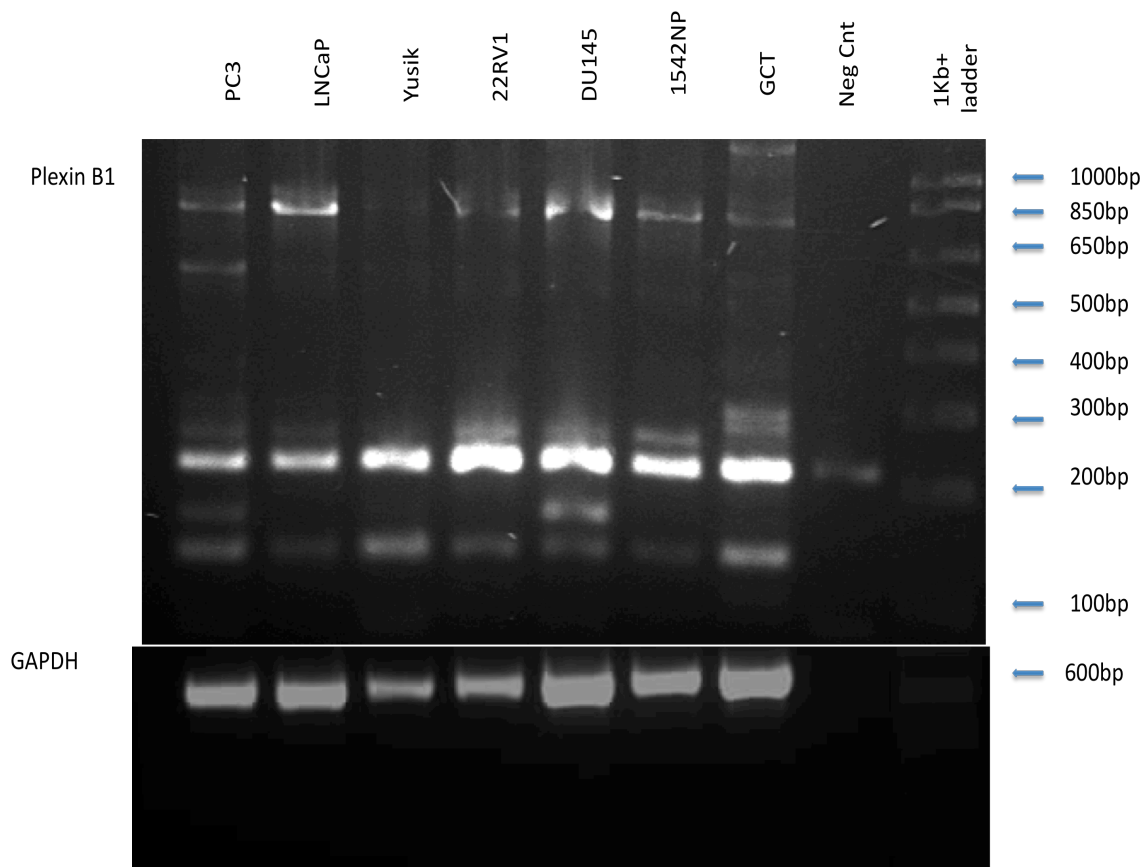
3. Alternative splicing of of plexin B1 exon 11 experiments

To investigate if these cell lines also express the two alternative spliced variants in question, variant R and the truncated variant, I initially aimed to investigate whether the cell lines expressed variants of plexin B1 that lacked Exon 11. This was because the NCBI gene reference sequence database indicates that exon 11 of the Plexin B1 mRNA may be alternatively spliced and is not present in both the truncated variant and variant R (NCBI, 2011). I extracted RNA using the Qiagen RNA extraction kit protocol from the

following cell lines; PC3/27, DU145/71, Yusik10, GCT27/75, 22RV1/5, LNCaP, 1542NP/51. I then performed RT-PCR using primers flanking exon 11 on the extracted RNA for each cell line. The cDNA PCR products were identified using agarose gel electrophoresis; they were cut out and sequenced to confirm their presence. The image of the agarose gel electrophoresis is shown in figure 17 below;

[Fig 17: The expression of plexin B1mRNA using exon 11 primers](#)

RNA from each the cell lines indicated was reverse transcribed to cDNA and amplified by PCR using primers flanking exon 11 of plexin B1. The products were separated on an agarose gel the band were subsequently cut out and sequenced. The same cDNAs were amplified in parallel using primers to GAPDH as a postive control.



As seen above most of the cell lines had at least four bands while there were two cell lines that each had an extra bands making 5 bands, these were PC3 and DU145. The bands that were common to all cell lines were equivalent to 3 sets of cDNA products of plexin B1 that were of ~800bp, ~250bp and ~200bp in size. The smaller 250bp and 200bp products, given their size, suggest they were the plexin B1 mRNAs that lacked exon 11. I confirmed these cDNA products by sequencing them using the Sanger DNA sequencing technique (provided by the UCL cancer institute, Eurofins Genomics or Beckman Coulter Genomics). The larger 800bp cDNA products contained the full sequence of exon 11 of Plexin B1. The 800bp band was found in all the cell lines tested therefore represents the plexinB1 transcript in which exon 11 is not spliced out. The band of approximately 250 baase pairs was identical to variant R sequence, in which part of exon 11 is spliced out. Thus this would be the first time that variant R mRNA, the ~250bp band seen on the electrophoresis image, has been identified by RTPCR in prostate cancer cell lines. The band of approximately 200 base pairs was identical to the truncated variant sequence. The ~200bp band is 34 nucleotides smaller than the variant R in keeping with the small space that separates the bands. The other bands are non specific amplified products as shown by the Sanger DNA sequence, this includes the small band only common to PC3 and DU145 cell lines.

An example is shown below of the nucleotide sequences of the ~800bp, ~250bp and ~200bp bands from the cell line LNCaP. The sequence of the 200bp band (truncated

version) is partially superimposed over the sequence of the 250bp band (variant R).

This is probably because the bands were so close together on the gel and so the 250bp band was contaminated with some of the 200bp band when the bands were cut out of the gel. Electropherograms are shown in the appendix.

LNCaP

Upper band: 766 nucleotides plexin B1 full length variant

```
AGGCAATGATCTAATAGATAGACCGATTTTTCCTAACTGGCGTTTTTGGCACCACC
AGAGCACCCACAAAGCCTCTTGTGATGCTGGGCCCATGGTTGCAAGCCATCATAG
CCCGCTTGTCTCCCCAAACCCTCCTGCAAGAGGTGGACCCAGCCCCTCCCCACCC
ACAGCCCCAAAAGCCCTGGCCACCCCTGCTCCTGACACCCTTCCCGTGGAGCCTG
GGGCTCCCTCCACAGCCACAGCTTCGGACATCTCACCTGGGGCTAGTCCTTCCCT
GCTCACCCCCTGGGGGCCATGGGCAGGTTCTGGCTCCATATCTTCCCCTGGCTCC
ACAGGGTCGCCTCTCCATGAGGAGCCCTCCCCTCCCAGCCCCCAAATGGACCTG
GAACCGCTGTCCCTGCCCCCAATGACTTCAGACCCTCAGCCACACCTGAGGACCT
CTTGGCCTCCCCGCTGTCACCGTCAGAGGTAGCAGCAATGCCCCCTGCAGACCCT
GGCCCCGAGGCTCTTCATCCCACAGTGCCCCTGGACCTGCCCCCTGCCACTGTTC
CTGCCACCACTTTCCCAGGGGGCCATGGGCTCCGTGAAGCCCGCCCTGGACTGGC
TCACGAGAGAAGGCGGCGAGCTGCCCGAGGCGGACAAGTGGACGGGGGGTGAC
GCACCCGCCTTTCTCCACTTCCACCCTCCTCACAAGTGATGGAGACTCAGCAGAG
CTTGAGGGGCCCTCCCGCCCCCCTCATCCTCCCGTCCAGCCTCGACTACCAGAA
```

LNCaP

250bp band: plexin B1 variant R

GTGCCGGCCTGTGTGAGCAGCCGCTGGGGGTGTAAGTGGTGTGTCTGGCAGCAC
CTGTGCACCCACAAGGCCTCGTGTGATGCTGGGCCCATG**GTTGCAAGCCA** **T C A**
G A/G T/G G/C A/C C/T G G/C A/T T/G G/A C/T C/T C/T A/C G/C C C/A A/G A/G A/G
C T/C T/C

LNCaP

200bp band: plexin B1 truncated variant

CTTTGAACAGGACTGTGTGAGCAGCCGCTGGGGGTGTAAGTGGTGTGTCTGGCAG
CACCTGTGCACCCACAAGGCCTCGTGTGATGCTGGGCCCAT**GTTGCAAGCCATC**
AGGTGATGGAGACTCAGCAGAGCTTGAGGGCCCTCCCGCCCCCCTCATCCTCCC
GTCCAGCCTCGACTACCAGA

The purple coloured nucleotides are missing in the 200bp band and have therefore been spliced out and indicate the missing exon 11. The experiment was done in triplicates for the cell lines using the next passage of cells to confirm the presence or absence of Exon 11. The results of these experiments show that all cells have at least three forms of plexin B1 i.e with or without Exon 11 and two different splice variants. I also checked the sequence for these specific variants using the NCBI genome database. I was able to confirm the existence of plexin B1 truncated variant due to its

sequence in all the cell lines. These were the smaller 200bp band as expected. An example is shown below;

Truncated variant reference sequence from NCBI

```
1801 agaggagccg actacgtatc cgtgagcgtg gagctcagat ttggcgctgt tgtgatcgcc
1861 aaaacttccc tctctttcta tgactgtgtg gcggtcactg aactccgccc atctgcgcag
1921 tgccaggcct gtgtgagcag ccgctggggg tgtaactggt gtgtctggca gcacctgtgc
1981 acccacaagg cctcgtgtga tgctggggcc atggttgcaa gccatcaggt gatggagact
2041 cagcagagct tgagggccct cccgcccccc tcctcctccc gtccagcctc gactaccagt
2101 atgacacccc cgggctctgg gagctggaag aggcgacctt gggggcaagc tcctgccctt
2161 gtgtggagag cggtcagggc tccacgttga tgccggtcca tgtggagcgg gaaatccggc
2221 tgctaggcag gaacctgcac ctttccagg atggcccagg agacaatgag tgtgtgatgg
2281 agctggaggg cctcgagggt gtggttgagg cccgggtcga gtgtgagcca cctccagata
2341 ccagtgcca tgcacctgc cagcagcacc agctcagcta tgaggctctg cagccggagc
```

The truncated splice variant has a different 5'splice site from the variant R, resulting in the splicing out of an additional 34bp. This results in a shift in reading frame and premature termination of translation, as shown below:

```
TGAT GCT GGG CCC ATG GTT GCA AGC CAT CAG GTG ATG GAG ACT CAG CAG
AGC TTG AGG GCC CTC CCG CCC CCC TCA TCC TCC CGT CCA GCC TCG ACT
ACC AGTATG ACA CCC CCG GGC TCT GGG AGC TGG AAG AGG CGA CCT
TGG GGG CAA GCT CCT GCC CCT GTG TGG AGA GCG TTC AGG GCT CCA CGT
TGA
```

The resultant truncated protein has amino acids 730-2135 missing and amino acids 677-729 are changed. The mRNA is 3,863bp, and the protein is 729 amino acids long. The splicing out of part of exon 11 in variant R does not result in a frameshift. Variant R produces a protein which has amino acids 688-870 missing to give a protein of 1952 amino acids. The agarose gel shows that the truncated variant is expressed at higher levels than variant R.

LNCaP

200 bp band: 184 nucleotides plexin B1 variant

CTTTGAACAGGACTGTGTGAGCAGCCGCTGGGGGTGTAAGTGGTGTGTCTGGCAG
CACCTGTGCACCCACAAGGCCTCGTGTGATGCTGGGCCCATGGTTGCAAGCCATC
AGGTGATGGAGACTCAGCAGAGCTTGAGGGCCCTCCCGCCCCCCTCATCCTCCC
GTCCAGCCTCGACTACCAGA

The red letters indicate identical sequences, therefore showing that the smaller 200bp LNCaP band was in keeping with the truncated variant.

4.The results of 3'RACE experiments for plexin B1 truncated variant and plexin B1 variant R

The next set of experiments undertaken was to confirm the presence of the truncated variant and variant R, using the 3'RACE protocol provided by Invitrogen as discussed in the second chapter. This was to search for a shortened 3'UTR end equivalent to variant R and the truncated variant. The cDNA products extracted from the gel electrophoresis as shown in figure 18 and 19 below, were sent for DNA sequencing. The analysis only showed the full 3' UTR end which is present in the known full length variants 1 and 2 and did not reveal the existence of a shortened 3'UTR thought to be contained in Variant R

Fig 18: Agarose gel electrophoresis of 3'RACE experiments in 6 cell lines

The Electrophoresis gel shows the expression of plexin B1 3'UTR region as determined by 3"RACE studies in the cell lines YUSIK, PC3, DU145, LNCaP, 1542NP. No shortened 3"UTR was found.

YUSIK	PC3	DU145	1542C	LNCAP	1542NP	RNA	No RNA	1kb + Ladder
						control	control	

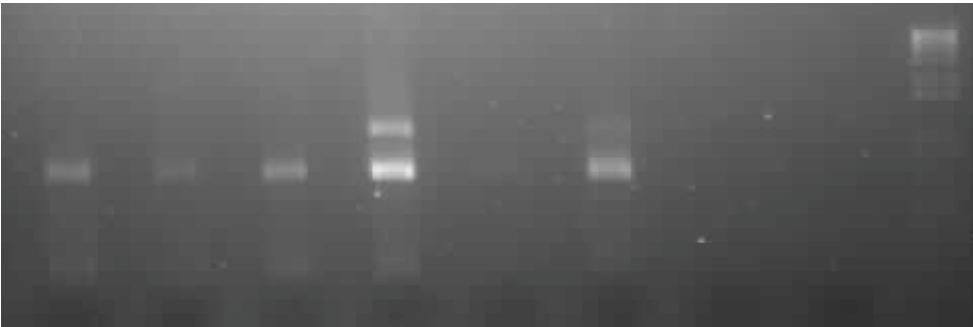
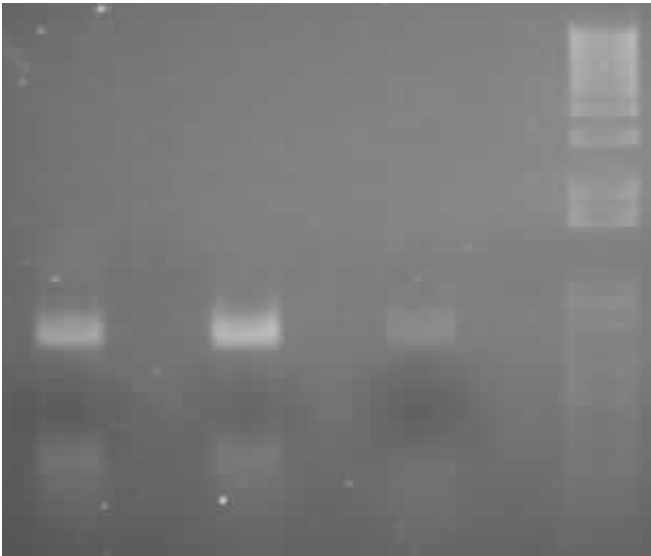


Fig 19: Agarose gel electrophoresis of 3'RACE experiments in 3 cell lines

The Electrophoresis gel shows the expression of plexin B1 3'UTR region as determined by 3"RACE studies in the cell lines 1542NP, 22RV1 and GCT. No shortened 3'UTR was found.

1542NP	22RV1	GCT27	1KB+ Ladder
--------	-------	-------	-------------



Discussion

Plexin B1 is an important receptor involved in the embryonic development and in the normal physiological process of the body due to the interaction with its ligand Sema 4D. It has been shown to occur in normal body tissue and studies have shown its existence in cancer cells. My first task was to use rtPCR to generate and multiply cDNAs from the extracted cellular RNA. I confirmed that all the cell lines did indeed express plexin B1 mRNA and I was able to show through quantitative PCR that plexin B1 was expressed at differing levels in the cell lines. It has previously been shown by Wong et al (Wong et al., 2007) that plexin B1 was highly expressed in LNCaP cells compared to PC3 cells and this was confirmed in this study. I was able to show for the first time that it is even more highly expressed in LNCaP LN3 cells and showed its varying levels in more cell lines such as VCaP. It has already been shown that plexin B1 expression is increased in prostate cancer and it has been suggested that it may have a role in the metastasis of the disease. It would appear that these expression levels might lend credence to its role in metastasis. LNCaP LN3 cells were generated by orthotopic transplantation of LNCaP into nude mice, followed by the serial (3x) orthotopic transplantation of the lymph node metastasis produced. LNCaP LN3 have a higher level of plexin B1 than LNCaP, suggesting that plexin B1 expression may contribute to metastasis in LNCaP cells in vivo. LNCaP was derived from prostate cancer lymph nodes and DU145 brain metastasis. These two aforementioned cell lines have a higher level of expression than PC3 cells that were derived from prostate cancer bony involvement.

Then section 3 of my study in this chapter confirms the existence of splice variants of plexin B1. The resultant protein products of these variants are likely to have different functions and their expression may be linked to prostate cancer metastasis. The full length variants of plexin B1 mRNA have a complete 3' UTR which can be regulated by miRNAs. The truncated variant and variant R plexin B1 mRNAs lack 3'UTR or incomplete 3'UTR respectively. Therefore their expressions is not likely controlled by miRNAs and this may be the link to their levels of expression in prostate cancer.

Tamagnone and colleagues (Tamagnone et al., 1999) have already discussed the existence of the truncated variant and my study has confirmed their findings. I was able to show in the cell lines PC3/27, DU145/71, Yusik10, GCT27/75, 22RV1/5, LNCaP, 1542NP/51 that a version of plexin B1 without exon 11 does exist due to splicing. On analysis of the nucleotide sequence of the smaller 200bp product I was able to confirm it was the truncated variant of plexin B1. Tamagnone and the NCBI genome database have shown that this variant is translated to protein. The protein lacks a transmembrane domain and an intracellular domain. This truncated variant of plexin B1 may act a dominant negative protein as it is predicted to bind to SEMA 4D but lacks an intracellular domain to transduce the signal. In addition evidence on the NCBI database showed that variant R, which has most of exon 11 spliced out, but no frame shift occurs and the rest of the protein is translated. I was able to show the existence of The Variant R, not mentioned by Tamagnone, though mentioned on the NCBI databases but hitherto not shown in the literature. This discovery was aided by the use of the exon 11 primers and the use of 3% agarose gel to separate out these bands that are quite

similar in size. The significance of this variant R is potentially a different function from the other proven variants of plexin B1 and is worth further analysis in future. PlexinB1 contains an insertion of 230 amino acids within PSI-2 domain. This insertion is not present in other plexins and is predicted to be heavily O-glycosylated and is similar in sequence to mucin domains. Mucins are heavily glycosylated and are components of many gel-like secretions. Exon 11, which is spliced out in both variants described here, encodes part of this mucin-like inserted sequence.

I then went on to discover if any of the cell lines expressed plexin B1 with shortened 3'UTR end as has been suggested in the NCBI genomic database(NCBI, 2011). I was not able to find a shortened plexin B1 3'UTR using the 3'RACE system. Although these results indicate that transcripts with a shortened 3'UTR are not transcribed in the cell lines tested, these variants may occur in other cell lines or primary prostate cancer tissue. This may be the case as it has been suggested that the truncated variant lacks a full 3'UTR end.

Chapter 4

Effect of 3' untranslated region (UTR) of plexin B1 on the expression of a reporter gene

Introduction

The effect microRNAs (miRNAs) have on gene expression is mainly through the inhibition of translation or the breakdown of mRNA transcripts. This effect is mainly due to the complementary binding of the seed region of the microRNA (usually at the very start of its 5'UTR), to the 3'UTR of the mRNA. It is known that microRNAs can be tumour suppressors and recent studies have shown that microRNAs have this function in prostate cancer(Qiang et al., 2011). Plexin B1 is a transmembrane receptor for semaphorin 4D that act as chemotactic cues for cell migration. There is overexpression and mutation of Plexin B1 in prostate tumours that suggests Plexin B1 has a role in prostate cancer and is a potential target for therapy perhaps by microRNAs mimics. Studies have shown the effect of plexin B1 overexpression in prostate cancer cells in vitro, whether through the transduction of the gene using a lentivirus or transfection into a cell of interest (Zhou et al., 2012, Wong et al., 2007). What is yet to be determined is if the overexpression of plexin B1 in prostate cancer in vivo results from the aberrant regulation of expression via the 3'UTR of plexin B1. The aim of this section of the study was to determine if expression of plexin B1 is controlled by its 3'UTR section.

Transfection is the process of introducing a foreign genetic material into an animal cell. In order to assess the effect of the plexin B1 3'UTR on gene expression, I cloned the 3'UTR of plexin B1 downstream of a luciferase gene to generate a LUC-plexin B1 3'UTR reporter construct. I carried out a series of transfection studies introducing this reporter construct into the cell lines and assessed the influence the plexin B1 3'UTR on expression of the luciferase relative to luciferase expression of a control construct

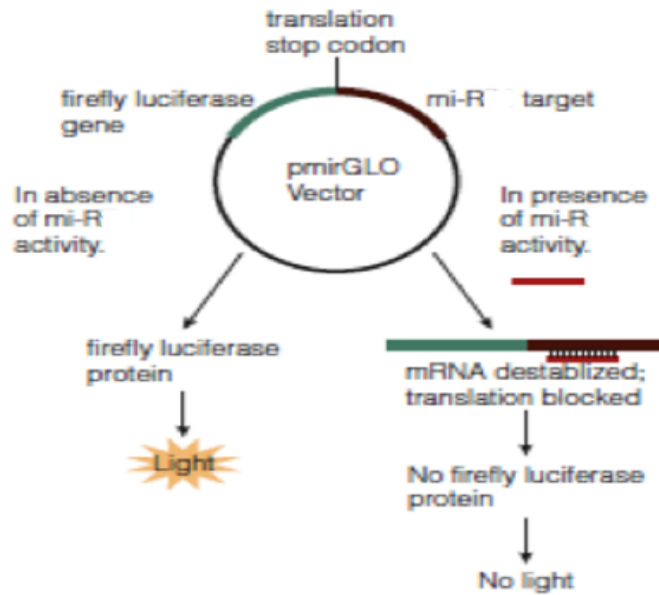
lacking the 3'UTR of plexin B1. A renilla luciferase reporter construct was co-transfected with the LUC-3'UTR or LUC-control construct to control for transfection efficiency. If we have a reduction in luminescence of the vector-construct (Luc-3'UTR) compared to the control construct (Luc-control) it means that there has been reduced mRNA translation of luciferase due to the presence of the 3'UTR of plexin B1. These co reporter genes are used because renilla luciferase acts as a control. If there was a generalised downward effect on the cells such as cell death, the expression both reporter assays would be reduced relative to renilla expression. In this way I was able to more accurately determine the effects of the Luc-3'UTR. I calculated the ratio of the luminescence of firefly to luminescence of renilla to standardise the reaction.

I first cloned the 3'UTR of the plexin B1, amplified by RACE into the luciferase reporter vector, making the construct LUC-3'UTR. The method has been described in the materials and method section in the second chapter. I then transfected this vector-construct versus control into my chosen cell lines PC3, LNCaP, 1542C, DU145, T24 and GCT and assessed the effect of the 3'UTR end. As shown in the schematic diagram below in figure 19, if the 3'UTR section of plexin B1 is actively repressed by potential microRNAs then there would be reduced activity of the reporter gene.

This transfection was done in triplicates each time, using 3 wells of a 96 well plate for Luc-control, 3 wells of a 96 well plate for Luc-3'UTR and 3 wells for no DNA controls. These experiments were repeated three times each times with 3 wells each to increase the accuracy of the final results. See the schematic diagram in figure 21.

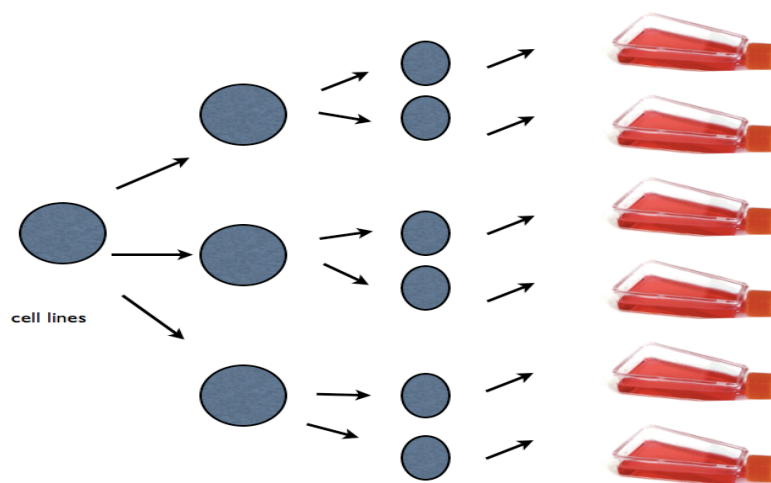
[Fig 20: The effect 3'UTR on the reporter gene expression](#)

Schematic diagram showing the effect of transfection of the vector construct into cell lines diagram modified from pmirGLO Dual-Luciferase miRNA Target Expression vector protocol.



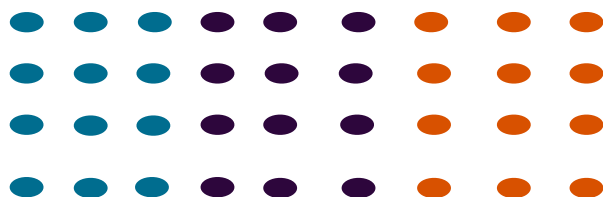
[Fig 21: Schematic diagram of the transfection in triplicates](#)

21a



21b

[Schematic of 96 well plates with triplicates](#)



Results

The results are described as follows;

- i- The results of plasmid vector-construct cloning experiments.
- ii- The results of transfection of cloned vector-construct versus control vector (including luciferase) experiments.

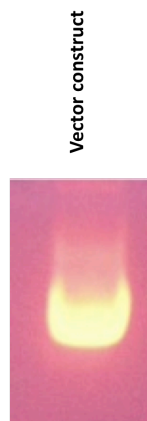
1.Plasmid vector cloning experiments

I successfully cloned the 3'UTR of plexin B1 into the plasmid vector creating a vector-construct containing the 3'UTR region of the plexin B1 downstream of the luciferase gene. The detailed methodology can be seen in the second chapter. I achieved this by first amplifying the 3'UTR of plexin by 3'RACE, cutting out the amplified product from an agarose gel and ligating this into the cut vector. I then replicated the construct by transforming E.coli and growing up the transformed bacteria. The plasmid was extracted from the bacteria and the DNA analysed by performing gel electrophoresis. This electrophoresis showed the supercoiled crescent like band, which is the particular type of band seen when an intact circular plasmid is run on an agarose gel. The cDNA sequences were sent for analysis. This gel electrophoresis is seen below in figure 22.

Fig 22: Agarose gel electrophoresis of the plasmid vector-construct

The image shows two crescent like band confirming the presence of the vector construct, luc-3'UTR

Agarose gel electrophoresis to confirm the presence of plasmid vector construct



The cDNA of the plasmid vector was successfully extracted from the gel and sent for sequencing. The sequence and BLAST results as displayed by the NCBI database is shown below

AD_B7_pGLOxhoS.ab1

NCBI blast result:

622 nucleotides Homo sapiens plexin B1 (PLXNB1), transcript variant 1, mRNA 99%

```
CTGACTTAGGCTTCGCCTGGCTGGGCAGCCCTGGAGCTCGGAGGAGAGGCCACC
TTCTTAGGTGCCTGTAGTGAAGCAAGCAGAGTTAGTGGAAGGTGACTCCCAGT
CTCCTGGTGGCTCTGGCCTCGGCCCTGCTGGATCCACCTCCTAGACCCGGGGCC
TCAAGGCTCATGGGGTAGTACCCAGCCTGCTCCCCGAGTCCAGCGACCCTGTGAC
ACCGGTCTGCAGGGAGTTGGGGACTAAGGGCTTCCAGAGAGTGGCTGGAAGAGA
CTCCAGGCCCTGGGGAGACTGTACTGTTCTGAACACTGGCCTTGGCCCACTG
GGATTTCGGAGAGGAAGGAGGAGAGCCCCATGCTTCCTGTCTGCCTCCTCCACCAT
CCCTGACCTCAGTTGAGCTGCCTCTGGCCTTGTTGCTGCTGCCACATCCTAGGTCT
AAGAGTTGAACGCCTCTCCTAGGCCACTACAACTGACCCCTCAGCAGGGGCTGGC
TGCCACAGGGCTGCCCTGCCTCATAGGTAGCCATGGTGAGGGCTATCTGCTGCAG
GGGGGTCTTGGGGAGAGTGGTGACTCCATTGACCCAGCTTTTCATTAAAGGATAA
CACACTGTCTTAGAGGGGA
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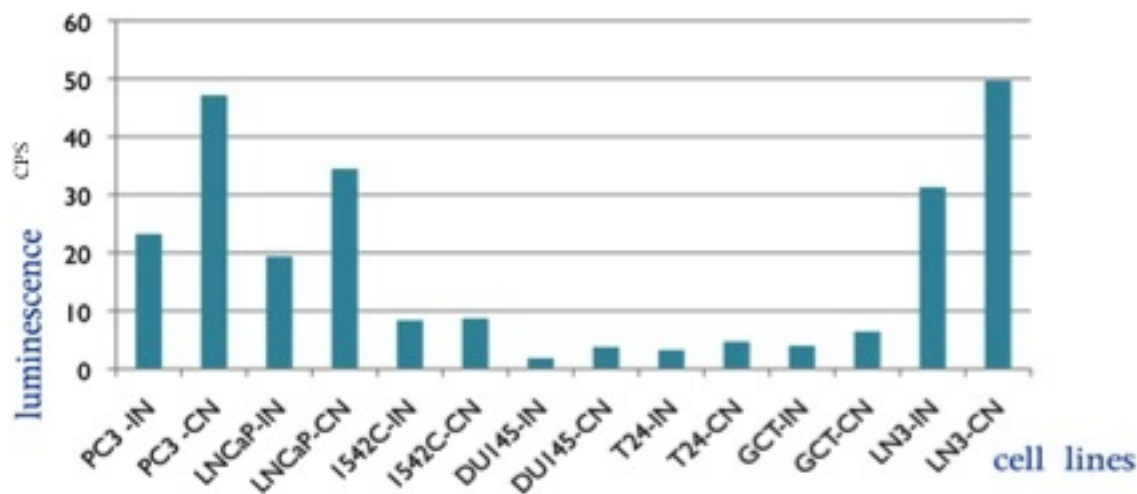
2. Transfection of cloned vector-construct versus control vector

The next step in this section of the study was to transfect the cloned vector-construct (Luc-3'UTR) I made into the chosen cell lines. The cells were co-transfected with either the vectors Luc-3'UTR and the Renilla construct or Luc- control and the Renilla construct. The expression of the luciferase was assessed after the addition of Dual Glo luciferase reagents (following the protocol of the Promega technical manual of the Dual Glo Luciferase assay system) using a Luminometer. The effect of the presence of the 3'UTR on Luciferase expression was determined by comparing the Luciferase activity of cells transfected with the Luc-3'UTR and control Luc constructs. The results show a difference in the effect of Luc- 3'UTR compared to the Luc-control across all cell lines

with a downward expression of the luciferase in cell lines transfected with Luc-3'UTR compared to Luc-control. This is shown in graph 3 below. This is the first time that it has been shown in prostate cancer cell line that the 3'UTR of plexin B1 mRNA has a direct effect on expression of the gene suggesting a role for miRNAs, which are known to bind the 3'UTR ends of mRNAs. As is necessary this stage of the investigation was done in triplicates. Each time a different passage of cells (i.e a different batch of the same cell line) was used for the transfection experiment involving both the LUC-3'UTR and the LUC-Control. I analysed these set of results statistically, with the data being normally distributed, using a one way ANOVA test on the SPSS 20 programme. The results are as shown in graph 4. I found that in DU145 and LNCaP cells, there were statistical significance decrease in luminescence due to transfection of the vector-construct Luc-3'UTR compared to the Luc-control with a P value of ≤ 0.05 .

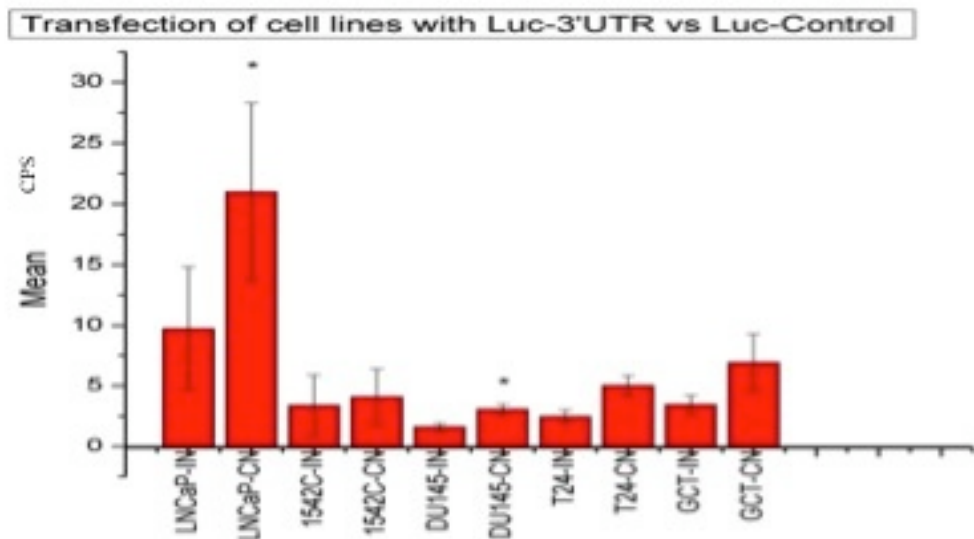
Graph 3: the effect of transfection of luc- 3'UTR versus luc-cont on cell lines

The graph shows the effect of transfection of vector construct containing plexin B1 3'UTR compared to control vector lacking plexin B1 3'UTR in a single experiment across various cell lines



Graph 4: transfection of cell lines with LUC-3'UTR VS LUC-Control average

The graph shows the effect of transfection after triplicate across various cell lines of vector construct containing plexin B1 3'UTR compared to control vector lacking plexin B1 3'UTR. There was a difference in the effect of transfected Plexin B1 Luc-3'UTR compared to Plexin B1 Luc-control across the cell lines as shown by reduced luciferase activity (luminescence). key * $p \leq 0.05$



Discussion

In this section of my work I was able to construct a plasmid vector that contained the plexin B1 3'UTR using set protocols and confirming my construct with the aid of cDNA sequencing. The process of making this plasmid vector involved cutting the plasmid vector with a restriction enzyme then attaching it to the 3'UTR of plexin B1 by ligation with a ligase to form the vector construct and then making multiple copies of them by multiplying the plasmid vector in treated E.coli bacteria.

It has been shown for the first time in prostate cancer cell lines that the 3'UTR of plexin B1 may have an effect on gene expression as exemplified by the reduced luminescence of the luciferase assay. In two cell lines DU145 and LNCaP this reduction is statistically significant with $P \leq 0.05$. These results suggest that the 3'UTR of plexin B1 does have an effect on gene expression and it suggests that some high level of plexin B1 in prostate cancer may result from aberrant regulation of expression via this region of plexin B1. As miRNAs regulate genes mainly through the 3'UTR, I suggest that in prostate cancer cells plexin B1 may be regulated by miRNAs binding to this region. This potentially provides another avenue for treatment of prostate cancer by identifying these potential miRNA. In order to investigate this potential regulatory mechanism I carried out a series of studies in the next set of chapters.

Chapter 5

Assessment of the regulation of plexin B1 expression by microRNAs

Introduction

It is known that the microRNAs regulate normal gene expression and they have also been shown to be involved in carcinogenesis. MicroRNAs are able to act both as tumour suppressors as well as oncogenes. As it has been shown that plexin B1 acts in an oncogenic capacity in prostate cancer I decided to investigate whether microRNAs negatively regulate plexin B1. This negative regulator or tumour suppressor microRNA would act by inhibiting the activity of plexin B1 and thus eventually be a candidate for therapeutic intervention against prostate cancer. There has been no discovery so far of such a microRNA. A precedent has already been set but in the case of cervical cancer as mentioned earlier in the hypothesis section of chapter one. It was suggested that the role of plexin B1 as an oncogene was negatively regulated directly by microRNA 214 (mir 214) in cervical cancer(Qiang et al., 2011). Therefore mir 214 was a good reference point for me to begin my search.

To perform this task, which involved qPCR analysis of a transcriptome array containing cDNAs of Hela cells pretreated with cancer specific microRNA, I started off by optimising my qPCR reactions in order to get as accurate a result as possible. Optimisation experiments included performing different qPCR reactions to ascertain which dye would be the most suitable for the transcriptome array from either Taqman or SYBR green dyes. SYBR green was chosen as it worked well with good CT values and was recommended by Qiagen the makers of the transcriptome PCR array. The optimisation process also included performing qPCR using cDNA initially from cancer

cell lines, then later cDNA from Hela cells to closer resemble the QPCR of the transcriptome array

This was because the array contained Hela cells. Another part of the optimization process was to perform qPCR using different annealing temperatures to find which temperature best suited the primers and probes. I then carried out the qPCR with SYBR green dye using Qiagen Sure FIND transcriptome PCR arrays to identify potential microRNA that regulate Plexin B1. On analysis of the optimization results GAPDH produced the best housekeeping gene results with a steady expression levels across the cancer microRNAs used in the array. The array contains Hela cells transfected with microRNA mimics and control Hela cells without microRNA mimics. Hela cells are treated with 90 cancer candidate microRNAs and also 6 controls. The relative levels of expression of Plexin B1 in Hela cells with the mimics relative to those controls without mimics were quantified and normalised using the housekeeping genes GAPDH. The data was analysed with the database software based on Microsoft Excel. This calculated the $\delta\delta$ CT fold change with significant values equivalent to those microRNAs that get above the cut off point of the log to -1 or 1 fold change. The figure 23 below shows a schematic diagram of the qPCR transcriptome process courtesy of Qiagen.

Figure 23: qPCR transcriptome analysis image courtesy of Qiagen

1. Prepare Reaction Mix with Gene-specific assay and Master Mix.

Target primer mix



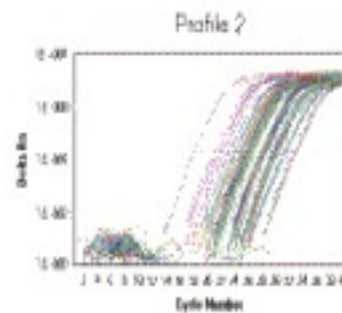
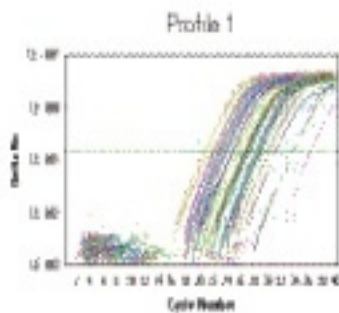
House-keeping primer mix



2. Load Transcriptome PCR Array with Reaction Mix.



3. Begin Thermocycling run in qPCR instrument.



4. Identify transcriptional regulators:
Export C_t values to Data Analysis software.

Results

This section is divided into two parts as follows:

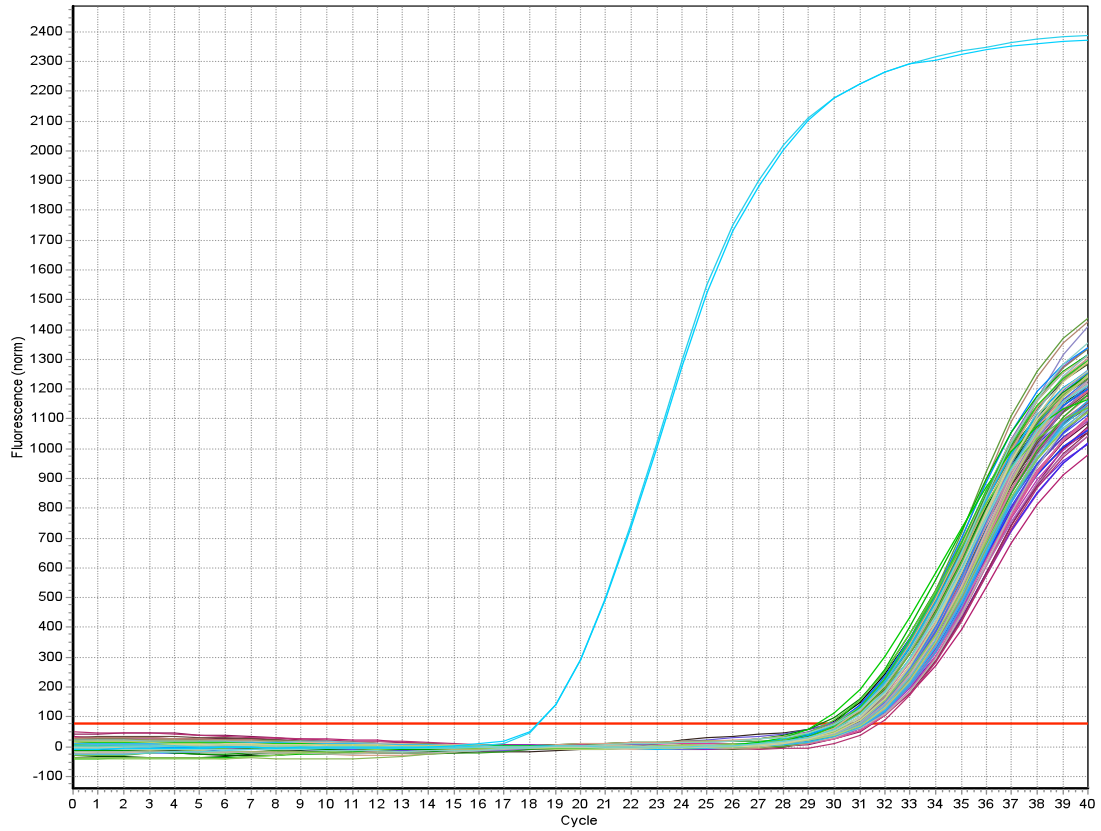
- 1- The results of qPCR optimisation experiments.
- 2- The results of miRNA qPCR transcriptome experiments

1.qPCR optimisation experiments

As stated in the introduction the aim of this section of the experiment was to get an accurate a reading as possible by finding the best conditions for qPCR. It involved altering the annealing temperatures and trying different housekeeping genes. The graphs in graph 5 and graph 6 below show the difference between the use of B2M and GAPDH as housekeeping genes. It can be seen that the B2M is further to the right of the curve than necessary further away from the control represented by the blue curve yielding higher CT values which reduces its accuracy. This indicates a low level of B2M expression in the cell line. It can be seen that GAPDH has good CT values indicating high expression closer to the left of the curve and nearer to the control blue curve and in further experiments to be shown in the next section yielded good steady readings.

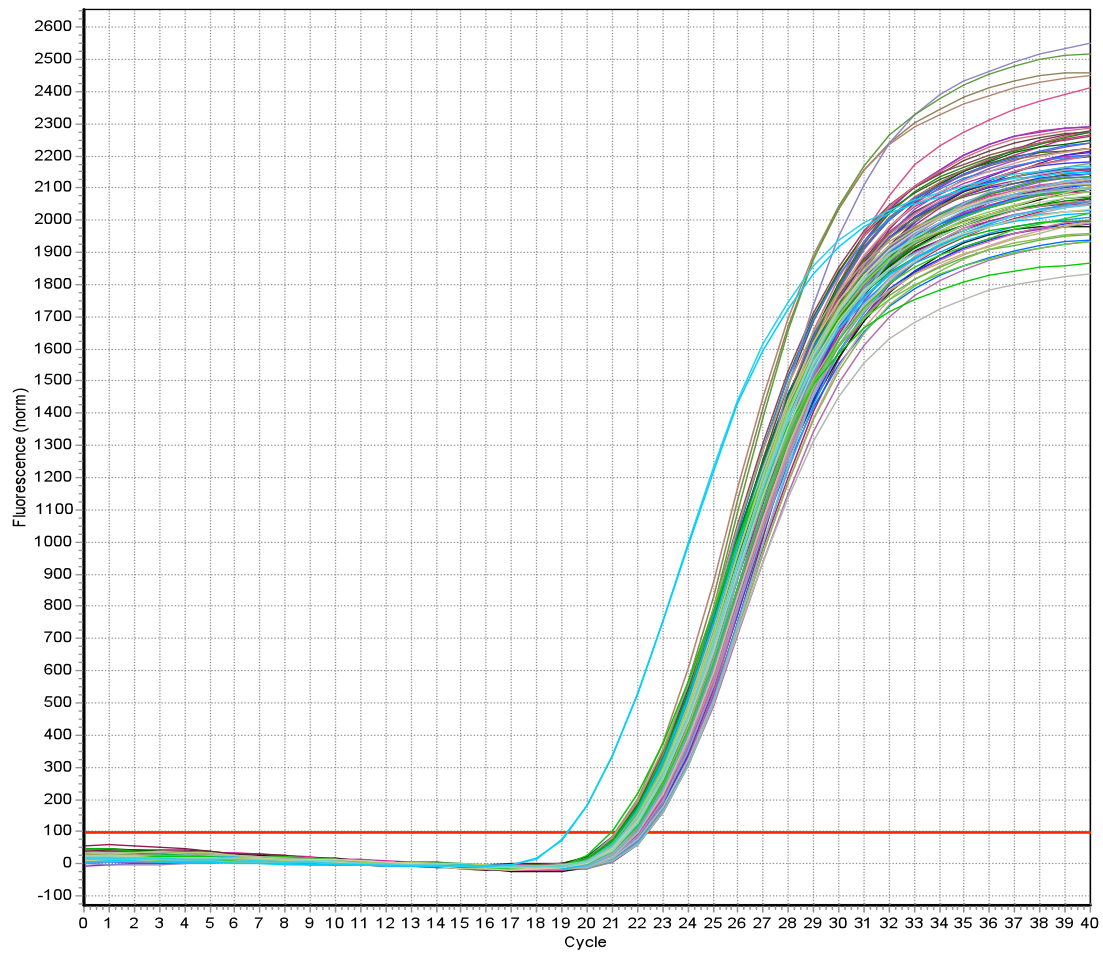
I settled on the parameters for the qPCR phases and cycles on the Mastercycler realplex qPCR machine as shown in table 4 in the qPCR methodology section in the second chapter.

Graph 5: B2M CT Curve



Threshold: 77 (Noiseband)
Baseline settings: automatic, Drift correction OFF

Graph 6: GAPDH CT curve



Threshold: 98 (Noiseband)

Baseline settings: automatic, Drift correction OFF

2.The miRNA qPCR transcriptome experiments

The Qiagen transcriptome array containing cDNA from Hela cells treated with 90 candidate cancer genes and 6 controls, yielded clear results with steady expression levels of the housekeeping gene GAPDH. See table 5 for Plexin B1 and table 6 for GAPDH below.

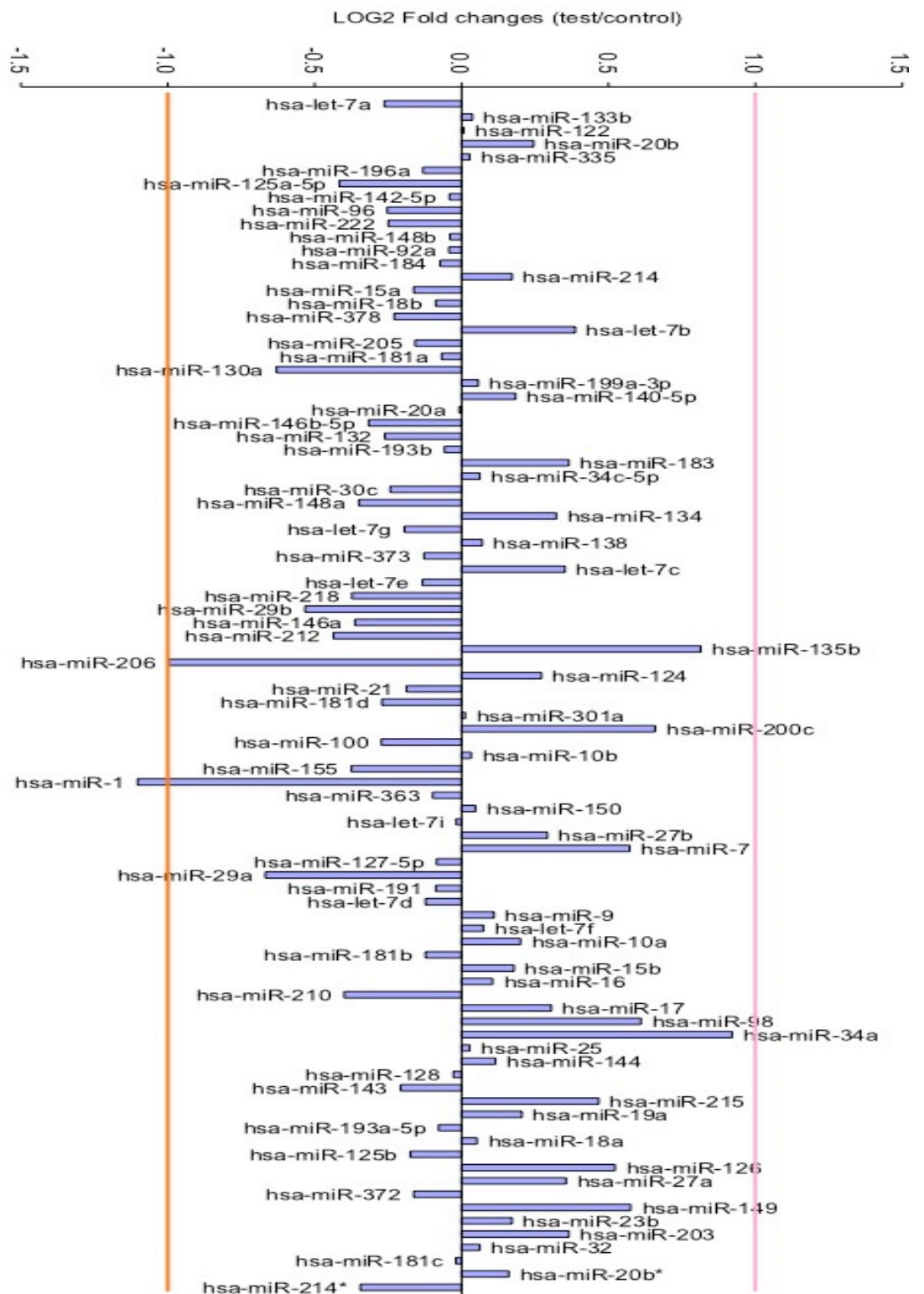
Table 5: qPCR of candidate miRNAs using plexin B1 primers

Symbol	Plate	Position	EXP1	EXP2	EXP3	Average	SD
hsa-miR-124	TCMB-201	D8	29.01	29.16	29.77	29.085	0.106
hsa-miR-21	TCMB-201	D9	29.17	29.25	29.12	29.180	0.066
hsa-miR-181d	TCMB-201	D10	28.72	28.92	29.13	28.923	0.205
hsa-miR-301a	TCMB-201	D11	28.67	28.88	28.70	28.685	0.021
hsa-miR-200c	TCMB-201	D12	28.13	28.12	28.15	28.133	0.015
hsa-miR-100	TCMB-201	E1	28.47	28.51	28.64	28.490	0.028
hsa-miR-10b	TCMB-201	E2	28.81	29.23	28.73	28.770	0.057
hsa-miR-155	TCMB-201	E3	28.62	28.72	28.96	28.767	0.175
hsa-miR-1	TCMB-201	E4	29.59	29.55	29.96	29.570	0.028
hsa-miR-363	TCMB-201	E5	28.34	28.33	28.73	28.335	0.007
hsa-miR-150	TCMB-201	E6	28.72	28.74	28.74	28.740	0.000
hsa-let-7i	TCMB-201	E7	28.91	29.01	29.35	28.960	0.071
hsa-miR-27b	TCMB-201	E8	28.60	28.60	28.57	28.600	0.000
hsa-miR-7	TCMB-201	E9	28.58	28.87	28.71	28.720	0.145
hsa-miR-127-5p	TCMB-201	E10	28.71	28.86	28.76	28.777	0.076

Table 6: qPCR of candidate miRNAs using GAPDH primers

Symbol	Plate	Position	EXP1	EXP2	EXP3	Average	SD
hsa-miR-135b	TCMB-201	D6	22.60	21.84	21.85	21.85	0.01
hsa-miR-206	TCMB-201	D7	22.55	21.93	21.91	21.92	0.01
hsa-miR-124	TCMB-201	D8	22.95	22.47	22.32	22.40	0.11
hsa-miR-21	TCMB-201	D9	22.87	21.95	22.11	22.03	0.11
hsa-miR-181d	TCMB-201	D10	22.34	21.69	21.69	21.69	0.00
hsa-miR-301a	TCMB-201	D11	22.67	21.73	21.74	21.74	0.01
hsa-miR-200c	TCMB-201	D12	22.54	21.85	21.81	21.83	0.03
hsa-miR-100	TCMB-201	E1	21.86	21.28	21.23	21.26	0.04
hsa-miR-10b	TCMB-201	E2	22.68	21.85	21.83	21.84	0.01
hsa-miR-155	TCMB-201	E3	22.02	21.38	21.48	21.43	0.07
hsa-miR-1	TCMB-201	E4	22.22	21.49	21.52	21.51	0.02
hsa-miR-363	TCMB-201	E5	22.18	21.19	21.36	21.28	0.12
hsa-miR-150	TCMB-201	E6	22.56	21.80	21.85	21.83	0.04
hsa-let-7i	TCMB-201	E7	22.92	22.00	21.96	21.98	0.03
hsa-miR-27b	TCMB-201	E8	22.72	21.93	21.93	21.93	0.00

Graph 7: Graph of transcriptome array showing mir1 a negative regulator



The qPCR transcription array I performed in triplicates (as shown in the table 5 and 6) produced potential candidate microRNAs that regulated plexin B1 expression. The next step is to verify the result. It was shown that microRNA 1 significantly negatively regulated plexin B1 a new finding that was hitherto unknown. This is shown graphically in graph 7 above.

Discussion

This section of my study has revealed a potential candidate microRNA. MicroRNA 1 appears to be a negative regulator of plexin B1 expression as shown clearly on the graph image above. It is also followed closely by mir 206, mir 29a and as suggested by Qiang et al mir214* although none these other microRNAs reach a significant level of regulation (Qiang et al., 2011). The candidates miRNAs used in this array are known to be cancer specific microRNAs. Expression of some miRNAs resulted in an increase in plexinB1 mRNA levels. This is most likely to be due to an indirect mechanism of expression control. These miRNAs may, for example reduce the expression of a plexinB1 inhibitor.

The study by Hudson et al in 2012 strongly suggests a role of microRNA 1 as a tumour suppressor in prostate cancer(Hudson et al., 2012). They showed that this microRNA prevents invasive properties in cancer cells and also the formation of filipodia. They

reveal that microRNA 1 is linked to prostate cancer metastasis and that there is an inverse relationship with reduction in its expression in metastatic tissue and that it directly and indirectly suppresses oncogenes involved in prostate cancer. It is therefore likely that microRNA 1 is a potential regulator of plexin B1 as shown by my transcriptome array studies.

The full list of the 90 cancer specific microRNAs used in the 96 well transcriptome array are listed in the appendix section.

Chapter 6

Verification of the effect of candidate microRNAs on plexin B1 expression

Introduction

The increased levels of plexin B1 in primary and metastatic prostate tissue when compared to normal tissue, as reported by our group, suggests a link between plexin B1 and prostate cancer metastasis (Wong et al., 2007). There have been several studies linking microRNAs specifically to prostate metastasis. What would be of interest is if any of these microRNAs may regulate plexin B1 expression. Aberrant miRNA control of plexinB1 expression could be one of the mechanisms by which plexinB1 is overexpressed in cancer. MicroRNA 1 has been linked to prostate cancer metastasis as shown by Hudson et al (Hudson et al., 2012), and the results of my transcriptome array studies in the previous chapter is in keeping with their findings. Some other microRNAs suggested to be linked to prostate cancer metastasis are mir221, mir154 and mir379 (Kneitz et al., 2014, Gururajan et al., 2014). I intended to discover whether any other microRNA also targeted plexin B1.

The next set of experiments were aimed at validating the findings of my results from the transcriptome array and to find other potential regulating microRNAs. I wished to determine if the expression levels of plexin B1 mRNA and protein had a direct correlation with the candidate microRNAs. I decided to investigate microRNA 1 (mir1) due to the transcriptome array results. I also chose microRNA 214 (mir214) because it was a regulator of plexin B1 in my transcriptome array studies, albeit not a statistically significant one, it has also been shown to negatively regulate plexin B1 in cervical cancer cells in the study by Qiang et al (Qiang et al., 2011). Interestingly in the transcriptome array study it can be seen that mir214 both positively and negatively

regulates plexin B1 depending on the variant of mir214. I chose to study the positive regulator mir214-3p (accession number MIMAT0000271). I expected mir214-3p to potentially increase the expression of plexin B1 by an indirect mechanism as suggested by the transcriptome array results as seen in figure 28 of the previous chapter . I also analysed the online microRNA databases (mirBASE, mirWALK, MiRanda) to assess microRNA candidates that are predicted to regulate plexin B1 (mirwalk, 2014, Mirbase) to look for potential miRNA binding sites. The above analysis revealed three microRNA candidates these microRNAs were mir320d, mir199a-5p, mir101-3p. The databases yielded quite a few microRNA candidates but these three were the most frequent occurring matches across the databases. Interestingly mir1 did not come up as a direct candidate microRNA regulator of plexin B1 on the databases and this may be perhaps because there are no obvious binding sites on the 3'UTR of plexin B1 for the known seed region of mir1. Notwithstanding based on my transcriptome experiments which revealed a significant negatively inhibiting role for mir1 on triplicate experiments I decided to investigate mir1 as well as mir214. I also made use of scramble short stranded microRNAs, from mirVana that served as a negative control, which I chose to call 'mirVana' for simplicity of terminology. I then obtained these microRNA candidates, the five of them in the form of microRNA mimics from Quiagen miScript miRNA mimics for my investigations.

I created the vector-construct (Luc-3'UTR) in which the 3'UTR is placed downstream of the luciferase gene (chapter 4) in order to investigate whether there were direct interactions between my candidate microRNAs and plexin B1 3'UTR by cotransfecting

the microRNA and the Luc-3'UTR and monitoring luciferase activity. I assessed the luciferase activity in these contranfections by using the luminometer Walac 1420. If the luciferase activity reduced in comparison to controls, then it meant the candidate microRNA halted the translation of the luciferase reporter mRNA implying that the binding of the miRNA to the 3'UTR of plexinB1 controls expression of the plexinB1 gene.

I then performed transfection control studies using the live imaging recording technique of an incucyte imaging system to assess the real time effect of transfection. This was in order to optimise the next set of transfection experiments which lacked the luciferase marker of the above co-transfection studies.

I then performed western blot experiments. These experiments were aimed at investigating whether the expression levels of plexin B1 protein varied depending on different candidate microRNAs. I assessed the levels of plexin B1 protein after I had transfected the different candidate microRNAs into the prostate cancer cell lines PC3 and LNCaP.

I then assessed the levels of plexin B1 mRNA levels by performing quantitative Polymerase Chain Reaction (qPCR) after transfection with the candidate microRNAs. This was to assess whether certain microRNAs had a greater effect than others on the expression level of plexin B1 mRNA.

In terms of the cancer cell lines for the transfection and also the co-transfection studies, I used DU145, PC3 and LNCaP cell lines. LNCaP cell lines tend not to adhere as well to the surface of a well as the others but as shown in the results of the qPCR studies in the third chapter they have the highest level of endogenous plexin B1 and therefore were of interest. I aimed to discover if microRNA transfection affected plexin B1 expression levels differently in PC3 cells which have the lowest levels endogenous plexin B1 compared to LNCaP cells.

Results

This section is divided into two parts

1. the results of the miRNA LUC-3'UTR co-transfection experiment.
2. The assessment of the efficacy of transfection with the transfection control investigation, the assessment of plexin B1 mRNA by qPCR, and plexin B1 protein expression levels by Western blotting.

1. The results of candidate miRNA co-transfection with pmirGlo LUC-plexinB1-3'UTR reporter vector into cell lines

The candidate microRNAs were co-transfected with the vector-construct into prostate cancer cell lines PC3 and DU145. Optimisation experiments were performed initially to determine the best conditions for co-transfection. The co-transfection experiment was performed following the Attractene transfection reagent handbook by Qiagen as well as searching the Qiagen transfection database protocol which I used to create specific protocols for the 96 well plate of the two cell lines (Qiagen). Qiagen recommended the attractene reagent for co-transfections while HiPerfect transfection reagent was recommended for transfection of single miRNAs. I used the Wallac 1420 luminometer to evaluate the luminescence of the cotransfection experiments. I co-transfected the vector construct LUC-3'UTR with the microRNAs, or with the scrambled microRNA, and compared this with the transfection of the vector construct LUC-3'UTR on its own.

The co-transfections were successful following optimisation of the protocols. In my optimising experiments I initially transfected using the fast forward protocol which

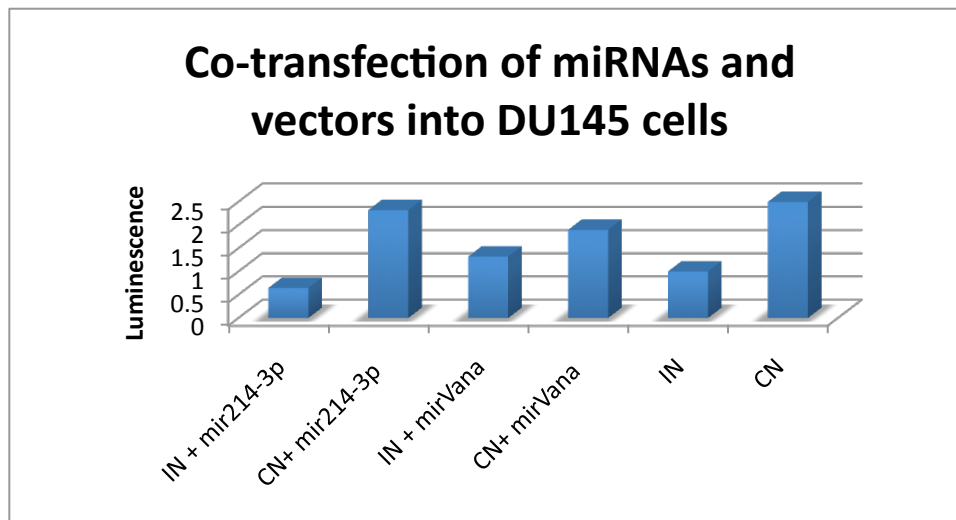
involved plating and transfection on the same day. I discovered that the traditional protocol involving plating the cells for a period of 24 hours before transfection the next day produced better results and so continued with the traditional approach in the main experiments. I found transfection most effective in the PC3 cells compared to DU145 cells and so I decided to focus on the cotransfection experiments using the PC3 cell line.

1a: Mir 214-3p cotransfections:

The graphs below contain the result breakdown of the co-transfection investigations for mir214-3p. Graph 8 shows the results of co-transfection of LUC-3'UTR construct or LUC-control construct with or without mir214-3p or scrambled miRNA into DU145 cells. Transfection of mir 214-3p decreased luciferase expression levels of LUC-3'UTR construct in comparison with co-transfection of the construct with scrambled miRNA or no miRNA co-transfection. This was also shown in PC3 cells, where I performed the cotransfection on three separate occasions as shown in graph 9 below.

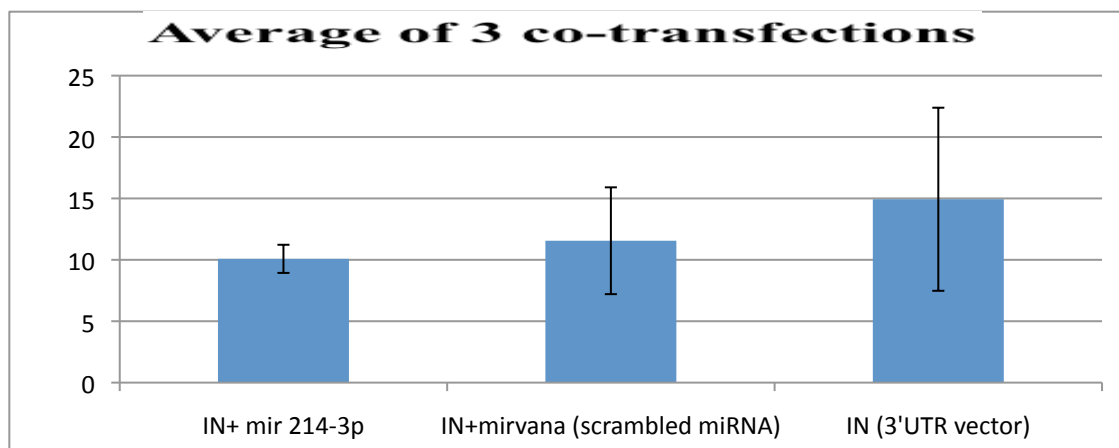
Graph 8: Co-transfection involving mir214-3p in DU145 cell line

the graph shows DU145 cells that have had; mir 214-3p co transfected with vector construct (IN + 214-3p), or with control vector (CN+ 214-3p). DU145 cells co transfected with scrambled mirRNA and vector construct (IN+ mirVana), or with scrambled mirRNA with control vector (CN+ mirVana), or DU145 transfected alone with vector construct (IN) or vector control (CN)



Graph 9: Average of Co-transfections in PC3 cells

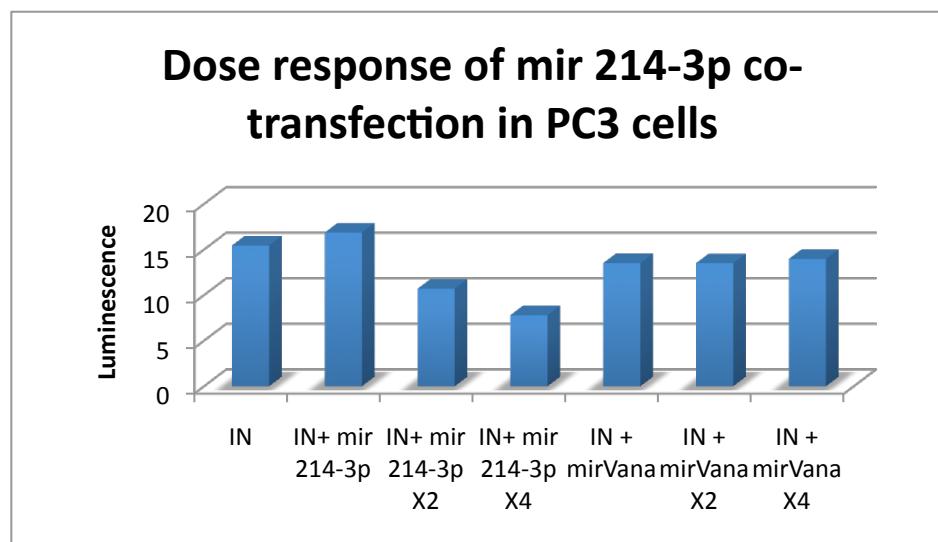
The graph shows the average of three separate co transfection experiments (each of the separate experiment were also done in triplicates) in PC3 cells. The cells were either transfected with vector construct and mir 214-3p (IN+ mir214-3p), vector construct and scramble miRNA(IN+ mirVana), or vector construct alone(IN).



There is a dose response when transfection is performed with the mir 214-3p. This is shown in the graph 10 below. I found that increasing doses of mir 214-3p reduced the expression of plexin B1 luciferase proportionally. There is a dose response of reduced luminescence as I increased the concentration of mir214-3p. In contrast, increased concentration of control scrambled miRNA had no effect on LUC-3'UTR luciferase activity. This suggests that mir214-3p has targeted the 3'UTR of plexin B1 and with increasing dosage there was more mir 214-3p available to prevent further translation of the luciferase mRNA. Thus mir 214-3p negatively regulated plexin B1 3'UTR.

[Graph 10: Dose response to increasing concentration of mir214-3p in PC3 cells](#)

The graph shows PC3 cells transfected with vector construct alone (IN), or along with mir 214-3p (IN+ MIR 214-3p) or with scrambled miRNA (IN+mirVana). Increasing concentrations of microRNAs were abbreviated in to X2- for double concentration and X4 for 4 times the concentration.

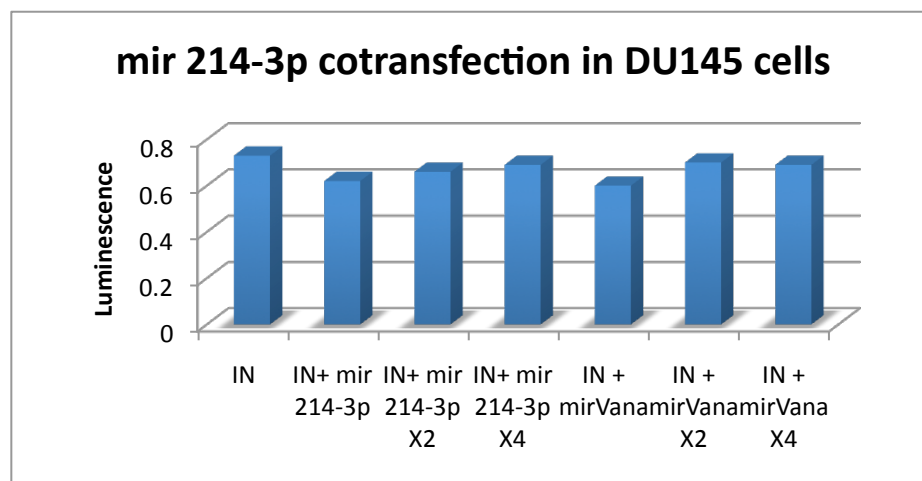


The next graph 11 shows the co-transfection of mir214 and LUC-3'UTR vector in DU145 cells. In theory there should be no difference as the cell lines in these co-

transfection experiments act as hosts for the interaction of both transfected substances but it appears that in DU145 cells there was no dose response in contrast to what was observed in PC3 cell lines it is important to note that the luminescence values of the DU145 experiments are much smaller so perhaps the response would be harder to detect with reduced luminescence overall. A possible explanation for this reduced luminescence was the fact that the measurement of the luminescence by the luminometer was done first in the PC3 cells before the DU145 and some decay of the luciferase may have occurred in the interval before the measurement was taken.

Graph 11: No dose response to increasing concentration of mir214-3p in DU145

The graph shows DU145 cells transfected with vector construct alone (IN), or along with mir 214-3p (IN+ MIR 214-3p) or with scrambled miRNA (IN+mirVana). Increasing concentrations of microRNAs were abbreviated in to X2- for double concentration and X4 for 4 times the concentration.



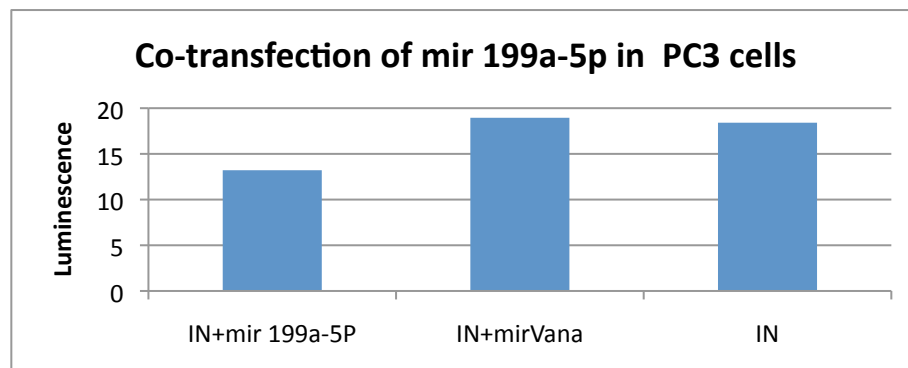
1b: Transfection with other microRNAs:

I then went on to carry out cotransfection experiments involving the other candidate microRNAs. The results were varied as shown by the graphs below. I found that mir 199a-5p also negatively regulates the expression of plexin B1, while the other microRNAs; mir 1, mir 101 and mir320d did not.

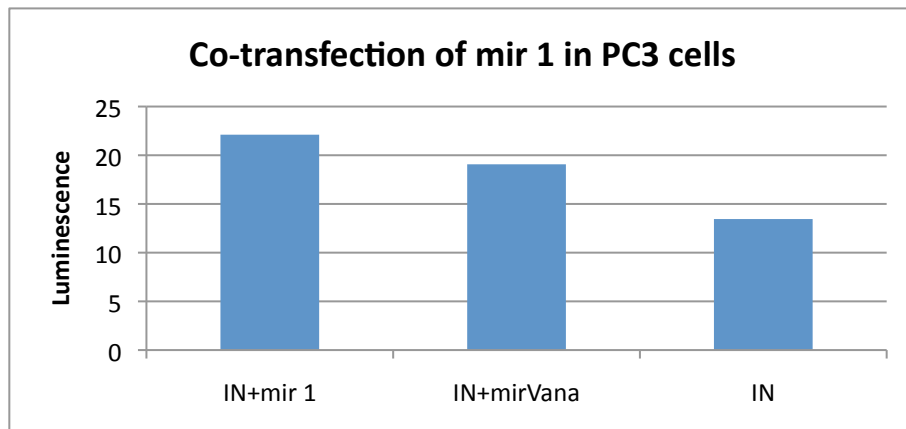
Graph 12: Co-transfections of microRNAs in PC3 cells

The graphs below show the effect of co transfection of the microRNAs into the PC3 cell lines. 12a shows transfection with vector construct alone (IN), or along with mir 199-5p (IN+ MIR 199-5p) or with scrambled miRNA (IN+mirVana). 12b shows transfection with vector construct alone (IN), or along with mir 1 (IN+ mir 1) or with scrambled miRNA (IN+mirVana). 12c shows transfection with vector construct alone (IN), or along with mir 101 (IN+ mir 101) or with scrambled miRNA (IN+mirVana). 12d shows transfection with vector construct alone (IN), or along with mir 320d (IN+ mir 320d) or with scrambled miRNA (IN+mirVana).

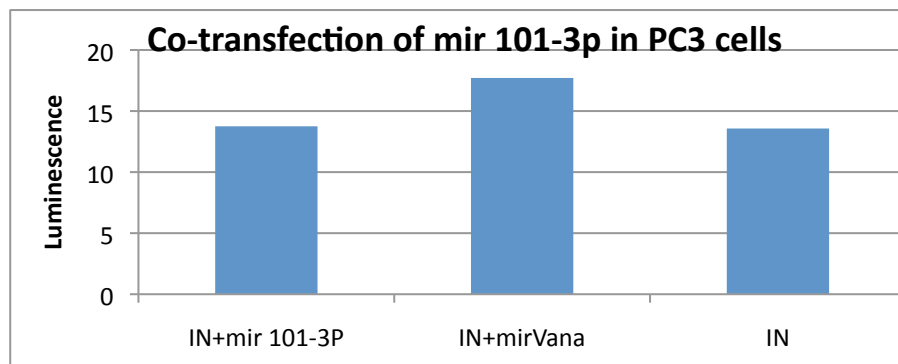
12a



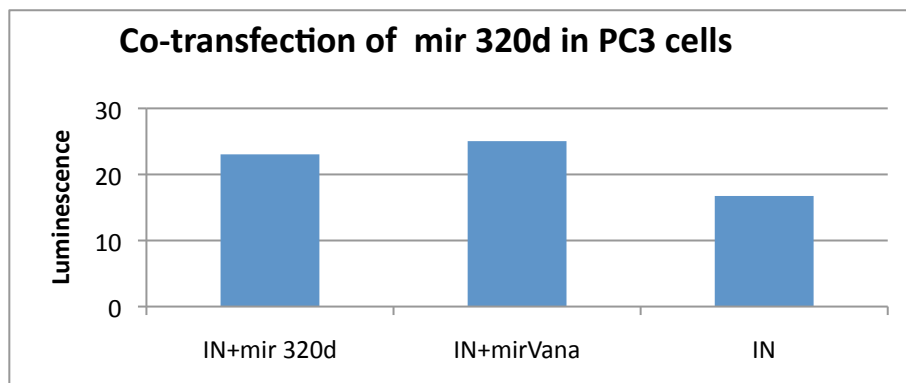
12b



12c



12d



To further verify the findings from my co transfection experiments I carried out transfection reactions assessing the mRNA and protein expression levels of plexin B1 as shown below in section 2.

2. The results of miRNA transfection alone into cell lines

2a: Transfection control experiments

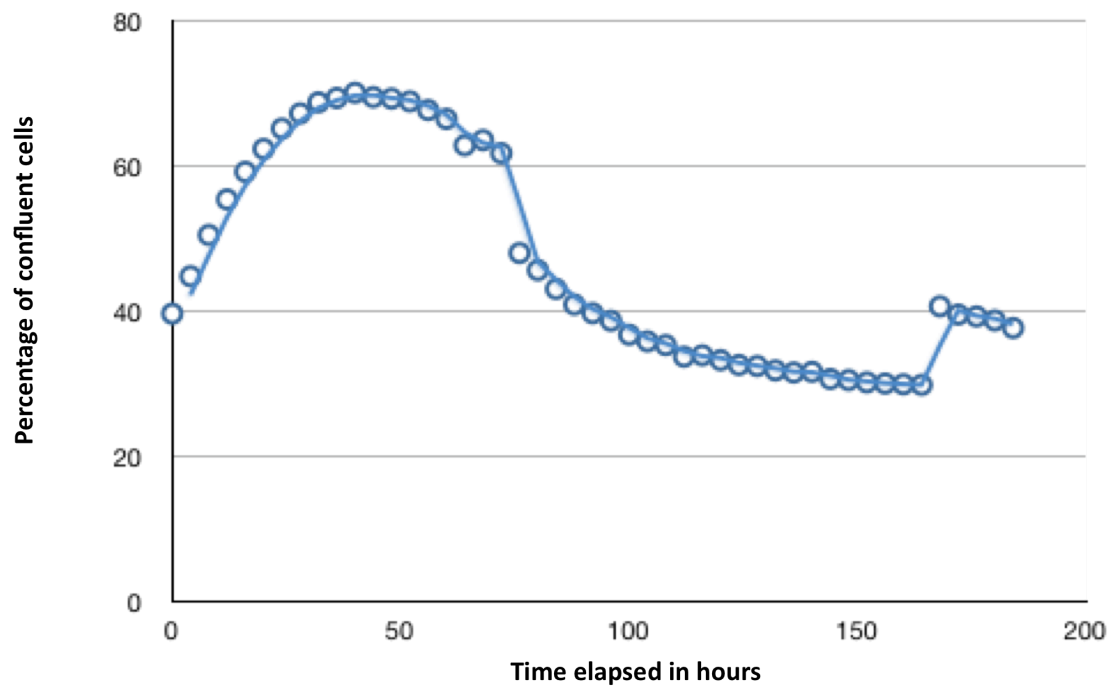
I initially carried out transfection control investigations to confirm that the transfection took place successfully. A siRNA (the Allstar Hs Cell Death control) that is lethal to cells in which it is expressed was used and cell death monitored using the Incucyte Imaging system. The results revealed effective transfection using siRNA control Allstar Hs Cell Death control (5nmol) following the Qiagen transfection protocol with HiPerfect reagent. The imaging system was set to take images every 4 hours for a duration of 184 hours to assess changes in each well. Cell death occurred in all wells transfected with two different concentrations of SiRNA in almost identical pattern while the control cells that were transfected with water or PBS remained fully viable. See graphs below

Graph 13: Transfection control of SiRNAs in PC3 cells

13a shows transfection of PC3 cells with 6ul SiRNA concentration; 13b shows transfection of PC3 cells with 12ul SiRNA concentration; 13c shows transfection of PC3 cells with PBS as control; and 13d shows transfection of PC3 cells with water as control

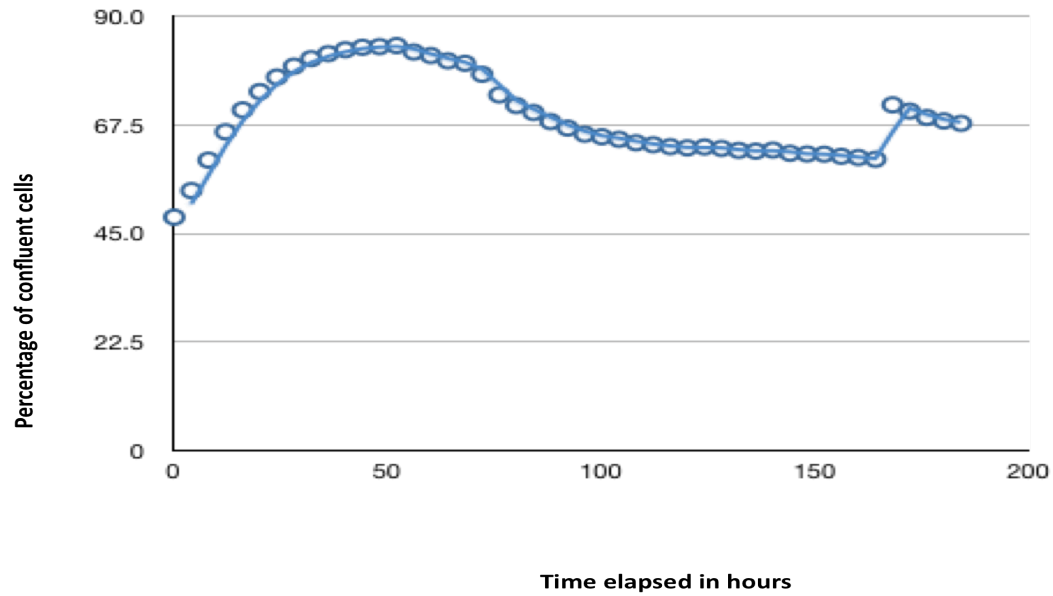
13a

Transfection of SiRNA 6ul concentration into PC3 cells



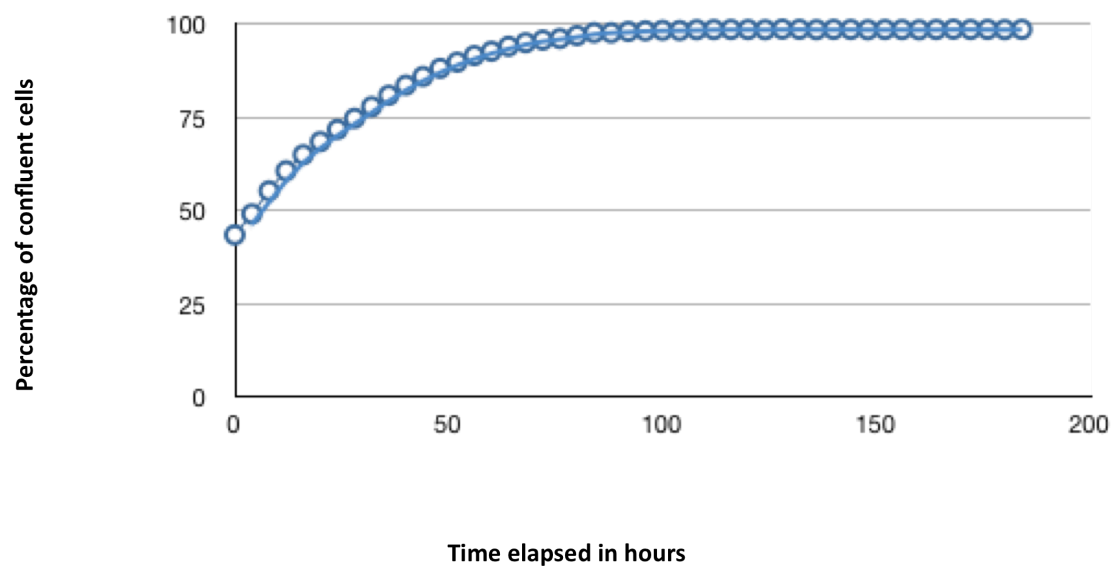
13b

Transfection of SiRNA 12ul concentration into PC3 cells



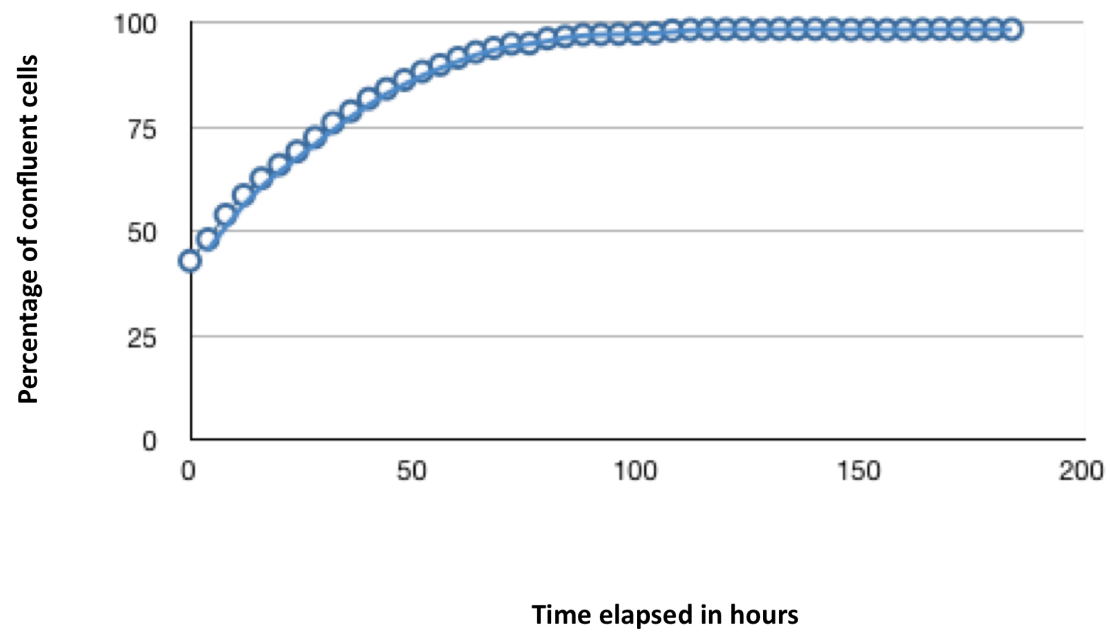
13c

Transfection of PBS control into PC3 cells



13d

Transfection of water control into PC3 cells



2b: Transfection of candidate microRNA into cell lines and assessment of plexin B1 mRNA expression by qRT-PCR

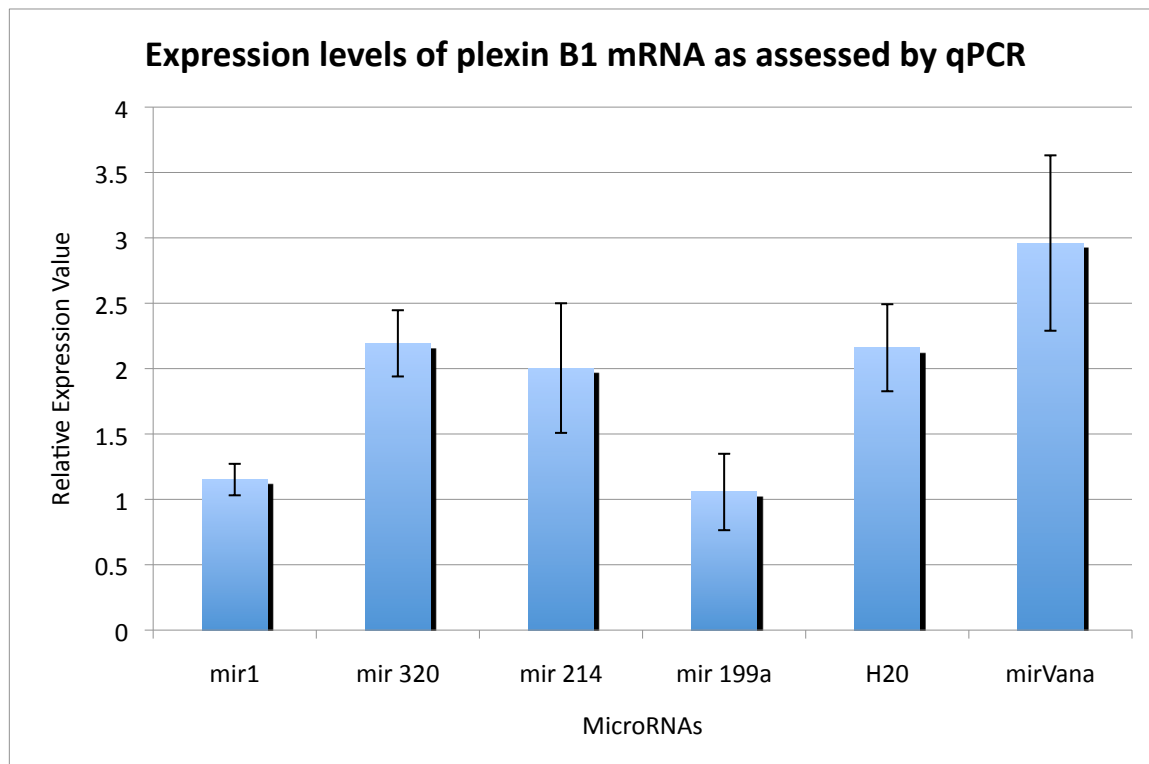
I then went on to transfect PC3 cells with the candidate microRNAs to confirm the interacts noted in section one in a different cell line. These experiments were designed to assess the effect of expression of the miRNAs on the levels of endogenous plexinB1 in prostate cancer cell lines and to determine if the candidate miRNAs had an indirect effect on plexinB1 expression levels in addition to their direct effects (ie by binding to the 3'UTR of plexinB1). Indirect effects on the mRNA levels of plexinB1 would not be detected by the LUC-3'UTR reporter construct studies. MiRNAs have been shown to both inhibit mRNA translation into protein or to result in degradation of the mRNA, depending on cellular context. Both mechanisms would be detected in the experiments using the reporter construct and luciferase activity. However only the miRNA-mediated mechanism resulting in mRNA degradation would be detected by monitoring changes in mRNA levels.

Transfection reactions were as per the Qiagen transfection protocol created from the protocol database using HiPerfect transfection reagent. The RNA was then extracted and after first strand cDNA synthesis was done using invitrogen superscript III protocol, qPCR performed as described in the methodology in the second chapter.

I performed three sets of experiments and in each experiment carried out duplicate reactions in each experiment. The results of the average of these experiments are shown in the graph below.

Graph 14: The average of 3 separate plexin B1 mRNA expression levels as assessed by qPCR after transfection of microRNAs in PC3 cells

The graph shows the average of the relative expression levels of plexin B1 mRNA after transfection with four microRNAs and two controls water and the scrambled microRNA (mirVana) in PC3 cells as assessed by qRT-PCR



The qRT-PCR results showed a down regulation of plexin B1 in mir 199a-5p, mir 1 and to a lesser extent mir 214-3p compared to water and the scrambled microRNA mirVana. There was no difference between the average results of transfected mir 320d compared to water and scrambled microRNA. The downward regulation of plexin B1 expression by microRNA 1 in keeping with the transcriptome array results. Mir 199a-5p appears to have a greater negative regulatory effect than mir1. Either miRNA 1 or 199a-5p may have may have clinical utility if their effects are verified and it is considered as a therapeutic option.

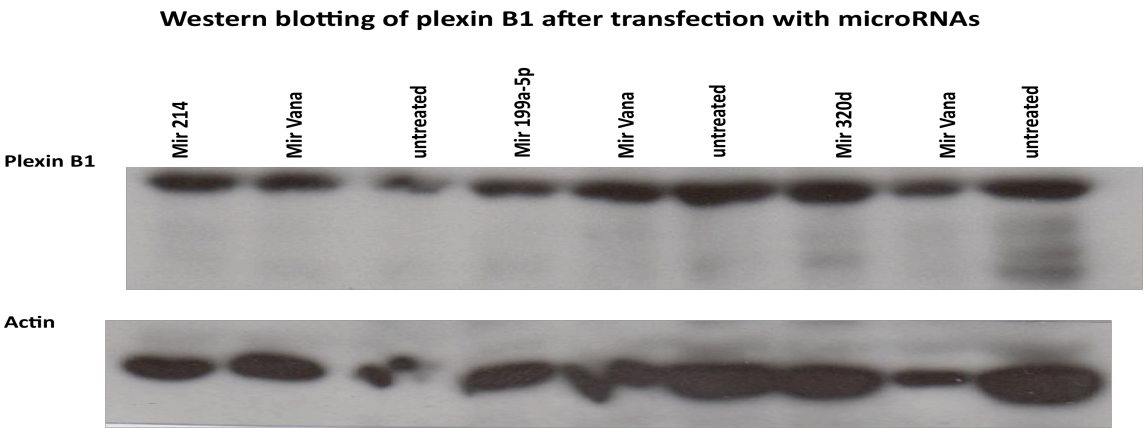
2c: Transfection of candidate microRNA into cell lines and assessment of plexin B1 protein expression by western blotting

In order to clarify the findings from the qRT-PCR and co-transfection studies I performed another set of transfection investigations this time assessing the plexin B1 protein expression levels. I used the LNCaP cells as hosts for transfections in these set of experiments as they have the higher amounts of endogenous plexin B1. I repeated this test until I obtained 3 satisfactory Western blots, as seen below in figure 24.

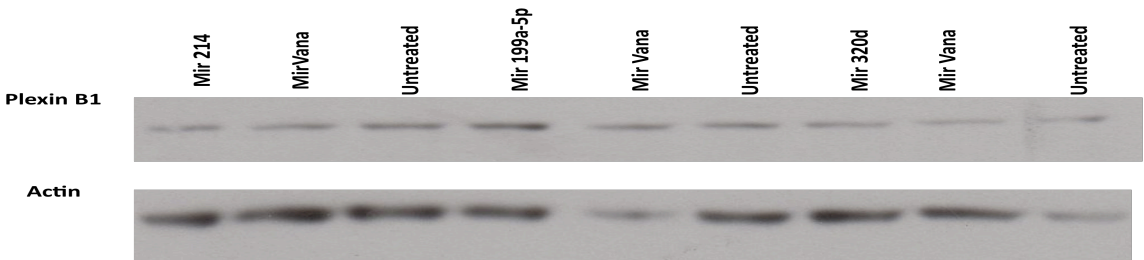
Figure 24: Western blots of plexin B1 protein after transfection of microRNAs

24a and 24b show images of western blottings of LNCaP cells transfected with a series of microRNAs with plexin B1 protein on the upper section and actin control on the lower section of each consecutive image.

24a



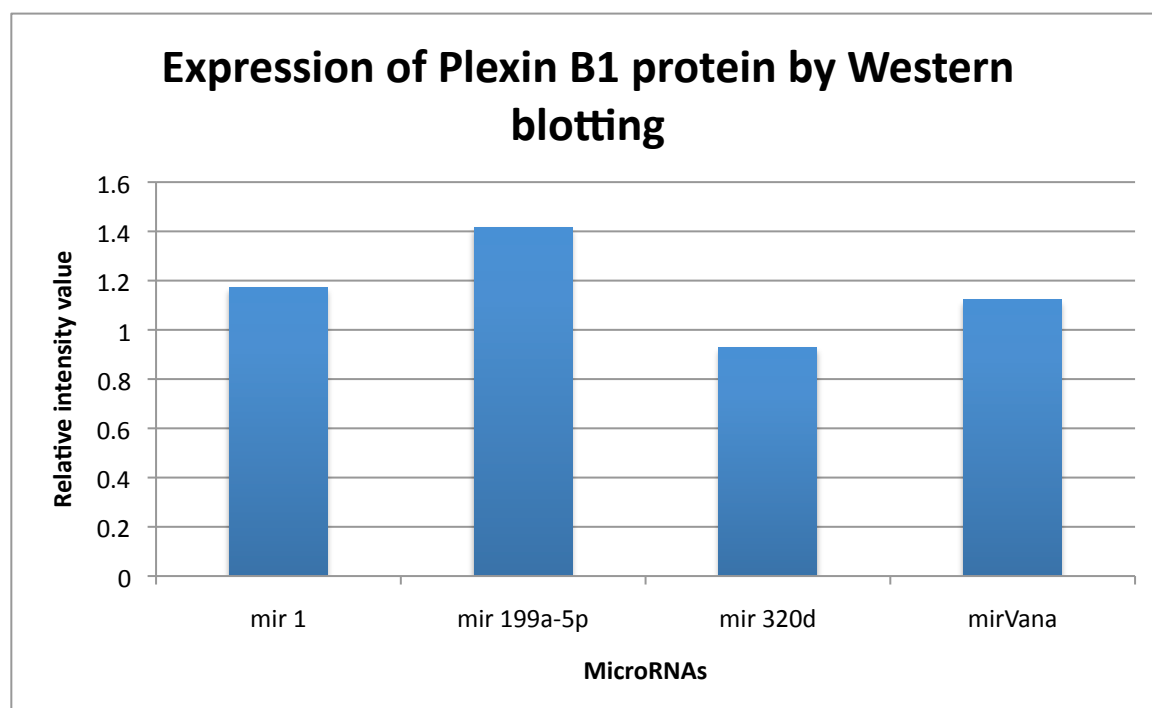
24b



I used the ImageJ programme to measure the intensity of plexin B1 Western blot bands relative to actin control for each of the microRNAs transfected. This was to obtain a more accurate value of the level of plexin B1 protein expressed. It involved measuring the intensity of both plexin and actin bands; the ratio of the intensity of plexin to actin bands was then calculated, I then normalised this ratio with the control. I found that there was a variation in the values depending on the transfected microRNA. I took the average of the best three Western blots studies to further increase the overall accuracy of the Western blots as shown in the graphs below.

[Graph 15: The average of 3 separate plexin B1 protein expression levels by Western blotting after transfection of microRNAs in PC3 cells](#)

The graph shows the average of the plexin B1 protein levels after transfection of microRNAs in LNCaP cells as obtained by Western blotting and measured using the ImageJ software.



The results of the Western blot studies as shown by graph 15 suggests mir 320d is the only negative regulator of plexin B1 protein. It is difficult to draw any conclusions because my Western blots are at best semi quantitative and not accurate either the qRT-PCR used for the plexin B1 mRNA analysis or the luciferase reporter construct and luminometer used for the co transfection.

Discussion

The results of this section does suggest that plexin B1 expression may be regulated by microRNAs. I investigated five different microRNAs that according to electronic databases and my previous transcriptome results potentially regulated plexin B1 levels. The luciferase-reporter cotransfection studies revealed that plexin B1 was negatively regulated by miR 214-3p and it was a dose response relationship. I was able to assess the effect of mir 214-3p more accurately by performing study in triplicates. As this co-transfection study assesses the control of the reporter gene by the 3'UTR of plexinB1, resulting from a direct interaction of the microRNA with the 3'UTR section of plexin B1. It would appear that mir 214-3p is a direct regulator of plexin B1. The other microRNAs to show a negative regulatory effect on plexin B1 in this LUC-3"UTR miRNA co-transfection study was mir 199a-5p although this was a single experiment not a triplicate study as with mir 214-3p. It is important to note at this point that according to my transcriptome array studies, which measure the effect of miRNAs on endogenous plexinB1 levels in Hela cells, mir 214-3p was predicted to positively regulate plexin B1.

MiRNA-214-3p may have a dual effect on plexinB1 expression – it may act indirectly to enhance plexinB1 expression (as shown in the transcriptome studies in Hela and in PC3 cells) and to directly inhibit expression (as shown in the LUC-3'UTR reporter gene studies), and the net effect is due to a balance between these two effects. The other microRNAs, mir 101, mir 320d and mir 1 did not negatively regulate plexin B1, but again these were single experiments. It would not be surprising if these other microRNAs either bind to other regions of the plexinB1 mRNA not represented in the LUC-3'UTR construct, or were indirect regulators of plexinB1 expression (for example by inhibiting an inhibitor of plexinB1 expression).

The next set of studies, the transfection control studies, showed very clearly that transfections occurred with the set of reagents being used following the prescribed protocol. Armed with this information I went on to transfect the candidate microRNAs and assessed the level of plexin B1 mRNA using qRT-PCR and protein levels using western blotting. The qPCR results confirmed the transcriptome experiments showing the negative regulatory effect of miR 1. Mir 214-3p was also a negative regulator of plexin B1 mRNA like the co transfection studies but again unlike the transcriptome array results. Finally the mir 199a-5p had the largest negative regulator effect, and this negative regulator effect was again in keeping with the co transfection studies. The mir 320d did was not found to negatively regulate plexin B1 mRNA in the qRT-PCR studies.

The results of the Western blots were at variance with the other studies and this is possibly due to a variety of reasons. Western blots are known to have many short-

comings (Taylor et al., 2013, Murphy and Lamb, 2013). Although I optimised my reactions and used the right controls and antibodies there were still a varied level of image quality. The Western blot though being practiced for many years still requires a high level of expertise. I feel that with further experiments the results may be different.

Another point of caution in the overall picture of this chapter is that on reflection I did not use a positive control such as mir 214-5p. Mir214-5p unlike mir 214-3p, has been shown to negatively regulate plexin B1 in cervical cancer cells as reported by Qiang (Qiang et al., 2011). To obtain a more accurate reading a known negative regulator such as mir214-5p should have been used to validate the results of my studies.

Therefore a repeat of the studies in this chapter including mir 214-5p is a suggestion for future studies especially as it was also found to be a negative regulator in my transcriptome studies in the fifth chapter.

Chapter 7

Discussion

Discussion

This section is divided into the following sections

- I. Review and significance of this study
- II. Future work

Review and significance of this study

Plexin B1 is the main receptor for Semphorin 4 D and this receptor- ligand complex have important functions in the body from the development of the nervous system where they act as guidance cues to developing neurones, to the immune system where it activates B cells and semaphorin 4D is also known as CD100 an important cell surface marker. While the role of plexin B1 in prostate cancer has become clearer with the increased expression of plexin B1 protein and plexin B1 mutations in prostate cancer the reason for the upregulation of plexin B1 has yet to be elucidated.

I therefore set out to discover the possible mechanism for this increased expression of plexin B1 to investigate what regulatory mechanisms may exist that leads to this upregulation in its expression. I hypothesized that a possible mechanism of the regulation of plexin B1 expression is due to microRNAs, the ubiquitous small RNA molecules that were discovered in the early 1990s and have grown in significance as the scope and importance of their functions have been revealed. I hypothesized that plexin B1 was upregulated either due to a lack of binding sites to the regulating microRNAs or a lack of these microRNAs themselves.

To set out to answer my questions I first carried out experiment in chapter 3 to confirm the existence of plexin B1 in a chosen set of cancer cell lines. I was able to show that plexin B1 was expressed in all the cell lines including PC3/27, DU145/71, Yusik10, GCT27/75, 22RV1/5, 1542NP/51 and LNCaP/73 by RT-PCR and I was also able to accurately quantify the relative levels of expression of plexin B1 in these cell lines using qRT-PCR. I found that plexin B1 is most abundant in 1542CP cells. LNCaP-LN3, a derivative of LNCaP selected by serial transplantation of metastases in mice, had increased levels of plexinB1 mRNA relative LNCaP, suggesting that the increase in expression may be associated with metastasis. LNCaP cells specifically LNCaP LN3. It is least expressed in PC3 cells. I hypothesize that the varying levels of expression as seen on qRT-PCR may be linked to the metastatic origins of the cell lines with LNCaP cell lines being derived from lymph node metastases and PC3 from bone metastases.

I then went on to investigate the variants of plexin B1 to search for variants of that lacked binding sites to microRNAs. As microRNAs bind mainly to the 3'UTR of mRNA I performed 3'RACE experiments that did not reveal any plexin B1 variants lacking in the 3'UTR section. Thus I did not find any variants that would be resistant to complementary binding effects of microRNAs that possibly regulate plexin B1 expression through that common mechanism. This is not to say plexin B1 cannot be regulated by binding to other sites along the main regions of its mRNA or even indirectly regulated. In terms of other possible binding sites for microRNAs there are two variants of plexin B1 shown by the NCBI database to lack exon 11, variant R and the truncated variant of plexin B1. I was able to show the existence of these two by performing RT-PCR using primers for

exon 11 of plexin B1. Of the two variants the truncated variant has been shown to exist in literature by Tamagnone (Tamagnone et al., 1999) while this I believe is the first time that variant R was identified through invitro analysis outside of the online databases. The NCBI attributes the Tamagnone paper with showing variant R but on review of the paper only the truncated variant is mentioned. This finding has significance not just in terms of a binding site to microRNAs using the main body of mRNA route but also the fact that this variant may have altered functions that needs to be explored. The truncated variant is predicted to produce a small extracellular protein with a sema domain and a novel 52 amino acid sequence. This is predicted to bind to sema4D and full length plexinB1 protein and could act as a dominant negative inhibitor of plexin signaling. The R variant has a mucin-like domain missing and is predicted to have altered function. All cell lines tested expressed both variants with a much higher abundance of the truncated version than the variant R at the mRNA level.

I then went on as stated in the fourth chapter to create a plasmid vector construct containing the 3'UTR of plexin B1 which I would then use to confirm that this section of plexin B1 is active through transfection studies by comparing the vector construct containing the 3'UTR of plexin B1 to control vector. Lower levels of reporter gene activity was seen in cells transfected with the LUC-3'UTR construct compared to the LUC-control vector, showing that the 3'UTR exerts some repression on reporter gene expression. I then performed transcriptome array experiments, which revealed plexin B1 mRNA was negatively regulated by mir 1 and to a less significant level by mir 214-5p as well as mir 206. I selected a number of candidate miRNAs from these studies and from

analysis of potential binding sites in the DNA sequence of the 3'UTR of plexinB1 for further analysis. I transfected 5 candidate microRNAs together with the LUC-3'UTR vector construct and assessed the level of reporter gene expression using a luminometer to detect the luciferase reporter activity. I also performed qRT-PCR and Western blots to determine the effect of the candidate miRNA on expression of endogenous plexinB1 mRNA and protein in prostate cancer cells.

In summary, my results indicate three potential miRNA candidates for regulation of plexinB1 expression:

MiRNA199a negatively regulated expression of the LUC-3'UTR reporter, suggesting a direct effect on plexinB1 gene expression, and decreased endogenous levels of plexinB1 mRNA in prostate cancer cells. It had little effect in the transcriptome studies which assessed endogenous levels of plexinB1 in Hela cells. MiRNA199a may therefore have both a direct effect of plexinB1 expression and indirect effects which are cell context specific.

MiRNA 1 repressed endogenous expression of plexinB1 in both Hela and PC3 cells, but had little effect on the LUC-3'UTR reporter assay. Hence miRNA 1 may have indirect effects on endogenous plexinB1 expression but no direct effect via binding to the 3'UTR. This is consistent with the lack of obvious miRNA binding sites in the 3'UTR of plexinB1. Alternatively the miRNA 1 may bind to a region of the plexinB1 mRNA not cloned into the LUC-3'UTR vector used in these experiments.

MiRNA 214-3p positively regulated expression of plexinB1 in Hela cells, although the increase was not significant, and like miRNA 199a, negatively regulated LUC- 3'UTR construct and endogenous plexinB1 in PC3 cells. Mir 214-3p may, like miRNA 199a, have direct effects on plexinB1 expression by binding to the 3'UTR of plexinB1, as well as indirect effects that are specific to the type of cell.

To make any firm conclusions there is a need for a positive control to validation of my transfection work, I suggest mir 214-5p again as explained in chapter 6.

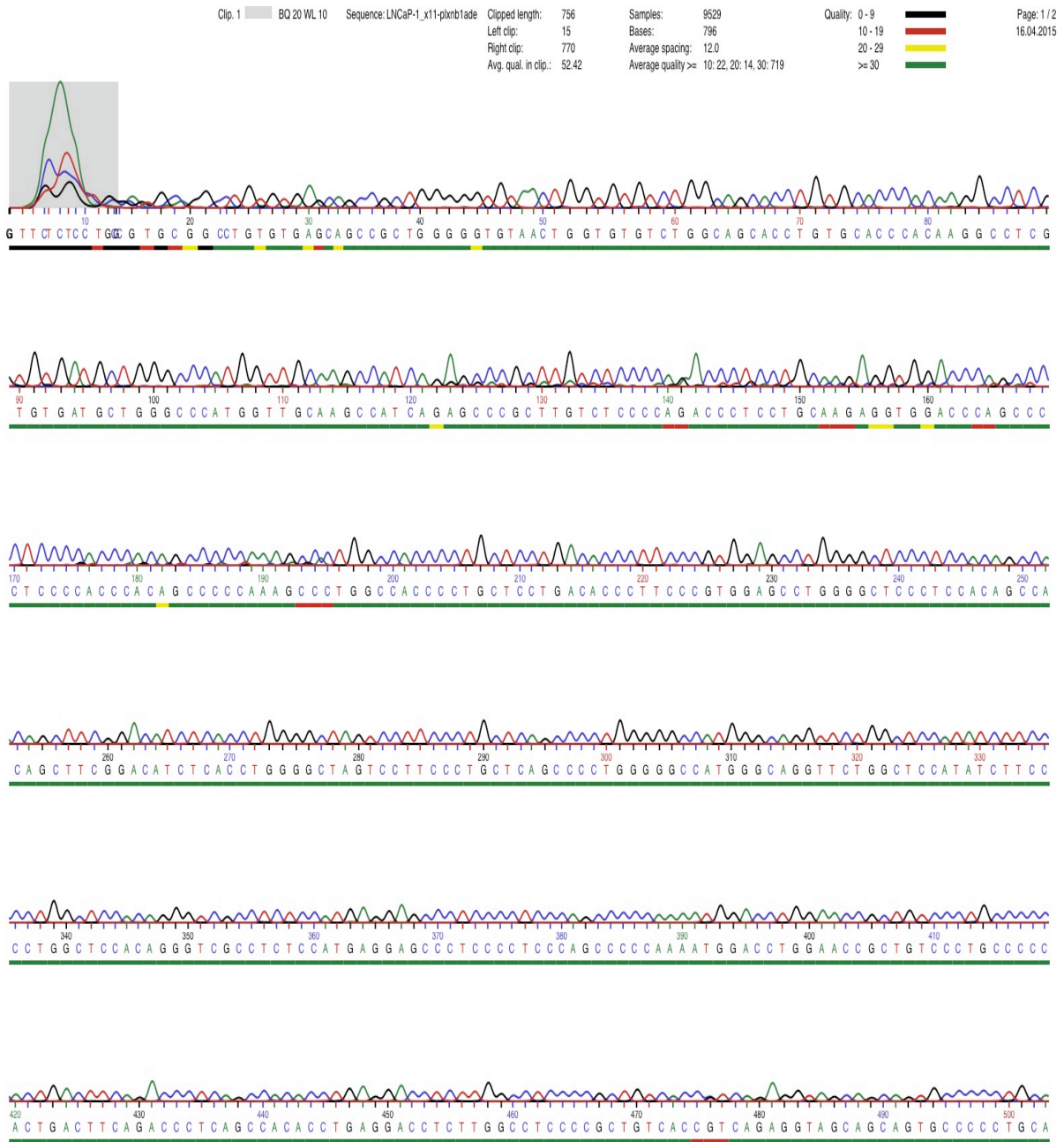
Future work

There is a lot of scope for further work in this study. Once the results are validated, the next stage should be tissue studies evaluating the levels of these candidate microRNAs relative to plexin B1 looking especially for an inverse relationship. I expect an inverse relationship, with plexin B1 levels increasing with distance metastasis compared to the primary prostate tumour, while the candidate microRNAs levels should decrease in distant metastasis compared to the primary prostate tumour. This would be assessed by Insitu hybridization for the microRNA levels in tissue and immunohistochemistry for the plexin B1 protein levels in tissue. The LUC-3'UTR experiments could be validated by mutation of the postulated miRNA binding sites in the construct. Further evidence for the role of the candidate miRNAs in regulation of plexinB1 mRNA expression could be gained by expressing anti-MIRS into PC3 cells; this is predicted to increase expression of plexinB1.

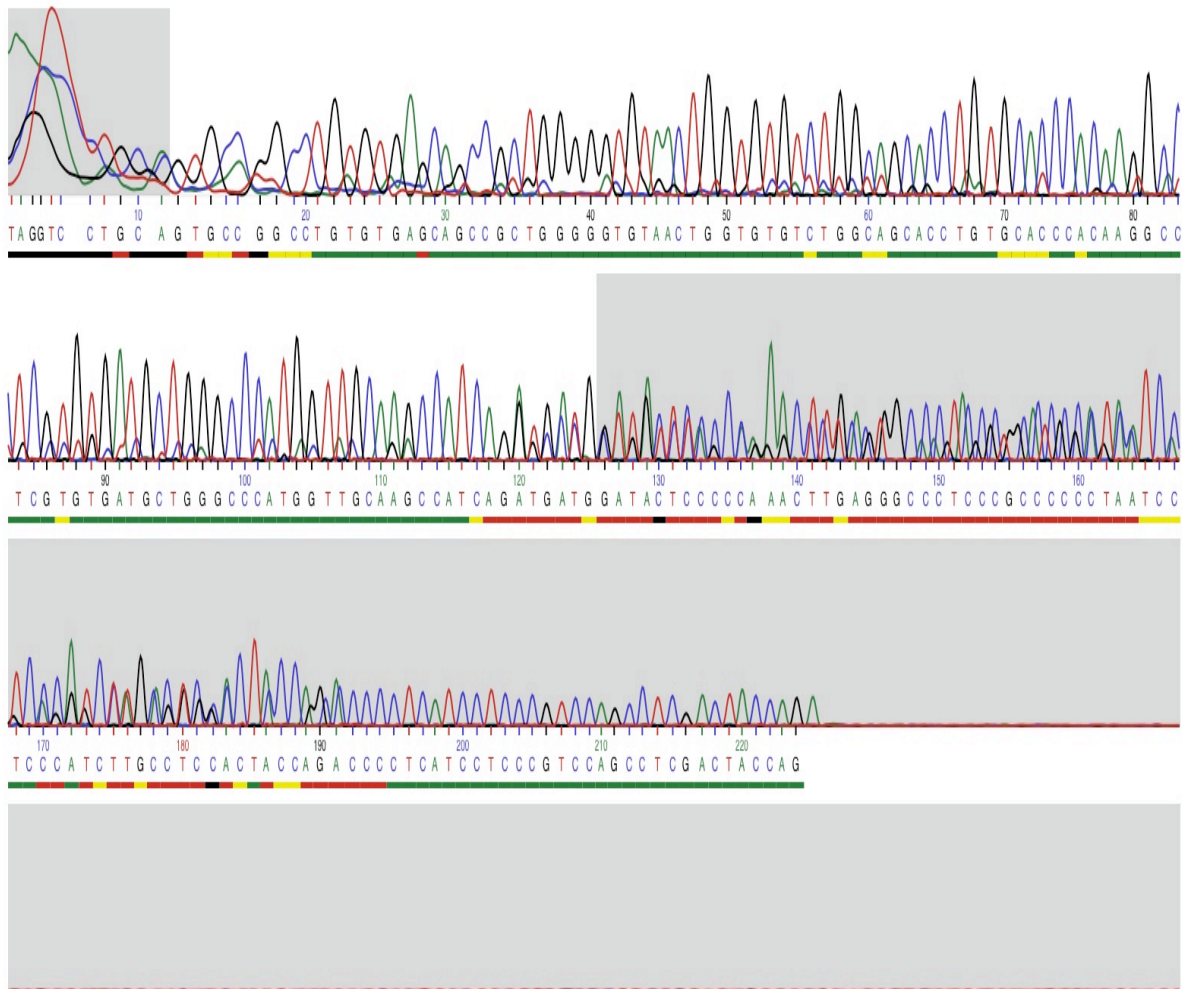
If the results are in keeping with my preliminary findings then the obvious next step would be to test out the candidate microRNAs for the therapeutic potential in the subset of prostate cancer associated with plexin B1. Preliminary mice studies being carried out by our group, which are yet to be published suggest that plexin B1 also affects primary prostate cancer formation in mouse models of prostate cancer as mice deficient of plexin B1 in their prostate show delayed development of primary tumours as compared to the same mouse models with the plexin B1 gene intact (personal communication). This adds further impetus to discovering pharmaceutical agents that can block plexin B1 in the prostate. My hope is that microRNAs could be one of these therapeutic agents and perhaps some of the candidate microRNAs I have identified.

Appendix

An example is shown below of the nucleotide sequences of the ~800bp, ~250bp and ~200bp bands from the cell line LNCaP. Electropherograms are shown below.



Clip: 1	BQ 20 WL 10	Sequence: LNCaP-2_x11-plxmb1ade	Clipped length: 114	Samples: 12961	Quality: 0 - 9	Page: 1 / 3
			Left clip: 12	Bases: 225	10 - 19	16.04.2015
			Right clip: 125	Average spacing: 58.0	20 - 29	
			Avg. qual. in clip.: 38.3	Average quality >=	>= 30	



Clip: 1 BQ 20 WL 10

Sequence: LNCaP-3_x11-plx1b1ade

Clipped length: 180

Samples: 12965

Quality: 0 - 9

Page: 1/3

Left clip: 12

Bases: 191

10 - 19

16.04.2015

Right clip: 191

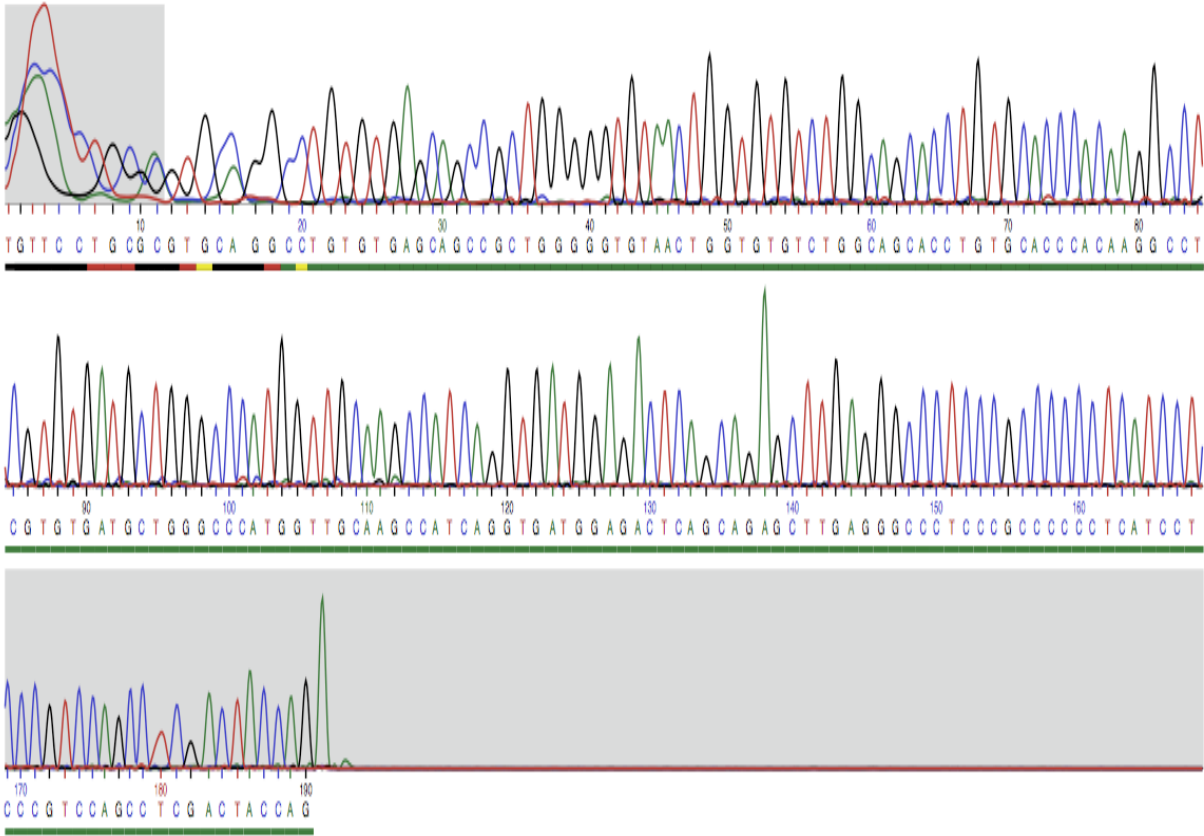
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20 - 29

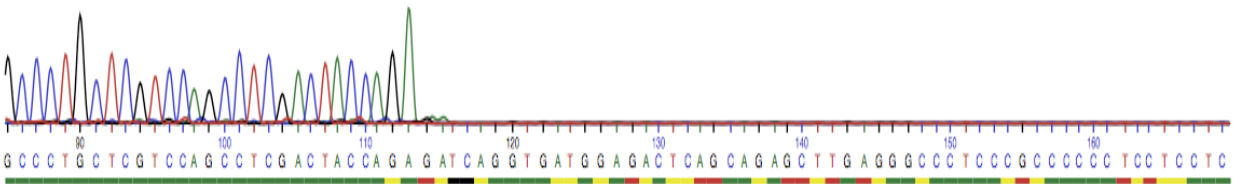
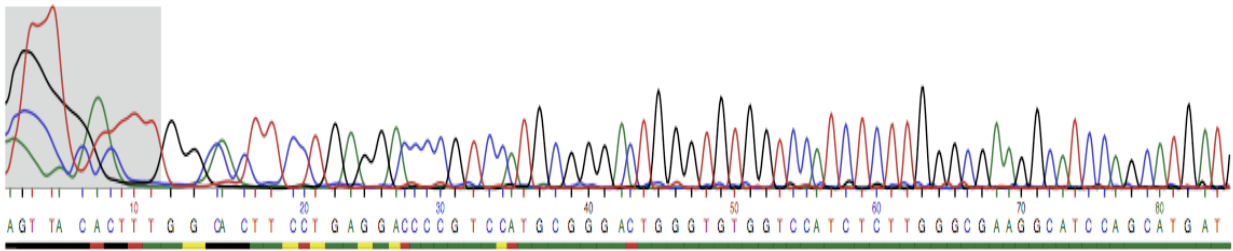
Avg. qual. in clip: 56.77

Average quality >= 10: 5; 20: 3; 30: 171

>= 30



Clip: 1 BQ 20 WL 10 Sequence: LNCaP-5_x11-plxmb1ade Clipped length: 168 Samples: 12962 Quality: 0 - 9 Page: 1 / 3
 Left clip: 12 Bases: 196 10 - 19 16.04.2015
 Right clip: 179 Average spacing: 67.0 20 - 29
 Avg. qual. in clip.: 42.02 Average quality >= 10: 29, 20: 30, 30: 122 >= 30



170
 C C G T C C A G C C T C T A C T A C C A G A G A T C
 180
 190

The full list of the 90 cancer specific microRNAs used in the 96 well transcriptome array

Position	Symbol	Description
A01	hsa-let-7a	MIMAT0000062
A02	hsa-miR-133b	MIMAT0000770
A03	hsa-miR-122	MIMAT0000421
A04	hsa-miR-20b	MIMAT0001413
A05	hsa-miR-335	MIMAT0000765
A06	hsa-miR-196a	MIMAT0000226
A07	hsa-miR-125a-5p	MIMAT0000443
A08	hsa-miR-142-5p	MIMAT0000433
A09	hsa-miR-96	MIMAT0000095
A10	hsa-miR-222	MIMAT0000279
A11	hsa-miR-148b	MIMAT0000759
A12	hsa-miR-92a	MIMAT0000092
B01	hsa-miR-184	MIMAT0000454
B02	hsa-miR-214	MIMAT0000271
B03	hsa-miR-15a	MIMAT0000068
B04	hsa-miR-18b	MIMAT0001412
B05	hsa-miR-378	MIMAT0000732
B06	hsa-let-7b	MIMAT0000063
B07	hsa-miR-205	MIMAT0000266
B08	hsa-miR-181a	MIMAT0000256

B09	hsa-miR-130a	MIMAT0000425
B10	hsa-miR-199a-3p	MIMAT0000232
B11	hsa-miR-140-5p	MIMAT0000431
B12	hsa-miR-20a	MIMAT0000075
C01	hsa-miR-146b-5p	MIMAT0002809
C02	hsa-miR-132	MIMAT0000426
C03	hsa-miR-193b	MIMAT0002819
C04	hsa-miR-183	MIMAT0000261
C05	hsa-miR-34c-5p	MIMAT0000686
C06	hsa-miR-30c	MIMAT0000244
C07	hsa-miR-148a	MIMAT0000243
C08	hsa-miR-134	MIMAT0000447
C09	hsa-let-7g	MIMAT0000414
C10	hsa-miR-138	MIMAT0000430
C11	hsa-miR-373	MIMAT0000726
C12	hsa-let-7c	MIMAT0000064
D01	hsa-let-7e	MIMAT0000066
D02	hsa-miR-218	MIMAT0000275
D03	hsa-miR-29b	MIMAT0000100
D04	hsa-miR-146a	MIMAT0000449
D05	hsa-miR-212	MIMAT0000269
D06	hsa-miR-135b	MIMAT0000758
D07	hsa-miR-206	MIMAT0000462
D08	hsa-miR-124	MIMAT0000422
D09	hsa-miR-21	MIMAT0000076
D10	hsa-miR-181d	MIMAT0002821

D11	hsa-miR-301a	MIMAT0000688
D12	hsa-miR-200c	MIMAT0000617
E01	hsa-miR-100	MIMAT0000098
E02	hsa-miR-10b	MIMAT0000254
E03	hsa-miR-155	MIMAT0000646
E04	hsa-miR-1	MIMAT0000416
E05	hsa-miR-363	MIMAT0000707
E06	hsa-miR-150	MIMAT0000451
E07	hsa-let-7i	MIMAT0000415
E08	hsa-miR-27b	MIMAT0000419
E09	hsa-miR-7	MIMAT0000252
E10	hsa-miR-127-5p	MIMAT0004604
E11	hsa-miR-29a	MIMAT0000086
E12	hsa-miR-191	MIMAT0000440
F01	hsa-let-7d	MIMAT0000065
F02	hsa-miR-9	MIMAT0000441
F03	hsa-let-7f	MIMAT0000067
F04	hsa-miR-10a	MIMAT0000253
F05	hsa-miR-181b	MIMAT0000257
F06	hsa-miR-15b	MIMAT0000417
F07	hsa-miR-16	MIMAT0000069
F08	hsa-miR-210	MIMAT0000267
F09	hsa-miR-17	MIMAT0000070
F10	hsa-miR-98	MIMAT0000096

F11	hsa-miR-34a	MIMAT0000255
F12	hsa-miR-25	MIMAT0000081
G01	hsa-miR-144	MIMAT0000436
G02	hsa-miR-128	MIMAT0000424
G03	hsa-miR-143	MIMAT0000435
G04	hsa-miR-215	MIMAT0000272
G05	hsa-miR-19a	MIMAT0000073
G06	hsa-miR-193a-5p	MIMAT0004614
G07	hsa-miR-18a	MIMAT0000072
G08	hsa-miR-125b	MIMAT0000423
G09	hsa-miR-126	MIMAT0000445
G10	hsa-miR-27a	MIMAT0000084
G11	hsa-miR-372	MIMAT0000724
G12	hsa-miR-149	MIMAT0000450
H01	hsa-miR-23b	MIMAT0000418
H02	hsa-miR-203	MIMAT0000264
H03	hsa-miR-32	MIMAT0000090
H04	hsa-miR-181c	MIMAT0000258
H05	hsa-miR-20b*	MIMAT0004752
H06	hsa-miR-214*	MIMAT0004564
H07	VTC	Negative Control
H08	VTC	Negative Control
H09	VTC	Negative Control
H10	VTC	Negative Control
H11	PPC	SA_00104
H12	PPC	SA_00104

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