IDENTIFICATION OF BIOMARKERS FOR DEVELOPMENT OF NF1-ASSOCIATED MALIGNANT PERIPHERAL NERVE SHEATH TUMOURS

Ellie Rad

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of Doctor of Philosophy

I dedicate this work to Rienk Doetjes

Always loved, never forgotten, forever missed

PUBLICATIONS

Published

1. STAT3 and HIF1α Signaling Drives Oncogenic Cellular Phenotypes in Malignant Peripheral Nerve Sheath Tumors. **Ellie Rad**, Kayleigh Margaret Dodd, Laura Elizabeth Thomas, Meena Upadhyaya and Andrew Robert Tee. "Molecular Cancer Research 13(7):1149-60, 2015".

2. Evaluation of copy number variation and gene expression in neurofibromatosis type-1-associated malignant peripheral nerve sheath tumours. Laura E Thomas, Jincy Winston, **Ellie Rad**, Matthew Mort, Kayleigh M Dodd, Andrew R Tee, Fionnuala McDyer, Stephen Moore, David N Cooper and Meena Upadhyaya "Human Genomics 9(1):3, 2015".

3. Neurofibromatosis Type 1: fundamental insights into cell signalling and cancer. **Ellie Rad** and Andrew Tee. "Seminars in Cell and Developmental Biology pii: S1084-9521(16)30049-0, 2016".

In progress

4. Targeting the STAT3 signalling pathway as a therapeutic target in Tuberous sclerosis models. Kayleigh Dodd, **Ellie Rad,** Jens Mattern, Andrew Tee.

CONTENT

FIGURES AND TABLES

ACKNOWLEDGMENTS

SUMMARY

Therapeutic options are currently limited for Neurofibromatosis type 1 (NF1) associated-malignant peripheral nerve sheath tumours (MPNSTs). MPNSTs are characteristically aggressive and the major cause of death in NF1 patients. Clinical trials using single drug agents to treat MPNSTs have so far been unsuccessful, which could be attributed to high levels of intra-tumoural molecular heterogeneity. To explore common cellular migratory and invasive signalling properties within the heterogeneous NF1-MPNST population, we utilised four different MPNST-derived cell lines, ST8814, S462, S1844.1 and S1507.2.

MET has previously been shown to be elevated in MPNST cells and is thought to promote their cellular migration and invasion. Interestingly, we report variation in MET gene expression and protein levels in a variety of MPNST derived cell lines. MET inhibitors were effective at suppressing the migration and invasion of cell lines with elevated MET protein levels but not those without. Importantly, targeted inhibition of STAT3 suppressed cell migration, invasion and tumour formation in all cell lines tested, regardless of MET expression levels. Herein, we demonstrate that STAT3 functions as a common driver of tumourigenesis in multiple NF1-MPNST cell lines with varying signalling profiles. STAT3 is activated downstream of a variety of receptor tyrosine kinases which are associated with NF-1-tumourigenesis, including MET, IL-6 and EGFR, making it an appealing therapeutic target for the heterogeneous NF1-MPNST population. We also demonstrate that cellular migration, invasion and tumour formation through STAT3 is highly dependent on the transcription factor HIF-1α, where knockdown of HIF-1α ablated these oncogenic facets of STAT3. Our research demonstrates that aberrant signalling through STAT3 and HIF-1α drives tumour progression within MPNSTs, indicating that inhibition of the STAT3/HIF-1α /VEGF-A signalling axis could be a viable therapeutic strategy in this context.

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ABBREVIATIONS

3'UTR	3' untranslated region
5'UTR	5' untranslated region
AKT	Protein kinase B
AP2	Activator protein-2
Bcl2	B-cell lymphoma 2
cAMP	cyclic adenosine 3',5'-monophosphate
CNAs	Copy number alterations
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor
CRE	Response element
CSRD	N-terminus cysteine/serine-rich domain
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptors
ERK1	Extracellular signal-regulated kinases 2
ERK2	Extracellular signal-regulated kinases 2
Ets	E-twenty six
FTI	Family (Farnesyl protein transferase inhibitor
G protein	GTP-Binding proteins
GAB1	Adaptor protein GRB2-associated binding protein 1
GAP	GTPase activating protein
GDP	Guanosine diphosphate
GEFs	guanine nucleotide exchange factors
GPCRsG	Protein coupled receptors
GRB2	Growth factor receptor-bound protein 2
GRD	GTPase activation protein related domain
GRDs	GAP-related domains
GTP	Guanosine-5'-triphosphate

HBM	Recurrent glioblastoma
HGF	Hepatocyte growth factor
HIF-1α	Hypoxia-inducible factor 1-alpha
HIF-1β	Hypoxia-inducible factor 1- beta
HIF-2α	Hypoxia-inducible factor 2-alpha
HIFs	Hypoxia-inducible factors
HREs	Hypoxia response elements
HSP70	70 kilodalton heat shock proteins
HSP90	Heat shock protein 90
IDPs	Intrinsically Disordered Proteins
IGF-IR	insulin-like growth factor I-receptor
IHC	Immunohistochemistry assays
IL-11	Interlukin-11
IL-6	Interlukin-6
IPT	Immunoglobulin–plexin–transcription
IR	Insulin receptor family
JAK2	Janus-activated kinase-2
JAKs	Janus-activated kinase family kinases
LIF	leukemia inhibitory factor
MAPKs	Mitogen-activated protein kinases
MET	Proto-oncogene MET
mLST8	Mammalian lethal with SEC13 protein 8
MMPs	Matrix Metalloproteinases
MPNST	Malignant Peripheral Nerve Sheath Tumour
mRNA	Messenger RNA
mTOR	Mechanistic target of rapamycin
mTORC1	Mechanistic target of rapamycin complex 1
mTORC2	Mechanistic target of rapamycin complex 2
NF	Neurofibromatoses
NF1	Neurofibromatosis type 1
NF2	Neurofibromatosis type 2

NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLS-CTD	Nuclear localization sequence
ODDD	Oxygen dependent degradation domain
OOS	overall survival
OPG	Optic Pathway Glioma
SPP1	Osteopontin
PABP	poly-A binding protein
Pax3	paired box 3
PDGF	Platelet-derived growth factor
PDGFB	Platelet-derived growth factor subunit B
PDGFRA	Alpha-type platelet-derived growth factor receptor
PDK1	3-phosphoinositide-dependent protein kinase-1
PFS	Progression-free survival
PHD2	Prolyl hydroxylase-domain enzyme 2
PHDs	Dependent prolyl hydroxylases
PI3Ks	Type I phosphatidylinositol 3-kinases
PIGF	Placenta growth factor
PKC	protein kinase C
PTEN	Phosphatase and tensin homolog
PTPs	Protein tyrosine phosphatases
RALGDS	RAL guanine nucleotide dissociation stimulator
Raptor	Regulatory-associated protein of mTOR
RNA	Ribonucleic acid
RTK	tyrosine kinases
SF	scatter factor
SH2	Src homology 2 domain
SH2	Src-homology-2
SHC	Src homology-2-containing
SOS	Son of Sevenless
SPP1	secreted phosphoprotein 1
SS	splice site

STAT3	Signal transducer and activator of transcription 3
STATs	Signal Transducer and Activator of Transcription
TAD	Transcriptional activation domain
Tcf-4	Transcription factor 4
TPA	12-O-tetradecanoyl-phorbol-13-acetate
TPR	Translocated promoter region
TSS	Transcription start site VEGF
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor Receptor
VHL	Von Hippel-Lindau tumour suppressor protein
VPF	Vascular permeability factor

1   Chapter 1: General introduction

The neurofibromatoses (NF) refer to a group of inherited autosomal dominant disorders that prompt tumour formation, predominantly within the nerve sheath, which results in a profound impact on the nervous system. There are currently three recognised types of neurofibromatoses: Neurofibromatosis type 1 (NF1) (MIM_162200), Neurofibromatosis type 2 (NF2) (MIM_101000) and Schwannomatosis (MIM_162991) (SCH) which share a common neuronal origin of tumour formation. Furthermore, in addition to tumour formation, NF1 has a pigmentary component while the clinical manifestations of NF2 are mainly associated with the nervous systems. There are several signalling pathways that play a role in cellular growth and proliferation in various cancers. In this study the aim is to identify which of these pathways are involved in the transformation of benign tumours in to malignant peripheral nerve sheath tumour (MPNSTs). The main focus of this study will be on investigating protein signalling pathways involved in invasion, migration, metastasis and tumour progression in MPNST cell lines. A combination of small molecule inhibitors and targeted shRNA knockdown of cell components will be used to block specific protein pathways in order to better understand the aggressive behaviour of MPNSTs.

1.1   Neurofibromatosis Type 1 (NF1)

Neurofibromatosis type 1 (NF1) (MIM 162200) is a complex neurocutaneous, dominantly inherited, autosomal disorder affecting multiple systems of the body. The descriptions of individuals with NF1 have been documented through history going back as far as the thirteenth century (Table 1). NF1 became synonymous with von Recklinghausen's disease after Friedrich Daniel Von Recklinghausen (1833-1911) gave a classic description of Neurofibromatosis in 1882 (Crump 1981).

The *NF1* gene has a high mutation rate, approximately one mutation occurring per 10,000 gametes per generation (100 times greater than many other genes); in individuals effected, approximately 400 mutations have been observed leading to protein truncation and

abnormalities. Furthermore in 30-50% of NF1 patients new mutations occur resulting in a birth incidence of approximately 1 in 3500 people and an overall occurrence worldwide of 1 in 5000 people (Evans et al. 2010; Ars et al. 2000). The high mutation rate in the *NF1* gene could possibly be due to its large size (350 kb with 60 exons).

Table 1.1. A summary of the   most important events in the history of NF1 research (Gutmann & Collins 1993; Morse 1999; Antônio et al. 2013; Ratner & Miller 2015).

Date	Description
13th Century	Sketch of a man found containing skin nodules suggesting he was a NF1 patient drawn by Heinricius a Cistercian monk.
1592	The initial clinical description of a man with a possible plexiform neurofibroma reported by Ulisse Aldrovandi an Italian Physician
1768	Mark Akenside (1721-1770) a British Physician wrote and published the first description of NF1 in English.
1793	Ludwig and W. G. Tilesius, described a patients with multiple fibrous tumours of the skin, countless growths on the skin, cafe´ au lait spots, macrocephaly, and scoliosis.
1847	Rudolf L. Virchow, reported NF1 disease in several family members.
1849	Robert William Smith cited 75 references in a NF1 literature review. He also presented two new case however he was not able to prove that the tumours had developed from fibrous connective tissue of small nerves.
1882	Friedrich Daniel von Recklinghausen, confirmed that the origin of the NF1 tumours was the nerves and gave them the term neurofibroma.
1978	National Neurofibromatosis Foundation was established this foundation is now known as the Children's Tumour Foundation.
1987	National Institutes of Health (NIH) established the classification for NF1 and also the diagnostic criteria and treatment guidelines. Baker *et al* and Seizinger et al . Mapped the *NF1* gene to a region on chromosome 17.
1990	Cawthon *et al.* Viskochil *et al.* and Wallace *et al.* mapped the *NF1* gene to 17q11.2 and preformed experiments that enabled the isolation and cloning of the *NF1* gene.
1993	Gutmann and Collins 1993; Viskochil *et al*, 1993, gave a description of the protein encoded by the *NF1* gene.
1994	Jacks *et al.* 1994; Brannan *et al.* 1994, developed Nf1-mutant mice.
1995	A direct testing for NF1 disease was made available
1997	Dr. Andre Bernards (Massachusetts General Hospital Cancer Center and Harvard Medical School, generated a Drosophila melanogaster model of NF1.
2012	Upadhyaya *et al.* Examined the number of gene copies per microarray in NF1 patients.
2014	Research on NF1 disease and its effects on patients is an ongoing process.

1.2   NF1 diagnostic and clinical features

NF1 is recognised for the variety of tissues and organs it can affect and also for its variation in disease expression within different individuals. Even amongst family members the manifestations of the disease can vary. The diagnosis of NF1 is made by the existence of two or more of the seven criteria established at the National Institutes of Health (NIH) in 1987 and further confirmed by Gutmann *et al.* (1997) (Table 2) (Ferner et al. 2007; Boyd et al. 2009; Gutmann et al. 1999; Gutmann 1997).

Table 1.2. The diagnostic criteria for NF1 (Debella et al. 2000).

Diagnosis criteria for NF1
1- Six or more café-au-lait spots (0.5 cm in children and 1.5 cm in adults)
2- Two or more neurofibromas (Cutaneous or Sub Cutaneous) or one plexiform neurofibroma
3- Axillary or groin freckling
4- Optic pathway glioma
5- Two or more Lisch nodules (iris hamartomas)
6- Bony dysplasia (sphenoid dysplasia or thinning of the long bone cortex with or without pseudarthrosis)
7- A first degree relative suffering from NF1

1.2.1   Clinical diagnostic features

1.2.1.1   Café-au-lait spots

The café-au-lait spots are hyperpigmented macules that begin to appear at a younger age in individuals with NF1 (99% of patients). They continue to increase until around two years.

This Hyperpigmentation is a result of an increased in the number of melanocytes and the existence of large melanosomes (von Deimling et al. 1995). The café-au-lait spots do not cause clinical complications in NF1 patients, however many patients feel distressed due to the appearance of these spots. Advice regarding skin camouflage is given to individuals to try and help them cope better with this feature (Ferner 2007).

1.2.1.2 Axillary freckling

Axillary freckles are smaller and tend to develop later in life than café-au-lait spots. They are usually seen in clusters that commonly form in the axillae as well as intertriginous areas of the body, for example upper eyelids, the base of the neck, under the breasts etc. Freckling is seen in 80% of children with NF1 by the age of 6 (Williams et al. 2009).

1.2.1.3 Lisch nodules

Lisch nodules are pigmented benign hamartomas within the iris that do not affect the vision. They are seen in less than 10% of patients under the age of 6, however they can be found in the majority of patients older than 10, making them a main characteristic feature of NF1 patients (Williams et al. 2009; Nichols et al. 2003).

1.2.1.4 Optic pathway glioma

The Optic Pathway Glioma (OPG) are pilocytic astrocytomas seen in 15-20% of NF1 patients and are responsible for morbidity in younger children suffering from the disease. These tumours occur on the optic nerve causing an expansion of the nerve. Even though in 1/3 of patients no associated symptoms are developed, these tumours can occasionally cause vision loss and abnormality, precocious puberty and proptosis (Williams et al. 2009).

1.2.1.5 Specific osseous lesions

Osseous lesions are congenital skeleton lesions associated with NF1 patient. These lesions can cause informalities within the spinal cord, thinning of the long bones, Pseudarthrosis or the growth of a false joint and dysplasia of the sphenoid wing. Furthermore, the development of neurofibromas can also affect the skeleton, these being tumours could cause enlargement of the intervertebral foramina. Also they could invade neighbouring skeleton structures (von Deimling et al. 1995; Williams et al. 2009).

1.2.1.6 Neurofibromas

Neurofibromas are benign peripheral nerve sheath tumours and they are one of the most common and distressing features of NF1. Neurofibromas are responsible for morbidity,

deformity and functional impairment. There are currently three clinically known neurofibromas: 1) Cutaneous (dermal) neurofibromas, 2) Subcutaneus (nodular) neurofibromas, 3) plexiform neurofibromas (Ferner et al *. 2007).

1) Cutaneous (dermal) neurofibromas, are soft nodules with a pink/red colour that are comprised of, Schwann cells, fibroblasts, perineural cells, mast cells and axons. In some cases they can cause discomfort, itchiness and disfigurement in NF1 patients. These tumours can be seen in the majority of NF1 patients, first occurring during puberty and throughout adolescence (Kimura et al. 1974; Le & Parada 2007). There is an increase in the size and number of cutaneous neurofibromas during puberty and pregnancy.

Even though there has been no response to hormonal contraceptives, the timing of cutaneous neurofibroma occurrence does suggest a hormonal role in their formation and growth (Dagalakis et al. 2014; McLaughlin & Jacks 2003). There has been no evidence regarding the transformation of cutaneous neurofibromas in to MPNSTs. At the moment there are no ultimate treatment for cutaneous neurofibromas except for surgical removal for cosmetic reasons or if they are causing the patient pain and discomfort because of their excessive size or their location. However the surgical removal of these tumours can cause scarring and the recurrence of neurofibromas after surgery. Laser surgery has been used recently as an alternative way of treatment for smaller lesions (Fox et al. 2012).

2) Subcutaneus (nodular) neurofibromas, are firm and oval shape tumours found below the skin and cause pain and neurological deficit in patients (Fox et al. 2012). Subcutaneus neurofibromas are described as superficially positioned tumours in which the skin can move freely over the lesion (Boyd et al. 2009). Malignant transformation of these tumours is very rare. Similar to the cutaneous neurofibromas, surgical removal is the only treatment available, however seeking advice from a specialist is highly recommended as the removal of these tumours can cause neurological deficits (Boyd et al. 2009; Fox et al. 2012; Jett & Friedman 2010).

3) Plexiform neurofibromas, are comprised of various cell types, including Schwann cells, local elements of supporting nerve fibres including perineural cells, neurons, fibroblasts, and blood vessels in addition to infiltrating mast cells (Ferner 2007). Usually plexiform neurofibromas are larger than other types of neurofibromas, involving several nerve fascicles, and are able to effect surrounding tissue and blood vessels (Upadhyaya et al. 2008). Plexiform neurofibromas are typically congenital (effecting 60% of NF1 patients) and can be seen during early years of life and adolescence. Plexiform neurofibromas have the ability to grow to a very large size, affecting an entire body section. This can cause significant discomfort, disfigurement and neurological deficits in patients. The surgical removal of these tumours has proven to be more difficult than other types of neurofibromas due to its infiltrative nature (Dogra & Rana 2013). In general the treatment of plexiform neurofibromas is more challenging due to their large size, location and involvement with several other nerves (Dogra & Rana 2013; Nguyen et al. 2013). One of the most important features of neurofibromas is their ability to transform in to malignant peripheral nerve sheath tumours (MPNST) (15%) causing an additional problem to treatment of these tumours.

Therefore it is crucial that plexiform neurofibromas are closely monitored for any unusual changes and transformations. Chemotherapeutic agents have been used in earlier studies to treat these tumours however the success has been very limited (Le & Parada 2007; Ferner 2007).

1.3   Malignant Peripheral Nerve Sheath Tumours (MPNSTs)

Malignant Peripheral Nerve Sheath Tumours (MPNSTs) are rare (3-10% of soft tissue sarcomas) high grade sarcomas of neuroectodermal differentiation, typically developing in deep soft tissue and are characteristically aggressive with a low prognosis (Kahn et al. 2014). Patients with NF1 disease have a 15 year reduction in their life expectancy due to mortality related to NF1-associated malignancies (Evans & Ingham 2013). MPNST can occur sporadically and in a later stage of life within the general population, however almost 50% of MPNSTs are associated with NF1, developing in

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approximately 10-15% of patients (Evans 2002). MPNSTs usually initiate in the nerve roots, extremities and pelvis, however they can also develop in other sites of the body. MPNSTs can cause pain, discomfort, disability and neurological insufficiency in patients due to the pressure they cause on to the surrounding areas (Sughrue et al. 2008). Studies have shown that a pre-existing plexiform neurofibroma has the ability to transform in to a malignant tumour resulting in an enlargement of the neurofibroma, which then will become painful for the patient (Farid et al. 2014). Individuals with subcutaneous neurofibroma have a 20 fold more chance of developing MPNSTs compared to other patients, therefore it is crucial for patients with these neurofibromas to be monitored and closely observed, in order to identify any malignant changes (Tucker et al. 2005).

Treatment options for MPNSTs are extremely limited, subsequently causing a low survival rate in patients suffering from these tumours. At the moment the main treatment for MPNSTs is complete surgical removal with clear margins (Kahn et al. 2014). Chemotherapy has also been used in several pre-clinical and clinical trials, however it is still not fully known how well MPNSTS respond. Some studies have suggested that the MPNSTs occurring in NF1 patients might respond differently to individuals with non-related NF1 MPNSTs (Korf 2000; Zehou et al. 2013; Farid et al. 2014). Radiotherapy has also been used, and have shown some success (Ducatman et al. 1986). However radiotherapy is tried to be avoided as it seems to cause the development of other malignant symptoms (Sharif et al. 2006).

1.3.1 Atypical Neurofibroma: An intermediate between Neurofibroma and MPNST

Loss of heterozygosity of NF1 within Schwann cells promotes hyper activation of the RAS pathway as neurofibromin is unable to switch Ras to an inactive GDP-bound form (Ratner & Miller 2015). Consequently, this then results in heightened cell proliferation and growth. Even though the loss of neurofibromin function is sufficient for the development of neurofibromas that are characteristically benign, additional genetic events are essential for the progression of these neurofibromas to MPNSTs (Beert et al. 2011).

Atypical neurofibromas are symptomatic, hypercellular PNSTs. These PNSTs contain cells which have hyperchromatic nuclei with no sign of mitosis. There isn't much known regarding the origin of these atypical neurofibromas. However it seems that these are premalignant tumours that can transform into MPNSTs (Beert et al. 2011).

There are several genomic abnormalities within MPNSTs that are absent in the neurofibromas. It is likely that transformation of neurofibromas to MPNSTs involve key genomic alternations that alter the cells within neurofibromas to malignancy (Beert et al. 2011). To support this idea , it has been observed that in 15 out of 16 atypical neurofibromas there is a frequent homozygous loss of the *CDKN2A* locus located on chromosome 9, which is absent in being neurofibromas (Beert et al. 2011). However, the adjacent locus *CDKN2B* does not show any sign of frequent deletion. *CDKN2A* encodes a tumour suppressor protein known as p16 that acts as a cell cycle inhibitor via the inhibition of the cycling dependent kinase 4 and 6 (CDK4/6). Moreover *CDKN2A* also encodes ARF tumour suppressor (p14^{ARF}) that plays a role in the stabilization of p53 via Mouse double minute 2 homolog (MDM2) inhibition (Beert et al. 2011).

The early loss of the *CDKN2A* locus, along with the loss of the *NF1* locus itself, supports the hypothesis that atypical neurofibroma is indeed an intermediate between neurofibroma and MPNST. This proposes that the loss of *CDKN2A* is one of the key steps in malignant transformation of neurofibromas in to MPNSTs (Beert et al. 2011).

In addition a study on NF1 mouse models has revealed that the Nf1 +/-; p16/ARF -/-, develop MPNSTs and not neurofibromas (Joseph et al. 2008). A previous study carried out by Spurlock *et al.* showed that the loss of TP53, RB1 and also *CDKN2A* occurs during the transformation from a benign neurofibroma to atypical neurofibroma, and then the transformation in to a malignant tumour (Spurlock et al. 2010).

1.3.2 Cell of origin in MPNST

It is not yet known, exactly what cell type the MPNSTs originate from. Several cells such as the neural crest, Schwann cell precursors, immature Schwann cells and also tumour cells that differentiate from a mature Schwann cell could possibly be the cell type that give rise to MPNSTs.

Within earlier studies concerning the origin of neurofibromas the involvement of various cell types such as the neural crest in the development of neurofibromas has been reported (Chen et al. 2014). A gene expression analysis on human MPNST tumours and also MPNST cell lines has revealed that MPNSTs express marker of neural crest cells (Miller et al. 2009). In addition other gene expression studies have shown two neural crest markers *SOX9* and *TWISTS*, to be significantly upregulated within MPNSTs (Pytel et al. 2010; Carbonnelle-Puscian et al. 2011). Of interest the downregulation of *SOX9* resulted in cell death and also the downregulation of *TWISTS* caused a decrease in cell migration (Miller et al. 2009).

Moreover other studies have reported the present of Schwann-like cells with a biallelic NF1 mutation in human neurofibromas (Joseph et al. 2008; Wu et al. 2008; Zheng et al. 2008), suggesting that neurofibromas are initiated in Schwann cells.

Knowing that most of the plexiform neurofibromas in human are congenital, and are identified either at birth or at early stages of infancy, it can be concluded that they have originated from the early Schwann cell lineage. In support of this, genetically modified mouse models have revealed that deletion of *Nf1* within the embryonic Schwann cell lineage can result in the development of neurofibromas (Joseph et al. 2008; Wu et al. 2008; Zheng et al. 2008). Although it is still not known in what development stage Schwann cell lineage initiate neurofibroma formation.

Furthermore frequent development of plexiform neurofibromas has been observed within the para-spinal region at the neural foramina associated with the dorsal root ganglion

(DRG) in NF1 patients (Chen et al. 2014). Further supporting this, development of neurofibromas in the DRG region was also detected in mouse models of NF1 (Chen et al. 2014).

Scientist hope that by identifying the cell of origin and by examining the interactions between the cell of origin and its microenvironment, they will be able to provide therapies that can help delay or if possible prevent the formation of neurofibromas and MPNSTs in NF1 patients.

1.3.3   The role of MicroRNAs (miRNA) in the formation of MPNSTs

miRNAs are a type of non-coding RNA, which play a role in regulating functions associated with gene expression (Sedani et al. 2012). Since their discovery approximately 20 years ago, miRNAs have shown to play an important role in cancer progression, including involvement in the progression of NF1-associated tumours (Sedani et al. 2012; Masliah-Planchon et al. 2013).

Due to the complex histopathology of MPNSTs and the intricate molecular mechanism involved in MPNST progression, the pathogenesis of MPNSTs is not fully understood (Kahn et al. 2014). As mentioned before biallelic *NF1* gene inactivation is necessary for the development of tumours, however other molecular mechanism and changes within the micro-environment involved in the MPNST progression are not well defined (Sedani et al. 2012).

An uncontrolled cell growth is one of the general characteristics of cancer; miRNAs can both stimulate or suppress this growth through various cellular processes, such as apoptosis, cell differentiation, proliferation etc. (Sedani et al. 2012). In addition approximately 50% of miRNA-coding genes have been detected within genomic regions of tumour cells (Calin et al. 2004) and have shown to be involved in tumour metastasis, invasion as well as tumour progression. They also contribute to the chemo-resistance and radio-resistance

features of tumours (Sedani et al. 2012) making them an attractive biomarker and therapeutic targets in cancer.

Even though studies regarding the role of miRNAs in NF1 tumour development is still at progress, studies have shown that a number of miRNAs could possibly play a role in NF1 tumourgenesis (Subramanian et al. 2010; Sedani et al. 2012; Presneau et al. 2013). A microarray analysis based study carried out by Presneau *et al.* revealed that miR-29c was the most significantly down regulated miRNA in patient MPNSTs compared to neurofibromas. Using a prediction software's/algorithms a list of genes that are targeted by miR-29c where identified. One of these genes was the matrix metalloproteinase (MMP)-2, which is known to be involved in cell migration and invasion (Presneau et al. 2013). To further investigate the role of miR-29c an MPNST cell line was transfected with miR-29c which resulted in reduced cell invasion (Presneau et al. 2013). In an earlier study Subramanian *et al.* demonstrated that miR-34a was downregulated in MPNST in comparison to neurofibromas. The forced expression of miR-34a in MPNST cells resulted in cell death through apoptosis (Subramanian et al. 2010). miR-34a is also known to be a direct target of the tumour suppressor protein p53 (Sedani et al. 2012).

Moreover studies have shown that miR-204 could play a role in the growth of MPNSTs (Gong et al. 2012). It has also been shown that this miRNA acts as a tumour suppressor in other forms of cancer as well as NF1-associated tumours, such as breast, prostate and kidney cancers (Wang et al. 2010). A study analysing differentially expressed genes between PNFs and MPNSTs, revelled the upregulation of a group of miRNAs (miR-301a, miR19a and miR-106b) in MPNSTs that directly target PTEN (Masliah-Planchon et al. 2013). It is important to note that loss of PTEN has been observed in human MPNSTs (Bradtmöller et al. 2012). The research on miRNAs and revealing the important role that they play in tumourgenesis has opened a new aspect for future therapies.

1.4   Genetics of NF1

1.4.1   The identification of the *NF1* gene

The *NF1* gene was discovered by gene linkage studies in 1987 and was assigned to the pericentromeric region of chromosome 17. Later on an advanced mapping located the gene in a region close to the 17q11.2 (Barker et al. 1987; Seizinger et al. 1987; Wallace et al. 1990). Subsequently, the gene was mapped to within 10-15 cM and in further work within 3cM of 17q11.2, which covers a region of 2-6x106 bp (Barker et al. 1987; Seizinger et al. 1987; Fain et al. 1989). In additional cytogenetic studies of two unrelated NF1 patients with balanced translocation, information to a more specific definition of the *NF1* gene position was provided. One patient involved chromosome 1 and 17 and the other 17 and 22 balanced translocation, the breakpoints in both individuals was in 17q11.2 region (Schmidt et al. 1987; Collins et al. 1989; Menon et al. 1989). Several other genes where discovered in the 17q11.2 region these include: NF1-c2 (NF1 candidate gene 2), the human oncogene homolog ERBA1, ERBB2, EV12 and NGFR. However none of these genes contributed to NF1 disease nor were they disrupted via the translocation (Seizinger et al. 1987; Collins et al. 1989; Fountain et al. 1989; O'Connell et al. 1990). This suggests that all these genes are embedded within the *NF1* gene. Finally the actual *NF1* gene, originally known as NF1LT (Wallace et al. 1990) was identified by positional cloning experiments at 17q11.2 in 1990 (Cawthon et al. 1990; Viskochil 2002; Wallace et al. 1990).

1.4.2   The *NF1* gene structure

The *NF1* gene is one of the largest human genes, spanning approximately 350 kb of genomic DNA, located on chromosome 17q11.2. This gene contains 60 exons, of which three are alternative exons (Yap et al. 2014; Li et al. 1995). The *NF1* gene encodes a 12 kb mRNA transcript (Griffiths et al. 2007). The 8457 bp open reading frame, codes for a 2818 amino acid protein, known as neurofibromin with an estimated molecular mass of 327 KD (Yap et al. 2014). Intron 27, one of the largest introns within the *NF1* gene, contains three embedded genes, *EV12A*, *EV12B* and *OMGP* (Li et al. 1995). All three genes are

transcribed in the opposite direction of the *NF1* gene and the protein products of these genes seem to have very little or no interaction with neurofibromin (Viskochil et al. 1990; Upadhyaya et al. 2008).

1.4.3 Embedded genes

Intron 27 is larger than rest of the introns within the *NF1* gene. This intron contains three embedded genes: EV12A, EV12B and OMGp (Cawthon et al. 1991; Johnson 2000)

1.4.3.1 *EV12A and EV12B*

The EV12A and EV12B are human homologues of the murine proto-oncogenes known as Evi-2A and Evi-2B respectively. These genes have been associated with murine myeloid leukemias (O'Connell et al. 1990; Vourc'h & Andres 2004). Both EV12A and EV12B encode for transmembrane proteins. The EV12A has two exons and encodes a 232 amino acid polypeptide, expressed within the peripheral blood, brain and bone marrow. The EV12B also has two exons and encodes for a 448 amino acid protein, expressed within the bone marrow, fibroblasts and peripheral blood (D Viskochil et al. 1990; Cawthon et al. 1991).

1.4.3.2 *OMG*

The OMG gene is a developmentally regulated gene mainly expressed in oligodendrocytes of the CNS prior to myelination. This gene contains two exons and encodes an extracellular adhesion protein known as oligodendrocyte myelin glycoprotein (OMgp) (Mikol & Stefansson 1988; Habib et al. 1998). OMgp has the ability to function as an adhesion molecule, contributing to the interactions between the plasma membrane of oligodendrocyte and axons essential for myelination. Studies have shown that OMgp act as a neurite outgrowth and neural stem cell proliferation inhibitor via the OMgp/NOGO receptor (NgR), as a result the OMgp/NgR pathway could possibly allow the degeneration of damaged axons after injury *in vivo* (Cawthon et al. 1991; Vourc'h & Andres 2004). Furthermore studies on murine models has revealed a tumour suppressor role for the OMgp (Habib et al. 1998).

The overexpression of OMG within the NIH3T3 fibroblasts causes growth suppressive properties also the loss of OMG due to deletions at the NF1 gene locus could possibly play a role as a mediator of the neurological defects associated with NF1 microdeletions (Habib et al. 1998; Martin et al. 2009).

1.5 *NF1* gene expression

1.5.1 Alternative splicing

There are 3 main alternative splicing exons within the *NF1* gene, in addition to several other splice variants with a lower expression level (Gutmann & Collins 1992; Gutmann, Geist, Wright, et al. 1995). Alternative splicing is an essential mechanism that results in proteasome diversity. This mechanism also plays a vital role in regulating gene expression at various developmental phases within different tissue types. There are 5 recognised alternative splicing mechanisms models: cassette exons, mutually exclusive exons, alternative 5' splice site (SS), alternative 3'SS and intron retention. The deregulation of alternative splicing has shown to contribute to tumourgenesis; therefore it has been examined as a potential mechanism of genotype-phenotype correlation in individuals with NF1 disease.

There are three main neurofibromin isoforms that are created via the alternative use of the 23a and 48a exons of the *NF1* gene (type 1, 2 and 3 neurofibromin) (Gutmann, Geist, Rose, et al. 1995).

1.5.1.1 *Type 1 neurofibromin*

The NF1 exon 23a is known as an alternative exon and lies within the GAP-related domain of neurofibromin. This exon is skipped in the neurons of the CNS. Type I neurofibromin lacks the 23a exon in addition to the 48a exon protein sequence. This isoform has a higher Ras-Gap activity than isoforms with these exons (Xu et al. 1990).

1.5.1.2 Type 2 neurofibromin

The type II neurofibromin involves the insertion of 21 amino acids in-between exon 23a and 24 within the GAP (GTPase activation protein) related domain (GRD). Exon 23a is differently spliced in various tissues. The stimulation of type 2 neurofibromin has been seen in neuroblasts and Schwann cells that seem to differentiate *in vitro*, in order to alternate its functional properties (Nishi et al. 1991; Andersen et al. 1993; Gutmann & Collins 1993; Gutmann, Geist, Wright, et al. 1995). Furthermore due to Gap function interruption, this isoform plays a less important role in Ras activation (Andersen et al. 1993; Skuse et al. 1996).

1.5.1.3 Type 3 neurofibromin

Within the type 3 neurofibromin transcript, exon 48a inserts an extra 18 amino acids (additional 54 nucleotides from exon 48a) into the carboxyl terminus of the protein transcript. This isoform is dominantly expressed in human heart and skeletal muscle (Gutmann, Geist, Rose, et al. 1995).

1.5.1.4 Other neurofibromin isoforms

Other splice isoforms of the *NF1* gene have also been identified. These include an isoform containing both the 23a and 48a exons in addition to a new exon known as 10a-2. The 10a-2 exon is an alternative exon added between exons 10a and 10b, this exon adds an additional 15 amino acids to the protein transcript. It appears that this neurofibromin isoform contains a transmembrane segment that is absent in the wild-type neurofibromin .Even though the exact function of this isoform is not known, due to its overall expression, it is believed that it has a housekeeping function on an intercellular membrane, for example the endoplasmic reticulum (Barron & Lou 2012).

Furthermore, another neurofibromin isoform is produced via insertion of 10 amino acids by the 9br exon into the amino terminal of neurofibromin after residue 1,260 (Danglot et al. 1995; Gutmann et al. 1999). The expression of this isoform is restricted to the CNS

with little or no expression in the cerebellum, brainstem, or spinal cord. This suggests that the 9br isoform is found in the neurons of the forebrain during the initial stages of postnatal development (Gutmann et al. 1999).

1.5.2   Neurofibromin

1.5.2.1   The cDNA sequence of the NF1 gene

The cDNA sequence of the *NF1* gene was initially defined by Gutmann and Collins (1993), and Viskochil *et al.* (1993) (Viskochil et al. 1993). The main protein product of the *NF1* gene is neurofibromin (Figure 1.1), a relatively large protein (~250 KDa) with 2818 amino acids. This protein is ubiquitously expressed this is somewhat associated with the diverse clinical manifestations of the disease (Barron & Lou 2012).

1.5.2.2   Tissue distribution of neurofibromin

As mentioned in section 1.5.6.1, the neurofibromin protein (Figure 1.1), is ubiquitously expressed, the NF1 mRNA can be found in most human tissues in a lower concentration. For example the lungs, spleen, skin fibroblast etc. (Wallace et al. 1990). A more dominant expression of neurofibromin has been identified through its GAP activity, western blotting and immunostaining, within the neurons; Schwann cells CNS and peripheral nervous system (PNS) (Daston et al. 1992; Golubić et al. 1992; Barron & Lou 2012). Furthermore, neurofibromin is seen to be expressed within the non-myelinating Schwann cells, however it has not been identified in the myelin forming Schwann cells, this correlates with studies showing NF1 somatic mutations occurring in Schwann cells derived from neurofibromas (Daston & Ratner 1992; Maertens et al. 2006). Based on the diverse expression of neurofibromin within neuronal and non-neuronal tissues, it can be established that the mutations occurring in the *NF1* gene can cause a wide range of pathologies.

1.5.2.3   The Intracellular distribution of neurofibromin

Specific cell signalling pathways and biological responses can be triggered depending on the localisation of a specific protein within the cell. Studies have shown localisation of the

neurofibromin protein in to various subcellular compartments, such as the actin and tubulin cytoskeleton structures and the cytosol can also generate different biological responses (Gregory et al. 1993; Li et al. 2001; Vandenbroucke et al. 2004; Leondaritis et al. 2009).

The localisation of neurofibromin has been associated with protection against some NF1 phenotypes, Studies on the NF1 mutant, NF1-DE43, has shown an inability of the protein product to localize in to the nucleus (Vandenbroucke et al. 2002). Moreover RASSF1c (microtubule-binding Ras effector) is typically found within the nucleus, however subsequently to DNA damage the RASSF1c shuttles in to the cytosol, where it is able to activate the RAS/MAPK signalling pathway and also JNK1/2 (Kitagawa et al. 2006).

Under normal conditions the nuclear neurofibromin is dephosphorylated, however the cytosolic neurofibromin is highly phosphorylated (Leondaritis et al. 2009). In order for the subcellular shuttling of neurofibromin, the Ser2808 located at the C-terminus of neurofibromin has to become phosphorylated via protein kinase C (PKC). In addition a phorbol ester: 12-O-tetradecanoyl-phorbol-13-acetate (TPA), a diacylglycerol analogue, has the ability to activate the Ras/MAPK signalling pathway via the C-terminus phosphorylation of neurofibromin which then results in cytosolic localisation. Therefore, regulating the nucleocytoplasmic shuttling of neurofibromin. Interestingly this is a common characteristic of tumour suppressor proteins (Fabbro & Henderson 2003; Leondaritis et al. 2009).

The neurofibromin phosphorylation is also responsible for the stabling and regulating of the protein function via interactions with other proteins and components, which include Ras, scaffold proteins, mitochondria, microtubules and the actin cytoskeleton (Gregory et al. 1993). In conclusion the C-terminus phosphorylation of neurofibromin is essential for the Ras/MAPK pathway activity, by the recruitment of neurofibromin from the nucleus to the
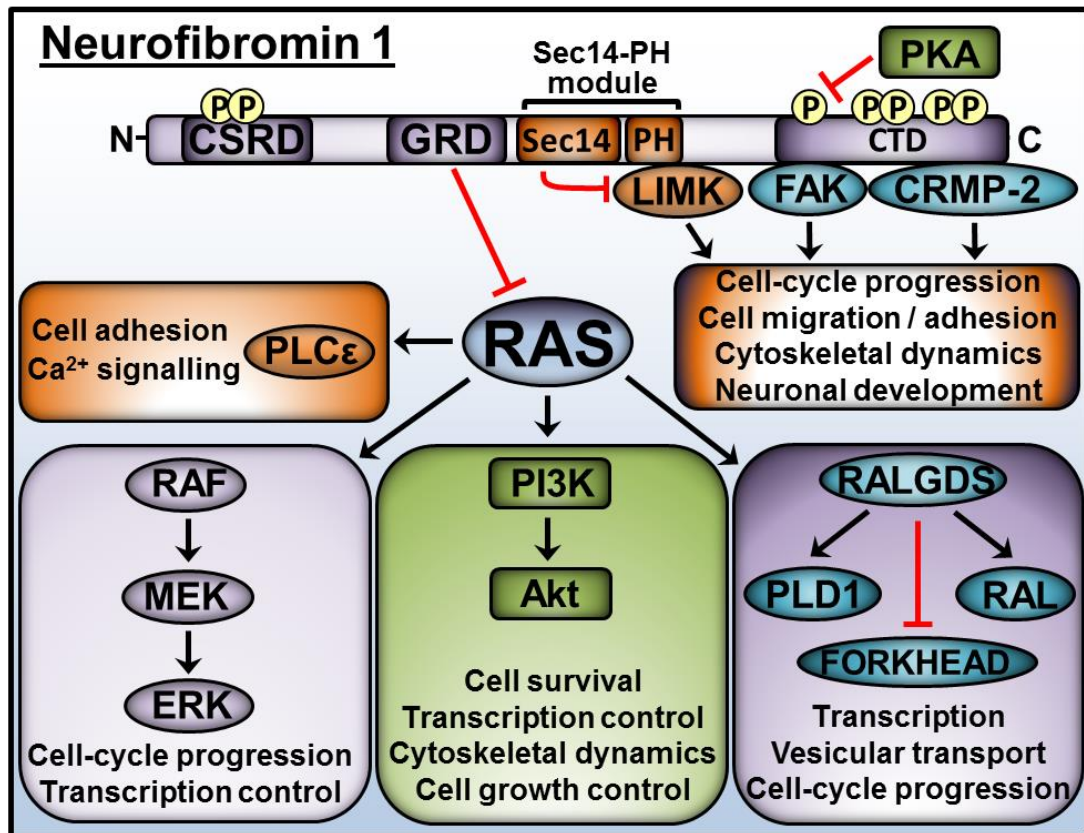
Figure 1.1. Functional domains of NF1 : Functional domains of NF1 include the GRD, which inhibits RAS GTPases (by converting them to an inactive GDP-bound status). Consequently, loss of NF1 function causes constitutive RAS activation towards an array of downstream effectors that include RAF, PI3K, RALGDS and PLCε (as well as others). The SEC14-PH module interfaces with both the cytoskeleton and membrane structures and is necessary for NF1 to function as a RAS-GAP as well as inhibition of LIMK. NF1 is a substrate of PKA, which inhibits NF1 through phosphorylation of the CTD. At the N-terminus, CSRD is also highly phosphorylated. FAK and CRMP-2 interact with the CTD which is required for neuronal development through regulation of cell adhesion and the cytoskeleton.

1.5.3 The Ras/GAP function

The *NF1* gene has approximately 98% homology between human and mouse and also approximately 60% homology between human and Drosophila, in particular within the GAP-related domains (GRDs), making it a highly conserved gene (Bernards et al. 1993; The et al. 1997).

Neurofibromin shows a large sequence homology to p120GAP, one of the first mammalian GAP recognised, Drosophila Gap1 protein and also the IRA proteins (Ira1p/Ira2p), which act as genitive regulators of the Ras-cyclic AMP signalling pathway identified within the yeast Saccharomyces cerevisiae (Trahey & McCormick 1987; Xu et al. 1990; Andersen et al. 1993; Jacks et al. 1994; Weinberg 1995).

Approximately 160 genes have been discovered that have the ability to encode GAP like proteins (McTaggart 2006). Catalytic domains are an essential in these proteins, in order to bind to Ras protein and to stimulate the GTPase activity, transforming GTP-bound Ras to GDP-bound Ras. In conclusion neurofibromin is a key regulator of Ras signalling pathway under normal cell conditions, increasing the hydrolysis of GTP in to GDP (Ballester et al. 1990; Xu et al. 1990; DeClue et al. 1991; McTaggart 2006).

1.5.4 Neurofibromin Non-GAP function

In order to determine new and applicable therapies for NF1 patients, it is crucial to investigate all roles and interactions of the neurofibromin protein *in vivo*. As mentioned above most studies are mainly dedicated to investigating the RasGAP function of the neurofibromin protein. Nevertheless there are indications of neurofibromin functioning in a non-GAP manner. Other than the GRD domain (10% of neurofibromin) neurofibromin has several other regions including an N-terminus cysteine/serine-rich domain (CSRD), a C-terminal helical domain that comprises of a functional nuclear localization sequence (NLS-CTD) and also a leucine-repeat domain located upstream of the extreme carboxyl terminus domain (Martin et al. 1990; Li et al. 2001; Vandenbroucke et al. 2004).

Furthermore using Ras inhibitors has only shown to be effective against some NF1 phenotypes, also neurofibromin has shown to have the ability to bind to other proteins resulting the co-localisation (Martin et al. 1990).

1.6 NF1 associated pathways

1.6.1 G proteins

GTP binding proteins (G proteins), are cytoplasmic proteins involved in the regulation of various cellular processes, such as, cell differentiation, proliferation and migration (Denayer et al. 2008). The abnormal regulation of the G proteins causes cellular transformation. The G protein family includes two different types: 1) small guanosine nucleotide bound G proteins, which include Rho, Rab, Ran and Ras (Bos 1989; Mitin et al. 2005). 2) Heterotrimeric G proteins.

1.6.2 The Ras family

The Ras proteins belong to a large subfamily of GTP-binding proteins (G proteins) with a low molecular weight. Depending on their sequencing conservation these proteins can be divided into several families. These various families are important for controlling different cellular processes. The Ras family has three different members: HRAS, KRAS and NRAS. All three members are stimulated via binding to tyrosine kinases (RTK), including G protein-coupled receptors, epidermal growth factor receptors (EGFR) etc. These three members are very closely related with an 85% amino acid identity. The HRAS, KRAS and NRAS protein are widely expressed, with KRAS nearly expressed in all human cell types. Studies on knockout mice have revealed that Kras is vital for the normal development of these mice, whereas Hras and Nras are not essential for mice development (Goodsell 1999; Downward 2003).

In order for the Ras proteins to function normally, they need to undergo post-translational modification to enable them to localise to the accurate subcellular compartment.

If the Ras mislocalisation of the Ras proteins results in the inactivation of these protein since they cannot recruit their target enzymes (Prior & Hancock 2012).

1.6.2.1 Signalling upstream of Ras

Dependent upon the intracellular protein partner Ras is associated with, it can exist in two forms: A GTP-bound activated form and a GDP-bound inactivated form. Within normal cells Ras protein activity is closely controlled via the ratio of bound GTP or GDP to this protein (Campbell et al. 2010). The GTP-GDP cycle is controlled through catalytic processes via specific proteins within the cell, these include: the GAPs (p120GAP and neurofibromin) or GEFs (guanine nucleotide exchange factors) including SOS, RasGRF and RasGRP (Mitin et al. 2005; McTaggart 2006). The activation state of the Ras protein and its downstream targets depends on the levels of these proteins and their balance within the cell.

In a normal cell, following the binding of growth factor receptors such as EGFR, the receptor autophosphorylates and interacts with the Src homology 2 domain (SH2) domain on the GRB2 adaptor protein (Mitin et al. 2005; Tidyman & Rauen 2009). SOS binds to the GRB2 via the Src homology 3 domain (SH3) (Mitin et al. 2005). Therefore receptor activation results in the recruitment of GRB2 and consequently SOS to the plasma membrane where they interact with Ras. The levels of Ras activity is increased via the GEF activity of SOS which catalysis the exchange of GDP for GTP (Downward 2003).

1.6.2.2 Signalling downstream of Ras

When bound to GTP, Ras has the ability to bind and activate effector enzymes, resulting in activation of pathways that control cell survival and proliferation. Furthermore therapeutic strategies that have the ability to target Ras and its downstream effectors, can be a possible effective solution to treating tumours in which Ras is aberrant (Downward 2003)(figure 1.2).

1.6.2.3 The Ras/MAPK pathway

One of the main effectors of Ras is the protein serine/threonine kinase RAF. Studies have shown that the active GTP-bound Ras binds to and activates all three related Raf proteins

(c-RAF1, BRAF and ARAF). The interaction between Ras ad Raf results in the re-localisation of Raf to the plasma membrane where it is then activated. The activation of Raf causes phosphorylation and subsequently activation of both protein kinase kinases 1 and 2 (MEK1and MEK2). Moreover MEK1 and MEK2 phosphorylate and activate the mitogen-activated protein kinases (MAPKs) ERK1 and ERK2 (extracellular signal-regulated kinases 1 and 2). ERK1/2 can be localised in to the nucleus, where they are able to stimulate transcription factors. As a result essential cell-cycle regulatory proteins are expressed allowing cell proliferation and cell cycle progression (Aramini et al. 2015; Lavoie & Therrien 2015)(Figure 1.3).

1.6.2.4 The Ras/PI3K pathway

Ras has the ability to directly interact and activate the catalytic subunit of type I phosphatidylinositol 3-kinases (PI3Ks). PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) to produce phosphatidylinositol 3,4,5triphosphate (PI(3,4,5)P3) and phosphatidylinositol 3,4bisphosphate (PI(3,4)P2). This then binds and causes translocation of 3-phosphoinositide-dependent protein kinase-1 (PDK1) and AKT (Also known as PKB). AKT acts as an anti-apoptotic protein and phosphorylates several targets crucial for the Ras survival signal (Castellano & Downward 2011).

Furthermore PI3K can also activate the RAC and RHO protein families. These families play an important role in regulation of the actin cytoskeleton and also the transcription factor pathway (Yang et al. 2012; Castellano & Downward 2011).

1.6.2.5 The Ras/RALGDS pathway

The third family of Ras effectors are the three members of the RAS-related (RAL) proteins: RAL guanine nucleotide dissociation stimulator (RALGDS), RALGDS-like gene (RGL/RSB2) and RGL2/RLF that interact with Ras-GTP via their Ras association (RA) domains (Neel et al. 2011).

1.6.2.6 The Ras/phospholipase Cε pathway

Ras can interact with phospholipase Cε. This protein contains two Ras associated domains and a RAS/GEF domain in addition to a phospholipase C domain.

The combination of all signalling pathways related to RAS allows the cell to promote cell survival, proliferation and cell cycle regulation many characteristics seen in tumour progression and malignant transformation (Harden & Sondek 2006).
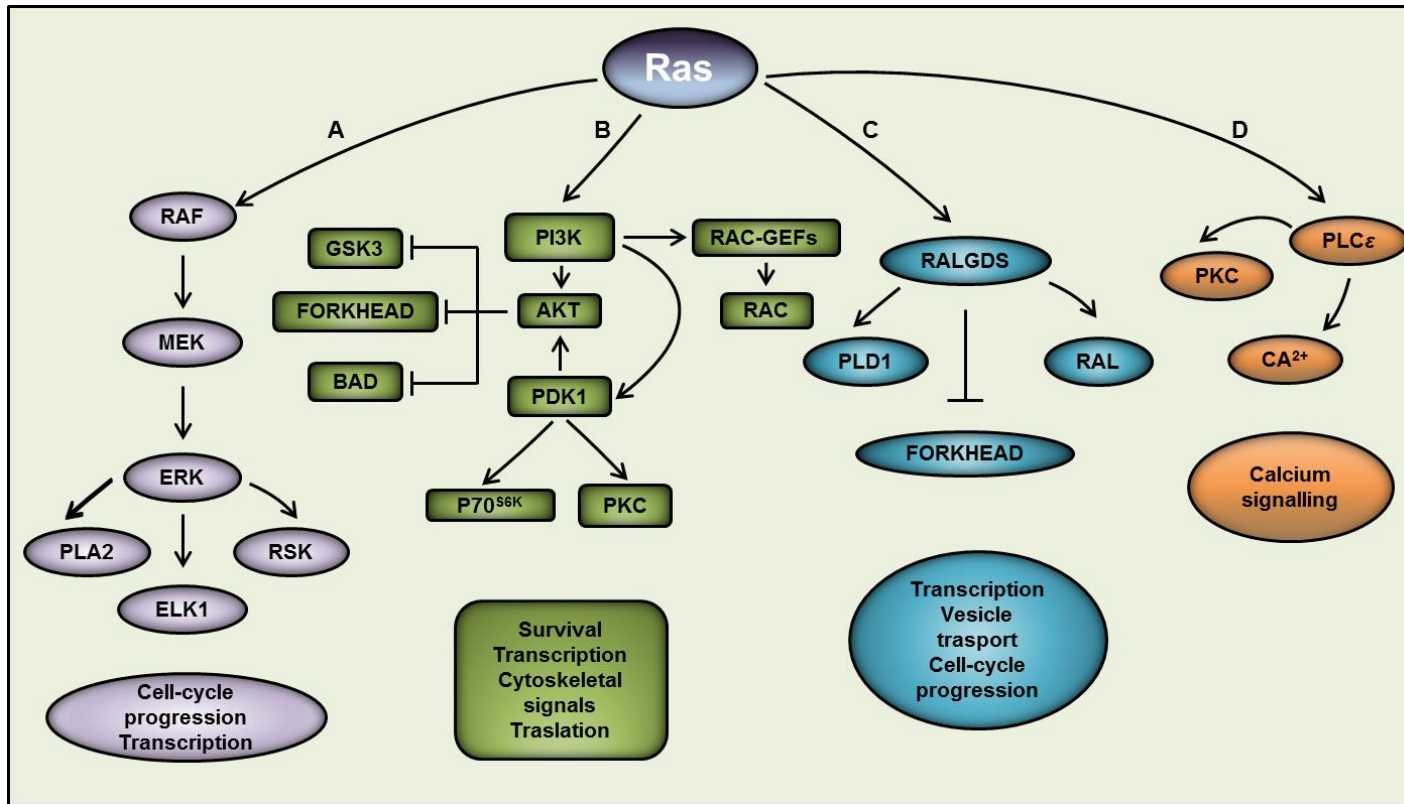
Figure 1.2. Ras downstream signalling: After activation of Ras, Ras has the ability to regulate several downstream effectors. A) The Ras/MAPK signalling pathway, RAF is known as one of the key effectors of Ras. Active Raf regulates the phosphorylation and subsequently activation of MEK1and MEK2, causing the activation of ERK1/2. Which results in the activation of several transcription factors, contributing to cell cycle progression. B) The PI3K signalling pathway, resulting in the activation of PDK1, which than activates several kinases in the AGC family such as, AKT, P70s6k and PKC. Activation of PI3K also activates the RAC-GEFs resulting in the activation of RhoGTPases, leading to cell survival. C) The activation of RALGDS via RAS, results in the regulation of phospholipase D1 (PLD1) and activation of CDC42/RAC-GAP-RAL binding protein 1 (RALBP1). Furthermore RALGDS inhibit the FORKHEAD transcription factors that are known to be involved in reducing cell cycle and inducing apoptosis through their gene targets. D) Phospholipase Cε is said to be involved in the activation of PKC, after being stimulated via Ras. Moreover Ras has been linked to calcium mobilisation through PLCε. (Downward 2003; Rajalingam et al. 2007; Castellano & Downward 2011).
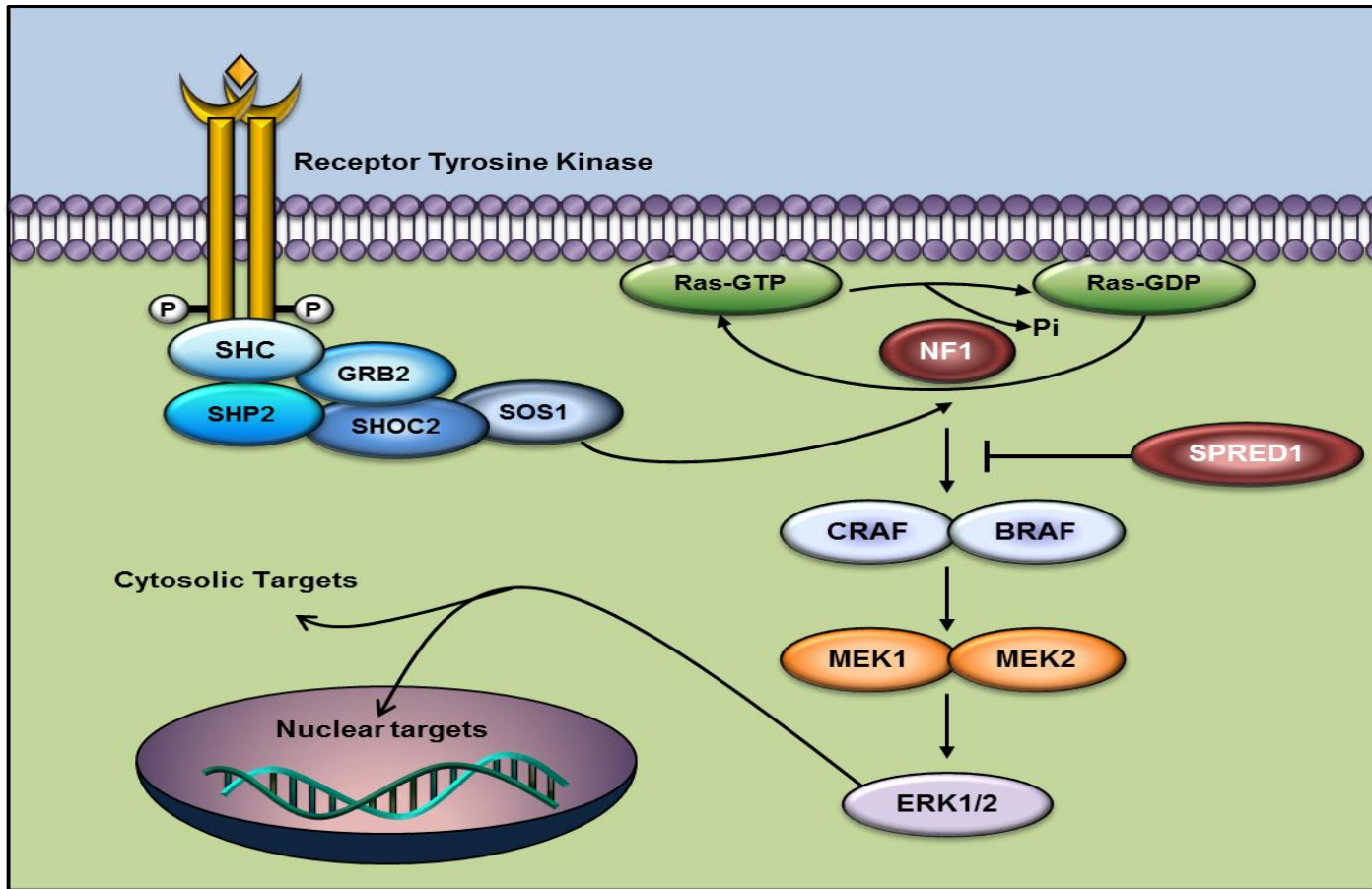
Figure 1.3. NF1 and Ras signalling pathway: The active GTP-bound Ras binds to and activates the Raf proteins. The interaction between Ras and Raf results in the re-localisation of Raf to the plasma membrane where it is then activated. The activation of Raf causes phosphorylation/activation MEK1and MEK2. MEK1/2 activates the ERK1 and ERK2. ERK1/2 can be localised in to the nucleus, resulting in stimulation of transcription factors (Gottfried et al. 2010; Feldkamp et al. 1999; Ratner & Miller 2015).

1.7 Growth factors amplified in MPNSTs

There is indications within the literature suggesting that the abnormal expression and activation of growth factors and their receptors could possibly be contributing to the MPNST pathogenesis (Yang et al. 2011; Du et al. 2013; Byer et al. 2013). The amplification of the *EGFR* , is observed frequently in MPNSTs and has been associated with poor prognosis (Tabone-Eglinger et al. 2008; Du et al. 2013). Furthermore It has been previously determined that the HGF, MET, and PDGFRA genes are frequently amplified in MPNSTs (Mantripragada et al. 2008; Upadhyaya et al. 2012). In a study carried out by Torres *et al.* it was demonstrated that a short pin RNA targeting the MET, resulted in decrease of cell growth in MPNSTs (Torres et al. 2011). Moreover the amplification of insulin like growth factor-1 receptor (IGF1R) was reported after carrying out a genomic and molecular characterization of MPNSTs. This study suggested that alternation in the IGF1R pathway and the increased IGF1R protein expression is involved in tumour progression in NF1 patients (Yang et al. 2011). Therefore making these signalling pathways a potential therapeutic target for patients with MPNST.

1.8 Protein signalling pathways altered in MPNSTs

1.8.1 HGF/MET signalling pathway

1.8.1.1 Receptor Tyrosine Kinases

Ever since the discovery of Receptor Tyrosine Kinases (RTK), numerous members of this family have been reported as main regulates of critical cellular processes. There are 58 known RTKs within the human body that can be divided in to 20 subfamilies. All RTKs share a similar structure comprised of an extracellular ligand binding domain, a transmembrane domain and a cytosolic section that contains the protein tyrosine kinase (TK) domain in addition to the carboxy (C-) terminal and juxtamembrane regulatory regions. RTKs function through their protein tyrosine kinase domain. In order to preform signal transduction RTKs

need to become phosphorylated. This occurs due to binding of a specific ligand to the extra cellular region of the receptor causing a selective trans-autophosphorylation of the tyrosine residues, some of the phosphorylated sites help maintain the active conformation of the receptor whereas some act as docking sites for other proteins and enzymes (Lemmon & Schlessinger 2010).

The phosphorylation of the RTK results in dimerisation of the receptor, transmitting the signal to the target protein. RTKs either form homodimers or heterodimers in order to regulate specific cell signalling pathways. Except for the insulin receptor family (IR) other RTKs are presented as monomers that form a non-covalently dimer in the membrane. The IR family, which are comprised of the IR, the insulin-like growth factor I-receptor (IGF-IR) and the insulin receptor-related receptor (IRR), are expressed as monomers however after undergoing transformation these receptors form two, α and β, polypeptide chain, which are stabilised by disulphide bonds creating a heterotetramer receptor in the membrane. The intercellular RTK signalling is essential for cellular processes such as cell cycle, survival, proliferation, differentiation and motility (Maruyama 2014).

1.8.1.2 Structure and function of MET

MET proto-oncogene, receptor tyrosine kinase (MET) is a proto-oncogene located on chromosome 7q21-31. The transcription of MET is controlled via Ets (E-twenty six), Pax3 (paired box 3), AP2 (activator protein-2) and Tcf-4 (transcription factor 4). The MET mRNA is expressed as several transcripts (8, 7, 4.5, 3 and 1.5 kb). The protein product of this gene is the MET tyrosine kinase which is expressed in the epithelial cells of various organs in the human body such as the kidney, bone marrow, liver, prostate, pancreas and muscle (Organ & Tsao 2011).

MET was first identified as an oncogene in the early 1980s. Initially the isoform of the MET receptor was isolated as a mutation containing the MET tyrosine kinase attached to the translocated promoter region (TPR). The proto-oncogene was known to encode a RTK however its ligand was not identified. The MET ligand was discovered by experiments

following two independent studies, one showing a potent motility factor, and the other reporting a factor that was mitogenic towards hepatocytes. Both studies were referring to the same molecule now known as hepatocyte growth factor (HGF) or scatter factor (SF) (Birchmeier et al. 2003).

The receptor tyrosine kinase MET is comprised of a short α- and a long β-chain forming a heterodimer via disulphide bonds. The extracellular part of the receptor contains 500 residues of the N-terminal that forms the large semaphorin (Sema) domain, this includes the entire α-subunit and part of the β-subunit followed by a PSI domain (seen in plexins, semaphorins and integrins) with 50 residues and four disulphide bonds. Attached to this domain are four immunoglobulin–plexin–transcription (IPT) domains connecting the extracellular part of the protein to the transmembrane helix. The intracellular part of the protein is comprised of a tyrosine kinase catalytic domain surrounded by a juxtamembrane adjacent to the membrane and a carboxy-terminal sequences facing towards the cytoplasm (Organ & Tsao 2011).

HGF is the only known ligand for the MET receptor. It is secreted as a single chain by the mesenchymal cells. HGF is activated when the bond between Arg494 and Val495 is broken via extracellular proteases. The activated form of HGF is comprised of an α- and β-chain held together by a disulphide bond. The α-subunit has an N-terminal hairpin loop attached to four kringle domains stabilized by three disulphide linkages. The β-subunit contains a serine proteinase homology domain. During cell development MET and HGF provide crucial signals that result in cell survival and proliferation (Luigi Naldini et al. 1991)(Figure 1.4).
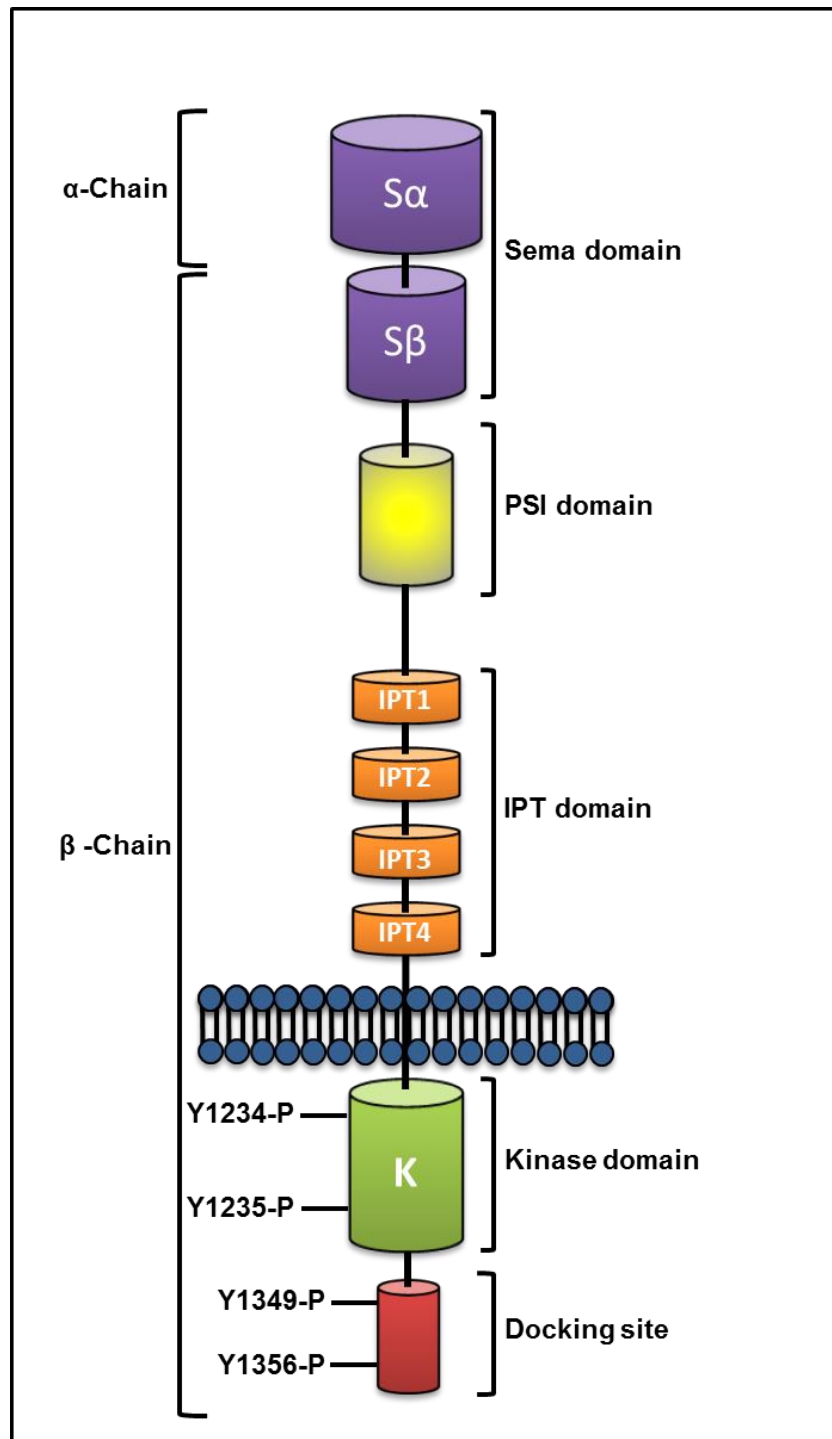
Figure1.4. Schematics of MET: The MET extracellular domain is comprised of two subunits, α and β. The N-terminal forms the large semaphorin (Sema) domain which includes the entire α-subunit and part of the β-subunit. This is followed by the PSI domain Attached to this domain are four immunoglobulin–plexin–transcription (IPT) domains connecting the extracellular part of the protein to the transmembrane helix. The intracellular part of the protein is comprised of a tyrosine kinase catalytic domain surrounded by a juxtamembrane adjacent to the membrane and a carboxy-terminal sequences facing towards the cytoplasm(Organ & Tsao 2011).

1.8.1.3 HGF/MET activation

HGF binding to the MET receptor results in the activation of the kinase by causing dimerisation and a trans-phosphorylation of two tyrosine residues (Tyr1234 and Tyr1235) within the catalytic domain. Subsequently two additional Tyrosine residues (Tyr1349 and Tyr1356) located within the carboxy-terminal tail are also phosphorylated forming a unique SH2 recognition motif for MET (Y1349VHVX3Y1356VNV). This results in the recruitment of various effectors and regulation of several signalling pathways, such as the transcription factor signal transducer and activator of transcription (STAT3), the adaptor proteins Growth factor receptor-bound protein 2 (GRB2), Src homology-2-containing (SHC), the effector molecules phosphatidylinositol 3-kinase (PI3K). Furthermore the adaptor protein GRB2-associated binding protein 1 (GAB1) unique to MET can act as a multi-adaptor protein creating a binding site for many downstream adaptors after binding directly to MET or indirectly through GRB2. There are two additional tyrosine phosphorylation within MET receptor, Tyr1313, which results in activation of the PI3K pathway and Tyr 1365, that contributes to cell morphogenesis (Organ & Tsao 2011).

In addition a negative regulation of MET is crucial for its activity. This can be achieved via different mechanisms such as the recruitment of casitas B-lineage lymphoma (c-CBL) by the Tyr1003 a negative regulator of MET, located in the juxtamembrane domain. This is also possible through the binding of several protein tyrosine phosphatases (PTPs) to the MET receptor. The PTPs balance the MET signalling through dephosphorylation of either the tyrosine residues located within the kinase domain or the docking domain. Moreover an increase in intracellular calcium levels and the activation of protein kinase C (PKC) via PLCγ /MET binding can contribute to the negative regulation of MET (Trusolino et al. 2010; Organ & Tsao 2011).

MET activation can turn on the Mitogen activated protein kinase (MAPK) cascades, by stimulating the rat sarcoma viral oncogene homolog (RAS) guanine nucleotide exchanger

Son of Sevenless (SOS) through binding to SHC and GRB2. This result in the activation of RAS followed by regulation of v-raf murine sarcoma viral oncogene homolog B1 (RAF) kinases. RAF then activates MAPK effector kinase MEK finally activating MAPPK. Once activated MAPK can translocate from the cytoplasm in to the nucleus and increase the transcription of various genes responsible for cell cycle, survival, motility and proliferation.

The MAPK cascade can also be regulated through Src homology 2 domain-containing phosphatase-2 (SHP2) interactions with MET. Accumulation of SHP2 to GAB1 helps prolong the MAPK phosphorylation (Organ & Tsao 2011).

The PI3K/AKT signalling pathway is also regulated via MET. PI3K binds to MET by its p85 subunit, either directly or indirectly through GAB1, resulting in AKT/protein kinase B activation. The activation of this pathway plays an important role in cell survival.

STAT3 has been associated with transformation downstream of c-MET. It can bind directly to the MET receptor resulting in tyrosine phosphorylation and dimerisation causing the translocation of STAT3 in to the nucleus.

Interestingly several upstream signalling co-receptors can closely associate with the MET receptor at the cell surface insuring the activation of MET-regulated pathways. For example MET can bind to integrin α6β4, this results in the formation of a docking site allowing the binding of signalling adaptors which then leads to the activation of PI3K, RAS and SRC pathways. In addition MET signalling is connected to the actin cytoskeleton through GRB2 and the ezrin, radixin and moesin (ERM) family of proteins via the v6 splice variant of the hyaluronan receptor CD44 resulting in the recruitment of SOS which helps switch on the RAS-ERK signalling pathway (Organ & Tsao 2011)(Figure 1.5).
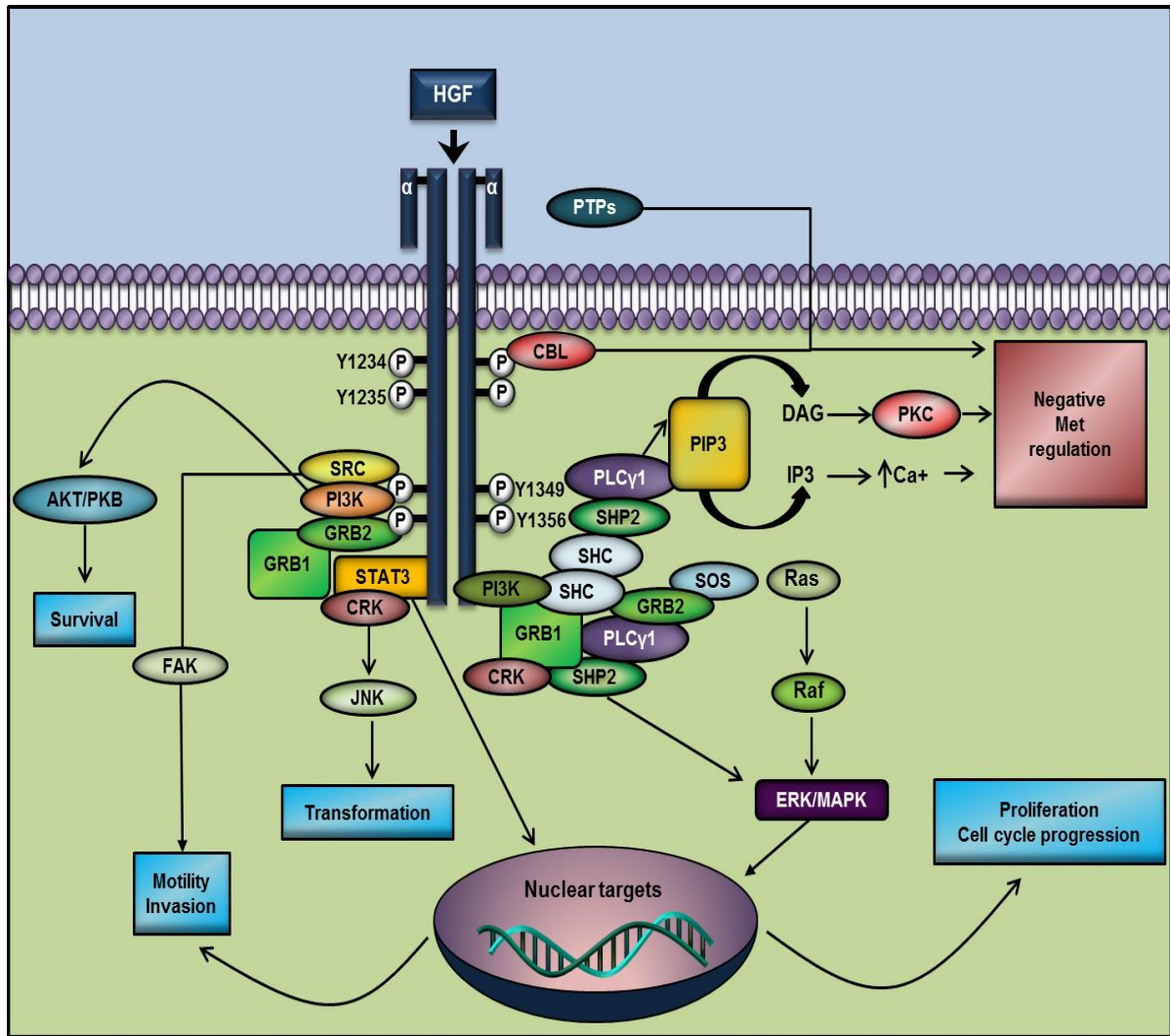
Figure 1.5. HGF/MET signalling pathway: The binding of HGF to it MET receptor activates the kinase via the phosphorylation of four tyrosine residues (Tyr1234 and Tyr1235, Tyr1349 and Tyr1356).This results in the formation of a unique SH2 recognition motif for MET (Y1349VHVX3Y1356VNV), recruiting several effectors and regulation of other signalling pathways. This includes the activation of STAT3, GRB2, SHC, PI3K, the MAPK cascade through Ras/SOS/SH2/GRB2. In addition GAB1 unique to MET acts as a multi-adaptor protein, creating a binding site for many downstream adaptors after binding directly to MET or indirectly through GRB2. MET can also be negatively regulated through various mechanisms. This includes the recruitment of (c-CBL) by the Tyr1003 a negative regulator of MET. Furthermore several protein tyrosine phosphatases (PTPs) can bind to MET negatively regulating this pathway. The PTPs balance the MET signalling through dephosphorylation of either the tyrosine residues located within the kinase domain or the docking domain. In addition the increase in the calcium levels which results in the activation of PKC through the PLCγ /MET binding also has the ability to contribute to the negative regulation of MET (Trusolino et al. 2010).

1.8.2   MET signalling cooperation

MET can interact with several membrane proteins located at the cell surface. These include semaphorin receptors, G protein coupled receptors (GPCRs), laminin receptor-α6β4 integrin, epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR). The cross talk between MET and other protein members contributes to the activation of MET and MET components resulting in integrations of signalling pathways present within the extracellular environment (Lan et al. 2013).

1.8.2.1   MET and semaphorin receptors

The semaphorin receptors are a big group of either the membrane-bound or secreted protein family. They were initially found in the nervous system, in which they involved and contributed to the formation of the neuronal network however these proteins are known to be involved in tumour growth and Metastatization, immune response, epithelial morphogenesis and cardiac and skeletal development. The semaphorins share a structural homology with a family of single-pass transmembrane molecules known as Plexins, which activate Semaphorins by binding to them allowing cooperation of MET with plexins. MET and the class B plexins have a common homology in their extracellular domain. They both carry a sema domain that has the ability to form a β-propeller structure, a cysteine-rich motif and immunoglobulin-like domains. The β-propeller structure stimulates protein-protein interaction therefor it is thought that the MET interacts with the class B plexins through this domain independent of HGF (Lai et al. 2009).

Studies have revealed that MET receptor can interact with all three members of the plexin B family, moreover sema4D the ligand for plexin B1 induces tyrosine kinase activity of MET which leads to the phosphorylation and activation of MET receptor by forming a complex, resulting in an invasive response (Giordano et al. 2002; Conrotto et al. 2004; Lai et al. 2009).

In addition studies have shown that the stimulation of semaD4 enhances phosphorylation of Gab1 as well as MET, promoting invasion through the Rac signalling pathway (Giordano et al. 2002). Also semaD4 can trigger a pro-migratory response

downstream of the HER2-plexin B1 complex via PDZ-RhoGEF (A guanine nucleotide exchange factors that upregulated Rho GTPases) recruitment, this results in the activation of Rho-A signalling pathway (Longenecker et al. 2001; Swiercz et al. 2008).

1.8.2.2 Integrin's and MET

Integrin's are a large family of αβ heterodimaric cell surface, transmembrane proteins that can interact with various extracellular ligands via an ion-dependent interaction (Hynes 1992; McDonald 2000). Both MET and integrin's initiate invasive behaviour within tumours, during embryonic development, MET is involved in the formation of normal tissues, this is done through mediating proliferation, migration and protection against apoptosis (Trusolino et al. 2001). In normal cells this process is crucial for maintaining the cell activity however in abnormal or cancer tissues this process causes invasion and metastasis. HGF-MET receptor has the ability to interact with integrin α6β4 resulting in the tyrosine phosphorylation and activation of integrin α6β4 (Trusolino et al. 2001). Accumulation of integrin's triggers MET activation and phosphorylation within mouse melanoma cells (Wang et al. 1996).

Furthermore a study has shown that the laminin receptor, α3β1 integrin forms a complex with MET which is essential for papilla maturation during kidney development (Liu et al. 2009). MET can also interact directly with focal adhesion kinase (FAK), a main mediator of integrin's, through its phosphorylated Tyr-1349 and Tyr-1356 site (Chen & Chen 2006).

1.8.2.3 CD44 and MET

CD44 is a large family of transmembrane glycoproteins that consists of a variety of different isoforms acquired through different splicing and post-translational modification. CD44 is seen in many different cell types within the body. This adhesion molecule plays an important role in signal submission, cell-cell and cell matrix interactions through the ezrinradixin, moesin (ERM) protein family (Hertweck et al. 2011). Two of the most important ligands of CD44 are hyaluronic acid (HA) (10) and Osteopontin (SPP1). Studies have shown that HGF

has the ability to bind to CD44v3 isoform (containing heparin sulfate proteoglycans and heparin sulphate), allowing the accumulation of HGF and promoting the binding of HGF to its receptor MET which then results in the activation of signalling pathways connected to the RTKs (van der Voort et al. 1999). The co-expression of MET and Cd44v3 has been reported in colon cancer leading to increased MET signalling and poor prognosis (Wielenga et al. 2000). In different established carcinoma cell lines the association of HGF/MET and CD44v6 is needed for the phosphorylation and activation of MET (Orian-Rousseau et al. 2002). It has also been reported that CD44v10 isoform is involved in the recruitment of MET via HGF into the caveolin-enriched microdomains, promoting activation of Rac signalling pathway (Singleton et al. 2006).

Further studies have shown an important role of MET and CD44 co-operation in the central and peripheral nervous systems development. Experiments on cd44-null mice with a heterozygous mutations in MET, HGF or GAB1 where found to be lethal to the embryo, causing a decrease in the synaptic transmission within the respiratory and phrenic nerve systems (Matzke et al. 2007).

1.8.3 MET and other RTKs

1.8.3.1 MET and EGFR

The EGFR is a member of the RTK that belongs to the ErbB family. The ErbB family has four subfamilies including EFGR (erbB1Her1), HER2/c-neu (ErbB-2), Her3 (ErbB-3) and Her4 (ErbB-4) (Zaczek et al. 2005). High levels of EGF receptor family have been observed in various human carcinomas. The overexpression of EGFr and HER2 has been linked to higher aggressive behaviour of tumours (Mendelsohn & Baselga 2000). Furthermore EGFr is involved in migration, invasion, proliferation and apoptosis inhibition, resulting in tumour formation and progression. MET and EGFR crosstalk has been detected in many carcinomas leading to tumorigenesis (Lan et al. 2013).

The co-operation between MET and EGFR can be regulated via different mechanisms: an increase of EGF production is caused through activation of Ras/Erk signalling pathway Mediated by MET, this causes a relocation of EGF to the outside of the cell where it binds to EGFR in an autocrine or paracrine manner (Reznik et al. 2008). EGFR activates the MAPK signalling pathway, resulting in the shortening of MET via Metalloproteinase (TIMP)-3 (Nath et al. 2001), the truncated MET can cause cell proliferation and transformation (Merlin et al. 2009). MET and EGFR can cross talk via trans-phosphorylation, EGFR and Ron can bind to MET creating a receptor complex, this the leads to the trans-phosphorylation and activation of MET (Jo 2000; Hu et al. 2007).

1.8.3.2 MET and PDFGR

PDGFR and its ligand PDGF are growth factors and receptor tyrosine kinase involved in the development of various tissues for instance it can contribute to proliferation of mesenchyme cells. Furthermore PDGFs play an important role in migration, differentiation and function of various mesenchymal cell types (Shim et al. 2010). Studies on uroepithelial cells have shown cooperation between MET and PDGFR-α, AXL, discoidin domain receptor tyrosine kinase 2 (DDR2), and/or insulin-like growth factor I receptor (IGF1R), this coexistence suggests a crosstalk between MET and other partners (Yeh et al. 2011).

Overexpression of MET/PDGFR has also been reported in human bladder cancer cell lines (Black et al. 2008). A cDNA microarray analysis revealed an association between PDGFR and MET; this was then confirmed by using specific siRNA knock down. Unlike EGFR, PDGFR cannot directly bind to MET, it is shown to be activated through the MEK/ERK signalling pathway (Yeh et al. 2011).

1.8.4 HGF/MET signalling in cancer

The activation of MET stimulates a crosstalk between the epithelial and the stromal sections, leading to a normal physiological process. Under these normal conditions the HGF/MET signalling pathway is tightly control by the activation of ligands at the targeted cell

surface, the internalisation and degradation of activated receptors and ligand delivery (Cecchi et al. 2010). In spite of various controls, MET signalling pathway deregulation can occur through many different mechanisms such as point mutations, receptor amplification, over expression of MET etc. (Sierra & Tsao 2011).

The over expression of MET is seen in numerous carcinomas such as lung, colorectal, breast prostate, pancreatic, head and neck, gastric, hepatocellular, ovarian, renal, glioma, melanoma, and also in a number of sarcomas, such as epithelial and mesenchymal (Ma, Jagadeeswaran, et al. 2005; Cecchi et al. 2010). The upregulation of MET in these tumours is caused via several epigenetic mechanisms, such as growth factors secreted due to tumourgenesis, hypoxia caused by tumour progression and several other activated oncogenes. MET gene amplification is one of the main causes of metastasis; this has been reported in a subset of lung cancers that have shown resistance to gefitinib and erlotinib, two inhibitors targeting the epithelial growth factor families (Modjtahedi et al. 2014).

Furthermore an abnormal paracrine or autocrine ligand production (Birchmeier et al. 2003), activation of kinases in the presence or absence of the amplified MET gene (Smolen et al. 2006) and MET gene mutations are other oncogenic mechanism used by MET signalling pathway resulting in metastasis and tumour progression. The initial MET mutation was first seen in renal cell carcinoma, subsequently other mutations were seen in lung cancer and in head and neck cancers (Diamond et al. 2013). Of interest MET can be over expressed due to transcriptional mechanism such as the activation of the Ras pathway (Organ & Tsao 2011). Overall the established characteristics of MET including migration, invasion, proliferation, anti-apoptosis and angiogenesis, make MET a suitable marker for determining malignancy in many different cancers.

1.8.5   STAT3 signalling pathway

1.8.5.1   The STAT family

STAT3 is a member of the STAT protein family. STATs belong to a family of transcription factors that have the ability to control the important aspects of cell survival, motility, growth and differentiation. The STAT protein is comprised of an N-terminal domain, a coiled-coil domain, a DNA-binding domain, a Src homology 2 (SH2) domain and a transactivation domain. There are currently seven recognised STAT proteins, which have similar regulation and activation mechanisms: STAT-1, 2, 3, 4, 5a, 5b and 6 (Ihle 2001).

The stimulation of various cytokines and growth factors results in the recruitment of STAT proteins to their receptor through their highly conserved Src-homology-2 (SH2) domain. Within an inactive cell the STAT proteins remain primarily as homodimers in the cytoplasm. Upon stimulation via cytokine/growth factor, associated Janus-activated kinase family kinases (JAK kinases) are activated causing phosphorylation of a specific tyrosine residues within the STAT protein. The phospho-tyrosine residue then can interact directly with the SH2 domain creating STAT protein dimers. Which then results in the translocation of the active STAT in to the nucleus. Where it can activate various other genes (Levy & Darnell 2002).

1.8.5.2   STAT3 Structure and function

STAT3 protein is ubiquitously expressed however the levels of activated STAT3 protein increases following cytokine and growth factor stimulation. The activated STAT3 is highly involved in various cellular processes such as cellular survival, growth, differentiation, wound healing and the acute phase immune response. It can also enable neuronal development in the brain in response to Ciliary Neurotrophic Factor (CNTF) (Levy & Lee 2002).

STAT3 has been reported to be activated in several human cancers. It can act as a carcinogen playing an important role in tumour formation through its transcriptional activation

of genes that encode for cell cycle regulators, apoptosis inhibitors, and also inducers of angiogenesis (Carpenter & Lo 2014). Several STAT3 target genes are reported to be upregulated during tumour formation, including Bcl-XL, surviving, cyclin-D1, Mcl-1, VEGF, Hsp70, c-myc (Kamran, Patil & Rajiv P. Gude 2013).

Several genes have been reported to be regulated through STAT3, however not all these genes are direct targets of STAT3. Microarray analysis and chromatin Immunoprecipitation (ChIP) looking at STAT3 DNA binding have identified genes regulated by STAT3 (Snyder et al. 2008)(Figure 1.6).

The structure of STAT3 is very similar to the other members of its STAT family. STAT3 becomes activated by binding to the gp130 receptor. The gp130 is a signal-transducing receptor which is activated by interleukin 6 and 11, oncostatin-M, cardiotrophin-1, leukemia inhibitory factor and ciliary neurotrophic factor, resulting in the dimerisation of gp130 receptor subunit. The dimerisation of gp130 causes JAK kinases recruitment which allows tyrosine phosphorylation of the gp130 receptor, this provides a docking site for STAT3, resulting in the phosphorylation and activation of STAT3 (Levy & Darnell 2002; Levy & Lee 2002).

STAT3 has two main phosphorylation sites, Tyr705 and Ser727. Both these sites are located within the transcriptional activation domain (TAD) (Rawlings et al. 2004). Originally it was thought that the dimerisation and activation of STAT3 protein was through the tyrosine705 phosphorylation which was thought to be an essential step towards the activation of the JAK-STAT3 signalling pathway. However now further studies have shown that STAT3 contains a second serine phosphorylation (727) site located within the C-terminus of the protein which helps regulate the transcriptional activity of the protein (Carpenter & Lo 2014). In the STAT1 protein, the serine phosphorylation is essential for the maximum activation of the protein via recruitment of its transcriptional cofactors, MCM5 and CamKI (Zhang et al. 1998; Nair et al. 2002). In addition there is evidence for a similar positive role of Ser727 phosphorylation in STAT3. The STAT3 protein also has two possible

TOS motifs, FDMDL located at amino acids 26-30 and FDMDL located at amino acids 756-760 (Lee et al. 2008)(Figure 1.6).
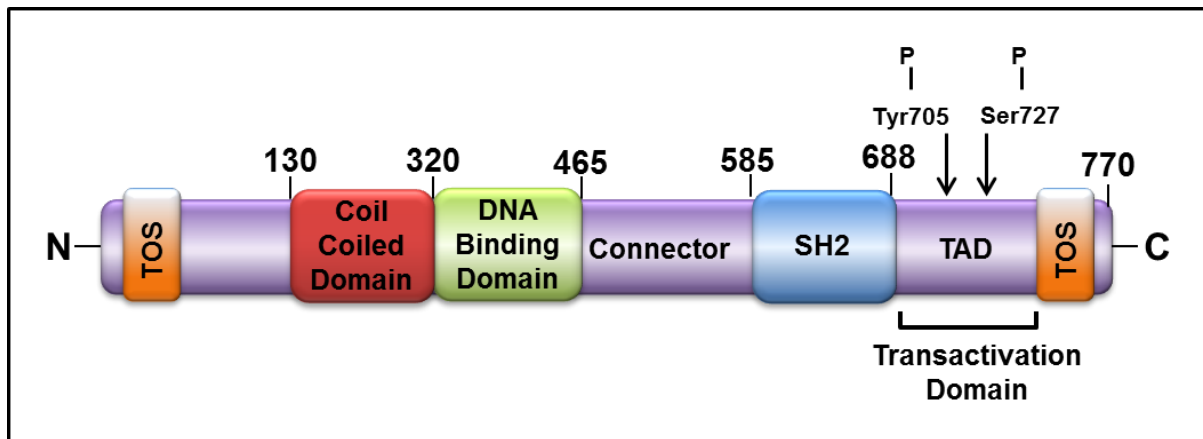
Figure 1.6. Schematic of STAT3: STAT3 has an N-domain located at the N-terminus which is involved in the oligomerization of the STAT3 protein. This region has the ability to form hook-like structure with 8 α-helices. This region is adjacent to the coiled-coil domain, the DNA binding domain, a linker region, the Src-homology 2 (SH2) domain and a carboxy-terminal transactivation (TA) domain that consists of a conserved tyrosine that has the ability to phosphorylate and plays an important role in STAT3 dimerization. The SH2 domain allows interaction between STAT3 and gp-130 receptor via the tyr705 phosphorylation. tyr705 phosphorylation is mediated by the JAK2 kinases whereas the Ser727 phosphorylation is mediated by mTORC1.
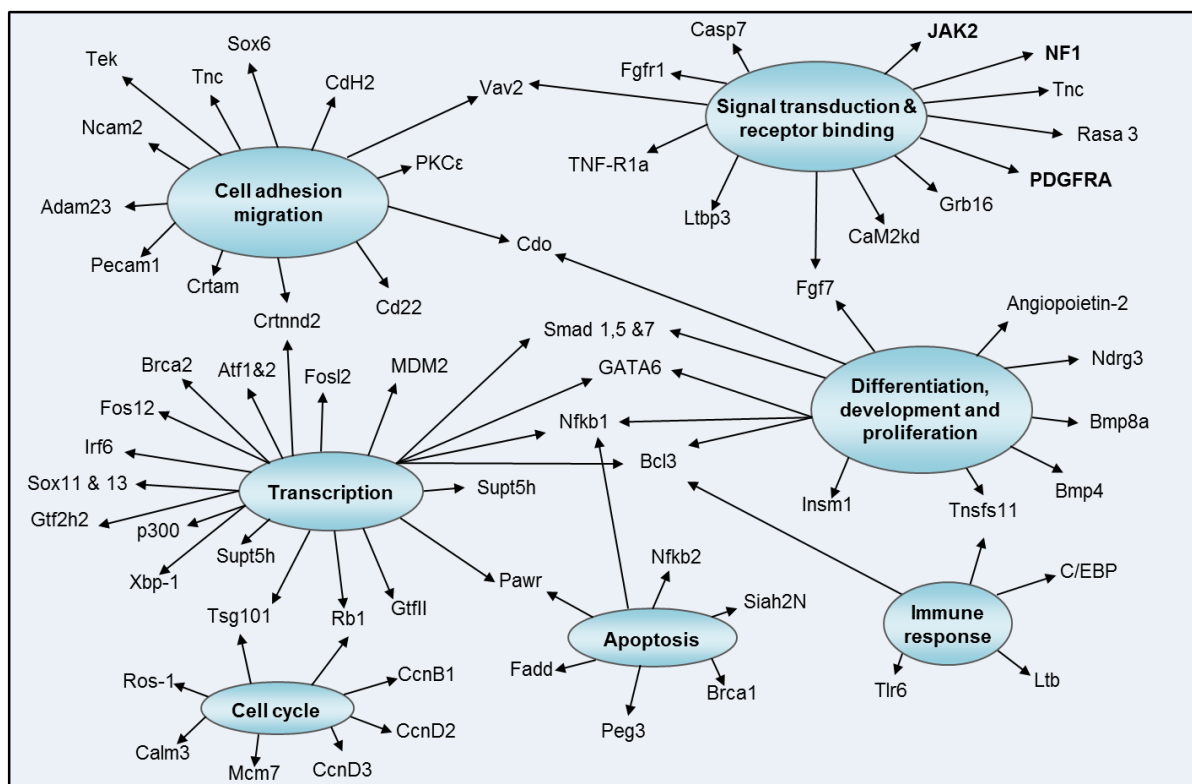
Figure 1.7. Gene targets of STAT3. The potential gene targets of STAT3 are shown in the diagram above. Genes have been classified in to 7 groups according to their function. This figure has been adapted from Kayleigh Dodd's thesis (Snyder et al. 2008).

1.8.5.3 STAT3 activation

1.8.5.3.1 Tyrosine phosphorylation of STAT3

Cellular localisation of proteins is an essential process for the effective function of numerous signalling pathways, and the nuclear translocation is the main process for the function of transcription factors (See figure 1.8).

The activation of STAT3 has known to rely particularly on the Tyr705 phosphorylation, regulated through the JAK2 signalling pathway, resulting in the dimerization of this protein and its translocation in to the nucleus, where it binds to the DNA regulating several genes such as Bcl-xL, Myc etc. (Figure 1.7) that are mainly involved in cellular processes such as cell survival, proliferation, and cell cycle (Dauer et al. 2005; Sakaguchi et al. 2012).

In the first studies performed it was assumed that the abnormal expression of Tyr705 phosphorylated STAT3 was responsible for tumour characterisations. As a result the main focus of STAT3 activation and its impact on tumours has been dedicated to Tyr705 phosphorylation. Moreover the success of STAT3 inhibitors in preventing cell survival and cell cycle have been widely linked to their ability to downregulation the Tyr705 phosphorylation of STAT3. Consequently therapies and clinical trials carried out using STAT3 inhibition should be only used in tumours with abnormal expression of Tyr705 phosphorylated STAT3.

Recent studies however, have revealed that the activity of STAT3 does not merely rely on its Tyr705 phosphorylation, but a second phosphorylation site located in the C-terminal domain (Serine 727) is also responsible for activation of STAT3 protein.

The Ser727 residue is phosphorylated in response to cellular stress despite of the Tyr705 phosphorylation. This suggests that STAT3 protein can exist in different forms within a cell and could possibly have different functions according to its site of phosphorylation. Of interest Ser727 phosphorylated STAT3 can interact with NFκB subunit and can play a role in

regulating other cancer related genes through this pathway. This cooperation between STAT3 and NFκB has been observed in colorectal and also breast cancer. Ser727 phosphorylated STAT3 has been seen in chronic lymphocytic leukemia and has shown to play an important role in cell survival in the absence of Tyr705 phosphorylation.

1.8.5.3.2 Serine phosphorylation of STAT3

Studies on engineered mice expressing a mutant STAT3 in which the serine727 is replaced with an alanine (S727A) showed that several of these mice died very shortly after birth, the mice that survived were approximately 50% smaller than the wild type, these mice also showed altered insulin-like growth factor 1 (IGF-1) serum levels and an increase in apoptosis. This suggests that the serine phosphorylation of STAT3 is essential for the growth and development of the embryo (Shen et al. 2004).

The STAT3 gene is often related to as the acute phase response gene, because of its fundamental role in this process (Alonzi et al. 2001). Conversely the engineered mice mentioned above had a normal STAT3 dependent liver acute phase response, suggesting that the acute phase response is controlled through the Tyrosine phosphorylation of STAT3 (Figure 1.9). Studies have suggested that the effects of STAT3 within the cell are dependent on phosphorylation of both sites (Ser727 and Tyr705), indicating a more important role for the Ser727 phosphorylation (Shen et al. 2004). On the contrary other studies on mutant mice (S727A) showed an increase level of Tyr705 phosphorylation, suggesting that Ser727 phosphorylation acts as a negative regulator of Tyr705 phosphorylation (Chung et al. 1997). This could possibly indicate that the Ser727 phosphorylation might be playing a role in the negative feedback loops for STAT3.

A study on neuroblastoma cell lines, showed that stimulation by CNTF (a neuropoeitic cytokine that stimulates the JAK/STAT signalling pathway as well as the ERK1/2, PI3K and mTOR signalling), induced both Tyr705 and Ser727 phosphorylation of STAT3 resulting in maximal transcriptional activation of STAT3. Additionally it was reported that mTORC1 was possibly the kinase that controlled the Ser727 phosphorylation of STAT3

(Yokogami et al. 2000); In support of this a more recent study reported that Ser727 phosphorylation of STAT3 could be activated through amino acid stimulation in a rapamycin sensitive manner (Kim et al. 2009).

Finally a study by Dodd *et al.* on Tsc2+/ − mouse model demonstrate that Ser727 phosphorylation of STAT3 is directly regulated by mTORC1 during hypoxia (Dodd et al. 2014).
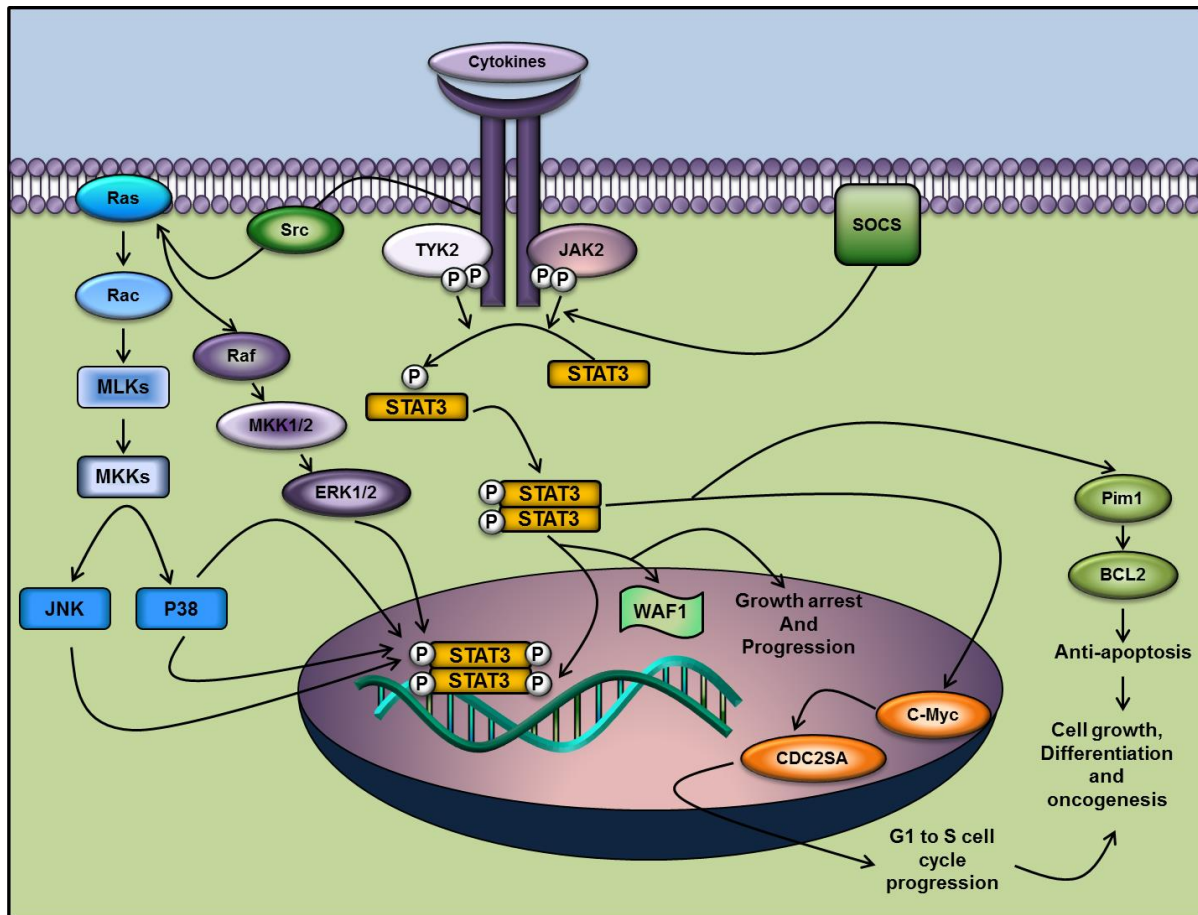
Figure1.8. The JAK/STAT3 signalling pathway: The activation of STAT3 has known to rely particularly on the Tyr705 phosphorylation, regulated through the JAK2 signalling pathway, after the activation of the receptor via growth factors, interleukins etc. The JAK2 is recruited to the receptor and autophosphorylates. This results in the binding of STAT3 to JAK2, this time causing the phosphorylation of STAT3 which then resulting in the dimerization of this protein and its translocation in to the nucleus, where it binds to the DNA regulating several genes that are envolved in cell invasion, migration and tumourgenesis.
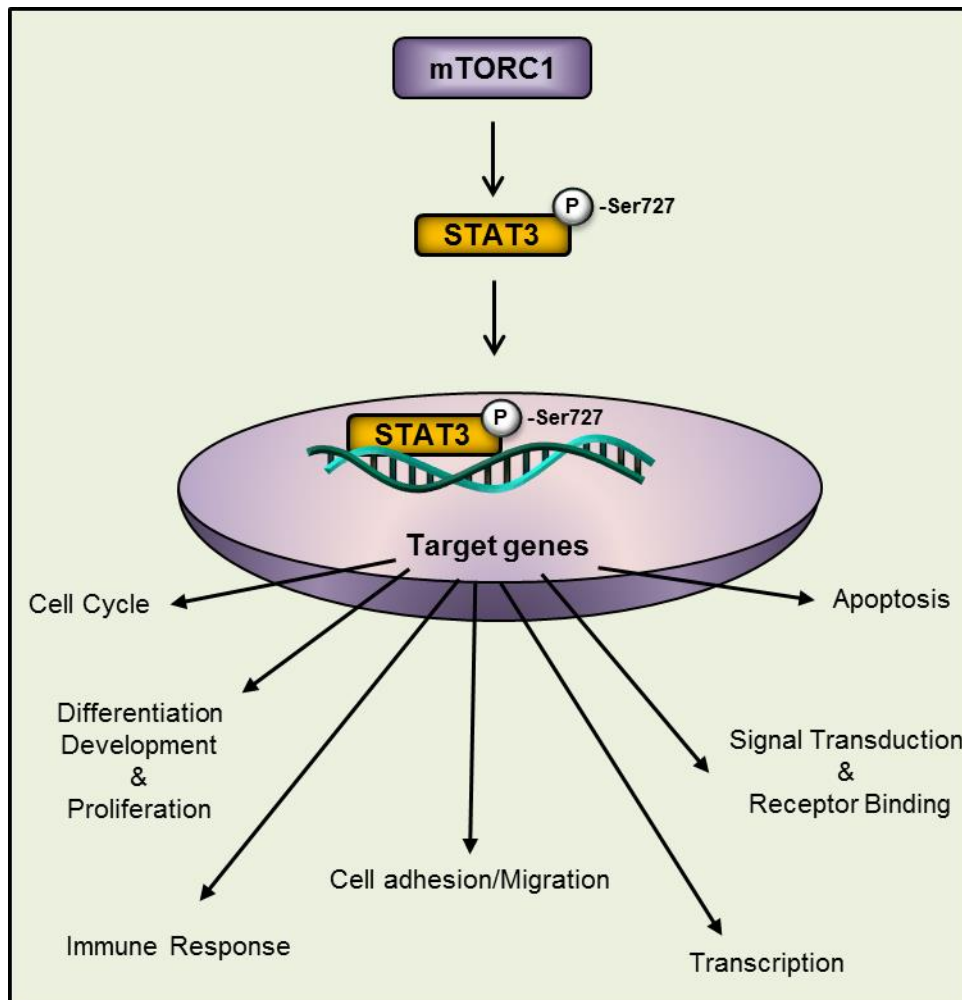
Figure1.9. The mTOR/STAT3 signalling pathway: Various cytokines or growth factors stimulate the activation of STAT3 through is phosphorylation at the Tyr705 via the Janus kinase (JAK) or via receptor tyrosine kinases (RTKs), enabling STAT3 dimerization, nuclear translocation and DNA binding. In addition to this STAT3 can also be phosphorylated on the Ser727 residue allowing its localization in to the nucleus and promoting its transcriptional activity, this can be achieved via mTORC1.

1.8.6 mTOR signalling pathway

The mammalian target of rapamycin (mTOR) is a conserved serine/threonine kinase (Cargnello et al. 2015). mTOR was discovered after rapamycin, an immunosuppressant drug, was identified. Rapamycin is an immunosuppressant drug produced from the bacterium Streptomyces hygroscopicus, this bacteria was first found in the soil samples taken from Easter Island (Rapa Nui). Rapamycin is a macrolide and acts as an immunosuppressive component via decreasing the growth cycle of T-lymphocytes. This drug is being given to patients that need transplant in order to reduce the chances of graft rejection. Rapamycin has the ability to bind to FKBP12 and form a complex which then inhibits the function of mTORC1 (Heitman et al. 1991; Brown et al. 1994; Sabatini et al. 1994). (Figure 1.10).

mTOR consists of two different complexes: mTORC1 and mTORC2. mTORC1 is comprised of mTOR itself along with regulatory-associated protein of mTOR (Raptor), mammalian lethal with SEC13 protein 8 (mLST8), PRAS40,DEPTOR, FKBP38 and Protor-1/2. mTORC2 contains mTOR bound to rapamycin-insensitive companion of mTOR (Rictor), mLST8, Sin-1 and Protor-1/2 (Cargnello et al. 2015). The two mTOR complexes affect different downstream targets. In this study the main focus will be on downstream effectors of mTORC1.

1.8.6.1 mTORC1

mTORC1 plays an important role in cell proliferation and growth. It also has the ability to manipulate metabolic process by regulating transcription of pathways such as autophagy, ribosomal biogenesis, glucose transport and angiogenesis (Polak & Hall 2009; Sengupta et al. 2010). mTORC1 is activated via several feedback loops and upstream components.

Numerous factors such as growth factor, insulin, nutrients available in the cell and many different mitogens can cause activation of this complex. As mentioned above this complex is comprised of mTOR, Raptor, mLST8, PRAS40, DEPTOR, FKBP38 and Protor-1/2, which can be directly activated through GTP-Rheb. The small G protein Rheb (Ras

homologue enriched in brain) is one of the well-known proteins that has the ability to stimulate mTORC1 signalling pathway activation (Tee et al. 2005; Tee & Blenis 2005). (Figure 1.10).

1.8.6.2 mTOR

mTOR is a part of the phosphoinositide kinase-related kinase (PIKK) protein kinases family, and consists of a serine/threonine kinase domain which lies within the C-terminal (Fingar & Blenis 2004). The N-terminal part of mTOR contains 20 tandem HEAT repeats with two α-helices (approximately 40 amino acids) that are arranged in a hydrophilic and hydrophobic manner, and it's involved in protein-protein interaction by providing a docking site for mTOR (Fingar & Blenis 2004).

The FRB (FKBP12/rapamycin binding) domain is located adjacent to the HEAT repeats. This is the site where the FKBP12/rapamycin heterodimer can bind to (Fingar & Blenis 2004; Hay & Sonenberg 2004). Studies have shown that mutations occurred within mTOR that cause lack of the FRB domain don't have the ability to regulate cell cycle progression into the G1 phase suggesting that this domain is absolutely essential for mTOR kinase activation (Vilella-Bach et al. 1999).

mTOR has two conserved FAT domains, in addition to the HEAT repeat and the FRB domain. There is a smaller FAT domain located at the C-terminus (FATC) and a larger FAT domain comprised of α-helices structures. Both these domains have the ability to interact with one another after mTOR activation. FATC plays a crucial role in mTOR kinase activity (Fingar & Blenis 2004).

1.8.6.2.1 Raptor

Raptor is a 150kDa protein that has the ability to bind to mTORC1, and also interact with both 4E-BP1 and S6K1. Raptor is highly conserved and is comprised of an N-terminal domain known as RNC (Raptor N-terminal conserved) domain which is linked to three HEAT repeats followed by seven WD40 repeats. The RNC domain is necessary for

mTORC1/Raptor interactions with substrates and also can form α-helices. Studies have demonstrated that a Raptor mutant 4 can bind to mTORC1 however it loses its ability to enable substrate phosphorylation. mTORC1 substrate phosphorylation is dependent on the mTOR signalling motif (TOS). TOS is a conserved regulatory motive observed within the N-terminal of all S6K's and also the C-terminal of the 4E-BP's. TOS forms a docking site for mTORC1 and Raptor to interact (Fingar & Blenis 2004; Cargnello et al. 2015).

Studies have shown that Raptor can be phosphorylated via mTORC1 at the Ser863 residue after stimulation by insulin or other mTORC1 upstream activators. The phosphorylation at this site triggers phosphorylation in five other sites within Raptor (Ser696/Thr706/Ser855/Ser859/Ser877). Mutations to the Ser863 site, reduces mTORC1 activity both *in vitro* and *in vivo* (Wang et al. 2009).

1.8.6.2.2 mLST8

mLST8 is a 36kDa protein, comprised of seven WD40 repeats. The knockdown of mLST8 caused disruption within the mTOR/Rictor interaction but did not affect the mTOR/Raptor complex. As a result it is though that mLST8 is crucial in the mTORC2 complex but not in the mTORC1 (Loewith et al. 2002).

1.8.6.2.3 PRAS40

PRAS40 is a 40 kDa proline enriched Akt substrate. PRAS40 is known to be a negative regulator of the mTORC1 when the cell is deprived of insulin via binding to the C-terminal kinase domain and blocking its ability to interact with Rheb. After insulin stimulation PRAS40 is directly phosphorylated by Akt and mTORC1, resulting in the detachment of PRAS40 from the complex, allowing interaction of mTORC1 with other substrates through the TOS motifs.

Phosphorylated PRAS40 binds to 14-3-3 chaperone proteins, allowing full activation of mTORC1 (Fonseca et al. 2007; Oshiro et al. 2007; Cargnello et al. 2015).

1.8.6.2.4 DEPTOR

DEPTOR is a 48kDa protein. It has the ability to bind directly to mTORC1 regardless of the mLST8 expression. Its interaction with mTORC1 is regulated through the PDZ domain which lies within the C-terminal of mTORC1, neighbouring the kinase domain. Cells with reduced DEPTOR expression have a higher kinase activity of mTORC1 substrates 4E-BP1 and S6K1. mTORC1 is shown to be a negative regulator of DEPTOR through phosphorylating DEPTOR at one of its serine/threonine residues that are located between the C-terminal DEP domain and the PDZ domain. this results in the detachment of DEPTOR from the complex similar to PRAS40 as described above (Peterson et al. 2009).
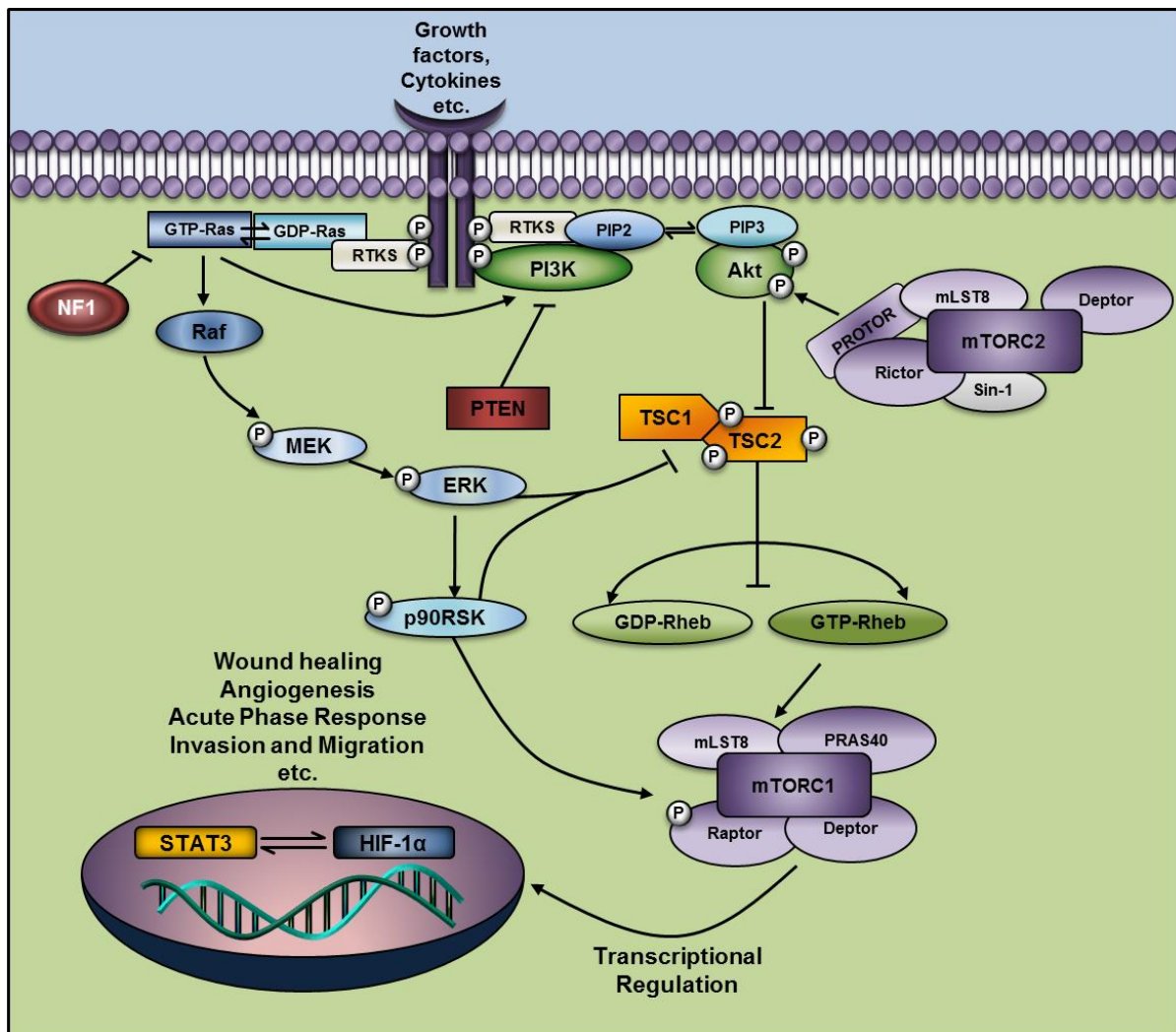
Figure1.10. The mTOR signalling pathway: PI3k is activated via growth factors/Insulin. PI3K the catalyses the transformation of PIP2 to PIP3. This provides a docking site for AKT, that has been phosphorylated via mTORC2. After the activation of AKT results in the direct phosphorylation of TSC2, which causes disruption of the TSC1/TSC2 complex. The TSC1/TSC2 complex is responsible for the activation of Rheb through GTPase activity, converting it to a GDP-bound state, unabling the activation of mTORC1. The activation of AKT and phosphorylation of TSC2 prevents this, resulting in the activation of mTORC1. Furthermore growth factors/Insulin can aslo cause activation of PI3K which activates the Ras/Erk pathway, disrupting the phosphorylation of TSC2 via Erk.

1.9 HIF-1α signalling pathway

1.9.1 Hypoxic Regulation

The cell is constantly coordinating a balance between having a sufficient oxygen concentration for its metabolic purposes without permitting the levels to reach toxicity. In an event of O2 deficiency, the cell has the ability to compensate for the lack of O2 by using systemic and intercellular changes in order to stop hypoxic damage and renovate adequate oxygenation within the cell. It has been identified that the cell does this by increasing the production of hypoxia-inducible factor (HIF) family of transcription factors.

1.9.2 HIF family of transcription factors

HIF functions as a heterodimer consist of a constitutively expressed HIF-β subunit and a highly inducible α sub-unit. The α-subunit has 3 different isoforms, HIF-1α (Figure 1.11), HIF-2α and HIF-3α. While HIF-1α and HIF-2α are very similar in their structure and function HIF-3α seems to perform an opposite role to the other 2 isoforms, acting as an inhibitor of hypoxia induced genes. Even though both HIF-1α and HIF-2α seem to be activated in the same oxygen dependents manner, HIF-1α is the only isoform ubiquitously expressed. HIF-2α is predominantly expressed in endothelium of blood vessels, lung, liver, heart, brain, pancreas, intestine and the kidneys (Ke & Costa 2006).

The hypoxic response of HIF is mainly regulated by prolyl hydroxylase-domain enzyme 2 (PHD2).The degradation of HIF is initiated through the Von Hippel-Lindau tumour suppressor protein (VHL) (Ke & Costa 2006).

1.9.2.1 HIF-1α

Hypoxia-inducible factor-1 (HIF-1α) is primarily responsible for the hypoxic response within cells and tissues. HIF-1α is an oxygen sensitive ubiquitously expressed HIF protein that acts as a transcriptional activator (Hu et al. 2003). HIF-1α is essential for the formation of new blood vessels.

Even though HIF-1β is constitutively expressed, keeping the mRNA and protein levels stable irrespective of O2 availability, the HIF-1α subunit has a short half-life (5 min) and its mRNA and protein levels are highly dependent on the O2 level in the cell (Huang et al. 1998).

HIF-1α is synthesised regardless of O2 availability however in normoxia HIF-1α gets degraded. This results in no HIF-1α protein within the cell. When hypoxic conditions occur HIF-1α stabilizes and moves from the cytoplasm in to the nucleus; HIF-1α then demonizes with the HIF-β subunit, causing the HIF complex to become transcriptionally active.

The activation of HIF-1α is mainly controlled by its post-translational modifications including phosphorylation, hydroxylation, ubiquitination and acetylation (Brahimi-Horn & Pouysségur 2009). The activated HIF-1α associates with the functional hypoxia response elements (HREs) embedded within the regulatory section of the target gene resulting in induced transcription and gene expression (Ke & Costa 2006). As mentioned before HIF-1α undergoes post-translational modifications, this can be seen in several domains of the HIF-1α protein. Under normoxia, two of the proline residues become hydroxylated as well as the acetylation of a lysine residue, this results in the interaction of HIF-1α protein with the von Hippel-Lindau (pVHL) ubiquitin E3 ligase complex (Mole et al. 2001; Masson & Ratcliffe 2014). Additionally the degradation of HIF-1α protein via 26S proteasome becomes possible when pVHL complex binds ubiquitin with HIF-1α. Moreover the transcriptional activity of HIF-1α in prohibited when an asparagine residue located within the C-TAD becomes hydroxylated, inhibiting the association between HIF-1α and CBP/p300 (Ke & Costa 2006).
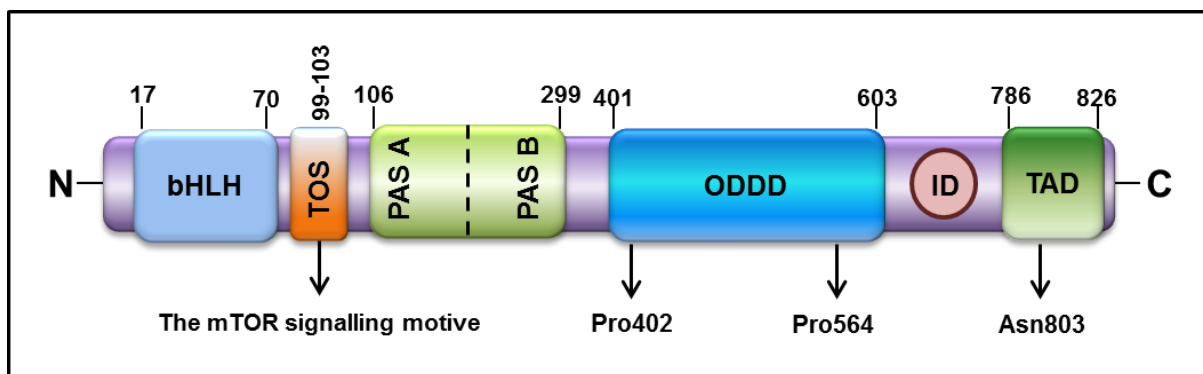
Figure1.11. HIF-1α Schematic: The image above demonstrates the conserved domains of HIF-1α. HIF-1α contains the basic β-helix-loop-helix (bHLH) and the PER-ARNT-SIM (PAS) domains. These domains allow the binding of HIF-1α to the DNA. The two transacting domains (TAD) and also the inhibitory domain (ID) are located within the c-terminal of HIF-1α. There's also an Oxygen Dependent degradation domain (ODDD), the proline residue within this domain becomes hydroxylated in the presence of O2 allowing ubiquitination via the Von Hipple-Lindau tumour suppressor. (Numbers specify the amino acid positions within the protein)(Krock et al. 2011).

1.9.2.2 HIF-2α

HIF-2α has a 48% amino acid sequence identity with HIF-1α consequently it also shares several biochemical and structure similarities with HIF-1α. HIF-2α was identified shortly after the cloning of HIF-1α. However while HIF-1α is ubiquitously expressed, HIF-2α is predominantly expressed in carotid body, endothelium and the lungs (Flamme et al. 1997; Tian et al. 1998).

1.9.3 HIF-1α and HIF-2α in tumourgenesis

Malignant tumours tend to grow and proliferate quite rapidly, compelling them to need a bigger supply of nutrients and oxygen. As a result malignant growths require the formation of new blood vessels.

Within the very first stages of malignant tumour progression, tumour cells could maintain an inactive state, resulting in a balance between cell apoptosis and cell proliferation. The tumour cells then enter the active state, where they start the process of angiogenesis. As mentioned previously this stage provides nutrients and oxygen for cells, making it a crucial step for the survival of malignant tumour cells. Studies have shown that the inhibition of angiogenesis in the early stages of tumour progression reduces tumourgenesis, suggesting a role for angiogenesis in tumour formation and progression. Interestingly, the vessels formed through angiogenesis in malignant tumour cells function quite poorly. This results in the stabilization of the hypoxic domains including HIF-1α even when the tumour is highly vascular (Krock et al. 2011).

In addition to hypoxic conditions, HIF can also be activated via several other oncogene pathways and tumour suppressors (Masson & Ratcliffe 2014). The abnormal activation of HIF-1α can be clearly observed in the Von-Hippel Lindau (VHL) syndrome which is a result of mutations within the Von-Hippel Lindau tumour suppressor protein. Individuals with this disease suffer from both benign and malignant highly vascularised tumours, and also an increased risk of developing hemangioblastoma and clear-cell renal carcinoma. The HIF-1α is comprised of an oxygen dependent degradation domain which can

be modulated via VHL. Imbedded within this domain are two proline residues (Pro564 and Pro402). In the presence of oxygen the two proline residues become hydroxylated via 2-oxoglutarate and Fe (II) dependent prolyl hydroxylases (PHDs), resulting in an increase of VHL/HIF-1α binding. VHL is a part of the E3 ligase complex that affects HIF-1α, causing ubiquitination of HIF-1α and preparing it for degradation at the proteasome (Kaelin 2004).

Moreover the activation of several growth factors can stimulate pathways such as the PI3K/PTEN/AKT that have an effect on the activation of HIF. The abnormal activation of the PI3K/PTEN/AKT signalling pathway results in the synthesis of HIF-α subunits. Furthermore AKT can also effect the activation of HIF-α through the mTOR signalling pathway (Jiang & Liu 2009).

The Ras/RAF/MAPK signalling pathway hyper-activation can cause the stabilization of HIF-1α. This is achieved by effecting the transactivation of HIF-1α. Studies have shown that the inhibition of the MAPK signalling pathway interrupts the interaction between HIF-1α and p300, also inhibits the transactivation of p300. On the contrary hyper expression of the MEK1 (upstream activator of MAPK) induces HIF-1α and p300 transactivation. This suggests that the RAS/RAF/MAPK pathway can cause activation of HIF-1α by endorsing the formation of a HIF-1α/p300 complex and also by increasing the transactivation of p300 (Sang et al. 2003).

The abnormal expression of HIF-1α and HIF-2α has substantial impact on tumour biology and has been linked to increase in tumour aggressiveness, therapeutic resistance and mortality in numerous cancers, including liver, lung, pancreas, skin, breast, brain, head and neck cancers. Furthermore the HIF-1α and HIF-2α gene targets also play an important role in angiogenesis, apoptosis resistance and metastasis (Keith et al. 2012).

The activation of HIF-1α and HIF-2α control the expression of various pro-angiogenic genes, such as VEGF, Tie-2, and Ang-1/2 etc. which are also considered as biomarkers for tumour hypoxia. Of importance HIF-1α has the ability to regulate VEGF directly. The

expression of VEGF plays a crucial role in the angiogenic effects caused by HIF-1α (Krock et al. 2011).

Moreover studies have shown that hypoxic conditions can also activate additional effector molecules that contribute to the activity of HIF-1α and HIF-2α. For example several microRNAs are activated through the HIF signalling pathway (Krock et al. 2011).

1.9.4   Osteopontin signalling pathway

1.9.4.1   *Structure of SPP1*

Osteopontin (OPN), also known as secreted phosphoprotein 1 (SPP1), is a 34 kDa, arginine–glycine–aspartate (RGD)-containing adhesive glycoprotein that was identified as a major sialoprotein in bone (Rangaswami et al. 2005). SPP1 is comprised of numerous conserved domains, including heparin- and calcium-binding domains, a thrombin-cleavage site, a CD44 binding site, and two integrin-binding sites (Tuck et al. 2007).

SPP1 undergoes a substantial posttranslational modification. *SPP1* gene is located on chromosome 4 (4q13) and has 7 exons .SPP1 does not fold in to a single defined structure , and belongs to a family of proteins that do not have a secondary and tertiary structure, known as the Intrinsically Disordered Proteins (IDPs)(Shevde & Samant 2014).

SPP1 contains a polyaspartic acid motif, which plays a role in the binding of the protein to hydroxyapatite, calcium ions and an RGD (Arg-Gly-Asp) sequence that mediates cell attachment. The SPP1 protein contains a thrombin cleavage site, several Serine and Threonine phosphorylation sites and sites for N- and O-linked glycosylation. The cleavage of SPP1 by thrombin (SPP1-R) modifies the protein and exposes an epitope for the cryptic C-terminal _α4β1 and _α9β1 integrin-binding motif (SVVYGLR) (Sodek et al. 2000).

Moreover different phosphorylation's, sulphation and also glycosylation gives SPP1 the ability to act and preform a verity of functions, allowing it to have a versatile tissue specific and function specific roles (Sodek et al. 2000).

The *SPP1* gene possesses an alternative translation start site, allowing SPP1 protein to exist as two variants, intercellular and extracellular. Of interest SPP1 can be exposed to alternative splicing, causing different isoforms of SPP1 with different functions to exist (SPP1-a, SPP1-b and SPP1-c). The existence of SPP1-b and SPP1-c within the blood are known as biomarkers for distinct cancers such as breast and prostate cancer (Shevde & Samant 2014).

1.9.4.2 *SPP1 and tumourgenesis*

SPP1 plays a role in apoptosis inhibition, contributing to cell survival and tumour progression. Nemours studies have shown the involvement and over expression of SPP1 in a variety of malignancies. High levels of SPP1 are used as a biomarker in cancers such as osteosarcoma, breast cancer, prostate cancer glioblastoma and melanoma (Rodrigues et al. 2007).

Furthermore it has been demonstrated that patients suffering from prostate, breast and lung cancer have a higher level of serum SPP1 then individuals with no cancer (Rodrigues et al. 2007). In breast cancer cells the over expression of SPP1 increases the metastatic and tumorigenicity of these cells via pathways that regulate angiogenesis (Rodrigues et al. 2007). Knockdown of SPP1 expression has shown to reduce growth and angiogenesis of colon cancer cells (Wu et al. 2014). In addition DNA microarray analysis has revealed that SPP1 is one of the highest expressed genes within metastatic melanoma (Zhou et al. 2005). This suggests that SPP1 could possibly be developed in very early stages of melanoma and causes an increase in tumour growth and invasion(Zhou et al. 2005).

1.9.5 The Wnt signalling pathway

The Wnt signalling is a central pathway involved in embryonic development and the regeneration of tissues (Klaus & Birchmeier 2008); When altered, Wnt signalling has an

important role in the progression of several cancers (Angers & Moon 2009), such as lung, breast, ovarian, prostate, brain, liver and colorectal cancer (Watson et al. 2013).

Wnt signalling is a highly complex. Three main signalling pathways that are activated through Wnt have been characterised: (i) the canonical Wnt pathway involves β-catenin and the LEF/TCF transcription factor (Luscan et al. 2013), (ii) the noncanonical planar cell polarity pathway that involves the jun N-terminal kinase (JNK) and cytoskeleton rearrangement (Huelsken 2002), and (iii) the Wnt/calcium pathway (Luscan et al. 2013).

The Wnt pathway can be activated in cancer cell lines via several mechanisms (Polakis 2012). In the context of cancer, activation of Wnt can occur either through the overexpression of Wnt ligand genes, activation mutation in the β-catenin gene or inactivating mutations within genes involved in the destruction of β-catenin (Such as AXIN1, GSK3B, and APC) (Curtin & Lorenzi 2010). The Wnt pathway can also be activated via crosstalk with several other signalling pathways (Reilly 2013), such as the PI3K/AKT/mTOR signalling pathway, where the loss of PTEN results in hyperactivation activation of the PI3K/AKT pathway. Akt activation then promotes phosphorylation and inactivation of glycogen synthase kinase 3 beta (GSK3B), which causes stabilization of β-catenin (Watson et al. 2013). It is important to note that loss of PTEN has been observed as a common event in human MPNSTs (Bradtmöller et al. 2012). Furthermore, growth factors such as EGFR can also play a role in the activation of the Wnt signalling pathway. The stimulation of EGFR causes the transcription of genes such as cyclin D1 (Watson et al. 2013). EGFR and other growth factors are also known to be overexpressed in human MPNSTs (Perrone et al. 2009; Wu et al. 2013).

Using comparative transcriptome analysis it was previously demonstrated that PI3K and β-catenin signalling are both involved in the promotion of MPNST growth (Mo et al. 2013). In addition to this, using a sleeping beauty forward genetic screen to identify new genes and pathways involved in malignancy, the Wnt pathway was found to be a driver of MPNST development (Watson et al. 2013). Of interest, in a recent study, 20 genes involved

in Wnt signalling exhibited altered expression in MPNST biopsies and cell lines in comparison to benign neurofibromas (Luscan et al. 2013).

Moreover in human colon cancer, Osteopontin (SPP1) is a transcriptional target of aberrant Wnt signalling, and SPP1 expression alone predicts survival (Reilly 2013). It has also been shown that the anti-apoptotic role of Wnt is mediated via SPP1, a direct Wnt target gene, and SPP1 was reduced by Wnt antibody administration *in vivo* (Viñas et al. 2010). These studies concur with recent results observed in a study carried out within our lab, showing that after examining copy number alterations (CNAs) and gene expression, SPP1 showed to exhibit the most significantly elevated differential mean expression level between the benign and malignant tumours. Also pathway analysis revealed that the canonical Wnt signalling pathway likely drives MPNST development (Thomas et al. 2015). Taking into account these findings, members of the Wnt pathway may not only constitute potential biomarkers of MPNST tumorigenesis but also represent potential therapeutic targets for small molecule inhibitors (Thomas et al. 2015).

1.9.6 The SUZ12 signalling pathway

Research into dysregulated pathways within MPNSTs has led to some major advances regarding mechanisms of malignancy. One such breakthrough was the discovery that components of the chromatin-modifying polycomb repressive complex 2 (PRC2), SUZ12 and EED are frequently inactivated through mutations within MPNSTs (De Raedt et al. 2014). De Raedt *et al.* uncovered that PRC2 functions as a tumour suppressor, that when lost, causes marked epigenetic reprogramming that can lead to malignancy of plexiform neurofibromas.

PRC2 is known to be dysregulated in several human tumours, including colon, ovarian, breast and liver (Kirmizis et al. 2003; Li et al. 2012). SUZ12 lies proximal to the NF1 gene, and is considered to cooperate with NF1 to suppress tumour growth. Indeed, more seriously affected NF1 patients carry a microdeletion of 17q within their germline that contains NF1, SUZ12 and 12 neighbouring genes (De Raedt et al. 2014). Such individuals with NF1 microdeletions have a higher prevalence of benign tumours and malignancies.

Highlighting this cooperative tumour suppressor function between both NF1 and SUZ12, knockdown of SUZ12 led to enhanced tumour colony growth in NF1-deficient malignant glioma cells but not glioblastomas with wild-type NF1 (De Raedt et al. 2014). Furthermore, Nf1+/-; Suz12+/- mice had higher tumour burden. Loss of Suz12 is known to modify gene-expression through reducing trimethylation of Histone 3 K27 (H3K27me3) and increasing its acetylation (H3K27Ac), which results in recruitment of bromodomain proteins and associated transcription factors. By blocking this transcriptional switch with a bromodomain inhibitor, JQ1, De Raedt *et al.* showed that they were able to restore the epigenetic status of Suz12-deficient cells to a more normal setting and reduced tumour size (up to 68%) in Nf1/TP53/Suz12 heterozygous mice (De Raedt et al. 2014). While this work implicates PRC2 as a tumour suppressor in the background of loss of function of NF1, others have reported that PRC2 also acts as an oncogene, where SUZ12 is upregulated in several human cancers, including colon, ovarian, breast and liver (Li et al. 2012). De Raedt *et al.* hypothesised that loss of function of both SUZ12 and NF1 synergised to amplify oncogenic Ras signalling to drive malignancy. This recent study provides a new therapeutic strategy that can be used in other types of cancer as well as in NF1 patients.

1.10 Pathway interaction in MPNSTs

1.10.1 STAT3 and HIF-1α

The initial link between STAT3 and HIF-1α was suggested in 2002. VEGF is a gene target of HIF-1α. An analysis of the VEGF promoter region showed a putative STAT3 binding site revealing that VEGF could indeed be activated through STAT3 in addition to HIF-1α (Niu et al. 2002). In this study a correlation between STAT3 and VEGF activity was observed, and VEGF expression was seen to be upregulated when a constitutively active STAT3 mutant was expressed (Niu et al. 2002). Furthermore, the link between VEGF and STAT3 was confirmed, when a direct up regulation of VEGF by STAT3 was seen in pancreatic cancer cell lines (Wei et al. 2003).

Later on studies revealed that both STAT3 and HIF-1α have the ability to bind to the VEGF promoter simultaneously via the transcriptional co-activators p300 and APE complex. This study suggested that in order to gain maximal activation of VEGF, both STAT3 and HIF-1α are required (Gray et al. 2005). In addition another group demonstrated that STAT3 phosphorylation can be induced via hypoxia, allowing the recruitment of HIF-1α and p300 alongside the acetylation of histone H3 resulting in the transactivation of the VEGF promoter (Jung et al. 2005).

The link between STAT3 and HIF-1α was later confirmed by STAT3 knockdown. Revealing that knockdown of STAT3 prohibits basal and also growth-factor induced HIF-1α protein expression (Xu et al. 2005). Jung, J.E., *et al.* demonstrated that STAT3 can directly bind to the C-terminal domain of HIF-1α. STAT3 then competes with VHL to bind to HIF-1α. Jung, J.E., *et al.* also showed that the over expression of constitutively active STAT3 has the ability to prohibit binding of VHL to HIF-1α (Jung et al. 2008). All studies above present strong evidence that STAT3 has the ability to modulate HIF-1α and both proteins work in synergy.

1.10.2 SPP1 and STAT3

The pathway in which SPP1 affects the JAK2/STAT3 signalling pathway is not well defined. A study performed on breast cancer cell lines has shown that SPP1 plays a role in promoting JAK2 phosphorylation and also the JAK2-dependent STAT3 activation within breast cancer cell lines. This study has demonstrated SPP1 effects cell mortality and tumour growth via the JAK2/STAT3 signalling pathway. Furthermore studies have also demonstrated an association between an increase in p-STAT3 levels and SPP1 levels that result in higher cancer progression, in patients. SPP1 regulates the JAK2/STAT3 activation via αvβ3-integrin-mediated signalling pathways, which then results in the regulation of further pathways such as Bcl2 and cyclin D1. Both Bcl2 and Cycling D1 contribute to breast cancer progression (Behera et al. 2010).

As mentioned before, the STAT3 protein acts as a transcription factor, by dimerizing and translocation in to the nucleus. The study carried out by Behera *et al.* on MDA-MB-468 breast cancer cell lines has revealed that SPP1 induces both STAT3 phosphorylation and its translocation in to the nucleus. In this study it was observed that using JAK2 inhibitors or αvβ3-integrin blocking antibody, significantly inhibited SPP1-induced STAT3 activation and localization in to the nucleus where it has the ability to bind to the DNA (Behera et al. 2010). Furthermore this this study also revealed that SPP1 plays a role in reducing and protecting the cell from Staurosporine (STS)-induced apoptosis via the JAK2/STAT3 signalling pathway. In clinical cases it has been observed that overexpression of phosphorylated STAT3 and SPP1 is highly associated with aggressive tumour growth in breast cancer patients.

1.10.3 SPP1 and MET

As previously describe SPP1 is involved in the progression of several cancer types, by promoting cell migration. It has been reported that SPP1 can induce tumourgenesis via the HGF/MET signalling pathway (Bramwell et al. 2005).

There are numerous cellular functions that are regulated by both SPP1 and HGF/MET via their interaction with αvβ1, αvβ3, and αvβ5 integrins. SPP1 plays an important role in cell attachment, cell spreading and migration similar to those of the HGF/MET signalling pathway. SPP1 and MET overexpression has been observed in several different malignant cell types, and both seem to contribute to tumour progression. In addition both SPP1 and MET increase the invasive behaviour of tumour cells. Tumour cells have to prohibit anoikis and apoptosis in order to become progressive and metastatic, both SPP1 and MET protect the tumour cells from apoptosis, contributing to the invasive migration of tumour cells (Bramwell et al. 2005).

A study performed by Tuck *et al.* has revealed that cell migration caused by abnormal activity of SPP1 involves the activation of the HGF receptor and is regulated via cross-talk between integrin's and growth factors. This study has shown for the first time that depending

on the level of tumour malignancy, various integrins couple with MET and regulate cell migration through SPP1. This study indicated that in metastatic breast cancer cell lines SPP1 and HGF interaction was mostly via αvβ3 rather than αvβ1 or αvβ5 in comparison to non-metastatic cell lines, where cell migration was regulated through a αvβ1 or αvβ5 dependent manner (Tuck et al. 2000).

1.11 Aims of thesis

NF1 disease is associated with severe phenotypic variability. This disease usually results in an increased risk of forming benign and also malignant peripheral nerve sheath tumours (MPNSTs). MPNSTs are characteristically aggressive and the major cause of mortality in NF1 patients. Clinical trials using single drug agents to treat MPNSTs have so far been unsuccessful. The molecular mechanisms underlying the signalling pathways associated with tumour malignancy in NF1 patients are poorly understood. Targeting signalling pathway thought to be responsible for tumour progression and combination therapy aiming for not one but several signalling pathways is needed for individuals with NF1-MPNSTs. The primary aims of this thesis are:

1- To determine important signalling pathways involved in progression of benign tumours into malignant peripheral nerve sheath tumours (MPNSTs).

2- To identify the role of MET and its downstream target STAT3 in the aggressive behaviour of MPNSTs. Knowing that MET is over expressed in NF1-related MPNST compared to benign tumours it was hypothesised that blocking MET signalling could possibly reduce cell migration in MPNST cell lines.

3- To investigate the relationship between STAT3 and HIF-1α in migration and invasion of MPNSTs. It was hypothesised that the knockdown of STAT3 (which lies downstream of MET) could reduce the aggressive behaviour of the MPNST cell lines.

4- To examine HIF-1α regulation in tumour progression. It was hypothesised that HIF1-α is regulated via the STAT3 pathway in these MPNST cell lines, and the blockage of STAT3 could cause a reduction in cell invasion and tumourgenesis through inhibition of HIF-1α.

5- To investigate potential therapeutic options for NF1-associated malignant peripheral nerve sheath tumours (MPNSTs). It was hypothesised that attempting to block different protein signalling pathways at the same time, could be more effective than inhibition of only one protein pathway involved in NF1 associated malignancies.

2   Chapter 2: Materials and Methods

2.1   Materials

2.1.1   Buffers and solutions

NP40 lysis buffer

50 mM Tris-HCl, 0.5 M NaCl, 50 mM β-glycophosphate, 5 mM MgCl2, 10% (v/v) glycerol

and 1% (v/v) , Adjust pH to 7.4, Nonidet-P40

Phosphate-Buffered Saline (PBS) (1X), liquid

One tablet dissolved in 200 mL of deionized water (Sigma, cat no: P4417)

Radio-Immunoprecipitation Assay (RIPA Buffer)

(Sigma, cat no: R0278)

Running buffer for SDS PAGE

2-8% gels: NuPAGE® Tris-Acetate SDS Running Buffer (Life technologies, cat no: LA0041)

4-12% gels: NuPAGE® MES SDS Running Buffer (Life technologies, cat no: NP0002) (1X:

50 mM MES, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.3)

Sample buffer

62.5 mM Tris-HCL, 50 mM DTT, 2% (w/v) SDS, 10% (v/v) Glycerol and 0.1% (w/v)

Bromophenol blue, pH 7.6

TBS

Add 2.42g Tris-base and 8g NaCl to 1l of dH20. Adjust pH to 7.6.

TBS-T

Add 2.42g Tris-base and 8g NaCl to 1l of dH20. Adjust pH to 7.6 and add 1ml Tween-20 to

give 0.1% (w/v).

Transfer buffer (10x)

144.07g Glycine, 30.285g Tris-Base, 2g SDS, Make up to 1l with dH20

2.1.2   Reagents and chemicals

Acetone (VWR, cat no: 20165.298)

Agar, Difco Noble (Becton Dickinson, cat no: 214230)

Crystal Violet (hexamethyl pararosaniline chloride) (Sigma, cat no: C0775-25G)

Dimethyl sulfoxide (DMSO) (Sigma, cat no: D8418)

Dithiothreitol (DTT) (Sigma, cat no: 43816/646563)

Ethanol (Fisher Scientific, cat no: E/06500F/17)

Glycerol (Sigma, cat no: G6279)

Hydrochloric acid (HCL) (Sigma, cat no: 320331)

Magnesium chloride, anhydrate (Sigma, cat no: M8266)

Magnesium chloride, hexahydrate (Sigma, cat no: M2670)

Mercaptoethanol (Sigma, cat no: M3148)

Methanol (Fisher Scientific, cat no: M/3900/17)

Methylated spirits (Fisher, cat no: M/4450/17)

Nonidet P40 Substitute (Sigma, cat no: 74385)

Powdered Milk (Marvel) Tesco

Sodium chloride (Fisher, cat no: S/3160/60)

Sodium Dodecyl Sulfate (SDS) (Sigma, cat no: 5030)

Tris (Sigma, cat no: 154563)

Tween-20 (Sigma, cat no: P4780)

2.1.3 Cell culture reagents

<u>β-Heregulin (rhNRG-1-β1/HRG-β1)</u>

0.2mM stock: 50µg in 31.25µl PBS and 1%BSA (R&D Systems, cat no: 396-HB)

<u>Cell freezing medium</u>

10% DMSO, DMEM, 10%FBS

<u>Cell freezing medium</u>

(Sigma cat no: C6164)

<u>Collagenase (278U/mg)</u>

2780U/ml stock: 10mg/1ml in DMEM and 10% FBS (Sigma, cat no: C0130-100MG)

<u>Dispase I (6U/mg)</u>

30U/ml: 5mg/1ml in dH2O (Roche, cat no: 04942086001)

<u>Dulbecco's Modified Eagle Medium (DMEM)</u>

L-glutamine, 4500 mg/L D-glucose, 110mg/L sodium pyruvate (Gibco, Invitrogen, cat no: 41966-029)

<u>Fetal Bovine Serum (FBS)</u>

(Sigma, cat no:)

<u>Forskolin</u>

2µM stock in 96% ETOH. (Sigma, cat no: F-6886)

<u>Human Fibronectin</u>

(R&D systems, cat no: 1918-FN)

Insulin

1mg/ml stock in PBS plus 5µl of 1M HCL (Sigma, cat no: 16634-50MG)

3-Isobutyl-1-METhylxanthine (IBMX)

Powder dissolved in 50% Ethanol and used at 0.5M (Sigma cat no: 15879)

N2 supplement

(Gibco, cat no: 17502-018)

Penicillin/Streptomycin (Pen/Strep)

10 000 units of penicillin (base) and 10 000 units of streptomycin (base)/ml utilising penicillin

G (sodium salt) and streptomycin sulphate in 0.85% saline (Invitrogen, cat no:15140-122)

Poly-L-Lysine

0.1 mg/ml stock (Sigma, cat no: P4707)

Poteinase K

1mg/ml stock in dH2O (Sigma, cat no: P2308-500MG)

Puromycin

(Invitrogen, cat no: A11138-03)

Trypsin-EDTA solution

(Invitrogen, cat no: R-001-100)

2.1.4   Stimuli and Inhibitors

Hepatocyte growth factor (HGF) (R&D systems, cat no: 3386-HG/CF)

Intelukin-6 (IL-6) (R &D systems, cat no: Q75MH2)

Platelet-derived growth factor (PDGF) (R&D systems, cat no: 120-HD)

Cucurbitacin I (Sigma, cat no: C4493)

FLLL31 (Sigma, cat no: F9057)

5, 15 DPP (Sigma, cat no: D4071)

SU11274 (Sigma, cat no: S9820)

PF-04217903 (Pfizer, cat no: S1094)

Rapamycin (Merck Millipore, cat no: 553212)

KU-0063794 (sigma, cat no: SML0382)

2.1.5   Antibodies

STAT3 (F-2)/ Mouse Monoclonal (Santa Cruz, cat no: sc-8019)

STAT3 / Rabbit mAb (Cell Signalling, cat no: 4904S)

p-STAT3 (Tyr705)/ goat polyclonal (Santa Cruz, cat no: sc-7993)

p-STAT3 (Tyr705)/ Rabbit IgG (Cell Signalling, cat no: 9145)

Phospho-STAT3 (Tyr705) (3E2) Mouse mAb (Cell Signalling, cat no: 9138)

Phospho-STAT3 (Ser727) Antibody (Cell Signalling, cat no: 9134)

MET(c-28) / Rabbit Polyclonal IgG (Santa Cruz, cat no: sc162)

Anti-MET antibody (abcam, cat no: ab10728)

Anti-phospho-MET (Tyr1234/1235)/rabbit monoclonal IgG (Millipore, cat no: 50-900)

HIF-1α (cell signalling, cat no: 14179)

HIF-2α (cell signalling, cat no: 7096)

VEGF (Millipore, cat no: GF25)

β-actin (Cell Signalling, cat no: 4967)

Phospho-rpS6 (Ser235/236) (cell signalling, cat no: 4858)

2.2   Methods

2.2.1   Cell culture

2.2.1.1   Cell lines

ST8814 MPNST cells, derived form a NF1 patient, were purchased from ATCC (distributed by LGC Standards, Middlesex, UK). The S462 MPNST cell line, a cell line established from MPNST 24472, which was taken from a 19 year old female NF1 patient; S1844.1 and S1507.2 cells, derived form a NF1 patient, were a kind gift from Prof. Mautner, (University of Hamburg, Germany) and the late Prof. Guha, (University of Toronto, Canada). All cell lines mentioned were cultured in 75 cm2 flasks unless stated otherwise, with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) Foetal Calf Serum (FCS) and 1% (v/v) Pen Strep. Cell lines were incubated at 37 oC in 5% CO_2 and split 3 times a week.

2.2.1.2   Lentivirus generation and shRNA knockdown of STAT3 and HIF-1α in MPNST cell lines

STAT3 shRNA (Clone ID: NM_003150.3-458s21c1), HIF-1α shRNA (Clone ID: NM_001530.x-3867s1c1) or SPP1 shRNA (Clone ID: NM_000582.2-597s1c1) and non-target control MISSION shRNA (Clone ID: SHCO16) in pLKO.1 vector (Sigma-Aldrich Company Ltd.) were packaged into lentivirus using HEK293T cells and co-transfected (lipofectamine 2000, Life Technology) with pLP1, pLP2, and pLP (VSVG). Confluent MPNST cell lines were infected with shRNA containing lentivirus (STAT3, HIF-1α, and SPP1 and non-target control) and selected over 2 weeks with 5 mg/mL puromycin (Life Technology). Puromycin selected mixed cell populations were then used for further experiments.

2.2.1.3 Establishment of benign cell line (Schwann cell culture) from patient tumour (protocol provided by Dr. Laura Thomas)

Neurofibromas were received from the NF1 patients who had consented to help with Prof. Upadhyaya's research on "NF1 tumorigenesis" and this project was ethically approved in 1997.

The presence of forskolin in the medium promotes the proliferation of Schwann cells. This occurs through elevation of intracellular cAMP and suppression of fibroblast growth. Replacement of proliferation medium by serum-free N2 medium (Bottenstein and Sato, 1979) followed by medium without forskolin, promotes schwann cell differentiation. N2 medium is a serum-free synthetic medium supplemented with insulin, transferrin, progesterone, selenium, and putrescine. Cells respond to the removal of serum through extensive process formation and retention of other differentiated properties. Growth of neuronal cells is stimulated with the N2 medium and non-neuronal growth is suppressed. Cells were passaged when cultures were confluent and harvested not earlier than at passage eight.

Preincubation

3ml of preincubation medium (DMEM +10% FBS, 1% Pen/strep Forskolin [2☐M]) filtered once through Millex syringe driven 0.22µM filters (Millipore, cat no:SLGS033SS) was added to each well on a 6 well plate (Nunclon, cat no: 140675). The tumours were dissected in DMEM + 10% FBS and a small piece from the centre of the tumour was dissected out and put into the pre-incubation medium in the 6 well plate. The tumour sections were then left to incubate for 10-14 days with the medium changed every 3 days without changing the plate or washing with PBS.

Incubation

Following the 10-14 day incubation in the preincubation medium, the tissue was transferred to a new 6 well plate and dissected into smaller pieces using a scalpel. Samples were then

incubated for 24 hours in 3ml of DMEM + 10% FBS, 1% PenStrep, Collagenase [160U/ml] and Dispase I [0.8U/ml] filtered once with the Millex 0.22µM filter.

Coating flasks and plates

A T25 flask (Nunclon, cat no:136196) was coated with 1.2ml of sterile Poly-L-Lysine solution. This was left for 1 hour at room temperature and was then washed with sterile PBS. 1.2ml of 4µg/ml laminin was then filtered and added to each flask. This was incubated for 1 hour at 37ºC, excess removed and then the flask was washed with PBS. The tissue in the 6 well plate was broken up using a pipette, transferred to a falcon tube and centrifuged for 10 minutes at 1200rpm. The supernatant was discarded and the pellet was resuspended in 5ml of DMEM + 10% FBS, 1% Pen/Strep, IBMX, [0.5mM], β-Heregulin [10nM], Forskolin [2µM] and Insulin [2.5µg/ml] and incubated for 24 hours. The medium was removed the following day and the N2 medium was prepared (10ml DMEM F12, 100µl/10ml N2 Supplement and 1% Pen/Strep) filtered as before, added to the 6 well plate and incubated for 24 hours. The following day the N2 medium was removed and replaced with 5ml of medium without Forskolin: DMEM +10% FBS, 1% Pen/Strep, IBMX [0.5mM], β-Heregulin [10nM] and Insulin[2.5µg/ml] and incubated for 6 days under standard conditions, with the medium replaced after 3 days. After 6 days the cells were confluent and so were transferred to a larger T75 flask, coated with laminin and poly-l-lysine as previously described. The cultures were then harvested following the 8th-12th passage, the Schwann cell content of each culture being established by S100 immunohistochemistry (Serra et al ., 2000). S100 is a calcium binding protein specifically found in Schwann cells of the peripheral nerve (Mirsky and Jessen, 1996). Immunohistochemical analysis of Schwann cell lines used in all experiments was carried out to ensure that the population of cells that had been cultured was pure. Due to the Method of fixation used for these cultures, while differential S100 staining could be identified, the morphology of the cells was often disrupted.

2.2.1.4 Splitting cells

Once cell were confluent, cells were passaged, all four MPNST cell lines (ST8814, S462, S1844.1 and S1507.2) were washed three times in EDTA/Trypsin. This was then removed from flask via aspiration and cells were incubated in 37oC for 3-5 mins. Cells were then examined under the microscope to insure that all cells had detached from flask. Cells were then resuspended in DMEM and transferred to a new flask.

2.2.1.5 Freezing cells for long term storage

For long term storage, cells were first trypsinized and resuspended in 6 ml of DMEM. Cells were then span down for 5 min at 2000gp. The cell pellet was then resuspended in freezing media and put in to cryogenic freezing container (FCS supplemented with 8% (v/v) DMSO) and stored in cryogenic vials in liquid nitrogen.

2.2.1.6 Cell lysis

Protein extraction:

In Sample buffer:

Under normal conditions

Confluent cells were starved for 4 hours prior to lysing. (Cells were treated with stimuli's or inhibitors appropriately before lysing). In order to harvest cells, cells were first washed once in PBS, before being resuspended in sample buffer. Cells were the sonicated for 3 x 5 s cycles on full power (30microns) before centrifugation at 13,000rpm for 5 min.

Under hypoxic conditions

Lysates were parped as described in section? . However cells were incubated in a hypoxic chamber (37 ^{o}C, 5% CO2 and 1% O2). A binder CB-150 hypoxic chamber was used. After appropriate treatments, cells were then placed into the hypoxic chamber for the indicated times. The incubator was then adjusted to 1% O2 for the whole duration of the experiments.

The binder series of hypoxic incubators allows precise control of oxygen concentrations, making it possible to preform experiments under hypoxic conditions.

In lysis buffer for Immunoprecipitation (IP)

Confluent cells were starved for 4 hours prior to lysing. (Cells were treated with stimuli's or inhibitors appropriately before lysing). In order to harvest cells, cells were first washed once in PBS, and lysed lysis buffer supplemented with protease inhibitors 1mM Na_3VO_4, 2µM antipain, 10µM leupeptin, 1µg/ml pepstatin, 0.1mM PMSF, 1mM DTT and 1mM benzamidine (The protease inhibitors were either added separately to lysis buffer or protease inhibitors tablets were used). All steps mentioned are performed on ice. Cells were then centrifuged for 13.000rpm, 8 min at 4^oC.

mRNA extraction

Lysates for Q-PCR were lysed in RNA protect buffer (no protease inhibitors) then centrifuged at 5,000 rpm for 5 min at 4oC, supernatant was aspirated and removed. The cell pellet was then stored at -80oC until mRNA extraction.

2.2.2 Immunoprecipitation (IP)

Immunoprecipitation uses the antigen-antibody reaction principle to identify a specific protein of interest. A protein can therefore be purified from a mixture of proteins to assess its quantity or physical characteristics. Cells were lysed in 1ml of lysis buffer (see above). Samples were put through several freeze thaw cycles to aid in cell lysis and finally sonicated for 3 x 5 s cycles on full power (30microns) to break up cells. The cells were then centrifuged at 13,000rpm for 8 minutes at 4ºC to pellet cell debris and remove air bubbles. Protein concentrations were assessed with use of Bradford reagent, in accordance with the manufacturer's protocol (Sigma-Aldrich Company Ltd.). For each Immunoprecipitation, lysates were diluted up to a 1ml volume in lysis buffer then rotated for 2 h at 4oC with 0.4% (v/v) antibody. 40µl of a 50:50 slurry of protein-G beads was then added and samples were

rotated for 1 h at 4oC. Beads were then washed 3 times in lysis buffer (plus protease inhibitors) before elution in 40µl of x1 sample buffer.

2.2.3 Bradford protein assay

For each measurement, 12.5µl of sample solution was diluted in 750µl of Bradford reagent and absorbance was measured and a standard curve generated using the Nanodrop spectrophotometer at 570nm. Three measurements were taken per concentration to generate a standard curve.

2.2.4 Western blot

The Invitrogen NuPage Novex gel system was used for electrophoresis. A 3-8% or 4-12% Tris-acetate gels was used (depending on sample size). Samples were loaded in to the wells and the Gel was run at 150 V for 1 h. Proteins were then transferred to a polyvinylidene fluoride membrane (PVDF), (Millipore, cat no: IPVH 000 10), The membrane was briefly pre-treated with 100% Methanol and then washed off in 1x transfer buffer for 5 minutes, and run at 25 V for 2 h in transfer buffer using the wet transfer system. The membrane was then blocked in 5 % (w/v) dry milk powder in TBS-T for 4 h (could be less or more usually leave for at least 2 hours). Membrane was incubated at 4 oC (cold room) overnight in primary antibody (1:1000 dilution in 2 % BSA (w/v) TBS-T). The membrane was then washed twice for 4 min in TBS-T and incubated in appropriate HRP-conjugated secondary antibody (1:10,000 dilution in TBS-T) for 30-60 min (Milk is added to the secondary antibody if needed) at room temperature. The membrane was then washed four times for 3 min with TBS-T and then incubated in Enhanced Chemiluminesence (ECL) solution (Amersham) for 1 min. Membranes were then exposed to photo-reactive film (Fujifilm, cat no: 4741008389) to visualize the signal.

2.2.5 Quantitative-PCR

For the extraction of mRNA, as mentioned before cells were harvested in RT-protect buffer and centrifuged at 5,000 rpm for 5 min to produce a pellet, the supernatant was discarded

and the pellet saved for mRNA extraction (or stored at -80oC for future experiments). mRNA was extracted using the Qiagen (West Sussex, UK) mRNA extraction kit in accordance with manufacturer's protocol, QIA shredders (Qiagen) were utilised to homogenise the pellet. Resulting mRNA concentration was determined using the nanodrop spectrophotometer.

Total RNA from each sample (1 µg) was transcribed into complementary DNA using a Quantitect reverse transcription kit (Qiagen) in a thermal cycler (Applied Biosystems). Quantitative real-time PCR reactions were conducted in 96-well plates using appropriate primer assays and Sybr Green PCR Master mix (Qiagen). Assays were performed as follows:

1.   Initial denaturation step (95 °C, 15 min),

2.   40 cycles of denaturation (94 °C, 15 s),

3.   Annealing step (55 °C, 30 s)

4.   Extension step (72 °C, 40 s).

The amplification products were quantified during the extension step in the fortieth cycle. The results were standardized to β-actin control. A dissociation step was performed, which verified that only one PCR product was produced with each primer set and shows their specificity. The correct amplicon size of PCR products was also verified by resolution on 1% (v/v) agarose gels.

2.2.5.1 Primers

MET primers used were forward 50-CCACCACAGTCCCCAGAGT- 30 and reverse 5'-AGATCACATGAACACAGGA-3'. VEGF-A primers used were forward 5'-GGAGAGCAGAAGTCCCATGA-3' and reverse 5'-ACTCCAGGGCTTCATCGTTA-3'. The following primer sets were purchased from Qiagen, who have the right to withhold primer sequence information: HIF-1α (cat no. QT01039542), β-actin (cat no. QT01680476).

2.2.6 Wound healing

Cells were seeded in 60 mm plates and left to reach 100% confluency. Cells were then synchronised in 1% (v/v) FBS DMEM for 24 h and "wounded" with a pipette tip. Dead cells were removed with PBS wash and then subsequently replaced with DMEM (10% (v/v) FBS). Cells were pre-treated for 30 min with either rapamycin, MET or STAT3 inhibitors (where indicated) before cytokine stimulation. Pictures were taken before treatment and 12-18 h after treatment using an inverted AMG EVOS microscope equipped with an Olympus camera.

2.2.7 Migration and Invasion Assays

Transwell permeable supports with 6.5 mm diameter inserts, 8.0 µm pore size and a polycarbonate membrane (Corning, cat no: 3428) were used to perform migration assays. Cells were grown in a $75cm^2$ flask with standard medium (10% (v/v) FBS) until confluent. Cells were then harvested using Trypsin-EDTA. Cells were counted using a haemocytometer. $1x10^6$ cells were resuspended in DMEM containing 1% (v/v) FBS. These cells were then seeded in the upper chamber of the Transwell; the lower chamber was filled with 600 □l of standard culture medium (10% (v/v) FBS) and 5 µg/ml fibronectin (R&D systems, Abingdon, UK), as an adhesive substrate. Cells were incubated at 37 oC 5% CO_2 for 24 h. The percentage of adherent cells was then determined by fixing the cells with Methanol and acetone (1:1) for 20 min at -20°C. Cells were then stained with Crystal Violet (5 mg/ml) in ethanol for 10 min, followed by a stringent wash with dH_2O until the water ran clear. Crystal violet stained cells were eluted with 1% (w/v) SDS and the absorbance was read at 550 nm on a Genova MK3 Lifescience Analyser (Jenway Scientific, Straffordshire, UK). For invasion assays, a similar protocol was employed; however the top chamber of the Transwell was filled with 300 µl of BD Matrigel Basement Membrane Matrix (1 mg/ml). The Matrigel was incubated at 37 ºC for 4 h to allow it to gel. Cells were then seeded and incubated as described for migration assay for 3 days. The number of invaded cells was determined by fixation staining and elution of crystal violet with 1% (w/v) SDS, as before.

2.2.8 Tumour spheroid assays

Two-layered soft agar assays were carried out in six-well plates. MPNST cell lines were plated in complete DMEM media in 0.35% (v/v) agar at (3 x 10x5) over a 0.6% (v/v) agar layer. The agar was then overlaid with complete DMEM media supplemented with 0.1 μM puromycin (Life Technology, Paisley, U.K.), and then colonies of MPNSTs were grown for 14 days at 37 °C in 5% CO2. Media was changed twice a week. Pictures were taken using an inverted AMG EVOS microscope equipped with an Olympus camera. Volume of tumour spheroids was measured using ImageJ software.

2.2.9 Cyquant cell proliferation assay

Cells were seeded at 4000 per well of a 96 well plate. Cells were treated with appropriate drug for 24 h or 48 h, the media was then removed and the plate was frozen at –80 ºC. Cell proliferation rate was determined using the CyQuant® Cell Proliferation Assay Kit (Life Technologies) following the manufacturer's protocol. Essentially, lysis buffer containing CyQUANT GR dye was added to each well and gently pipette to allow lysis of the cells and incorporation of the dye to the DNA. Fluorescence was detected by excitation at ~485 nm and emission detection at ~530 nm using a plate reader. Cell number was calculated using a standard curve generated from 250 to 50000 cells.

3 Chapter 3: Involvement of hepatocyte growth factor and MET in the development of malignant peripheral nerve sheath tumours (MPNSTs)

3.1 Introduction

MET activation promotes a multifaceted system of intercellular signalling cascades that leads to cell proliferation and migration. HGF the sole ligand of MET is secreted by mesenchymal cells adjacent to MET-expressing epithelial cells throughout embryogenesis or during tissue injury, functioning as a paracrine signalling mechanism. MET activity is involved in the genesis and progression of various cancers and it has been suggested that MET activation also plays an important role in tumourigenesis. Current evidence implies that the HGF/MET signalling pathway plays a vital role in the tumour growth and invasive properties of MPNSTs and enhancement of MET expression has previously been observed (Sharif et al. 2006; Rao et al. 1997; Torres et al. 2011).

An earlier study by Thomas *et al.* on 14 malignant and 7 benign patient tumours revealed that in many of the malignant tumours MET expression was relatively high when compared to the benign tumours (unpublished data by Meena Upadhyaya's group). From this initial data, I hypothesised that MET is likely involved in malignant transformation of some MPNST cell lines. Using different MET inhibitors in conjunction with western blot analysis, migration and invasion assays, this chapter aims to characterise the involvement of the HGF/MET signalling pathway in tumour progression within MPNST cell lines.

There is also apparent cross talk between MET and other receptor tyrosine kinases in other cancers (Viticchiè & Muller 2015). Therefore, one aim in this chapter was to address whether MET signalling to downstream targets such as STAT3 could be influenced through other receptor tyrosine kinases that are also linked to malignancy in MPNST cells, such as the PDGFR and IL-R. In this chapter it was also uncovered that inhibition of mTORC1 by treatment with rapamycin was sufficient to block HGF-induced activation of MET. This novel finding suggests that mTORC1 activation is required for HGF-induced activation of MET.

Therefore, it is possible that mTORC1 and MET might cooperate to drive malignant signalling within MPNST cells. To examine this possibility, rapamycin was used to inhibit mTORC1 as a single agent and also in combination with MET inhibitors. Although the involvement of mTORC1 in malignancy of MPNSTs has been previously implicated the connection between mTORC1 and MET has not been analysed in detail. It is currently known that Nf1-deficient astrocytes have an abnormally activated mTORC1 signalling pathway as apparent by aberrant levels of phosphorylated ribosomal S6 protein in NF1 mutant mice and NF1 human patient samples (Dasgupta et al. 2005). In this study by Dasgupta *et al.* inhibition of mTORC1 restored growth and proliferation to a more normal level within mutant NF1 mice (Dasgupta et al. 2005).

3.2 Results

3.2.1 Variable expression of MET in multiple MPNST cell lines

MET gene expression was examined by Thomas *et al.* using qPCR in 14 NF1-MPNST tumour samples and 7 benign neurofibromas (figure 3.1 A). 8 out of the 14 NF1-MPNST tumour samples showed elevated levels of MET mRNA in comparison to the 7 benign tumours. Having this preliminary data the first aim was to investigate the mRNA expression levels of MET in 4 different MPNST cell lines, ST8814, S462, S1844.1 and S1507.2 compared to a benign control. To do this mRNA was extracted from all 4 MPNST cell lines and quantitative-PCR was utilised to determine MET mRNA levels. The ST8814 MPNST cell line showed the highest level of MET expression (2.7 fold) compared to the benign cell line, followed by the S1844.1 (1.9 fold) and S1507.2 (1.6 fold) cell lines. The S462 cell line showed very low levels of MET expression and expressed similar MET to the benign cell line (figure 3.1 B). Such variance in MET expression in MPNSTs indicates that MET expression (as a single readout of malignancy) would not be a reliable readout of malignancy in the heterogenous MPNST population.

Due to the biological diversity of proteins within a cell, the correlation of protein levels with the associated mRNA can vary. Therefore, we also analysed the protein levels of MET in the MPNST cell lines. Similar to the MET mRNA levels, we observed variation in the level of MET protein expression. MET was elevated in the ST8814, S1844.1 and S1507.2 MPNST cell lines (highest expression in the ST8814 cells), but not the S462 cells (figure 3.1 C). This variation in MET expression, both at mRNA and protein levels, highlights the heterogeneous status of MPNSTs.
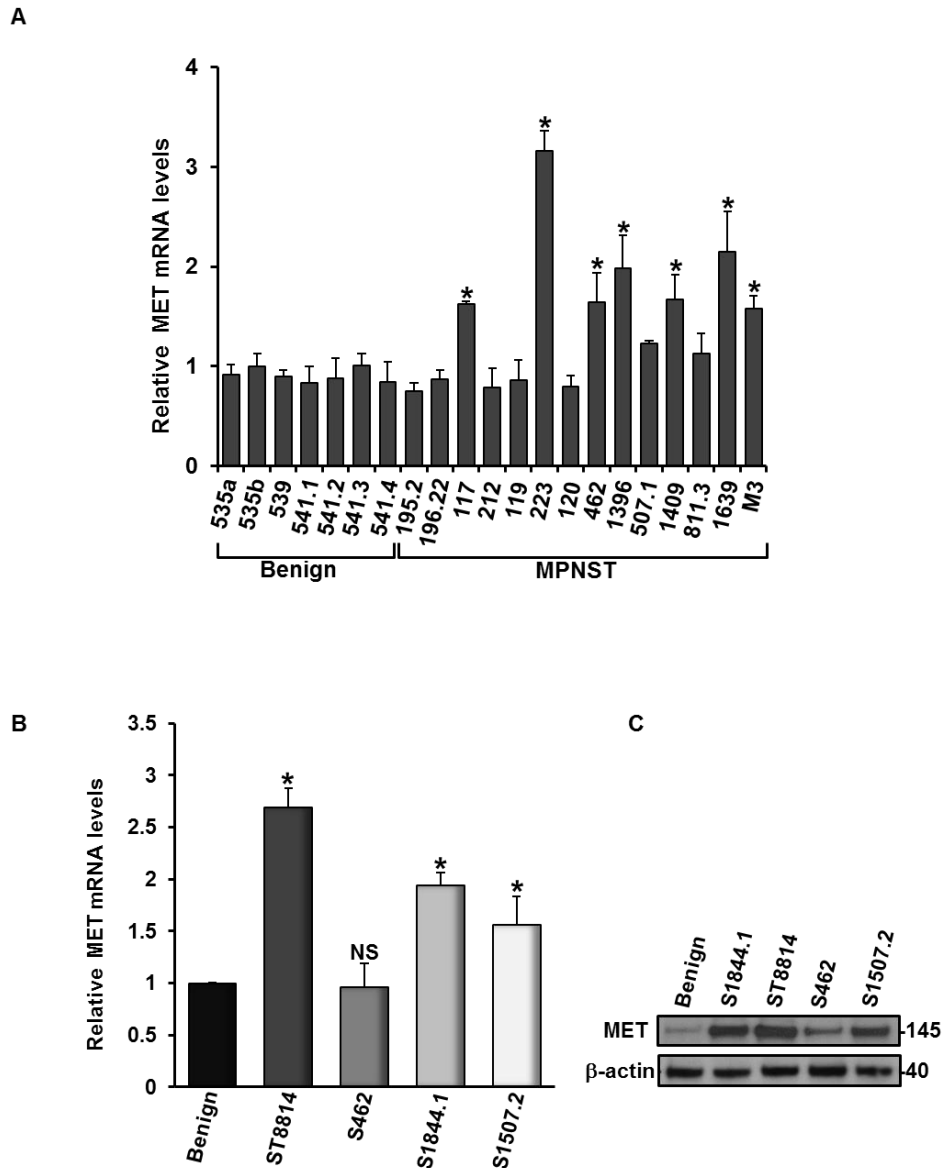
Figure 3.1. Variable expression of MET in multiple MPNST cell lines. Gene-expression of MET is frequently elevated in MPNSTs. **A)** MET mRNA levels, standardised to β-actin, was assessed in benign versus malignant tumours from NF1 patients. Error bars indicative of standard deviation from three independent experiments each performed in triplicate, * $p < 0.05$ **B)** MET mRNA levels, standardised to β-actin, was assessed in 4 MPNST cell lines (ST8814, S462, S1844.1 and S1507.2), Error bars indicative of standard deviation from three independent experiments each performed in triplicate **C)** MET protein levels were compared in four MPNST cell lines (ST8814, S462, S1844.1 and S1507.2) and a benign cell line (Schwan cells). β-actin protein serves as a control. Three independent experiments were performed.

3.2.2 Sensitivity to MET inhibition in multiple MPNST cell lines

Two MET inhibitors, SU11274 and PF-4217903 were used to block MET signalling. SU11274 is a first generation MET inhibitor that competes with ATP to bind to the activation loop of MET. PF-4217903 is a novel ATP-competitive small molecule inhibitor of MET and is clinically viable. PF-4217903 showed efficacy in a patient with MET driven papillary renal cell carcinoma (RCC) (Kawachi et al. 2013) and subsequently reached phase I clinical trials.

In all four MPNST derived cell lines, the MET inhibitors, SU11274 and PF-4217903, were sufficient to block HGF induced MET activation, as observed by a reduction in Tyr1234/1235 phosphorylation of MET (Figure 3.2). Of interest, both platelet-derived growth factor (PDGF) and interleukin-6 (IL-6) caused MET activation in the ST8814, S1507.2 and S462 cells (Figure 3.2) revealing cross talk between MET and the PDGF receptor (PDGF-R) and Interleukin receptor (IL-R) in MPNST cells. This is in concordance with previous studies identifying crosstalk between multiple receptor tyrosine kinases and MET in cancer cell lines (Yeh et al. 2011; Lan et al. 2013). This data reveals that MPNSTs do not require HGF to activate MET, but instead can employ a range of receptor tyrosine kinases that are linked to malignant progression of MPNST cells.
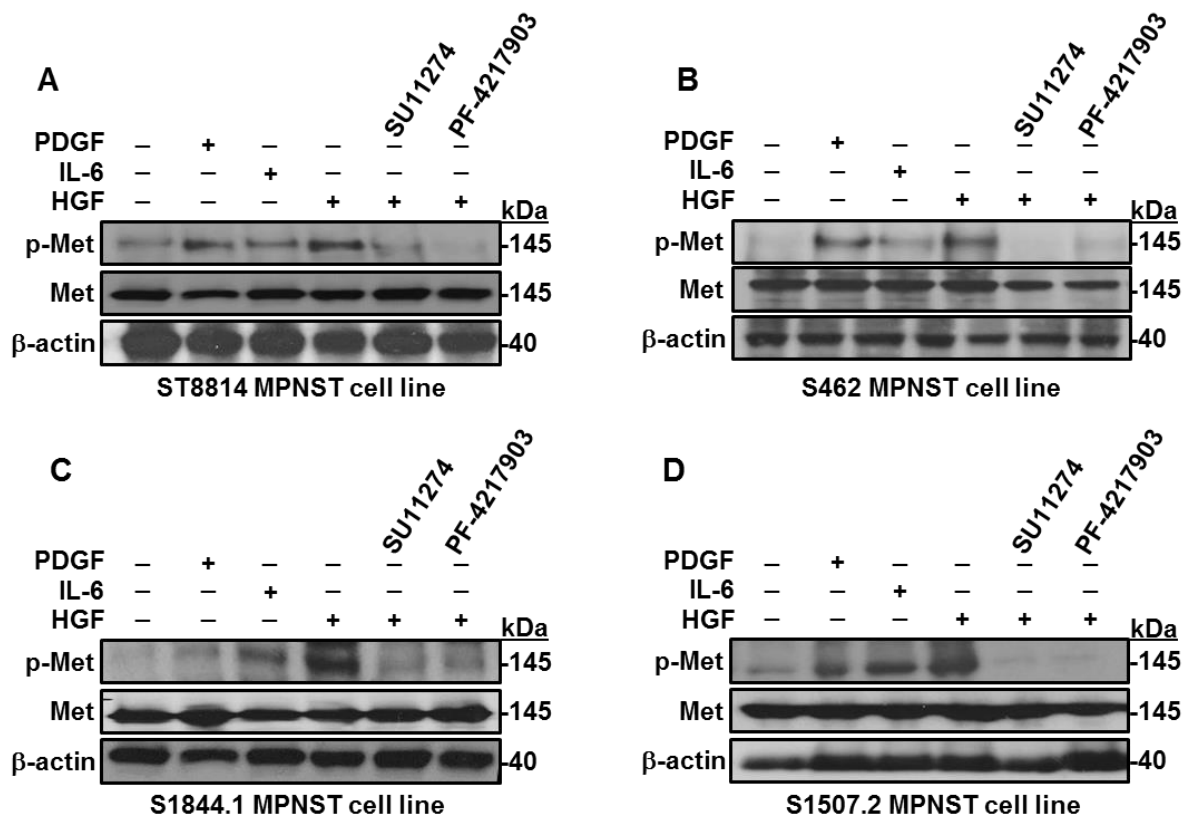
Figure 3.2. Sensitivity to MET inhibition in multiple MPNST cell lines. Where indicated, starved **A)** ST8814, **B)** S462, **C)** S1844.1 and **D)** S1507.2 cells were pre-treated with 10 nM SU11274 and 7.5 ng/µl PF-4217903 for 30 min prior to 30 min stimulation (10 ng/ml PDGF, 20 ng/ml IL-6, and 20 ng/ml HGF). DMSO vehicle only was used as a control. Total and phosphorylated MET (try1234/1235) and β-actin was analysed. Three independent experiments were performed for each cell line.

3.2.3 The role of MET/HGF signalling pathway in wound healing of MPNST cell lines

In this study, it was hypothesised that the 4 MPNST cell lines may have differential dependency on migratory cell signalling pathways due to the heterogeneous nature of MPNSTs. To test this hypothesis, initially the effects of MET inhibition on signalling pathways that could contribute to malignancy were analysed. This study shows that HGF induced wound healing in the ST8814, S1844.1 and S1507.2 cells was significantly reduced after inhibition of MET with both the SU11274 and PF-4217903 inhibitors (Figure 3.3). In contrast wound healing in the S462 cell line was insensitive to MET inhibition during the 18 h wound healing assay (Figure 3.3). These results suggest that instead of utilising the migratory HGF/MET signalling pathway for wound healing, the S462 cell line is more dependent on other migratory signalling pathways. Critically, this data reveals that MPNST cell lines can exhibit varied sensitivity to MET inhibition, suggesting that therapeutic targeting of MET may not be a suitable treatment strategy for all NF1 patients.

The significance of MET inhibition during wound healing was analysed by preforming a one way ANOVA. The percentage of migrated cells were determined by measuring the area of the wound before and after drug treatment in each cell line (figure 3.4).
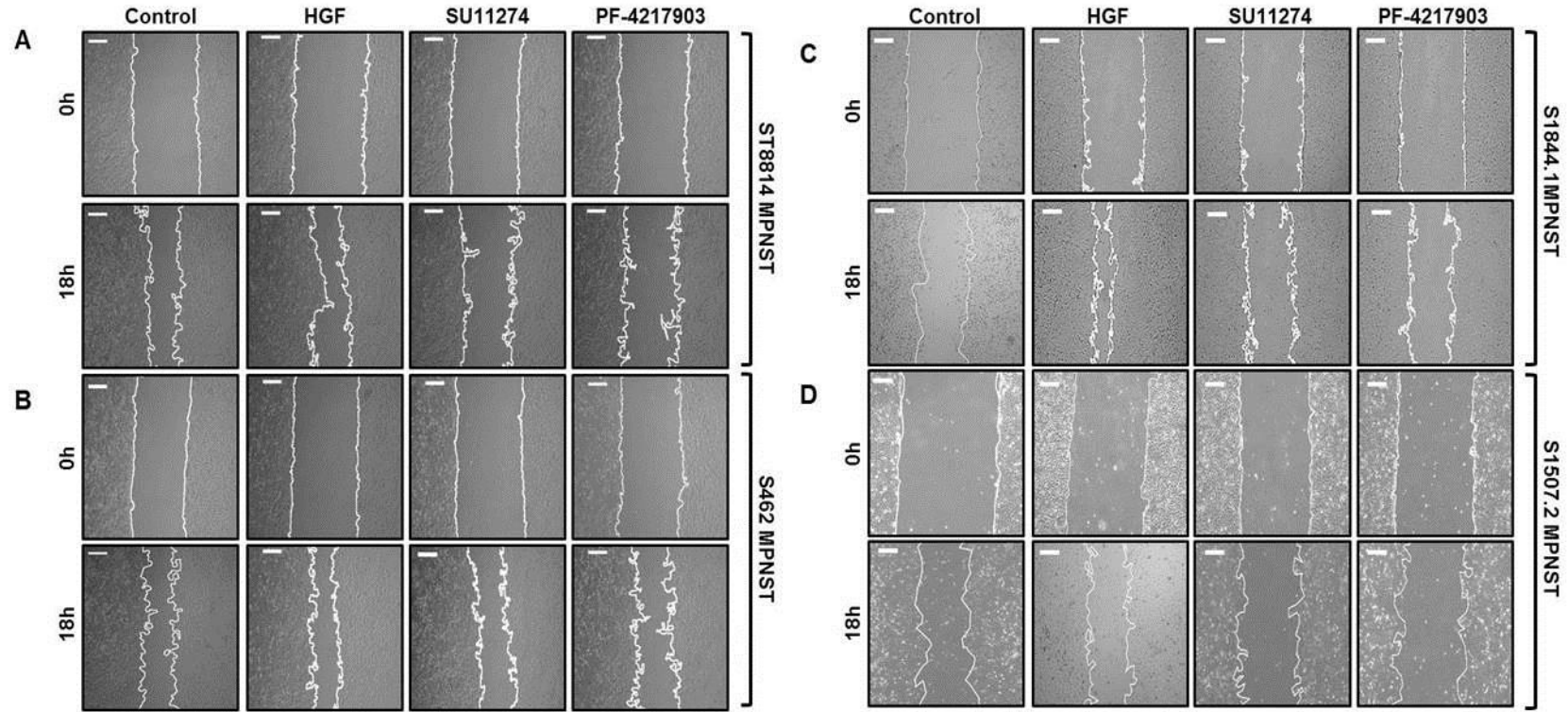
Figure 3.3. The role of MET/HGF signalling pathway in wound healing of MPNST cell lines. Where indicated, **A)** ST8814, **B)** S462, **C)** S1844.1 and **D)** S1507.2 MPNST cell lines were seeded in 60 mm plates, once cells were 90% confluent, cells were starved (1% FBS) for 24 h prior to wound healing assay. Each plate was scratched using a pipette tip, pictures were taking at 0 h. Cells were then stimulated using 20 ng/ml HGF in the presence or absence of 10 nM SU11274 and 7.5 ng/µl PF-4217903 where indicated and placed in the incubator (5% CO_2 /37 ^{O} C) for 18 h before taking pictures. (Scale bar represents 250 µm. Cells were imaged on a microscope at x10 magnification). 3 Individual experiments were performed. Pictures shown here are representative of the 3 individual experiments carried out.
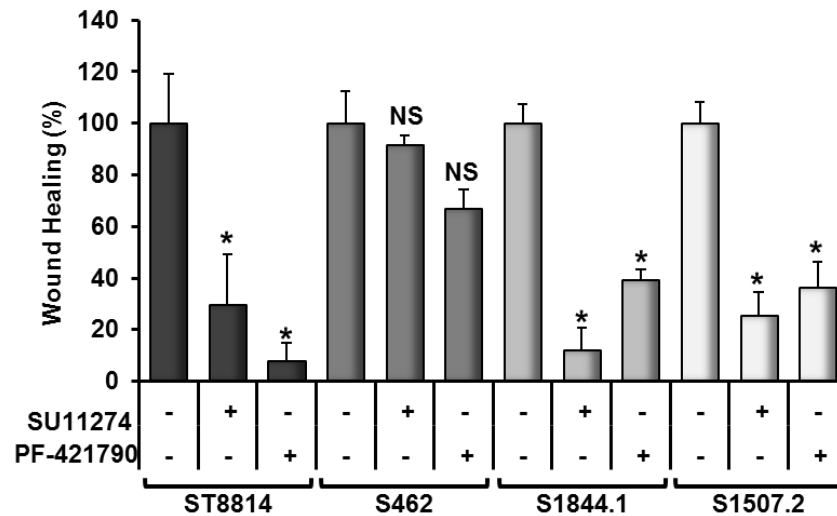
Figure 3.4. MET inhibition potently impairs HGF induced wound healing in 3 MPNST cell lines while the S462 cell line show resistance to MET inhibition. The significance of MET inhibition on wound healing was determined by one way ANOVA. The percentage of migrated cells were compared in all 4 MPNST cell lines. Error bars are indicative of standard deviation from three independent experiments * $p < 0.05$ when comparing wound closure of treated versus untreated cells.

3.2.4 Inhibition of MET impairs cell migration and invasion in 3 of the 4 MPNST cell lines

The next step in this study was to analyse the effectiveness of MET inhibition upon cell migration and invasion under HGF stimulation. For cell migration, cells were seeded on a Transwell permeable supports with 6.5 mm diameter inserts, 8.0 μm pore size and a polycarbonate membrane. The same protocol was used for invasion assays, however the top chamber of the Transwell was filled with 300 μl of BD Matrigel Basement Membrane Matrix. Cells were then treated with the MET inhibitor, SU11274, prior to HGF stimulation.

The results clearly demonstrate that in the ST8814, S1844.1 and S1507.2 cell lines, inhibition of MET (SU11274) significantly decreased both cell migration and invasion (Figure 3.5).

Conversely, cell migration and invasion in the S462 cells was not significantly inhibited by the MET inhibitor (Figure 3.5). The insensitivity of the S462 cells to MET inhibition indicates that the migratory and invasive properties of these cells are less dependent on HGF/MET signalling as mentioned before. These cell lines depend on other signalling pathways for their migratory characteristics.
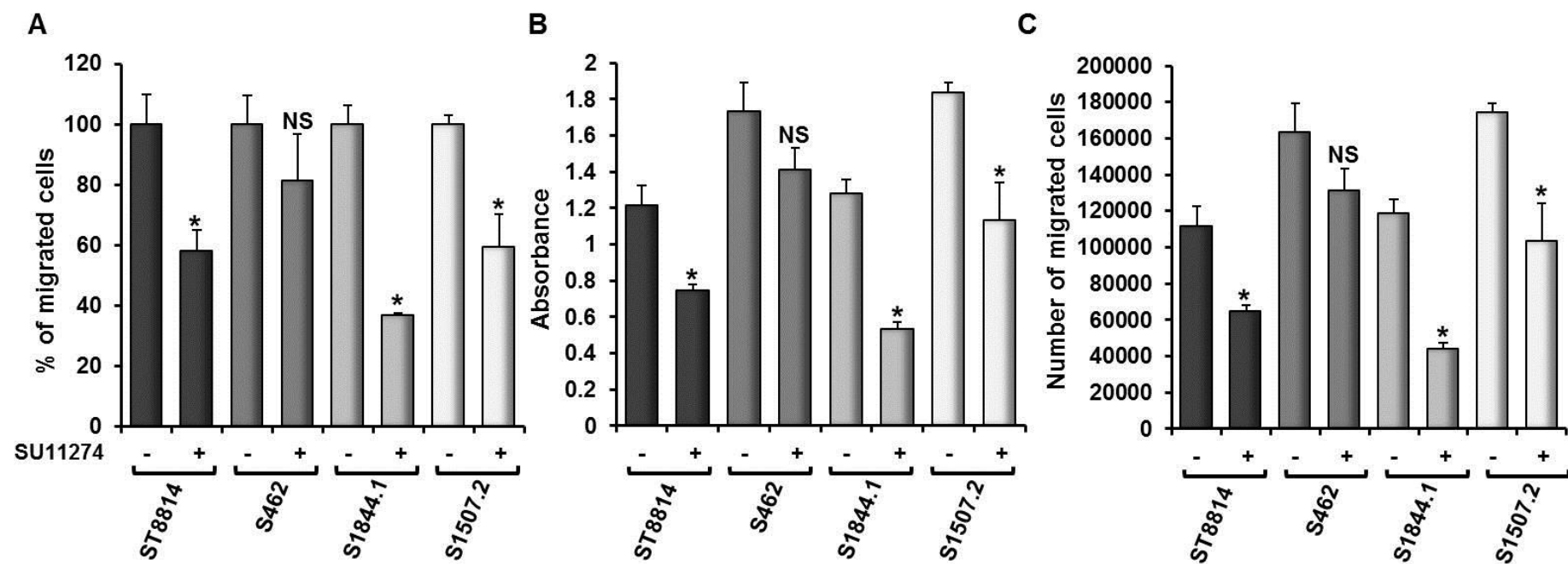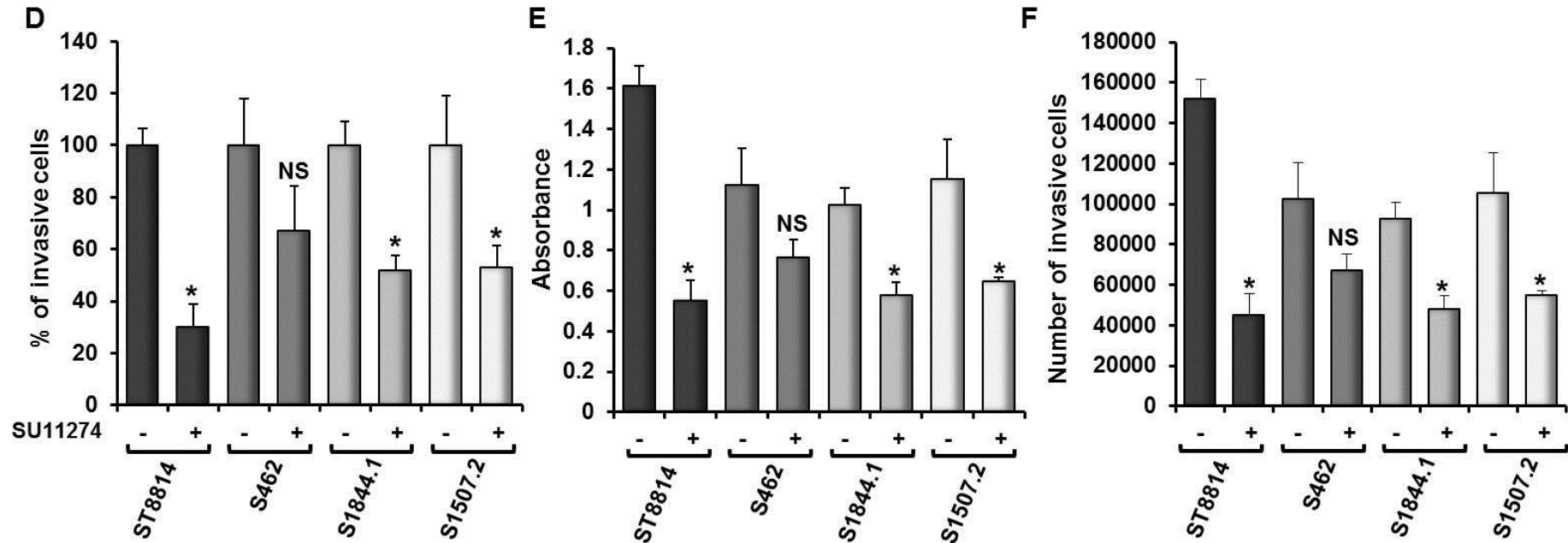
Figure 3.5. Inhibition of MET can impair cell migration and invasion in 3 MPNST cell lines. Cell migration and invasion assay was carried out on ST8814, S462, S1844.1 and S1507.2 cell lines in the presence or absence of 10 nM SU11274 (MET inhibitor) prior to 20 ng/ml HGF stimulation in accordance to the protocol described in section 2.2.7. **A)** Graph demonstrates the percentage of migrated cells in each cell line **B)** Graph demonstrates the absorbance read on the Genova MK3 Lifescience Analyser of migrated cells (see section 2.2.7). **C)** This graph demonstrates the number of cells migrated in control vs treated cell lines, as seen from the graphs above these MPNSTs have different migratory rates. **D)** Graph demonstrates the percentage of invasive cells in each cell line **E)** Graph demonstrates the absorbance read on the Genova MK3 Lifescience Analyser of invasive cells (see section 2.2.7). **F)** This graph demonstrates the number of invasive cells in control vs treated cell lines.

3.2.5 Inhibition of MET reduces cell proliferation in 3 of the MPNST cell lines

MET and its only ligand HGF play a key role in cell proliferation and survival in various human cancers (Hong et al. 2013). Therefore it was of interest to investigate the role of MET inhibition on cell proliferation. The MET inhibitor SU11274 was used to carry out this assay.

Results showed that the SU11274 inhibitor had a significant effect reducing proliferation in all MPNST cell lines except for the S462 cell line (Figure 3.6). These results are consistent with what was observed during cell migration and invasion in these MPNSTs cell lines. Once again showing that the S462 cell line does not rely on the HGF/MET pathway for its invasive and survival behaviour.
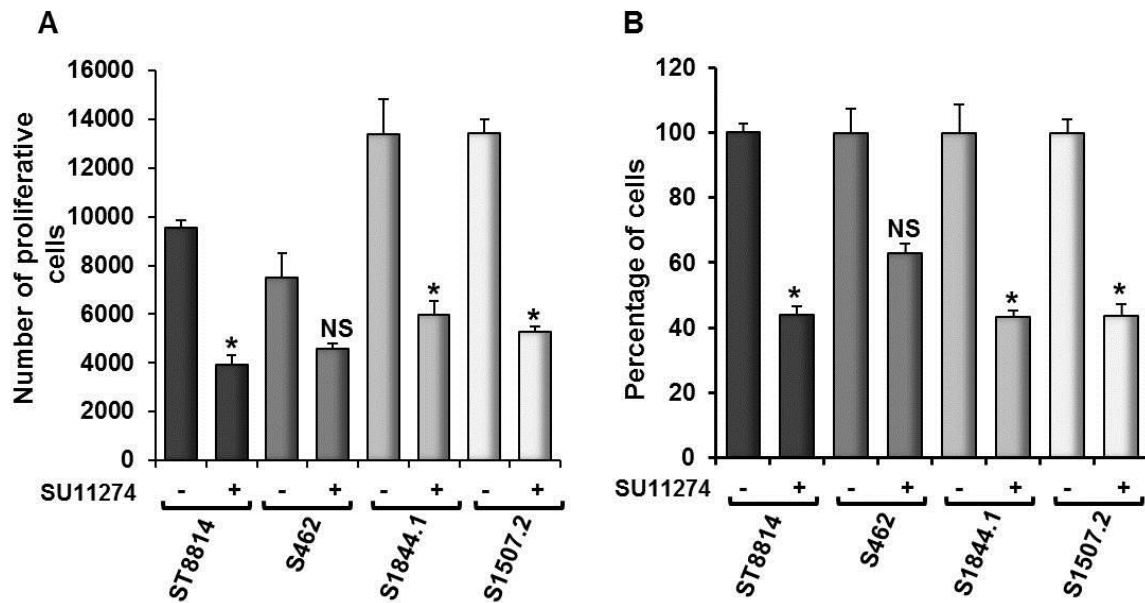
Figure 3.6. Inhibition of MET reduces cell proliferation in 3 MPNST cell lines. A cell proliferation assay was carried out on ST8814, S462, S1844.1 and S1507.2 cells. Cells were seeded at 4000 per well of a 96 well plate. Cells were treated with 10 nM SU11274 (MET inhibitor) prior to 20 ng/ml HGF stimulation, where indicated. Cell proliferation rate was determined after 24 h, using the CyQuant® Cell Proliferation Assay Kit (Life Technologies) following the manufacturer's protocol. Essentially, lysis buffer containing CyQUANT GR dye was added to each well and gently pipette to allow lysis of the cells and incorporation of the dye to the DNA. Fluorescence was detected by excitation at ~485 nm and emission detection at ~530 nm using a plate reader. **A)** Cell number was calculated using a standard curve generated from 250 to 50000 cells. **B)** Percentage of proliferative cells was calculated from cell numbers. Error bars indicative of standard deviation from three independent experiments; each experiment was performed in triplicate. * $p < 0.05$ when comparing treated versus untreated cells.

3.2.6 Rapamycin treatment inhibits MET phosphorylation in MPNST cell lines

Given the limited success of clinical trials using single drug agents, and the heterogeneous nature of MPNSTs, the next approach in this study was to investigate the effects of drug synergy on MPNSTs cell lines. Initially the effects of MET inhibition on signalling pathways that could contribute to malignancy in these cell lines were analysed (Figure 3.7).

Rapamycin was used singly and in combination with one of two MET inhibitors, SU11274 or PF-4217903, prior to stimulation of the cells with HGF. Results show a decrease in MET Tyr1234/1235 phosphorylation in all 4 MPNST cell lines after treatment with rapamycin. The combination of MET inhibitors with Rapamycin didn't show a substantial difference compared to when the MET inhibitors were used by themselves (figure 3.7).
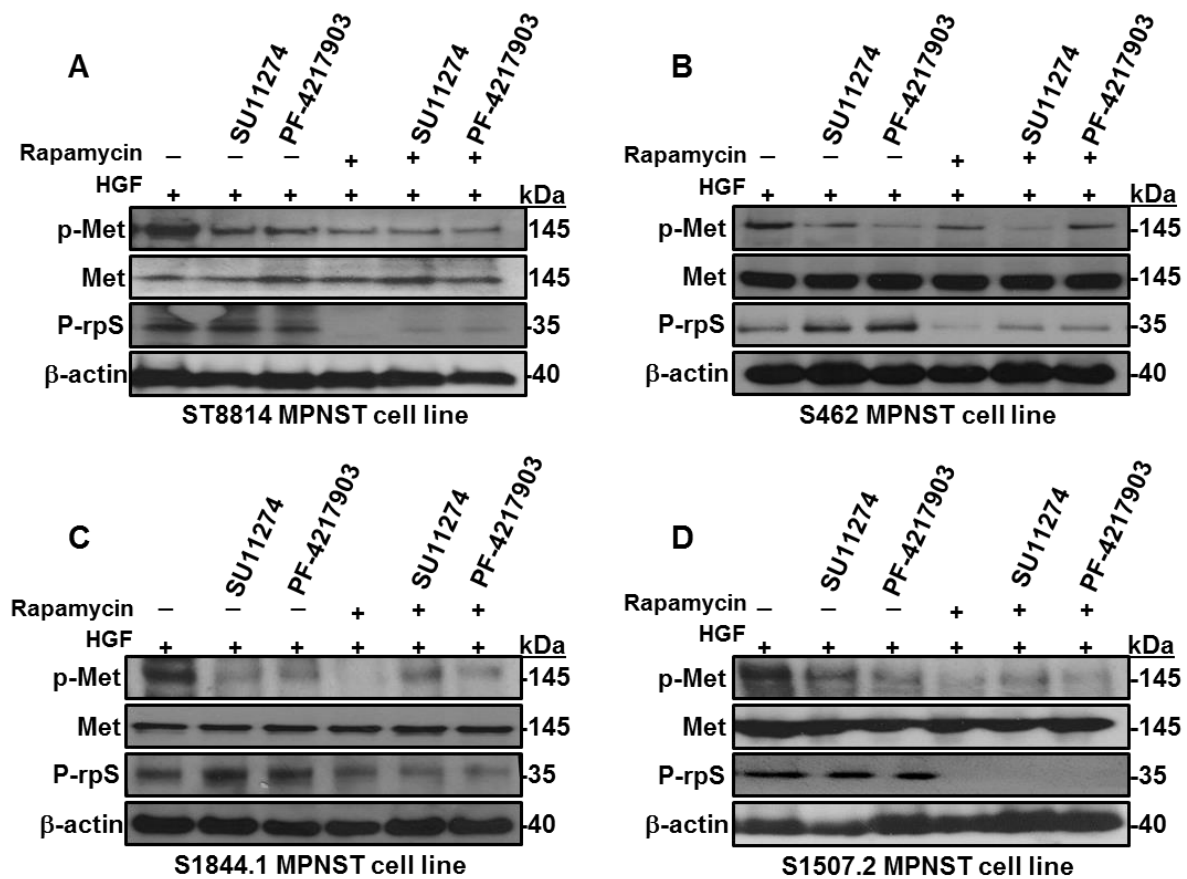
Figure 3.7. Rapamycin treatment inhibits MET phosphorylation in MPNST cell lines.
Where indicated, starved **A)** ST8814, **B)** S462, **C)** S1844.1 and **D)** S1507.2 cells were pre-treated with 10 nM SU11274 and 7.5 ng/µl PF-4217903, 50 nM rapamycin or a combination of both rapamycin and the MET inhibitors for 30 min prior to 30 min stimulation (20 ng/ml HGF). Total and phosphorylated MET (try1234/1235) and β-actin was analysed. Three independent experiments were performed for each cell line.

3.2.7 Rapamycin treatment reduces wound healing in 3 MPNST cell lines

In order to investigate the effects of rapamycin treatment on cell movement a wound healing assay was carried out (Figure 3.8) (See section 2.2.6). The results show that HGF induced wound healing in the ST8814, S1844.1 and S1507.2 cells was significantly reduced after treatment with rapamycin (Figure 3.9). There was no significant difference in wound healing reduction when rapamycin was used in combination with both the SU11274 and PF-4217903 inhibitors. In contrast wound healing in the S462 cell line was insensitive to rapamycin treatment (figure 3.9).
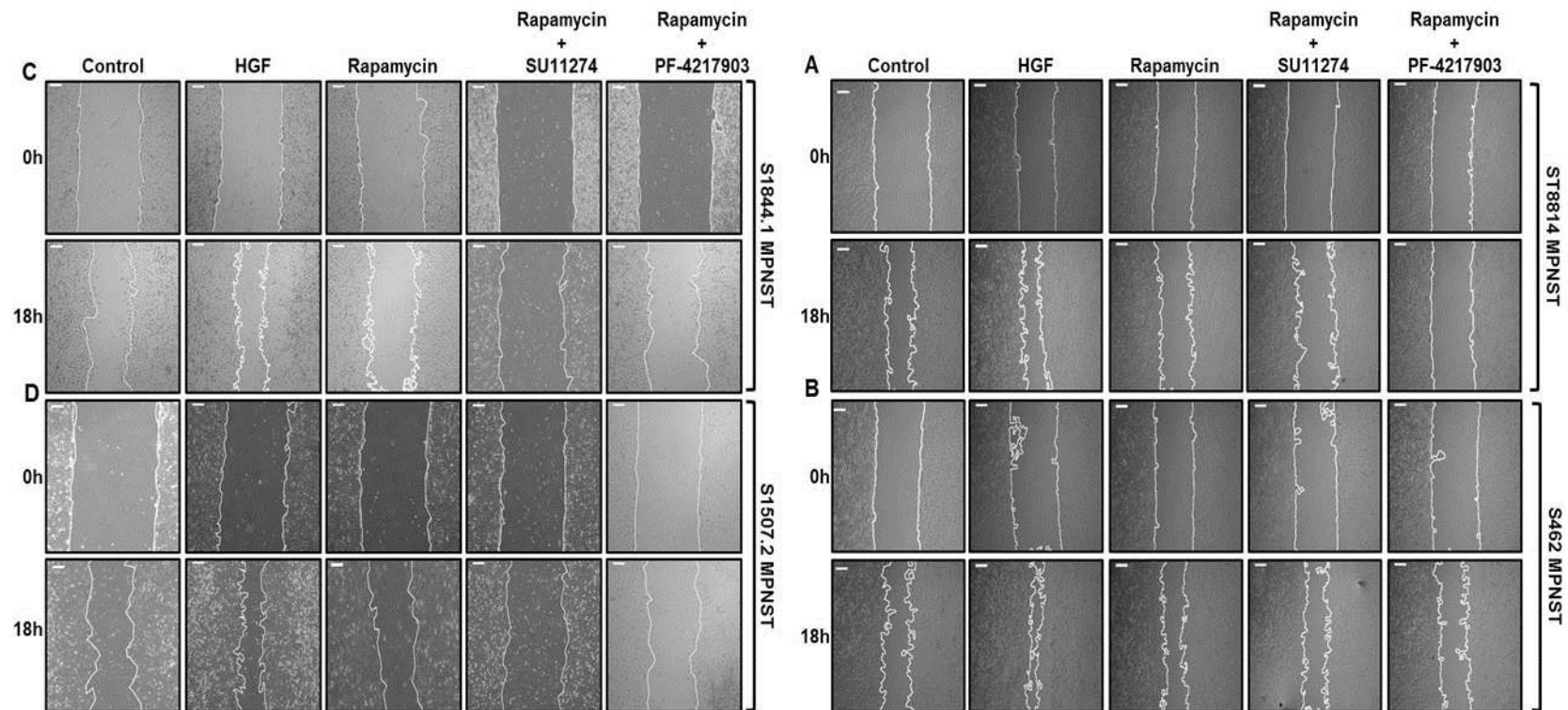
Figure 3.8. Rapamycin treatment reduces wound healing in 3 MPNST cell lines. Where indicated, **A)** ST8814, **B)** S462, **C)** S1844.1 and **D)** S1507.2 MPNST cell lines were starved (1% FBS) for 24 h prior to wound healing assay. Cells were stimulated using 20 ng/ml HGF 18 h in the presence or absence of 50 nM rapamycin, or a combination of 50 nM rapamycin with either 10 nM SU11274 or 7.5 ng/µl PF-4217903 as indicated. Pictures were taking at 0 and 18 h. (Scale bar represents 250 µm).
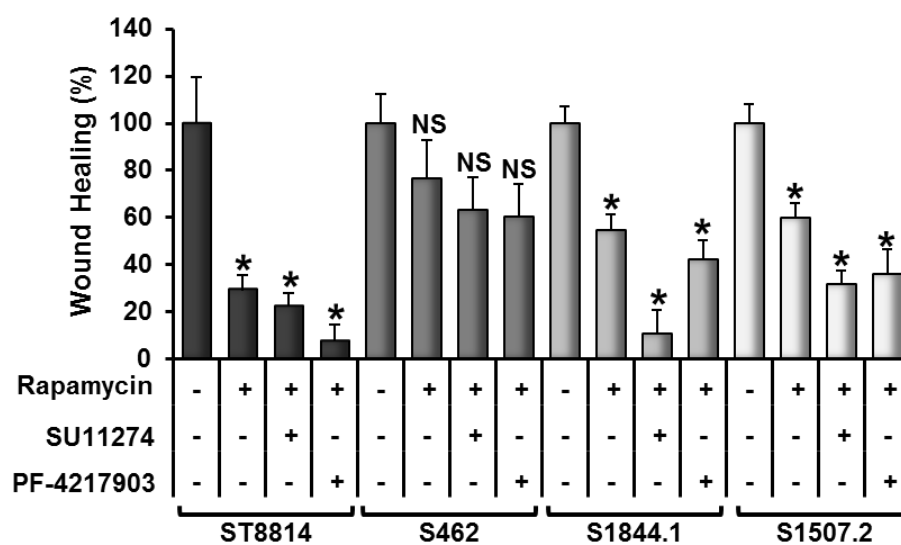
Figure 3.9. Rapamycin impairs HGF induced wound healing in all MPNST cell lines while the S462 cell line show no resistance rapamycin. The significance of rapamycin treatment on wound healing was determined by one way ANOVA. The percentage of migrated cells were compared in all 4 MPNST cell lines. Error bars indicative of standard deviation from three independent experiments * $p < 0.05$ when comparing wound closure of treated versus untreated cells.

3.2.8   Rapamycin treatment inhibits cell migration and invasion in 3 MPNST cell lines

The next step in this study was to analyse the effectiveness of rapamycin upon cell migration and invasion under HGF stimulation (Figure 3.10). For this purpose, migration and invasion assays were carried out (See section 2.2.7). Cells were treated with rapamycin prior to HGF stimulation. The results clearly demonstrate that rapamycin significantly decreased both cell migration and invasion in the ST8814, S1844.1 and S1507.2 cell lines. Conversely, cell migration and invasion in the S462 cells was not significantly inhibited by rapamycin (Figure 3.10).
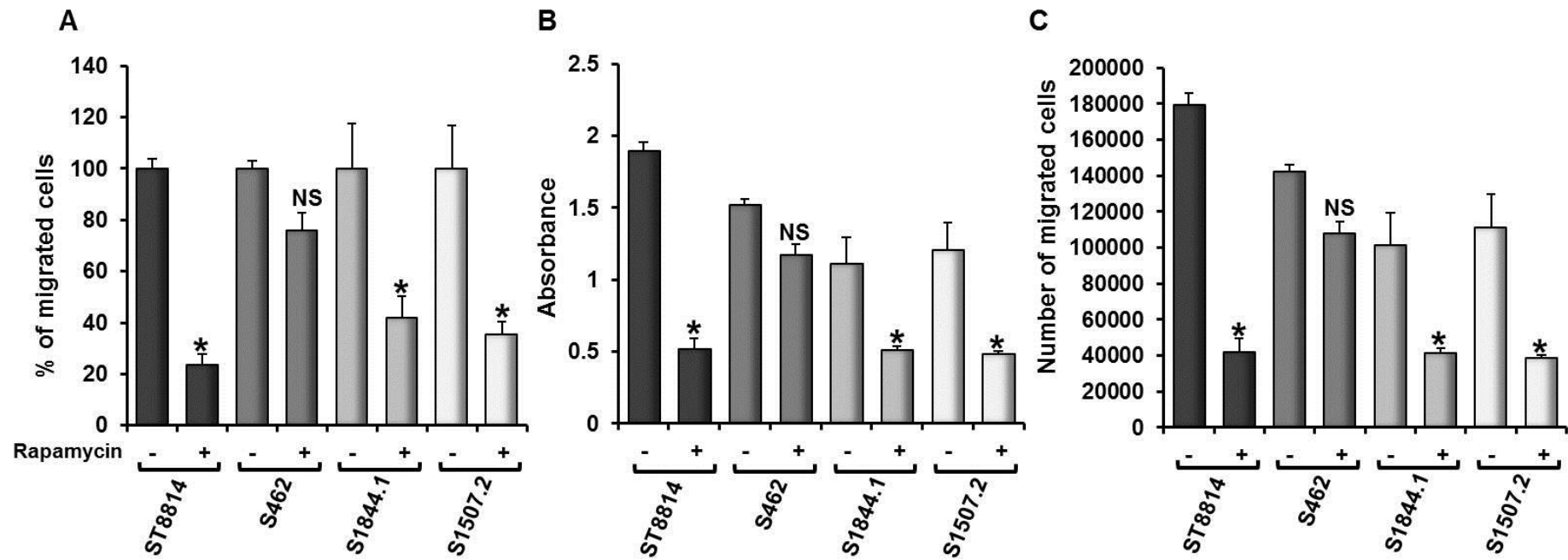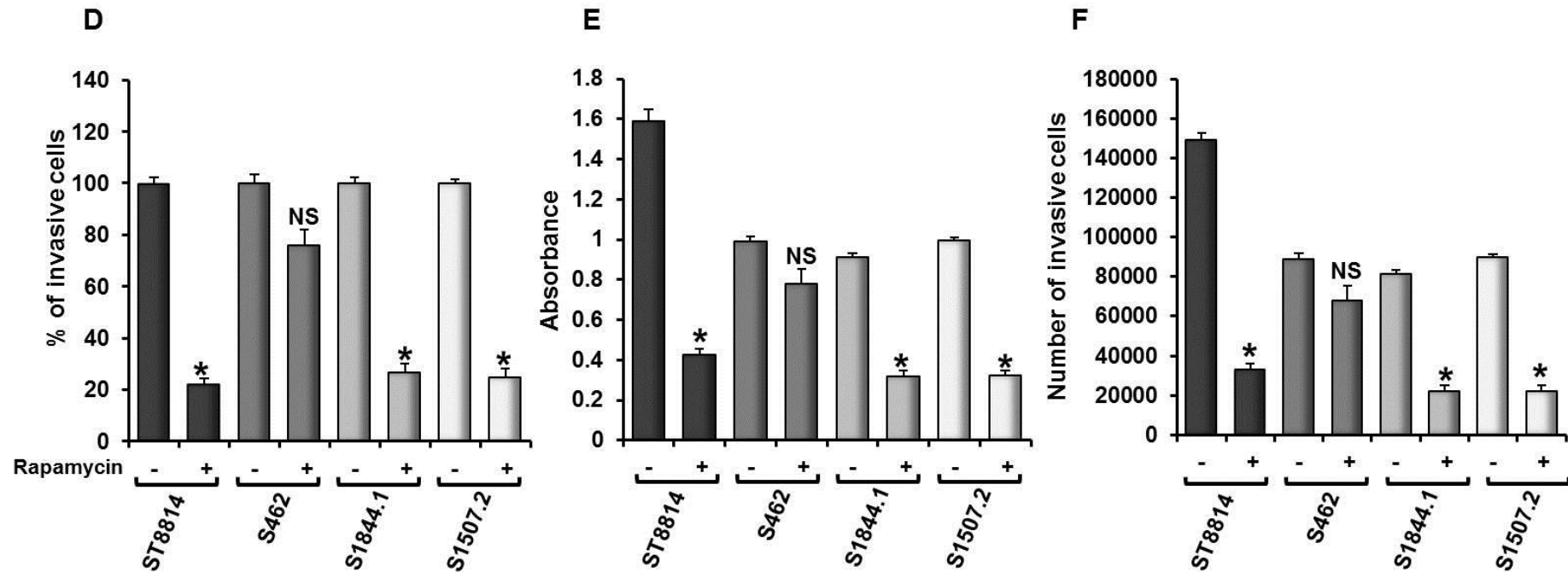
Figure 3.10. Rapamycin treatment inhibits cell migration and invasion is 3 MPNST cell lines. Cell migration and invasion assay was carried out on ST8814, S462, S1844.1 and S1507.2 cell lines in the presence or absence of 50 nM rapamycin prior to HGF stimulation where indicated in accordance to the protocol described in section 2.2.7. **A)** Graph demonstrates the percentage of migrated cells in each cell line **B)** Graph demonstrates the absorbance read on the Genova MK3 Lifescience Analyser of migrated cells (see section 2.2.7). **C)** This graph demonstrates the number of cells migrated in control vs treated cell lines **D)** Graph demonstrates the percentage of invasive cells in each cell line **E)** Graph demonstrates the absorbance read on the Genova MK3 Lifescience Analyser of invasive cells (see section 2.2.7). **F)** This graph demonstrates the number of invasive cells in control vs treated cell lines.

3.2.9   Rapamycin impairs cell proliferation in 3 MPNST cell lines.

As described in section 3.3.5, inhibition of the HGF/MET pathway significantly reduced cell proliferation of the MPNST cell lines. In this section, the aim was to examine the effects of rapamycin on cell proliferation, and also to observe whether a combination of a MET inhibitor and rapamycin would show a synergic effect on impairing cell proliferation in these MPNST cell lines. Results demonstrate that rapamycin reduces cell proliferation in all MPNST cell lines. However after treating the cells with both rapamycin and the MET inhibitor SU11274, no synergic or additive effects were observed (2-4% difference between rapamycin treatment and combination of both rapamycin and SU11274 inhibitor).
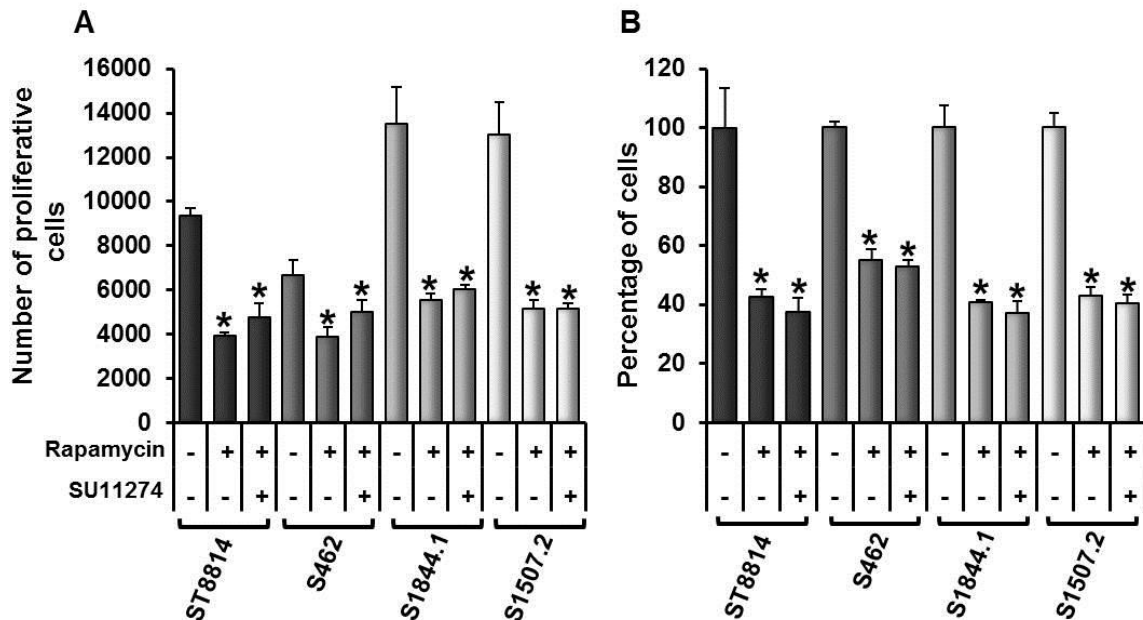
Figure 3.11. Rapamycin impairs cell proliferation in all MPNST cell lines. A cell proliferation assay was carried out on ST8814, S462, S1844.1 and S1507.2 cells. Cells were seeded at 4000 per well of a 96 well plate. Cells were treated with 50 nM rapamycin or a combination of 50 nM rapamycin with 10 nM SU11274 prior to 20 ng/ml HGF stimulation, where indicated. Cell proliferation rate was determined after 24 h, using the CyQuant® Cell Proliferation Assay Kit (Life Technologies) following the manufacturer's protocol. Essentially, lysis buffer containing CyQUANT GR dye was added to each well and gently pipette to allow lysis of the cells and incorporation of the dye to the DNA. Fluorescence was detected by excitation at ~485 nm and emission detection at ~530 nm using a plate reader. **A)** Cell number was calculated using a standard curve generated from 250 to 50000 cells. **B)** Percentage of proliferative cells was calculated from cell numbers. Error bars indicative of standard deviation from three independent experiments; each experiment was performed in triplicate. * $p < 0.05$ when comparing treated versus untreated cells.

3.3 Discussion

Although it is known that somatic *NF1* gene inactivation results in aberrant Ras signalling, Ras activation is insufficient to induce malignant transformation alone. Even though many advances have been made in determining the molecular pathophysiology of MPNSTs, it is still unclear which pathways are common drivers of malignancy in the heterogeneous MPNST population. This study provides evidence that HGF/MET signalling is involved in the tumourgenesis and invasive behaviour of selected MPNST cell lines.

MET is a transmembrane tyrosine kinase receptor and HGF acts as its sole ligand (L Naldini et al. 1991; Cooper 1992). The signalling pathway occurring between MET receptor and its ligand HGF plays an important role in embryogenesis, regeneration, wound healing and tumour progression, therefore suggesting that the MET signalling alteration could possibly contribute to the transformation of benign tumours to MPNSTs in NF1 patients (Weidner 1993).

Studies using DNA copy number analysis in NF1 tumours have revealed an amplification of HGF, MET and PDGFR-A in MPNSTs. MET targeting has previously been shown to inhibit the migratory, invasive, and angiogenic characteristics of MPNST cells (Torres et al. 2011). Studies have also shown the presence of active HGF/MET pathway in neurofibromas of NF1 patients and Rat Schwann cells (Krasnoselsky et al. 1994; Rao et al. 1997). Moreover the HGF/MET signalling pathway has been identified as a mitogen in many cancers (Peruzzi & Bottaro 2006).

Results of this chapter demonstrate that MET signalling could be involved in tumourgenesis and metastasis within three of the MPNST cell lines examined. In the S462 MPNST cell lines, MET inhibition with drug inhibitors blocked tyrosine phosphorylation of MET (figure 3.2), however no significant effect was observed when testing wound healing (figure 3.3), migration or invasion (figure 3.4). This was expected as the S462 MPNST cell line showed the lowest level of MET protein compared to the three other MPNST cell lines.

Moreover, the insensitivity of the S462 cell line indicate that targeting MET al one might not be suitable for treating the heterogeneous NF-1-MPNST population.

Of Interest, figure 3.2 shows that PDGF stimulation also caused robust activation of MET, this is suggestive of signalling cross-talk between PDGF-R and MET. PDGFR expression is known to transform Schwann cells lacking NF1 (Badache & De Vries 1998; Upadhyaya et al. 2012), implying that PDGF-R amplification might be involved in Schwann cell hyperplasia. In a more recent study using a panel of 11 MPNST tumours, PDGF-R, PDGF-R and EGF-R were shown to be over-expressed when compared to benign controls correlating with a higher level of PI3K/Akt/mTORC1 signal transduction (Perrone et al. 2009; Wu et al. 2013), It is therefore possible that some of the transforming potential through PDGF-R might be via activation of MET. There is also evidence supporting a role for the HGF/PDGF/P70S6K signalling pathway in tumourgenesis. Studies have shown that mTOR and the P70S6K pathway are involved in the progression of prostate cancer (Dai et al. 2009). Furthermore, Interleukin stimulation was also sufficient to induce MET phosphorylation in the MPNSTs, which reveals that there is cross talk between MET and various tyrosine receptor kinases.

There are reports in the literature indicating that the mTOR pathway could possibly be regulated via MET signalling. Studies have shown that using rapamycin reduces the cell growth of the murine pre-B cell line (BaF3) containing TPR-MET, where TPR-MET is a fusion oncoprotein resulting from a chromosomal translocation (TPR3 locus on chromosome 1 upstream of a cytoplasmic region of MET on chromosome7). Of interest this study has also shown that the MET inhibitor, PHA665752, cooperates with rapamycin in inhibiting the growth of the TPR-MET transformed cells (Ma, Schaefer, et al. 2005). It is thought that PHA665752 could possibly be modulating the AKT/mTOR pathway. It was proposed that the effects observed after combining both rapamycin and the PHA665752 inhibitor could be due to inhibition of other complementary pathways that lie downstream of MET (Ma, Jagadeeswaran, et al. 2005).

Further studies have reported that treatment of genetically engineered murine MPNST models with rapamycin resulted in a delay of malignant tumour growth and progression (Johannessen et al. 2008). However in contrast to this study, a preclinical trial carried out on mouse models of NF1 showed that treatment of the mice using RAD001 (mTORC1 rapalogue inhibitor) did not have any effect on decreasing the size of tumours (Wu et al. 2012). Further supporting this study a clinical trial carried out on 12 NF patients reported that rapamycin had no effect on the shrinkage of non-progressive NF1 associated tumours (Weiss et al. 2014). To further investigate whether rapamycin would delay the progression of NF1-associated progressive tumours in patients additional work by Weiss *et al.* was carried out. Results of this study showed that the treatment of rapamycin decreased the time of growth of progressive tumours by 4 months. Further studies are needed to investigate the effects of rapamycin on other groups of plexiform neurofibromas (Weiss et al. 2015). Clinical trials and studies have also suggested that rapamycin could possibly be more effective if used in combination with other inhibitors for example inhibitors of the MEK signalling pathway (Weiss et al. 2015). Moreover recent studies have shown that the inhibition of both the MEK and mTOR pathway has synergistic effects in both sporadic and NF1-associated MPNST (Watson et al. 2014). Studies have shown that rapamycin effects the growth of malignant NF1 tumours via suppression of the mTOR target cyclin D1 (Chong et al. 2010).

Knowing that both MET and mTOR signalling pathways could possibly be involved in promoting the invasive behaviour of MPNSTs, it was of interest to investigate whether combination of MET and mTOR inhibitors might have additive effects on reducing the cell migratory properties of these MPNST cell lines. Furthermore, within this study rapamycin showed inhibition of MET phosphorylation, similar to that of the MET inhibitors treatment (figure 3.2).

Similar to MET inhibition, rapamycin did not reduce wound healing, migration and invasion in the S462 cell line. Rapamycin was also used in combination with SU11274

inhibitor, however no substantial difference was observed between using rapamycin or SU11274 inhibitor and using these two drugs in combination. Even though rapamycin affects the migration and invasion properties of selected MPNST cell lines, it does not appear to have an additive effect when combined with MET inhibitors. It is likely that much of the inhibitory effect of rapamycin to repress these migration and invasion properties is through rapamycin's ability to inhibit MET activation. Further work is clearly needed to investigate how mTORC1 inhibition could negatively regulate MET. Given that rapamycin was sufficient to block HGF induced MET phosphorylation, it suggests that inhibition of mTORC1 might either be preventing membrane presentation of MET or is causing dephosphorylation of MET (or both). mTORC1 has been implicated in the regulation of phosphatases (Harwood et al . 2008) as well as receptor regulation (Wang & Proud 2010). It is also possible that inhibition of mTORC1 might be resulting in recruitment of an undetermined negative regulator of MET that interferes with MET phosphorylation. Therefore rapamycin or similar drugs that have the ability to block the mTOR pathway could possibly be used in cancers that express MET.

4 Chapter 4: Characterisation of STAT3 and its involvement in the development of MPNSTs

4.1 Introduction

The mortality of cancer is mainly due to the primary tumours spreading throughout the body via metastatic processes. Research over the last decade has verified several genes and also micro RNAs (miRNA) that are involved in invasion and metastasis. However, in spite of these reports associating single genes with metastasis, there appears to be a group of main regulators that manage the metastasis processes. Specifically the activation of the JAK/STAT3 signalling pathway has frequently been reported as a trigger for increased migration and invasion in several cancer types (Teng et al. 2014). Moreover STAT3 has been described as an oncogene and its abnormal activation is associated with pathogenesis (Dodd et al. 2014). It has been reported in many studies that STAT3 is constitutively active in human tumours causing cell transformation and cancer progression (Bromberg et al. 1999).

STAT3 can be activated via a variety of stimuli that leads to activation of multiple biological processes such as, cell growth, angiogenesis, cell survival, cell migration, tumourgenesis, cell cycle progression, apoptosis and acute phase immune response (Hirano et al. 2000; Levy & Lee 2002; Qin et al. 2008). The direct response of STAT3 is first mediated by the activation of the gp130 receptor subunit via cytokines (Hirano et al. 2000). Many receptor tyrosine kinases, including MET, can phosphorylate and activate JAK2, which lies immediately upstream of STAT3. JAK2 is a tyrosine kinase that phosphorylates STAT3 on the tyrosine residue, leading to its nuclear localisation and activation as a transcription factor which then promotes transcription of genes linked to cancer progression, driving cell migration, invasion, and survival (Avalle et al. 2012).

In chapter 3 it was demonstrated that the HGF/MET signalling pathway could possibly be involved in the invasive behaviour of some but not all MPNST cell lines. In this chapter the focus of study will be on STAT3 a downstream target of MET and also several

other receptor tyrosine kinases. Knowing the role that the JAK2/STAT3 signalling pathway plays in stimulating cell migration and invasion, this chapter was designed to determine the importance of STAT3 regarding cancer progression in the context of MPNST.

4.2 Results

4.2.1 MPNST cell lines have markedly different STAT3 signalling profiles in response to HGF stimulation

In the ST8814 cells, tyrosine phosphorylation of STAT3 was robustly induced after just 30 min of HGF stimulation and maintained throughout the 3 h time course. However in the S462 cell lines, cells responded to HGF stimulation after 2 h. Within the S1844.1 and S1507.2 HGF stimulation was observed after 30 mins of HGF stimulation however these cells did not contain their stimulation after 2-3 h. These results clearly demonstrate a difference in the HGF/MET signalling pathway as expected from results shown in chapter 3. Cells were also stimulated with interleukin-6 (IL-6) for 30 min as a positive control for STAT3 activation as it is known that IL-6 has the ability to activate STAT3. PDFG did not show a notable effect in STAT3 activation (Figure 4.1).

This experiment was carried out due to the fact that after 30 min stimulation with HGF, no changes or stimulation in the S462 cell lines was detected. Results in this section determine the time course of HGF stimulation needed in each cell line. In further experiments the S462 cell lines were treated for 2 h with HGF while the other cell lines were treated for 30 mins.
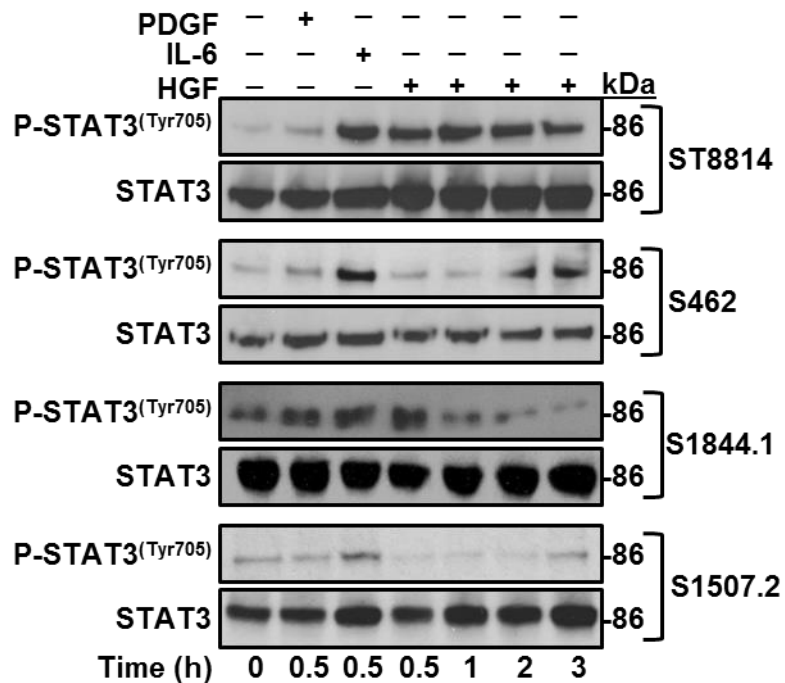
Figure 4.1.MPNST cell lines have markedly different STAT3 signalling profiles in response to HGF stimulation. Where indicated, starved ST8814, S462, S1844.1 and S1507.2 cells were pre-treated with 20 ng/ml HGF for 0.5-1-2 or 3 hours before being lysed in lyses buffer. From protein lysates, total and phosphorylated STAT3 (Tyr 705) were analysed. Cells were also stimulated for 30 mins with IL-6 and PDGF. IL-6 was used a positive control for STAT3 activation.

4.2.2 Sensitivity to STAT3 inhibition in multiple MPNST cell lines

After demonstrating that MPNST cell lines have markedly different STAT3 signalling profiles, the next step in this study was to investigate the sensitivity of these 4 MPNST cell lines to a panel of STAT3 inhibitors. Three different STAT3 inhibitors were employed; Cucurbitacin-I, 5,15-diphenylporphyrin (5,15-DPP) and FLLL31. Cucurbitacin-I is derived from cucurbitane and FLLL31 is derived from curcumin, the bioactive compound in the spice turmeric. Both of these inhibitors selectively bind to and inhibit the tyrosine kinase JAK2, which is immediately upstream of STAT3. 5,15-DPP is a selective STAT3 Src homology-2 domain (SH2) antagonist, preventing STAT3 SH2 domain-mediated ligand binding, dimerisation and signal transduction. Results demonstrate that all three inhibitors suppressed HGF-induced JAK2-mediated tyrosine 705 phosphorylation of STAT3 in all cell lines (figure 4.2). Ser727 phosphorylation of STAT3, which is directly phosphorylated by mTORC1 (Reynolds et al. 2004), was less sensitive to drug treatments (Figure 4.2).
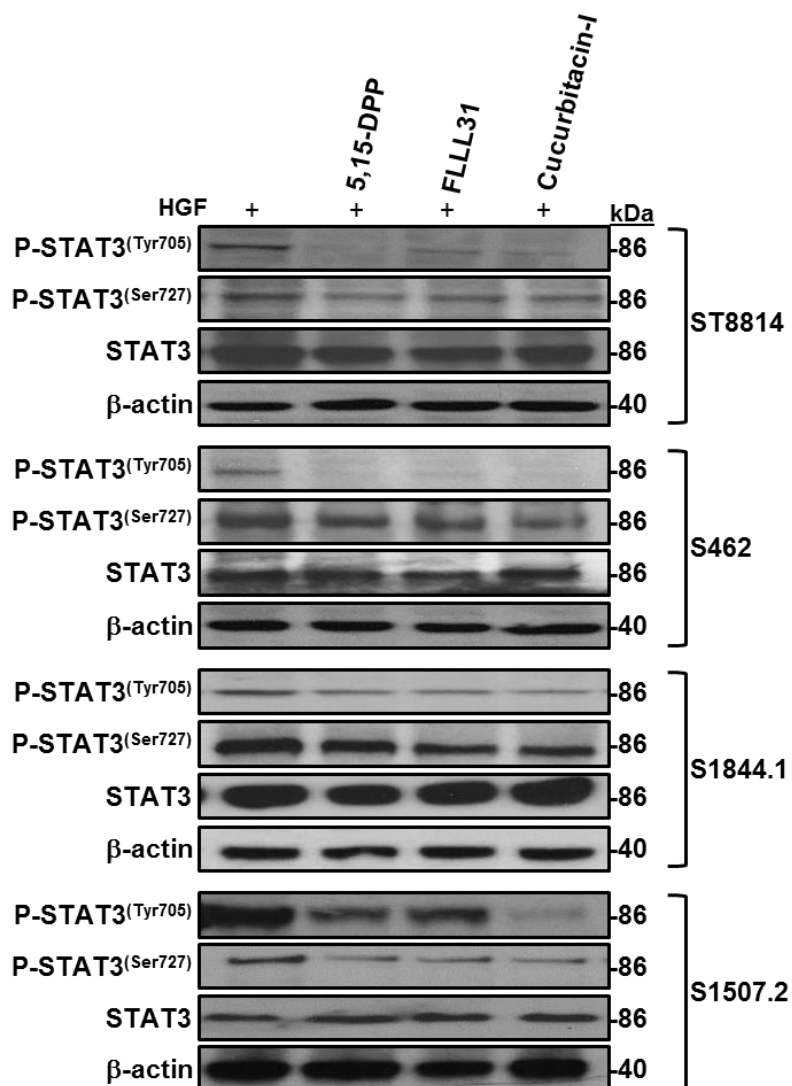
Figure 4.2. Sensitivity to STAT3 inhibition in multiple MPNST cell lines. Where indicated, starved ST8814, S462, S1844.1 and S1507.2 cells were pre-treated with 5 nM Cucurbitacin-I, 50 nM 5,15-DPP or 5 nM FLLL31 for 30 min prior to 20 ng/ml HGF stimulation. The ST8814, S1844.1 and S1507.1 MPNST cell lines where stimulated with HGF for 30 mins, while the S462 MPNST cell lines were stimulated with HGF for 120 mins to insure maximum stimulation. From protein lysates, total and phosphorylated STAT3 (Tyr 705 and Ser727) where analysed. β-actin was used as a control .

4.2.3 The inhibition of STAT3 robustly impairs wound healing in multiple MPNST cell lines

The next stage of this study was to determine what impact STAT3 inhibition could have on wound closure of these MPNST cell lines. In order to do this wound heal assays were carried out (See section 2.2.6) (Figure 4.3). The three STAT3 inhibitors where used prior to HGF stimulation. As figure 4.5. Shows, FLLL31 significantly blocked wound healing in all cell lines, suggesting that STAT3 is a key mediator of cell migration in MPNST derived cell lines.

5,15-DPP was ineffective at suppressing wound healing in 3 out of the 4 MPNST cell lines (ST8814, S462 and S1507.2). While cucurbitacin-I significantly blocked wound healing, it also greatly altered the morphology of the S1844.1 and S1507.2 cell lines (figure 4.4), causing cellular detachment. This suggests that cucurbitacin-I likely has additional non-specific drug interactions that does not involve JAK2.For these reasons, the curcumin analogue FLLL31 was utilised for the next step of this study.
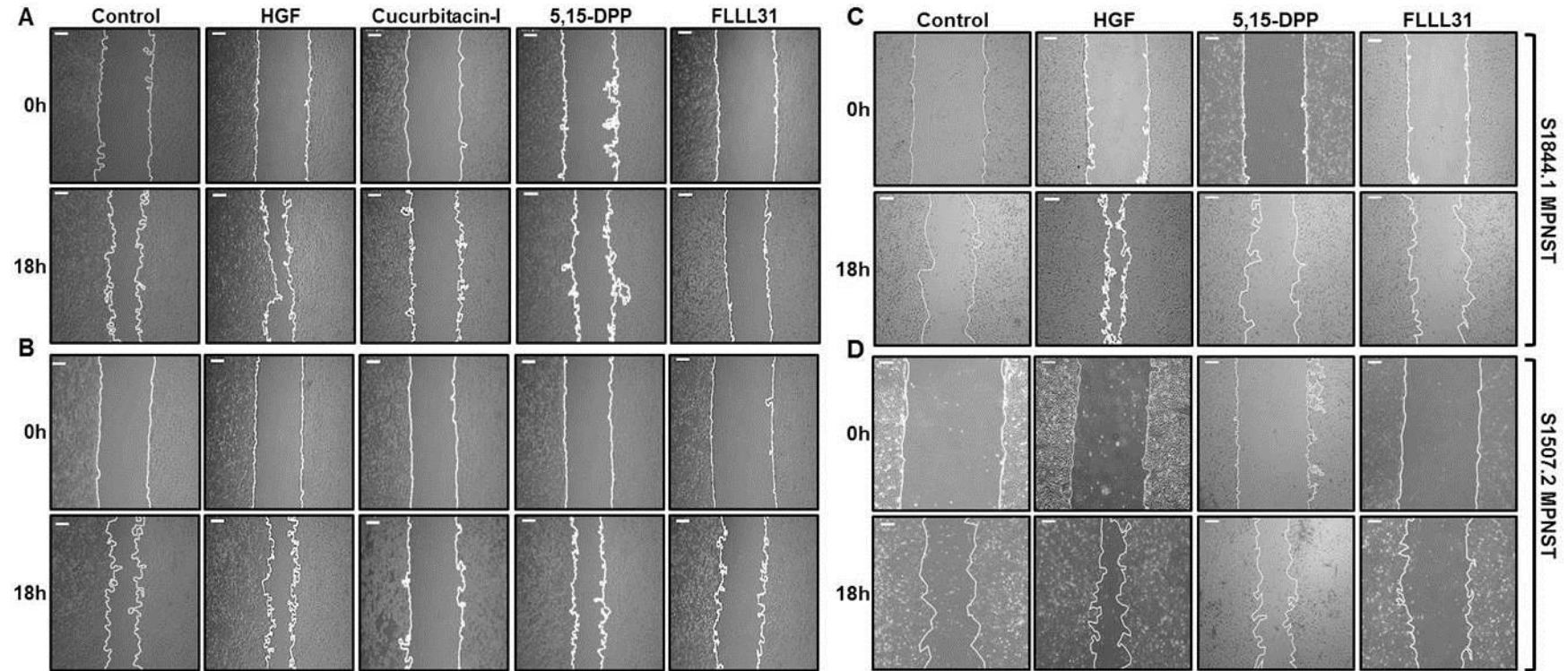
Figure 4.3. The inhibition of STAT3 robustly impairs wound healing in MPNST cell lines. Where indicated, **A)** ST8814, **B)** S462, **C)** S1844.1 and **D)** S1507.2 MPNST cell lines were starved (1% FBS) for 24 h prior to wound healing assay. Cells were stimulated using 20 ng/ml HGF 18 h in the presence or absence of 5 nM Cucurbitacin-I, 50 nM 5,15-DPP and 5 nM FLLL31. Pictures were taking at 0 and 18 h. (Scale bar represents 250 μm). n=3.
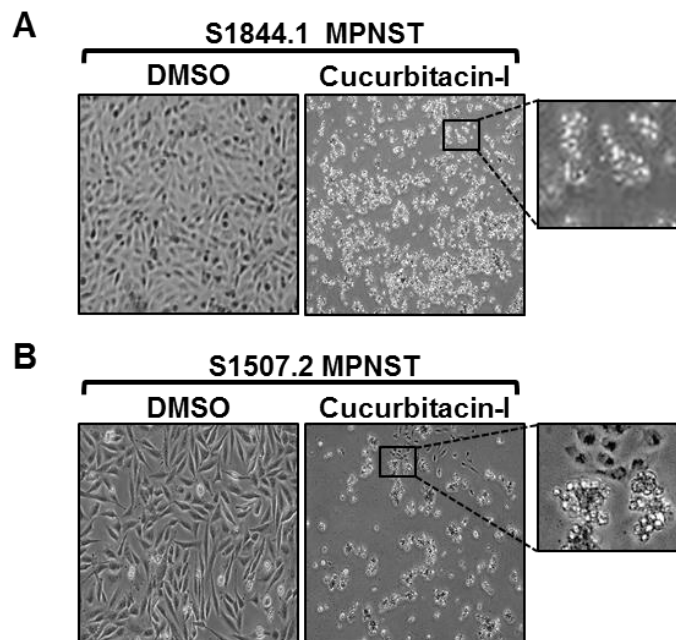
Figure 4.4. Cucurbitacin-I greatly altered the morphology of the S1844.1 and S1507.2 cell lines causing cellular detachment. Treatment with 5 nM Cucurbitacin-I for 30 min prior to 20 ng/ml HGF stimulation caused detachment of cells and a change in the cell morphology of **A)** S1844.1 and **B)** S1507.2 MPNST cell lines.
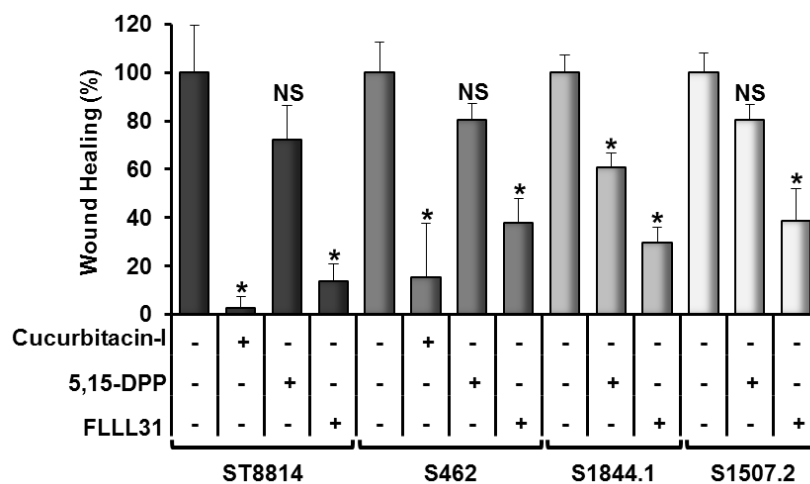
Figure 4.5. The significance of STAT3 inhibitors in reducing wound healing in MPNST cell lines. The significance of the STAT3 inhibitor effect on wound healing was determined by one way ANOVA. The percentage of migrated cells were compared in all 4 MPNST cell lines. Error bars are indicative of standard deviation from three independent experiments * $p < 0.05$ when comparing wound closure of treated versus untreated cells.

4.2.4 STAT3 inhibition impairs migration and invasion in all 4 MPNST cell lines

After investigating the effects of STAT3 inhibitors on wound closure in these cell lines, the next series of experiments were to evaluate the effect of STAT3 inhibition in these MPNST cell lines on cell migration and invasion. For this experiment only the FLLL31 STAT3 inhibitor was chosen, since 5,15 DPP only showed efficiency on one cell line and also cucurbitacin-I altered the morphology of 2 of the MPNST cell lines suggesting non-specific effects. Cell invasion and migration assays were carried out as described in chapter 2 Cells were then treated with FLLL31 inhibitor prior to HGF stimulation. Results demonstrate that in all 4 cell lines STAT3 inhibition was sufficient to block both migration and invasion (figure 4.6).
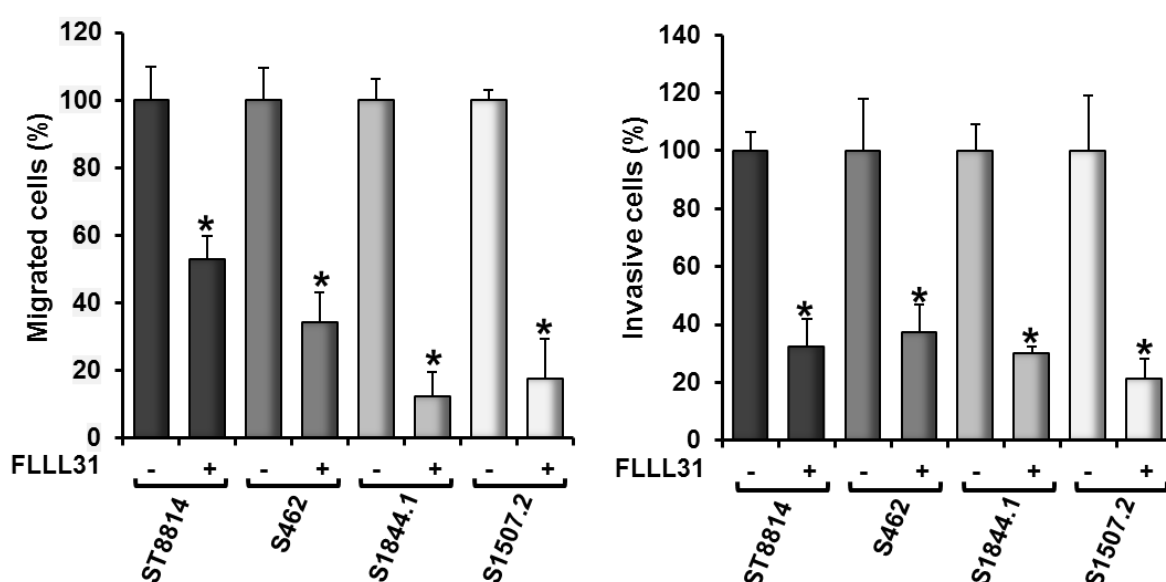
Figure 4.6. STAT3 inhibition impairs migration and invasion in all 4 MPNST cell lines. **A)** Cell migration and **B)** cell invasion assays were carried out on ST8814, S462, S1844.1 and S1507.2 cells in the presence or absence of 5 nM FLLL31 prior to 20 ng/ml HGF stimulation, where indicated. n = 3. * $p < 0.05$ when comparing treated versus untreated cells.

4.2.5 STAT3 inhibition using the FLLL31 inhibitor significantly reduces cell proliferation in all MPNST cell lines.

The FLLL31 STAT3 inhibitor was used to investigate the effects of STAT3 inhibition on cell proliferation. As mentioned before the inhibitor Cucurbitacin-I caused detachment of cells and a change in the cell morphology of the S1844.1 and S1507.2 cell lines. Furthermore 5, 15 DPP inhibitor did not show significant effect in reducing wound healing. Therefore these two inhibitors were not used for the proliferation assay. Results clearly demonstrate that FLLL31 significantly reduces proliferation in all MPNST cell lines.
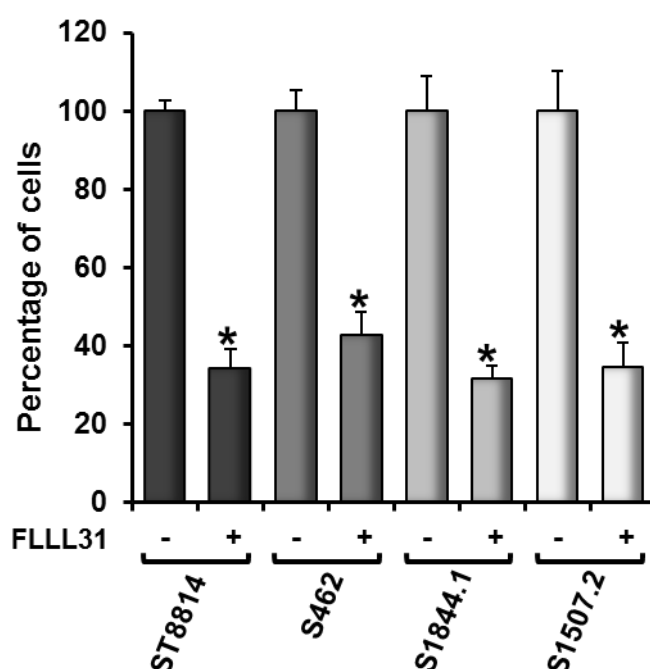
Figure 4.7. STAT3 inhibition using the FLLL31 inhibitor significantly reduces cell proliferation in all MPNST cell lines. Cell proliferation assay was carried out on ST8814, S462, S1844.1 and S1507.2 cells in the presence or absence of 5 nM FLLL31 prior to 20 ng/ml HGF stimulation, where indicated. n = 3. * $p < 0.05$ when comparing treated versus untreated cells.

4.2.6 STAT3 knockdown in multiple MPNST cell lines significantly impairs wound healing

After demonstrating that drug inhibition of STAT3 blocked migration of all MPNST cell lines, it was important to further validate these results. To do this, STAT3 knockdowns were created using shRNA in all 4 MPNST cell lines. Both STAT3 shRNA and non-target control MISSION shRNA were packaged into lentivirus and confluent MPNST cell lines were infected with shRNA containing lentivirus to produce the stable knockdown cells (see section 2.2.1.2). The efficiency of STAT3 knockdown was confirmed at the level of protein expression via western blot (Figure 4.8A), and at mRNA level via qPCR (Section 2.2.5) (Figure 4.8B).

The next stage of this study was to determine the effects of STAT3 knockdown on wound healing. Validating the STAT3 drug inhibition study on wound healing, knockdown of STAT3 also robustly inhibited wound closure when compared to the control shRNA MPNST cell lines (figure 4.9). Statistical analysis of the wound healing assay show that STAT3 knockdown in all cell lines inhibited wound closure by over 50% (figure 4.10).
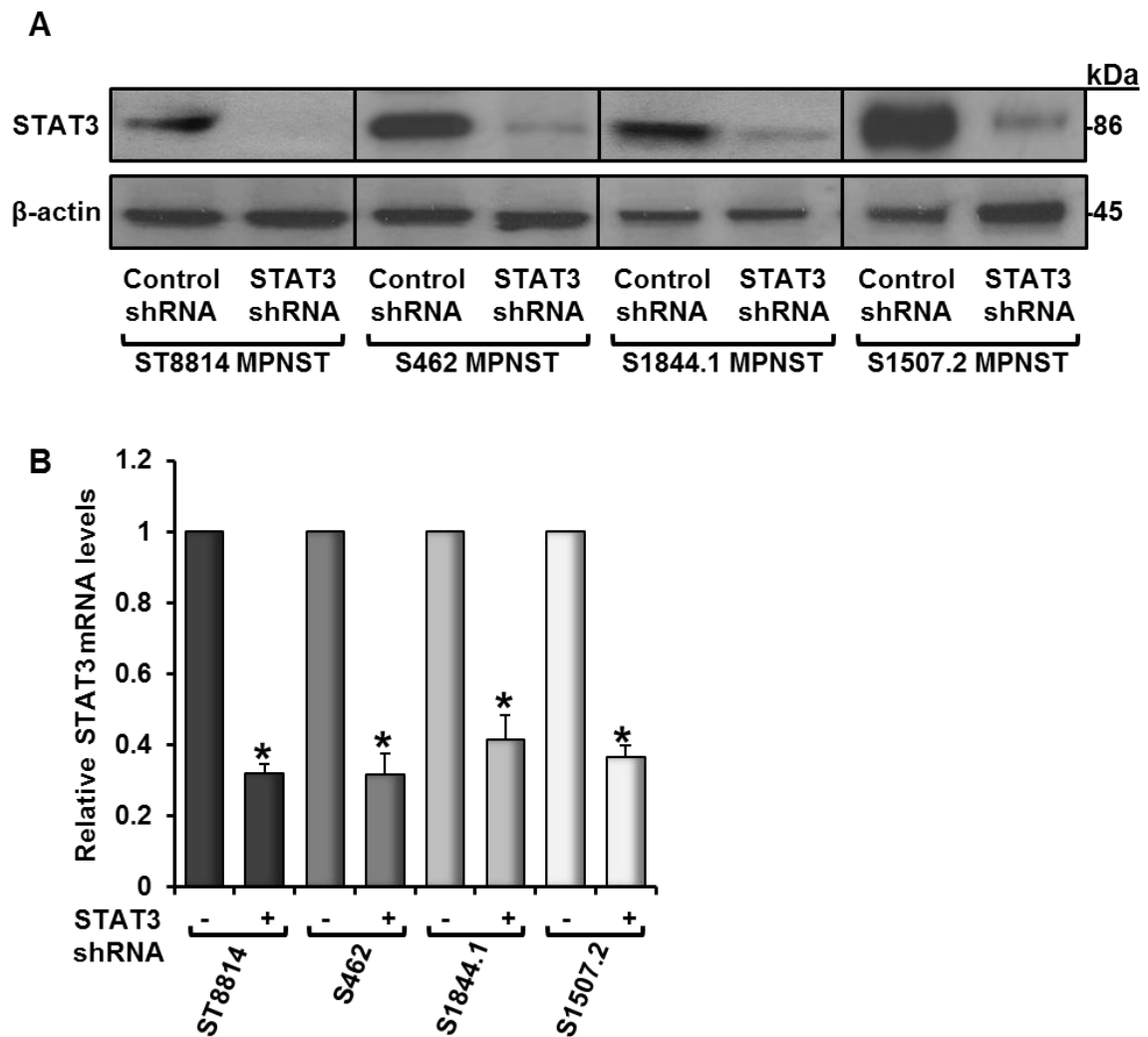
Figure 4.8. Western blot and qPCR assay was carried out to confirm efficient knockdown of STAT3. **A)** STAT3 protein levels were compared between cell lines by western blot, and β-actin serves as a loading control. n = 3. **B)** STAT3 mRNA levels were compared between control and knockdown c3ell lines via qPCR, n=3.
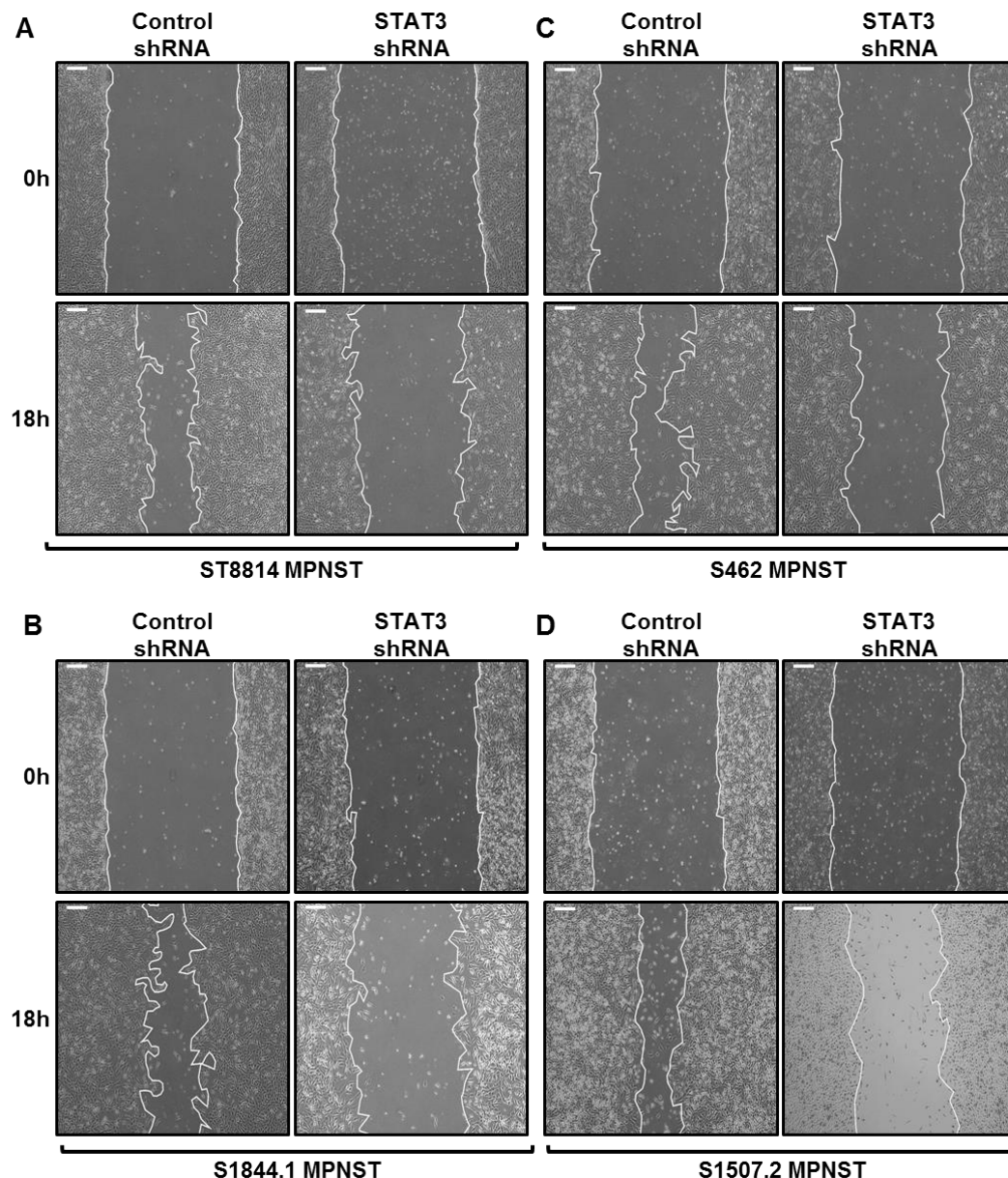
Figure 4.9. STAT3 knockdown in multiple MPNST cell lines significantly impairs wound healing. Wound healing assay on MPNST cell lines **A)** ST8814, **B)** S462, **C)** S1844.1 and **D)** S1507.2 Cells were carried out. Cells were seeded and synchronised in 1%FBS for 24hours and wounded with a pipette tip. Wounds were followed up for 18 h. Reduction in the rate of wound healing can be seen in the cell lines carrying STAT3 knockdown. Pictures were taking using an inverted AMG EVOS microscope equipped with an Olympus camera. (Scale bar represents 250 µm).
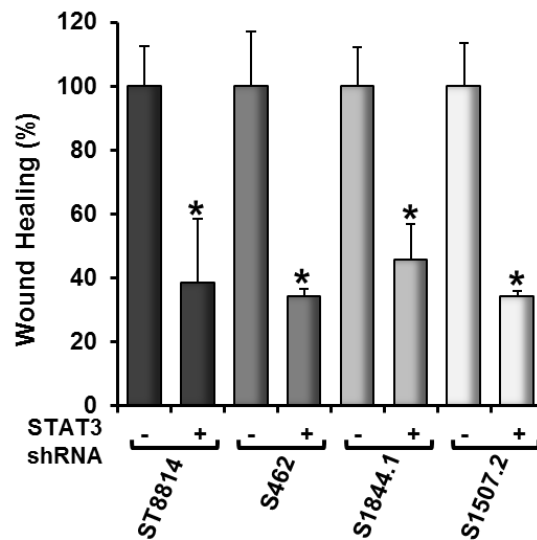
Figure 4.10. STAT3 knockdown reduces wound closure by over 50% in all MPNST cell lines. The effects of STAT3 knockdown on wound healing was determined by one way ANOVA. The percentage of migrated cells were compared in all 4 MPNST cell lines. Error bars are indicative of standard deviation from three independent experiments * $p < 0.05$ when comparing wound closure of treated versus untreated cells.

4.2.7 STAT3 knockdown in multiple MPNST cell lines impair migration and invasion

Following the wound healing experiment preformed in section 4.3.6, the migratory and invasive behaviour of MPNSTs after STAT3 knockdown was utilised via migration and invasion assay (see section 2.2.7). Results demonstrate once again that STAT3 knockdown significantly reduces migration and invasion of all 4 MPNST cell lines when compared to the control cells that utilised the non-target shRNA (Figure 4.11).
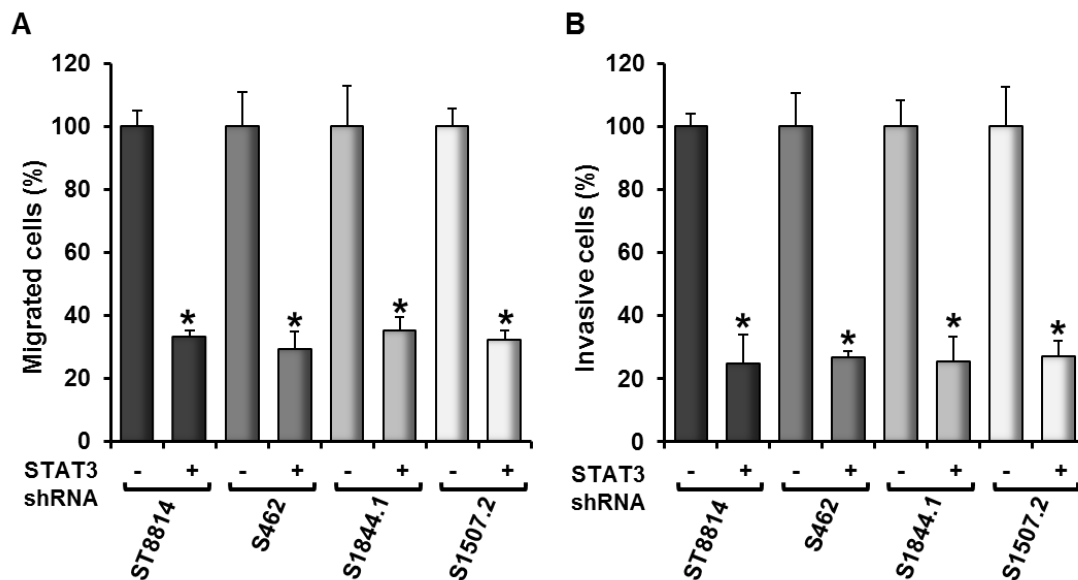
Figure 4.11. STAT3 knockdown in multiple MPNST cell lines impair migration and invasion in MPNST cell lines. A) Migration and **B)** Invasion of cells transfected with STAT3 and control shRNA was determined by seeding cells on Matrigel basement membrane on polycarbonate membranes. A significant reduction in the migration and invasion activity of all MPNST cell lines transfected with STAT3 shRNA can be observed in comparison to the non-target control. Error bars indicative of standard deviation from three independent experiments * $p < 0.05$.

4.2.8 STAT3 knockdown reduces cell proliferation in all MPNST cell lines by over 65%

As described in section 4.3.5, STAT3 inhibition significantly reduced cell proliferation in all MPNST cells. To further investigate the role of STAT3 in cell survival and proliferation a proliferation assay was carried out using control and STAT3 knockdown cell lines. Results show that in STAT3 knockdown cells, cell proliferation is reduced by over 65% in all MPNST cell lines. Showing the most effect in the S462 cell line (74% reduction in proliferated cells).
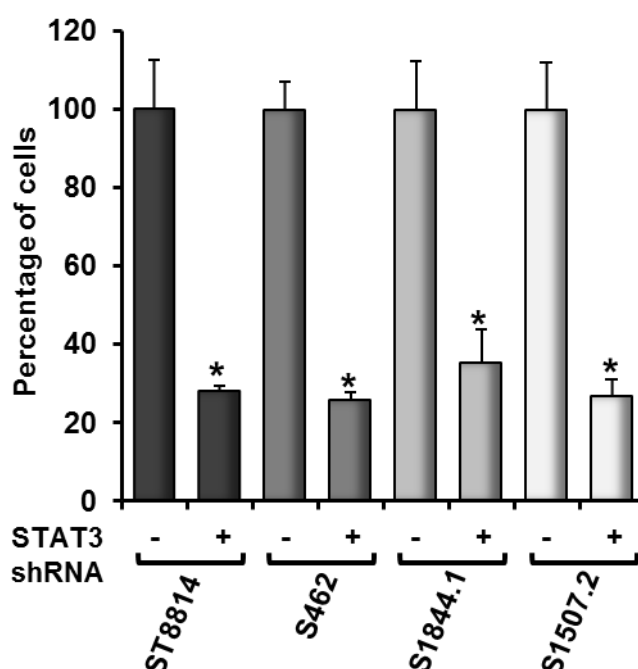
Figure 4.12. STAT3 knockdown reduces cell proliferation in all MPNST cell lines by over 65%. A proliferation assay was carried out on all MPNST cell lines transfected with either STAT3 or control shRNA (ST8814, S462, S1844.1 and S1507.2). Percentage of live cells was determined. A significant reduction in cell proliferation of all MPNST cell lines transfected with STAT3 shRNA can be observed in comparison to the non-target control. Error bars indicative of standard deviation from three independent experiments * $p < 0.05$.

4.2.9　STAT3 knockdown in multiple MPNST cell lines impair tumour formation

After demonstrating that STAT3 knockdown significantly reduces the migratory properties of all 4 MPNST cell lines it was important to investigate the ability of MPNSTs to form tumours before and after STAT3 knockdown. Therefore these MPNST cells were subjected to tumour spheroid assays (See section 2.2.8). Tumour growth and formation of all cell lines were closely monitored over 2 weeks. Tumour volume was greatly reduced within the STAT3 knockdown cell lines (Figure 4.13A). Statistical analysis revealed a significant reduction in the average tumour spheroid volume in all MPNST cells upon STAT3 knockdown, with the S1507.2 cell lines showing the biggest reduction of approximately 80% (Figure 4.13B). This data supports the central involvement of STAT3 in promoting tumourgenesis in multiple MPNSTs.
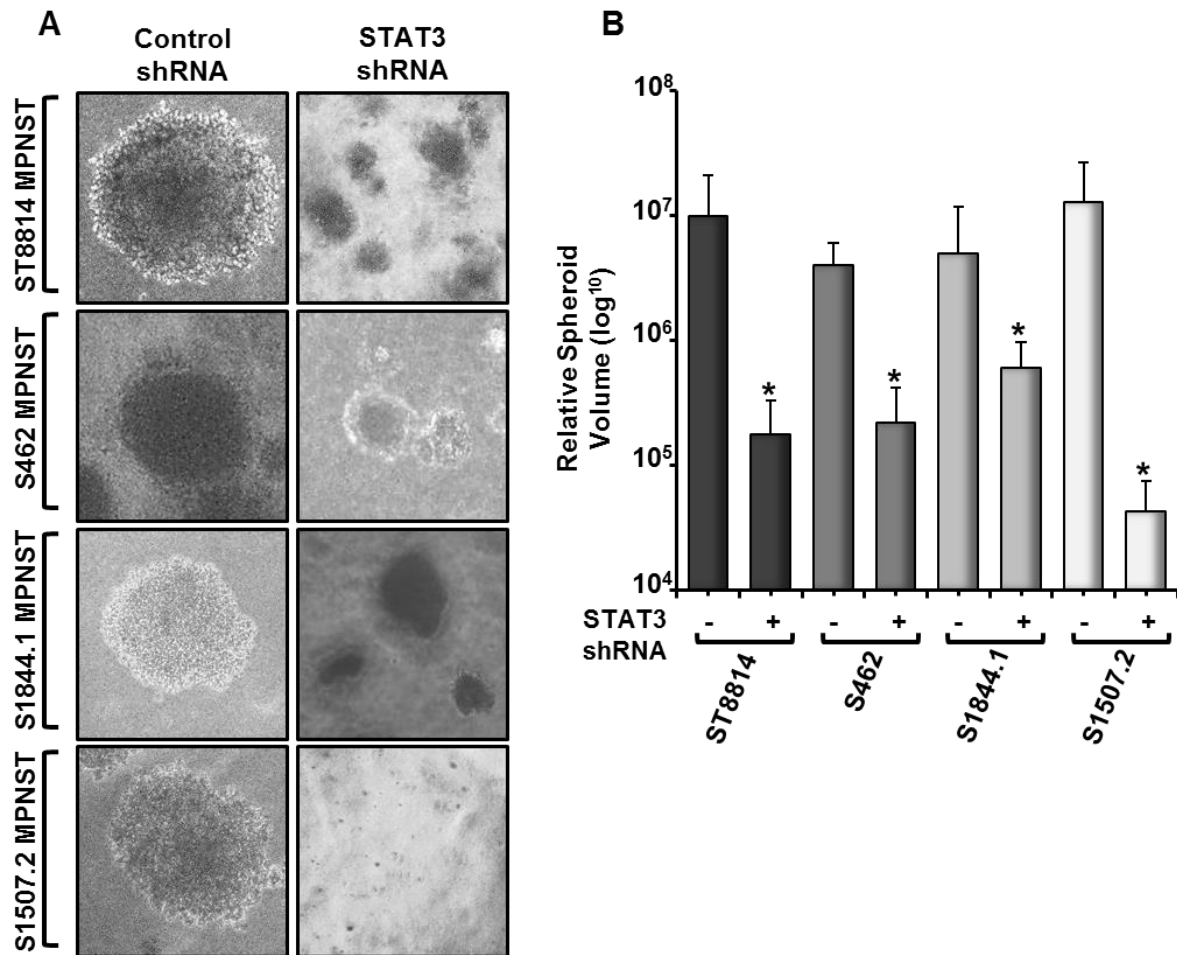
Figure 4.13. STAT3 knockdown in multiple MPNST cell lines impair tumour formation.

A) Stable ST8814, S462, S1844.1 and S1507.2 cell lines, expressing either non-target or STAT3 shRNA, as indicated, were subjected to tumour spheroid growth assays. Tumour growth was monitored over 2 weeks (scale bar represents 250 µm), **B)** the volume of 40 tumours from each cell line were randomly measured and the tumour size of the STAT3 knockdown cells were compared to the control cell line. Statistical analysis shows that the knockdown of STAT3 significantly reduces tumour growth in all 4 MPNST cell line.

4.3 Discussion

As demonstrated in chapter 3, The HGF/MET signalling pathway is involved in numerous cellular processes. A wide range of downstream signalling pathways have been associated with HGF/MET tumourgenesis and Metastatic properties. The JAK2/STAT3 is a downstream signalling pathway from MET thought to play a role in cell transformation. STAT3 activation has been observed in several primary tumours and also tumour cell lines (Pensa et al. 2000).

As mentioned before there are several other receptor tyrosine kinases that have the ability to activate the JAK2/STAT3 signalling pathway such as PDGF-R (Perrone et al. 2009). Interleukin receptors (IL-R) (Kawachi et al. 2013; Park et al. 2013) and EGF-R (Perrone et al. 2009; Park et al. 2013), that could result in tumourgenesis.

Further work by Wu *et al.* has shown an increase in levels of the EGF-R within peripheral nerve glial cells that cause the transformation of benign tumours to aggressive tumours in mice. EGF-R also stimulates STAT3 activation both *in vivo* and *in vitro.* Therefore abnormal activation of STAT3 was also observed in both mouse PNSTs and human MPNSTs. Reports have also shown that the JAK2/STAT3 inhibitor, FLLL32, decreases the time of MPNST growth in mice (J. Wu et al. 2014). STAT3 also initiates tumourgenesis and cell proliferation in other types of cancers.

This study provides an insight in to how STAT3 activation, could effects the Metastatic characteristics of MPNST cell lines. It has been clear that the HGF/MET signalling pathway is not responsible for the malignant behaviour of all the MPNST cell lines tested in this study. The 4 MPNST cell lines studied had varied response to inhibition of the HGF/MET pathway. To explore signal transduction from MET towards STAT3 in more detail, a time course experiment was carried out on these cell lines to determine the HGF response. Figure 4.1 demonstrates that the MPNST cell lines used in this study responded differently to HGF stimulation. In the ST8814 cells, tyrosine phosphorylation of STAT3 was robustly induced after just 30 min of HGF stimulation and maintained throughout the 3 h time course.

While in contrast, HGF stimulation of the S462 cells resulted in a much slower and less pronounced STAT3 response after 30 mins. The S462 cell lines needed 2-3 h stimulation to show a response to HGF. The S1844.1 and S1507.2 cell lines showed a quicker response to HGF stimulation after 30 mins; however unlike the ST8814 cell line they did not maintain their response throughout the 3 h stimulation. This difference likely reflects increased HGF/MET signalling (through increased MET and potentially pro-HGF expression) in the ST8814 cells when compared with the S462, S1844.1 and S1507.2 cell lines.

Cells were also stimulated with interleukin-6 (IL-6) for 30 min as a positive control for STAT3 activation; also illustrating that signalling through multiple receptor tyrosine kinases in MPNSTs (such as interleukin receptors) can activate JAK2/STAT3 signalling. Of interest, IL-6 serum levels were reportedly elevated in NF1 patients with MPNST (Park et al. 2013). It is therefore likely that STAT3 is activated via multiple mechanisms in NF-1, making it an appealing therapeutic target.

When investigating the effects of STAT3 inhibitors on STAT3 phosphorylation (Tyr705 and Ser727), the Ser727 phosphorylation of STAT3 (figure 4.3) was less affected by these inhibitors. This was expected as the inhibitors used are mainly JAK2/STAT3 inhibitors that would block JAK2-mediated phosphorylation of the Tyr705 residue. Furthermore, the Ser727 phosphorylation of STAT3, is known to be directly phosphorylated by the serine/threonine kinase, mTORC1, and not the tyrosine kinase, JAK2 (Dodd et al. 2014).

Wound healing, migration and invasion assays have previously been used to determine the tumour invasive behaviour of cancer cells (Hulkower & Herber 2011). These assays were utilised within this study to determine the effects of STAT3 repression via inhibitors on the migratory properties of MPNST cell lines. For wound healing, all three STAT3 inhibitors, cucurbitacin-I, 5,15 DPP and FLLL31, were used. However cucurbitacin-I caused the S1844.1 and S1507.2 cell lines to round up and eventually detach from the plate. This finding implies that cucurbitacin-I does not only target JAK2/STAT3, but rather is likely to also have off drug-target effects on these cell lines (figure 4.4), making it a less reliable

JAK2 inhibitor to use. With the ST8814 and S462 cell lines cucurbitacin-I did not cause detachment of cells however after 24h it did change the morphology of the cells slightly also causing them to roundup and look unhealthy. The 5,15 DPP inhibitor did not show any significant reduction in cell movement within three of the cell lines examined. This could be due to the fact that the 5,15 DPP inhibitor is not a JAK2 inhibitor but a selective STAT3 Src homology-2 domain (SH2) antagonist.

Therefore, the follow-up migration and invasion assays were carried out using only the FLLL31 inhibitor. This showed significant reduction in both migration and invasion in all MPNST cell lines tested.

As the STAT3 inhibitors showed variability in decreasing cell migratory behaviour, and to be sure that STAT3 inhibition is causing a decrease in cell migration, STAT3 gene expression was initially attempted to be knockdown in the ST8814 cell lines using lipofectamine transfection with STAT3 shRNA expressing DNA plasmid. However after 3 attempts, STAT3 knockdown was not achieved and likely is due to poor transfection efficiency. Therefore, to enhance knockdown of STAT3, stable cell lines were established using lentivirus infection of STAT3 shRNA. STAT3 protein levels were analysed to ensure significant knockdown (figure 4.). It was also noted that proliferative rates of STAT3 knockdown cells were lower, when compared to the non-target control cells. Consequently, STAT3 knockdown cells were seeded at a higher density to maintain them in tissue culture. In particular, knockdown of STAT3 within the S462 MPNST cell line resulted in a decrease of cell adherence as well as cell growth compared to the other three MPNST cell lines examined (ST8814, S1844.1 and S1507.2). The S462 cells became unhealthy and died 3-4 weeks after the STAT3 knockdown whereas the other cell lines survived for 7-8 weeks after knockdown. All experiments carried out using the STAT3 knockdowns were preformed within the first month after insuring successful knockdown of the STAT3 gene. STAT3 expression returned after 4-8 weeks after infection and selection, which implies that STAT3 expression likely gives these MPNST cell lines a selective advantage in tissue culture. Consequently,

control blots for STAT3 were routinely carried out to verify that STAT3 was still being efficiently knocked-down. Also given the higher level of cell death in the S462 cell lines, loss of STAT3 expression likely reduces the viability of these cells through loss of STAT3-driven cell survival pathways. A known survival pathway of STAT3 is through metabolic transformation through HIF-1α (Which is further explored in chapter 5).

In addition wound healing, migration and invasion assays were also carried out on STAT3 knockdown cell lines to determine the functional effect of STAT3 on the malignant behaviour of MPNSTs. Rapid wound healing in the control MPNST cell lines clearly shows aggressive and migratory characteristics of these cells that was lost upon STAT3 knockdown. To further confirm this, invasion assays indicated that the control non-target shRNA infected MPNST cell lines were able to invade through a basement membrane matrix and find their way toward a chemo attractant (In this case fibronectin), suggesting that these cells have a high ability to breakdown extracellular matrix and invade neighbouring tissues through metastasis processes. Furthermore STAT3 has been reported to regulate genes involved in metastasis and invasion, including the matrix Metalloproteinases (MMPs). The MMPs have the ability to degrade the basement membrane along with the extracellular matrix. In more metastatic and aggressive cancers MMPs are usually seen to be over expressed allowing the intravasation of cancer cells in to the vasculature system. Apart from MMPs, there are other STAT3 targets that play important roles in the invasive behaviour of tumour cells including HIF-1α, VEGF, HSP70, HSP90 etc. (Teng et al. 2014).

Moreover a significant reduction in wound healing, migratory and invasive behaviour of cells was identified in all 4 MPNST with efficient STAT3 knockdown. These results coincide with what was observed when STAT3 was blocked using JAK2/STAT3 drug inhibitors.

There is evidence in the literature indicating that STAT3 promotes the early stages of tumour growth in MPNST cells (J. Wu et al. 2014). Furthermore STAT3 has been known as an oncogene that promotes tumour formation and tumourgenesis during cancer progression,

helping to maintain tumour cell proliferation in numerous cancers (Yu et al. 2009; J. Wu et al. 2014).

To examine whether or not STAT3 also has an effect on tumour growth in these MPNSTs cell lines, a tumour formation assay was carried out. In this experiment the control MPNST cell lines started forming tumours 5 days after being seeded into soft agar (data not shown) in contrast to this the STAT3 knockdown cell lines did not show any sign of tumour formation. The growth of the tumours were monitored closely over 2 weeks and pictures were taken on day 14. The control MPNSTs had formed fewer but large tumours whereas the STAT3 knockdowns had formed more but much smaller clusters of cells in soft agar. Statistical analysis was carried out comparing the volume of 40 tumour spheroids from the control and knockdown cell lines. These spheroids are not perfect spheres; therefore the length of the widest point of the spheroid was taken to measure the approximate radius and tumour volume for all the tumour spheroids. Figure 4.12 clearly demonstrates that STAT3 knockdown affects the ability of these aggressive MPNST cell lines to cluster and form tumours in soft agar, once again suggesting that STAT3 plays a fundamental role in driving malignancy within a range of MPNST cell lines and possibly other tumours.

5 Chapter 5: Hypoxic signalling downstream of STAT3 drives oncogenic cellular phenotypes in malignant peripheral nerve sheath tumour cells

5.1 Introduction

Cell signalling pathways that drive NF1 tumour formation and malignancy have not yet been clearly delineated. In the last result chapter, work presented showed that STAT3 was centrally involved in an array of oncogenic cellular phenotypes within MPNSTs. This chapter aims to ascertain what downstream signalling component(s) of STAT3 likely attribute to these malignant characteristics.

Research over the last decade indicates a correlation between STAT3 activation and HIF-1α protein expression. For instance, STAT3 activation is known to enhance HIF-1α protein expression. Furthermore, STAT3 and HIF-1α are both recruited to the VEGF-A promoter under hypoxic conditions resulting in histone H3 acetylation proximal to the promoter and VEGF-A expression (Jung et al. 2005).

Given the link between STAT3 and HIF, it is possible that HIF signalling might be involved in the malignant phenotypes of MPNSTs. This chapter examines the involvement of STAT3 in the regulation of HIF and VEGF-A.

When solid tumours grow larger than approximately 2 mm, their proximity to the blood supply is not close enough. Consequently, tumour growth becomes restricted as a result of low oxygen levels within the centre of the tumour, causing hypoxia. Hypoxia signalling within the tumour core leads to angiogenesis, which re-feeds the tumour core with essential oxygen as well as nutrients and growth factors. Hypoxia can play a role in resistance to cancer therapies, where the hypoxic regions within the tumour can be less sensitive to radiotherapy. The transcription factor, HIF-1α, is responsible for increasing the oxygen levels in physiological and pathophysiological conditions. Making it a valuable target for therapy (Gilkes et al. 2014).

In this part of the study, the effects of HIF-1α on migration, invasion and tumourgenesis within MPNST cell lines was examined through a series of experiments.

To identify previously unrecognized differently expressed genes between benign and malignant NF1 tumours, an analysis of copy number variation (CNVs) and gene expression was carried out by Upadhyaya and her team. Results showed that the *SPP1* gene [NCBI: NM_001040058] (Osteopontin; SPP1) was found to exhibit the most significantly elevated differential mean expression level between the benign and malignant tumours (Table 5.1). Furthermore studies have shown that SPP1 plays an important role in the motility of several cancer cells, such as breast cancer, prostate cancer and melonoma. In addition SPP1 has the ability to mediate JAK2/STAT3 actiation via the avb3 integrin in breast cancer cell lines (Behera et al. 2010). SPP1 was therefore selected in this chapter for further functional analysis to assess its possible role in malignant transformation. This work aims to elucidate the role of SPP1 in cell migration and invasion via the knockdown of SPP1 in 4 different MPNST cell lines.

Table 5.1. Top 20 genes identified as being significantly differentially expressed between MPNSTs and PNFs (Thomas et al. 2015).

Gene symbol	Mean expression benign	Mean expression Malignant	Fold change Benign vs Malignant	p-value
FAP	4.87	8.75	14.75	0.0001
NSBP1	5.22	3.67	-2.92	0.0001
KRTAP13n4	3.77	3.13	-1.55	0.0001
PTGIS	4.58	6.60	4.06	0.0002
KIAA0746	4.33	5.82	2.80	0.0002
SPP1	**5.65**	**12.06**	**85.19**	**0.0002**
FAM177A1	8.01	7.11	-1.86	0.0003
ADH1B	6.50	3.18	-9.99	0.0003
GRIK3	7.20	3.85	-10.19	0.0004
FLJ42200	3.32	4.05	1.65	0.0004
COL10A1	3.56	4.67	2.17	0.0004
CSRP1	9.09	7.28	-3.50	0.0005
GRIK2	7.02	3.47	-11.66	0.0005
CD72	4.08	5.08	1.99	0.0005
EFHC2	4.84	3.42	-2.67	0.0005
SLC25A12	5.56	6.30	1.68	0.0006
GTDC1	6.95	5.92	-2.05	0.0006
PEG3	5.58	4.17	-2.65	0.0006
CCT5	8.62	9.33	1.64	0.0006
TDP1	4.17	5.49	2.50	0.0007

5.2   Results

5.2.1   STAT3 Knock down in multiple MPNST cell lines results in a reduction of HIF-1α, HIF-2α and VEGF-A protein expression

The initial stage of this investigation was to analyse the STAT3/HIF-1α/HIF-2α/VEGF-A signalling axis in MPNSTs. To do this, 4 stable STAT3 knock down MPNST cell lines were utilised (See section 4.2.6). In these STAT3 knockdown MPNSTs, protein expression of HIF1- α, HIF-2 α and VEGF-A was examined and compared to the non-target control cells. These experiments were carried out in hypoxic conditions (1% O_2) to establish the protein expression and activity of HIF-1α/HIF-2α.

The data clearly demonstrates that HIF-1α, HIF-2α and VEGF-A protein expression is reduced in the absence of STAT3 activation within 4 MPNST cells (figure 5.1). STAT3 protein expression was ablated by STAT3 shRNA and confirms that STAT3 was efficiently knocked down in these MPNST cells (figure 5.1).
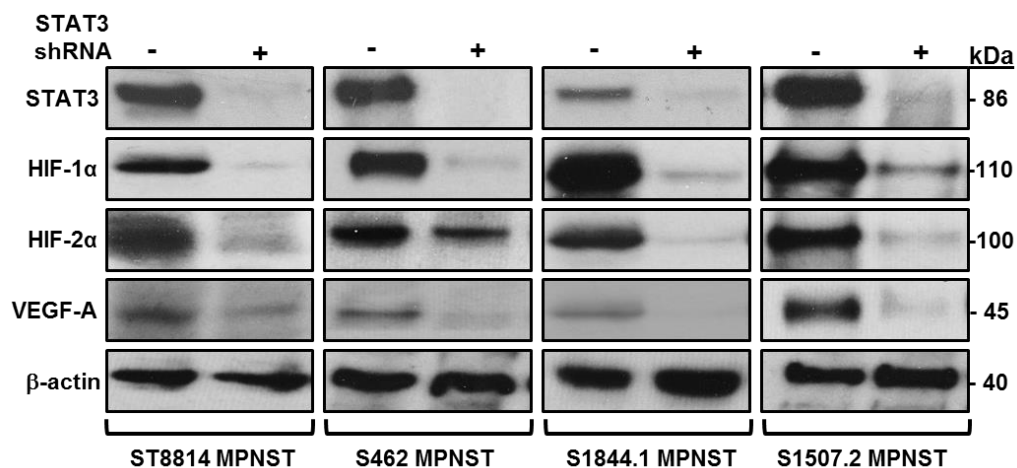
Figure 5.1: STAT3 Knock down in multiple MPNST cell lines results in a reduction of HIF-1α, HIF-2α and VEGF-A protein expression: ST8814, S462, S1844.1 and S1507.2 MPNST cell lines where transfected using lentivirus packages either with STAT3 shRNA or non-target control MISSION shRNA. Cells were serum starved and cultured under hypoxia (1%) overnight. Cells were then harvested in sample buffer and total lysates were analysed for HIF-1α, HIF-2α and VEGF-A levels using a western blot assay. Western blot analysis shows that HIF-1α, HIF-2α and VEGF-A protein expression are potently impaired upon STAT3 knockdown. β-actin serves as a loading control. n = 3.

5.2.2 mTORC1 inhibition with rapamycin is not sufficient to block signal transduction through STAT3/HIF-1α in multiple MPNSTs.

It was previously shown in our lab by Dodd *et al.* that mTORC1 is a pivotal kinase involved in HIF-1α/VEGF-A signalling in HEK293 cells and an upstream kinase of STAT3 (Dodd et al . 2014). In addition a study has indicated that HIF-1α expression levels in MPNSTs were fairly resistant to mTORC1 inhibition with rapamycin (Johannessen et al. 2008), indicating that mTORC1 is less involved in promoting HIF-1α in MPNSTs. Therefore in this study it was hypothesised that JAK2/STAT3 might be the dominant pathway that promotes signal transduction through HIF-1α in MPNSTs. To confirm this new JAK2/STAT3/HIF-1α signalling axis in MPNSTs, the HIF-1α protein expression after treatment with the JAK2 inhibitors, cucurbitacin-I, 5,15-DPP and FLLL31, and with the mTORC1 inhibitor, rapamycin was examined (Figure 5.2). Results show that Inhibition with Cucurbitacin-I and FLLL31 significantly reduce HIF-1α protein levels in all MPNST cell lines. Consistent with our previous observations, the 5,15-DPP inhibitor was less effective. Apart from in the S1507.2 cells, inhibition of mTORC1 with rapamycin was insufficient to decrease the protein levels of HIF-1α in these MPNST cell lines. Phosphorylation of ribosomal protein S6 (rpS6) was used as a readout for mTORC1 activity and confirmed efficacy of rapamycin. In all MPNST cell lines, STAT3 and mTORC1 inhibition reduced VEGF-A protein levels (Figure 5.2), where presumably the mTORC1 input through VEGF-A is likely through its translational regulation as previously reported by our lab (Dodd et al. 2014).
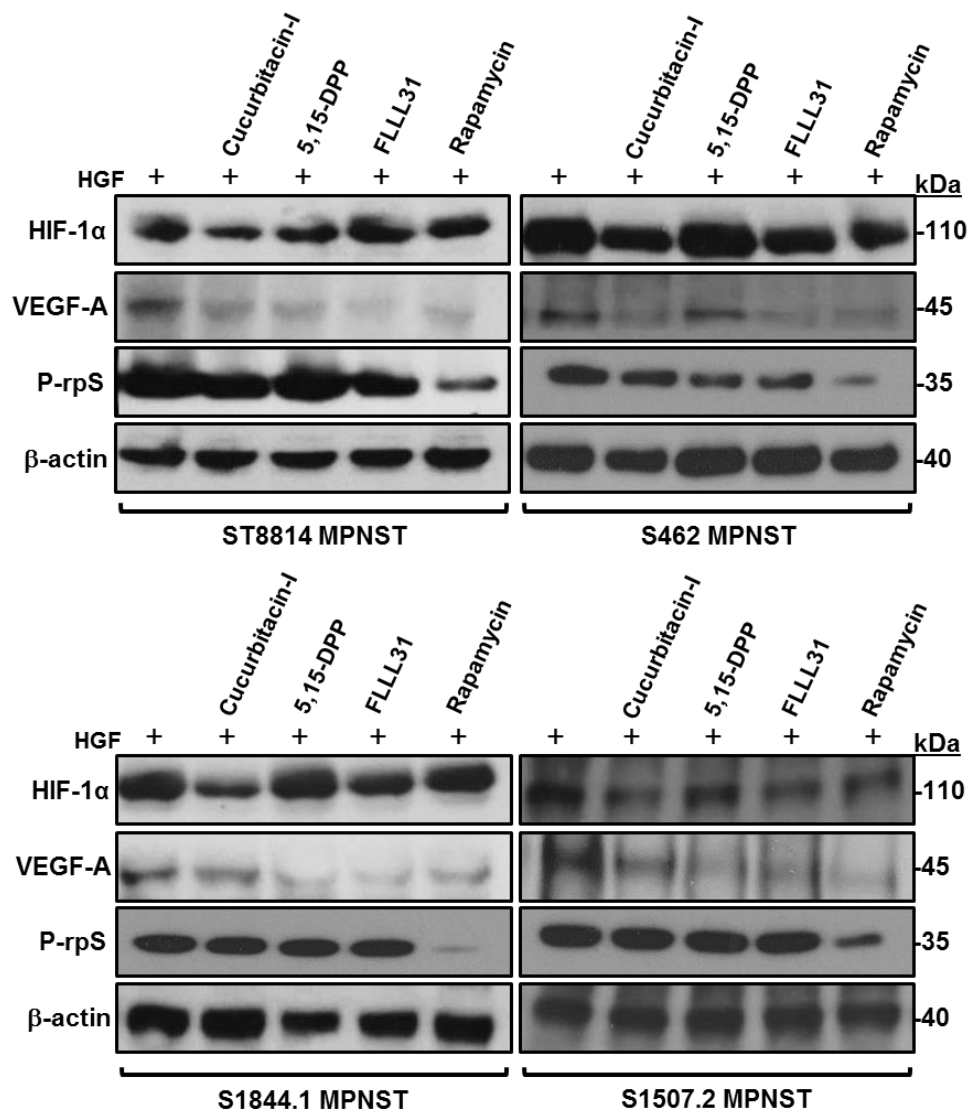
Figure 5.2: mTORC1 inhibition with rapamycin is not sufficient to block signal transduction through STAT3/HIF-1α in multiple MPNSTs: 4 MPNST cell lines (ST8814, S462, S1844.1 and S1507.2) were pre-treated with 5 nM Cucurbitacin-I, 50 nM 5,15-DPP and 5 nM FLLL31 or Rapamycin for 30 min prior to 30 min 20 ng/ml HGF stimulation, Western blot analysis shows that HIF-1α and VEGF-A protein expression is impaired after treatment with STAT3 inhibitor

5.2.3 HIF-1α knockdown in the 4 MPNST cell lines impair wound healing

It is a well-known fact that one of the key processes driving malignancy is indeed hypoxia. In addition, HIF-1α inactivation has been shown a significantly inhibit tumour growth and malignancy within mouse models (Rohwer et al. 2013).

In order to examine the involvement of HIF-1α in oncogenic signalling downstream of STAT3 in the MPNST cell lines, HIF-1 α shRNA was used to generate HIF-1α knockdown MPNST cells. HIF-1α protein expression and mRNA levels (section 2.2.5) were analysed, confirming that HIF-1α was efficiently knocked down in these MPNST cells before proceeding to further experiments (figure 5.4). The growth of the control and HIF-1α knock down cells were closely monitored in all four MPNST cell line. Interestingly, HIF-1α knockdown in the S462 MPNST cell line caused a reduction in the growth and proliferation rate, and cells started to appear stressed after 3-4 weeks, while the other MPNST cell lines were less effected and continued to grow as normal for 2-3 months.

In order to examine the dependency of HIF-1α on cell motility, a wound healing assay was carried out on these HIF-1α. Cells were scratch and placed in hypoxia (1% O2) in order to activate HIF protein for 18 h. Pictures of cells were taken at 0 and 18 h. Pictures clearly demonstrate that the knockdown of HIF-1α reduces the ability of all 4 MPNST cell lines to close the wound (figure 5.5). A statistical analysis was carried out to investigate the significance of HIF-1α knock down in prohibiting cell motility in these malignant cell lines. A one way ANOVA was performed and a significant P-Value was observed for all cell lines (figure 5.6).
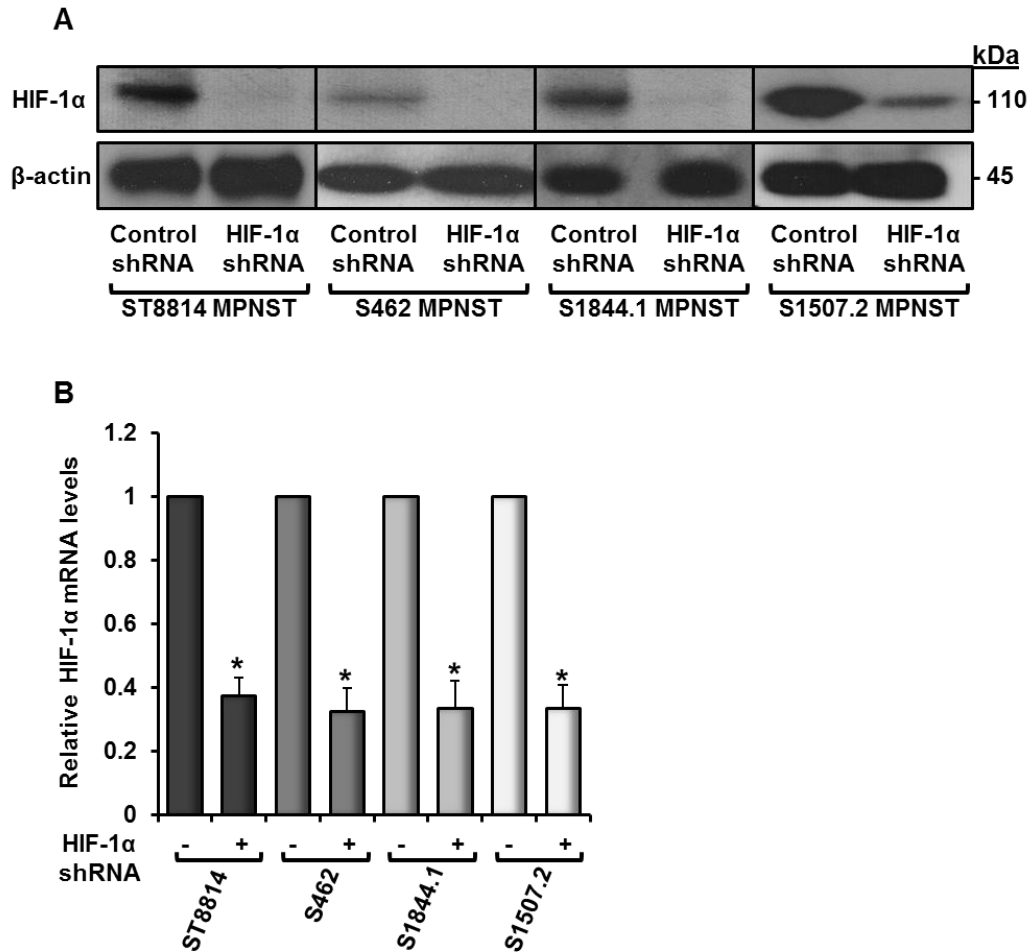
Figure 5.4: HIF-1α knockdown in the 4 MPNST was confirmed using western blot assay and qPCR: ST8814, S462, S1844.1 and S1507.2 MPNST cell lines where infected with lentivirus packaged with either HIF-1α shRNA or non-target control MISSION shRNA. Cells were serum starved and cultured under hypoxia (1% O2) overnight. Cells were then harvested in sample buffer and total lysates were analysed for **A)** HIF-1α protein expression levels and **B)** mRNA level. β-actin serves as a loading control. n = 3.
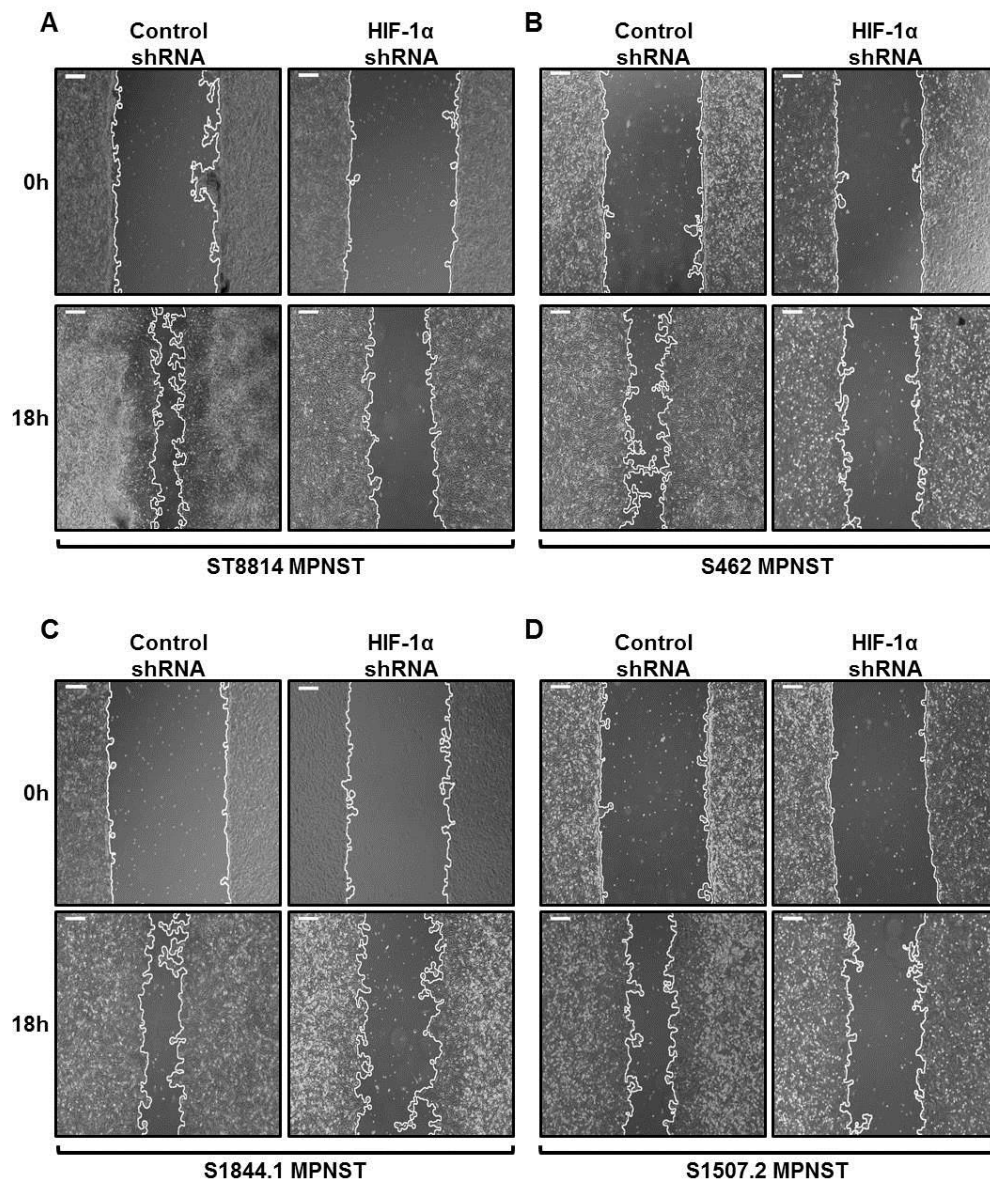
Figure 5.5: HIF-1α knockdown in the 4 MPNST cell lines impair wound healing: Stable **A)** ST8814, **B)** S462, **C)** S1844.1 and **D)** S1507.2 cell lines, expressing either non-target or HIF-1α shRNA as indicated were serum starved overnight and subjected to cell wound as described in section 4.2. Cells were grown in hypoxia (1% O2), pictures were taken at 0 and 18 h after wounding.
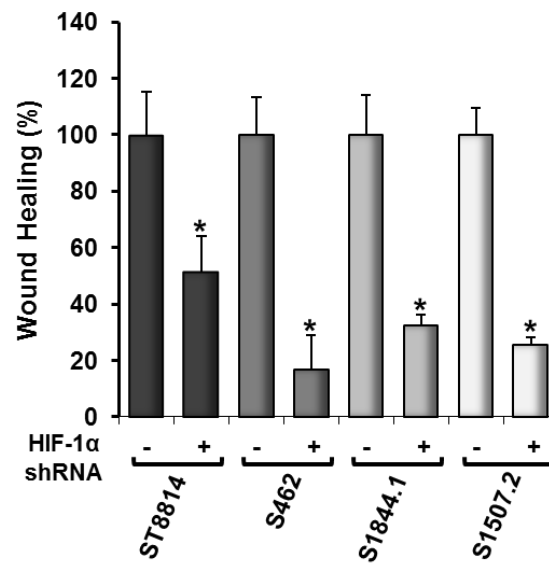
Figure 5.6: HIF-1α knockdown reduces wound closure in all MPNST cell lines. The effects of HIF-1α knockdown on wound healing was determined by one way ANOVA. The percentage of migrated cells were compared in all 4 MPNST cell lines. Error bars are indicative of standard deviation from three independent experiments * $p < 0.05$ when comparing wound closure of treated versus untreated cells.

5.2.4 HIF-1α knockdown in the 4 MPNST cell lines impair cell invasion and migration

The next stage of this line of experimentation was to investigate the effect of HIF-1α on cell migration and metastasis. Cell migration and invasion assays were carried out to examine whether HIF-1 α was involved in these malignant traits.

With both migration and invasion assay 10^6 cells were cultured in hypoxia (1% O_2) and the migrated cells were determined by Genova MK3 Lifescience Analyser (see section 2.2.7). The results demonstrate that HIF-1α knockdown in all the MPNST cells tested significantly reduces cell migration and invasion (Figure 4.6). HIF-1α knockdown had the most effect on the S462 MPNST cell line with approximately 80% reduction in cell migration and invasion. Furthermore the knockdown of HIF-1α caused the S462 cell lines to grow considerably slower than the control cell line. The ST8814 cell lines were less affected by HIF-1α knockdown, with approximately 40% reduction in the cell migratory and invasive behaviour of the cell lines (Figure 5.7).
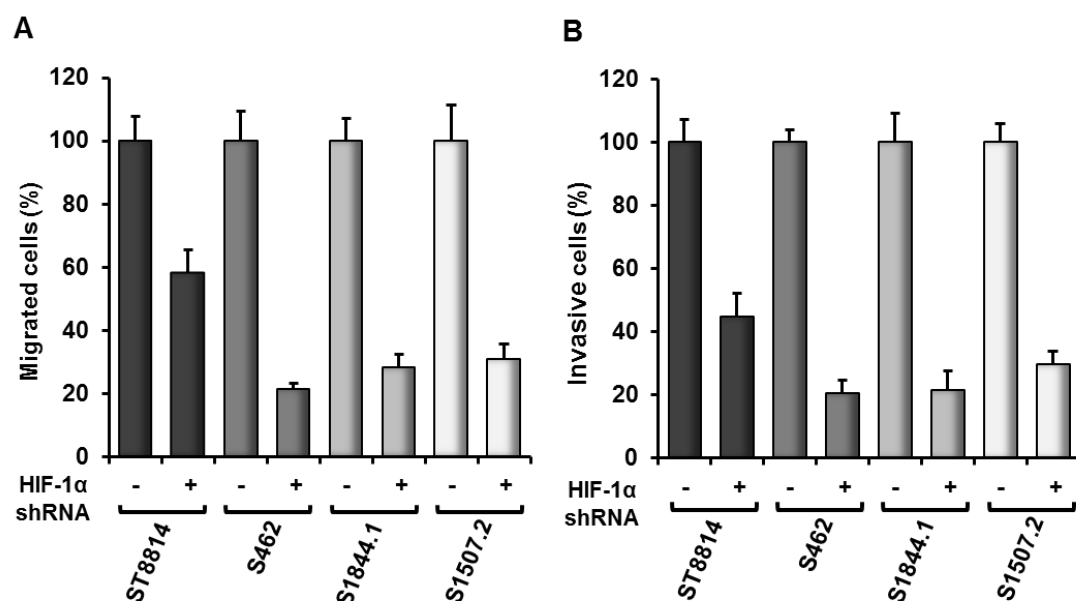
Figure 5.7: HIF-1α knockdown in the 4 MPNST cell lines impair cell invasion and migration: Stable ST8814, S462, S1844.1 and S1507.2 cell lines, expressing either non-target or HIF-1α shRNA as indicated were subjected to either **A)** Migration or **B)** Invasion assays. 106 Cells were seeded in transwell permeable supports as described in section … and were cultured in hypoxia (1% O2) Cells were stained and absorbance was read at 550 nm on a Genova MK3 Lifescience Analyser .The number of migrated cells were calculated by plotting the absorbance against a standard curve. The percentage of invasive or migrated cells were calculated in 3 individual experiments.

5.2.5 HIF-1α knockdown reduces cell proliferation in all MPNST cell lines by over 68%.

The knockdown of HIF-1α affected the growth of the S462 MPNST cell line. After knockdown, the growth of these cells reduced significantly and cells started to look rounded and unhealthy after a couple of weeks. Therefore it was of interest to examine the effects of HIF-1α knockdown on cell proliferation in these cell lines. In order to do so a cell proliferation assay was carried out. Results demonstrate that cell proliferation is significantly impaired after HIF-1α knockdown in all MPNST cell lines with the highest effect on the S462 cell line (approximately 76% reduction) (Figure 5.8).
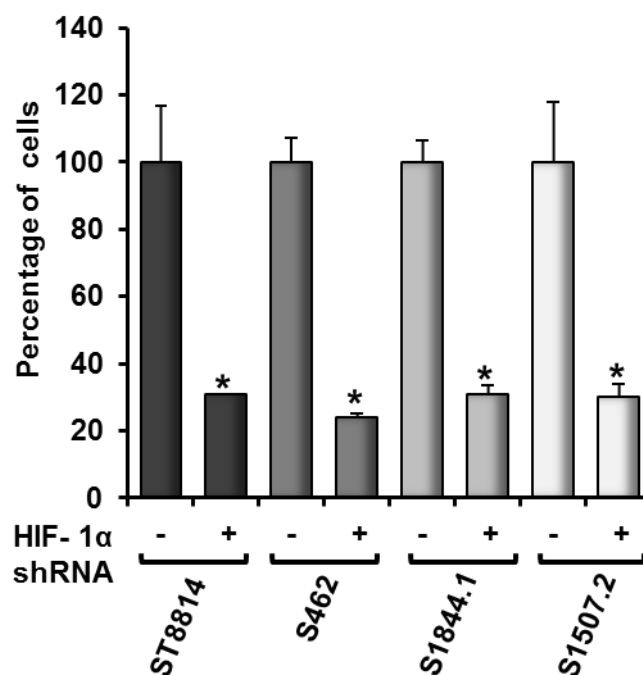
Figure 5.8.HIF-1α knockdown reduces cell proliferation in all MPNST cell lines by over 68%. A proliferation assay was carried out on all MPNST cell lines transfected with either HIF-1α or control shRNA (ST8814, S462, S1844.1 and S1507.2). Percentage of live cells was determined. A significant reduction in cell proliferation of all MPNST cell lines transfected with HIF-1α shRNA can be observed in comparison to the non-target control. Error bars indicative of standard deviation from three independent experiments * $p < 0.05$.

5.2.6 HIF-1α knockdown reduces the ability of MPNST cell lines to form tumours

After confirming the involvement of HIF-1α in wound healing, migration and invasion, I was interested in observing the role that HIF-1α plays in tumour formation. Therefore, both HIF-1α knockdown and non-target MPNST cell lines were subjected to tumour spheroid assays (See section 2.2.8). The growth and tumour formation of cells were monitored over two weeks and tumour formation in HIF-1α knockdown and non-target cells were compared in all 4 MPNST cell lines. It was observed that HIF-1α knockdown cell lines were significantly compromised in their ability to form tumours compared to the non-target MPNST cell lines (Figure 5.9A).

Statistical analysis was carried out and results demonstrate a significant reduction in the average tumour spheroid volume in all MPNST cells after HIF-1α knockdown (Figure 5.9B).
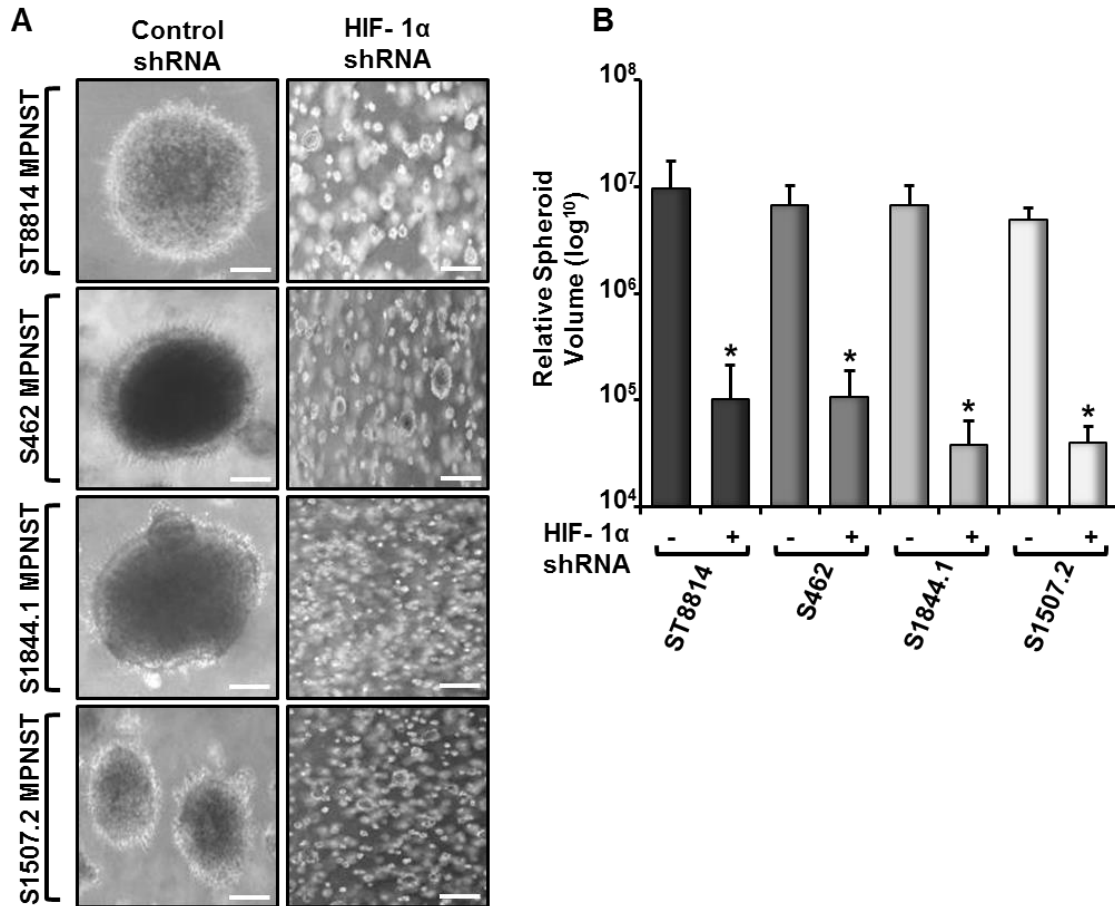
Figure 5.9. HIF-1α knockdown reduces the ability of MPNST cell lines to form tumours.
Stable ST8814, S462, S1844.1 and S1507.2 cell lines, expressing either non-target or HIF-1α shRNA, as indicated, A) were subjected to tumour spheroid growth assays. Tumour growth was monitored over 2 weeks (scale bar represents 250 µm) pictures were taken after 2 weeks , B) the volume of 40 tumours from each cell line were randomly measured and the tumour size of the HIF-1α knockdown cells were compared to the control cell line. Statistical analysis shows that the knockdown of HIF-1α significantly reduces tumour growth in all 4 MPNST cell line.

5.2.7 SPP1 knockdown in multiple MPNST cell lines impairs wound healing

Given that we observed significant differences in the expression level of SPP1 in MPNSTs compared to the benign plexiform neurofibromas (Table 5.1) (Thomas et al. 2015) and also that SPP1 has previously been shown to play an important role in tumorigenesis and metastasis (Rodrigues et al. 2007), SPP1 gene expression was knocked down using a similar approach to STAT3 and HIF-1α knockdown (See section 2.2.1.2). Validation of specific gene knockdown using shRNA clones was completed by western blot analysis. A β-actin control blot was performed to confirm that there were no shRNA off-target effects (Figure 5.10). Furthermore a wound healing assay was carried out to examine the effect of SPP1 knockdown on the ability of the MPNST cells to wound heal. As observed in figure 5.11, SPP1 knockdown impairs the cells ability to migrate and close the wound compared to the control cell line where the cells migrate faster and start to close the wound at an earlier stage. Statistical analysis clearly demonstrate that the knockdown of SPP1 gene significantly reduces wound healing in all MPNSTs tested (Figure 5.12).
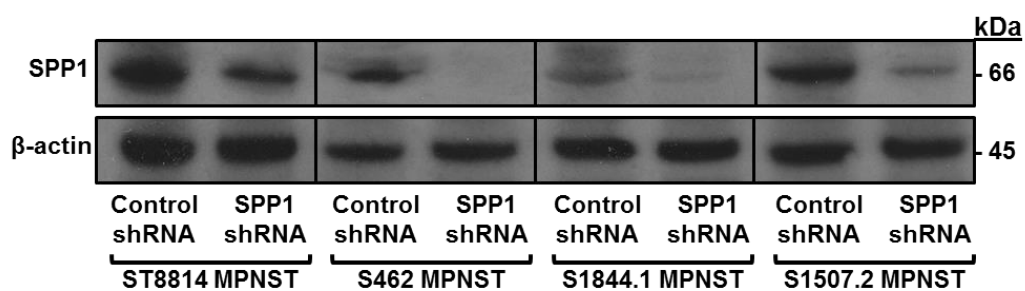
Figure 5.10 SPP1 knockdown in the 4 MPNST cell lines was confirmed using western blot assay: ST8814, S462, S1844.1 and S1507.2 MPNST cell lines where transfected using lentivirus packages either with SPP1 shRNA or non-target control MISSION shRNA. Cells were serum starved and cultured (5% CO_2 37°C) overnight. Cells were then harvested in sample buffer and total lysates were analysed for SPP1 protein expression levels. β-actin serves as a loading control. n = 3.
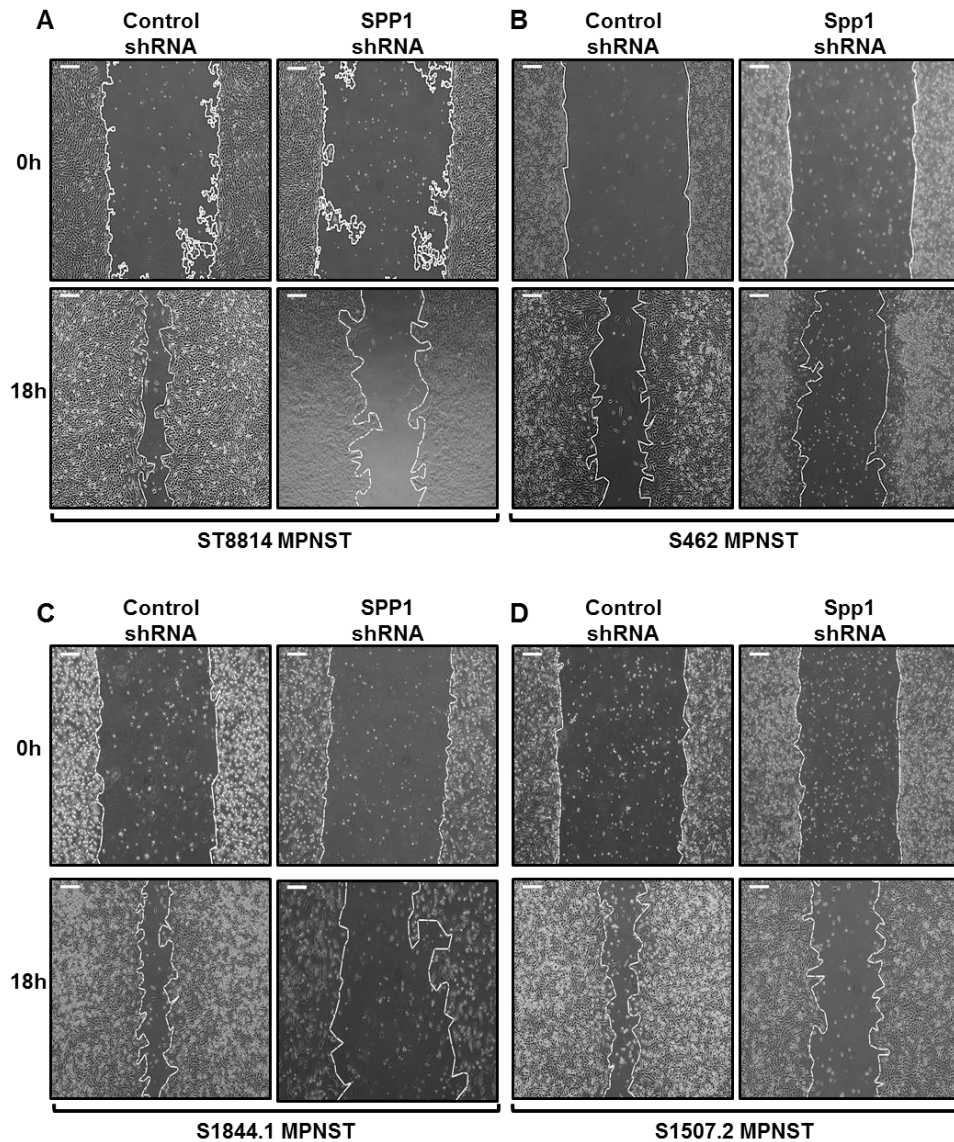
Figure 5.11. SPP1 knockdown in the 4 MPNST cell lines impair wound healing: Stable **A)** ST8814, **B)** S462, **C)** S1844.1 and **D)** S1507.2 cell lines, expressing either non-target or SPP1 shRNA as indicated were serum starved overnight and subjected to cell wound as described in section 4.2. Cells were grown in 5% CO2 and at 37oC. Pictures were taken at 0 and 18hafter wounding. E) The wound area was measured at 0 and 18h using imageJ software and the percentage of wound healing was calculated. SPP1 knockdown significantly reduces wound healing in all MPNST cell lines. Error bars are indicative of standard deviation from three independent experiments. * $p < 0.05$
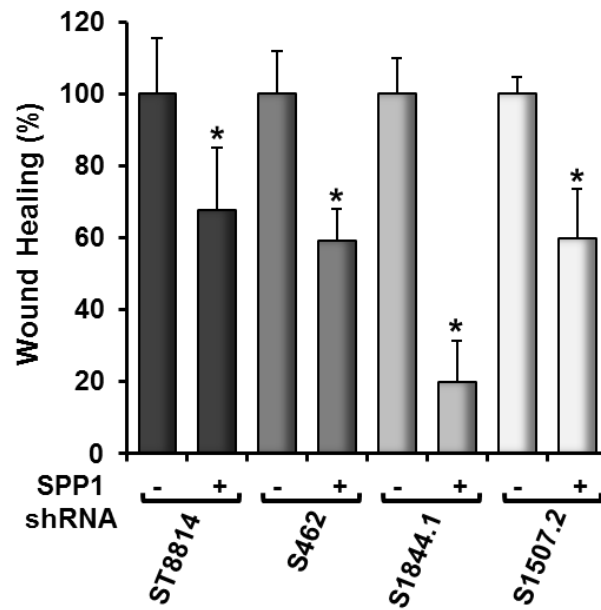
Figure 5.12: SPP1 knockdown reduces wound closure in all MPNST cell lines. The effects of SPP1 knockdown on wound healing was determined by one way ANOVA. The percentage of migrated cells were compared in all 4 MPNST cell lines. Error bars are indicative of standard deviation from three independent experiments * $p < 0.05$ when comparing wound closure of treated versus untreated cells.

5.2.8 SPP1 knockdown in the 4 MPNST cell lines impair cell invasion but not cell migration

As mentioned before in all cell lines, knockdown of SPP1 robustly inhibited wound closure suggesting a possible role for SPP1 in metastasis. In order to further support this, an invasion and also migration assay was carried out. Interestingly results revealed that SPP1 knockdown significantly reduces the cells' invasive properties in all 4 MPNST cell lines however it had no significant effect on reducing cell migration (Figure 5.13).

There are several factors involved in cell invasion, which include, cell motility, cell adhesion, and the activation of enzymes that have the ability to degrade the extracellular matrix. SPP1 is known as a metastatic gene that can change the gene expression of several genes including EGFR, MMPs and uPA. The MMP family of proteins and uPA (plasminogen activator, urokinase) have the ability to breakdown the extracellular matrix allowing the cells to invade other tissues, resulting in metastasis in abnormal cells. Studies have also shown that SPP1 is responsible for the PI3K-dependent NFκB activation which once again results in the activation of uPA and cell invasion (Das et al. 2005; Song et al. 2009). Moreover it has been shown that SPP1 also can play a role in cell migration via its interactions with integrins.

It is possible that in the MPNSTs tested in this study SPP1 only affects the signalling pathways related to cell invasion but not cell migration. It also could be possible that these cells carryout their migratory characteristic through positive feedback loops after SPP1 knockdown, resulting in no significant effect in cell migration after SPP1 knockdown.
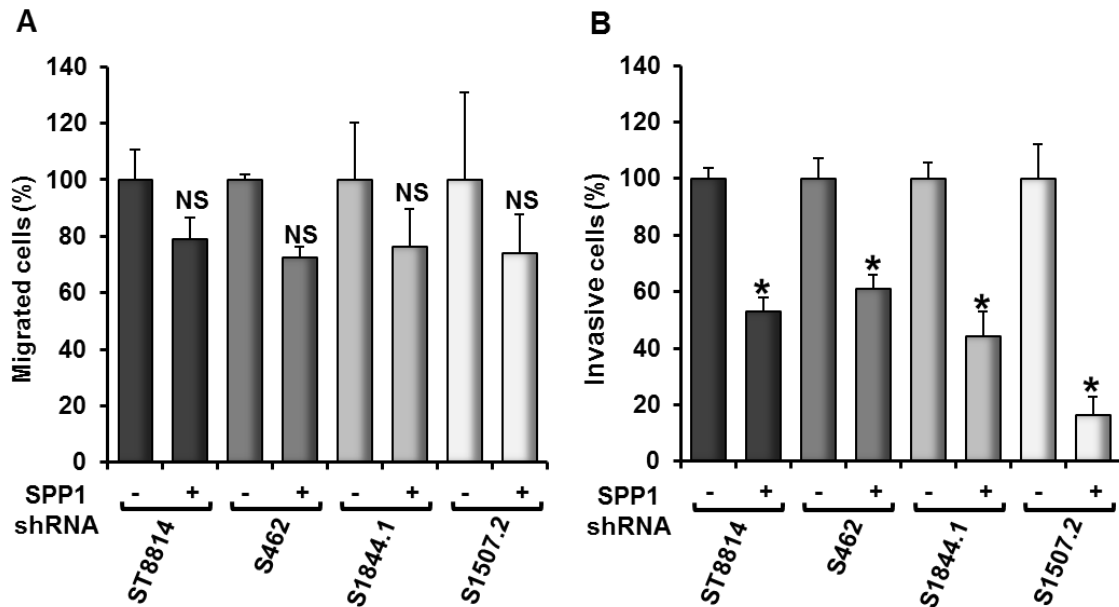
Figure 5.13. SPP1 knockdown in the 4 MPNST cell lines impair cell invasion but not cell migration. Stable ST8814, S462, S1844.1 and S1507.2 cell lines, expressing either non-target or SPP1 shRNA, as indicated, were subjected to A) Migration and B) Invasion assay. SPP1 knockdown significantly reduced cell invasiveness; Crystal violet was used to stain invasive cells. Cells were then eluted with 1% (w/v) SDS and the absorbance was read at 550nm on a Genova MK3 Lifescience Analyser; the number of cells was calculated by plotting the absorbance against a standard curve. The percentage of invasive cells was calculated in 3 individual experiments. SPP1 knockdown significantly reduced cell invasion in all four MPNST cell lines, suggesting a potential role for SPP1 in metastasis. Similar method was used for migration assay, however SPP1 knockdown showed no significant effect in reducing cell migration in these MPNST cell lines.

5.2.9 SPP1 knockdown in the 4 MPNST cell lines impair tumour formation in all MPNST cell lines

it has been reported that the expression of SPP1 plays an important role in tumourgenesis and invasion of cancer cells. The mechanism behind SPP1 causing tumourgenesis is not fully known, however it has been shown that SPP1 expression increases the ability of cells to from tumours in soft agar (Rittling & Chambers 2004). In order to investigate the role of SPP1 in tumour formation within MPNST cell lines, a tumour formation assay was also carried out. Of interest results showed that both control and SPP1 knockdown cell lines formed tumour colonies in soft agar. However, SPP1 knockdown caused a significant reduction in tumour spheroid size in all MPNST cell lines tested (Figure 5.14).
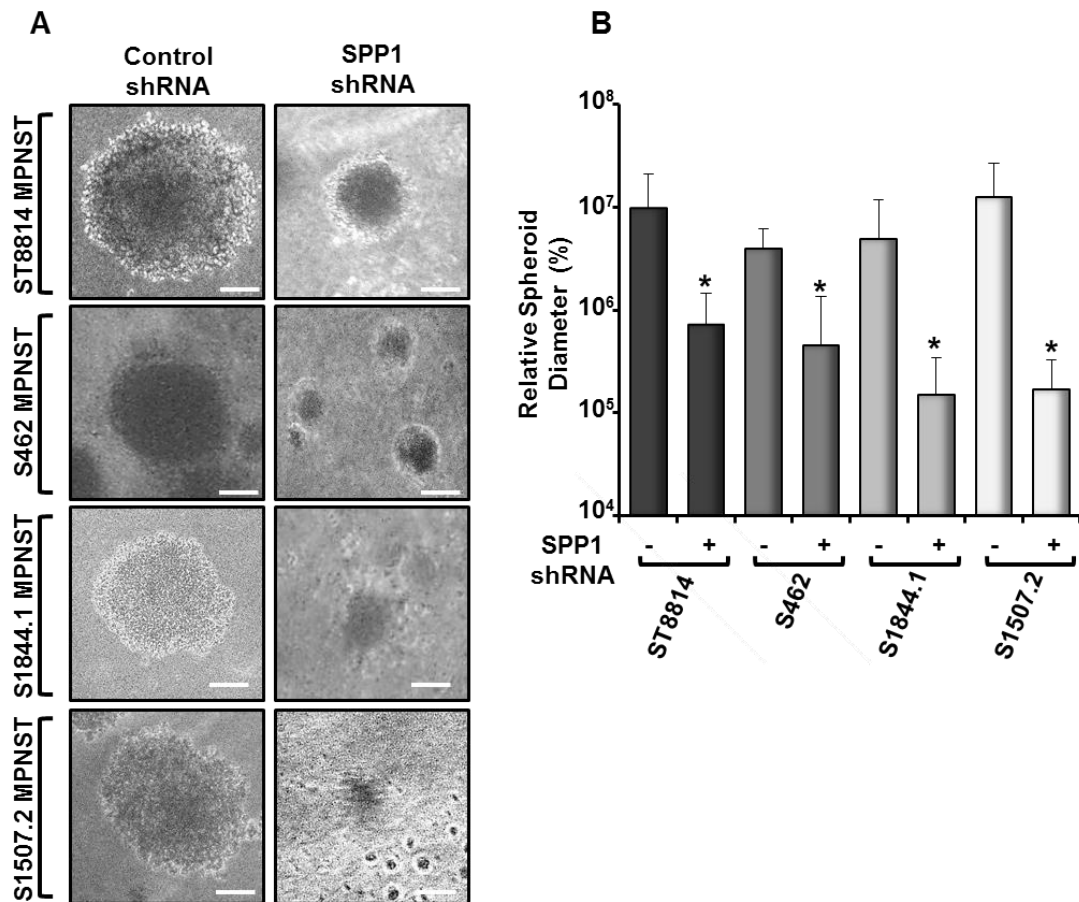
Figure 5.14. SPP1 knockdown in the 4 MPNST cell lines impair tumour formation in all MPNST cell lines. A) Stable ST8814, S462, S1844.1 and S1507.2 cell lines, expressing either non-target or SPP1shRNA as indicated, were subjected to tumour spheroid growth assays. a) Photographs of 40 tumours were taken after 2 weeks of incubation. (Scale bar represents 250 μm B) the volume of 40 tumours from each cell line were randomly measured and the tumour size of the SPP1 knockdown cells were compared to the control cell line. Statistical analysis shows that the knockdown of SPP1 significantly reduces tumour growth in all 4 MPNST cell line, consistent with a role for SPP1 in tumour growth (P = 0.0001), * P< 0.05 when comparing treated versus untreated cells

5.3 Discussion

5.3.1 HIF-1α/STAT3

The first part of this study provides evidence that both STAT3 and HIF-1α could be feasible drug targets for the treatment of MPNSTs. The study indicates that STAT3 and HIF-1α functions as a 'common' migratory/invasive signalling pathway within the heterogeneous population of MPNSTs. Given the high level of dependency on both STAT3 and HIF-1α for cell migration, invasion and tumour formation in multiple MPNSTs, therapeutic strategies that target the STAT3/HIF/VEGF-A pathway or pathways that converge on STAT3 could be a viable option for treating NF1 patients.

There are several studies that demonstrate that hypoxia can lead to STAT3 activation in various cell types (Lee et al. 2006; Pawlus et al. 2014; Kim et al. 2015). Furthermore, studies have shown that STAT3 can physically interact with HIF-1α, which then results in the activation of HIF-1α target genes via functional cooperation of both STAT3 and HIF-1α (Pawlus et al. 2014; Kim et al. 2015). Recently, Upadhyaya *et al.* (2012) used Affymetrix SNP 6.0 Array analysis to examine the genetic profile of MPNSTs compared with benign neurofibromas (Upadhyaya et al. 2012).

The study reported MPNST-specific upregulation of seven Rho-GTPase pathway genes which are thought to be critically involved in MPNST development and metastasis. Signal transduction through STAT3 is also a critical driver of Rho and therefore may be contributing to this elevation (Aznar & Lacal 2001; Debidda et al. 2005).

It is apparent that many genetic alterations found in MPNSTs lead to amplification of signal transduction pathways that enhance either JAK2/STAT3 or mTORC1/STAT3 signalling, where HIF-1α lies downstream of both STAT3 and mTORC1 (Dodd et al. 2014). For instance, PTEN loss is known to occur in both MPNSTs and epithelioid sarcomas (Xie et al. 2011; Keng et al. 2012), which gives rise to aberrant signalling through PI3K/mTORC1. mTORC1 activation has also been implicated in MPNST tumourigenesis (Johannessen et al.

2008). While mTORC1 inhibitors have shown some success in NF1 patients (De Raedt et al. 2011; Watson et al. 2014), tumour regression did not occur via the usual mechanisms, with the pro-angiogenic factor HIF-1α remaining elevated under rapamycin treatment (Johannessen et al. 2008). Of interest, I also observed that HIF1-α protein levels were not completely ablated with rapamycin treatment in all 4 MPNST cell lines tested (Figure 5.2).

Given that HIF-1α, HIF-2α and VEGF-A protein levels were markedly reduced after STAT3 knockdown (Figure 5.1), this data indicates that the HIF/VEGF pathway is predominantly regulated through JAK2/STAT3 in a range of MPNST cell lines and not the mTORC1 signalling pathway. It was previously demonstrated that both the mTORC1/STAT3 and JAK2/STAT3 signalling pathways converge on STAT3 for maximal STAT3 activation, with JAK2 mediating Tyr705 phosphorylation and mTORC1 mediating Ser727 phosphorylation.

Previous work carried out by our laboratory has shown that the JAK2/STAT3 inhibitor, FLLL31, significantly reduced both the transcription and translation of HIF-1α by blocking mTORC1 as well as the STAT3 signalling pathway (Dodd et al. 2014). As mentioned in section 4.3.2, FLLL31 is derived from curcumin, the bioactive compound in the spice turmeric. The anticancer effects of curcumin have been demonstrated *in vivo* (López-Lázaro 2008), however clinical trials carried out have shown no success using curcumin compound, this has mainly been due to the poor bioavailability and solubility of this substance. FLLL31 and FLLL32 are analogues of curcumin that were developed to have better bioavailability. OPB-31121 is a STAT3 inhibitor that was used in a phase 1 clinical trials for advanced solid tumours to identify the Maximum Tolerated Dose (ClinicalTrials.gov Identifier: NCT00955812), but the results of the trial have yet to be posted.

Although evaluation of molecular abnormalities in tumours on an individual basis might help design tailor made therapy, there are limitations to this approach with regards to therapy with MPNSTs. As a result of intra-tumoural molecular heterogeneity in MPNSTs, tumour profiling may not be possible. Instead, it may be more feasible to develop a

therapeutic strategy that targets multiple signalling pathways which are commonly dysregulated in MPNSTs. Given the high level of dependency on both STAT3 and HIF-1α for cell migration, invasion and tumour formation in multiple MPNSTs, therapeutic strategies that target the STAT3/HIF/VEGF-A pathway or pathways that converge on STAT3 could be a viable option for treating NF1 patients.

As well as blocking cell migration, invasion and tumour spheroid growth, STAT3 knockdown was sufficient to block gene-expression of VEGF-A, which is a readout of angiogenesis. As both the JAK2/STAT3 and the mTORC1/STAT3 pathways converge on HIF1-α and HIF-2α to drive cancer progression, it is very probable that targeting both the JAK2/STAT3 and mTORC1/STAT3 pathways in parallel would be required to fully suppress STAT3, HIF-1α and HIF-2α in MPNSTs. Consequently, a better response could be achieved with combination therapies that target both the JAK2/STAT3 and mTORC1/STAT3 pathways.

In addition, studies have suggested that inhibition of mTORC1 via rapamycin prevents the growth of malignant tumours by suppressing the cell cycle component that arrests proliferative drive and an mTOR target, cyclin D1 (Chong et al. 2010).

5.3.2 SPP1/STAT3

The functional loss of neurofibromin, due to *NF1* gene inactivation, leads to increased cell growth and proliferation through constitutive Ras pathway signalling. Although this is necessary for benign neurofibroma formation, it is insufficient to explain the malignant transformation of a benign PNF to an MPNST, since additional genetic lesions are required for this to occur (Upadhyaya et al. 2012). The heightened proliferative drive through Ras pathway activation will enhance the rates of genetic mutations, which over time will develop these benign neurofibromas into MPNSTs with a varied range of genetic signatures that contribute to their malignancy. Within our lab, Upadhyaya *et al.* aimed to identify genes that contribute to the malignant transformation of benign neurofibromas by establishing which genes were differentially expressed between benign and malignant tumours in NF1 patients.

Furthermore, the *SPP1* gene was found to exhibit the most significantly elevated differential mean expression level between the benign and malignant tumours. Exon array analysis identified a mean expression level of 12.06 (log2) for SPP1 gene in malignant tumours and a mean expression level of 5.65 (log2) in benign plexiform neurofibromas. This equates to an 85-fold increase (based on anti-logged LS means) in expression in MPNSTs compared to benign plexiform neurofibromas (P = 0.0002). Therefore, SPP1 was selected for further analysis to assess its possible role in migration and invasion within MPNST cell lines (Thomas et al. 2015).

The work presented in this study demonstrates that SPP1 knockdown in four different MPNST cell lines revealed a significant reduction in the growth of the tumour colony, wound healing and cell invasion. This data supports a role for the increased expression of SPP1 in the malignant transformation and invasion of cells during NF1-MPNST development (Thomas et al. 2015).

SPP1 is an extracellular matrix protein with cytokine properties. It is involved in extracellular matrix (ECM) and adhesion related pathways where it performs key roles in cell-cell communication, focal adhesion, immune cell activation and immune cell migration. It plays an essential role in the pathway that leads to type I immunity, thereby enhancing the production of interferon-gamma and interleukin-12 and reducing interleukin-1 synthesis. In terms of an association with cancer, SPP1 has been shown to promote the growth of different tumours (Furger et al. 2001; Tilli et al. 2012). Cells harbouring activated RAS have been found to exhibit a higher level of SPP1 (Thomas et al. 2015). It could therefore be possible that RAS activation through the functional loss of neurofibromin (due to *NF1* gene inactivation) could contribute towards the overexpression of SPP1. Furthermore, the use of SPP1 inhibitors such as agelastatin A has successfully reduced colony formation, migration and invasion in human breast cancer cell lines (Mason et al. 2008). It would have been of interest to have tested agelastatin A within the MPNST cell lines.

6 Chapter 6: General discussion

Although many potential therapies have been put forward for NF1 patients, there has been very little success. This limitation regarding therapy is likely due to tumour heterogeneity, with variance in growth factor receptor amplification and mutations to oncogenes and tumour suppressors in MPNST cells. A lot of research has been carried out showing differences in genetic mutations and gene-expression; however very little has been done regarding the investigation of cell signalling at the protein level.

6.1 Candidate signalling pathways

In the NF1 field, an important knowledge gap is which cell signalling pathways are critically responsible for driving tumour growth and malignancy within the heterogenic population of MPNSTs. Therefore, the initial aim of this study was to first identify signalling pathways that could possibly be contributing to the transformation of benign neurofibromas to MPNSTs and characterising the role of these pathways in malignancy. Identifying such signalling pathways common to driving their tumourgenesis and malignancy may also be relevant therapeutic targets for treating not only NF1 related but also other similar malignancies.

Previous studies have shown amplification of the HGF/MET signalling pathway in NF1-MPNST patient samples compared to the NF1 benign patient samples (Torres et al. 2011). Studies have demonstrated that HGF, MET and p-MET are over expressed in human cell lines (ST8814 and STS26T). The activation of MET results in an increase of MPNST tumourgenesis, migration and invasion. Also overexpression of the HGF/MET pathway regulated the expression of VEGF and METalloproteinase-2 (MMP2). In addition the overexpression of p-MET was observed to have a direct effect on the patient survival. (Torres et al. 2011). The abnormal activation of MET has been associated with various other tumour types (Yoshinao et al. 2000; Kong et al. 2009). Studies have also shown HGF to be a mitogen within Schwann cells, increasing cell proliferation in these cells. It has been hypothesised that Schwann cells could be the origin of MPNST cell lines (Torres et al. 2011).

Furthermore Torres et al have shown that MET has a significant effect on the migration, invasion and angiogenesis of MPNST cells both *in vitro* and *in vivo*. This study suggests that HGF-induced MMP2 expression could possibly play a role in the malignant behaviour of MPNSTs. Other studies have also shown that HGF can contribute to the malignancy of tumours through direct activation of MET expression as well as cooperating with VEGFR2 (Saucier et al. 2004). SU *et al.* have shown that HGFA, HGF, MET and CD44 are co-expressed in MPNSTs. In this study it was observe that the MPNST cell line (ST8814), that expressed all proteins mentioned, has a high level of MET activity and also has the ability to transform the existing pro-HGF in to active HGF which could be blocked via a HGF-neutralizing antibody. Inhibition of this autocrine loop showed a significant decrease in the incisiveness of the MPNST cells. However the blockage of this pathway did not affect cell proliferation (Su et al. 2004).

In addition, a study was carried out by Crosswell *et al.* , in order to investigate the effects of HGF/MET expression on neurofibroma (NBL) cell lines and also tumours extracted from patients suffering from NBL. Results demonstrated that in patients with stage 3 and 4 NBL (metastatic tumours and locally advance tumours respectively), there was a higher level of MET expression compared to those patients with stage 1 and 2 NBL (localised disease). The MET inhibitor PHA665752 was used in order to investigate the effects of HGF/MET blockage on tumourgenesis of NBL cells. Results show that PHA665752 has the ability to inhibit cell migration and proliferation in a dose-dependent manner (Crosswell et al. 2009).

Other studies have aimed to target the HGF/MET pathway in other types of tumours, in order to investigate its role in tumourgenesis (Rao et al. 1997; Torres et al. 2011). However previous studies are typically limited to two MPNST cell lines, where the ST8814 cell line is most commonly used. This is mainly due to the difficulty in culturing MPNST cell lines and in creating robust knockdowns. In order to address the issue of heterogeneity at the protein cell signalling level the use of several MPNST cell lines side by side is essential. Therefore, in order to help better understand the malignant behaviour of these cells, this

study used 4 different MPNST cell lines for all experiments. One of the limitations within this study however was a lack of a benign control cell line. Initially, neurofibroma derived Schwann cells were created to be use as a benign control. These cells were established from a benign tumour donated by a NF1 patient. Unfortunately these benign control cells died after 2 weeks of culture. The poor survival of these benign control cells might be attributed by their relatively untransformed status. Due to a lack of fresh NF1 patient samples, additional attempts to generate a benign control cell line were not possible. In chapter 3, the benign control protein and mRNA samples used were kindly provided by Meena Upadhyaya laboratory (figure 1A).

There is much clinical interest regarding drug inhibitors of MET (Table 1). An important aspect in MET inhibition is that, the downstream signalling pathways are not only regulated via MET, but can also be controlled through other upstream regulators. Therefore it is essential to select patients for clinical studies of MET inhibitors that have high expressed and activated MET, where MET is driving tumourgenesis. However detecting whether or not MET is activated in patients is a challenge. The expression of MET by itself is not an indication of its activity, in order for MET to become active it has to become phosphorylate. The phosphorylation site of MET degrades rapidly making it hard to detect within patients.

Genentech Company carried out a phase II clinical trial of Onartuzumab, a single arm antibody that disrupts the interaction of MET with HGF (Merchant et al. 2013), in combination with Erlotinib (Small-molecule TK inhibiter, targeting the EGFR) in order to investigate the effects on patients suffering from advanced non-small-cell lung cancer (NSCLC). Patients were selected by measuring the MET protein expression from patient tumour samples using immunohistochemistry assays (IHC). Results showed that Onartuzumab in combination with erlotinib had a positive impact on the Progression-free survival (PFS) and overall survival (OS) within the patients with overexpressed MET. This was in contrast to results seen in MET-negative patients treated with Onartuzumab (Spigel et al. 2012). In addition Roche Company started a Phase III clinical trial of Onartuzumab.

However this study was terminated due to lack of clinically meaningful efficiency. This study also investigated the effects of Onartuzumab in combination with erlotinib on MET-positive patients with NSCLS in comparison to patients treated only with erlotinib. The overall results were similar for both groups (Spigel et al. 2013).

In addition a phase II clinical trial was carried out to investigate the effects of Rilotumumab (AMG 102), a human monoclonal antibody against HGF, in patients suffering from recurrent glioblastoma (GBM). Results showed that Rilotumumab did not have antitumour activity in patients with recurrent GBM. This could be due to the fact that all patients that participated in this study were already pre-treated with other anti-cancer drugs (Wen et al. 2011). Amgen Company is carrying out a phase III clinical trial of Rilotumumab in MET-positive patients with Gastroesophageal Junction Adenocarcinoma (ClinicalTrials.gov Identifier: NCT02137343). This trial is ongoing.

A phase II clinical trial was carried out in order to examine the effect of Cabozantinib (XL 184), an orally bioavailable tyrosine kinase inhibitor with activity against MET and VEGF2 on patients with Castration-resistance prostate cancer (CRPC). Results of this study reported that Cabozantinib caused reduction of soft tissue lesions, bone turnover markers and pain (Smith et al. 2013). As a follow-up to this study, Exelixis Company carried out a phase III clinical trial on patients with Metastatic castration-resistant prostate cancer (mCRPC), to test the effects of Cabozantinib. Unfortunately this study did not meet its primary endpoint of demonstrating overall survival for patients treated with Cabozantinib as compared to prednisone (synthetic corticosteroid drug that is particularly effective as an immunosuppressant drug). Furthermore a phase III clinical trial of Cabozantinib on patients with Hepatocellular Carcinoma is ongoing (Exelixis/ ClinicalTrials.gov Identifier: NCT01908426). Another phase II clinical study aimed to examine the effects of Tivantinib, a highly selective MET inhibitor, in patients diagnosed with advanced hepatocellular Carcinoma specifically patients with MET-positive tumours. Tivantinib caused stabilization of the disease in 53% of the patients being monitors (Santoro et al. 2013). In order to further

investigate the effects of Tivantinib, a phase III clinical trial is being carried out using tivantinib, by Arqule Company on patients suffering with Hepatocellular Carcinoma and NSCLC.

Table 6.1 Information regarding clinical development of HGF/MET inhibitors (Garber 2014; Zhang et al. 2015).

Agent	Company	Phase	Cancer Type
Onartuzumab (MetMab)	Genentech/Roche	III	Lung
Rilotumumab	Amgen	III	Gastric, lung, colon,brain, ovary, renal
Cabozantinib (Cometriq)	Exelixis	III	Lung
Tivantinib	Arqule	III	Lung, colon, breast, liver, prostate, myeloma
LY2875358	Eli Lilly	II	Gastric
Ficlatuzumab	Aveo Pharmaceuticals	II	NSCLC
Foretinibl	GlaxoSmithKline	II	Gastric
Crizotinib (Xalkori)	Pfizer	II	NSCLC
INC280	Novartis/Incyte	II	Recurrent Gliobastoma
AMG337	Amgen	II	Gastric/Esophageal
MSC2156119J	Merck Serono	II	Sold tumours with MET amplification or over expression
ABT-700	AbbVie	I	Sold tumours with MET amplification or over expression
MGCD265	Mirati Therapeutics	I	Advanced malignancy
Volitinib (HMPL-504)	Hutchison MediPharma	I	
SAR125844	Sanofi	I	Sold tumours, may have MET amplification or c-MET overexpression
Altiratinib	Deciphera Pharmaceuticals	I	Sold tumours, may have MET amplification or c-MET overexpression
LY2801653	Eli Lilly	I	Advanced cancer

6.2 STAT3 in tumourgenesis

The work carried out in chapter 4 indicated that STAT3 could possibly be a common' therapeutic target for MPNSTs with heterogeneity in their migratory/invasive signalling profiles.

STAT3 plays an important role in the progression of numerous cancers; STAT3 also plays a key role in stromal cells, such as immune cells. Immune cells are known to be recruited in to the tumour microenvironment resulting in tumour progression (Yu et al. 2007; Yu et al. 2014). The abnormal activation of STAT3 results in an increase of cell growth, proliferation, angiogenesis and metastasis (Bromberg 2002; Hleb et al. 2004). The upregulation of STAT3 has been detected in various types of cancer, including breast, panceatic, melanomas etc. Furthermore, several tumours, such as lymphocytic leukemia, Hepatocellular Carcinoma etc., tend to produce an abnormal amount of IL-6 and IL-10 that function as STAT3 activators resulting in a 'feed-forward' mechanism of STAT3 (Yu et al. 2007). In addition to driving cell migration, STAT3 activation leads to the regulation of several target genes that play an important role in Cell migration and invasion, apoptosis, proliferation and cell cycle progression. The involvement of STAT3 in cellular migration during embryonic development and cancer metastasis has been reported (Teng et al. 2009). Initial studies have shown that STAT3 is essential for wound healing and also cell migration in keratinocytes *in vitro* (Sano et al. 1999; Ng et al. 2006; Teng et al. 2014). However wound healing solely measures the rate of cells that move collectively and not the migratory behaviour. Further studies have shown that the STAT3 has the ability to increase migratory behaviour via the integrinβ6 in prostate epithelial cells (Kamran, Patil & Rajiv P Gude 2013).

In addition one of the characteristics of tumour metastasis is the ability to invade in to the extracellular matrix. There are several studies indicating that STAT3 plays an important role in invasion (Xiong et al. 2008; Yang et al. 2009). It has been demonstrated that STAT3 is involved in the regulation of matrix metalloproteinases (MMPs) which are known to be involved in tumour invasion and metastasis (Xie et al. 2004). Other studies have observed

that the knockdown of STAT3 caused a reduction in the invasive behaviour of pancreatic cancer cells and decreased the expression of MMP-7 in nude mice (Li et al. 2011). Furthermore STAT3 activation has the ability to bind directly to the MMP-2 promoter, resulting in MMP-2 expression (Xie et al. 2004).

Moreover the over expression of phosphorylated STAT3 has been detected in cutaneous squamous cell carcinoma and has been shown to contribute to the invasive and Metastatic behaviour of the cells (Suiqing et al. 2005). Correspondingly the inhibition of STAT3 resulted in a decrease of tumour growth both *in vitro* and *in vivo* (Judd et al. 2014). Similar results were observed in chapter 4, when MPNST cell lines were treated with STAT3 inhibitors, wound healing, migration and invasion of these cells were significantly affected.

There is evidence in the literature indicating that STAT3 is involved in the malignant transformation of cells via activation of several oncogenes, protein tyrosine kinases etc. (Kamran, Patil & Rajiv P Gude 2013; Xiong et al. 2008). In chapter 4, I also demonstrate that the knockdown of STAT3 significantly reduces the ability of the MPNST cell lines to form tumours in soft agar, indicating an important role for STAT3 in tumour formation and malignant transformation of cells. Additionally other studies have reported that the activation of STAT3 via the Src could possibly be contributing to malignant transformation of the cells by inhibiting apoptosis (Turkson et al. 1998).

Another aspect of tumour formation is the formation of new blood vessels. The most important protein that contributed to angiogenesis is VEGF. VEGF binds to the transmembrane receptor tyrosine kinases which then results in neovascularisation. STAT3 is known to be directly involved in the transcriptional activation of the VEGF gene. Studies have reported that the overexpression of STAT3 in melanoma cells and also human pancreatic cancer cells causes overexpression of VEGF leading to tumour angiogenesis and tumour progression. Also in this thesis it is demonstrated that the knockdown of STAT3 in MPNST cell lines robustly increases the expression of VEGF-A (see figure 5.1). In addition to VEGF, STAT3 has been reported to also regulate expression of HIF-1α, one of the crucial

mediators of angiogenesis in tumour cells (Xu et al. 2005; Rad et al. 2015). Both STAT3 and HIF-1α bind to the VEGF promoter, resulting in hyper activation of VEGF, therefore contributing to angiogenesis of tumour cells. In this thesis I have also shown that STAT3 knockdown significantly reduces the expression of HIF-1α in MPNST cell lines.

Even though it is known that STAT3 has the ability to switch on many genes (approximately 300) (figure 6.1), it is still unclear which STAT3-regulated genes are involved in the tumour progression and result in tumour migration, invasion, tumourgenesis and angiogenesis of the MPNST cell lines being studied in this thesis. One way to investigate what these genes are and how STAT3 regulates them, is to use next generation sequencing, using STAT3 knockdown cell lines and compare them to a control cell line. Dr Andrew Tee has secured funding in order to furthered pursue this study (See section 6.6, future directions).

The activation of the JAK2/STAT3 signalling pathway has been observed in many solid tumours, making it a suitable target for the development of anticancer drugs. In order to block STAT3 several hypothesis have been suggested, such as the inhibition of upstream effectors to block STAT3 activation, or blocking STAT3 phosphorylation in order to prevent its nuclear translocation, therefore prohibiting its transcription effect on target genes. As mentions before due to its high impact in several cancer types, the development of an anti-STAT3 component has been identified as a promising therapeutic target. There have been studies investigating the effects of STAT3 inhibitors however up till now clinical trials have not been successful.

OPB-31121 was first acknowledged by the Otsuka pharmaceuticals Co. Ltd to prohibit the growth of several types of tumour cell lines. OPB-31121 is recognised as an inhibitor of STAT3 phosphorylation however it does not inhibit the upstream kinases that play a role in regulating STAT3 (Hayakawa et al. 2013; Kim et al. 2013).

The first study using the OPB-31121 inhibitor was carried out on gastric cancer cells showing that OPB-31121 inhibited JAK2 phosphorylation and also blocked gp130 expression, both leading to inhibition of STAT3 phosphorylation. Suggesting that the OPB-31121, inhibits STAT3 through its upstream effectors. Moreover, it was reported in this study that the expression levels of constitutively active JAK2/STAT3 protein has no influence on the effectiveness of the OPB-31121 inhibitor, as it might have been expected. On the contrary gastric carcinoma cell lines that showed to have the lowest levels of constitutively active JAK2/STAT3 protein, showed the highest sensitivity to OPB-31121 inhibitor compared to the other cell lines (Kim et al. 2013). A second study was performed to further investigate the mechanism in which OPB-31121 inhibitor acts (Hayakawa et al. 2013). In this study it was observed that OPB-31121 selectively inhibited STAT3 tyrosine phosphorylation however OPB-31121 did not block residually phosphorylated STAT3 translocation in to the nucleus as observed by immunofluorescent staining (Hayakawa et al. 2013).

There are ongoing phase I/II clinical trials using this inhibitor in Japan, Korea and also Hong Kong (Hayakawa et al. 2013). The phase I clinical trial carried out in Korea, showed evidence that OPB-31121 inhibitors could possibly be used safely and restively well in patients suffering with solid tumours (Oh et al. 2015). The phase I/II clinical trials carried out in Hong Kong and Japan are ongoing (Hayakawa et al. 2013).

Moreover, a recent phase I clinical trial was carried out to test the effects of OPB-31121 in patients suffering from advanced hepatocellular carcinoma (HCC). OPB-31121 had previously shown promising results in preclinical studies carried out on liver fibrosis and hepatitis model. However, unfortunately OPB-31121 showed no significant results in acting as an antitumour drug in patients with HCC. In addition OPB-31121 caused nervous system related toxicities, making it hard to continue with clinical trials using this drug on patients with advanced HCC (Okusaka et al. 2015).

In a previous study carried out by Banerjee *et al.* , cucurbitacin-I was used in order to investigate the effects of STAT3 inhibition on growth of NF1-deficient cells. Results of this

study showed a decrease in cell proliferation of the NF1-deficient MPNSTs *in vitro* after treatment with cucurbitacin-I. In this study however the treatment of the ST8814 cell line with cucurbitacin-I resulted in reduction of STAT3 Ser727 phosphorylation but not the STAT3 Tyr705 phosphorylation (Banerjee et al. 2010). This is in contrast to what I observed in this study, after treatment of all 4 MPNST cell lines, including the ST8814 cell line, with cucurbitacin-I, a reduction in STAT3 Tyr705 phosphorylation was observed however STAT3 Ser727 phosphorylation was not significantly reduced. It is worth mentioning that unlike this study, in the study performed by Banerjee *et al.* cells were not stimulated with HGF after drug treatment. Further investigation is needed in order to develop potential STAT3 inhibition therapies to treat NF1 patients.

6.3   HIF1-α in tumourgenesis

As described in chapter 5, the study performed in this thesis presents evidence to suggesting that HIF-1α is possibly involved in the malignant behaviour of MPNST cells. Moreover of particular interest in this study was the association between HIF-1α and STAT3. In chapter 5, I analysed the relationship between STAT3 and HIF-1α. Of interest the knockdown of STAT3 robustly blocked not only HIF-1α but also HIF-2α and VEGFA expression. This is in concordance with work done by Dodd et al within our laboratory, revealing that STAT3 knockdown results in a significant reduction to HIF-1α mRNA level (HEK-293 cell lines were used in this study). Furthermore this study also showed that mTORC1 stimulates Ser727 phosphorylation of STAT3 under hypoxic conditions, resulting in the HIF-1α activation. Of interest this study also observed that the inhibition of STAT3 blocks HIF-1α transcription in the same way rapamycin does (Dodd et al. 2014).

Dodd *et al.* has also shown that STAT3 inhibition using the FLLL31 STAT3 inhibitor also reduces HIF-1α transcription and translation making this inhibitor an attractive small molecule for treatment of vascularised tumours. The of HIF-1α in the HEK293 cell lines was driven both by STAT3 and mTORC1 inhibition (Dodd et al. 2014), however Dodd *et al.* did not observed inhibition of mTORC1 in the TSC cell lines after treatment with FLLL31 (Data

not shown). FLLL31 is a derivative of curcumin which is found in the spice turmeric. However even though the antitumour properties of curcumin have been shown in previous studies *in vivo* unfortunately clinical trials using this substance have shown very little bioavailability and solubility of this compound (Tee et al. 2005). Interestingly in this thesis after treatment with FLLL31 inhibitor, there was a significant reduction in VEGFA protein levels, detected by western blotting; however levels of HIF-1α were not as effected after treatment with this inhibitor. This could be due to the fact that in the MPNST cell lines HIF-1α is not mTORC1 driven unlike the HEK293 cell lines used in Dodd et al study where HIF-1α is driven by both mTORC1 and STAT3.Studies have shown that the inhibition of mTORC1 using rapamycin treatment does not affect HIF-1α in NF1-associated malignancies in a genetically engineered murine model which supports the hypothesis that HIF-1α is not driven via mTORC1 in MPNST cell lines. During my investigation in to the regulation of HIF-1α, I also observed that rapamycin treatment doesn't have a major effect on the HIF-1α protein levels. In addition VEGF-A is known to be regulated via several transcription regulators, including STAT3, explaining why inhibition and STAT3 in the MPNST cell lines has an inhibitory effect on VEGF-A.

There has been numerous studies regarding the activity and of transcription factors and the signalling pathway they affect, this has been most important in the field of biology and cancer. Despite all research carried out in this field, there is still very little known about the interaction between the transcription factors, DNA and other proteins (Fontaine et al. 2015). Determining techniques to target transcription factors have proven to be hard, due to the lack of identified molecular targets of transcription factors. Transcription factors mainly act through protein-protein interactions. Moreover one obstacle facing the development of molecules that mimic the endogenous ligands for inhibition of transcription factors is the lack of chemical libraries that meet the structural requirements to interfere with the protein-protein interaction modulated via transcription factors (Fontaine et al. 2015).

Regardless of challenges facing the development of pharmacological transcription factor inhibitors, several academic groups and pharmaceutical companies have aimed to develop HIF-1α inhibitors. As discussed before the intra-tumour hypoxia is known to be involved in tumourgenesis. The HIFs are responsible for the changes in the oxygen levels within the tumour cells, studying the microenvironment and the mechanism and signalling pathways mediate through the HIF transcription factor has opened a new therapeutic target for patients suffering from cancer. Normal cells do not undergo variations in the levels of oxygen that tumour cells experience, these abnormal tumour cells are exposed to abnormal oxygen levels that regulate the activation of numerous signalling pathways involved in cell survival . Therefore these activated pathways in tumour cells due to oxygen level variation could be a valuable potential target for therapy (Onnis et al. 2009). Depending on the putative mechanism of action HIF-1α inhibitors could target HIF-1α mRNA expression, HIF-1α protein translation, HIF-1α protein degradation, HIF-1α DNA binding, HIF-1α transcriptional activity (Onnis et al. 2009).

Furthermore a recent pilot study was carried out in order to investigate and test the effects of the HIF-1α inhibitor EZN-2968 on patients suffering from refractory solid tumours. EZN-2968 is an antisense oligodeoxynucleotide that hybridizes with HIF-1α mRNA and blocks HIF-1α protein expression. This study aimed to assess the antitumour response of EZN-2968 and also to measure the variation in HIF-1α mRNA levels and its target genes. Unfortunately this study was terminated in its early stages, due to its lack of success in reducing the levels of HIF-1α mRNA levels, Protein expression and targeted genes. This could possibly be due to the lack of patients and the small sample size used in this study (Jeong et al. 2014). As it has proven difficult to target transcription factors, perhaps it will be more feasible to target downstream targets of HIF-1α, such as VEGF.

6.4   Conclusion

This study is the first to analyse 4 MPNST cell lines in depth regarding cell signalling pathways linked to malignancy. This study demonstrates the differences of sensitivity to

MET inhibition in the panel of MPNSTs, highlighting for the first time the variation of cell signalling pathways and sensitivity within multiple MPNSTs.

In this study, the mTORC1 inhibitor rapamycin was examined, showing in a manner similar to MET inhibitors, that all but the S462 cell line was sensitive to mTORC1 inhibition for wound healing, cell migration and invasion. This is the first report of differential sensitivity to rapamycin depending on the MPNST cell-line. This further supports the finding showing that there are differences in cell signalling pathways involved in NF1 malignancy. This study also showed that rapamycin treatment blocked VEGF-A expression in all MPNST cell lines, but is less effective at blocking HIF protein expression, when compared to STAT3 inhibition. This further supports our laboratory previous observations (Dodd et al. 2014) that VEGF-A is regulated via both HIF-dependent and -independent mechanisms.

In addition this study confirmed that JAK2/STAT3 inhibition is effective in cell lines regardless of MET expression levels, suggesting it would be a better therapeutic target for a wider range of the heterogeneous MPNST population.

This work reveals that, in a range of MPNST cell lines, multiple growth factor receptors can potently activate the JAK2/STAT3 pathway, which is linked to known genetics and expression studies in MPNST cells showing that EGFR, PDGFR, and interleukin receptors are all elevated. This data highlights that STAT3 is commonly activated by multiple receptor tyrosine kinases linked to malignancy in NF1 patients. Furthermore, through analysis of multiple growth factors and cytokines, the work herein shows that both Interleukin and PDGF can both potently activate MET signalling within multiple MPNSTs. This finding describes how these different tyrosine kinase receptors are able to cooperate with one another to activate STAT3 and malignancy. Although HGF is the sole ligand for MET, MET can still be activated via signalling cross talk between receptors in these MPNST cell lines. Such redundancy within the signalling network from these receptor tyrosine kinases likely contributes to the lack of success when receptor tyrosine kinases inhibitors were tested in

NF1 patients. It is possible that targeting the JAK2/STAT3/HIF pathway that is downstream from all these receptor tyrosine kinases might have more success.

Prior to this study, the mechanism promoting HIF-1α activity in MPNSTs was unknown, with one study indicating that HIF-1α was unlikely to be an mTORC1-dependent mechanism (Johannessen et al. 2008). Previous studies carried out by our team indicated that mTORC1 and cytokine signalling pathways converge on STAT3 to promote HIF-1α expression. It was, therefore, hypothesised that the JAK2/STAT3 pathway might be more involved in HIF elevation in this context. As initially predicted; complete knockdown of STAT3 in all 4 MPNST cell lines ablated HIF-1α and VEGF-A expression. Such a finding has possible implications to new better strategies to target angiogenesis, of which current antiangiogenic drugs are pretty ineffective at blocking VEGF-A signalling through VEGF receptors. Current angiogenic therapies include: monoclonal VEGF-A antibody (bevacizumab), 4 tyrosine kinase inhibitors targeting VEGF receptors (axitinib, pazapanib, sorafenib, and sunitinib), 2 allosteric inhibitors of mTORC1 (everolimus, and sirolimius) and high-dose recombinant interleukin (IL)-2. These current therapies are all related, in that they impact vascular endothelial growth factor A (VEGF-A) signalling within the mammalian target of rapamycin complex 1 (mTORC1)/hypoxia inducible factor (HIF) signalling pathway.

Moreover, chapter 5 clearly demonstrates that STAT3 is also essential for HIF-2α expression. This finding is significant when considering that HIF-2 is thought to be more centrally involved in proliferative drive and malignancy when compared to HIF-1(Loboda et al. 2012). For instance, enhanced proliferative drive might be as a consequence of higher levels of CCND1 expression, which is a direct gene target of HIF-2α, and not HIF-1α. I also show that STAT3 inhibitors markedly repress HIF-1α and VEGF-A expression, which infers that drug inhibition of STAT3 in NF1 patients would be beneficial. This work also suggests that we could possibly utilise blood secreted VEGF-A as a biomarker for drug effectiveness, when targeting the JAK2/STAT3 pathway.

This study also shows that HIF-1α knockdown mimics the effects of STAT3 knockdown in all cell lines. This data identifies HIF-1α as a key mediator of the oncogenic outputs of STAT3. This is a substantial finding, and could have future clinical relevance as there is much interest with drug development of better HIF-1α inhibitors. Although there has been much investment into drug discovery and development of HIF inhibitors, currently there are no clinically viable drugs that directly targets HIF. The work herein describes an indirect way to block HIF-1α, i.e., via inhibition of STAT3.

6.5   Limitations of this study

As mentioned before, one of the limitations of this study is the lack of a benign control cell line for comparison. Initially, a neurofibroma derived Schwann cell-line was created from a benign tumour kindly donated by a NF1 patient for use as a benign control. Unfortunately, these cells failed to thrive *in vitro* and entered cellular senescence, a common problem with the culturing of non-malignant cells. Additional attempts to generate a benign control cell line were not possible due to limited access to NF1 patient samples.

The clinical diversity of cancer as a pathology is one of the biggest challenges faced in the development of effective therapeutics. This work indicates that targeting further downstream signalling components involved in malignancy may be more effective therapy in a broader range of MPNSTs. Unfortunately, the study is limited by the number of cell lines available. To fully validate our findings, a wider panel of cell lines could be tested, with greater variability in their signalling profiles. Another limitation of this work may be in the therapeutic signalling of STAT3 and MET. Whilst targeting STAT3 has shown significant success in a range of cell lines and animal models, as a transcription factor STAT3 was previously considered as an 'undruggable-target' in the clinic. Whilst we have promising information from our collaborators in Indianopolis regarding the phase I testing of the new STAT3 inhibitor next year, there is still limited information at this time on whether targeting STAT3 in patients is clinically viable. This is one of the limitations of using cell culture as a

means of drug discovery, given that bioavailability, side effect profiles and pharmacokinetics are not considered.

Due to time and financial constraints of the project there were some avenues of investigation which could have been followed up with more time. For instance, in this study the effects of HGF/MET inhibition in combination with mTORC1 inhibition was investigated. However due to time limitations of this PhD project the combination of STAT3 and mTORC1 inhibition has not been examined. Given that mTOR inhibitors have shown success in xenograft models for NF1, it would be interesting to monitor the ability of MPNST cell lines to form tumours in soft agar after treatment with both STAT3 and mTORC1 inhibitors and compare this to when these inhibitors are used singly. mTOR inhibitors are ineffective at blocking HIF-1 expression in NF-1 (Johansson et al. 2008), therefore targeting STAT3 in parallel could enhance the anti-tumour efficacy of the mTOR inhibitors in this setting.

Correspondingly further work is required regarding the interaction between SPP1 and other signalling pathways in this thesis. A western blot assay could be used to observe the levels of p-STAT3 in SPP1 knockdown cell lines.

Overall taking in to consideration the time and financial constraints of this PhD program, it can be concluded that these limitations could be addressed in future work carried out on NF1-associated MPNSTs.

6.6 Future direction

There are still many potential areas regarding NF1 research and the discovery of potential effective treatments for the prevention or treatment of NF1-related tumours. This study represents one of many steps taken towards providing an insight in to protein signalling pathways involved in NF1 tumourigenesis.

Of interest the results of this study that the inhibition of mTORC1 using rapamycin blocked MET activation in MPNST cell lines. This effect was also observed in the breast cancer cell line MDA-MB-231 (This data is not shown in this thesis). This newly identified

feedback mechanism from mTORC1 to the MET receptor could have important clinical implication that links to upstream signalling through the JAK2/STAT3/HIF angiogenic signalling pathway. It was hypothesised that mTORC1 regulates MET through the vesicular trafficking between the plasma membrane and endosomes/lysosomes. Further work is required to confirm this mechanism, however time and financial constraints prohibited this within the scope of the PhD project. To test whether this hypothesis is correct, several experiments could be carried out. Creating Raptor knockdowns in MPNST cell lines (To prevent mTORC1 signalling) could be used in order to confirm the importance of mTORC1 in driving HGF/MET/STAT3 induced cell migration, invasion and tumour formation. A Cell Surface Protein Isolation Kit can be used to determine the location of MET before and after mTORC1 inhibition. The dependence of MET localisation and activation can also be determined via immunofluorescence.

In this thesis it has been demonstrated that STAT3 is required for tumour formation in MPNST cell lines. This work has led to pilot study aimed at identifying STAT3 downstream targets essential for tumourigenesis in MPNSTs. In order to do so we will utilise RNA-sequencing to profile gene-expression at a high resolution to study the differences between various MPNST cell lines with and without STAT3 or HIF-1α knockdown. Funding for this pilot project has already been kindly provided by Prof. Julian Sampson, which is currently underway. Our lab is collaborating with the Wales Gene Park Genomic Facility to take forward this project. The TruSeq RNA Library Preparation Kit will be used to generate mRNA-focused libraries for this project. I will be working closely with our bioinformaticians in order to analyse the data collected from the next generation sequencing. In this project I plan to evaluate genes found to be differentially regulated within the knockdown cell lines (STAT3 or HIF-1α) compared to the control MPNST cell lines to identify key targets involved in malignancy.

Evidently, a transcriptional programme is orchestrated by an array of receptor tyrosine kinases within the MPNSTs, of which STAT3 seems to be a pivotal downstream component

driving malignancy. Given the genotypic variation between MPNST cells and the redundancy of receptor signalling pathways (and signalling cross-talk) that converge on STAT3, therapy at the level of the receptors has not shown much success. By targeting STAT3, or further downstream components, a broader range of genotypically different MPNSTs might respond to therapy. The rationale to profile gene-expression of MPNST cell lines with and without STAT3 and/or HIF-1α, is to identify new cancer targets and biomarkers of malignancy.

Furthermore, the publication gained from this thesis (STAT3 and HIF1α Signalling Drives Oncogenic Cellular Phenotypes in Malignant Peripheral Nerve Sheath Tumours) has led to a collaboration between our laboratory and Dr Mark Kelley and Dr Melissa Fishel (Indiana University School of Medicine, Indianapolis), who have provided us with a panel of drug inhibitors that target the STAT3 signalling pathway which they would like us to test on the MPNST cell lines used in this study. This group has been working on STAT3 and related interactions to STAT3 control via redox signalling in pancreatic cancer and aim to take the STAT3 inhibitor drug in to clinical trials early 2016.

As part of this STAT3 inhibitor drug program, novel STAT3 inhibitors will be screened for their effectiveness to block angiogenic signalling pathway through STAT3/HIF-1α/VEGF-A, as well as inhibiting cell migration, invasion and tumour formation in not only MPNST cell lines but also TS (Tuberous sclerosis) and RCC cell lines. Comparisons between these malignant and benign models may also help identify key factors involved in driving metastasis and malignancy.

Studying STAT3 in this thesis, has also helped to better understand the mechanisms behind tumour angiogenesis. There are currently several cancer therapies available which specifically target VEGF to inhibit tumour growth, this work indicates that targeting STAT3 may be an effective mechanism for blocking VEGF signalling and could be used as anti-angiogenic therapy. This could have implications for patients suffering from highly vascularized tumours such as renal cell carcinoma (RCC) where inappropriate stabilization of HIF is a key mediator of the malignant phenotype. Further work is required to determine

whether targeting STAT3 may have anti-angiogenic effects in a broader range of malignancies.

Moreover MPNSTs can also be used as a model of malignancy and help understand tumour progression in other cancers. The Genetic alteration, driven through loss of NF1 function and hyper-proliferation and consequential genetic instability, leads to key signature events that are commonly found in other malignancies. Revealing those 'usual suspects' of cancer, loss of heterozygosity (LOH) analysis linked Tumour Protein P53 (TP53), Retinoblastoma protein 1 (RB1) and Phosphatase and tensin homolog (PTEN) as key gene markers of MPNST tumour progression; where these tumour suppressor genes are commonly inactivated in sporadic cancer. In addition mutations in the NF1 gene have also been detected in other cancers such as lung and colon cancer (cacev et al 2005. Ding et al 2008).

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