THE ROLE OF PLATELET-DERIVED GROWTH FACTOR RECEPTOR SIGNALING IN MEDULLOBLASTOMA METASTASIS

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DOCTOR OF PHILOSOPHY

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ABSTRACT

Medulloblastoma is the most common brain tumor in children and one third of the patients remain incurable. Tumor metastasis is one of the primary reasons for its high mortality rate. Despite evidence of overexpression of PDGFRα and PDGFRβ in metastatic medulloblastoma, their individual roles remain controversial and equivocal. Analysis of their specific signaling pathway in medulloblastoma cells revealed that PDGFRα and PDGFRβ signaling events lead to distinct cellular functions: while PDGFRβ stimulated cell proliferation and invasion, the expression of CD44 to regulate progression via c-Myc and inhibited cell death, PDGFRα displayed the opposite effects. Studies also revealed that c-Myc plays an intermediary role by regulating the downstream molecules in PDGFRβ signal pathway such as CD44 and NFκB. NFκB activity was found to be down- regulated in the absence of PDGFRβ pathway, with its activity restored by the overexpression of c-Myc. Analysis of medulloblastoma patient tissues without a prior knowledge of their metastatic nature further confirmed that PDGFRβ-CD44 axis regulate medulloblastoma metastasis.

Co-inhibition studies performed by simultaneous inhibition of both PDGFRβ and c-Myc either by using siRNAs or by using pharmacological inhibitors demonstrated an enhanced inhibitory effect on medulloblastoma cell proliferation and migration. Using miRNA profiling of Daoy cells lacking either PDGFRβ or c-Myc alone or both, a set of miRNAs regulated by both PDGFRβ and c-Myc in common were identified. Integrative analysis of these miRNAs and their targets revealed that activation of PDGFRβ signaling and overexpression of c-Myc may enhance medulloblastoma progression via modulating

the expression of several miRNAs such as miR-1280, -1260 and consequently regulating the expression of oncogenic molecules, such as Jagged 2 and CDC25A, respectively. Specific inhibition of miRNAs, miR-1280 and -1260, and JAG2 demonstrated their vital roles in medulloblastoma cell proliferation and migration.

These findings suggest that the PDGFRβ-CD44 is a regulatory axis modulating medulloblastoma progression via c-Myc and targeting PDGFRβ/c-Myc/CD44 may provide a novel therapeutic strategy for the treatment of metastatic medulloblastoma.

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LIST OF ABBREVIATIONS

ANK - Ankyrin repeats

APC - *Adenomatous polyposis coli*

ATCC - American Type Culture Collection

BDNF - Brain-derived neurotrophic factor

BMI1 - B lymphoma Mo-MLV insertion region 1 homolog

Cbf1 - C-promoter binding factor 1

CDC25 - Cell division cycle 25 homolog A

CDK6 - Cell division protein kinase 6

Ch - Chromosome

ChIP-PCR - Chromatin immunoprecipitation – polymerase chain reaction

CK1 - Casein kinase 1

CKK1 - Calmodulin-dependent protein kinase 1

CNS - Central nervous system

CO_2 - Carbon dioxide

CSF - Cerebro spinal fluid

CSL - Cellulose synthase-like gene

CT - Computed tomography

Dhh - Dessert hedgehog

DLL - Delta-like ligands

DMEM - Dulbecco's minimal essential medium

DNA - Deoxyribonucleic acid

DTT - Dithiothreitol

DVL - Dishevelled

EDTA - Ethylenediaminetetraacetic acid

EGF - Epidermal growth factor

EGFR - Epidermal growth factor receptor

EGL - external germinal layer

FZD - Frizzled receptor

GAB1 - GRB2-associated-binding protein 1

GABA - Gamma-Aminobutyric acid

GNP - granule neuron precursor

Gray - Gy

Grb2 - Growth factor receptor-bound protein 2

Grb5 - Growth factor receptor-bound protein 5

GSK3 - Glycogen synthase kinase 3

HDAC - Histone deacetylase

Hes1 - Hairy and enhancer of split-1

IgG - Immunoglobulin G

IGL - Internal granule layer

Ihh - Indian hedgehog

IkBα - I-kappa-B-alpha

IP - Immuno precipitation

IRB - Institutional review board

Isochrom - Isochromosome

JAG2 - Jagged 2

KCl - Potassium chloride

KDM6A - Lysine (K)-specific demethylase 6A

LCA - Large cell/anaplastic

LDH - Lactate dehydrogenase

LRP5/LRP6 - Low-density lipoprotein receptor-related protein 5/6

Lys - Lysine

MAML1 - Mastermind-like protein 1

MB - Medulloblastoma

MBEN - MB with extensive nodularity

MEM - Minimal essential medium

$MgCl_2$ - Magnesium chloride

miRNA - MicroRNA

MRI - Magnetic resonance imaging

MTS - (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)

Mut - Mutation

NaCl - Sodium chloride

Nck - Non-catalytic region of tyrosine kinase adaptor protein

NEXT - Notch extracellular truncation

NFκB - Nuclear Factor-KappaB

NICD - Notch intercellular domain

NPR3 - Natriuretic peptide receptor C/guanylate cyclase C

NT3 - Neurotropin-3

PBS - Phosphate buffer saline

PDGF - Platelet-derived growth factor

PDGFR - Platelet-derived growth factor receptor

PI3K - Phosphatidylinositol 3-kinase

PKCα – Protein kinase C alpha

PLC-γ1 - Phospholipase C-gamma 1

PTCH – Patched receptor

PTEN - Phosphatase and tensin homolog

qRT-PCR - Quantitative real time – polymerase chain reaction

Ras/GAP - Ras GTPase activating protein

RAS/MAPK - RAS/mitogen activated protein kinase

Rb - Retinoblastoma protein

RNA – Ribonucleic acid

SDS-PAGE - Sodium dodecyl sulphate – poly agarose gel electrophoresis

SFRP1 - Secreted frizzled-related protein 1

SHH- Sonic hedgehog

Smo - Smoothened receptor

Stat5 - Signal transducer and activator of transcription 5

SUFU - Suppressor of fused homolog

TCF/LEF - T-cell factor/lymphoid-enhancer factor

TE - Tris EDTA

TGFβ/BMP - Transforming growth factor beta/ bone morphogenetic protein

trkB - Tyrosine-related kinase B

UDG - Uracil DNA glycosylase v-fms

WHO - World Health Organization

YAP1 - Yes-associated protein 1

CHAPTER 1. INTRODUCTION

1.1. Medulloblastoma (MB)

MB is a highly malignant childhood brain tumor comprising 20% of all pediatric central nervous system (CNS) tumors and 40% of all cerebellar tumors [1-3]. Every 9.6 children per million is estimated to be affected by MB [4]. Despite the advances in its diagnosis and treatment, MB still remains a common malignant tumor responsible for significant (~10%) childhood morbidity and mortality with an overall 5-year survival rates for children to be just around 60% [4, 5]. One of the primary causes for significant mortality rates in children with MB is the metastasis of the tumor. Approximately 30-35% of the children have disseminated disease at the time of clinical presentation [6, 7]. Metastasis occurs along the spinal cord and later spreads to other organs like bone marrow, lungs, bones and lymph nodes [8]. No specific therapy for the treatment for metastatic MB is available till date. Deciphering signal pathways and molecular mechanisms that regulate MB might provide crucial therapeutic targets and strategies in the treatment of metastatic MB.

1.1.1. MB history

World Health Organization (WHO) has categorized MB as a grade 4 tumor that consist of highly aggressive type of cells [9]. James Homer Wright, a pathologist at Massachusetts General Hospital, Boston was the person who first described MB in 1910 as neurocytoma as it resembled neuroblastoma [10-12]. Based on the observation from the tumor tissue, he elucidated MB as a rosette, which is a characteristic feature of classic

MB, a variant of MB. In 1897, Shaper put forth his views about MB origin indicating that MB originated from a hypothetical multipotential cell called as “Medulloblast”, which was believed to be one of the cells that populated primitive neural tumor [13]. Later in 1925, Bailey and Cushing coined the term “Medulloblastoma” favoring MB derivation from medulloblast and described it as a highly malignant glioma arising in the cerebellum or fourth ventricle and had the tendency to extend to other parts of the CNS [9, 10, 14, 15]. Cushing performed numerous operations on MB patients and studied their characteristic clinical, pathological and epidemiological features. Cushing experimented in various ways to treat MB patients; however, all patients died except one, named Jack Hagan, who survived for five years post incomplete resection. The treatment regimen that was administered to this patient consisted of three treatments of cranial irradiation followed by posterior fossa resection thrice, to ensure and limit recurrence of the local disease. Eventually, he died because of spinal metastasis. The success of saving his patient for comparatively prolonged period of time was sufficient for Cushing to believe that MB could be treated [13].

1.1.2. MB cells of origin

Studies have indicated that MB arises from neuroepithelial stem cells differentiating along glial and neuronal pathways present in or near the cerebellum **(Figure 1A)** [16]. Evidence now postulates a close relationship between the normal development of cerebellum and the initiation of MB. Cerebellum is a part of the brain structure which takes almost seven months after birth to be mature, and this prolonged maturation period increases the chances for abnormalities during development [17, 18]. Aberrations arising in cell differentiation and signal pathways that produce different elements of cerebellum have been indicated to trigger the induction of MB [14, 18].

The process of normal cerebellum development involves two specific germinal zones that give rise to the cells of the cerebellum – primary germinal zone and the nuclear transitory zone [19]. Primary zone consists of Purkinje cells and interneurons that are precursor cells of gamma-aminobutyric acid (GABAergic) neurons which are positioned above the IV ventricle [20, 21]. Nuclear transitory zone assists in the migration of glutamatergic neurons located in the deep cerebellar nuclei to rise within the rhombic lip [22]. The external germinal layer (EGL) is formed when the granule neuron precursor (GNP) cells, which also rise from the rhombic lip, migrate across the cerebellar anlage. The internal granule layer (IGL) is formed when granule neuron precursor cells proliferate inside the EGL and later migrate inwards **(Figure 1B)** [16, 19].

Numerous signaling pathways have been demonstrated to play critical roles in the development of cerebellum. Sonic hedgehog (SHH) pathway with its receptor patched (PTC) is known to be expressed by neuronal precursors in the EGL [23, 24]. Similarly, other molecules like neurotrophils such as $p75^{NTR}$ and brain-derived neurotrophic factor (BDNF) along with its receptor tyrosine related kinase B (trkB), have been shown to be involved in EGL cell divisions; studies have also shown that neurotropin-3 (NT3) and its receptor trkC might have a crucial role in the terminal differentiation of EGL cells into IGL neurons [25-27]. Notably in MB, the expression of trkC is a potential marker for good prognosis [16, 28, 29].

Studies performed recently have disclosed numerous other gene mutations that play a crucial role in cerebellar developmental process that trigger MB origination when deregulated [30]. PI3K/Akt signaling pathway promotes growth and survival of neuronal

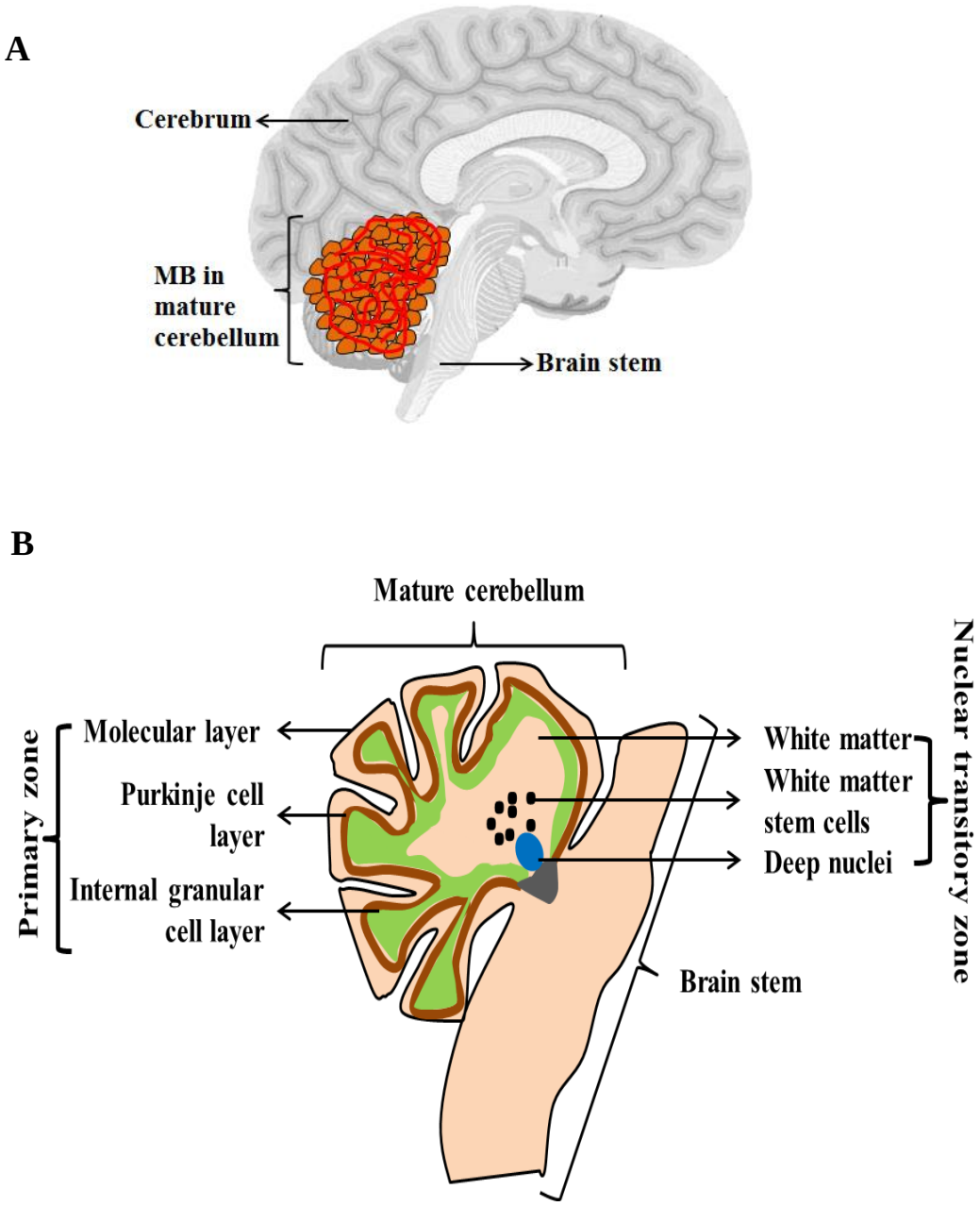

Figure 1. Precursor cell populations in normal mature cerebellum development and MB location. A) Location of MB formation in brain. B) The normal cerebellum development is made up of two germinal zones - primary germinal zone and the nuclear transitory zone. Primary zone consists of gamma-aminobutyric acid ergic neurons and nuclear transitory zone assists in the migration of glutamatergic neurons located in the deep cerebellar nuclei to rise within the rhombic lip. The IGL is formed when granule neuron precursor cells proliferate inside the EGL and later migrate inwards [14, 16, 19].

precursor cells during the normal cerebellum development. Animal studies on PTEN, a negative regulator of PI3K/Akt signaling pathway, which when deleted have demonstrated abnormalities in the cerebellar tissues [31]. Similarly, complete absence in the formation of cerebellum in animal models was observed in the presence of Wnt-1 proto-oncogene mutants [32, 33]. In addition, β-catenin which is a vital molecule regulating the canonical WNT pathway, when deleted, has been shown to produce abnormalities in mice cerebellar morphogenesis [34].

1.1.3. MB signaling pathways

Studies performed in genetically engineered *in vivo* models of MB provide evidence indicating that signal pathways play a very crucial role in the initiation of MB by promoting proliferation and differentiation of neural progenitor cells during normal cerebellar development [35]. Mutations in molecules involved in pathways such Sonic-Hedgehog-Patched, Notch, and Wnt have been shown to play an important role in the induction of MB [36-38].

1.1.3.1. Sonic hedgehog signaling

Sonic hedgehog (SHH) signaling plays a crucial role in the growth and development of the cerebellum by regulating both progenitor and stem cells in the CNS [39-41]. SHH signaling is initiated by three ligands namely Sonic hedgehog (SHH), Indian hedgehog (IHH) and Dessert hedgehog (DHH) [41]. The ligand has a receptor complex comprising of a twelve-pass transmembrane protein called Patched (PTCH) with a characteristic transporter-like structure, which is secreted by the Purkinje cells present below (a) EGL and (b) Smoothened (SMO), [24, 42, 43]. In the absence of Shh, Smo activity is repressed

by Ptch1; whereas in its presence, Ptch1 undergoes certain conformational changes and can no longer suppress Smo, leading to the activation of its downstream molecules such as Gli family of transcription factors GLI1, GLI2, and GLI3 (named after glioblastoma) **(Figure 2)** [23, 42, 44].

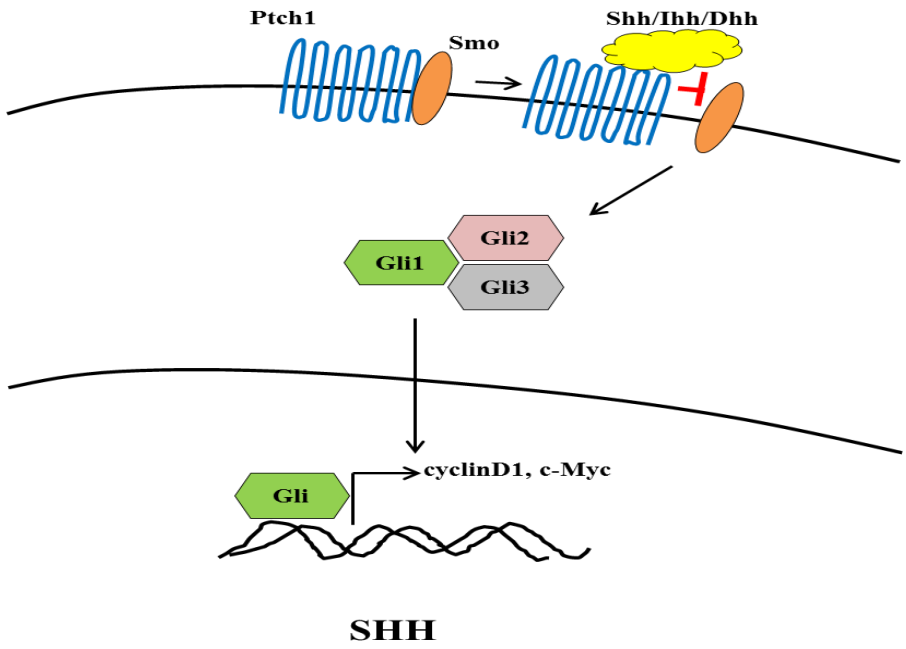

Figure 2. Schematic representation of the SHH signaling pathway in normal cerebellum development. SHH ligand binds to its receptor activating Smo which then activates the downstream molecules. The importance of SHH pathway has been demonstrated in embryogenesis; however, alterations in this pathway have the potential to induce MB formation in cerebellum.

Genetic study has demonstrated that mutations in the key regulatory molecules of Shh pathway induce MB formation [45, 46]. Ptch, a tumor suppressor gene located on chromosome 9q22.3, is mutated in an autosomal dominant disorder called Gorlin's syndrome whose characteristic feature is abnormalities in various developmental processes with a predisposition to numerous diseases such as MB [47]. Mutations in molecules such as PTCH, Suppressor of fused homolog (SUFU), and SMO were

identified in more than 25% of sporadic MB, especially in desmoplastic MBs [45, 46]. An elevated expression of BMI1, target gene of SHH signaling pathway has been observed in MB tumors that deregulate both Rb and p53 pathways leading to the development of MB [48]. . Also the requirement of BMI1 for Hedgehog pathway-driven MB expansion has been demonstrated [48-51]. A significant growth reduction in mice model [Ptch1(+/-)p53(-/-)] of MB, post treatment with SHH pathway inhibitors demonstrated the importance of SHH pathway in MB [52]. A conclusive proof for the role of SHH pathway in MB induction came from studying the Ptch1 knockout mice models [53, 54].

1.1.3.2. Wnt signaling

Involvement of WNT signaling has been elucidated in numerous developmental processes like CNS progenitor growth, cell expansion, differentiation, and tissue homeostasis [55-58].Canonical pathway is activated by the interaction of Wnt with a heterodimeric receptor complex – seven-pass transmembrane receptor Frizzled (FZD) and co-receptor LRP5/LRP6. The interaction leads to the accumulation of β-catenin and consequently activating its downstream signaling cascade [49, 59, 60].. In the absence of WNT protein, β-catenin, a key molecule in this pathway, gets phosphorylated at the amino terminal region by Axin complex made up of APC (*adenomatous polyposis coli* gene product), CK1 (casein kinase 1) and GSK3 (glycogen synthase kinase 3) leading to its ubiquitination and proteosomal degradation [61-63]. However, in the presence of WNT ligand, a receptor (FZD-LRP5/LRP6)-ligand interaction occurs, phosphorylating Dishevelled (DVL) leading to the interaction of DVL with FZD and releasing β-catenin [64, 65]. The stable β-catenin then travels to the nucleus interacting with T-cell

factor/lymphoid-enhancer factor (TCF/LEF) transcription factors transcribing downstream molecules such as cyclin D1 and c-Myc **(Figure 3)** [66-69].

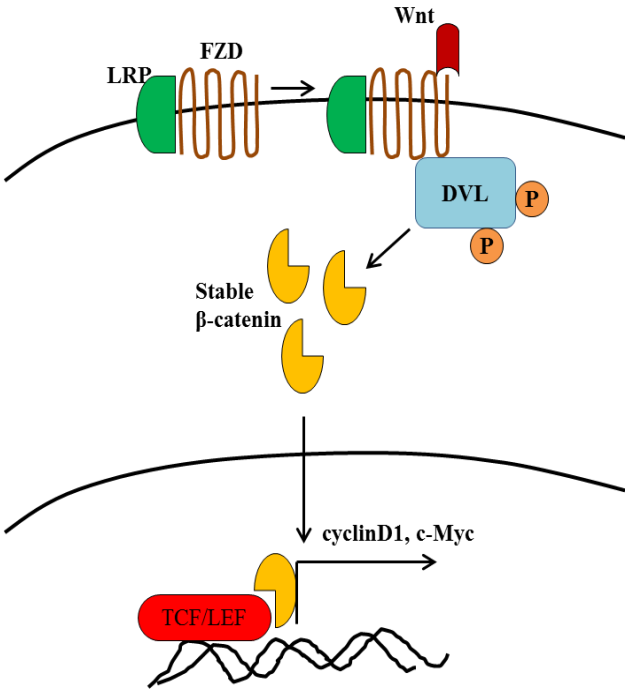

Figure 3. Schematic representation of the WNT signal pathway in normal cerebellum development. Interaction of Wnt ligand with its receptor FZD-LRP5/LRP6 complex results in the phosphorylation of DVL which then release β-catenin.

Mutations in certain specific genes involved in WNT pathway have been identified to induce MB. APC, an important gene in this pathway, has germline mutations in patients with Turcot's syndrome, those that have a predisposition to give rise to various cancer forms along with MB [70, 71]. Inactivated APC leads to the accumulation of β-catenin, which consequently elevates the expression of genes involved in the induction of cell proliferation and apoptosis [71, 72]. However, the most common mutation in WNT signaling of MB (~15%) has been associated with the CTNNB1 gene encoding for the β-catenin protein molecule, is correlated with a good prognosis [70, 73-76]. To further

study the importance of this pathway in MB, various *in vivo* experiments were performed. Transgenic mice with overexpressed stable β-catenin and in the presence/absence of p53, demonstrated no formation of MB tumors, indicating that WNT signaling by itself is incapable of tumorigenesis [77, 78].

1.1.3.3. Notch signaling

Notch signaling plays a vital role in critical developmental processes like embryonic and postnatal cell fate specification events and maintaining homeostasis in stem and progenitor cells during embryogenesis [79-81]. This pathway has mainly four receptors – Notch1, Notch2, Notch 3, and Notch 4 and numerous ligands such as Jagged1, Jagged2 (homologues of serrate), delta-like ligands such as DLL1, DLL3 and DLL4, DNA-binding protein Cbf1 and effector molecules such as Hes1 and Hes5 [5, 80, 82, 83]. The Notch receptor is a single-pass transmembrane protein consisting of a short extracellular, a single-pass transmembrane domain and a small intercellular domain. The extracellular domain has 36 EGF-like repeats, of which 11^{th} and 12^{th} are capable of forming interactions with Delta ligand [84-86]. The Notch intercellular domain (NICD) is made up of a high affinity RAM region and a low affinity ANK region (seven ankyrin repeats) both necessary for CSL (CBF1/RBPjκ/Su(H)/Laf-1) transcription factor activation, a long nuclear localization sequence that links RAM and ANK, followed by a transactivation domain and a C terminus consisting of conserved amino acid proline/glutamic acid/serine/threonine-rich motifs known as PEST sequence [87, 88].

Notch signaling is activated when either Delta or Jagged protein binds to a Notch receptor on the other cell [5]. This interaction results in 2 proteolytic cleavages – First, ADAM-17 (ADAM: a disintegrin and a metalloproteinase) cleaves the transmembrane

region that sheds the ectodomain region of the Notch receptor leaving behind a membrane-tethered intermediate named as NEXT (Notch extracellular truncation) [89, 90]. γ-secretase then cleaves NEXT that acts as a substrate, to release the soluble NICD in to the cytosol [88, 91, 92]. The NICD then translocate to the nucleus where it binds to CSL via the RAM domain displacing HDAC (histone deacetylase) co-repressor and activating a co-activator complex (MAML1, SKIP, and histone acetyltransferase). This process leads to the elevated transcription of Notch signaling downstream molecules like HES1 and HES5 **(Figure 4)** [79, 93-96].

Expression of Notch1 in precursor cells of a developing brain and a significant expression of Notch2 in the actively multiplying GNPs in EGL have been definite indications to suggest the role of Notch signaling in MB [97-100]. Evidence for the significance of Notch signaling in MB came from experiments inhibiting various Notch pathway key regulators, where a reduction in cell proliferation and an increase in cell apoptosis were observed [97, 101, 102]. A poor survival rate in MB patients has been linked to high expression of HES1 protein [101, 103]. All these studies have demonstrated that targeting Notch signaling can be beneficial towards the treatment of MB.

1.1.3.4. Other signaling pathways

Recent studies have identified various other signaling pathways that might have a crucial role in MB tumorigenesis. PI3K/Akt and TGFβ/BMP (bone morphogenetic protein) signaling are two other pathways that regulate important cellular functions such as cell proliferation, differentiation, survival, angiogenesis, and tumor growth [36, 104-110]. It has been demonstrated that PI3K/Akt signaling regulates MB cancer stem cell

survival post irradiation *In vivo* [111, 112]. A study has indicated that BMP production in the TGFβ signaling induce apoptosis in MB cells [113]. Reports also revealed that BMP2 inhibits proliferation of GNP cells, presumed cells of MB origin, induced by SHH pathway [114].

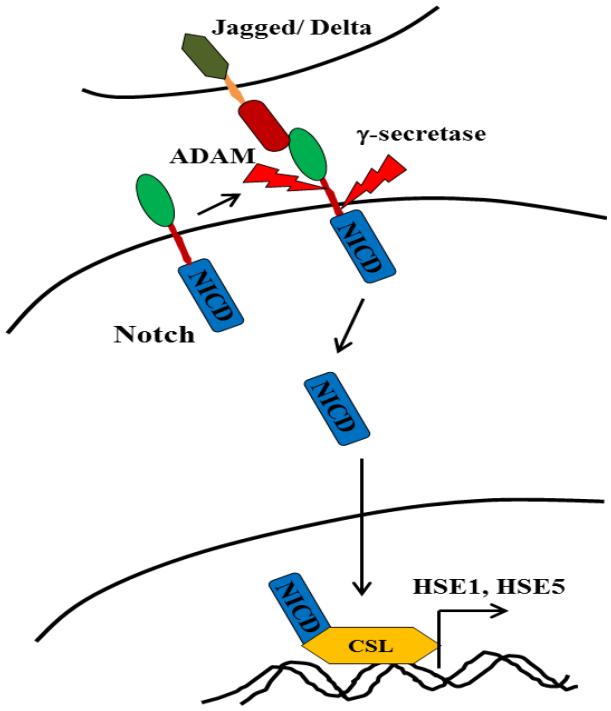

Figure 4. Schematic representation of Notch signal pathway in normal cerebellum development. The Notch signal pathway is activated when Jagged or Delta binds to a Notch receptor, where the ADAM and the secretase cleave the membrane protein to release NICD in to the cytosol [100].

In the last decade, another important receptor molecule and its signaling pathway have been shown the potential to be a therapeutic target for the treatment of MB – Platelet-Derived Growth Factor Receptor (PDGFR) **(Figure 5)**. Reports have demonstrated that PDGFR α/β are highly expressed in MB and that when subjected to inhibition have resulted in tumor reduction [115-118]. However, extensive studies are yet to be performed to identify which PDGFR isoform, among α or β or both play a crucial

role in MB progression. MacDonald in 2001 identified PDGFRα to be highly expressed in MB by gene expression analysis; however in 2003 Gilbertson put down this notion and reported that it was PDGFRβ that was vital in MB metastasis [117, 118]. Thus, extensive study of these signaling pathways and the molecules involved can be beneficial in providing novel approaches in MB treatment.

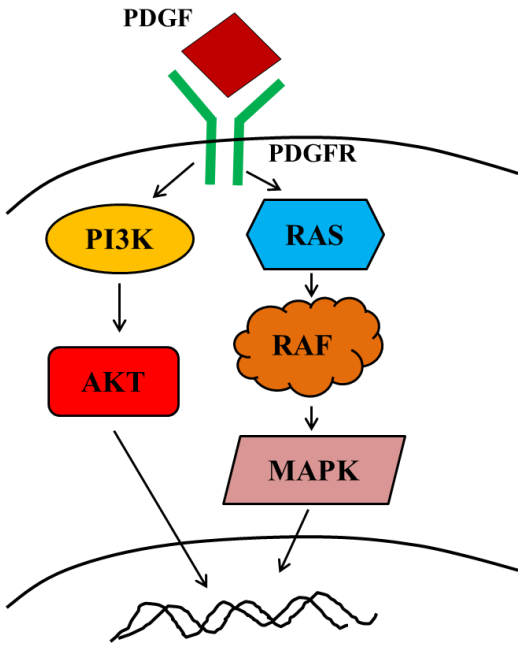

Figure 5. PDGFR signaling pathway. The binding of PDGF ligands to PDGFRs leads to the activation of PDGFR pathway and its downstream molecules such as PI3K, Ras, MAPK. Targeting an alternative pathway as a therapeutic strategy might be a potential strategy in the treatment of MB.

1.1.4. MB histological classification

MB is one of the most common malignancies of childhood brain tumor derived from the aberrations arising in the small embryonal neuroepithelial cells of the cerebellum [14, 119, 120]. Initial classification of CNS tumors distinguished MB from other tumors as they had the ability to differentiate along neuronal and glial lines [14, 15]. Another

closely related CNS tumors are the Primitive Neuroectodermal Tumors (PNETs) [121]. While MB is a tumor of the posterior fossa or the cerebellum, PNETs arise in the supratentorial regions [122]. Although both PNET and MB have common characteristics such as tendency to disseminate via cerebrospinal fluids or clinical attributes such as age at presentation, they differ largely based on pathological diversity, morphological features and biological behaviors demonstrated in MB and its variants along with the lack of PNETs to differentiate via glial and neuronal lines [14, 119, 123-126]. Further studies performed on the genetic nature of MB versus PNET have distinctly shown frequent chromosome 17 aberrations in MB unlike in PNET [119, 127]. Based on all the histological studies World Health Organization (WHO) in 2007 classified MB into four variants – classic, desmoplastic, anaplastic or large-cell and nodular [3].

1.1.4.1. Classic MB

Classic variant of MB is made up of undifferentiated, small, round or ellipsoidal, blue cells with very little cytoplasm and largely composed of dense hyperchromatic nuclei [119, 128]. 40% of classic MB exhibit neuroblastic differentiation which appears as Homer-Wright rosettes [10, 14]. Staining of these cells with Ki-67 antibody demonstrated mitotic cell divisions in 80% of tumors [129]. Hence, mitotic percentage index have been used as prognostic indicators for calculating survivability in pediatric MB [130]. Invasion of tumor cells have been seen in the cortex, white matter, nuclei of the cerebellum and also in the subarachnoid space. Classic MB makes up approximately 64-78% of MBs whose mean age of onset is 9 years [14, 131].

1.1.4.2. Desmoplastic MB

Tumors classified on the basis of pathological factors describe desmoplasia as a large mass of collagenous tissue present within a neoplasm [132]. A biphasic architecture consisting desmoplastic variant of MB is made up of lightly stained reticulin-free nodules/islands that are surrounded by compactly packed, reticulin-rich, actively proliferating, and mitotically active areas [131, 133]. Reticulin staining clearly shows nodules or linear trabaculae being separated by collagen [132]. The incidence of desmoplastic MB is up to 50% in adults when compared to 15% in children and whose onset mean age is 17 years [128, 132].

1.1.4.3. Anaplastic/large cell MB

Tumor cells belonging to large cell/anaplastic (LCA) variant of MB have ample cytoplasm with prominent and pleomorphic nuclei, cell-cell wrapping and extensive mitotic activity [134, 135]. It is the most malignant and aggressive form of MB that can be easily distinguished from other MBs on the basis of its histological and cytological features in H&E stained sections [136]. Anaplastic/large cell MB, with a poor prognosis, occurs rarely and they comprise about 4 – 15% of all MBs [10, 14].

1.1.4.4. Nodular MB

This variant of MB is also referred to as MB with extensive nodularity (MBEN) and is very similar to desmoplastic MB. The two differ in their reticulin-free zones where these regions become extensively large and abundant in neurophil-like tissue in nodular MB [14, 137]. These enlarged nodules consist of neurocytic cells that stream across

neurofibrillary matrix. It occurs in children less than 3 years of age and the incidence of nodular MB is around 3% but with good prognosis [14].

Table 1. MB histological classification. Few similarities and differences between classical, desmoplastic, LCA, and MBEN subgroups of MB [10, 14, 119, 128, 131, 132, 137-139].

	Classic	**Desmoplastic**	**Large cell anaplasia**	**MBEN**
Percentage of incidence	64-78%	50% in adults, 15% in children	4-15%	3%
Mean age of onset	9 yrs	17 yrs	10 yrs	< 3 yrs
Reticulin	Negative	Positive	Negative	Positive
CSF spread	Yes	Yes	Yes	yes
CTNNB1 mutation	Yes	Yes	Yes	yes
Prognosis	Moderate	Good	Poor	Good
Nuclei	Dense hyperchromatic	Dense hyperchromatic	Prominent and pleomorphic	Dense hyperchromatic
Aggressiveness	Moderate	Less	More	Less

1.1.5. MB subgroups

Availability of high-throughput techniques for transcriptomics led to an in-depth study of MB that discovered the biological distinction prevailing between two MB variants like classic and nodular MB, offering the first indications for the presence of subgroups such as SHH MB subgroup [140, 141]. While the above mentioned subgroups were classified purely on the basis of histological features, MB transcriptomic studies

highlighting differences in genetical, transcriptional, demographical, and clinical inferences in MB performed in the last ten years utilizing large sample sizes and various statistical methods, the researchers in the MB field have come to consent that MB can be majorly divided into four subgroups – WNT, SHH, Group 3/C, and Group 4/D based on molecular signatures [142-152]. Evidence also suggests the existence of subtypes within the subgroups that are molecularly and clinically distinct as listed in (**Table 2)** [144, 149, 153-155].

- Cho *et. al.* classification: c1 - MYC, c2, and c4 - neuronal differentiation markers, GRM1 and GRM8, c3 - SHH signaling, c6 - WNT, c5 - photoreceptor transcriptional programs, and expression of GABRA5 [154].
- Kool *et. al.* classification: A - WNT, B – SHH, C - neuronal differentiation genes, D - neuronal differentiation genes and photoreceptor genes, E - photoreceptor genes [144].
- Thompson *et. al.* analyzed subgroups A through E on the basis of mutations in specific genes in WNT and SHH signal pathway [149].

As mentioned in **(Table 2)**, to further characterize and study the MB subgroups in detail, researchers have developed subgroup-specific animal models which provide the investigators a practical platform to test various ideologies for the treatment of MB. Various techniques were adopted to identify these subgroups, of which genomic technologies were widely used [144, 147, 170-174]. Complete understanding of the biology and complexity of MB becomes necessary to find novel approaches for the treatment of MB. In-depth study regarding MB subgroup and their specific biomarkers can assist in providing MB patients with group-targeted treatment that might drastically enhance the chances of prolonged MB survival.

Table 2. MB subgroup classification. Based on the studies performed by several research groups, MB subgroups can be classified into four main subgroups, WNT, SHH, Group3/C, and Group 4/D on the basis of its prevalence, cellular origin, age, genetics, histology, immunohistochemical markers, metastasis, and outcome, and available animal model for in-depth study of the subgroups [16, 46, 53, 74-76, 141, 149, 156-169].

MB classification Northcott *et.al.*[147] Taylor *et. al.*[145]	**WNT**	**SHH**	**Group 3/C**		**Group 4/D**	
Cho *et. al.*[154]	c6	c3	c1	c5	c4	c2
Kool *et. al.*[144]	A	B	E		C	D
Thompson *et. al.* [149]	B	C', D	E, A		A, C	
Prevalence	7-8%	28-32%	26-27%		34-38%	
Cellular origin	Dorsal brainstem progenitor	Cerebellar GNP	Cerebellar stem cell		Unknown	
Age	Child Adult	Infant Child Adult	Infant Child		Infant Child Adult	
Genetics	CTNBB1 mut TP53 mut Monosomy 6	PTCH1 mut SMO mut SUFU mut 9q del MYCN amp	MYC amp Gain OTX2 Gain Ch. 1q, 8, 14, 17, 18 Loss Ch. X, 10, 11, 13, 16		CDK6 amp Gain Ch. 7, 12q, 18 Loss Ch. X, 8 Isochrom. 17q	
Histology	Largely classic Rarely LCA	Desmoplastic Classic LCA MBEN	Classic LCA		Classic LCA	
Immunohistochemical markers	CTNNB1 CKK1 FilaminA YAP1	GLI1 SFRP1 GAB1 FilaminA YAP1	NPR3		KCNA1 KDM6A	
Metastasis	Rare	Variable	Frequent		Variable	
Outcome	Favorable	Favorable to intermediate	Poor		Intermediate	
Animal model	*Blbp-Cre:Ctnnb1$^{lox(ex3)}$;TP53^{flx}*	*Ptch1$^{+/-}$Tp53$^{-/-}$*	*c-Myc^{+}Tp53$^{-/-}$*		Unknown	

1.1.6. MB metastasis and classification

Risk stratifications classify MB into majorly two groups based on age, degree of surgical resection and Chang's operative staging system [175] – Standard-risk MB and High-risk MB **(Table 3)**. The characteristic features of standard-risk MB are patients with age 3 years and older, absence of dissemination in the cerebrospinal fluid (CSF) and spinal cord, diagnosis performed post operation showing a residual tumor of less than 1.5 cm^2; while high-risk MB comprises of patients with age less than 3 years, presence of CSF and spinal cord metastasis, evidence of residual tumor greater than 1.5 cm^2 post operation and of LCA histology [9, 176, 177]. Studies have shown that 5-year survival rates of patients with standard-risk MB is ~70% while it is 40% for the patients with high-risk MB [178].

Metastasis is a process in which tumor cells detach from their primary location, invade and travel to any other location in the body establishing a secondary tumor. It has been demonstrated that metastasis is one of the key features that forms an obstacle and resists therapy in MB resulting in poor outcome [118, 179]. Pediatric MB at clinical presentation are commonly found disseminated to CSF; and at diagnosis are often present with metastasis in the spinal cord or with leptomeningeal, bone, bone marrow, lung, and liver metastasis [179-186]. Currently, disseminated MB patients have been grouped under operative staging system with International TNM staging designation by Chang *et. al.* where letter T stands for primary tumor (based on the size and extent of involvement, it has been subdivided into T_1, T_2, T_3, T_4), N has been omitted in MB staging system, and M stands for metastasis (subdivided into M_0, M_1, M_2, M_3, M_4) **(Table 3)** [175].

Table 3. Chang's classification of metastatic MB. Based on Chang's classification of metastasis, MB can be divided into T_1-T_4 and M_0-M_4 [175].

Classification	Characteristic features
T_1	Tumor <3 cm in diameter; located in midline position in the vermis, roof of fourth ventricle and cerebellar hemispheres
T_2	Tumor >3 cm in diameter; further invading one adjacent structure or partially filling the fourth ventricle
T_3	**T_{3a}:**Tumor invading two adjacent structures or completely filling the fourth ventricle with extension into the aqueduct of Sylvius, foramen of Magendie or foramen of Luschka, thus providing marked internal hydrocephalus
	T_{3b}: Tumor arising from the floor of the fourth ventricle or brain stem and filling the fourth ventricle
T_4	Tumor further spreading through the aqueduct of Sylvius to involve the third ventricle or midbrain or tumor extending to the upper cervical cord
M_0	No evidence of gross subarachnoid or hematogenous metastasis
M_1	Microscopic tumor cells found in cerebrospinal fluid
M_2	Gross nodule seedings demonstrable in the cerebellar, cerebral subarachnoid space or in the third or lateral ventricles
M_3	Gross nodule seedings in the spinal subarachnoid space
M_4	Extraneuraxial metastasis

1.1.7. MB therapies

Sir William Macewen, in 1879, was the first surgeon to successfully operate a 14-year old girl with pediatric brain tumor [187]. In 1925, the importance of surgery along with radiation in MB was first demonstrated by Cushing and Bailey [9, 15]. Since then innumerable modern technologies in the field of surgery, chemotherapy, and radiology have greatly influenced brain tumor treatments, especially MB. The common symptoms of MB patients at presentation are nausea or vomiting, headaches, ataxia, diplopia, nystagmus, papilledema, setting sun sign and lethargy [9, 188]. The initial diagnosis of an increasing homogenous mass in the midline posterior fossa is performed by computed

tomography (CT) scan, with subsequent verification with magnetic resonance imaging (MRI) [9, 10, 181, 189].

Surgical resection even today is the initial and standard treatment for MB patients. Today, surgery is being performed with highest precision, achieving negligible damage to normal brain and avoiding neurological damage [2, 189] and the standard regimen for standard-risk MB consisted of surgical resection followed by conventional doses of radiotherapy comprising of 54-56 gray (Gy) in total (36 Gy to the craniospinal axis along with 18-20 Gy to the posterior fossa) **(Table 4)** [6, 190-192]. The standard treatment for MB involves surgery, radiotherapy at craniospinal axis along with chemotherapy (eg. vincristine, cisplatin, carboplatin, etopside and cyclophosphamide in different combinations) [191, 193]. This combination treatment has been able to achieve a favorable overall 5-year survival rate of 70% for standard-risk MB patients [6, 176, 194]. Nevertheless, to further achieve absolute MB survival rates stringent and appropriate MB treatments are in dire need.

A high rate of survival has been demonstrated with MB therapies; however, complications or side effects are often observed with surgical procedure, radiotherapy or chemotherapy. With surgery, there are high chances of infection, bleeding and dysfunction. Also, irradiation to posterior fossa results in a syndrome with characteristic symptoms of ataxia, mutism, irritability and truncal hypotonia [191, 195-197]. Another major concern with surgery is the residual tumor left behind post-operation that frequently supports relapse of the disease [198]. Innumerable side effects have been monitored in patients subjected to irradiation such as hair loss, radiation dermatitis, hearing, endocrine and growth defects, clumsiness and gonadal dysfunction [191, 199, 200] . Neurotoxicity, bone marrow aplasia, loss of appetite, hepatotoxicity, pulmonary

Table 4. MB treatment regimen. Based on the studies performed by several researchers in MB treatment, treatment regimen in childhood MB can be basically divided into three groups, standard-risk MB (>3 years), high-risk MB (>3 years) and MB in infants.

	Investigator	Treatment
Standard-risk MB (> 3 years)	Packer *et. al.* [190]	23.4 Gy craniospinal and posterior fossa irradiation + chemotherapy (vincristine; vincristine, cisplatin, lomustine; cisplatin); 5-year survival rate ~85%
	Gajjar *et. al.* [161]	23.4 Gy craniospinal irradiation, followed by 55.8 Gy to tumor bed + high dose chemotherapy + autologous stem cell rescue; 5-year survival rate ~85%
High-risk MB (> 3 years)	Evans *et.al.* [201]	36 Gy craniospinal irradiation with posterior fossa boost + chemotherapy (prednisone; cisplatin, vincristine, lomustine; vincristine); 5-year survival rate ~48%
	Packer *et. al.* [190]	36 Gy craniospinal and posterior fossa irradiation + chemotherapy (vincristine; vincristine, cisplatin, lomustine; cisplatin); 5-year survival rate ~85%
MB in infants and young children	Van Eys *et. al.* [202]	Chemotherapy (nitrogen mustard, vincristine, procarbazine and prednisone); long term survivors
	Head Start I & II [203-205]	Cisplatin, vincristine, etopside, cyclophosphamide and methotrexate; myeloblative chemotherapy; autologous stem-cell transplant; 5-year survival rate ~79%

toxicity, and many more have been noticed in patients with chemotherapy [191]. Additionally, a constant effort has to be done to monitor the patient for MB relapse. In most cases MB relapse have been detected within two years of initial treatment. Salvage therapy in the form of irradiation can be adopted for young children and infants with local relapse treated with just chemotherapy at the time of diagnosis [206, 207]. Effective use of high-dose chemotherapy with subsequent stem-cell rescue as salvage therapy have also been tried however with no significant results [9, 208, 209]. All these reports

suggest the need for high surveillance of MB patients or alternative methods to minimize or to avoid all the challenges confronted during MB treatment.

1.2. MB and PDGFR

Platelet-Derived Growth Factor Receptors (PDGFRα and PDGFRβ) signaling activated by its specific ligands (PDGF-AA, - BB, -CC, -DD, and -AB) have been demonstrated to trigger numerous cellular processes such as wound healing, cell proliferation, cell migration, survival and growth in both normal cells and various cancer forms, including MB [117, 118, 210-213]. Cell proliferation and migration studies performed using PDGFR inhibitors such as cambogin, Imatinib and Suntinib have clearly identified PDGFR as a potential target for MB treatment [115, 116, 214].

PDGFR signaling has been linked to MB metastasis; however, the dispute as to which of the two PDGFR isoforms, PDGFRα or PDGFRβ, is imperative for MB metastasis, still remains unresolved. Earlier, efforts were made to define the roles of individual PDGFR in MB metastasis. Gene profiling identified both PDGFRα and PDGFRβ to be highly expressed in metastatic MB [118, 140, 215-217]; *in vivo* MB model system developed to further verify the gene profile study also demonstrated similar results [218]. Notably, one study specifically demonstrated that PDGFRα was highly expressed in metastatic MBs by array analysis and it was further proposed to be a bona fide therapeutic target for metastatic MB based on the results obtained using a PDGFRα neutralizing antibody and a MAP2K1/2 inhibitor[118]. A concern was raised; however, since the PDGFRα probe set used in the microarray analysis was subsequently shown to detect PDGFRβ instead, leading to the possibility that PDGFRβ rather than PDGFRα is preferentially expressed in metastatic MB [117].

Hence, in this study we aim to resolve the unsettled argument and to identify the isoform of PDGFR that plays a crucial role in MB metastasis possibly by regulating specific downstream molecules and miRNAs. We also aim to provide a novel therapeutic approach and molecules for targeted MB therapy.

1.3. PDGFR

PDGFRs were first identified from studies on human fibroblasts that exposed the existence of 180 kDa receptor, stimulated by its ligand that resulted in the tyrosine kinase activity [219-221]. The precursor receptor protein molecule is initially composed of a 120 kDa protein core which undergoes rapid glycosylation to form a 160 kDa precursor molecule, finally maturing into a 180 kDa cell surface receptor [222-224]. The presence of two forms of PDGFRs, PDGFRα and PDGFRβ, was demonstrated by performing the cross-competition and the saturation binding studies in between the PDGF ligand isoforms showing different affinity towards these receptors [225-227]. Also, studies performed indicated that the individual PDGFR subunits, α and β, homo-dimerize or hetero-dimerize to form three distinct receptor forms – PDGFRαα, PDGFRββ and PDGFRαβ [228, 229]. The binding affinity of the ligand isoforms towards these receptors are highly restricted such that PDGF-AA, -BB, -AB bind to PDGFRα having similar binding affinities of K_d, 0.1-0.5 nM; while PDGFR-BB, -AB bind to PDGFRβ with higher and lower binding affinities of 0.5 nM and 2.5 nM, respectively [211, 226, 228]. It has also been identified that the chromosome location for PDGFRα gene is 4q11-q12 and for PDGFRβ gene is 5q23-q31 [222, 227, 230]. Both PDGFRα and PDGFRβ have been shown to play vital roles in the regulation of embryonic development along with various other functions such as cell proliferation, survival, and chemotaxis [231, 232].

Table 5. Comparison between PDGFRα and PDGFRβ. Table 5 provides similarities and differences in between PDGFRα and PDGFRβ based on their structural moiety and functional roles, chromosomal location, amino acid arrangement, and ligand-binding affinities [227, 232-253].

	Alpha	**Beta**
Gene	6.5 kb	5.7 kb
Amino acids	1089	1106
Cysteines	10	10
ATP binding site	Present	Present
Ig family	Present	Present
Ligand binding	PDGF-AA, -BB, -AB	PDGF-BB, -AB
Signal peptide	23 aa	32 aa
Intracellular signaling molecules	Src, PI3K, SHP-2, Crk, PLC-γ1	Src, Shc, Grb2, SHP-2, Grb7, PLC-γ1, Stat5, Nck, PI3K, RasGAP
Chromosome location	4q11-q12	5q23-q31
Unique phosphorylation site	Tyr 762 (Crk)	Tyr 771 (RasGAP)
Expression in body organs	Lung alveolar septa and intestinal villi, dermis layer of the skin, interstitial mesenchymal cells of testis and kidney, lens epithelium, astrocytes of retina, palate mesenchyme	Pancreatic stroma cells, glomerulus in kidney, stromal cells of breast tissue, human connective tissue cells, arachnoid tissue of brain, pericytes and endothelial cells in blood vessels
Function in normal cells	Mitogenicity, chemotaxis, embryonic development, development of oligodendrocyte compartment of the brain	Mitogenicity, chemotaxis, embryonic development, kidney development, development of peripheral neuronal system
Function in cancerous cells	Proliferation, differentiation, metastasis	Metastasis, transformation, angiogenesis, proliferation

1.3.1. PDGF ligands

PDGF ligands were first identified as a component in serum which was later purified from human platelets, present as a di-sulphide bonded protein consisting of two

homologous polypeptide chains, A and B, that are arranged in various combinations (PDGF-AA, BB, AB) and making up a molecular weight of 30 kDa [244, 254-258]. In addition to PDGF-A and B, two new ligands were identified in this family, PDGF-C and D, which initiated PDGF-PDGFR signal pathway [259-262]. The PDGF ligands (PDGF-AA, -BB, -CC, -DD, -AB) interact with PDGFRα or PDGFRβ receptor selectively, initiating receptor dimerization and finally signal transduction [225, 229, 263-265].

PDGF acts as an important mitogen for many mesenchymal cells such as smooth muscle cells, fibroblasts and glial cells [266-268]. Various studies have demonstrated the importance of PDGF and its receptors as a vital growth factor for the animal development. Studies using knockout mouse have demonstrated that the development of vasculature cells are assisted by PDGF-B and its receptor while most of the processes during embryogenesis development is supported by PDGF-A and its receptors [241, 269-273]. Alongside the knockout studies, mutation studies were also performed to testify the roles of these growth factors by mutating tyrosines residues in the cytoplasmic tail regions of the PDGFRs to phenylalanines that resulted in abrogated PDGFR signaling functions [241, 274-276]. All the above mentioned mouse studies clearly testify the roles of PDGF and its receptors in animal development. Although PDGF signaling and its functions play a critical role in growth and development of numerous cells in animals, their aberrations or overexpression can lead to drastic consequences. The first indications of PDGF involvement in tumorigenesis emerged when studies revealed 92% homology in between PDGF-B and v-sis (an oncogene present in simian sarcoma virus) [267, 277-280]. Clinical studies indicated the association of the aberrations in the expression of PDGF and PDGFRs with the induction and progression of diseases in kidneys, lungs and

joints and also in atherosclerosis, pulmonary hypertension and organ fibrosis [267, 279, 281-285]. Studies performed in the last decade have investigated and identified that PDGF-PDGFRs play critical roles in tumor processes such as wound healing, angiogenesis, migration, invasion, metastasis [234, 286-290]; especially in brain tumors such as glioblastoma and MB [117, 118, 210, 291]. Together, these evidence portray PDGF-PDGFRs to be a potential therapeutic target in the treatment of numerous tumors, more specifically MB for our studies.

1.3.2. Identification of PDGFRs

cDNA cloning studies of PDGF receptors from murine 3T3 cells identified amino acid sequences indicating this surface molecule to be a tyrosine kinase protein consisting of a split tyrosine kinase domain [222]. A human homologue of this murine PDGF receptor was cloned using DNA probes which further assisted in the study of this gene [236, 237, 292]. This receptor molecule was composed of 1106 amino acids containing a 32 amino acid leader sequence and termed as the beta-subunit (now referred to as PDGFRβ). The beta-subunit is initially synthesized as a 160 kDa precursor which undergoes glycosylation to form a mature PDGFRβ of 180 kDa [224, 293]. Expression of beta-subunits in mammalian cells showed a higher binding affinity of these receptors towards PDGF-BB when compared to PDGF-AB (low affinity) and -AA (no affinity) [227, 236, 237]. These binding studies also confirmed the ideology of the presence of multiple PDGF receptors and that cloned PDGF receptor was of the beta-subunit type [225, 226].

The gene encoding for the other form of human PDGF receptor was cloned using stringency hybridization approach where a DNA probe from either the tyrosine kinase

domain of v-fms or the PDGF beta-subunit was used for identification [235, 239]. This receptor molecule was composed of 1089 amino acids containing a 23 amino acid leader sequence and termed as alpha-subunit (now referred to as PDGFRα). The precursor of an alpha-subunit is 140 kDa, the maturation of which results in the 170 kDa PDGFRα [293, 294]. Expression of alpha-subunits in mammalian cells showed a similar high binding affinity of these receptors towards all forms of PDGF ligands unlike the beta-subunit [227, 235, 239].

1.3.3. PDGFR structure and receptor activation

PDGFR is a cell surface protein tyrosine kinase receptor. Structurally, both PDGFRα and β consists of mainly three regions – extracellular domain consisting of five ligand binding immunoglobulin-like motifs, a single-pass transmembrane domain that separates the extracellular and the intracellular domain and an intracellular domain made up of a split tyrosine kinase domain and a carboxy terminal region with an overall structural amino acid homology of ~44% **(Figure 6)** [220, 227, 295]. The amino acid sequence homologies between different regions of the two receptors are ~30% in the extracellular domain, ~48% in the transmembrane domain, ~83% in the juxtamembrane region and ~28% in the C terminal region. A high percentage of homology is found in the kinase domain (~87% in kinase domain 1 and ~74% in kinase domain 2) with ~35% homology in the kinase insert region [227, 293, 295].

The presence of ten highly conserved cysteine residues in the extracellular domain of both receptors has been identified which acts as a spacer resulting in five Ig-like motifs for ligand binding [220, 227]. A series of experiments such as co-immunoprecipitation of PDGF receptors, competition binding of PDGF to PDGFRs and subunit cross linking

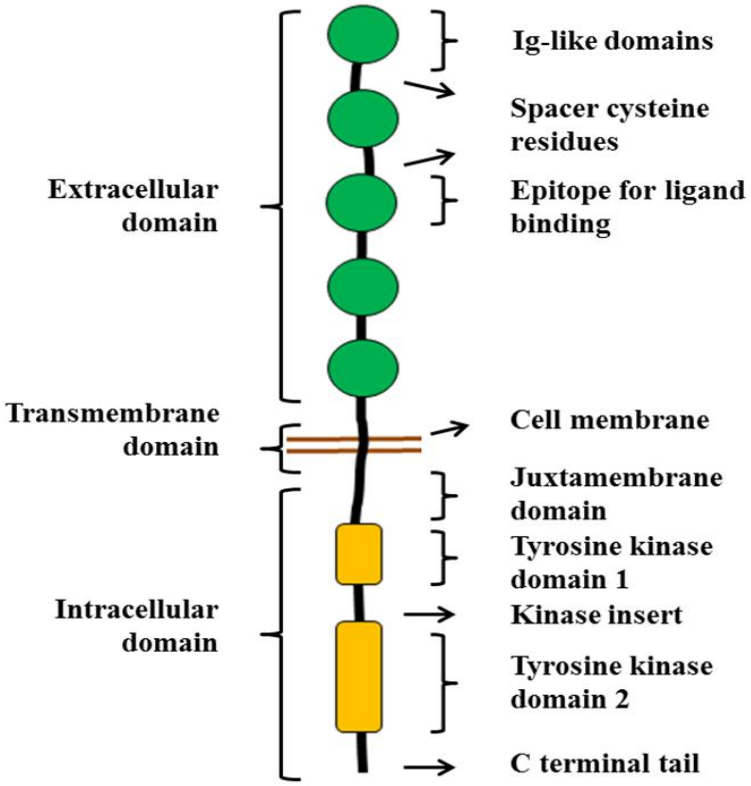

Figure 6. PDGFR structure. PDGFR can be mainly divided into extracellular domain, transmembrane domain and intracellular domain. The extracellular domain consists of five Ig-like domains and the intracellular region is made up of split tyrosine kinase domains and a C-terminal region [220, 227, 295].

were performed that defined the presence of two PDGF receptors, PDGFRα and β, the specific binding affinities of PDGF isoforms towards the individual PDGFRs and the mechanism of PDGF receptor dimerization that trigger PDGF-PDGFR signal transduction [224, 225, 228]. Studies have shown that PDGF ligand dimeric isoforms (PDGF-AA, -BB, -CC, -DD, -AB) with two receptor binding epitopes, are capable of binding to the extracellular domains of PDGFRs bringing the receptors together forming a receptor homodimer or heterodimer (PDGFRαα, PDGFRββ and PDGFRαβ) **(Figure 7)** [264, 296, 297]. Various studies on subunit cross-linking, coimmunoprecipitation of

receptor subunits and competition binding affinity studies have led to the model that the two isoforms of PDGF receptors homo or heterodimerize on the basis of high affinity binding sites present on the PDGF receptors for the ligands that assist the receptors to either form PDGFRαα, PDGFRββ or PDGFRαβ forms [227-229]. Mutational analysis have demonstrated that the amino acids making up the epitope in each PDGFR, most required for ligand binding, that leads to the dimerization of the receptors resides in the outermost three Ig-like motifs [244, 298-302]. PDGFRα bind to both PDGF-A and –B chains with high affinity whereas PDGFRβ bind to only PDGF-B chain with high affinity and PDGF-A with a low affinity; hence PDGF-AA induces the formation of PDGFRαα and PDGFRαβ whereas PDGFR-BB induces the formation of all three forms of PDGFR combinations [228, 297, 303, 304].

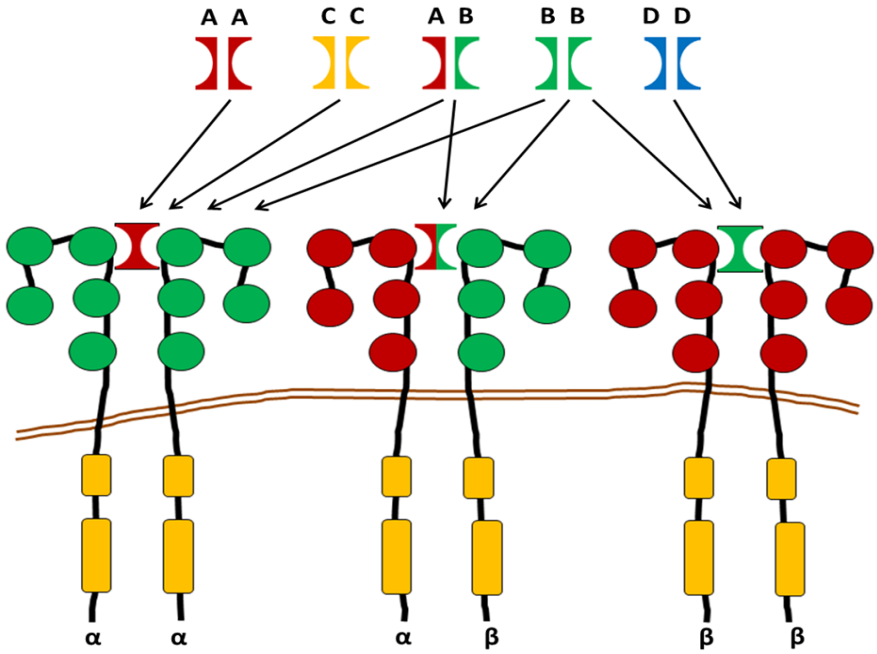

Figure 7. PDGFR ligand binding. PDGFRα and β can homo or hetero-dimerize to form PDGFRα, PDGFRαβ and PDGFRββ receptors. Each dimer specifically binds to ligand dimers; PDGFRαα binds to PDGF-AA, -CC, -AB, -BB, PDGFRαβ binds to PDGF-AB while PDGFRββ binds to PDGF-BB, -DD [264, 296, 297].

Transmembrane region of the PDGF receptor plays an insignificant role in receptor activation or signal transduction; however it plays a major role in anchoring the receptor in the plasma membrane linking the extracellular environment with the internal molecules of the cell [305]. The juxtamembrane region separates the transmembrane from the tyrosine kinase region in the intracellular domain. This region has been demonstrated to play a crucial role in initiating PDGFR kinase activation by phosphorylating its 579 and 581 tyrosine residues, leading to the trans-phosphorylation of 857 tyrosine residue in the activation loop, resulting in the conformational change that allows the substrates to have an access to them [306].

The protein tyrosine domains (kinase domain 1 and 2), unlike in other tyrosine kinase receptors like EGFR and insulin receptor, are split in PDGFR and separated from each other by ~100 hydrophilic amino acid containing kinase insert [293, 305, 307]. Mutation in autophosphorylation site (tyrosine 751 residue) of the kinase insert region has demonstrated its importance in the regulation of interactions with certain subsequent downstream cellular proteins such as PI3K [308]. The tyrosine kinase domain is a highly conserved region among all receptor tyrosine kinases and with ~80% amino acid homology in between PDGFR α and β [227, 293, 305]. The kinase domain is made up of two lobes, N terminal lobe consisting of a α helix and five β sheets and a C terminal lobe which is majorly helical in nature [309]. Both PDGF receptors consist of a conserved glycine-rich consensus sequence GlyXGlyXXGlyX(15-20)Lys , termed as the phosphate binding loop, located in between the N and C terminal lobes, that functions as a docking site for ATP [310-312]. Studies replacing the lysine residue in the consensus sequence of the ATP binding site have demonstrated a complete abolishment of the kinase activities highlighting the importance of this sequence in PDGF-PDGFR signal transduction [305,

313]. For the substrate to be phosphorylated by the tyrosine kinase, utilizing the energy obtained from ATP hydrolysis, it has to first have access to the active site located within the kinase domain, which is well protected by 20-30 amino acids stretch of activation loop. Phosphorylation of the tyrosine residue in the activation loop, tyrosine residue 849 for PDGFRα and 857 for PDGFRβ, is necessary for tyrosine kinase enzyme activation in PDGFR leading to conformational changes that open the activation loop exposing the active site to the cytoplasmic substrate molecules like Src homology2 and protein tyrosine phosphatase domains **(Figure 8)** [308, 309, 314]. C terminal region, the last portion of the intracellular domain, a highly divergent region among all receptor tyrosine kinases, has been found to play a vital role assisting in enzyme activation process [310]. Mutation studies have shown that phosphorylation of tyrosine residues 1009, 1021 in the C terminal region of PDGFRβ results in the conformational change ofthe activation loop exposing the active site to PLCγ [315-317].

1.3.4. Autophosphorylation and signal transduction

Dimerization of two PDGFRs brings the receptor tyrosine kinases adjacent to one other leading to the autophosphorylation of conserved tyrosine residues in them [318, 319]. Two different sets of autophosphorylation sites assist in increasing the catalytic efficacies of the enzyme kinase triggering PDGF-PDGFR signal transduction[244]. The first set of tyrosine residues reside in the activation loop of the kinase domain which when phosphorylated enhance kinase activity. Mutation studies have identified it to be Tyr 849 in the PDGFRα and Tyr 857 in the PDGFRβ receptor [244, 308, 320]. The second set of tyrosine residues (Tyr 579 and Tyr 581 in PDGFRβ) found outside the kinase domains, when phosphorylated provide docking sites for downstream cytoplasmic

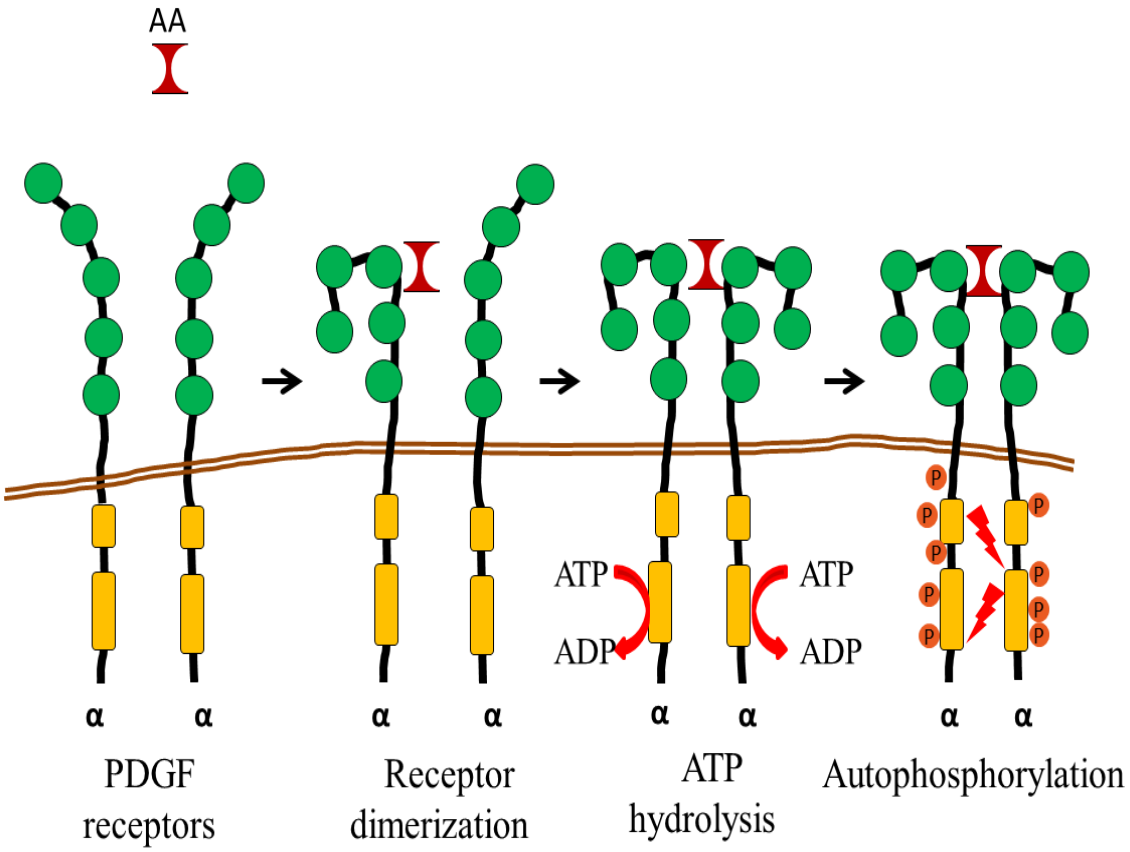

Figure 8. PDGFR activation. PDGF ligand binds to the Ig-like domain in the extracellular region of one isoform of the PDGFR, increasing the affinity for a second receptor in the vicinity to form a receptor dimer. Receptor dimerization leads to ATP hydrolysis resulting in autophosphorylation of the active sites of PDGFRs thereby activating the receptor [308, 309, 314].

molecules like Src-homology 2 (SH2) domains [321, 322]. A number of molecules containing the SH2 domains have been shown to be activated by PDGFRα and PDGFRβ tyrosine phosphorylation, such as SHP-2, PI3K, PLC-γ1, Grb 7, Crk, Src, RasGaP, Stat 5, Shc, and Grb 2 [244, 319, 323-329]. However, distinct binding of Crk molecules has been demonstrated with PDGFRα alone (Tyr 762) [319, 330]; similarly, RasGap has shown binding specificity towards PDGFRβ only (Tyr 771) and not PDGFRα [331-333] **(Figure 9)**. Interestingly, unique tyrosine residue phosphorylation was observed in hetero-dimeric PDGFRs induced by PDGF-AB that triggered greater mitogenic signaling

pathway when compared to homo-dimeric PDGFRs [334-336]. For example, an elevated level of phosphorylation was observed in Tyr 754 PDGFRα and Tyr 771 PDGFRβ in the hetero-dimeric form than in the homo-dimeric form [336, 337].

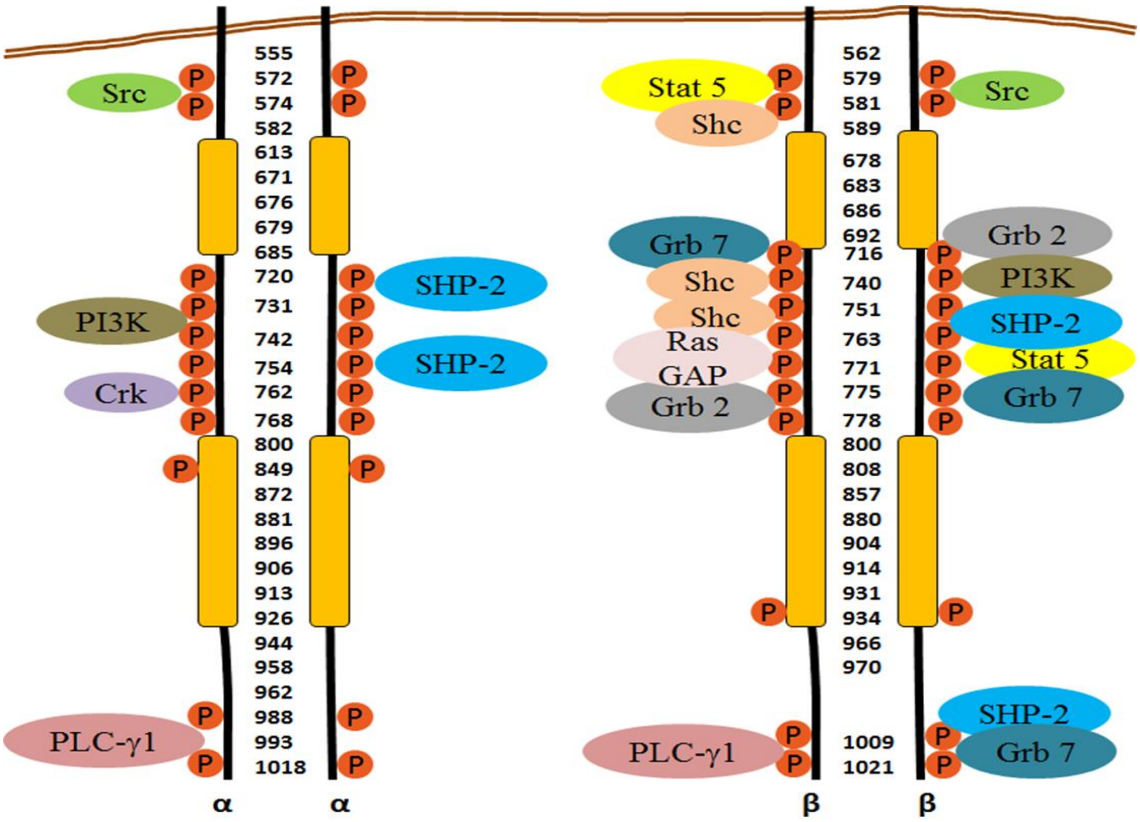

Figure 9. Phosphorylated tyrosine residues in PDGFRαα and PDGFRββ. Receptor activation results in phosphorylation of the specific tyrosine residue, phosphorylating the active site and triggering unique signal cascade [244, 319, 323-328]. Certain molecules like Crk and RasGAP get activated specifically by PDGFRαα and PDGFRββ, respectively [331-333].

1.3.5. Role of PDGFR signaling in MB progression

The significance of PDGFR signaling in MB was demonstrated by McDonald *et al*. by performing an expression profiling on the MB samples and identifying PDGFRα and RAS/MAPK pathway to be therapeutic targets for the MB metastasis [118]. However, it

was Gilbertson *et al*. that indicated that it was PDGFRβ and PDGFRα that was overexpressed in MB [117]. Till date the debate remains equivocal, thus encouraging our study to investigate and to decipher the right isoform of PDGFR molecule that can be treated as a potential therapeutic target MB progression.

In this study we specifically blocked PDGFRα or PDGFRβ using siRNA, shRNA or inhibitors in MB cells, Daoy, D283 and D425 that mostly represent SHH subgroups of MB, to identify the potential target molecule for MB. In the process, we deciphered a novel PDGFRβ-c-Myc-CD44 pathway along with downstream molecules like miR-1280 and -1260 and their target molecules JAG2 and CDC25A to play a crucial role in MB metastasis and progression. MB patient samples were analyzed that indicted the possibility of PDGFRβ and CD44 to be distinct target molecules for MB metastasis. We also targeted PDGFRβ and c-Myc and evaluated the rate of cell proliferation and migration when co-inhibited. In conclusion, our study reveals that PDGFRβ-c-Myc-CD44 signal pathway can be a potential therapeutic target in the treatment of metastatic MB.

CHAPTER 2. MATERIALS AND METHODS

2.1. Cell lines

Human MB cell lines, Daoy and D283 were purchased from American Type Culture Collection (ATCC) and D425 cells were a gift from Dr. Darell D. Bigner [338]. Daoy and D283 cells were maintained in modified Eagle's medium (MEM) containing 10% fetal bovine serum (FBS), along with 2 mM non-essential amino acids and 5 mM sodium pyruvate; D425 cells were maintained in improved MEM medium containing 10% FBS and cultured in 5% CO_2 incubator at 37°C.

2.2. Reagents

The following reagents were purchased from various manufacturers: anti-human PDGFRα rabbit antibody (Santa Cruz), anti-human PDGFRβ rabbit antibody (Epitomics), anti-human PDGFRα mouse neutralizing antibody (R&D Systems), anti-human PDGFRβ goat neutralizing antibody (R&D Systems) c-Myc rabbit antibody (Invitrogen), anti-human CD44 mouse IgG (Cell Signaling), Jagged 2 rabbit antibody (Cell Signaling), control siRNA (Invitrogen), PDGFRβ siRNA (Invitrogen), pGL4.32[luc2P/NF-κB-RE/Hygro] Vector (Promega), PDGFR inhibitor cambogin (a novel PDGFR inhibitor identified in our lab) [214], c-Myc siRNA (Santa Cruz), c-Myc inhibitor 10058-F4, (Z,E)-5-(4-Ethylbenzylidine)-2-thioxothiazolidin-4-one (Calbiochem), microRNA array (Exiqon), TaqMan microRNA assay kit (Applied Biosystems), miR-1280 and miR-1260 inhibitors (Applied Biosystems). The pBabe-puro CD44 vector, pBabe-puro control vector, packing vectors pUMVC and pCMV-VSV-G were obtained from Addgene, c-Myc expressing plasmid was purchased from Origene.

2.3. SiRNA transfections

SiRNAs are small non-coding ~22 nucleotide base pairs that were used for our transfections to knockdown specific genes for our study [339]. MB cells ($2x10^6$) were transfected with specific siRNA along with control (scrambled) siRNA using lipofectamine ltx (Invitrogen) in opti-MEM reduced serum medium for 4 h following the manufacturer's instruction. Scrambled siRNA served as the control. The serum starved cells were then fed with equal volume of MEM medium and kept in culture for 48 h at 37°C in a CO_2 incubator. After 48 h, cells were harvested for total RNA for PCR and protein for Western blotting analysis. List of siRNA used along with their sequences are presented in **(Table 6).**

Table 6. Gene specific siRNAs. Gene specific siRNAs used for gene knockdown in vitro with their sequence and source.

siRNA	Sequence (5' – 3')	Source
Scrambled	ACAUCACGUACGCGGAAUACUUCGA	Invitrogen
PDGFRα	GGAGGAUGAUGAUUCUGCCAUUAUA	Invitrogen
PDGFRβ	UCACGGAAAUAACUGAGAUCACCAU	Invitrogen
c-Myc	CCCAAGGUAGUUAUCCUUAtt	Santa Cruz
(siRNA pool)	GGAAACGACGAGAACAGUUtt	
	CCUGAGCAAUCACCUAUGAtt	
CD44	GUAUGACACAUAUUGCUUCUUUU	Dharmacon
JAG2	GCAAGGAAGCUGUGUGUAA	Dharmacon
(siRNA pool)	GCGUGUGCCUUAAGGAGUA	
	GAACGGCGCUCGCUGCUAU	
	GGUCGUACUUGCACUCACA	

2.4. Western blot analysis

Western blot analysis was performed to confirm level of protein expression either post gene knockdown or to identify effect of gene knockdown on the downstream molecules. The proteins were extracted using standard 50 mM tris lysis buffer (1 M Tris pH 7.4, 5 M NaCl, Triton x-100, Roche complete cocktail inhibitor) and its concentration was measured by BCA protein assay (Thermo scientific). 40 µg protein of each sample was loaded into each well of a gel and SDS-PAGE was performed using 1X running buffer (10 X stock – 30g tris base, 144g glycine, 10g SDS) at 80V for 4% acrylamide gel and 120V for 10% acrylamide gel. The proteins were then transferred using a transfer buffer (running buffer with 20% methanol) onto 0.45 µM nitrocellulose membrane (Bio-Rad) by Western blotting using 100V on an ice bath for 2 h. The membrane was initially incubated with specific primary antibody overnight at 4°C, washed and then incubated with secondary antibody adhered to a HRP-conjugate for 2 h. The results were visualized using SuperSignal West Pico chemiluminescence Substrate (Thermo Scientific) by exposure on the autoradiographic films (Gene Mase). Rabbit secondary antibody (BioRad, catalogue # 170-5046) was used at 1:2000 dilutions and mouse secondary antibody (Sigma, catalogue # A9917) at 1:5000 dilutions. Experiments were performed in duplicate. List of antibodies used, their sources and working dilutions are presented in **(Table 7)**.

2.5. Cell proliferation assay

Cell proliferation assay involves a colorimetric measurement of conversion of a tetrazolium dye to an insoluble purple colored compound called formazon. In our study this assay was performed to analyze the survivability of cells in different conditions. For

Table 7. Primary antibodies. Primary antibodies used with their source, catalogue numbers and working dilutions.

Antibody	Source	Catalogue #	Dilution
PDGFRα	Santa Cruz	sc-338	1:750
PDGFRα (neutralizing antibody)	R & D Systems	MAB322	1:500
PDGFRβ (neutralizing antibody)	R & D Systems	AF385	1:250
PDGFRβ	Epitomics	1469-1	1:8000
c-Myc	Sigma	AV32708	1:2000
	Cell signaling	9402S	1:1000
CD44	Cell signaling	3578	1:1000
PKCα	Cell signaling	2056	1:1000
NFκB	Cell signaling	3034S	1:1000
JAG2	Cell signaling	2205	1:1000
CDC25A	Cell signaling	3652	1:1000
β-actin	Sigma	A2228	1:6000

the experiments using siRNAs (control siRNA, PDGFRα siRNA, PDGFRβ siRNA, c-Myc siRNA, CD44 siRNA, JAG2 siRNA), MB cells ($2x10^4$/well) were placed in 70% MEM, 30% Opti-MEM with 15 pmol siRNA and 0.25 μl/well of lipofectamine (Invitrogen) in 96-well plates. Scrambled siRNA served as the control. For the experiments using inhibitors, cells ($2x10^4$/well) were plated in 96-well plates overnight. Then, cells were treated with the relevant controls (equal amount of solvent) or inhibitors (SJ001, 10058-F4, miR-1280 and -1260 inhibitors). After 48 h of treatment, cell proliferation rates were determined using MTS assay (Promega) as per the manufacturer's protocol. Experiments were performed in triplicate. The results are presented as percentage for cell proliferation.

2.6. Lactate dehydrogenase (LDH) assay

Lactate dehydrogenase assay involves a colorimetric measurement of conversion of NAD to NADH by the lactase dehydrogenase enzyme released in the medium to form a colored compound. In our study this assay was performed to analyze the cell deaths in different conditions. Cells ($2x10^4$/well) were placed in DMEM without penicillin and streptomycin a day before transfection in 24-well plate. After 48 h of transfection, heat inactivated culture media were harvested for assessment of released LDH using an LDH based in vitro toxicology assay kit (Sigma). The LDH released in the medium reduced NAD to NADH forming a colored tetrazolium dye which was read spectrophotometrically at 490 nm. The intensity depicted the amount of cell death in the sample Experiments were performed in triplicate. The results are presented as fold change for cell death.

2.7. Cell invasion assay

Cell invasion assay involves the invasion of tumor cells across the cell matrix coated boyden chamber which is then dyed and absorbance read colorimetrically. This asaay was performed in our study to analyze the invasive ability of cells in different conditions**.** 2.5×10^6 MB cells were seeded in a medium without antibiotics a day before the experiment. Daoy cells were transfected with control siRNA, PDGFRα siRNA, PDGFRβ and CD44 siRNA for 48 h. D283 cells were treated with either Control or retroviral vector containing CD44 cDNA for 24 h along with Wild Type. After which both Daoy and D283 cells were starved in the presence of reduced or serum-free medium (Gibco) for additional 24 h. On the day of the experiment, the cell invasion chamber assay that is performed using a Boyden chamber (Calbiochem InnoCyte cell invasion assay kit 24-

well) is placed in the CO_2 incubator to bring it to room temperature. Warm PBS is added to the upper chamber to rehydrate the basal membrane matrix and incubated for 1 h at 37°C. During the incubation, the treated Daoy and D283 cells are suspended to attain a concentration of 2.5 x 10^4 cells/ml in the serum-free media. PBS is then carefully aspirated from the upper chamber without disturbing the matrix-coated membrane. 500 µL of cell suspension is then added to the upper chamber. 750 µL of complete media along with PDGF-BB (chemo attractant – 50 ng/mL) was added to the bottom chamber. This set up was incubated for 24 h at 37°C in the CO_2 incubator. After incubation, 500 µL of cell staining solution (Calcein-AM in cell detachment buffer, 1:100 dilutions) was added to the unused wells. The cell suspension medium was carefully aspirated from the upper chamber and was placed in the cell staining solution. The cells were dislodged by gently tapping the bottom of the upper chamber. The chamber was then incubated for 30 min at 37°C in the CO_2 incubator. The upper chamber was then removed and the assay was incubated for an additional half hour. 200 µL of each sample of the dislodged cell suspension was then taken in the 96-well plate (black) in duplicates and the fluorescence measured spectrophotometrically at an excitation wavelength of 485nm and emission wavelength of 520 nm. Experiment was repeated thrice.

2.8. RT-PCR for tissues

RT-PCR is a technique employed to analyze the cell mRNA expression levels by using the cDNA got from RNA using reverse transcriptase. In our study it was performed to evaluate the mRNA levels of PDGFRα, PDGFRβ and CD44 in MB tissues. RNA was isolated from five MB tissues and also from Daoy and D283 cells by using the TRI reagent (Sigma) following the manufacturer's protocol. The quantity and purity of RNA

isolated was calibrated with the help of NanoDrop 1000 spectrophotometer (Thermo Scientific). 1 µg of total RNA was taken to prepare cDNA with the help of SuperScript first-strand synthesis system for RT-PCR (Invitrogen) following the instructions mentioned by the manufacturer. RT-PCR reaction mix was set up with the help of SYBR GreenER qPCR supeermix (Invitrogen) as given in the **(Table 8)**.

Table 8. RT-PCR reaction mix for tissue samples. RT-PCR reaction mix with its components and volume in 1 sample.

Components	**Volume in 1 sample (µl)**
SYBR GreenER qPCR supermix	12.5
Forward primer	1
Reverse primer	1
cDNA	2
Distilled water	8.5
Total	25

The thermal cycler program used to perform the PCR reaction is tabulated in **(Table 9)**. The experiment was repeated thrice and the Ct values obtained were analyzed by using $2^{-\Delta\Delta Ct}$ methodology [340].

Table 9. The thermal cycle protocol for ChIP-PCR. The thermal cycle protocol showing the PCR program consisting of temperature, time, number of cycles and the cycle description.

Temp.	**Time**	**Cycle**	**Description**
50°C	2 min	1	Uracil DNA glycosylase (UDG) incubation
95°C	10 min	1	UDG inactivation and Initial enzyme activation
95°C	15 sec	40	Denaturation
60°C	1 min		Annealing/extension

2.9. Immunohistochemistry

Immunohistochemistry is a technique to stain and evaluate protein expression levels in paraffin embedded tissues by unmasking and exposing the antigens present in the cells to our gene specific antibodies. In our study this technique was performed to evaluate the protein levels of PDGFRα, PDGFRβ and CD44 in MB tissues. MB tissues used in this study were collected from the primary cerebellar tumors at the University of Texas Southwestern Medical Center. Diagnoses were confirmed by pathologists and the use of patient tissue for research was approved by the institutional review board (IRB) of Texas Southwestern Medical Center and North Dakota State University. Five-micron sections sliced from the fresh frozen tissues were stained for PDGFRα, PDGFRβ and CD44 protein expression using a colorimetric method as described previously [341, 342]. Briefly, slides were fixed in -20°C cold acetone for 2 min, and pre-treated with H_2O_2 for 5 min at room temperature. After blocking the non-specific binding site using a phosphate buffer (PBS) containing 2% FBS, the tissue sections were incubated with the primary antibodies (anti-human PDGFRα rabbit antibody or anti-human PDGFRβ rabbit antibody or anti-human CD44 mouse antibody along with rabbit and mouse IgG as control) overnight at 4°C. Following 3 washes with 1xPBS containing 0.3% triton-100, a biotinylated anti-mouse IgG/anti-rabbit IgG (H+L) secondary antibody (Vector Laboratories) was applied to the tissue sections for 2 h at room temperature. The signal was visualized using ABC reagent from the Vector Laboratories by following the company's instruction. Images were captured by using a microscope (Olympus).

2.10. Overexpression of CD44

Retroviraal infections is a method that involves using the virus particles carrying the plasmid for gene expression of a gene of interest to the target cells. In our study CD44 overexpression was performed on the D283 cells to evaluate the effect of CD44 on MB cell invasion. $1x10^6$ HEK 293 cells were seeded in DMEM the previous night to attain 30% confluency on the day of the experiment. The cells were then co-transfected with 0.9 µg of pUMVC (gag/pol expression vector), 0.1 µg of VSV-G (expression vector) and 1 µg of either Babe puro (Control) or Babe CD44 (CD44 cDNA) vector (Addgene) using Lipofectamine (Invitrogen) and incubated overnight at 37°C. The medium was replaced with fresh medium to dilute out the cytostatic factor. It was further incubated for 48 h and 72 h after which the supernatants were collected after passing them through a 0.45 µM syringe filter. The supernatants were then diluted with the medium and placed on the $2x10^6$ D283 cells in the presence of polyprene (8 µg/ml). The set up was incubated for further 24 h after which either the cells were lysed to extract the proteins for western blotting to confirm the expression of CD44 in D283 cells or the cells were taken for invasion assay.

2.11. Wound healing assay

Wound healing assay involves the evaluation of the ability of cells to migrate and heal the artificially created wound. In our study this assay was performed to evaluate the effect of specific genes on MB cell migration. For the experiments using siRNAs, Daoy cells were grown up to 80% confluence and then transfected with specific siRNA (PDGFRβ siRNA, c-Myc siRNA, JAG2 siRNA) using lipofectamine ltx (Invitrogen) in opti-MEM reduced serum medium for 4 h following the manufacturer's instruction.

Scrambled siRNA served as the control. The cells were then fed with equal volume of MEM medium and kept in culture at 37°C in a CO_2 incubator for 36 h. For the experiments using inhibitors, Daoy cells were also treated with specific inhibitors (PDGFR inhibitor, c-Myc inhibitor, miR-1280, and -1260 inhibitors). Equal amount of solvent served as a control. At the 36 h time point, cells were detached, and equal number of cells was re-distributed in a 48 well plate. After 48 h incubation, an artificial wound was made using a 100 µl pipette tip by scraping across the bottom of the well. The medium was changed to remove all the detached cells. Movement of cells into the wound area was captured by taking images at 0 and 24 h using a phase-contrast microscope (Olympus). Migration rate in percentage was calculated by comparing the width of the wound at 0 and 24 h in each sample. Wound healing assay was not performed on D283 and D425 cells as they are half adherent/half suspension cells. Experiments were performed in triplicate. The results are presented as percentage for wound healing.

Percentage wound healing was calculated using the formula:

$$\left[\frac{Width\ of\ wound\ in\ sample\ (0\ h) - Width\ of\ wound\ remaining\ in\ sample\ (24\ h)}{Width\ of\ wound\ in\ control\ (0\ h) - Width\ of\ wound\ remaining\ in\ control\ (24\ h)}\right] x100$$

2.12. Creation of PDGFRα and PDGFRβ stable knockdown cell lines

Stable transfections are performed by transfecting cells with shRNA that incorporates with the host DNA to transcribe constitutively. In our study we stable transfected Daoy cells with Control (scrambled) shRNA, PDGFRα shRNA and PDGFRβ shRNA containing plasmids that were prepared using a pRNAT-CMV3.2/Neo vector from GenScript which included ampicillin resistance gene (for bacterial selection) and neomycin resistance gene (for mammalian cell line selection). ShRNA design consisted of the following parts: BamHI and XhoI restriction sites, sense sequence (specific to

either PDGFRα or PDGFRβ along with a scrambled sequence not complimentary to any genes acting as a Mock), a hair-pin loop sequence, an anti-sense sequence and a termination sequence.

The vector was initially restriction digested with BamHI (Invitrogen) and XhoI (Invitrogen) and the digested mixture was run on a 1.2% agarose gel. Interested band on the gel was excised off and DNA was extracted from it using the Qiagen gel extraction kit. The extracted DNA was then ligated with the shRNA using T4 DNA ligase (BioLabs), transformed into competent *E. coli* cells (One shot® Top 10 competent cells, Invitrogen) and plated on to the agar plate containing ampicillin (45 µg/ml). Plasmids were isolated from the bacterial culture using ChargeSwitch®- Pro plasmid maxiprep kit (Invitrogen) following the company's instructions. The plasmids were then introduced into Daoy cells by lipofectamine ltx (Invitrogen) transfection followed by neomycin selection (200 µg/ml). The knockdown of PDGFRα and PDGFRβ was confirmed by Western blot analysis.

2.13. miRNA profiling

miRNA profiling is an array performed on samples to analyze the differentially regulated miRNAs that are expressed as a heat map where green color represents low expression and red color represents high expression of a particular miRNA. In our study we used Control cells (harboring a control shRNA vector), PDGFRα^{KD}, PDGFRβ^{KD}, c-Myc^{KD} (using c-Myc specific siRNA), PDGFRβ^{KD}and c-Myc^{KD} double knockdown cells (using PDGFRβ^{KD} and c-Myc specific siRNA) and CD44^{KD} cells for miRNA profiling. The knockdown of all specific genes was confirmed using Western blotting analysis. Total RNA was isolated from control, PDGFRα^{KD}, PDGFRβ^{KD}, c-Myc^{KD}, PDGFRβ^{KD}

and c-Myc^{KD} double knockdown cells and CD44^{KD} using the miRCURY^{TM} RNA isolation kit (Exiqon) following the manufacturer's protocol. NanoDrop 1000 spectrophotometer (Thermo scientific) and agarose gel electrophoresis were used to assess the quality of the RNA isolated. The samples were labeled using the miRCURY LNA^{TM} microRNA Hi-Power Labeling kit Hy3^{TM}/Hy5^{TM} and hybridized on the miRCURY LNA^{TM} microRNA Array (6th Gen). Experiments were performed in duplicate. The quantified signals were normalized (background corrected) using the global Lowess regression algorithm and the analyzed data demonstrated differentially regulated miRNAs that are presented in the heat map.

2.14. miRNA validation

miRNA validation by TaqMan PCR using total RNA isolated from transiently transfected cells was performed to validate the heat map generated by miRNA profiling. In our study MB cells were treated with either PDGFRα, PDGFRβ, c-Myc, PDGFRβ and c-Myc, CD44 siRNA using scrambled siRNA as control for 24 h. PDGFRβ^{KD} Daoy cells were also transfected with increasing concentrations (30 nM, 60 nM, and 90 nM) of miR-1280 and miR-1260 specific inhibitors along with a negative inhibitor (60 nM) for 24 h. Total RNA was isolated using TRI reagent (Sigma) following manufacturer's protocol and NanoDrop 1000 spectrophotometer (Thermo Scientific) was used for RNA quantification. 1 µg of total RNA was used to prepare miRNA specific cDNA using TaqMan® microRNA reverse transcription kit (Applied Biosystems). In brief, primer pool was prepared by adding 10 µl of each miRNA, 5x RT primer and making the volume up to 1000 µl using 1X TE buffer. RT reaction mix was made by mixing the following components **(Table 10).**

3 µl of total RNA (1 µg) was added to each reaction to make the final volume to 15 µl. cDNA was prepared using the following parameters in a thermocycler – 30 min at 16°C, 30 min at 42°C and 5 min at 85°C after which the mixture was held at 4°C. The synthesized cDNA was then used to perform qRT-PCR using the following reaction mix to validate the regulated miRNAs in MB cells **(Table 11).**

Table 10. RT reaction mix for miRNA validation. The RT reaction mix showing the components with their volume taken for 1 sample.

Components	**Volume for 1 sample (µl)**
RT primer pool	6.0
dNTP (100 mM)	0.3
Multiscribe reverse transcriptase (50 U/µL)	3.0
RNase inhibitor (20 U/µl)	0.19
10x RT buffer	1.01
Nuclease-free water	1.50
Total	12

Table 11. RT-PCR mix for miRNA validation. The RT-PCR mix showing the components and the volume taken for 1 sample.

Component	**Volume for 1 sample (µl)**
20X TaqMan® MicroRNA assay	0.5
TaqMan Universal PCR master mix, no AmpErase UNG (Applied Biosystems)	5
Nuclease-free water	3.5
Total	9

1 µl of cDNA was added to make up the volume to 10 µl. RNU6B was chosen as an endogenous control. Experiments were performed in duplicates. PCR was performed using the following program shown in **(Table 12).** Fold change obtained from Ct values

(using $2^{-\Delta\Delta Ct}$ methodology) was converted into logarithmic base 2 for statistical analysis [340, 343]. miRNAs with P-value <0.05 was considered to be differentially expressed.

Table 12. The thermal cycle protocol for miRNA validation. The thermal cycle protocol showing the PCR program consisting of temperature, time, number of cycles and the cycle description.

Temp.	Time	Cycle	Description
95°C	10 min	1	Initial enzyme activation
95°C	15 sec	40	Denaturation
60°C	1 min		Annealing/extension

2.15. Chromatin Immuno-Precipitation – Polymerase Chain Reaction (ChIP-PCR) Assay

ChIP-PCR is a technique to analyze the physical interaction between a protein and a DNA by identifying the binding regions on the DNA. In our study Mock (scrambled shRNA), PDGFRα^{KD}, and PDGFRβ^{KD} (test samples) along with c-Myc positive HeLa and NFκB positive HEK 293 cells were plated the previous night in a 10 cm cell culture plate. The next day cells were induced with PDGF-BB (50 ng/ml) for 30 min and later cross-linked with 10 ml of 1% formaldehyde for 30 min at room temperature. The reaction was ceased by adding 1 ml of 1.37 M glycine and mixed immediately. The plates were then placed on ice and washed thrice with Buffer A **(Table 13)**. Cells were then

Table 13. Buffer components for ChIP-PCR. The buffer components for ChIP-PCR along with their volume in 50 ml stock solution.

Buffer A components	**Volume in 50ml stock (ml)**
1X PBS	50
Protein cocktail inhibitor	1 tablet
Buffer B components	**Volume in 50ml stock (ml)**
25 mM Hepes, pH7.8	1.25
50 mM $MgCl_2$	1.5
1 M KCl	0.5
0.1% Igepal CA-630	0.05
50 mM DTT	1
Distilled water	45.7
Protein cocktail inhibitor	1 tablet
Buffer C components	**Volume in 50ml stock (ml)**
1 M Hepes, pH7.9	2.5
140 mM NaCl	1.4
50 mM EDTA	1
20% Triton X-100	2.5
10% sodium deoxycholate	0.5
20% SDS	0.25
Distilled water	41.85
Protein cocktail inhibitor	1 tablet
Buffer D components	**Volume in 50ml stock (ml)**
50 mM Hepes, pH7.9	2.5
5 M NaCl	5
50 mM EDTA	1
20% Triton X-100	2.5
10% sodium deoxycholate	0.5
20% SDS	0.25
Distilled water	38.25
Protein cocktail inhibitor	1 tablet

Table 13. Buffer components for ChIP-PCR (continued).

Buffer E components	**Volume in 50ml stock (ml)**
50 mM Hepes, pH7.9	2.5
250 mM NaCl	2.5
50 mM EDTA	1
10% sodium deoxycholate	0.5
20% SDS	0.25
20% Triton X-100	2.5
Distilled water	40.75
Protein cocktail inhibitor	1 tablet
Elution buffer components	
1 M Tris, pH 8.0	2.5
50 mM EDTA	1
1% SDS	2.5
1 M sodium bicarbonate	2.5
Distilled water	41.5

trypsinized and the detached cells were harvested by centrifugation at 1000 rpm for 5 min at 4°C. The pellet ($2.5x10^6$ cells) was re-suspended in 500 µl of Buffer B **(Table 13)** and kept on ice for 10 min. The cells were dounced 10-15 times to release the nuclei. The released nuclei were then harvested by centrifuging at 2000 rpm for 5 min. The cells were re-suspended in 500 µl of Buffer C **(Table 13)**. Sonication was performed (using Branson Digital sonifier ®) at 30% amplitude for 10 sec each three times, making sure the sample was placed on ice at least for 1 min in between each sonication. The samples were then centrifuged at 14000 rpm for 15 min at 4°C. The supernatant was collected and pre-cleaned with Protein-A sepharose™ CL – 4B (GE Healthcare – 50 µl of the slurry (80 mg in Buffer C) per 500 µl of the lysate) with constant rotation for 1 h in a cold room. The samples were centrifuged at 2000 rpm for 5 min and the supernatant now containing

the pre-cleaned chromatin was collected. 50 µl aliquot of each sample was saved to serve as the Input DNA. To the rest of the sample 5 µl of primary antibody (c-Myc and NFκB – Cell signaling, control Rabbit IgG – Santa Cruz) was added and incubated in a cold room with constant rotation for overnight. Next day 50 µl of Protein-A sepharose slurry was added to each sample and incubated for 2 h with constant rotation in the cold room. Then, centrifugation was performed at 10000 rpm for 3 min. The beads were then washed twice each time with Buffer C, Buffer D, Buffer E **(Table 13)** and TE buffer**.** To the beads, 200 µl of Elution buffer **(Table 13)** was added and incubated at 65°C for 10 min. Centrifugation was performed at 14000 rpm for 1 min and the supernatant was collected. Beads were eluted again to obtain a total 400 µl of eluate. In parallel, the saved Input DNA was thawed and 350 µl of elution buffer was added to bring the total volume up to 400 µl. 16.5 µl of 5M NaCl was added to each tube and incubated at 65°C overnight for reverse-cross-linking. 2 µl of RNAse (Sigma) was added the next day and incubated at 37°C for 1 h. 4 µl of EDTA (0.5 M) and 2 µl of Proteinase K (Qiagen) was added and incubated at 42°C for 2 h. Post-incubation the DNA were extracted with chloroform/isoamyalcoholonce by centrifuging at 14000 rpm for 10 min. The aqueous phase was collected to which 40 µl of sodium-acetate (3M) and 1 ml of ethanol was added, vortexed and was incubated at -20°C overnight for precipitation. The samples were centrifuges at 14000 rpm for 30 min the next day and the pellet was washed once with 80% EtOH. The immuno-precipitate (IP) and the Input sample pellets were re-suspended in 50 µl of Tris, pH 8.5. The chromatin precipitates were then taken for qRT-PCR. The following primers were used for quantitative analysis **(Table 14).**

Table 14. ChIP-PCR gene specific primers. ChIP-PCR primers for IKBα (NFκB positive control), B23 gene (c-Myc positive control) and test primes for both NFκB and c-Myc along with their sequence.

Positive control primers: NFκB (IkBα gene promoter)	
CHIP-NFκB-primer - F	5'- GGA CCC CAA ACC AAA ATC G -3'
CHIP-NFκB primer - R	5'- TCA GGC GCG GGG AAT TTC C -3'
Positive control primers: c-Myc (B23 gene promoter)	
CHIP-c-Myc primer - F	5'- GCT ACA TCC GGG ACT CAC C -3'
CHIP-c-Myc primer - R	5'- GCT GCC ATC ACA GTA CAT GC -3'
Test primers	
CHIP-c-Myc-primer - F	5'- CCC TCC GTC TTA GGT CAC TG -3'
CHIP-c-Myc-primer - R	5'- TGC CAC CAA AAC TTG TCC AT -3'
CHIP-NFκB-primer - F	5'- AGA GAG GTG CCC ATT CAC AC -3'
CHIP-NFκB-primer - R	5'- TCC AAG TGG AAA GAG GGA GA -3'

The qRT-PCR was performed using the following program shown in (**Table 15).**

Experiment was performed in triplicates. Fold change obtained from Ct values (using $2^{-\Delta\Delta Ct}$ methodology) was converted into logarithmic base 2 for statistical analysis [340, 343]. Ct values with P-value <0.05 was considered to be significant.

Table 15. The thermal cycle protocol for ChIP-PCR. The thermal cycle protocol showing the PCR program consisting of temperature, time, number of cycles and the cycle description.

Temp.	Time	Cycle	Description
50°C	2 min	1	Uracil DNA glycosylase (UDG) incubation
95°C	10 min	1	UDG inactivation and Initial enzyme activation
95°C	15 sec	40	Denaturation
60°C	1 min		Annealing/extension

2.16. Statistical analysis

Each experiment was repeated at least three times. Data is presented as mean values ± standard deviation. Differences between 2 groups were analyzed using Student's t- test. $P < 0.05$ was considered statistically significant.

CHAPTER 3. TO DETERMINE DIFFERENTIAL ROLES OF PDGFRα AND PDGFRβ IN MB

3.1. Introduction

MB is one of the most common malignant brain tumors in children with an overall 5-year survival of just ~60% [5, 344]. Several studies have demonstrated that overexpression or over-activation of PDGFRs and c-Myc in the tumor tissues of patients with MB is associated with poor prognosis [117, 118, 345]. Inhibition of PDGFR signaling by siRNA or inhibitors such as cambogin, Suntinib, and Imatinib, induced cell death and limited cell migration/invasion in MB cells [115, 116, 214]. However, the specific molecular mechanisms that regulate PDGFRα and PDGFRβ directing them to distinct signaling pathways and controling MB development are yet to be fully understood.

The objective of this study was to elucidate distinct roles of PDGFRα and PDGFRβ in regulating MB metastasis. We hypothesized that individual PDGF receptors regulate specific downstream molecules and miRNAs to induce different functions in MB. To delegate specific roles of PDGFRα or β, RNA interference approach was used to knockdown PDGFRα or β specifically in MB cells using gene specific siRNAs and then subjected for function studies. Changes in expression of downstream proteins in PDGFRα or β specific pathway were evaluated by Western blot analysis. miRNA profiling was also performed to identify miRNAs differentially regulated by PDGFRs. Hence, this study aimed to identify certain molecules and possible miRNA targets which are involved in PDGFR signaling modulating MB metastasis that leads to new insight into MB therapy.

3.2. Results and discussions

3.2.1. Determining the baseline expression of PDGFRα and PDGFRβ in MB cell

To elucidate the role of PDGFRα and PDGFRβ in regulating MB metastasis, we used two cell lines, Daoy (metastatic) and D283 (uncertain for metastasis), as *in vitro* model systems for our experiments. Although D283 cells were isolated from a metastatic site, expression profiling by MacDonald *et al*. assigned this cell line as "uncertain" for metastasis as it did not consist of predictor genes analyzed for MB metastasis [118]. Primarily, we determined the baseline expression of PDGFRα and PDGFRβ in both these cell lines by Western blot analysis. Proteins from equal number of cells from both cell lines were extracted and their concentration was estimated using the BSA assay (Pierce). 40 µg of proteins from each cell line were used for the SDS-PAGE. Immunoblotting results showed that metastatic Daoy cells expressed both PDGFRα and PDGFRβ ten-folds higher than uncertain for metastasis D283 cells when normalized with the expression of β-actin **(Figure 10)**. This differential expression of PDGFRs in metastatic (Daoy) and uncertain for metastasis (D283) cell lines indicated that PDGFRs might play a crucial role in MB metastasis.

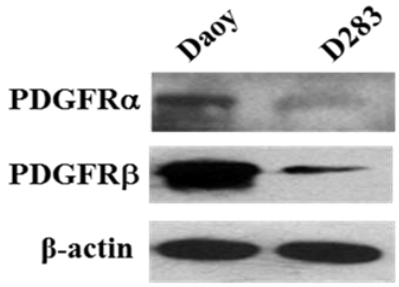

Figure 10. Baseline expression of PDGFRα and PDGFRβ in MB cells. Proteins extracted were analyzed by Western blot analysis and normalized by comparing with the expression of β-actin.

3.2.2. Knockdown confirmation of PDGFRα and PDGFRβ in MB cells

Till date, the individual role of PDGFRα and PDGFRβ in MB is not clearly understood. To elucidate this, we used gene specific siRNAs to specifically knockdown either PDGFRα or PDGFRβ in both MB cell lines. The transient knockdown was confirmed by Western blot analysis as shown in **(Figure 11)**.

A significant knockdown of PDGFRα in PDGFRα siRNA treated cells and PDGFRβ in PDGFRβ siRNA treated cells was observed in both MB cells. However, partial gene knockdown was obtained in D283 when compared to Daoy cells. We reason that, as D283 are largely suspension cells the rate of transfection in these cells was not very efficient. β-actin was used as the loading control.

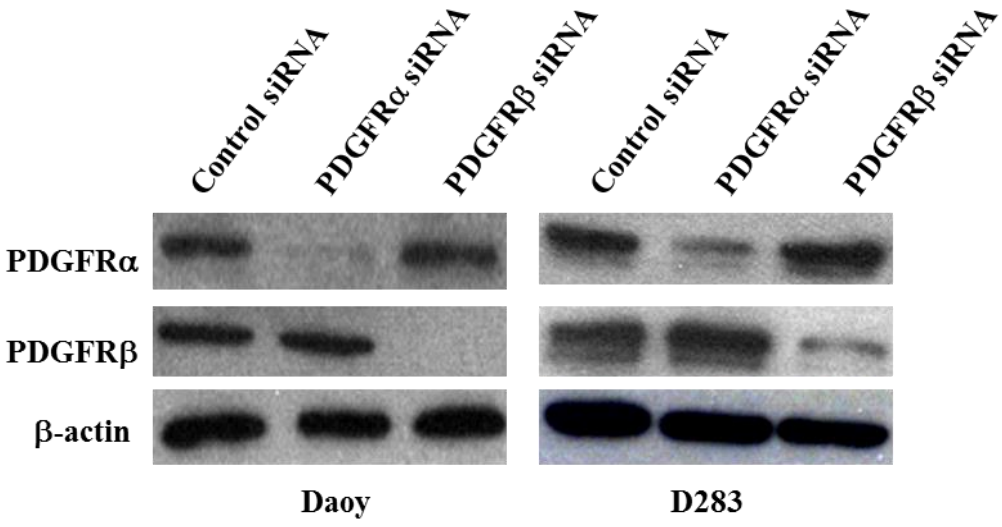

Figure 11. Knockdown confirmation of PDGFRα and PDGFRβ in MB cell lines. Proteins extracted from PDGFRα and PDGFRβ knockdown cells by using specific siRNAs after 48 h transfection in both Daoy and D283 were analyzed by Western blot analysis and normalized by comparing with the house keeping gene β-actin.

3.2.3. Determining the roles of individual PDGFRs on MB cell proliferation

Specifically knock down PDGFR in MB cells were then subjected to cell proliferation assay to analyze the effect of individual receptors on MB cell proliferation. MTS assay demonstrated that PDGFRα and PDGFRβ differentially regulated MB cell proliferation. PDGFRα siRNA treated cells demonstrated an increase (~20%) while PDGFRβ siRNA treated cells demonstrated a marked decrease (~30%) in MB cell proliferation when compared to control cells treated with scrambled siRNA **(Figure 12)**. Even though the results do not show drastic effects, they clearly indicated that in the presence of PDGFRβ (PDGFRα siRNA treated cells) and in the presence of PDGFRα (PDGFRβ siRNA treated cells) MB cells demonstrated completely different functions from each other. This result also indicated that individual PDGFRs might trigger different pathway regulating the expression of different downstream molecules.

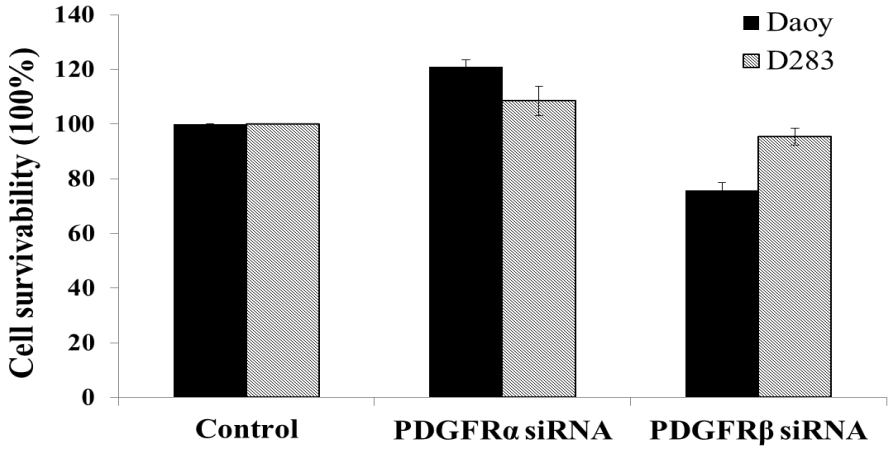

Figure 12. Effect of PDGFRα and PDGFRβ on MB cell proliferation. PDGFRα and PDGFRβ specifically knocked down in Daoy and D283 MB cells by siRNAs were analyzed for cell proliferation by MTS assay after 48 h transfection and presented as percentage cell survivability,**$p<0.01$, *$p<0.05$ compared to control group.

A significant difference in cell proliferation was observed in both Daoy and D283 cells as shown in **(Figure 12)**.

3.2.4. Determining the role of individual PDGFRs on MB cell death

To further confirm the above obtained results, specifically knocked down PDGFR cells were subjected to cell death assay with the anticipation of obtaining a contrary result. The assay provided an estimation of the amount of Lactate Dehydrogenase (LDH) released from the dead cells into the heat-inactivated medium from both the MB cell lines treated with either PDGFRα or PDGFRβ specific siRNAs. The results, as shown in **(Figure 13)** clearly demonstrated that a significant increase, if not dramatic, in fold change of LDH was obtained from PDGFRβ knockdown cells when compared to control cells. This was because of higher cell death of MB cells in the absence of PDGFRβ in both cell lines. Contrary to this, a significant decrease in fold change in the amount of released LDH was obtained in PDGFRα knockdown cells when compared to control cells due to the fact that PDGFRβ present in those cells induced MB cell proliferation.

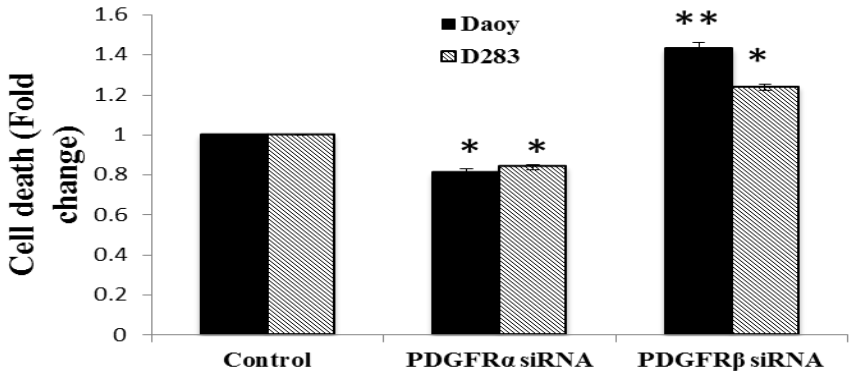

Figure 13. Effect of PDGFRα and PDGFRβ on MB cell death. PDGFRα and PDGFRβ specifically knocked down in Daoy and D283 MB cells by siRNAs were analyzed for cell proliferation by MTS assay after 48 h transfection and presented as percentage cell death,**$p<0.01$, *$p<0.05$ compared to control group.

This result further reinforced the notion that PDGFRα and PDGFRβ play different and contrary roles in MB and demonstrated that PDGFRβ assists cell proliferation while PDGFRα has opposite effects on MB cells *in vitro*.

3.2.5. Determining the role of individual PDGFRs on MB cell invasion

PDGFRα and PDGFRβ demonstrated opposite effects on MB cell invasion. To further analyze the role of individual receptors on MB cell invasion, we subjected the metastatic Daoy cells for invasion assay. Annie P. Moseman in our lab performed the following experiment. Two methods were applied to determine the roles of PDGFRα and PDGFRβ on MB cell invasion: 1) by blocking PDGFR signaling using PDGFRα or PDGFRβ specific neutralizing antibodies against a control antibody; and 2) by knocking down PDGFRs using PDGFRα or PDGFRβ specific siRNAs against a control siRNA. The treated cells were then used to analyze for cell invasion abilities with the aid of cell invasion chamber assay.

A significant decrease in MB cell invasion was observed in PDGFRβ knockdown/blocked metastatic Daoy cells. Both approaches demonstrated that PDGFRα knockdown/blockage promoted, whereas PDGFRβ knockdown/blockage inhibited cell invasion in Daoy cells **(Figure 14)**. Similar trends induced by individual PDGFRs were observed in both the methods adopted. From the above experiments we can conceive that PDGFRα and PDGFRβ not only play differential roles in MB cell proliferation and cell death but in cell invasion too; PDGFRβ assists cell proliferation and cell invasion, while PDGFRα has contradictory effects in MB.

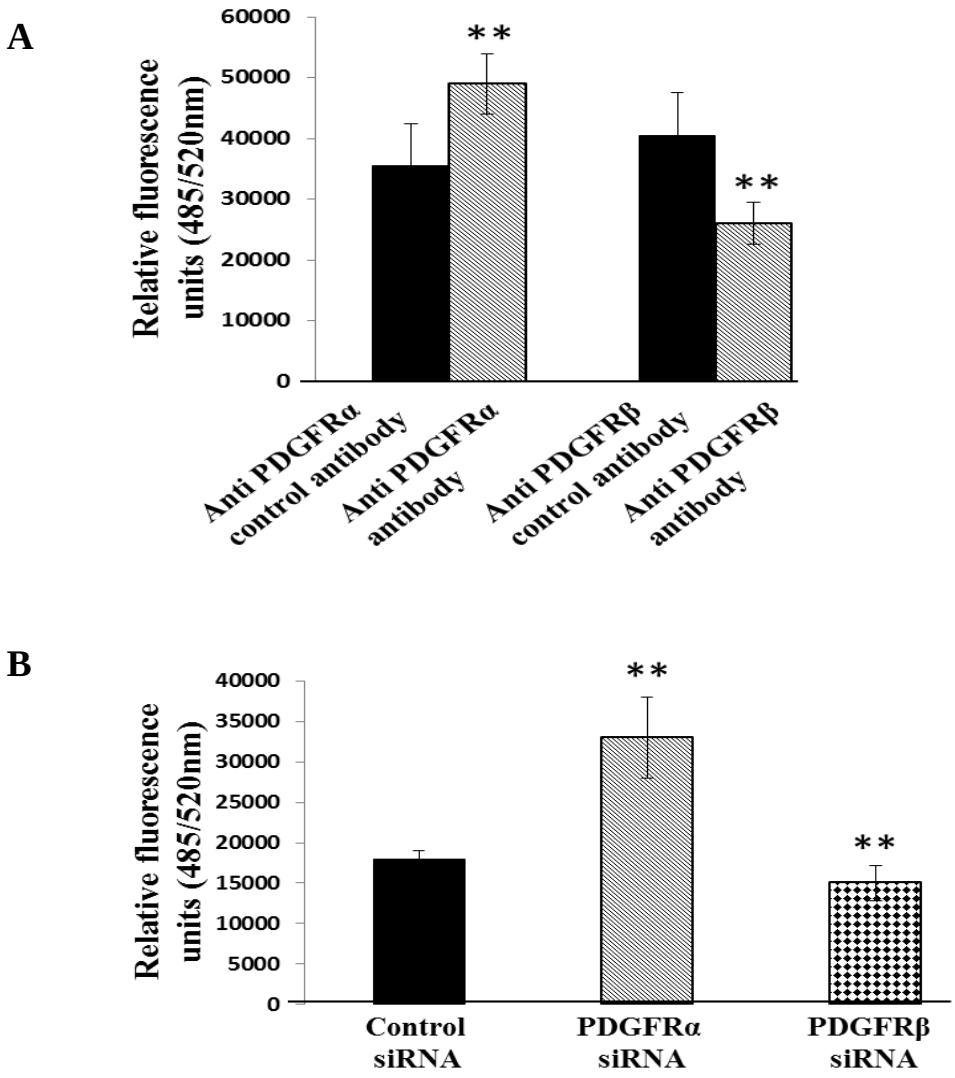

Figure 14. Effect of PDGFRα and PDGFRβ on MB cell invasion by A) using anti-human PDGFRα mouse neutralizing antibody and anti-human PDGFRβ goat neutralizing antibody along with their respective control antibodies B) using PDGFRα/β gene specific siRNAs with control siRNA after 48 h transfection. Cell invasion chamber analysis if cell invasion *in vitro* **$p<0.01$ compared to respective controls.

3.2.6. Determining specific downstream molecules regulated by individual PDGFRs in MB

Differential roles exhibited by PDGFRα and PDGFRβ in MB gave us the insight that these receptors might trigger different signal pathways that regulate specific downstream

molecules, consequently performing distinct functions in MB. Western blot analysis on certain proteins such as c-Myc, CD44 and PKCα extracted from PDGFRα or PDGFRβ siRNA treated cells demonstrated that the expression of these proteins were down-regulated in PDGFRβ knockdown cells alone, while no change in expression was observed in PDGFRα knockdown cells. This data clearly suggested that PDGFRα and PDGFRβ could possibly activate diverse pathways in MB.

Another important observation from this study was that a significant level of CD44 expression was observed in metastatic Daoy cells, whereas an undetectable level of CD44 was obtained in uncertain for metastasis D283 cells. This result highlighted the possible mechanism that might provide MB cells with invasive abilities for metastasis; which is PDGFRβ, not PDGFRα, regulating metastasis via modulating the expression of CD44. This data suggested that CD44 along with molecules like c-Myc and PKCα, which are regulated by PDGFRβ pathway alone might induce cell invasion causing MB metastasis **(Figure 15)**.

3.2.7. Determining NFκB activity regulated by individual PDGFRs in MB

To further identify if NFκB is selectively activated by either PDGFRα or PDGFRβ, we performed the NFκB luciferase assay. A co-transfection of either PDGFRα/β siRNA along with a plasmid containing the luciferase reporter gene (Promega) was performed. After 48 h transfection, the cells were assayed for NFκB activation using the ONE-Glo™ Luciferase Assay System (Promega). The results depicted in **(Figure 16)** clearly demonstrated that PDGFRβ siRNA significantly down-regulated the NFκB activity in the MB cells co-transfected with NFκB luciferase plasmid, while no change was observed in

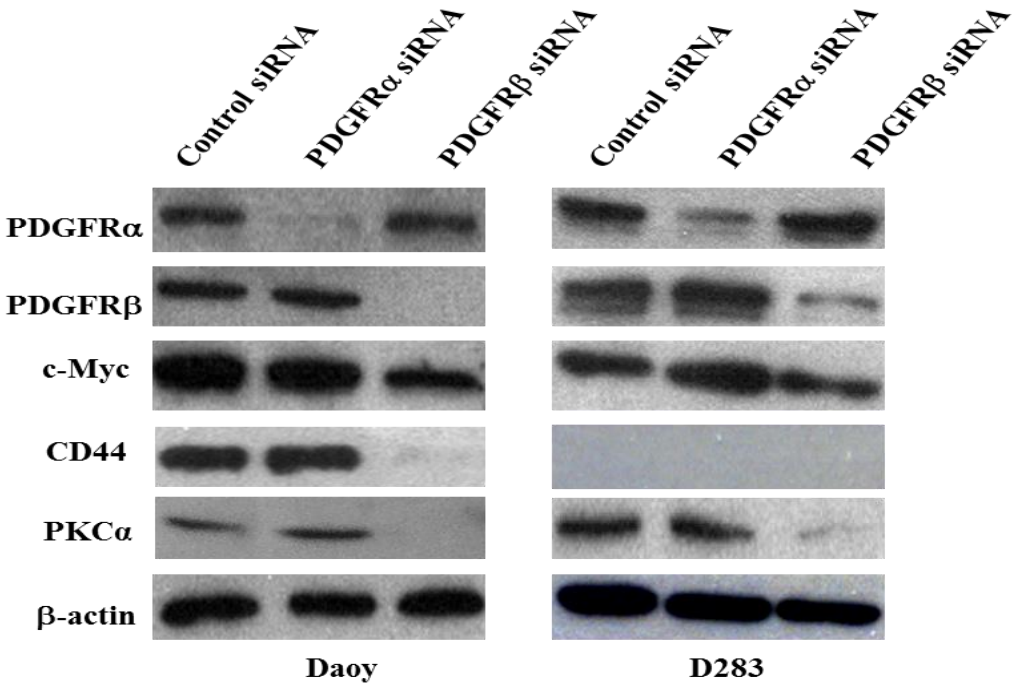

Figure 15. Effect of PDGFRα and PDGFRβ on downstream molecules. Protein extracts from PDGFRα and PDGFRβ specific knock down in both Daoy and D283 were analyzed for downstream target molecules such as c-Myc, CD44 and PKCα after 48 h transfection using β-actin as the internal loading control.

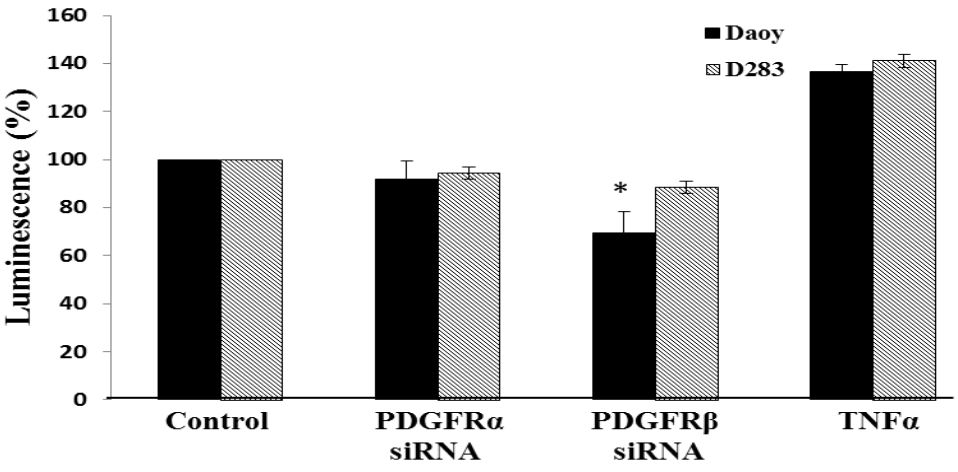

Figure 16. Effect of PDGFRα and PDGFRβ on NFκB activity. Daoy and D283 cells co-transfected with PDGFRα or PDGFRβ siRNA and NFκB response element reporter plasmid, pGL4.32 [luc2P/NFκB-RE], were monitored for luciferase activity after 48 h transfection and presented as percentage luminescence, *$p<0.05$ when compared to control cells. TNFα induced cells were taken as positive control.

MB cells treated with PDGFRα siRNA. A significant change in NFκB activity was observed in the Daoy cells, while non-significant but definite reduction was observed in D283 cells. We reason that the high expression of c-Myc downstream of PDGFRβ masks the effect of PDGFRβ knockdown on NFκB activity. TNFα induced MB cells were taken as the positive control. This data showed that only PDGFRβ and not PDGFRα regulate NFκB activity in MB cells.

From all the above data we can conclude that PDGFRα and PDGFRβ have discrete pathways and regulate distinct downstream molecules in MB. PDGFRβ specifically regulates the expression of c-Myc, CD44, PKCα, and NFκB activity in MB cells suggesting that PDGFRβ controls MB cell proliferation and invasion via these molecules. Also, further analysis of PDGFRβ-CD44 axis by determining the physical interaction between the two molecules, also by studying the role of PKCα might provide compelling result on limiting MB progression.

3.2.8. Determining the effect of c-Myc on CD44 expression in MB cells

Our result demonstrated that c-Myc regulates CD44 expression in MB. Our preliminary results demonstrated a PDGFRβ siRNA specific down regulation of c-Myc. To further elucidate the effect of c-Myc on other molecules like CD44 and also to identify the position of c-Myc in PDGFRβ-specific pathway in MB, we subjected MB cells to different treatments – control siRNA, PDGFRα siRNA, and PDGFRβ siRNA in the presence or absence of c-Myc plasmid along with single treatments of c-Myc siRNA and c-Myc plasmid.

As expected, from **(Figure 17)** we observed a significant down-regulation of c-Myc expression in the PDGFRβ knockdown cells. Interestingly with the overexpression of c-

Myc, we observed a proportional increase in CD44 expression. We also observed that, overexpression of c-Myc masked the effects of PDGFRα and PDGFRβ specific siRNAs on its target genes, indicating that PDGFRβ and c-Myc regulate each other in MB. Hence, in PDGFRβ specific pathway, c-Myc plays the role of an intermediary molecule between PDGFRβ and CD44 in MB.

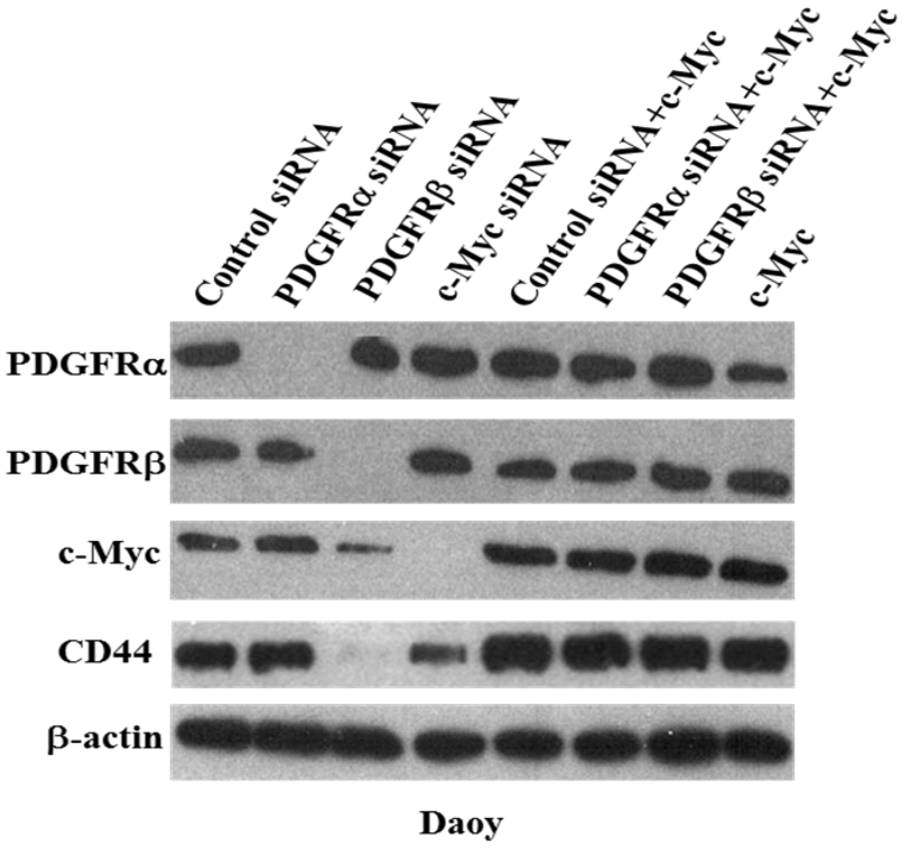

Figure 17. Effect of c-Myc on CD44 expression in MB cells. Proteins analysis from control siRNA, PDGFRα siRNA, PDGFRβ siRNA in presence or absence of c-Myc plasmid, c-Myc siRNA alone and c-Myc plasmid alone in Daoy cells after 48 h transfection by Western blotting. β-actin was taken as the loading control.

Notably, CD44 expression in c-Myc siRNA singly treated cells when compared to PDGFRβ siRNA singly treated cells where a complete abolishment of CD44 expression was observed, only a partial down-regulation was obtained indicating that both c-Myc

and PDGFRβ regulate CD44 expression in MB. MB cells treated with c-Myc plasmid alone served as a positive control.

3.2.9. Determining the effect of c-Myc on NFκB activity in MB cells

Our previous result demonstrated that c-Myc expression and NFκB activity was selectively regulated by PDGFRβ alone in MB cells. To further verify the relationship between PDGFRβ, c-Myc and NFκB, we either knocked down c-Myc alone or overexpressed c-Myc in the presence or absence of PDGFRβ siRNA, co-transfecting it with NFκB response element reporter plasmid, pGL4.32 [luc2P/NFκB-RE], to investigate its effect on NFκB activity in MB cells. TNFα induced MB cells acted as the positive control.

As demonstrated earlier, PDGFRβ siRNA down-regulated the NFκB activity in MB cells. However, with overexpression of c-Myc, we observed that NFκB activity in PDGFRβ knocked down cells was significantly restored. We also noticed a significant down-regulation of NFκB activity in the c-Myc siRNA treated cells, indicating that c-Myc regulates the expression of NFκB activity and that it is found upstream of NFκB in the PDGFRβ pathway in MB cells **(Figure 18)**. Notably, no change in NFκB activity observed in MB cells treated with c-Myc plasmid alone was also considered to be significant because of the overexpressed c-Myc in MB cells. We reason that overexpression of c-Myc in the already overexpressed environment becomes redundant and hence does not drastically modulate the NFκB activity.

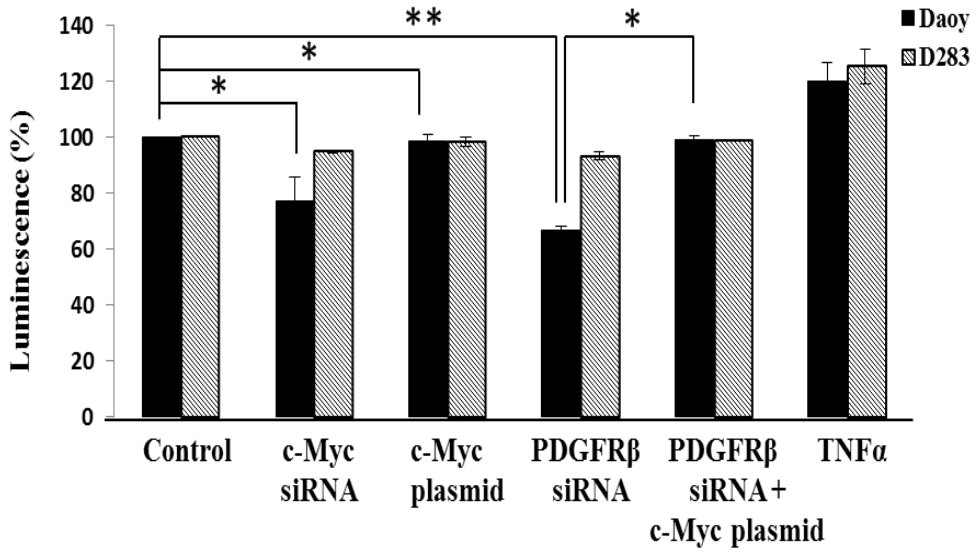

Figure 18. Effect of c-Myc on NFκB activity in MB cells. Luciferase assay was performed on Daoy cells co-transfected with control siRNA, c-Myc siRNA, c-Myc plasmid, PDGFRβ siRNA in the presence or absence of c-Myc plasmid after 48 h co-transfection and presented as percentage luminescence, *$p<0.05$, **$p<0.01$, when compared to control cells. TNFα induced cells were taken as the positive control.

From the above two figures **(Figure 16, Figure 17)** we can conclude that c-Myc acts as a transitional molecule in the PDGFRβ pathway, transmitting signal from PDGFRβ to either CD44 or NFκB in MB.

3.2.10. Stable knockdown of PDGFRα and PDGFRβ in Daoy cells

Studies performed thus far - cell proliferation, cell death, cell invasion, regulated downstream molecules, have all provided us with compelling results suggesting that PDGFRα and PDGFRβ have different functional roles in MB. To further decipher the mechanism and the molecules in the pathway that destine PDGFRα and PDGFRβ to direct MB cells in opposing directions, we designed PDGFRα and PDGFRβ specific shRNAs to generate $PDGFR\alpha^{KD}$ and $PDGFR\beta^{KD}$ Daoy cell lines. Mock cell line

consisting of scrambled shRNA non-specific to any genes was used as the control. The stable knockdown was confirmed by Western blot analysis. β-actin was used as the loading control **(Figure 19)**.

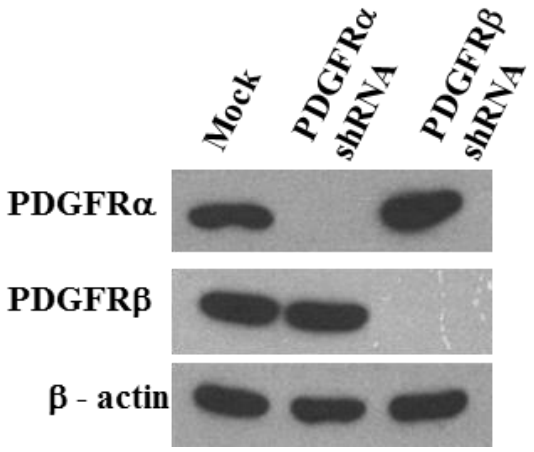

Figure 19. Stable knock down of PDGFRα and PDGFRβ in Daoy cells. Protein analysis from Daoy cells transfected with shRNAs specific to PDGFRα and PDGFRβ cells by Western blotting using β-actin as the house keeping gene.

3.2.11. Heat map presenting differentially regulated miRNAs

Heat map generated using the total RNA extracted from the either Mock, PDGFRα^{KD} or PDGFRβ^{KD} cells revealed ~30 differentially regulated miRNAs. The samples were prepared in duplicates. The conflicting functional roles of PDGFRα and PDGFRβ in MB cells indicated the existence of certain molecules, such as miRNAs, in the pathway that are differentially expressed which enforce PDGFRα and PDGFRβ signals to perform distinctly from each other. This led us to investigate the miRNA profiles of each stable knock down Daoy cell lines to further intricately analyze the differences persisting in

PDGFRα and PDGFRβ signal pathway in MB cells by generating the heatmap **(Figure 20)**.

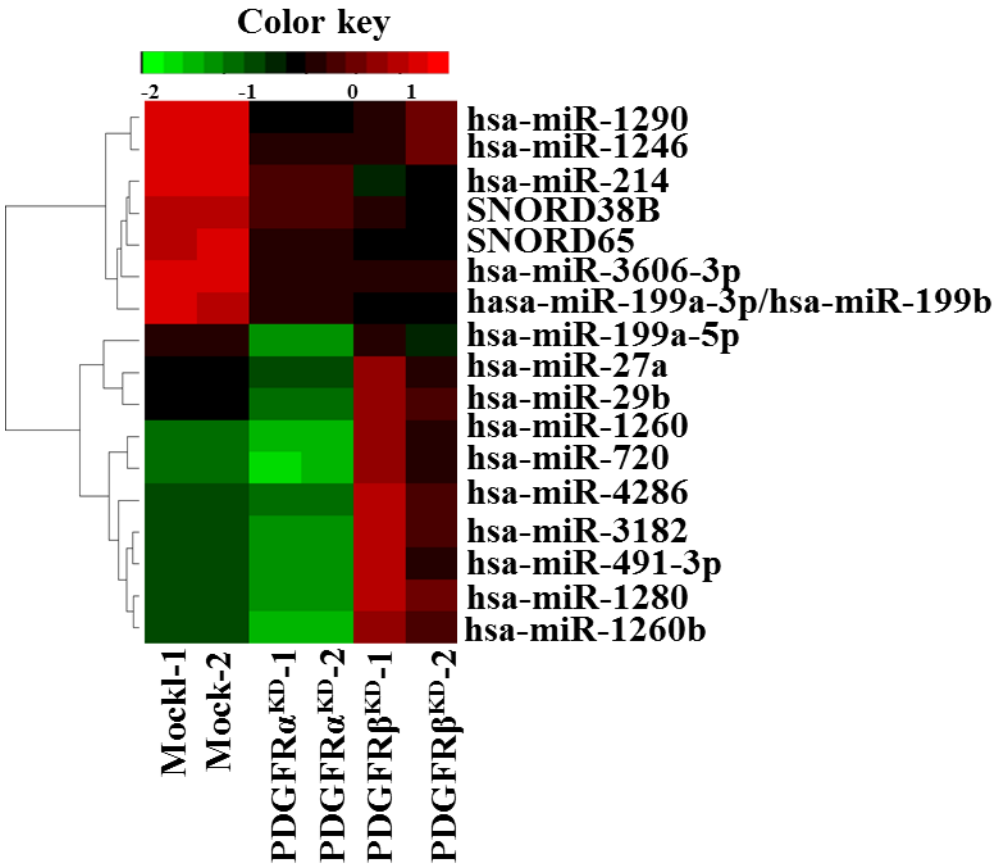

Figure 20. Differentially expressed miRNAs in PDGFRα and PDGFRβ stably knocked down Daoy cells. Total RNAs extracted from samples were subjected to miRNA profiling using 6th Gen miRNA array from Exiqon. The samples were labeled using the miRCURY LNA™ microRNA array. The normalized log ratio values obtained from the ImaGene®/Nexus™ microarray analysis software were used for analysis. The heat map diagram generated using the clustering algorithms show the result of a one-way hierarchical clustering of miRNAs and samples. Each row represents a miRNA and each column represents a sample. The color scale illustrates the relative expression level of miRNAs. Green color represents an expression level below the reference channel, and red color represents expression higher than the reference.

Heat map generated, as shown in **(Figure 20)**, clearly exhibits that certain miRNAs, such as miR-1280, -1260, -193a-3p, -491-3p, - 720, -4286, are differentially expressed by

PDGFRα and PDGFRβ. Expression pattern demonstrates that most of the miRNAs are under-expressed in PDGFRα^{KD} while they are overexpressed in PDGFRβ^{KD} when compared to Mock profile.

3.2.12. miRNA validation in MB cells

Two miRNAs, miR-1280, and -1260, were chosen to represent the miRNA profile generated from PDGFRα and PDGFRβ stable knock down Daoy cell lines. Initially, total RNA isolated from Daoy and D283 cells transiently transfected with PDGFRα and PDGFRβ specific siRNA, using scrambled siRNA as control, were subjected to TaqMan qRT-PCR for validation. Results demonstrated that similar expression patterns of the chosen miRNAs were obtained in both transiently transfected Daoy and D283 cell lines when compared with heat map profile obtained from stably knocked down Daoy cell lines, thus validating the expression patterns obtained from PDGFRα^{KD} and PDGFRβ^{KD} profiles in MB **(Figure 21)**.

All these data provided us with definite indications that indeed PDGFRα and PDGFRβ trigger disparate pathways in MB via regulating specific downstream molecules and miRNAs, assigning themselves with distinct functional roles. However, the exact mechanism by which PDGFRβ, and not PDGFRα, aids in MB cell proliferation and invasion is still unclear.

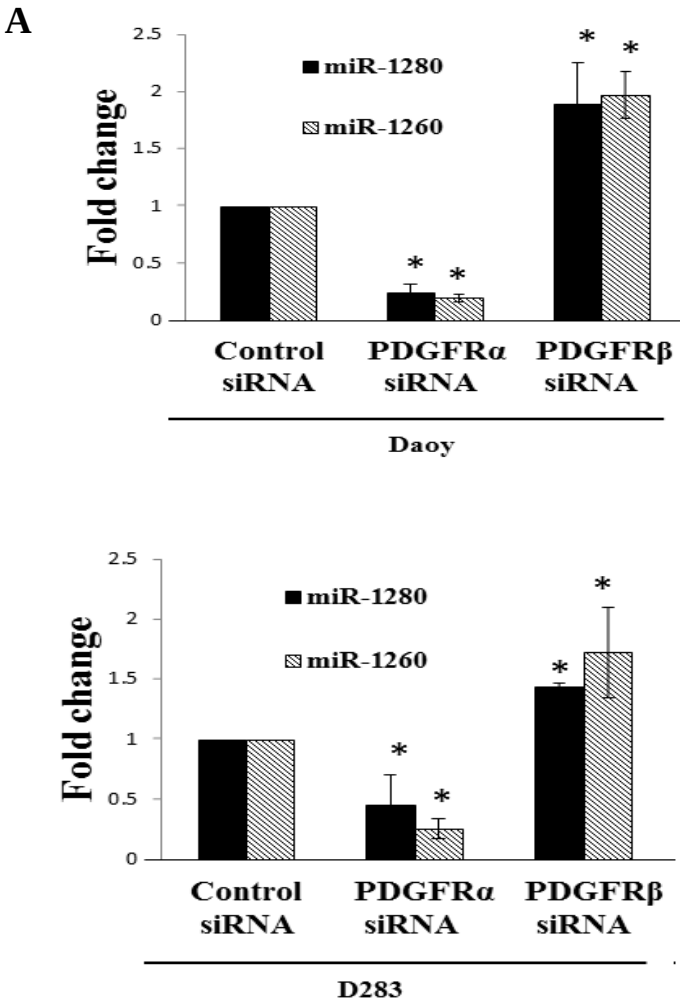

Figure 21. Validation of differentially expressed miRNAs in PDGFRα and PDGFRβ transiently transfected MB cells. The expression analysis of miR-1280 and miR-1260 in A) Daoy and B) D283 cells by real-time PCR and presented as fold change, *p<0.05 when compared to control.

3.3. Discussion and conclusion

In this study, we demonstrated that PDGFRβ signaling promotes while PDGFRα suppresses MB cell proliferation and migration/invasion via the differential regulation of their downstream targets **(Figure 12, Figure 13, Figure 14, and Figure 15)**. Recent studies have confirmed that PDGFRβ could play a vital role in MB development *in vitro* [115, 116]; however, the exact role of PDGFRα in MB metastasis remains unclear. In this study, we demonstrated that PDGFRα and PDGFRβ have distinct functions in MB cells

via differentially regulating their downstream targets and that PDGFRβ, not PDGFRα, plays a dominant role in the control of metastasis in MB. These results partially agree with previous findings [115, 116]. Furthermore, we present evidence supporting the notion that the expression of CD44 is required for PDGFRβ regulating MB metastasis, and that the PDGFRβ-CD44 regulatory axis, with participation of c-Myc is required for the control of metastasis in MB **(Figure 15, Figure 17)**.

Structure-function analysis shows that although the two PDGFRs have 70% homology in the N termini and 80% in the C termini [295], distinct differences exist in their ligand binding domain (31% identical) and in a sub-domain located at the c-terminal region (a 27-28% homology). These features presumably allow the two receptors to display different ligand affinities and/or to interact with different target protein sets to mediate distinct functions *in vivo* and *in vitro* [217, 295, 346, 347]. To further elucidate the differential functional roles of PDGFRα and PDGFRβ in MB cells, stable knockdown cells were generated using PDGFRα and PDGFRβ specific shRNAs using mock, non-specific to any genes, as the control **(Figure 19)**. miRNA profiling of these cells revealed a set of differentially regulated miRNAs **(Figure 20)**. To verify the heat map obtained, two miRNAs, miR-1280 and -1260, were chosen randomly and its expression in PDGFRα and PDGFRβ transiently knockdown MB cells were analyzed by RT-PCR **(Figure 21)**.

In this study, we have shown that PDGFRα and PDGFRβ play distinct roles in cell proliferation, survival, and migration/invasion in MB cells, with PDGFRα limiting and PDGFRβ promoting cell proliferation and migration/invasion. The disparate cellular outcomes support the importance of the inherent domains and the different interacting

protein partners that may be recruited to PDGFRα and PDGFRβ c-termini. Our results suggest that interference with PDGFRβ and/or its downstream targets may offer novel strategies for metastatic MB therapy.

CHAPTER 4. TO DETERMINE THE MECHANISM OF PDGFRβ-CD44 AXIS REGULATNG INVASION IN METASTATIC MB

4.1. Introduction

Our study so far has shown that PDGFRβ specifically regulates CD44, unlike PDGFRα **(Figure 17)**. Our study also showed that high expression of CD44 expression was found only in metastatic Daoy MB while no detectable levels of CD44 were found in uncertain for metastasis D283 MB cells [348]. From our preliminary data we hypothesize that PDGFRβ-CD44 might provide a potential regulatory axis that modulates MB metastasis. CD44 is cell surface adhesive protein that functions as a transmembrane receptor molecule for all the components making up the extracellular matrix (ECM) such as hyaluronic acid (HA) [349]. It undergoes alternative splicing between exon five and ten to give rise to various CD44 variants (CD44v) [350, 351], of which CD44 standard (CD44s) is the smallest variant of CD44 (80kDa) that has been shown to be highly expressed in MB cells [352, 353]. Earlier studies have demonstrated the importance of CD44 in disease metastasis such as breast cancer, prostate cancer and non-Hodgkin's lymphoma [354-357]. CD44 expression patterns in various primary brain tumors and brain metastasis have been evaluated [353]; However, till date, no study has been done to enumerate the importance of CD44 in MB metastasis. Hence, to evaluate the role of PDGFRβ-CD44 axis on MB metastasis, we plan to either knockdown CD44 in metastatic MB cells or overexpress CD44 in uncertain for metastasis MB cells and then evaluate their invasive abilities by performing cell invasion chamber assay.

4.2. Results and discussions

4.2.1. Baseline expression of CD44 in MB

Detectable levels of CD44 were observed only in Daoy and not in D283 cells. Two MB cell lines were chosen to determine the role of PDGFRβ-CD44 axis on MB metastasis – Daoy (metastatic) and D283 (uncertain for metastasis). Initially, the baseline expression of CD44 in both cell lines was analyzed by performing Western blotting. β-actin was used as the internal control **(Figure 22)**.

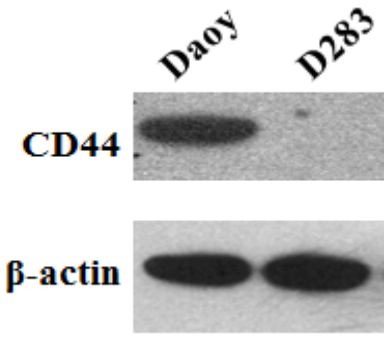

Figure 22. Baseline expression of CD44 in MB cells. Proteins extracted were analyzed by Western blot analysis and normalized by comparing with the expression of β-actin.

4.2.2. Determining the role of CD44 in MB metastasis

CD44 plays a crucial role in MB metastasis. Our earlier results demonstrated that detectable levels of CD44 were observed only in metastatic Daoy cells and not in uncertain for metastasis D283 cells and also that knockdown of PDGFRβ resulted in almost complete abolishment of CD44 in MB. To further verify the notion that PDGFRβ-CD44 axis drives metastasis in MB, we either knocked down PDGFRβ or CD44 in Daoy cells using gene specific siRNAs or overexpressed CD44 in D283 cells using cDNA

retroviral transfection. Overexpression and knockdown of CD44 was confirmed by Western blotting using β-actin as the loading control. These cells were then subjected to cell invasion chamber assay for 24 h to investigate the effect of CD44 in MB invasion.

We observed that in both PDGFRβ and CD44 knockdown cells, invasion of MB cells reduced drastically when compared to control cells treated with control siRNA. Similarly, a significant increase in invasive ability was observed in CD44 overexpressed D283 cells when compared to Wild type D283 and control retroviral cDNA **(Figure 23)**. These results clearly indicated that PDGFRβ via CD44 regulated metastasis in MB.

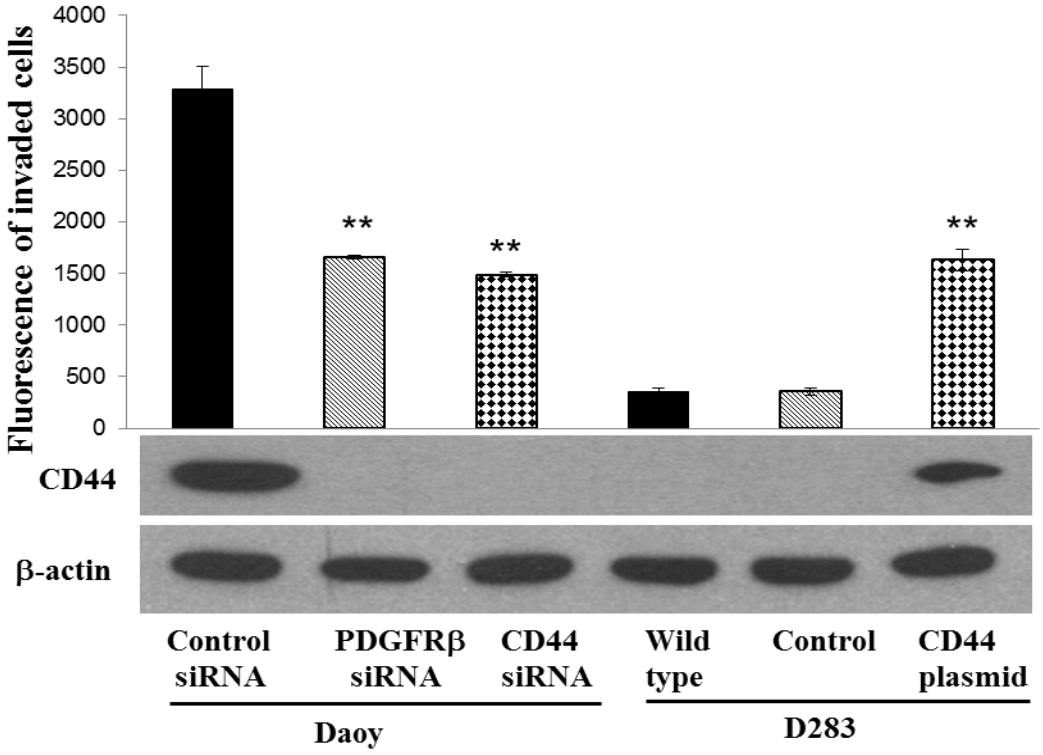

Figure 23. Effect of CD44 on MB metastasis. Protein analysis of Daoy cells transfected with control siRNA, PDGFRβ siRNA and CD44 siRNA along with D283 cells Wild type, retro-viral cell transfection with either control cDNA or CD44 cDNA after 48 h transfection by Western blotting. These cells were then subjected to cell invasion chamber assay *in vitro*, **$p<0.01$ when compared to their respective controls.

A complete reduction in invasion of CD44 knockdown Daoy cells or a complete attainment of invasive ability in the CD44 overexpressed D283 cells was not observed from the results obtained from the invasion assay. This suggests that there might be other molecules in this pathway that might also have a role to play in MB progression. The above outcome also demonstrates that PDGFRβ alone lacks the ability to control MB metastasis but can do so via regulating CD44 expression.

4.2.3. PDGFRβ-CD44 axis regulates MB progression via c-Myc

Our results so far demonstrated that CD44 is a mediating molecular coupler in the PDGFRβ signaling pathway that functionally integrates the events elicited by PDGFRβ and that both molecules play an active role in regulating invasion activity in MB cells. In addition, the observation that exposure to c-Myc siRNA reduced the NF-κB activities and the expression of CD44 in Daoy cells, while the overexpression of c-Myc restored the effect of PDGFRβ siRNA on the NF-κB activities and CD44 expression **(Figure 16, Figure 18)** suggesting that an integral link exists between the expression of c-Myc and CD44 in MB cells. These data suggest that the expression of c-Myc is required for PDGFRβ signaling to activate the NF-κB pathway and to control CD44s expression. To further determine whether PDGFRβ stimulating CD44 expression requires c-Myc and/or NF-κB in MB cells, the quantities of c-Myc and NF-κB molecules binding to the CD44 promoter in Daoy cells with or without stable knockdown of either PDGFRα or PDGFRβ in response to PDGF-BB stimulation were analyzed using chromatin immunoprecipitation (ChIP) coupled with real-time PCR.

As shown in **(Figure 24)**, the binding of c-Myc to CD44 promoter in PDGFRβ knockdown cells compared to the control or PDGFRα knockdown cells was largely reduced, while the binding of NF-κB to CD44 promoter had little change in both $PDGFR\alpha^{KD}$ and $PDGFR\beta^{KD}$ conditions. These results demonstrated that PDGFRβ enhances the expression of CD44 in MB cells via c-Myc.

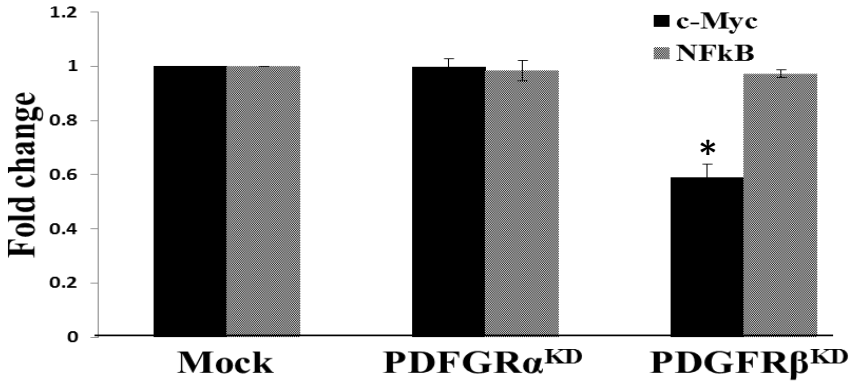

Figure 24. PDGFRβ-CD44 axis regulates MB progression via c-Myc, not NFκB. The specific binding of c-Myc and NFκB to the CD44 promoter region in wild type, $PDGFR\alpha^{KD}$ and $PDGFR\beta^{KD}$ Daoy cells determined by ChIP-PCR and presented as fold change, *$p<0.01$ when compared to respective controls.

4.2.4. Determining PDGFR-CD44 mRNA expressions in MB patient samples

Our results from the *in vitro* system clearly demonstrated that PDGFRβ-CD44 axis regulates MB metastasis. To further establish the relationship between PDGFR-CD44 with metastasis, total RNA extracted from five MB tissue samples (M-stage unknown at the time of analysis) obtained from the surgically resected primary cerebellar tumors from the University of Texas Southwestern Medical Center (one with cervical and lumbar metastasis and the rest four without metastasis) and also from Daoy and D283 cell lines

were subjected to RT-PCR. Differential levels of PDGFRα, PDGFRβ and CD44 levels were evaluated using GAPDH as the internal control by Dr. Fengfei Wang.

Blinded analysis (without a prior knowledge about the metastatic nature of the tumor samples) of the tissue samples showed moderate expression of both PDGFRα and PDGFRβ in all five samples; however an elevated expression of CD44 was noticed only in one sample which was later known to be from the metastatic tumor **(Figure 25)**. Similarly, high expression of CD44 was observed only in metastatic Daoy cells and not in uncertain for metastasis D283 cells.

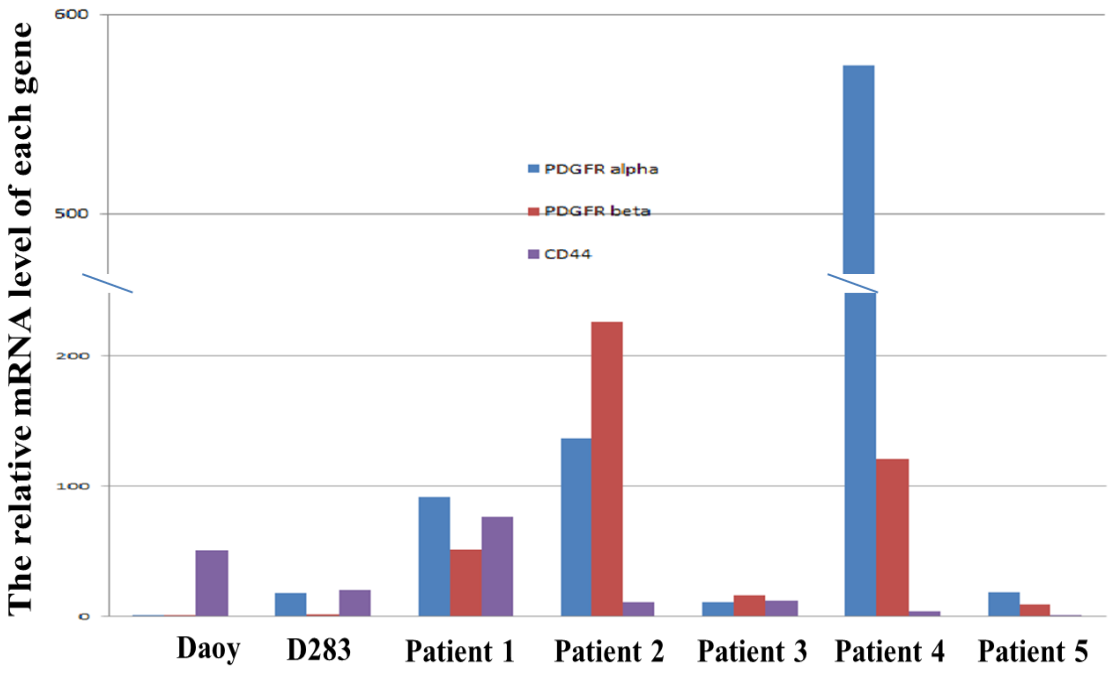

Figure 25. Relative expression levels of PDGFRα, PDGFRβ and CD44 in MB cells lines and tissue samples. The results of RT-PCR were summarized from the average Ct values of duplicated samples from two MB cell lines, Daoy and D283, and five MB patient tissue samples which were normalized using the house keeping gene, GAPDH.

These results also indicated that the expression levels of either PDGFRα or PDGFRβ alone is not sufficient to decide metastatic fate of the MB cells, and that PDGFRβ along with CD44 expression can infer the cells to probably give rise to metastasis.

4.2.5. Determining PDGFR-CD44 protein expressions in MB patient samples

To further verify our deduction that PDGFRβ regulates MB metastasis by modulating the expression of CD44, we analyzed the protein levels of PDGFRα, PDGFRβ and CD44 (metastatic nature of the tumor samples unknown at the time of analysis) in the five MB patient samples by immunohistochemistry by Dr. Fengfei Wang. Colorimetric analysis of the stained tissue samples either with PDGFRα, PDGFRβ or CD44 along with rabbit IgG as control, replicated the results obtained from the RT-PCR showing moderate expression of PDGFRα and PDGFRβ in all the samples, with only high expression of CD44 in one sample which was later known to be metastatic **(Figure 26)**. Although the results obtained with a few patients' tumor samples are very preliminary, as there was just one metastatic sample that demonstrated a higher expression of CD44 than the rest of the non-metastatic samples, they can be considered as a positive approach for further consideration in the treatment of MB metastasis.

The summary **(Table 16)** of both mRNA and protein expression in all five MB patient tissue samples provides us with rudimentary evidence that reinforces our deduction about PDGFRβ imposing its effect on MB metastasis via CD44.

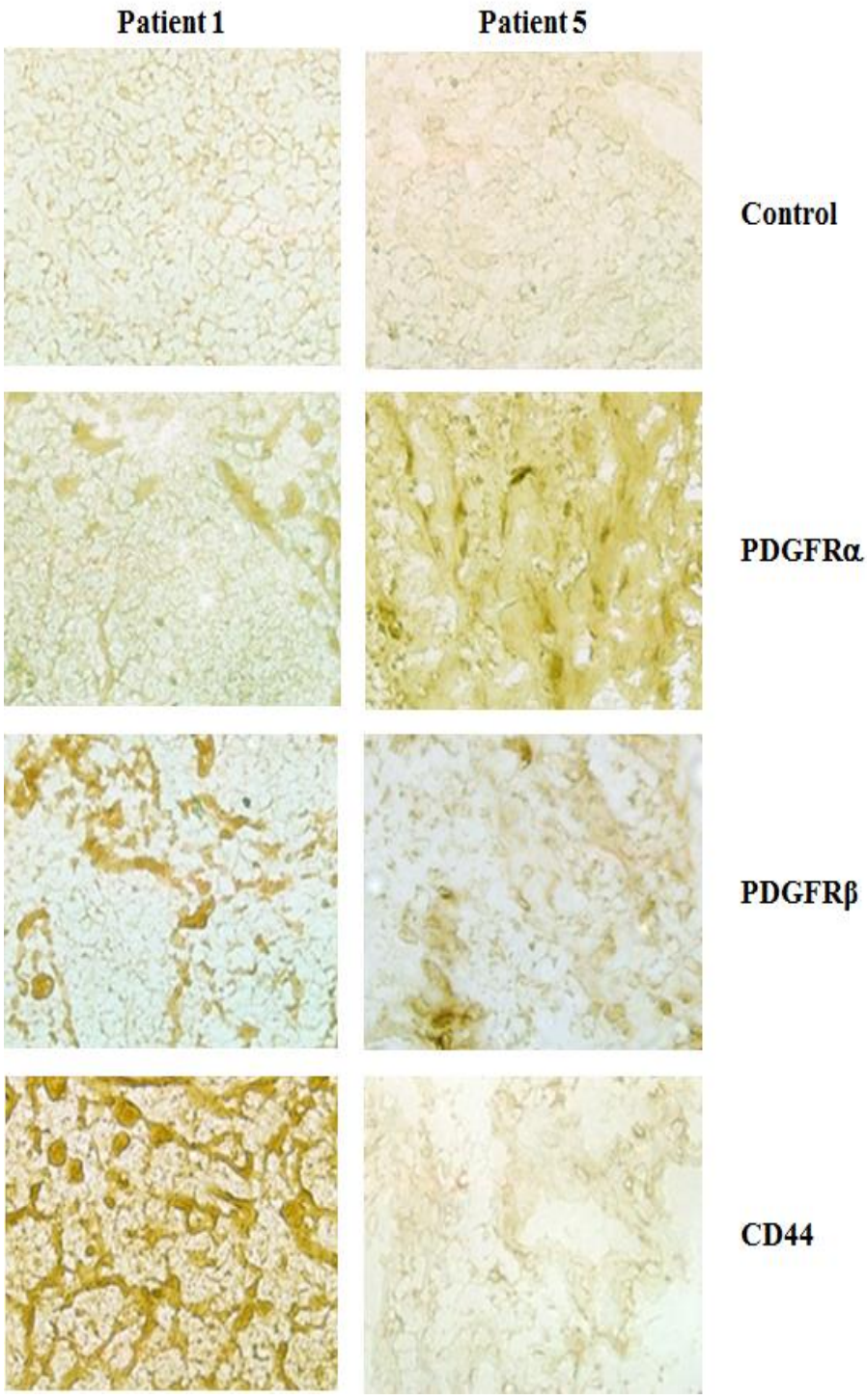

Figure 26. Expression levels of PDGFRα, PDGFRβ and CD44 in MB. Results are shown in two representative group; patient 1 (metastatic) and patient 5 (non-metastatic). The immunohistochemistry staining of each gene was performed twice and the results were analyzed by two investigators.

Table 16. Expression levels of PDGFRα, PDGFRβ and CD44 in all five MB tissue samples. The results of RT-PCR were summarized from the average Ct values of duplicated samples from five MB patient tissue samples which were normalized using the house keeping gene, GAPDH. When the actual Ct value for a particular gene from a sample was ≥ 33, it was considered as low expression (+/-, -). The protein levels after immunohistochemistry were scored according to the color and intensity of staining of whole tissue.

	metastasis	PDGFRα		PDGFRβ		CD44	
		mRNA	protein	mRNA	Protein	mRNA	Protein
Patient 1	Yes	+++	++	++	+++	+++	+++
Patient 2	No	+++	+	+++	-/+	+/-	-
Patient 3	No	+	-	++	-	+/-	-
Patient 4	No	++++	-/+	++++	++	-	-
Patient 5	No	++	+	++	+	+/-	-

4.3. Discussions and conclusions

In this study, we present evidence supporting the novel PDGFRβ-CD44 regulatory axis and contribution by transcription factors, i.e. c-Myc and NFκB, in the control of metastasis in MB. As mentioned, whether or not PDGFRα, β, or both, are required for MB metastasis has been contested [117, 118]. However, both metastatic and non-metastatic MB tissues expressed comparable levels of PDGFRs (PDGFRα compared with PDGFRβ) as analyzed by real time RT-PCR **(Figure 25)**. Thus, additional factors drive MB metastasis, not merely PDGFRα or β. A reasonable candidate, CD44, has surfaced from our studies; of note, the high expression level of CD44 was detected only in the metastatic MB tissue and not in the tissue from the other four patients without metastasis **(Figure 12)**. However these results are very preliminary as a higher expression of CD44 was noticed in only one metastatic sample when compared to the rest non-metastatic samples. It is worth noting that to minimize bias, the protein and gene expression data of MB tissues were obtained in the absence of information of the metastasis status of the

tissues. Equally important, the differential expression of CD44 can be replicated in the metastatic and uncertain for metastasis MB cell lines, Daoy and D283 cells, suggesting that the latter can serve as a reasonable *in vitro* model for further investigation of the mechanism of metastasis by PDGFR. Furthermore, the metastatic rate of MB cells was shown to be modulated by the knockdown of CD44 in the metastatic Daoy cells and overexpression of CD44 in the uncertain for metastasis D283 cells. These data provide further support that CD44 plays an important role in MB metastasis **(Figure 23)** and that the expression of CD44 is required for PDGFRβ regulating MB metastasis. Moreover, we also found that c-Myc is downstream from PDGFR signaling but upstream of CD44, which is in partial agreement with our microarray data from MEFs [217].

Because our data showed that PDGFRβ is involved in the control of c-Myc and NF-κB, and because NF-κB activation has been shown to activate c-Myc promoter in fibroblasts in response to PDGF signaling [358], it is likely that PDGFRα and PDGFRβ differentially regulate metastatic MB functions via several key downstream targets such as PKCα, c-Myc, NF-κB activity, and CD44 **(Figure 15, Figure 16)**. A new PDGFRβ-CD44 regulatory axis in MB cells has been put-forth from our studies. Among these targets, c-Myc and CD44 play a critical role in PDGFRs signaling to control MB metastasis. Though we firmly confirmed our hypothesis that PDGFRβ-CD44 regulatory axis controls metastasis in MB patients **(Figure 25, Figure 26)**, this study is a proof of concept (POC) in terms of clinical MB tissues and larger clinical MB specimens may be extended.

In summary, based on our preliminary results, we can clearly show that PDGFRβ, not PDGFRα, plays an essential role in MB metastasis, and that the PDGFRβ-CD44

regulatory axis might control metastasis in MB patients. Our study suggests that PDGFRβ signaling antagonists and/or inhibitors may find efficacy as novel therapeutic agents in the treatment of metastatic MB. Our findings that CD44 is a downstream target of PDGFRβ signaling and c-Myc is an important molecular mediator suggest that novel targets for the control of MB metastasis may lie in the co-targeting of PDGFRβ, c-Myc, and CD44. This approach may shift the focus from more commonly employed targets such as PI3K/Akt and MAPKs which, because of their global involvement in the functions of multiple cell types, may be more likely to produce off-target side effects.

CHAPTER 5. EFFECT OF CO-INHIBITING PDGFRβ AND C-MYC IN MB AND IDENTIFICATION OF NOVEL MB TARGETS

5.1. Introduction

Several studies have shown that over-expression/over-activation of genes such as PDGFRs and c-Myc in the tumor tissues of MB patients are correlated to MB patients with an extremely aggressive tumor phenotype and poor prognosis [117, 118, 345, 359]. Our recent study shows that only an elevated level of PDGFRβ in the patients correlated with a poor outcome [360]. Disrupting PDGFR signaling in MB cells using either PDGFRβ specific siRNA or PDGFR inhibitor such as cambogin (SJ001), Imatinib and Sunitinib have been demonstrated to decrease cell proliferation and migration of MB cells [115, 116, 214].

c-Myc, a proto-oncogene, is a transcription factor controlling multiple cellular events such as proliferation, cell cycle progression, apoptosis and differentiation, by regulating the expression of its target genes [361-364]. It was found that both genomic amplification and/or promoter activation of c-Myc gene are common events in MB cells and overexpression of c-Myc promotes tumorigenesis while inhibition of c-Myc induced tumor growth *in vitro* and *in vivo* [345, 359, 365, 366]. Although previous studies have shown both c-Myc and PDGFRβ to be potential therapeutic targets for MB, the effects of co-targeting these two molecules and the mechanism by which abnormal PDGFRβ signaling and overexpression of c-Myc enhance MB progress is not clear so far. In this study we co-inhibited both PDGFRβ and c-Myc using specific siRNAs and inhibitor molecules to investigate their effect on cell proliferation and migration. Additionally in this study, we performed miRNA profiling on PDGFRβ^{KD}, c-Myc^{KD} and PDGFRβ^{KD}c-

Myc^{KD} cells along with mock as control to identify differentially regulated miRNAs in MB. Here we have integrated miRNA profiling data and a bioinformatics aided target prediction tool to identify novel potent targets in MB [367, 368].

5.2. Results and discussions

5.2.1. Confirming double gene knockdown of PDGFRβ and c-Myc in MB cells

Our earlier studies clearly demonstrated that in MB cells PDGFRβ regulates cell proliferation and invasion via c-Myc and CD44. We also observed a partial down-regulation and a complete abolishment in the c-Myc and CD44 expression respectively, in the PDGFRβ knockdown cells **(Figure 15)**. We next aimed to co-target both PDGFRβ and c-Myc, two important genes involved in regulation of MB cellular function, by using gene specific siRNAs. Knockdown of genes was confirmed by Western blotting, as shown in **(Figure 27)** with β-actin as the loading control in all three MB cell lines, Daoy, D283 and D425. As a proof-of-principle we demonstrated the effects on an additional cell line, D425.

We observed a significant knockdown in PDGFRβ and c-Myc in both single and double knockdown cells. Also, as expected only a partial downregulation in c-Myc was observed in PDGFRβ knockdown cells.

5.2.2. Determining the effect of co-inhibition on MB cell proliferation

Our studies on PDGFR signal transduction revealed that out of the two isoforms, PDGFRβ plays a crucial role in MB progression by regulating specific downstream molecules such as c-Myc and CD44. All these molecules have been associated with cellular functions such as cell proliferation and migration. To further elucidate their roles

in MB, we hypothesized that co-inhibiting PDGFRβ and c-Myc can enhance inhibitory effects on MB cell proliferation. PDGFRβ and c-Myc were knocked down alone or in combination using siRNAs and evaluated for cell proliferation by performing MTS assay.

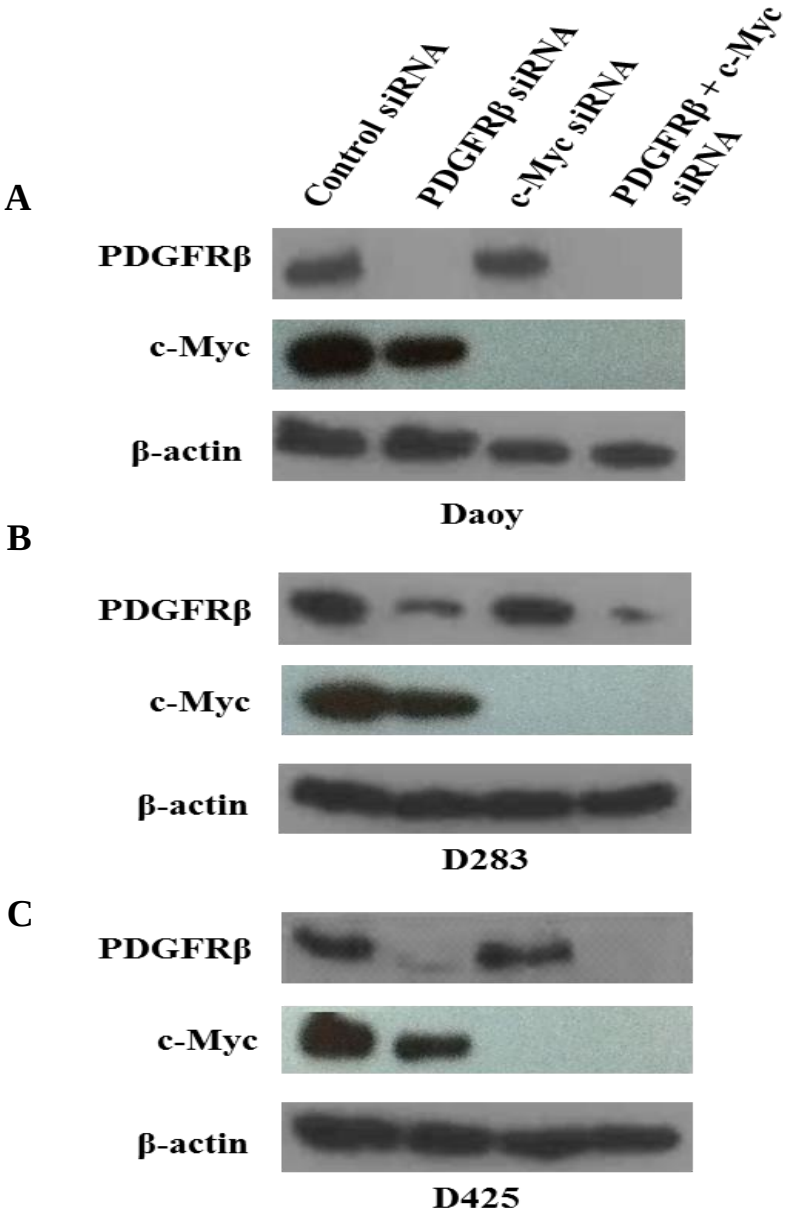

Figure 27. Double gene knockdown confirmation in MB cells. Protein analysis of PDGFRβ and c-Myc in A) Daoy, B) D283 and C) D425 cells extracted from control, PDGFRβ^{KD}, c-Myc^{KD} and PDGFRβ^{KD}c-Myc^{KD} samples after 48 h transfection by Western blotting using β-actin as the loading control.

From **(Figure 28)** we observed that c-Myc plays a greater role than PDGFRβ in MB cell proliferation, as evidenced by the results obtained which demonstrated that c-Myc knockdown alone inhibited MB cells more than PDGFRβ alone. However when in combination, an enhanced additive inhibitory effects on MB cell proliferation was observed, ~25% more than c-Myc alone. This indicates that co-targeting PDGFRβ and c-Myc might provide a better and alternative approach in the treatment of MB.

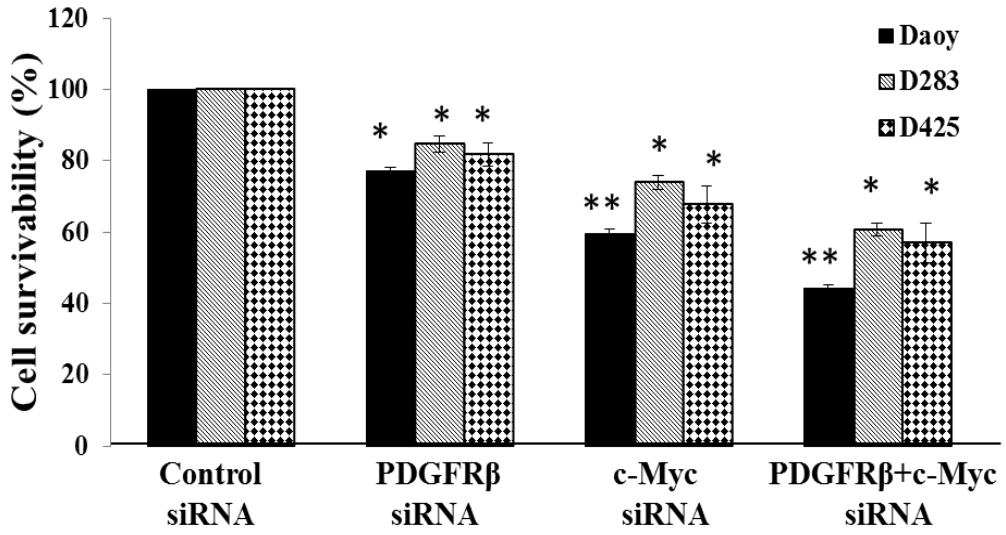

Figure 28. Effect of co-inhibition using siRNAs on MB cell proliferation. MB cells transfected with control siRNA, PDGFRβ siRNA, c-Myc siRNA and PDGFRβ+c-Myc siRNA were analyzed for cell proliferation after 48 h transfection by MTS assay and presented as percentage cell survivability, *$p<0.05$, **$p<0.01$ when compared to control.

5.2.3. Determining IC_{50} for c-Myc inhibitor on MB cells

Our results with gene knockdown clearly emphasized the efficacy of co-inhibiting two genes, PDGFRβ and c-Myc, in MB **(Figure 28)**. To further analyze the pharmacological effects with use of inhibitor molecules, we used 10058-F4 (a previously demonstrated effective inhibitor for c-Myc [369, 370]) to first evaluate the IC_{50} of this

drug molecule on MB cells. 10058-F4 was dissolved in DMSO and had a final concentration of 0.25% DMSO in cell medium which was non-toxic to cells. MB cells were initially treated with increasing concentration of 10058-F4 (0, 12.5, 25, 37.5 and 50 µM) for 48 h and then subjected to MTS assay to calibrate the IC_{50}.

We observed a dose-dependent inhibition of MB cell proliferation with gradient increase in 10058-F4 c-Myc inhibitor concentration. 50% inhibition of cell proliferation was observed at a concentration of 25 µM in all three MB cell lines; indicating that the IC_{50} of the drug molecule on MB cells is 25 µM **(Figure 29)**.

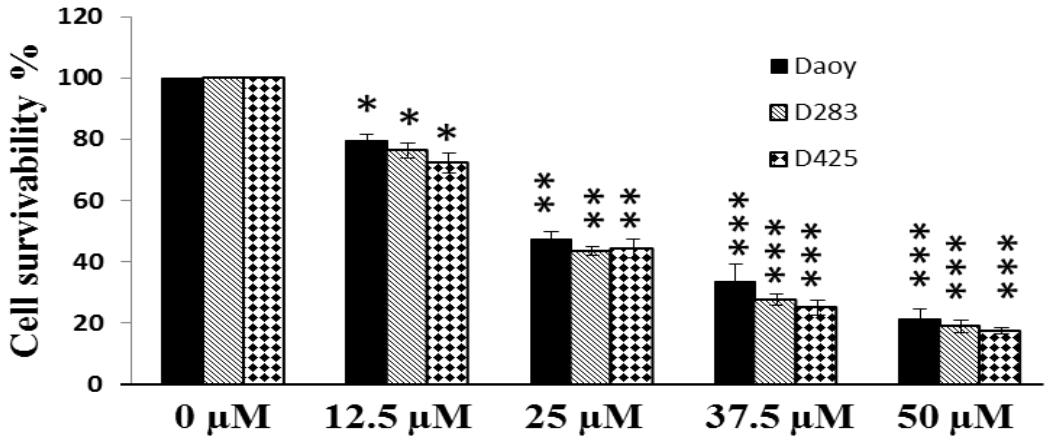

Figure 29. IC_{50} calibration of 10058-F4 c-Myc inhibitor on MB cells. MB cells treated with increasing concentrations of 10058-F4 for 48 h were subjected to MTS assay and the results presented as percentage cell survivability, *p<0.05, **p<0.01, ***p<0.001 when compared to respective controls.

5.2.4. Determining the pharmacological effect of co-inhibition on MB cell Proliferation

Our study using siRNAs demonstrated that knocking down both PDGFRβ and c-Myc resulted in more than 60% inhibition of cell proliferation **(Figure 28)**. We then aimed at verifying the pharmacological effects of co-inhibiting these two genes using PDGFR and c-Myc specific inhibitor such as SJ001 (cambogin) and 10058-F4 respectively. SJ001 is a

novel PDGFR specific drug whose IC_{50} is 5 µM in MB cells [214] and 25 µM for c-Myc inhibitor 10058-F4 (mentioned above). Although SJ001 has been demonstrated to inhibit both PDGFRα and PDGFRβ previously in our lab, we chose SJ001 for our experiments as it is a novel PDGFR inhibitor and its effect on MB cell regulation studies were yet to be studied [214]. The cells were treated with either SJ001 or 10058-F4 alone or in combination to test the pharmacological effects.

(Figure 30) demonstrated that co-inhibitory effects on MB cells using inhibitor molecules specific to PDGFR and c-Myc had greater effects, ~ 75%, than when treated alone. The results from both siRNAs and inhibitor molecules indicate that co-targeting of molecules to inhibit cell proliferation provides us with a better approach to limit tumor progression.

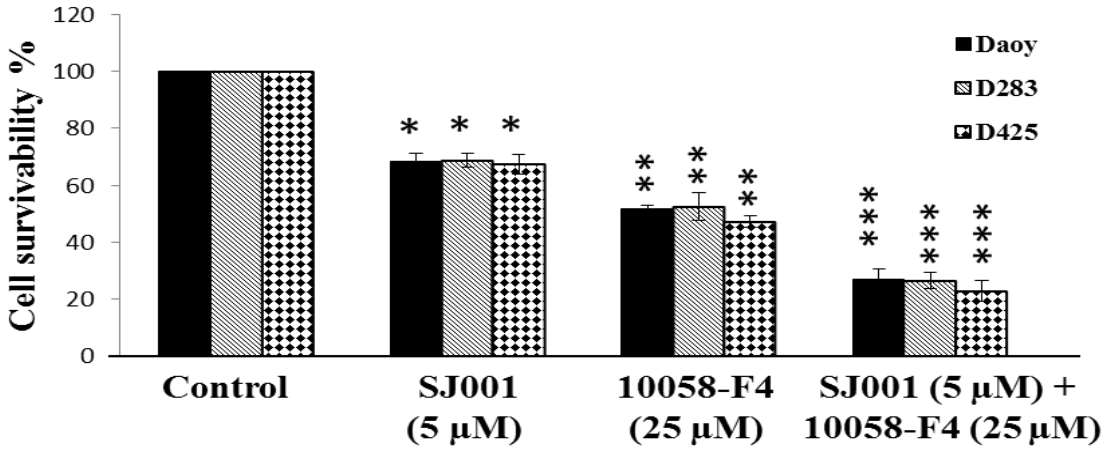

Figure 30. Pharmacological effect of co-inhibition on MB cell proliferation. MB cells treated with either SJ001 or 10058-F4 alone or in combination for 48 h were subjected to MTS assay and the results presented as percentage cell survivability, *p<0.05, **p<0.01, ***p<0.001 when compared to respective controls.

5.2.5. Determining the effect of co-inhibition on MB cell migration

Cell migration is an important process in tumor metastasis and progression. Our studies on cell proliferation demonstrated that co-inhibition enhanced the inhibitory effects on MB cell proliferation **(Figure 30)**. To further evaluate the effect of co-inhibition on cell migration, Daoy cells were treated with either PDGFRβ or c-Myc specific siRNAs and PDGFR and c-Myc specific inhibitors (SJ001 and 10058-F4, respectively) alone or in combination and the treated cells were subjected to wound healing assay. Images were captured both at 0 h and at 24 h after wound making. Wound healing assay was performed only in Daoy cells while cell lines such as D283 and D425, being half adherent/half suspension cells, were exempted from the migration study.

A significant down-regulation was noticed in cells treated with PDGFRβ siRNA and SJ001 when compared to c-Myc siRNA and 10058-F4 indicating that while c-Myc control MB cell proliferation, PDGFRβ is responsible for MB migration **(Figure 31A&B)**. However, in the double gene knockdown cells an additive inhibitory effect was observed which was stronger (~30%) than c-Myc^{KD} or PDGFRβ^{KD} taken alone as shown in **(Figure 31C)**. The above results clearly suggest that co-targeting PDGFRβ and c-Myc in MB cells can be beneficial in limiting both cell proliferation and migration and with further study can also be established as an alternative approach for the treatment of MB.

5.2.6. Heatmap showing differentially regulated miRNAs by PDGFRβ and c-Myc in MB

Our study so far has provided us with two important genes, PDGFRβ and c-Myc that regulates MB metastasis and progression, and has also proven that co-targeting these genes enhances inhibitory effects on MB cell proliferation and migration **(Figure 15, Figure 30, Figure 31)**. Our study specializes on the identification of potential therapeutic

targets for targeted inhibitory therapy for MB. The search for potential targets led us to further elucidate the mechanism by which these molecules control MB progression and to dissect their pathway to reveal new molecules regulating vital cellular processes; for this we performed miRNA profiling on stably transfected mock, PDGFRβ^{KD} (stable knockdown) , c-Myc^{KD} (knockdown using gene specific siRNA) and PDGFRβ^{KD}c-Myc^{KD} samples. Specific gene knockdown was initially confirmed by Western blot analysis **(Figure 32A)**. As shown in **(Figure 32B),** heat map generated by Dr. Fengfei Wang and Dr. Saeed Salem identified ~30 differentially regulated miRNAs that are regulated by both PDGFRβ and c-Myc in common.

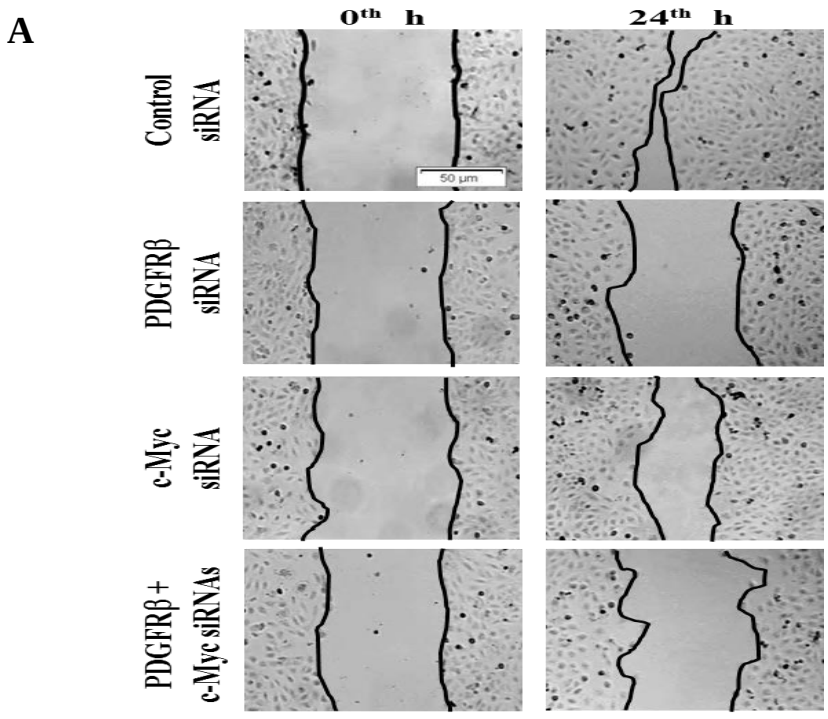

Figure 31. Effect of co-inhibition using siRNAs and inhibitors on MB cell migration. Wound healing assay performed on Daoy cells treated with A) PDGFRβ and c-Myc siRNA alone or in combination, B) PDGFR and c-Myc specific inhibitor alone or in combination, C) the results presented as percentage wound closure, *p<0.05, **p<0.01, ***p<0.001 when compared to respective controls.

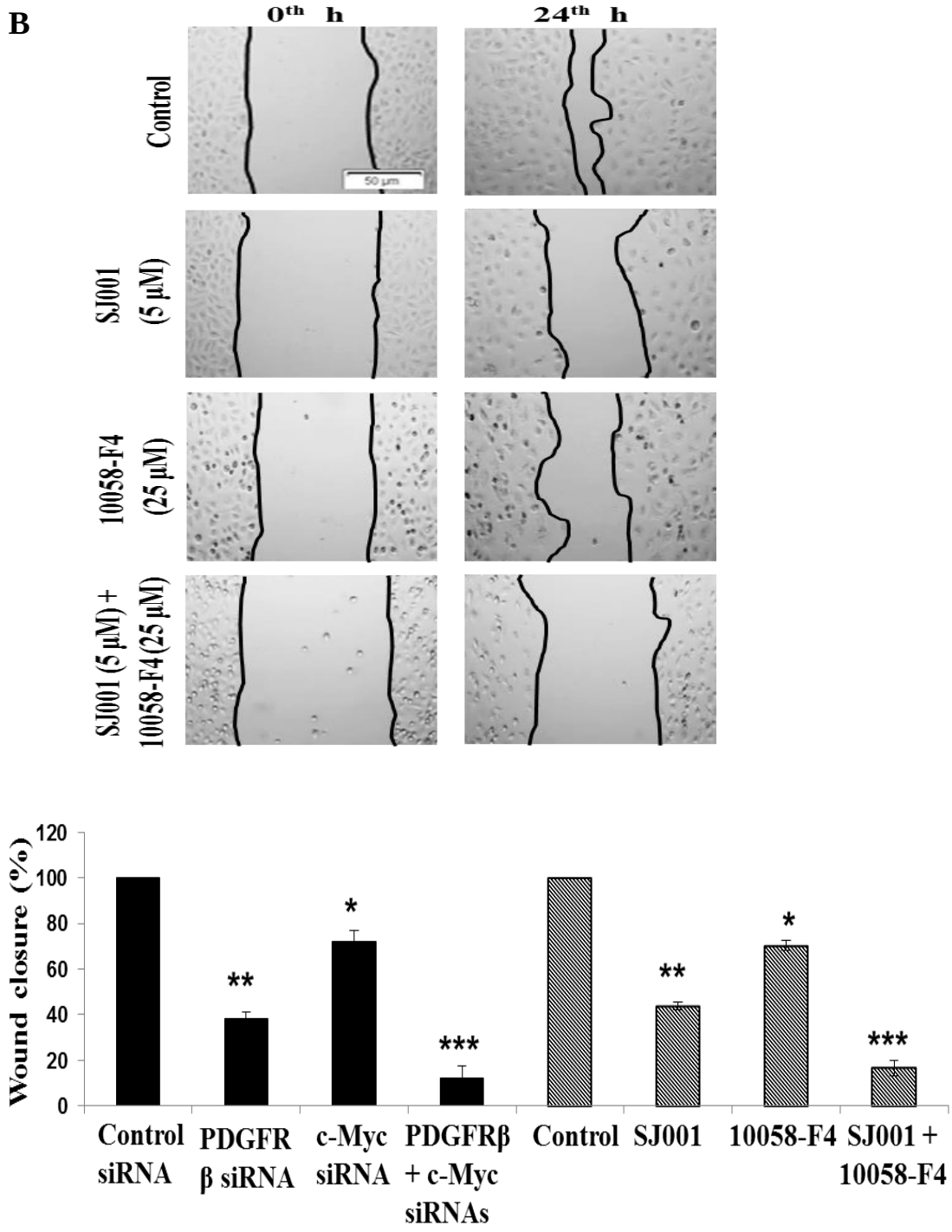

Figure 31. Effect of co-inhibition using siRNAs and inhibitors on MB cell migration (continued). Wound healing assay performed on Daoy cells treated with A) PDGFRβ and c-Myc siRNA alone or in combination, B) PDGFR and c-Myc specific inhibitor alone or in combination, C) the results presented as percentage wound closure, *$p<0.05$, **$p<0.01$, ***$p<0.001$ when compared to respective controls.

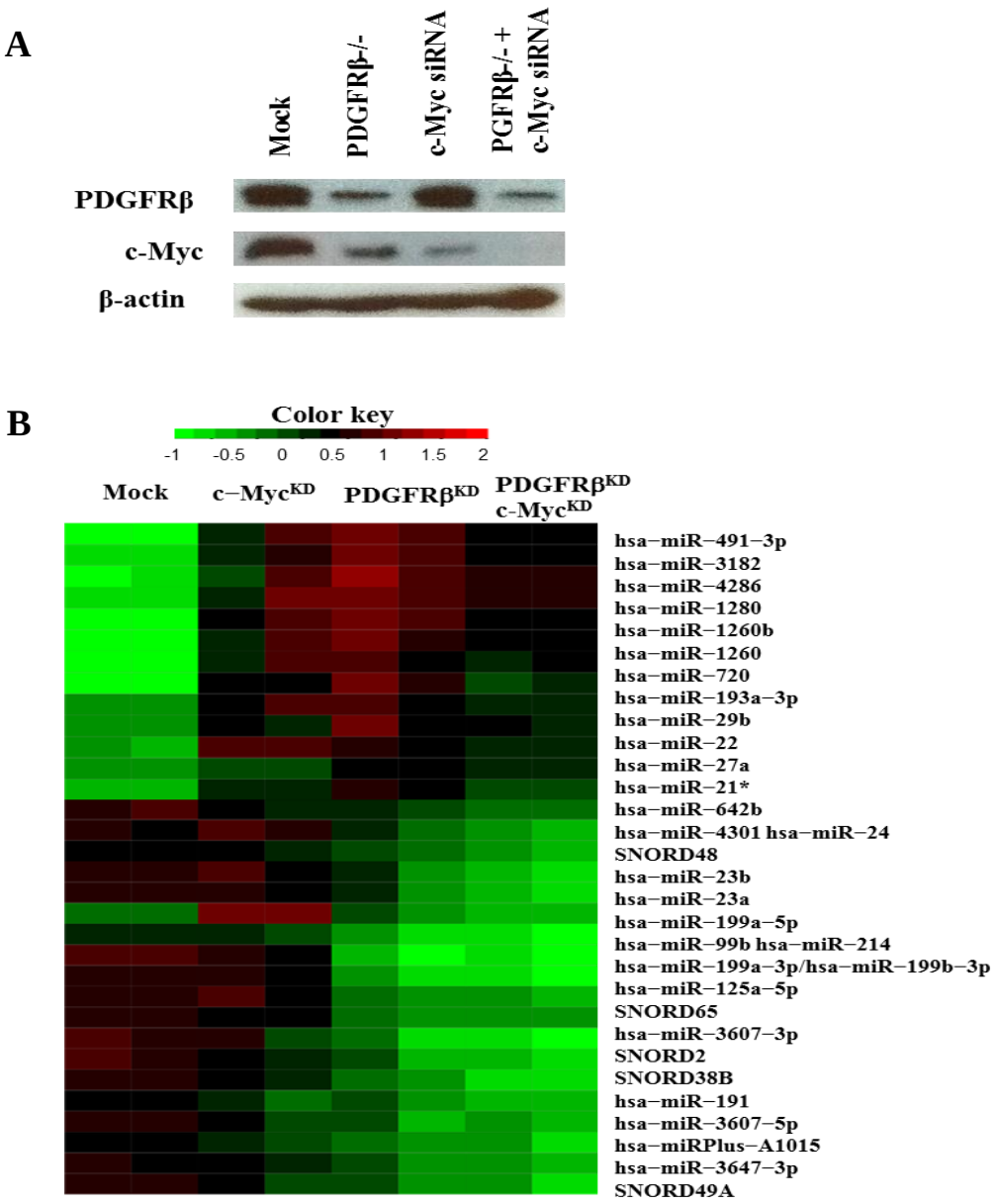

Figure 32. Differentially regulated miRNAs by PDGFRβ and c-Myc in MB. A) Protein analysis of PDGFRβ and c-Myc in PDGFRβ^{KD} stable knockdown cells and c-Myc siRNA treated cells. B) Heat map generated after data analysis representing the differentially regulated miRNAs by mock, PDGFRβ^{KD}, c-Myc^{KD} and PDGFRβ^{KD}c-Myc^{KD} cells. Total RNAs extracted from samples were subjected to miRNA profiling using 6th Gen miRNA array from Exiqon. The samples were labeled using the miRCURY LNA^{TM} microRNA array. The normalized log ratio values were used for analysis. Each row represents a miRNA and each column represents a sample. The color scale illustrates the relative expression level of miRNAs. Green color represents an expression level below the reference channel and Red color represents expression higher than the reference.

We noticed that a set of miRNAs in PDGFRβ^{KD}, c-Myc^{KD} , and PDGFRβ^{KD}c-Myc^{KD} samples showed similar trends of regulation when compared to mock cells suggesting mainly two things – that both PDGFRβ and c-Myc lie in the same signal pathway and that both regulate a particular miRNA in a similar manner in MB.

5.2.7. miRNA validation in MB cells

To further validate the array data obtained by profiling Daoy mock, PDGFRβ^{KD}, c-Myc^{KD} and PDGFRβ^{KD}c-Myc^{KD} cells, we randomly selected three miRNAs – miR-1280, and -1260 and verified their expression regulation in three MB cell lines – Daoy **(Figure 33A)**, D283 **(Figure 33B)** and D425 **(Figure 33C)**, by transiently transfecting them with gene specific siRNAs. Total RNA extracted from PDGFRβ and c-Myc alone or double knockdown cells were subjected to TaqMan RT-PCR to confirm miRNA regulation.

miR-1280 and -1260 expression levels obtained from three MB cell lines, were in accordance with the array data; thus confirming the specific regulation of miRNAs by both PDGFRβ and c-Myc.

5.2.8. miRNA target prediction and validation

Our next aim was to identify specific target molecules for the chosen miRNAs. An interesting miRNA target prediction database has been made available to the users by Wang *et. al.* that uses wiki interface to predict both conserved and non-conserved miRNA targets in animals (http://mirdb.org/miRDB/) [326, 327]. **(Table 17)** lists the top five predicted targets with highest target score for miR-1280 and -1260. To further validate the regulation of the predicted targets, Jagged 2 and CDC25A were chosen as the target molecule for miR-1280 and -1260 respectively. Expression patterns of these targets

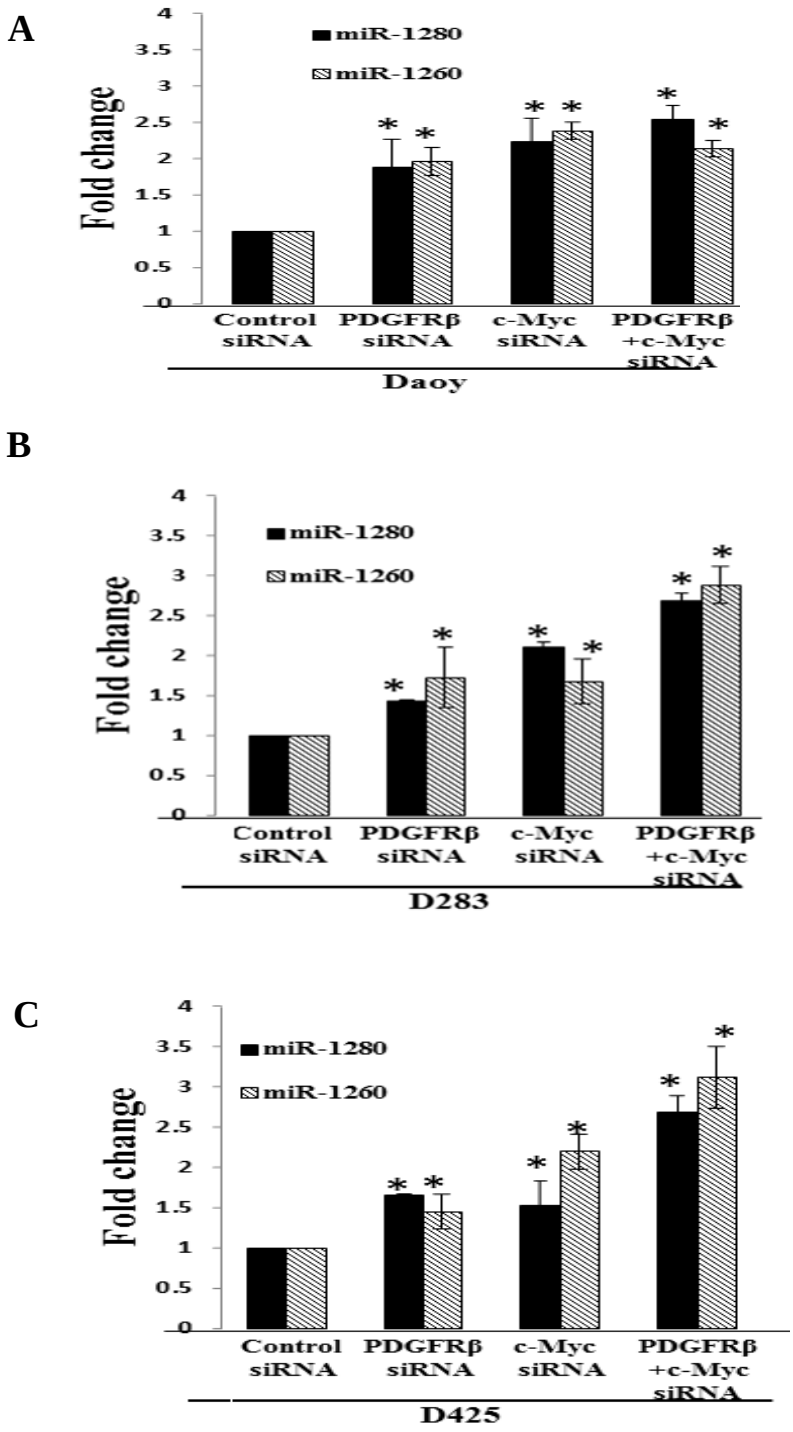

Figure 33. Validation of differentially expressed miRNAs by PDGFRβ and c-Myc in MB cells. Total RNA extracted from PDGFRβ^{KD}, c-Myc^{KD} and PDGFRβ^{KD}c-Myc^{KD} transiently transfected MB cells for 24 h were analyzed for the expression levels of miR-1280 and -1260 in A) Daoy, B) D283 and C) D425 by TaqMan® microRNA assay from Applied Biosystems and presented as fold change, *$p<0.05$ compared to respective controls.

molecules in PDGFRβ^{KD}, c-Myc^{KD} and PDGFRβ^{KD}c-Myc^{KD} cells were evaluated in three MB cell lines, Daoy **(Figure 34A)**, D283 **(Figure 34B)** and D425 **(Figure 34C)**, by Western blotting.

Table 17. The potential targets of miRNAs in MB cells regulated by PDGFRβ and c-Myc. The miRNAs significantly regulated by c-Myc and PDGFRβ in Daoy cells were listed in the table and their potential target genes were predicated using a miRNA target predication database.

Name	Gene Symbol	Predicted target gene
miR-1280	JAG2	jagged 2
	UBTF	upstream binding transcription factor, RNA polymerase I
	RALGAPB	Ral GTPase activating protein, beta subunit (non-catalytic)
	PDE8A	phosphodiesterase 8A
	ZNF544	zinc finger protein 544
miR-1260	EIF2C1	eukaryotic translation initiation factor 2C, 1
	CTAGE1	cutaneous T-cell lymphoma-associated antigen 1
	CDC25A	cell division cycle 25 homolog A (S. pombe)
	ABL2	v-abl Abelson murine leukemia viral oncogene homolog 2
	MYRIP	myosin VIIA and Rab interacting protein

Protein expression analysis demonstrated that both PDGFRβ and c-Myc single knockdown partially down-regulated the expression of Jagged 2 and CDC25; however a significant down-regulation in both the target molecules was observed in co-inhibited cells when compared to control cells in all three MB cell lines. Thus validating the

predicted targets and also indicating that both PDGFRβ and c-Myc simultaneously inhibit Jagged 2 and CDC25A to produce a synchronized effect in MB.

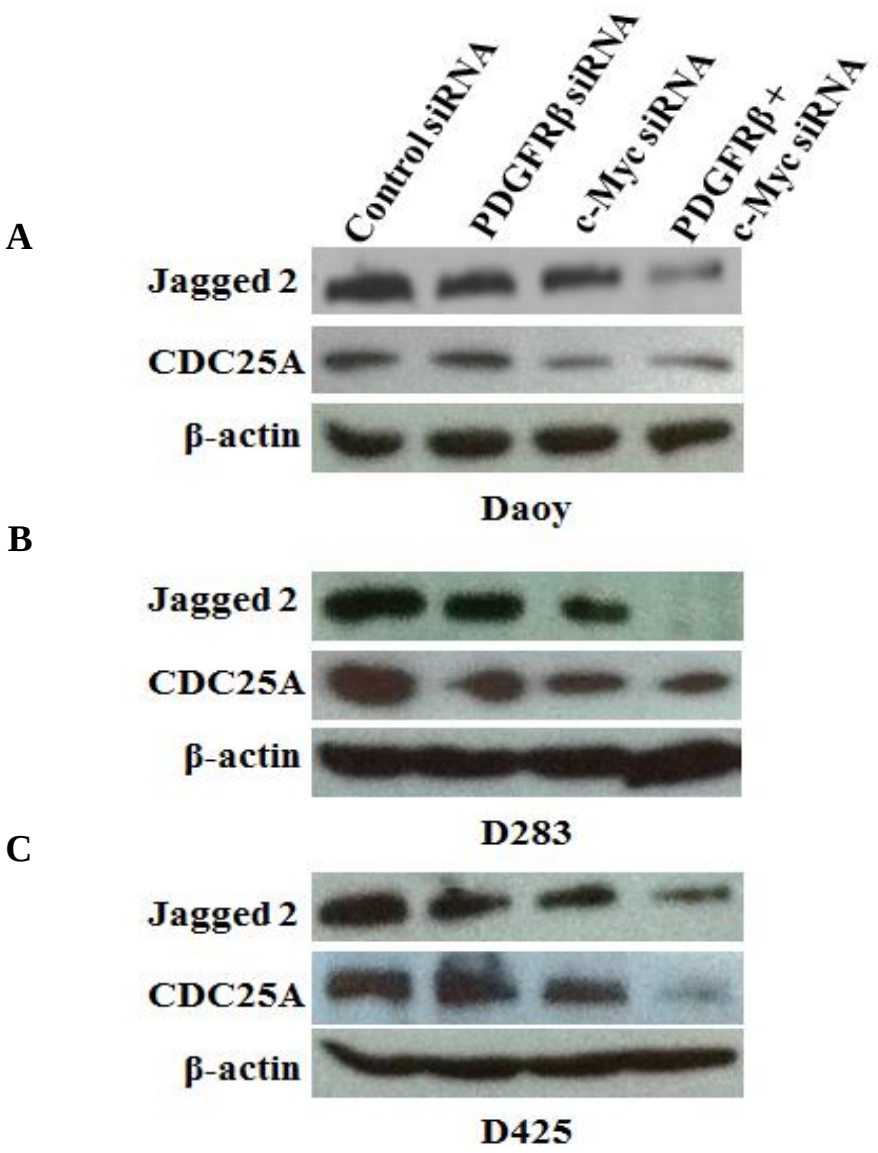

Figure 34. miRNA target validation. Protein analysis of Jagged 2 and CDC25A in PDGFRβ^{KD}, c-Myc^{KD} and PDGFRβ^{KD}c-Myc^{KD} transiently transfected MB cells using gene specific siRNAs for 48 h in A) Daoy, B) D283 and C) D425 cells by Western blotting using β-acting the internal loading control.

5.2.9. miRNA - inhibitor testing

Our earlier results enumerated that MB cells treated with PDGFRβ siRNA expressed higher level of miR-1280 and -1260 when compared to control cells **(Figure 32B, Figure 33)**. Our results also demonstrated that both PDGFRβ and c-Myc regulate miR-1280 and -1260 and Jagged 2 and CDC25A molecules in MB cells **(Figure 34)**. However, the miRNA and its target specificity in MB cells still remained unanswered. Hence, we then aimed at verifying the miRNA-target specificity by initially transfecting the $PDGFR\beta^{KD}$ stable cells with increasing concentrations (30 nM, 60 nM, 90 nM) of miR-1280 specific and miR-1260 specific inhibitors, along with negative inhibitor (60 nM, non-specific to all miRNAs) as the control. Inhibitor-miRNA specificity was evaluated by subjecting the total RNA extracted from miR-inhibitor transfected cells to TaqMan qRT-PCR. Ct values obtained were analyzed according to $2^{-\Delta\Delta Ct}$ methodology [340, 343].

From **(Figure 35)** we observe a dose-dependent decrease in the expression level of miR-1280 and -1260 with the gradient increase in the concentration of miR-inhibitor -1280 and -1260 respectively. This indicated that the inhibitors used to suppress the expression levels of the target miRNAs in MB cells were highly specific and that these miR-inhibitors can be used further to evaluate the miRNA-target specificity in MB cells.

5.2.10. miRNA-target specificity

miR-1280 and -1260 specifically target Jagged 2 and CDC25A respectively. In 2010, Schopman *et al*. studies indicated that miR-1280 might be a fragment of the $tRNA^{Leu}$ and thus the miRNA should be validated in more detail [371]. As a justification for our miRNA studies, we initially validated miR-1280 sequence by using miR-1280 specific inhibitors and evaluated its effect on the predicted target molecule, Jagged 2. Hence, to

further demonstrate the miRNA and target specificity, miR-inhibitors were chosen for specific down-regulation of miR-1280 and miR-1260 to verify if they specifically acted upon their target molecules, Jagged 2 and CDC25A respectively, in MB cells. Proteins extracted from miR-inhibitor-1280 and -1260 (30 nM, 60 nM, and 90 nM) and negative inhibitor (60 nM) transfected PDGFRβ^{KD} stable knockdown cells were analyzed for change in protein expression of Jagged 2 and CDC25A by Western blotting in MB.

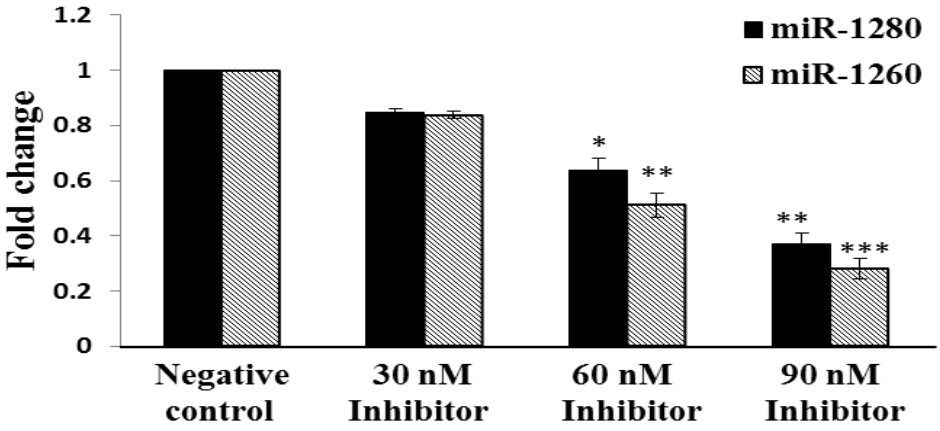

Figure 35. miRNA-inhibitor testing. Total RNAs from PDGFRβKD stable knockdown cells transfected with miR-1280 and miR-1260 inhibitors (30 nM, 60 nM, 90 nM) for 24 h were isolated and subjected to TaqMan® microRNA assay from Applied Biosystems and presented as fold change, *p<0.05, **p<0.01, ***p<0.001 compared to respective controls.

Our results clearly demonstrated a dose-dependent increase in the expression level of Jagged 2 and CDC25A with gradient increase in miR-1280 and -1260 inhibitor concentration (30 nM, 60 nM, and 90 nM) respectively in both Daoy and D283 cells as shown in **(Figure 36)**. β-actin was used as the internal control. From our earlier results

we know that PDGFRβ^{KD} cells express higher miR-1280 and -1260 when compared to control cells **(Figure 33)**. As Jagged and CDC25A are targets of miR-1280 and -1260 respectively, a down-regulation of their expression was observed in the cells treated with negative inhibitor. However, with gradient increase in the inhibitor concentration, dose-dependent suppression of its specific target molecule was observed. Thus, verifying the specificity of miR-1280 and -1260 to its target molecules Jagged 2 and CDC25A respectively.

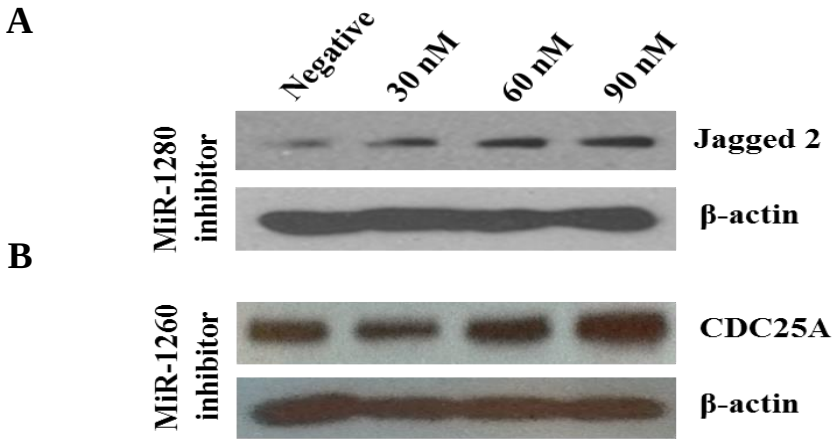

Figure 36. miRNA-target specificity. Protein analysis of Jagged 2 and CDC25A in PDGFRβ^{KD} stable knockdown Daoy cells transfected with miR-1280 and miR-1260 inhibitors (30 nM, 60 nM, 90 nM) for 48 h by Western blotting using β-actin as the internal control.

5.2.11. Role of miR-1280 and -1260 in MB cell proliferations

Experiments performed so far have indicated that both PDGFRβ and c-Myc regulate the expression of miR-1280 and -1260 in MB **(Figure 33)**. miR-inhibitor experiments have also demonstrated that miR-1280 and -1260 regulate the expression of Jagged 2 and CDC25A in MB **(Figure 34)**. However, the functional importance of these miRNAs in MB is still unclear. To evaluate the role of miR-1280 and -1260 in MB cell proliferation,

PDGFRβ^{KD} stable knockdown cells transfected with specific miR-inhibitors (60 nM) along with a negative inhibitor (60 nM) were subjected to MTS assay.

When compared to control cells, we observed ~25% to ~15% increase in cell proliferation in MB cells treated with miR-1280 and -1260 respectively **(Figure 37)**. These results clearly suggest that mir-1280 and -1260 play a definitive role in MB cell proliferation.

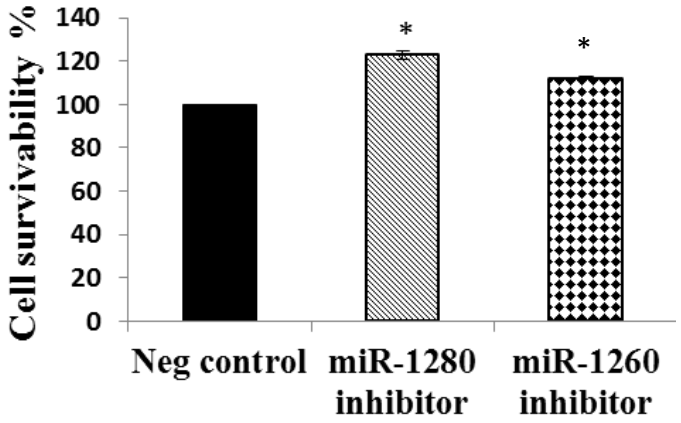

Figure 37. Role of miR-1280 and -1260 in MB cell proliferation. PDGFRβ^{KD} stable knockdown Daoy cells treated with either miR-1280 or -1260 inhibitor (60 nM) for 48 h were subjected to MTS assay and the results presented as percentage cell survivability, *$p<0.05$ when compared to respective controls.

5.2.12. Role of miR-1280 and -1260 in MB cell migration

Both miR-1280 and -1260 induce MB cell migration. Our promising cell proliferation results **(Figure 37)** prompted us to verify the importance of miR-1280 and -1260 in MB cell migration. As mentioned above, PDGFRβ^{KD} stable knockdown cells were used and transfected with either miR-1280 or -1260 (60 nM) along with negative inhibitor as the

control (60 nM). The transfected cells were then used to perform the wound healing assay to evaluate the functional role of miR-1280 and -1260 in MB progression.

Our results from **(Figure 38A)** demonstrated that an increase in cell migratory ability was displayed by PDGFRβ^{KD} cells transfected with specific inhibitors for either miR-1280 or miR-1260; inhibition of miR-1280 showed a marked increase in cell migration (~55%), while an moderate increase (~15%) was observed in miR-1280 inhibited cells **(Figure 38B)**. Cellular functions such as proliferation and migration results **(Figure 37, Figure 38)** clearly enumerated the importance of miR-1280 and miR-1260 in MB.

5.2.13. Role of Jagged 2 in MB cell proliferation

Our previous results have identified that miR-1280 targets *JAG2* in MB **(Figure 36)**. Also, we have demonstrated that both PDGFRβ and c-Myc regulate the expression of Jagged 2 in MB **(Figure 34)**. To further evaluate the role of Jagged 2 in regulating MB cellular functions, we initially knocked down Jagged 2 in three MB cell lines – Daoy, D283 and D425, using Jagged 2 specific siRNAs along with control siRNA as control. The knockdown was confirmed by Western blot analysis. These cells were then subjected to MTS assay to evaluate their role in MB cell proliferation.

Western blot analysis demonstrated a significant knockdown of Jagged 2 in all three MB cell lines transfected with specific siRNA when compared to control siRNA **(Figure 39A)**. Also, from **(Figure 39B)** we noticed that in the absence of Jagged 2 a marked decrease in MB cell proliferation was observed, ~25% in Daoy, ~20% in D283 and ~23%

in D425 compared to control, indicating that Jagged 2 along with mir-1280 and PDGFRβ play a crucial role in MB cell proliferation.

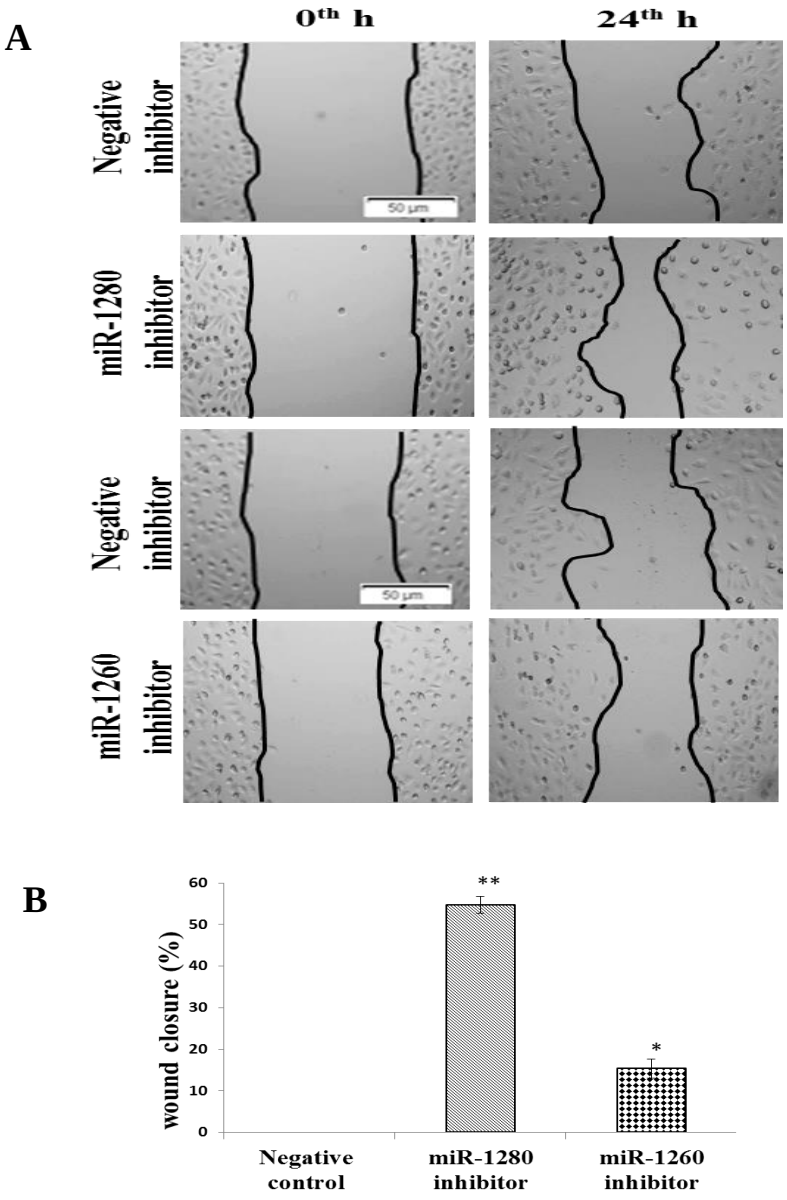

Figure 38. Role of miR-1280 and -1260 in MB cell migration. A)Wound healing assay performed on PDGFRβ^{KD} stable knockdown Daoy cells treated with miR-1280 and miR-1260 inhibitor, B) the results presented as percentage wound closure*p<0.05, **p<0.01, when compared to control.

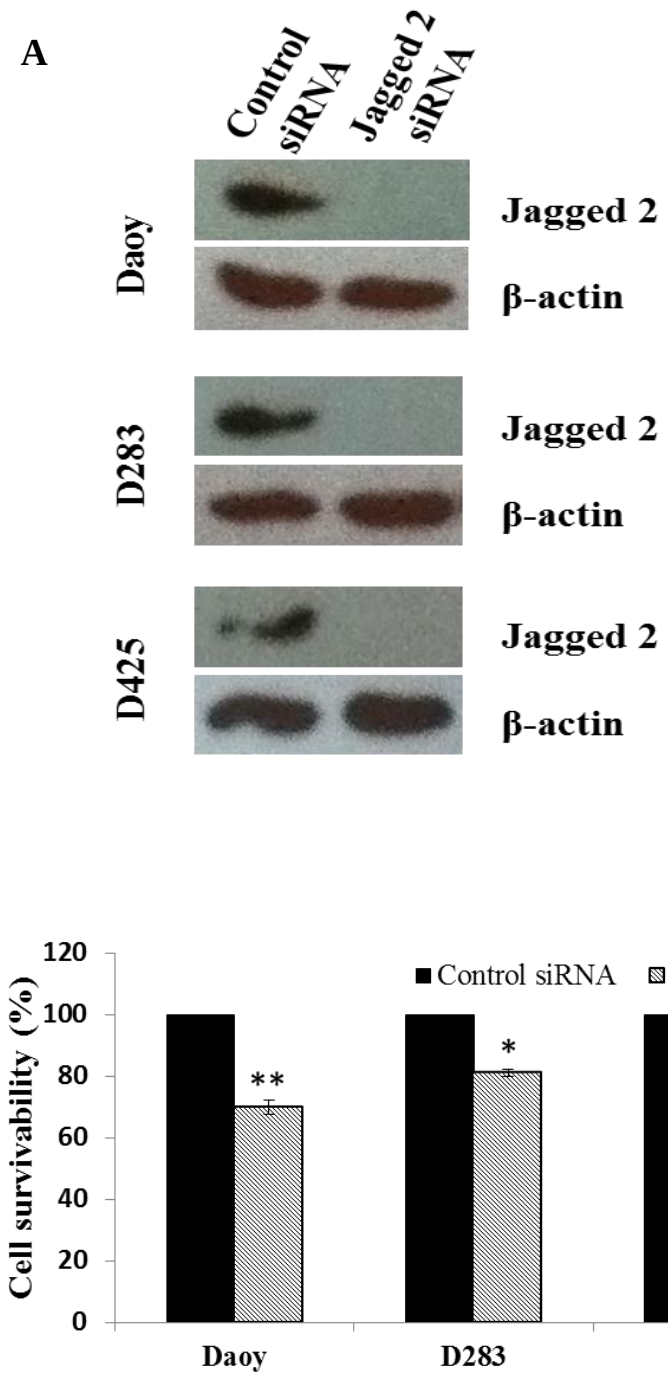

Figure 39. Role of Jagged 2 in MB cell proliferation. A) Protein analysis of Jagged 2 in MB cell lines after transfection with control siRNA and Jagged 2 specific siRNA for 48 h by Western blotting using βactin as the loading control, B) MB cells treated with either control or Jagged 2 siRNA for 48 h were subjected to MTS assay and the results presented as percentage cell survivability, *p<0.05, **p<0.01when compared to respective controls.

5.2.14. Role of Jagged 2 in MB cell migration

Jagged 2 regulate MB cell migration. Our earlier results have demonstrated that Jagged 2 is regulated by both miR-1280 and PDGFRβ pathway in MB **(Figure 34, Figure 36)**. We have also seen that Jagged 2 plays a role in regulating MB cell proliferation **(Figure 39B)**. To further enumerate its role in MB cell migration, we specifically knocked down Jagged 2 in MB and evaluated its effect by performing wound healing assay.

Wound healing assay demonstrated that in the absence of Jagged 2 a significant down-regulation (~42%) in MB migration was observed when compared to control cells **(Figure 40)**. This result indicated that Jagged 2 regulates MB cell migration along with proliferation. Our earlier studies indicated that PDGFRβ regulates MB progression by modulating its downstream molecules like c-Myc, and CD44 and certain miRNAs like miR-1280 and -1260 **(Figure 15, Figure 33)**. Put together, all the data reinforce our notion that PDGFRβ pathway regulates MB metastasis and progression.

5.3. Discussion and conclusion

Published reports, including our own, have shown that inhibition of either PDGFRβ or c-Myc decreased cell proliferation and/or migration/invasion [344, 372-375] **(Figure 12, Figure 14)**. In this study, we determined the effects of co-targeting both c-Myc and PDGFRβ using either gene specific siRNAs or inhibitors on MB cell proliferation and migration. Additive inhibitory effects on both MB cell proliferation (~60%) and migration (~85%) were observed in MB cells knocking down both PDGFRβ and c-Myc when compared to single knock down of either PDGFRβ (~25% and ~60%) or c-Myc (~40% and 25%). PDGFRβ siRNA or inhibitor had a greater reduction effect on

migration, while c-Myc siRNA or inhibitor largely inhibited MB cell proliferation; however, the combined inhibition of both PDGFRβ and c-Myc offers synergistic pharmacologic effects in limiting MB progression **(Figure 28, Figure 30, Figure 31)**.

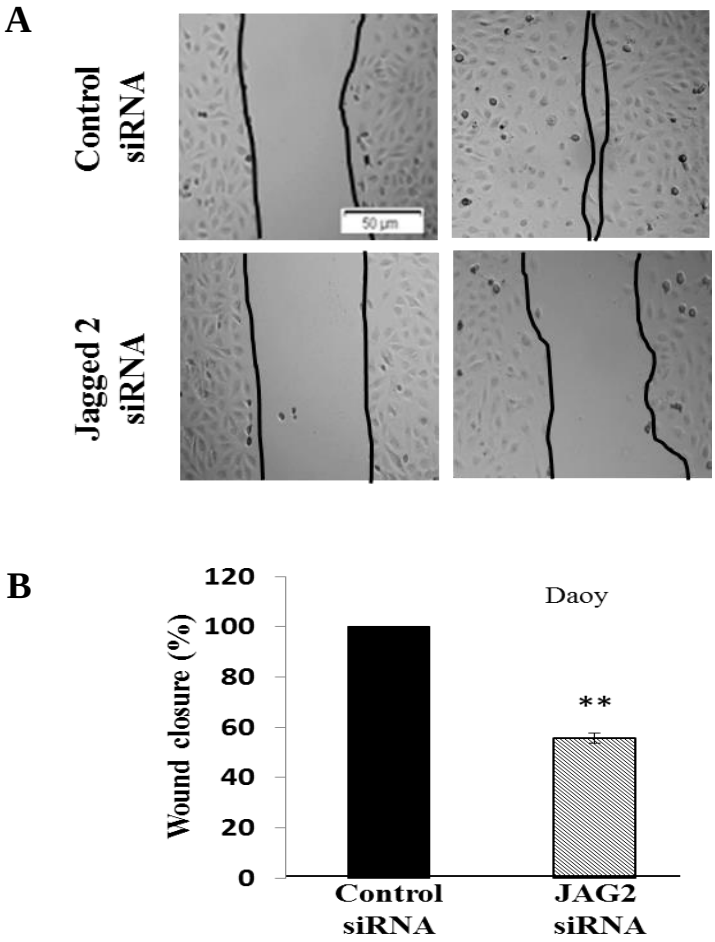

Figure 40. Role of Jagged 2 in MB cell migration. A)Wound healing assay performed on Daoy cells transfected with control or Jagged 2 siRNA for 48 h B) the results presented as percentage wound closure, **p<0.01when compared to control.

Recently, non-coding microRNAs have been shown to play crucial roles in cancer progression [293, 294]. miRNA profiling on MB tissues from patients has revealed

signature patterns to differentiate between childhood and adult MBs, identify their molecular subtypes, and also miRNAs which play important roles in MB [301-304]. We reasoned that specific miRNAs might be regulated in the PDGFRβ-CD44 regulatory axis that controls MB metastasis. To evaluate such miRNAs, we performed miRNA profiling to identify differentially regulated miRNAs in MB which might further assist in understanding the molecular mechanisms regulating metastatic MB **(Figure 32B)**.

In this study, by using miRNA profiling, we demonstrated that PDGFRβ and c-Myc cells cumulatively regulate MB growth and migration by limiting the expression of a set of miRNAs, such as miR- 1260, -1280 and consequently stimulating the expression of molecules related to tumor proliferation and metastasis, e.g., CDC25A and Jagged 2 respectively **(Figure 36, Figure 37, Figure 38)**. To decipher the mechanism that synergistically inhibits MB progression by co-targeting PDGFRβ and c-Myc, we analyzed the miRNA profiles in wild type, $PDGFR\beta^{KD}$, $c\text{-}Myc^{KD}$ or $PDGFR\beta^{KD}c\text{-}Myc^{KD}$ Daoy cells, and identified a set of miRNAs that are commonly regulated by both PDGFRβ and c-Myc, indicating the existence of a cross-talk prevailing in between the two molecules **(Figure 32B)**. With the aid of miRNA predication data base **(Table 15)**, several oncogenes and tumor suppressors have been revealed to be potential targets of miRNAs regulated by PDGFRβ and c-Myc in the metastatic MB cells. By validating the regulated miRNAs and their targets **(Figure 33, figure 36)**, we revealed that PDGFRβ and c-Myc regulate the MB cellular events via multiple pathways which include miRNAs and their target molecules, such as Jagged 2 that can be a potential target for MB.

Jagged 2 is a transmembrane glycoprotein that binds to notch receptors regulating cell proliferation and differentiation in both normal and pathological conditions [376-381].

Notch signaling plays a vital role in maintaining homeostasis in stem and progenitor cells during embryogenesis, aberrations in which can result in MB formation [79, 80]. All these studies indicate that targeting Notch signaling can be beneficial towards the treatment of MB. CDC25A is modulator protein that controls cell cycle transition from G1 to S phase with dual phosphatase activity [382-386]. Several studies have indicated that CDC25A is an action molecule that mediates c-Myc signaling for cell proliferation [387, 388]. In the siRNA knockdown of either PDGFRβ or c-Myc condition, we observed a reverse correlation between the mir-1280 and miR-1260 and their expected targets, Jagged 2 and CDC25A, respectively **(Figure 36)**. This result confirmed that Jagged 2 and CDC25A are genuine targets of miR-1280 and miR-1260, respectively. Notably, either miR-1280 inhibitor or miR-1260 inhibitor had less effect on cell proliferation but significantly enhanced cell mobility. This could be explained by reasoning that multiple miRNAs are involved in PDGFR and c-Myc activated signaling in MB cells. However, surprisingly, the effects of Jagged 2 siRNA showed similar effects as that obtained by miR1280 inhibitor on Daoy cell proliferation and migration. These data indicate that Jagged 2 is a potential therapeutic target for MB.

In conclusion, our results have demonstrated that co-targeting two vital genes limits cell proliferation and metastasis. We revealed that PDGFRβ and c-Myc have additive effects in promoting MB progression via miRNAs and their targets such as Jagged 2 and CDC25A. Our data indicate that blockage of PDGFRβ and c-Myc signaling in MB cells simultaneously will co-inhibit cell migration and cell proliferation via modulating a set of miRNAs and consequently down-regulating the expression of tumorigenic factors, e.g. Jagged 2 and CDC25A and tyrosine kinase receptors, e.g. EGFR, ERBB4. Our results

suggest that co-targeting of PDGFRβ and c-Myc provide a novel therapeutic strategy for the treatment of metastatic MB and indicate that Jagged 2 is a potential new target of MB.

CHAPTER 6. DISCUSSION AND CONCLUSIONS, CLINICAL IMPLICATIONS AND FUTURE DIRECTIONS

6.1. Discussions and conclusions

In this study, we demonstrated that PDGFRβ but not PDGFRα signaling leads to cell proliferation and migration/invasion in MB cells **(Figure 12, Figure 14)**. We present evidence supporting the notion that the expression of CD44 is essential for PDGFRβ regulating MB migration/invasion, and that the PDGFRβ-CD44 regulatory axis, along with the participation of c-Myc, is essential for the metastasis in MB **(Figure 17, Figure 23)**. We further determined the effects of co-targeting both c-Myc and PDGFRβ using either gene specific siRNAs or inhibitors on MB cell proliferation and migration. Additive inhibitory effects on both MB cell proliferation (~60%) and migration (~85%) were observed in MB cells knocking down both PDGFRβ and c-Myc when compared to single knock down of either PDGFRβ (~25% and ~60%) or c-Myc (~40% and 25%) **(Figure 28, Figure 30, figure 31)**. PDGFRβ siRNA or inhibitor had a greater inhibitory effect on migration while c-Myc siRNA or inhibitor largely inhibited MB cell proliferation; however, the combined inhibition of both PDGFRβ and c-Myc offers additive pharmacological effects in limiting MB progression **(Figure 28, Figure 30, figure 31)**. To decipher the mechanism that additively inhibits MB progression by co-targeting PDGFRβ and c-Myc, we analyzed the miRNA profiles in wild type, $PDGFR\beta^{KD}$, $c\text{-}Myc^{KD}$ or $PDGFR\beta^{KD}c\text{-}Myc^{KD}$ Daoy cells, and identified a set of miRNAs that are regulated by both PDGFRβ and c-Myc in common, indicating the existence of a cross-talk prevailing in between the two molecules **(Figure 32B)**. With the aid of miRNA predication data base **(Table 17)**, several oncogenes and tumor suppressors have been

revealed to be potential targets of miRNAs regulated by PDGFRβ and c-Myc in the metastatic MB cells. By validating the regulated miRNAs and their targets **(Figure 33, Figure 36)**, we revealed that PDGFRβ and c-Myc regulate the MB cellular events via multiple pathways which include miRNAs and their target molecules, such as Jagged 2, that can be a potent target for MB.

PDGFRα was reported to be highly expressed in metastatic MBs by array analysis and it was further proposed to be a bona fide therapeutic target for metastatic MB based on the results obtained using a PDGFRα neutralizing antibody and a MAP2K1/2 inhibitor [118]. A concern was raised, however, since the PDGFRα probe set used in the microarray analysis was subsequently shown to also detect PDGFRβ, leading to the possibility that PDGFRβ rather than PDGFRα is preferentially expressed in metastatic MB [117]. Structure-function analysis shows that although the two PDGFRs have 70% homology in the N termini and 80% in the C termini of kinase domains [295], distinct differences exist in their ligand binding domain (31% identical) and in a sub-domain located at the c-terminal region (a 27-28% homology). These features presumably allow the two receptors to display different ligand affinities and/or to interact with different target protein sets to mediate distinct functions *in vivo* [295, 346, 347] and *in vitro* [217]. In this study, we have shown that PDGFRα and PDGFRβ play distinct roles in cell proliferation, survival, and migration/invasion in MB cells, with PDGFRα limiting and PDGFRβ promoting cell proliferation and migration/invasion **(Figure 12, Figure 14)**. The disparate cellular outcomes support the importance of the inherent domains and the different interacting protein partners that may be recruited by PDGFRα and PDGFRβ c-

termini. Our results suggest that interference with PDGFRβ or rather its downstream targets and regulators may offer novel strategies for metastatic MB therapy.

We present evidence supporting the novel PDGFRβ-CD44 regulatory axis and contribution by transcription factors, i.e. c-Myc and NFκB, in the control of metastasis in MB **(Figure 15, Figure 16)**. As mentioned before, predicament as to which isoform of PDGFRα, β, or both, are required for MB progression is still being contested [117, 118]. However, both metastatic and non-metastatic MB tissues expressed comparable levels of PDGFRs (PDGFRα compared with PDGFRβ) as analyzed by real-time RT-PCR **(Figure 25)**. These results indicate that additional factors drive MB progression, not merely PDGFRα or β. A potential candidate, CD44, has surfaced from our studies; of note, the high expression level of CD44 was detected only in the metastatic recurrent MB tissue and not in the tissue from the other four patients without metastasis **(Figure 26)**. It is worth noting that we have ruled out the possible bias as the protein and gene expression data of MB tissues were obtained in the absence of information of the metastasis status of the tissues. Equally important is that the differential expression of CD44 can be replicated in the metastatic and uncertain for metastasis MB cell lines, Daoy and D283 cells, suggesting that the latter can serve as a reasonable *in vitro* model for further investigation of the mechanism of metastasis by PDGFR. Furthermore, the metastatic rate of MB cells was shown to be modulated by the knockdown of CD44 in the metastatic Daoy cells and overexpression of CD44 in the uncertain for metastasis D283 cells **(Figure 23)**. These data provide further support that CD44 plays an important role in MB metastasis and tumor progression, and that the expression of CD44 is required for PDGFRβ regulating MB progression. Moreover, we also found that c-Myc is downstream from PDGFRβ

signaling but upstream from control of CD44 **(Figure 24)**, which is in partial agreement with our microarray data from MEFs [217]. Additionally, it has been demonstrated by co-immunoprecipitation experiments that PDGFRβ and CD44 form a complex in fibroblast cells [389].

Based on the results from this study, PDGFRα and PDGFRβ differentially regulate MB cellular functions via several key downstream targets such as PKCα, c-Myc, CD44, and NFκB activity **(Figure 15)**. Among these targets, c-Myc and CD44 play a critical role in PDGFRs signaling to control MB metastasis. NFκB activation has been shown to activate c-Myc promoter in fibroblasts in response to PDGF signaling [358]; We demonstrated that PDGFRβ is involved in regulating the expression of c-Myc and the activity of NFκB in MB cells and that c-Myc plays a critical role in the alterations of NFκB activity and the expression of CD44 in MB cells in response to PDGFRβ signaling **(Figure 15, Figure 16, Figure 17, Figure 18)**.

To further decipher the molecular mechanisms along the PDGFRβ pathway that play vital roles in regulating MB progression, we focused our study on miRNAs. Our aim was to identify specific miRNAs that are differentially regulated by PDGFRα and PDGFRβ. To achieve this goal, total RNAs extracted from Daoy cells, stably knocked down in either PDGFRα or PDGFRβ were analyzed for differential expression of specific miRNAs. Microarray analysis from Exiqon provided us with two potential candidates, namely miR-1280 and miR-1260, along with their specific target molecules, Jagged 2 and CDC25A, respectively that might additionally play a part in directing PDGFRβ to its distinct pathway.

Jagged 2 is a transmembrane glycoprotein that binds to notch receptors regulating cell proliferation and differentiation in both normal and pathological conditions [376-381]. However the contribution of Notch signaling in MB development is still controversial [390-393], Notch signaling plays a vital role in maintaining homeostasis in stem and progenitor cells during embryogenesis, aberrations in which can result in MB formation [79, 80]. Significance of Notch signaling in MB has been demonstrated by inhibiting numerous Notch pathway key regulators, where a reduction in cell proliferation and an increase in cell apoptosis were observed [97, 101, 102]. *In vivo* studies have also verified the importance of ligand activated notch signaling in wound healing [394, 395]. Abnormal notch signaling initiated by Jagged 2 has also been demonstrated to play an important role in tumor initiation and progression in other tumor types including human B-cell lymphoma [395], multiple myeloma [396-398], and breast cancer [399]. All these studies indicate that targeting Notch signaling can be beneficial towards the treatment of MB. In the siRNA knockdown of either PDGFRβ or c-Myc condition, we observed a reverse correlation between miR-1280 and miR-1260 and their expected targets, Jagged 2 and CDC25A, respectively **(Figure 33, Figure 34, Figure 36)**. These results confirmed that Jagged 2 and CDC25A are genuine targets of miR-1280 and miR-1260, respectively. Notably, either miR-1280 inhibitor or miR-1260 inhibitor had less effect on cell proliferation but significantly enhanced cell mobility **(Figure 37, Figure 38)**. This could be explained by reasoning that multiple miRNAs are involved in PDGFR and c-Myc activated signaling in MB cells. However, surprisingly, the effects of Jagged 2 siRNA showed similar effects as that obtained by miR1280 inhibitor on Daoy cell proliferation and migration. These data indicate that Jagged 2 is a potential therapeutic target for MB **(Figure 39, figure 40)**.

Cell cycle is a key event for cell proliferation and tumor growth, which is tightly controlled by many regulators. CDC25A is one of such an important modulator that controls cell cycle transition from G1 to S phase with dual phosphatase activity [382-386]. Several studies have indicated that CDC25A is an action molecule that mediates c-Myc signaling for cell proliferation [387, 388]. In this study, we noticed that a correlation existed in between the expression levels of CDC25A in MB cells either at conditions where PDGFRβ and c-Myc were down-regulated using gene specific siRNAs or at conditions where miR-1260 was inhibited using specific miR-inhibitor **(Figure 34, Figure 36)**. These results indicate that CDC25A is a downstream target of PDGFRβ and c-Myc. However, miR-1260 inhibitor showed only ~10% inhibition on MB cell proliferation or migration **(Figure 37, Figure 38)**. These results suggest that PDGFRβ and c-Myc promote MB growth via multiple targets as indicated in the **(Table 17)**.

In conclusion, our results have demonstrated that PDGFRβ, not PDGFRα, plays an essential role in MB, and that the PDGFRβ-CD44 regulatory axis controls progression in MB patients. Our findings that CD44 is a downstream target of PDGFRβ signaling and c-Myc is an important molecular mediator suggest that novel targets for the control of MB metastasis may lie in the co-targeting of PDGFR, c-Myc, and CD44. By co-targeting two vital genes in MB, we revealed that PDGFRβ and c-Myc have additive effects in promoting MB progression via miRNAs and their targets such as Jagged 2 and CDC25A. Our data indicate that blockage of PDGFRβ and c-Myc signaling in MB cells simultaneously will co-inhibit cell migration and cell proliferation via modulating a set of miRNAs and consequently down-regulating the expression of tumorigenic factors, e.g. Jagged 2 and CDC25A and tyrosine kinase receptors, e.g. EGFR, ERBB4. Our results

suggest that co-targeting of PDGFRβ and c-Myc will provide a novel therapeutic strategy for the treatment of metastatic MB and indicate that Jagged 2 is a potential new target of MB.

6.2. Overall results

Overall, our results suggest PDGFRβ-CD44 axis and not PDGFRα regulates MB metastasis and also that co-inhibiting PDGFRβ and its specific downstream molecule PDGFRα results in an enhanced reduction of MB cell proliferation and migration. We demonstrated that while PDGFRβ signaling pathway induced MB cell migration and invasion, PDGFRα had contradictory effects. We hypothesized and confirmed that distinct cellular function depicted by PDGFRα or PDGFRβ is due to the specific downstream target molecules they regulate. In the absence of PDGFRβ we observed a down-regulation of important molecules like c-Myc, CD44, PKCα along with reduced NFκB activity; however no such change was observed in the absence of PDGFRα. Interestingly, we found no detectable levels of CD44 in uncertain for metastasis D283 cells when compared to metastatic Daoy cells indicating that PDGFRβ-CD44 axis plays a crucial role in MB metastasis. Experiments that use siRNAs demonstrating knockdown effects appear minor due to a couple of reasons such as the short half-life of siRNAs in the medium and also the doubling time of the cells that decrease the efficiency of the siRNAs [400]; also because D283 cells are non-adherent cells. *In vitro* invasion assay using CD44 overexpressed D283 cells and CD44 knock down Daoy cells reinforced that PDGFRβ-CD44 axis controls metastasis in MB. We also observed that c-Myc acts as an intermediary molecule between PDGFRβ and CD44; while it is regulated by PDGFRβ, it regulates the expression of its downstream molecule CD44.

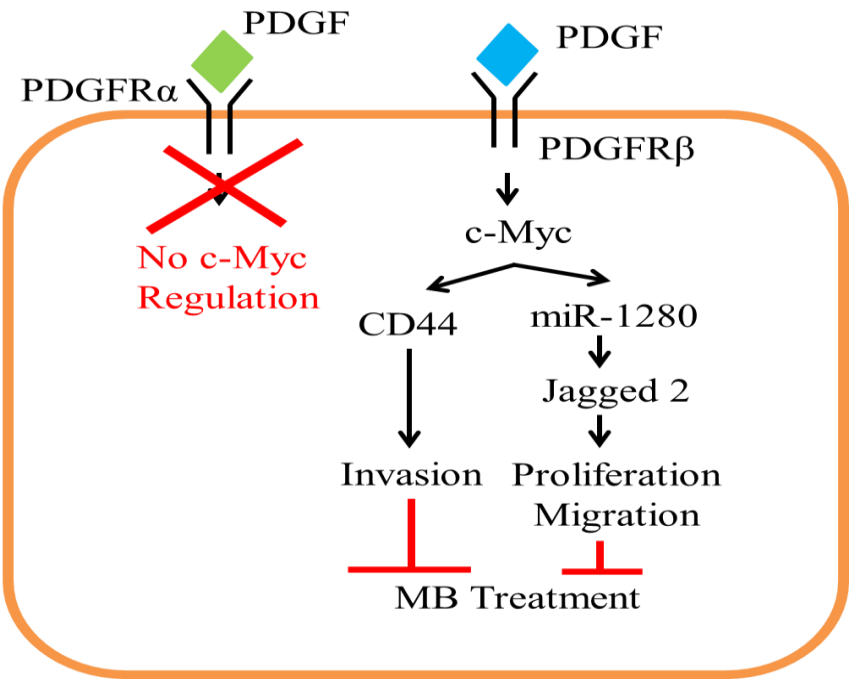

Figure 41. A panel of the proposed model. The panel depicts the PDGFRβ pathway specifically regulating c-Myc which then either regulates (a) CD44, an important molecule for MB invasion or (b) miR-1280, which then inhibits its target molecule Jagged 2, a vital molecule for MB cell proliferation and migration. Targeting this alternative pathway can hence be a potential therapeutic strategy in the treatment of MB.

Co-inhibiting PDGFRβ and c-Myc by both specific siRNA and inhibitor molecules exhibited additive inhibitory effect on MB cell proliferation and migration. These results suggested that for both targeted therapy and pharmacologically, co-inhibiting PDGFRβ and c-Myc might provide a novel therapeutic strategy for the treatment of MB. Our search on targeted inhibitory therapy intrigued us to further study the mechanisms in the PDGFRβ signal pathway that bring about this additive effect; the results revealed miRNAs, miR-1280 and miR-1260, that are regulated by both PDGFRβ and c-Myc, which in-turn regulate the expression of their target molecules such as Jagged 2 and CDC25A respectively in MB. We also observed that specific knockdown of Jagged 2 reduces MB cell proliferation and migration indicating that Jagged 2 plays a role in MB

progression. These molecules pose as potential therapeutic targets for MB inhibitory therapy.

To summarize, our study has contributed in identifying a distinct signal cascade initiated by PDGFRβ, followed by c-Myc and CD44. In parallel we also identified that both PDGFRβ and c-Myc regulate miR-1280, and miR-1280 regulates the expression of Jagged 2. All these data might assist in providing a novel therapeutic strategy for the treatment of MB.

6.3. Clinical implications and future directions

Several studies have demonstrated that PDGFRs are overexpressed in MB and that they are vital for tumor progression [117, 118]. It has also been shown that PDGFR inhibition reduces cell proliferation [115, 116, 214]. Our study presented that PDGFRβ instead of PDGFRα induce cell proliferation via c-Myc and regulate metastasis via CD44 in MB. Hence targeting PDGFRβ signal pathway to treat MB patients can be more beneficial for prolonged survival. Earlier results have demonstrated that while PDGFRαα specifically phosphorylates Crk molecules and RasGAP is specifically phosphorylated by PDGFRββ [331-333]. In-depth study of these molecules and their pathway might assist in deciphering the distinct functions played by PDGFRα and PDGFRβ in MB. Discovery of novel drugs specifically targeting PDGFRβ can distinctly limit MB cell migration and invasion. SJ001 is one such PDGFR inhibitor identified in our lab that has shown a great potential in inhibiting MB cell growth [214]. Also, *in vivo* studies by knocking out either PDGFRα or PDGFRβ to measure the effect on tumor progression can be performed to further elucidate the specific functions in MB.

Furthermore, study of specific downstream molecule like CD44, specifically regulated by PDGFRβ, has exhibited its crucial role in MB metastasis. Also, physical interaction between PDGFRβ and CD44 has been demonstrated in the fibroblast cells [389]. CD44 is a cell surface adhesion protein which has been linked to metastasis in numerous cancer forms [349, 354, 356, 357]. *In vivo* studies have also demonstrated down-regulation in metastasis by knocking down CD44 [401, 402]. In our studies we noticed that overexpressing CD44 in uncertain for metastasis MB cells induced them to have increased invasive ability when compared to control cells. By knocking out CD44 and measuring tumor metastasizing to other organs by *in vivo* studies can be performed to confirm that CD44 plays a crucial role in MB metastasis. Also, to further confirm the importance of CD44 in Mb CD44-expressing cell lines such as ONS76 and UW228 can be used to knockdown CD44 and check for the invasion *in vitro* [403, 404]. Simultaneously, non-adherent cell lines just like D283 such as D341 and D425 can be analyzed further for the baseline expression of CD44 [338, 405]; and overexpress CD44 if undetectable levels of CD44 are obtained to check for the change in their invasive abilities caused by CD44. Hence, targeting CD44 can prove to be beneficial in the treatment of MB.

In our results we identified that c-Myc regulates CD44 by interacting with its promoter region, thereby regulating MB invasion via CD44. We also demonstrated that by inhibiting c-Myc, MB cell proliferation reduces significantly. Co-inhibition of c-Myc along with PDGFRβ exhibited an additive inhibitory effect on both cell proliferation and migration both by using siRNAs and inhibitors. Co-inhibition experiments also verified that both PDGFRβ and c-Myc molecules regulate Jagged 2 and CDC25A in MB. This

result indicates that co-targeting two important molecules like PDGFRβ, which is crucial for MB cell migration, and c-Myc, which plays a vital role in MB cell proliferation, can provide an alternative approach and a better therapeutic strategy in the treatment of MB.

To further elucidate the mechanism by which PDGFRα and PDGFRβ induce distinct signal pathways in MB, we performed miRNA profiling in the absence of either PDGFRαr PDGFRβ. Few miRNAs such as miR-1280 and miR-1260 were identified that were differentially regulated by either of the receptors. Similarly, to identify specific miRNAs regulated by both PDGFRβ and c-Myc we performed miRNA profiling in PDGFRβ^{KD}, c-Myc^{KD} and PDGFRβ^{KD}c-Myc^{KD} cells. Interesting ~30 different miRNAs were identified, of which two were miR-1280 and miR-1260. Specific inhibition of these miRNAs demonstrated mainly two things, they regulate MB cell proliferation and migration and also that miR-1280 targets Jagged 2 and miR-1260 targets CDC25A specifically.

Jagged 2 is an important ligand that binds to notch receptor to induce notch signaling [378, 381]. Notch signaling is a crucial pathway during embryogenesis, aberrations in which can induce MB formation [79, 89, 95]. Our study demonstrated that specific knockdown of Jagged 2 resulted in down-regulation of both MB cell proliferation and migration. This data clearly indicates that Jagged 2 is a potential therapeutic target in MB.

The information gained from our study provides new insights into the possible mechanisms by which PDGFRs regulate MB metastasis and progression. It also provides us with novel therapeutic strategies in the treatment of MB. To our knowledge, this is the first study that indicates PDGFRβ-CD44 axis to control metastasis in MB and co-

targeting PDGFRβ and c-Myc to provide enhanced inhibition of MB cell progression via Jagged 2.

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