THE ROLE OF CELL ADHESION GENES IN THE PATHOGENESIS OF MEDULLOBLASTOMA

by

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Abstract

Medulloblastoma is the most common pediatric brain tumour, yet many of the underlying genetic and epigenetic factors have yet to be discovered. After a genome wide screen of a large cohort of primary medulloblastomas, we discovered that many of the genes within the cell adhesion family are affected by either copy number loss and/or decreased expression unexplained by copy number change. This led us to believe that both genetic and epigenetic factors were affecting this gene family. Through methylation-specific PCR, RT-PCR and high-throughput methylation status analysis, we have concluded that promoter CpG methylation plays a role in the expression of the PCDH10 protein in both medulloblastoma cell lines and primary tumours. Through functional validation with a stable cell line re-expressing PCDH10, I show that cell cycle and proliferation remain unchanged but migration is decreased in cells with PCDH10 re-expression. This suggests that PCDH10 has characteristics of a tumour suppressor in medulloblastoma.
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Table 1: Medulloblastoma subgroups

List of abbreviations

APC Adenomatosis Polyposis Coli
APS Ammonium Persulfate
CDH1 Cadherin 1
CDH2 Cadherin 2
CDK Cyclin-dependent kinase
CHIP Chromatin Immunoprecipitation
CNS Central Nervous System
CpG Cytosine/guanine dinucleotide
CTNNA1 Alpha-catenin 1
CTNNA2 Alpha-catenin 2
CTNNA3 Alpha-catenin 3
CTNNAL1 Alpha-catulin 1
DAC 5-aza-2'-deoxycytidine, Decitabine
DAPI 4',6-diamidino-2-phenylindole
DMEM Dulbecco modified Eagle's minimal essential medium
DNMTs DNA methyl transferases
dNTPs Deoxyribonucleotide triphosphate
EDTA Ethylenediaminetetraacetic acid
EGL External Granule cell Layer
EtOH Ethanol
FACS  Fluorescence-activated cell sorting
FBS  Fetal Bovine Serum
G418  Geneticin
GCPs  Granule Cell Precursors
GFP  Green Fluorescent Protein
GISTIC  Genomic Identification of Significant Targets in Cancer
HA  Hemagglutinin
HBSS  Hank’s Buffered Salt Solution
HDAC  Histone deacetylases
HGF  Hepatocyte Growth Factor
IF  Immunofluorescence
IGL  Internal Granule cell Layer
LOH  Loss of Heterozygosity
MALDI-TOF  Matrix-assisted Laser Desorption/Ionization Time-of-Flight
MB  Medulloblastoma
MeDIP  Methylated DNA immunoprecipitation
miRs  microRNAs
MQ  Milli-Q water from Milli-Q Purification System
MSP  Methylation Specific PCR
MTS  (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)
     2H-tetrazolium)
NSCs  Neural Stem Cells
P3  Postnatal day three
PBS  Phosphate Buffered Saline
PCDH10  Protocadherin 10
PCR  Polymerase Chain Reaction
PI  Propidium Iodide
PTCH1  Patched homolog 1
qRT-PCR  Quantitative Reverse-transcriptase Polymerase Chain Reaction
RIPA  Radio Immunoprecipitation Assay Buffer
RT-PCR  Reverse Transcriptase Polymerase Chain Reaction
SDS  Sodium dodecyl sulfate
SFM  Serum-free media
SHH  Sonic Hedgehog
SNP  Single Nucleotide Polymorphism
SOC  Super Optimal Broth
sPNET  Supratentorial Primitive Neuroectodermal tumour
TBST  Tris-Buffered Saline with Tween 20
TSA  Trichostatin A
TSG  Tumour Suppressor Gene
WNT  A combination of Wg (wingless) and Int, homologous genes
Chapter One: Introduction
1.0 Introduction

1.1 Medulloblastoma

Medulloblastoma, a tumour of the cerebellum, is the most common malignant brain tumour in children [1, 2]. The five-year survival rate of medulloblastoma patients has surpassed 60% [3]. Despite this hopeful statistic, many survivors of medulloblastoma face a diminished quality of life, due to detrimental effects to the developing brain. The cerebellum is the coordination centre of the brain, which uses sensory inputs from the periphery to fine-tune movement and balance [4, 5]. Medulloblastoma is more prevalent in males, and has an average age of onset of seven years of age [2, 6]. A dangerous characteristic of medulloblastoma is its predilection to metastasize through the cerebrospinal fluid [2]. There are several familial genetic conditions that predispose to medulloblastoma, including Turcot syndrome and Gorlin syndrome. Turcot syndrome results from a mutation in the APC gene, which leads to an accumulation of the gene Beta-catenin (CTNNB1) in the nucleus, allowing an increase in transcription of several known proto-oncogenes, including C-MYC [7]. Gorlin syndrome is characterized by frontal and temporoparietal bossing, hypotelorism and a predisposition to basal cell carcinoma and medulloblastoma [8]. Gorlin syndrome is caused by a mutation in the Patched gene, found on chromosome 9q [8]. Although these familial syndromes have helped fuel the discovery of several genetic pathways that are frequently affected in medulloblastoma, including the Wnt and Sonic Hedgehog (SHH) pathways, our SNP array studies suggest that this only accounts for 40% of cases. This means that the majority of cases are sporadic and genetic and epigenetic causes remain unknown [2, 9, 10].
1.1.1 Clinical behaviour of medulloblastoma

Medulloblastoma patients are currently stratified into two groups: standard-risk and high-risk. High-risk patients are children that are less than three years of age, have a residual tumour mass after surgery that is greater than 1.5 cm\(^2\), or metastatic disease at diagnosis [6]. Due to tumour growth and compression of the fourth ventricle, children with medulloblastoma frequently present with signs of intracranial pressure. Signs of intracranial pressure include early morning headaches with vomiting, irritability and lethargy. Ataxia, manifested as an unsteady gait, is another common clinical sign of medulloblastoma, since many paediatric medulloblastomas arise in the cerebellar midline. Medulloblastoma patients are treated with maximum surgical resection, radiation, followed by chemotherapy [1, 9]. Neurocognitive difficulties can occur in medulloblastoma patients after treatment, and is most common in patients under seven years of age. This age group shows a significant decline in IQ compared to control patients. Factors that can play a role in these neurocognitive deficits include gender, age at treatment, level of pre-treatment intellectual function and total dose of radiotherapy [1]. Few studies have looked at the long-term impact of these treatments. Most focus on three to five year survival and effects, but it has been shown that medulloblastoma patients are detrimentally affected by their therapy into their adult lives [11]. Affecting both the endocrine and nervous systems, there is only a small minority of patients that are free of sequelae [11]. In addition to the neurocognitive difficulties seen in survivors, interference with perception and cognition can contribute to social isolation and difficulties in achieving employment, as well as reduced fertility [11]. Genetically, not all medulloblastomas are similar; subgroups differ in their prognosis and likelihood of metastases [9, 10, 12].
1.1.2 Medulloblastoma cytogenetics

The most frequent genetic aberration in medulloblastoma is isochromosome 17q (i17q) [9, 13]. This i17q event appears to be somewhat unique to medulloblastoma, as it is not seen in any other neuroectodermal tumours, such as ependymomas, cerebellar astrocytomas, haemangioblastomas, supratentorial neuroblastomas and ependymoblastomas. This i17q event is seen in Acute Myeloid Leukemia (AML) [14]. Isochromosome 17q is the loss of 17p arm and gain of 17q, so the result is one p arm and 3 q arms. Even though this is the most frequent event, the target genes of this aberration are still unknown [15]. Chromosome 17p is lost in 30-50% of medulloblastomas [15]. In tumours with i17q, an extra copy of chromosome 7 is often seen as well [15]. Additionally, deletions of the chromosomal regions 10q and 11 are recurrent events in medulloblastoma. Loss of a sex chromosome is also common, as well as unbalanced translocations resulting in an extra copy of 1q and loss of 1p. Deletions or unbalanced translocations are also seen in chromosomes 8 and 16q. Loss of 9q is correlated with good prognosis and is not seen in combination with 17p deletions, suggesting that there are multiple pathways to the initiation of medulloblastoma [15]. Using molecular techniques, such as DNA-microarray analysis, it is possible to distinguish between different molecular subsets of the disease, which differ in terms of prognosis. This technology may aid the future of gene-based predictors of treatment plans and therapeutic response [16].
1.1.3 Genetic alterations in medulloblastoma

Cancer is a disease of the genome, and medulloblastoma is no exception, possessing a distinctive gene-expression profile [16]. Disruptions in genes involved in the SHH pathway, the Wnt pathway and the Notch signalling pathway are all common genetic alterations found in medulloblastoma (Figure 1 from Radtke et al, 2003 [17]).

![Diagram of Wnt and SHH pathways](image)

Figure 1: (A) The Wnt pathway. B-catenin is usually found in a complex with APC and GSK3B, which targets B-catenin for degradation in the cytoplasm. When any of the components of this pathway are not functioning properly, B-catenin enters the nucleus where it activates downstream targets. (B) The SHH pathway. In the absence of SHH, PTC (also known as PTCH1) represses SMO. When SMO is not repressed, it signals the transcription of GLI1 and other downstream targets. Figure from Radtke et al, 2003 [17].

When bound to the PTCH1 receptor, the Sonic Hedgehog protein allows the inhibition of Smoothened to be relieved, thus allowing transcription of genes such as BMI1, which increase
cell proliferation, since BMI1 is involved in cell cycle regulation through p16INK4A and p19ARF [18]. Mutations in the SHH pathway are detected in approximately 35% of sporadic medulloblastomas, including mutations in PTCH1, SUFU, SMO, leading to an increase in pathway activity [3]. The loss of chromosome 17p occurs in 30-50% of medulloblastomas, although it is unresolved which gene or genes are the important missing factors on this deleted chromosome arm [3]. Nuclear CTNNB1, indicative of a mutation in the Wnt pathway, is found in approximately 9% of sporadic medulloblastomas. The Wnt pathway leads to the breakdown of CTNNB1 within the cytoplasm. When CTNNB1 is not broken down in the cytoplasm, it enters the nucleus and causes an increase in downstream targets such as the oncogene, CMYC. Other mutations in the Wnt pathway include mutations in both pathway components APC and AXIN [3].

Medulloblastomas are genetically heterogeneous tumours which can be divided into four or five categories, based on unsupervised hierarchical clustering [9, 12]. This contradiction between research groups concerning the number of medulloblastoma subgroups may be a result of the batch effect, different methods of clustering or different use of statistical analysis. Both Northcott et al. and Kool et al. identify subgroups A and B to consist of the tumours with changes in the Wnt pathway and SHH pathway, respectively. The disagreement lies within subgroups C, D and E [9, 12]. This subgroup data is summarized in Table 1.

<table>
<thead>
<tr>
<th>Research group</th>
<th>Sample number</th>
<th>Subgroup</th>
<th>Associated genes/pathway</th>
<th>Clinical relevance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kool et al.</td>
<td>n=60</td>
<td>A</td>
<td>Wnt pathway</td>
<td>Best prognosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>SHH pathway</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>Neuronal differentiation genes</td>
<td></td>
</tr>
</tbody>
</table>
Table 1: Subgroup data from Northcott et al. and Kool et al., describing the putative subgroup divisions among medulloblastomas, based on genetic data.

<table>
<thead>
<tr>
<th>Group</th>
<th>Genetic Changes</th>
<th>Prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Wnt pathway</td>
<td>Best prognosis</td>
</tr>
<tr>
<td>B</td>
<td>SHH pathway</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>High MYC and miR-17/92 expression</td>
<td>Worst prognosis</td>
</tr>
<tr>
<td>D</td>
<td>Low MYC</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>Photoreceptor genes</td>
<td></td>
</tr>
</tbody>
</table>

Each group has specific genetic changes that make it unique and have an effect on clinicopathological features. Both studies by Kool et al. and Northcott et al. agree that individuals in group A, have a better prognosis, and thus may benefit from a less rigorous treatment, as to not have such damaging effects on the developing CNS. These patients have overexpression of genes in the Wnt pathway and its target genes, such as MYC, as well as mutations in APC and CTNNB1. The majority of these patients have lost a copy of chromosome 6 [9]. Tumours in Group B show overexpression of genes involved in SHH signalling, such as PTCH1 and GLI2. As well, many of these cases have lost part of chromosome 9q. Both clusters A and B share an increased expression of genes involved in cell cycle and the Notch pathway [9]. Using Kool et al.’s division of medulloblastoma subgroups, it was shown that groups C and D show increased expression of neuronal differentiation genes; D and E show increased expression of genes normally expressed in retinal photoreceptor cells; categories C, D and E are closely related, but currently no known pathways or biological processes are activated or
changed in these groups [9]. Northcott et al. have shown that group C and D frequently have a loss of chromosome 17p and gain of 17q. This is the most frequent genetic aberration in medulloblastoma, but the genes that drive medulloblastoma on this aberration are still unknown. The majority of female patients also showed loss of one of their X chromosomes in the tumour [9]. Kool et al. state that groups C, D and E are much more likely to develop metastases and this knowledge is clinically significant [9]. Since many recurrent chromosomal losses and gains have been reported, it is clear that a significant portion of medulloblastoma cases are genetically driven, however many patients do not have a clear genetic underlying cause to their disease, which must be further investigated for a better understanding of this disease, or diseases [9].

1.1.4 Aberrant expression in medulloblastoma

Mutations in the SHH pathway in PTCH1, SUFU or SMO can all lead to expression of an oncogenic transcription factor, GLI1 [19, 20]. It has been shown that inactivation of GLI1 significantly reduces spontaneous formation of medulloblastoma, and it was concluded that this gene plays a functional role in the development of medulloblastoma [20, 21].

Many tumours overexpress targets of the SHH pathway, including N-MYC, C-MYC, and CYCLIN D1 by at least five-fold [20, 22]. C-MYC and N-MYC genes are amplified in 10% of medulloblastomas [3, 20], aberrant expression of these genes has been shown to lead to tumourigenesis [23].

BMI1 is also regulated by the SHH pathway; BMI1 promotes cell proliferation in cerebellar granule cell cultures and was found to be overexpressed in a large percentage of human
medulloblastomas [3, 5]. *BMI1* is an identified oncogene in lymphoma, and is overexpressed in other cancers, such as colorectal cancer and non-small cell lung carcinoma [5, 24, 25]. Overexpression of *HER2* in medulloblastoma correlates with a poor clinical outcome, and this gene is overexpressed in approximately 40% of cases [3, 26]. *HER2* increases migration of medulloblastoma cells, thus increasing the likelihood of metastasis [3]. Also known as *ERBB2*, *HER2* is a therapeutic target for some breast cancers, but since the expression level of *HER2* is much lower in medulloblastoma than in breast cancer, medulloblastoma is not susceptible to the current monoclonal antibody treatment [26]. T-cells genetically engineered to express a *HER2*-specific antibody, recognize and kill medulloblastoma cell lines as well as an orthotopic, xenogenic mouse model. This promising therapy may be a plausible approach to treating *HER2*-positive tumours in the future [26]. Mice lacking both *P53* and *Ink4c* genes present with medulloblastomas. *Ink4* proteins inhibit the CDK family, which play a role in regulating exit from the cell division cycle. *Ink4c* seems to be detected mainly in proliferating neurons as they exit the cell cycle, and *Ink4c* has been shown to be methylated in the promoter region of medulloblastomas [27]. It was also shown that medulloblastomas rarely form in *P53*-null mice or *Ink4c*-null mice but when both genes are knocked out in combination, a significant number of medulloblastomas arise, suggesting that *Ink4c* is not an initiating event, but may play a role in progression [20]. The transcription factor *SOX4* is known to play a role in normal CNS development, and has been shown to be overexpressed in multiple malignancies, including medulloblastoma [28-30]. *SOX11* was also shown to be overexpressed in medulloblastoma compared to normal controls [28-30]. Another gene that is amplified in medulloblastoma is *OTX2*. One study found levels of *OTX2* to be 140-fold higher in medulloblastoma tumours then in normal cerebellum [28]. These findings
are similar to other studies on OTX2 levels in medulloblastoma [29]. OTX2 is expressed in neural cells during cerebellar development, and OTX2-/− mice are embryonic lethal [29].

There are a large number of identified genes that show aberrant expression in medulloblastoma, but the important underlying question that has yet to be answered, is whether these genes are driving the initiation or progression of the disease, or are mere passengers in driver events.

1.1.5 Medulloblastoma epigenetics

Epigenetic events that lead to deregulated cell growth have been shown to have a significant role in the pathogenesis of a multitude of human cancers, including medulloblastoma [31-34]. CpG islands are small stretches of DNA, 0.5 to several kilobases long, of repeating cytosine and guanines, commonly found in the promoter region of a gene [33]. A methyl group can be attached to the cytosine of a CG site by a covalent bond, thus blocking transcription factors from binding to their target sites, which inhibits transcription of the gene [35, 36]. Fifty to sixty percent of genes in the human genome have a CpG island in their promoter region, aberrant hypermethylation of these CpGs are common in human cancer [36]. Global hypomethylation along with hypermethylation of CpG islands in promoter region is seen, but it is unknown whether or not these two events are caused by a common underlying mechanism [36].

There are three genes that are known to comprise the methylation machinery, DNMT1, DNMT3a and DNMT3b. DNMT1 is for maintenance methylation, and de novo methylation is implemented by DNMT3a and DNMT3b [34, 36]. It has been shown that proper DNMT1 expression in the developing CNS is vital for survival, as Dnmt1 deletion in murine embryos resulted in DNA hypomethylation and death immediately after birth, but conditional deletion in postmitotic
neurons did not affect survival [34]. It has been shown that there is a slight increase in expression levels of all three of the DNMTs in cancer, although it is still under investigation as to what the initiation event of CpG hypermethylation in cancer may be [33, 37]. Investigating the epigenome through candidate gene approaches, as well as through the genome-wide search strategy, has revealed many genes showing an epigenetic-dependent regulation [31]. The candidate gene approach tests well-known cancer genes for methylation, which has brought to light the epigenetic silencing of many genes involved in cell cycle regulation, DNA repair, drug resistance, apoptosis, angiogenesis, invasion and metastasis [36]. Using a genome wide approach, new genes can be discovered, not previously known to play a role in the pathogenesis of cancer. For example, a whole-genome search for epigenetic alterations in metastatic breast cancer allowed researchers to map the changes in epigenetics of the metastases compared to the initial primary lesion, to better understand the transition to an invasive cancer [38]. Commonly methylated genes in medulloblastoma are RASSF1A include HIC1 [39, 40]. RASSF1A induces cell cycle arrest and HIC1 encodes a transcriptional repressor that is believed to play a role in targeting DNA damage response [39, 41]. These genes are also methylated in other CNS tumour types such as ependymoma, glioma and sPNET [39, 42, 43]. In medulloblastoma the methylation of p16, p14, TIMP3, p15, DAP4K, CASP8, MGMT is seen. CDH1 methylation is controversial [31, 39]. CDH1, also known as E-Cadherin, is a known tumour suppressor gene and is shown to be methylated in cancers such as breast, thyroid and gastric cancer [33]. In one study, CDH1 was shown to be unmethylated in 16 primary medulloblastoma samples, but methylated in three cell lines [31]. Contradictory to this, another investigation showed that CDH1 was methylated in 31/41 medulloblastoma samples, as seen through Methylation Specific PCR [39]. Three out of thirty-six medulloblastomas were
methylated at the CDH1 promoter in an additional study, including 1/4 cell lines [44]. In another small cohort of medulloblastomas, 0/16 primary tumours and 1/3 cell lines were shown to be methylated [45]. The question of whether or not the methylation of the promoter of CDH1 plays a role in the pathogenesis of medulloblastoma needs further interrogation, with larger cohorts of primary tumours.

CASP8, a key player in the apoptosis pathway, is another gene which comes under controversy for its methylation status in medulloblastoma. Multiple studies have shown that it is methylated in a proportion of medulloblastomas, but this ranges from 36% to 90% of primary tumour samples [39, 44, 46]. Epigenetic silencing of this gene has also been associated with poor prognosis in medulloblastoma patients, while an HDAC inhibitor, Valproic acid, and interferon gamma, seem to restore molecular CASP8 expression, suggesting that histone-mediated silencing may play a role [47]. The methylation status of MGMT, a DNA repair gene, has also come under scrutiny, with multiple cohorts ranging in methylation from 0% to 76% of primary medulloblastomas [44, 45, 48].

The S100 gene family of calcium binding proteins have been associated with multiple malignant tumour types. This gene family is involved in the regulation of cell cycle and cell growth and motility [32]. Four members of the S100 family, S100A2, S100A4, S100A6, S100A10, showed methylation changes in primary tumours and cell lines compared to normal cerebellar controls. Several X-linked genes showed a sex-specific disruption in their normal methylation patterns in medulloblastoma. These include MSN, POU3F4, and HTR2C [31]. These genes are hemi-methylated in normal female cerebellum from X-inactivation, and unmethylated in normal male cerebellum. In medulloblastoma, they show complete methylation. This is seen only in male
patients [31]. Further, other genes that show promoter CpG methylation in medulloblastoma include *COL1A2, HTATIP2, ZIC2* and *SGNE1* [31, 49, 50].

An inhibitor of the invasive growth HGF/Met pathway, *SPINT2* has been identified as a putative tumour suppressor in medulloblastoma. It is epigenetically silenced through promoter methylation in 34.3% of primary medulloblastomas examined [51]. Not only were there hemizygous deletions found where *SPINT2* maps to in the genome, there were also gains on chromosome 7 where HGF/Met loci are found [51]. Re-expression of this gene reduced proliferative capacity and increased overall survival in xenografts, suggesting that this gene plays a role in the pathogenesis of medulloblastoma [51].

1.2 Granule cells

Granule cells are the most abundant neuron in the CNS, they account for almost half of all of the neurons in the human central nervous system [52-54]. The murine cerebellum contains about $10^8$ granule cells [55]. Transplantation experiments have shown that granule cells arise solely from the External Granule Cell Layer (EGL) [56, 57].

1.2.1 Granule cell development

Granule cell precursors, originate from the rhombic lip in the developing cerebellum [5]. These cells migrate over the cerebellum and form the External Granule Cell Layer (EGL) at the end of the embryonic period [52]. From the outer EGL, granule neuron precursors migrate to inner EGL. These cells are postmitotic and premigration [52, 53]. From the inner EGL, they migrate to Internal Granule cell Layer (IGL). Adhesion molecules play a role in this migration [37, 52, 53, 58]. This is a unique feature of granule cells, in that other neuronal cells migrate outwards.
from the ventricles, instead of migrating inwards [55]. In the mouse, the Granule Cell Precursors (GCPs) leave the rhombic lip at E13, to migrate across the cerebellum to the External Granule Cell Layer (EGL). The EGL starts to form at E15 [54]. During the first two to three weeks after birth, the pool of granule cell precursors is significantly increased by extensive proliferation, to generate a large quantity of granule cells. The GCPs then exit the cell cycle and migrate into the Internal Granule cell Layer (IGL) [54, 59]. This increased mitotic rate of the GCPs decreases as development advances [54]. It was shown that granule cell precursors double once per day between E18 and P3, this declines to once every two days from P3 to P6, further declining after this point [54]. It was also shown that GCPs have an increased period of cell division just prior to the time that they differentiate [54]. Approximately three weeks after birth in the mouse, the EGL disappears and all of the GCPs have migrated into the IGL and differentiated into granule cells [55].

1.2.2 Granule cells and medulloblastoma

*MATH-1* is solely expressed in external granule cell precursors, cells which are committed to end up in the external granule cell layer in the cerebellum. The expression of *MATH-1* in medulloblastoma supports that granule cells are the putative cell of origin of medulloblastoma [60]. Further, multiple studies have implicated the SHH pathway in regulating the proliferation of granule cell precursors and as a major mutational target in medulloblastoma [55]. Treating cerebellar cultures with SHH inhibits granule cell differentiation and migration, as well as significantly increasing the proliferative response of the GCPs. In addition, injecting a SHH-blocking antibody intracranially into mice, leads to a decreased proliferation of GCPs, these observations suggest that SHH is a vital regulator of GCP proliferation [55].
One of the first oncogenic miRs to be validated was the miR-17-92 cluster [61]. miR-17-92 is expressed in the developing cerebellum and GCPs, but not in differentiated granule cells [62]. This miR-17-92 cluster is amplified in human medulloblastomas [62]. This study provides evidence the SHH pathway and the miR-17-92 gene cluster concomitantly contribute to the progression of GCPs into medulloblastoma [62].

Another putative cell of origin of medulloblastoma lies within cells from the subventricular zone, as it was shown that deleting Ptch1 in neural stem cells (NSCs) in the ventricular zone leads to medulloblastoma [63]. These Ptch1 deficient NSCs showed increased proliferation but could still differentiate into astrocytes, oligodendrocytes and neurons. Once they had left the ventricular zone and been committed to the granule cell lineage, they went through rapid expansion and formed medulloblastomas. Strengthening this argument is the observation that Ptch1 deletion does not lead to other tumour types [63]. Included in this study was an experimental Ptch1 deletion in GCPs, which also led to medulloblastoma, although at a slower rate then in NSCs [63]. Aside from this evidence, the mechanisms as to how SHH and other pathways regulate granule cells is still poorly understood and is worth investigating in the future, as many questions still remain [55]. Identifying the cell of origin for medulloblastoma is key, so that comparisons can be made between normal and cancerous cells, to gain insight into the changes that take place in initiation, progression and metastasis [63].
1.3 Cell Adhesion genes

Cadherins are a superfamily of genes that are involved in calcium-dependent cell adhesion seen in most tissue types [64]. The cadherin superfamily has over one hundred members, in vertebrates [65]. The majority of cadherins are type I integral membrane proteins and have extracellular domains with a variable number of a repeated domain [66]. These repeated sequence domains contain sequences which are involved in calcium binding [65]. The cadherin gene family can be subdivided into six subfamilies, including classical cadherins I, classical cadherins II, cadherins in desmosomes, protocadherins, cadherins with a short cytoplasmic domain (T-cadherin) and distantly related genes (FAT family cadherins and 7TM-cadherins) (Figure 2) [66, 67].
All classical cadherins contain a highly conserved domain which functions as a binding site for catenins, which anchor the cadherins onto the cytoskeleton [66]. The intracellular domain is not
conserved across subfamilies, and the current theory is that cadherins interact with each other through their cadherin repeats but generate different signals in the cytoplasm through their unique intracellular domains, thus leading to the superfamily’s wide range of functions within cells [65].

Protocadherins are also type I transmembrane proteins, which have weak adhesive properties. They are structurally much more diverse than classical cadherins, and research on their function is only limited to date [68].

An example of a classical cadherin would include E-cadherin (CDH1), a transmembrane protein, which is part of the adherens complex with beta-catenin and alpha-catenin, forming adherens junctions [69]. CDH2 plays a similar role in cell biology as CDH1, acting as part of the adherens complex, attaching to catenins [70]. These two cadherins show 46% similarity at the amino acid level.

1.3.1 The developmental role of adhesion genes

Cadherins are well known for their adhesive properties, but their functions extend to a wide range of roles within the cell. Functions include aspects of tissue morphogenesis, such as cell recognition and sorting, boundary formation and maintenance, as well as induction and maintenance of cell and tissue polarity [68]. In addition, they play a role in mechanically linking hair cells in the cochlea and are involved in neural circuit formation in brain development [68]. It has been implied that different cadherins control the cell sorting of different tissues during development, and both CDH1 and CDH2 are involved in this process in the brain. The mechanisms which underlie this process remain unknown [68]. Certain classical cadherins are
found in specific areas of the embryonic brain and although there is no known functional significance of this, some are even restricted to certain areas of the brain as development progresses. A suggested explanation is that these cadherins send signals to each other, creating a network of cell-cell interactions transmitting different signals and information [68]. Cadherins promote axon fasciculation (muscle twitching), neurite outgrowth, axon elongation, and the evidence for their participation in the formation of synapses, is mounting. Classical cadherins are also known to regulate synaptic plasticity [68]. Clearly, cadherins play a key role in the development of the central nervous system. Protocadherins are primarily expressed in the nervous system, and although many of their functions at the cellular and molecular level have yet to be interrogated, it is clear that they are involved in tissue development [65, 68]. Catenins are also important in the developing brain. Deletion of CTNNΔ2 in the developing mouse brain results in abnormal migration of Purkinje cell precursors in the cerebellum during development [71]. As well, knockout mice show a distorted anterior commissure and olivary nuclear complex, demonstrating that CTNNΔ2 is vital for proper CNS development [71].

1.3.2 Cell adhesion genes and cancer

Loss of adhesion is a hallmark of cancer cells, and cadherins and catenins have been shown to be deregulated in a multitude of human cancers [7, 64, 72]. Deregulation of adhesion molecules can promote many cancer-defining biological processes such as growth, survival, migration and metastasis. Furthermore, cell adhesion molecules have been seen to be overexpressed in drug resistant cells in multiple cancers and have been shown to protect cells from death by radiation, genotoxic chemotherapy, or targeted pathway inhibitors [73].
CDH1 is a known tumour-suppressor in many cancers [69, 74-76]. CDH1 downregulation has been implicated in metastasis; it decreases the strength of cellular adhesion within a tissue, resulting in an increase in cellular motility. This may allow cancer cells to invade surrounding tissues [74, 75]. Furthering this theory, transfection of CDH1 cDNA into mouse mammary tumour cells was shown to reduce cell proliferation in three-dimensional culture, while treatment with anti-CDH1 antibodies stimulated proliferation [77]. CDH1 has also been shown to be methylated in cancer [78]. In a study with 51 medulloblastoma samples, 8% of them were methylated at the CDH1 promoter [44]. An investigation including five medulloblastoma samples showed that all were negative for CDH1 immunoreactivity [64]. Further, a repression of CDH1 expression may also cause an increase in cytoplasmic CTNNB1, which could affect the Wnt pathway, implicating another pathway known to be involved in the pathogenesis of medulloblastoma [68]. Although these studies show that CDH1 may be involved in pathogenesis of medulloblastoma, more clarity on its role is needed. While a tissue-specific knockout of CDH1 in breast cancer did not initiate tumours, in combination with P53, more aggressive and metastatic tumours were seen, suggesting that although CDH1 might not be an initiating factor, it may play a role in progression [74].

When the extracellular domain of Neural-cadherin (CDH2) is deleted, the adhesive properties are gone, but the mutant outcompetes endogenous N-cadherin, taking its place in the adherens junctions [70]. As well, mutant CDH2 can non-specifically block multiple cadherin subtypes, because the cytoplasmic domain is conserved among classic cadherins. This blocks N-cadherin mediated cell-cell adhesion in two ways; it competes for CTNNB1 at the membrane, which would disconnect cadherins from the actin cytoskeleton, as well as downregulating cadherin
production [70]. This method of deleting the extracellular domain is utilized because complete knockout of N-cadherin causes early embryonic lethality, mainly because of heart defects [70]. It has been shown that loss-of-function mutations in alpha-catenin (CTNNA) can consequently result in tumourigenesis. CTNNA1 is part of the adherens junction, attached to CTNNB1 and anchoring the complex onto the cytoskeletal wall [79]. Expression of wild-type protein in cells with loss-of-function mutations leads to tumour suppression and a restoration of growth control [79]. The phenotypic effects in these tumours are generally thought to be due to loss of adhesion [79]. The proliferation from neural progenitor cells is tightly controlled, since CNS-specific deletion of CTNNA1 causes abnormal activation of the Hedgehog pathway, which shortens the cell cycle, decreases apoptosis and causes cortical hyperplasia, similar to supratentorial primitive neuroectodermal tumors (sPNET) [80]. CTNNA2 has a similar structure and function to that of CTNNA1 [79]. CTNNA3 is found mainly in testis and cardiomyocytes but low levels are found within the brain [81]. Similar to the function of CTNNA1, CTNNA3 attaches to CTNNB1 and the cytoskeleton, but CTNNA3 is necessary for the formation of stretch resistant cell-cell adhesion complexes in cells such as muscle cells [81]. CTNNA3 has been implicated in bladder cancer, as monoallelic and biallelic CTNNA3 expression patterns were demonstrated in tumour bladder tissue, whereas normal cases show only biallelic expression [82]. Additionally, it has been shown that CTNNA3 inhibits Wnt signalling, similar to the other catenins, which is a pathway that is commonly deregulated in medulloblastoma [83]. PCDH10 is another member of the cadherin gene family; it encodes a cadherin-related neuronal receptor thought to play a role in the establishment and function of specific cell-cell connections in the brain. In the literature, it is shown to be methylated in other cancers and a well known tumour suppressor gene in cervical, gastric, haematological, nasopharyngeal and esophageal cancers [84-86].
1.3.3 Cell adhesion genes and brain tumours

A small study showed that a significant percentage of meningiomas showed Loss of Heterozygosity (LOH) at the \textit{CDH1} locus, and along with others, has concluded that loss of \textit{CDH1} positively correlates with increased invasion and metastasis [87, 88]. One of the most troublesome characteristics of gliomas is their invasive capabilities; the reason behind this invasive behaviour is thought to be associated with the cadherin gene family. Throughout the literature, \textit{CDH1} is commonly dubbed an invasiveness suppressor, but little is known as to how it interacts with astrocytes that develop into migrating and malignant glioblastomas [87]. In a study by Nikuseva et al., no glial tumour showed LOH in \textit{CDH1}, despite their aggressive characteristics, which conflicts with a previous study showing that increased instability of the cadherin-catenin components was necessary for increased invasion and metastasis in glioblastoma cell lines [87, 89]. A study on meningiomas showed that \textit{CDH1} is present in recurrent tumours if the recurrence is benign, but is lost if the recurrent tumour is morphologically malignant [64]. A well known tumour suppressor in other cancers, \textit{CDH1} has been shown to be correlated with differentiation, invasiveness and metastasis in multiple carcinomas [64].

\textit{CTNNB1} is involved in both cell adhesion and cell signalling in the Wnt pathway, a pathway commonly affected in medulloblastoma [7]. Mutations in the Wnt pathway can lead to nuclear accumulation of \textit{CTNNB1}, leading to transcription of target genes such as oncogenic \textit{CMYC} and cell cycle progressor \textit{CYCLIND1} [7]. Additionally, \textit{CTNNB1} has been shown to be mutated in multiple sporadic medulloblastomas [7]. Although many of the other adhesion genes are involved in other cancers as well as in brain development, there is relatively less literature on
adhesion genes and brain cancer in comparison, in the future, these genes should be investigated for their putative role in the pathogenesis of medulloblastoma.
Chapter Two: An epigenetic screen of adhesion genes in medulloblastoma
Chapter 2: An epigenetic screen of adhesion genes in medulloblastoma

Introduction and Rationale

Our lab performed expression arrays and large copy number studies. Exon arrays from Affymetrix have probes for every exon so that we could assess the expression level of each individual exon, unlike normal gene expression arrays which show 3’ bias. Paul Northcott evaluated over 100 medulloblastoma primary tumours on this exon array; he also assessed over 200 on the Affymetrix 500K SNP array for copy number analysis. From these screens, we found numerous deregulated gene families, including genes involved in cell adhesion. In addition to this, we found numerous cases where decreased expression of cell adhesion genes was not copy number driven. This led us to hypothesize that these changes are caused by epigenetic events.

GISTIC is an algorithm that identifies the statistically significant regions of gain or loss in a cancer genome. $CDH1$ and $PCDH10$ are both frequent targets of focal loss in medulloblastoma, based on large copy number profiling studies done in our lab (Figure 3). The GISTIC formula is $\text{Frequency} \times \text{Amplitude} = \text{Significance}$ [90] (Figure 4). Both the 100K and the 500K SNP array platforms were used to identify copy number aberrations in the medulloblastoma genome and both found several of our candidate adhesion genes to fall in an area of significant focal loss.

Through copy number profiling, GISTIC and expression arrays, we have arrived at this subset of adhesion genes which may be important in medulloblastoma (MB) tumourigenesis: $CDH1$, $CDH2$, $CTNNA3$, $CTNNA1$ and $PCDH10$. $CDH1$ shows frequent single copy loss in our data, in addition to being a known tumour suppressor gene (TSG) in other cancers [69, 91-93]. $CDH2$ is methylated in other cancers [94, 95]. It was shown that in chickens, deleting the extracellular domain of $CDH2$ causes it to be non-functional and replaces endogenous cadherins [70]. $CTNNA1$ has been associated with numerous cancers, playing a role in the growth and
progression of human myelodysplastic syndrome (a preleukemic disorder) and acute myeloid leukemia, the tumorigenesis of thyroid carcinoma and the migration of colonic cancer cells [96-98]. Across a large cohort of primary medulloblastomas, the expression profile of $CTNNA1$ showed a significant decrease in tumours compared to normal controls. Additionally, $CTNNA3$ is homozygously deleted in one of our primary medulloblastoma samples (Figure 5). As the evidence implicating cell adhesion genes mounts in the development of medulloblastoma grows, further investigations of their role are essential.

![Figure 3: 100K and 500K SNP array GISTIC analysis identifying copy number aberrations, showing that $CDH1$ and $PCDH10$ are in regions of statistically significant loss. Each array analyzed a different subset of primary medulloblastomas, since we originally used the 100K when it was the highest resolution available, and took advantage of the 500K resolution when the technology was released. The subset of medulloblastomas used on the 500K SNP array were]
primary tumours that we did not have in our possession when we ran the 100K SNP array. The x-axis represents the Q-value, which is a measure of whether amplified or deleted regions are of statistical significance, according to the GISTIC algorithm.

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**GISTIC Formula: Frequency X Amplitude = Significance**

Figure 4: GISTIC algorithm for calculating statistically significant regions of gain and loss within the cancer genome [90].

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Figure 5: *CTNNAL3* region of genomic focal loss. The red and blue lines represent each allele, and the deletion maps to 10q21.3. FISH stands for Fluorescent in situ hybridization.
Hypothesis

I hypothesize that genetic and epigenetic aberrations in cell adhesion molecules contribute to the initiation, maintenance and progression of medulloblastoma.

Materials and Methods

**Methylated DNA Immunoprecipitation Arrays**

DNA was sheared into fragments of approximately 400-600 base pairs. It was then denatured into single stranded DNA, to improve binding affinity of the antibodies. 5-methylcytidine antibodies, which attach to a methyl cytosine, were added, and magnetic beads conjugated to anti-mouse-IgG were used to bind the methylcytidine antibodies. The unbound DNA can then be separated out in the supernatant, enriching for methylated DNA. Proteinase K is then added to digest the antibodies and release the DNA to be used as the methylated fraction. Each fraction, unbound (unmethylated) and bound (methylated) are treated with a different fluorescent dye (red or green) and unmethylated and methylated fractions are hybridized to an array, and presence and relative quantities of fluorescence is detected. Reagents were from Eurogentec. This technique is highly sensitive and can detect down to at least two CG dinucleotides [99].
**DAC (5-aza-2'-deoxycytidine) treatment**

DAC is a cytosine analogue that cannot be methylated. As the cell divides, more DAC is incorporated into the DNA and we can see a restoration of expression in genes that were silenced by methylation. Medulloblastoma cell lines were grown in DMEM mixture (which includes 500ml DMEM, 50ml FBS and 5ml antibiotic), 10cm dishes, in 8ml of media. Cells were split and passaged using 1ml trypsin/EDTA in each 10cm dish, left on for 5 minutes, then diluted and passaged to new, sterilized plates. Medulloblastoma cell lines were treated with 5’-methyl-azacytidine for three days at which point DNA, RNA and protein was extracted. Each 10cm dish was treated with 5uM of the drug each day. Control plates were grown in equivalent conditions, without drug treatment. For protein extraction, cells were lysed using RIPA lysis buffer, with the Complete Mini protease inhibitor tablet (Roche). Dishes were washed with 8ml of cold PBS while on ice and then incubated on ice for twenty minutes with RIPA and protease inhibitors. The dishes were then scraped and the cells in media were pipetted into Eppendorf tubes. Protein was then mixed with 2X loading gel at a 1:1ul ratio then boiled at 98°C for 5 minutes to stabilize the protein. The protein was then stored at -20°C.

**Reverse Transcriptase-PCR**

**RNA extraction:** Tissue culture cells in 3.5cm dishes were lysed with 1ml of Trizol (Invitrogen). Cells were spun at 12,000g for 10 minutes at 4°C. They were incubated for 5 minutes at room temperature, followed by the addition of 200ul chloroform (per 1ml Trizol), cells were shaken vigorously for 15 seconds, incubated two to three minutes at room temperature, then centrifuged at 12,000g for 15 minutes at 4°C. The aqueous phase (containing RNA) was then transferred to a new tube and 500ul isopropyl alcohol was added. After 10 minutes of incubation at room temperature, the tubes were centrifuged at 12,000g for 10 minutes at 4°C. The supernatant was
then removed and the pellet was washed with 1ml 75% EtOH, vortexed and centrifuged at 7500g for 5 minutes at 4°C. The pellet was then briefly dried, water was added and then concentration of RNA was measured.

**RNA to cDNA:** 2ug of RNA was combined with 8ul GIBCO H$_2$O, on ice. Add 10ul 2X Reverse Transcriptase buffer, 2ul Superscript III (SSRTIII) and run in the PCR machines using the following program. 1. 50°C for 2 minutes. 2. 94°C for 2 minutes. 3. 94°C for 15 seconds. 4. 63.3°C for 30 seconds. 5. Go to step 3, 39 times. 6. Store at 4°C.

The product was then transferred to an Eppendorf tube. 80ul of GIBCO H$_2$O was added, resulting in 100ul of 20ng/ul cDNA. The cDNA was stored at -20°C.

**RealTime PCR:** In each well, 0.5ul of 20ng/ul cDNA was combined with 11.0ul H$_2$O, and the combined product was added to a mixture of 12.5ul Sybr Green (Invitrogen) with 0.5ul of each the forward and reverse primer included. Real time is run using the following conditions.

The RT PCR was run under the following conditions: 1. Incubate 50°C for 2 minutes. 2. Incubate 95°C for 2 minutes. 3. Incubate 95°C for 15 seconds. 4. Incubate 59°C for 30 seconds. 5. Plate read. 6. Cycle to step 3 for 39 times. 7. Melting curve from 60-95°C, read every 1 degree, hold 1 second. 8. End.

Each of these experiments was performed in triplicate and repeated at least twice.

**Western Blotting**

8 or 10% Acrylamide gels were used, depending on the size of the expected protein. 8% gels consist of two entities, the separating gel and the stacking gel. Separating gel consists of the following: 30% Acrylamide (4ml), 4X Tris-HCl/SDS, pH 8.8 (3.75ml), MQ H$_2$O (7.25ml), 10% APS (50ul), Temed (10ul). Stacking gel is added after separating gel has solidified, in the
following concentrations, 30% Acrylamide (650ul), 4X Tris-HCl/SDS, pH 6.8 (1.25ml), MQ H$_2$O (3.05ml), 10% APS (25ul) and Temed (5ul).

Running buffer: 900ml MQ water + 100ml Tris 10x SDS. The gel is run according at 150Volts over one hour. Transfer buffer: 200ml Methanol, 700ml MQ water, 100ml Transfer buffer. Transfer at 90Volts for 90 minutes on ice. Blocking was carried out for one hour at room temperature in 5% milk (Bioshop). Membranes were then incubated overnight at 4°C in primary antibody. Following wash cycles in TBST, membranes were incubated for 30 minutes at room temperature in secondary antibody. After washing the membrane in TBST, it was incubated in 1ml of chemi-luminescence (500ul each), for two minutes. The membrane was then developed in dark room onto film.

**Building a CDH2 dominant negative construct**

N-cadherin was originally in the pCMVsport6 plasmid (Open Biosystems). Primers were designed so that the majority of the extracellular domain would be deleted. These primers were exceptionally long, to account for the addition of a Kozak site [66]. In order for the primers to have enough time to anneal, the PCR was adjusted accordingly. PCR conditions: 1. 94°C for three minutes. 2. 94°C for thirty seconds. 3. 57°C for thirty seconds. 4. 72°C for 1 minute 45 seconds. 5. Cycle to step 2, 39X. 6. 72°C for five minutes. 7. Store at 4°C. The following amounts of each were used in the PCR reaction.
<table>
<thead>
<tr>
<th>H₂O</th>
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<tr>
<td>MgCl₂</td>
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<td>dNTP</td>
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<td>PCR 10X buffer</td>
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<td>DNA</td>
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<td>HiFidelity taq polymerase (Invitrogen)</td>
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<td>Forward primer</td>
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<td>Reverse primer</td>
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The construct was sequenced to verify that it had been amplified in the correct sequence and then cloned into pcDNA3.1 topo vector using BamHi and HpaI restriction enzymes (New England Biolabs) and TOP10 E-coli competent cells (Invitrogen). Colonies were picked, and DNA was extracted using Qiagen mini prep kit. To verify proper directional insertion of the PCR product into the plasmid, DNA was digested by enzymes to check the size of the DNA fragments, which indicated the direction of the insert.

DNA was then transiently transfected into hek293 cells using the Fugene system (Roche), in a SFM:fugene:DNA, 97ul:3ul:1ug ratio. The cells were lysed using RIPA lysis buffer as previously described, and the protein was used for western blot. Western blots were carried out according to previous methods, probing for anti-HA to show expression of the construct. GFP was used as a transfection control, and cells were approximately 40% GFP positive.

Immunofluorescence to look for HA, the tag on the protein, was also done, to show expression of the construct.

To test for expression, cells were tested for immunofluorescence on cover slips. Cells were grown on coverslips in 6 well plates. After rinsing the cells twice with PBS, they were fixed with 4% paraformaldehyde in phosphate buffer for 15 minutes at room temperature. Cells were then rinsed with PBS three times, then permeabilized with 0.2% TritonX for 5 minutes. Slides were
then treated with blocking, 5% normal goat serum in PBS, for 30 minutes. After removing the maximum amount of blocking solution possible, anti-HA antibody at 1:500 was added for 1 hour at room temperature. Rinsing three times with PBS, fluorescent secondary antibody was then added for 30 minutes, the cells were then rinsed again with PBS and coverslips were added.

Results

Promoter hypermethylation at the CDH1 locus in medulloblastoma cell lines

In an effort to screen medulloblastoma cell lines for CpG methylation, we used a technique that enriches for methylated DNA called Methylated DNA immunoprecipitation [99]. This DNA was then hybridized to a promoter tiling array method called MeDIP-ChIP. This technique generated a promoter methylation profile for all Refseq promoters and UCSC annotated CpG islands. Upon examination of the CDH1 locus, we found enrichment in numerous medulloblastoma cell lines compared with normal brain control, including D425, D458, ONS76 and Med8A (Figure 6). Each peak is a statistically significant region of enrichment and we arbitrarily chose the peak score cut off of 2, which is a widely used threshold in the field. Since this threshold is arbitrary, we use MeDIP-ChIP as a screening tool and not a confirmatory technique. CDH1 promoter methylation and its effect on gene expression was then validated using RT-PCR on DAC-treated medulloblastoma cell lines.
Figure 6: MeDIP CHIP analysis for CDH1, identifying regions of enrichment in the promoter CpG island. This figure is from chromosome 16, where the top yellow panel represents the CpG site, the following blue line represents the transcriptional start site and the blue panel titled ‘tiled region’ represents the investigated area. This data is from medulloblastoma cell lines, where anything above a peak score of 2.0 is considered a significant amount of methylation.

Re-expression of silenced adhesion genes following DAC treatment

The RNA expression levels of six genes, CDH1, CTNNA3, CTNNA2, CTNNA1 and CTNNAL1 were tested, comparing the RNA expression level of DAC treated cell lines to that of normal, untreated controls, using qRT-PCR (Figure 7-11). Five medulloblastoma cell lines were used for
this experiment, including Med8A, ONS76, UW228, Daoy and D283. Each cell line showed different expression level changes in response to the treatment, but the majority of the genes showed an increase of expression in treated RNA, in at least one cell line. This indicates that these adhesion genes may be downregulated by hypermethylation events.

Figure 7: Every cell line tested showed marked increase in expression level in treated RNA, compared to that of normal RNA. Error bars for this graph were calculated using a calculation of standard error, by dividing the standard deviation by the square root of the number of replicates. Statistically significant differences are represented with the * symbol.

Figure 8: CTNNA3 showed a significant increase in RNA expression in four out of five cell lines, including UW228, ONS76, Daoy and D283. Error bars for this graph were calculated using a calculation of standard error, by dividing the standard deviation by the square root of the number of replicates. Statistically significant differences are represented with the * symbol.
Figure 9: CTNNA1 showed a significant increase in expression levels of CTNNA1 in treated RNA compared to normal controls in two cell lines, UW228 and ONS76. Error bars for this graph were calculated using a calculation of standard error, by dividing the standard deviation by the square root of the number of replicates. Statistically significant differences are represented with the * symbol.

Figure 10: While the majority of the medulloblastoma cell lines showed no significant change in CTNNA2 RNA expression levels, there was a marked increase in the treated RNA from the Daoy cell line. Error bars for this graph were calculated using a calculation of standard error, by dividing the standard deviation by the square root of the number of replicates. Statistically significant differences are represented with the * symbol.
Figure 11: While the majority of the medulloblastoma cell lines showed no changes in expression levels of CTNNAL1, both Daoy and UW228 showed increased expression in the treated cell line RNA, compared to untreated RNA. Error bars for this graph were calculated using a calculation of standard error, by dividing the standard deviation by the square root of the number of replicates. Statistically significant differences are represented with the * symbol.

**CTNNA1 protein re-expression following treatment with DAC**

In an effort to examine stable protein re-expression we sought to investigate the status of CTNNA1 following DAC treatment. A western blot was performed to compare expression in DAC treated cell lines and normal untreated controls (Figure 12). This blot shows that in three of six medulloblastoma cell lines, D283, UW228 and RES256, expression of the CTNNA1 protein was increased in DAC-treated cells, compared to untreated normal controls, suggesting that DNA methylation may have been the cause of decreased protein expression in the untreated normal controls. This could also be seen as a result of non-specific secondary effects of the drug treatment, such as the re-expression of other methylated targets that could be associated with
CTNNA1. The lack of concordance between the effect of DAC on RNA and protein for the CTNNA1 gene could be explained by degradation of post-translational modifications that affect the expression of CTNNA1 (Figure 9 and Figure 12).

Figure 12: CTNNA1 western blot, where (-) represents untreated, control protein and (+) represents the protein of DAC-treated cells. The DAC treatment was done on all cell lines concurrently, and that this is a representative blot, which was repeated three times.

Construction and functional validation of a dominant-negative CDH2 protein in medulloblastoma pathogenesis

The CDH2 dominant negative construct was generated with a HA-tag and sequenced accordingly. It was produced in concordance with the structure of a dominant negative CDH2 construct built by Fujimori et al [70] and shown below (Figure 13).
To ensure that the CDH2 dominant negative protein was being expressed in mammalian cells, I fixed transfected cells on coverslips and stained for HA, as well as DAPI, and demonstrated that CDH2 dominant negative was indeed being expressed. Seen in Figure 14 (A-C), the transfected CDH2 construct cells stain positive for HA, and the untransfected control does not. Both stain positive for DAPI, a fluorescent stain that binds to DNA. This should be confirmed in the future by western blot. In the literature, this dominant negative CDH2 has been shown to be non-functional, but also to replace endogenous cadherins [66]. In CDH2 knockout models, other cadherins can replace CDH2, so the effects of the loss of CDH2 cannot be elucidated [70]. In the future, a murine model will be made with the CDH2 dominant negative construct, to investigate the effect of a non-functional CDH2 that replaces endogenous cadherins in the cerebellum.
Discussion

The RNA expression of five adhesion genes was restored in medulloblastoma cell lines, when treated with DAC, relative to the RNA expression levels of untreated controls. This indicates that their original expression levels may be decreased due to promoter CpG methylation. DNA methylation plays a key role in the initiation and/or progression of many cancers, since promoter methylation can silence transcription of tumour suppressor genes. This phenomenon has been seen in over thirty-seven tumour types and plays a key role in many cancers [31, 33, 35, 36, 44, 45, 86, 100, 101]. Although each of these genes needs to be studied in further experiments to investigate their contribution to the pathogenesis of medulloblastoma, it has been suggested that
the genes within the adhesion gene family are important contributors [58, 64, 73, 78, 82, 87, 88, 102, 103]. Not only has our lab found aberrant genetic changes in these genes, but I have also shown that promoter methylation affects the level of expression in the candidate adhesion genes examined. This is significant because although these genes have been implicated in other cancers, their methylation status in medulloblastoma was uncertain. The next step in this process would be to investigate methylation patterns using a high-throughput methylation analysis technique, over a large panel of primary medulloblastomas. From there, functional work could be done, through the construction of stably expressing cell lines and mouse models. Functional assays should include cell proliferation and cell cycle analysis, as well as migration studies. Methodology of these experiments is discussed in Chapter 3. This would elucidate the functional role, if any, that these genes may play. Although there is still a large amount of work to be done in the future to establish the role that adhesion genes play in the progression of medulloblastoma, through our methylation studies, we see that methylation affects the expression of these genes in medulloblastoma cell lines. As this data looks solely at cell lines, it is important to remember that studies in cell lines have many caveats. For example, many of these cell lines may harbour cell culture artefacts in DNA methylation and portray a poor representation of primary medulloblastomas. Hence, it is vital to compare this data with data from primary tumours, to identify commonalities and rule out any important differences. That being said, this research is a step towards elucidating the role that candidate cell adhesion genes may play in the initiation and/or progression of medulloblastoma.
Chapter 3: *PCDH10* – a putative Tumour Suppressor Gene in Medulloblastoma
Chapter 3: PCDH10 – a putative Tumour Suppressor Gene in Medulloblastoma

Introduction and Rationale

PCDH10 is part of the cadherin gene family; it encodes a cadherin-related neuronal receptor thought to play a role in the establishment and function of specific cell-cell connections in the brain. In the literature, it is shown that PCDH10 is epigenetically silenced, linked to poor prognosis and a known tumour suppressor gene in a multitude of other cancers [84-86, 102, 103]. It was also shown to be a marker of poor prognosis when methylated in patients with gastric cancer, suggesting that PCDH10 could be an important clinical factor [102].

Based on large copy number studies done in our lab, we can see that the PCDH10 locus is a frequent target of focal deletion in medulloblastoma, and the GISTIC algorithm indicates that this loss is statistically significant (Figure 15).
Figure 15: Genomic homozygous focal loss of PCDH10 in a primary medulloblastoma sample. The red and blue lines represent each allele, and the deletion maps to 4q28.3. FISH stands for Fluorescent in situ hybridization.

From these compelling results, we planned to further investigate the methylation and functional role of PCDH10 in medulloblastoma through Methylation Specific PCR (MSP) and RT-PCR to investigate methylation status and level of expression.

Following MSP and RT-PCR results, I tested the tumour suppressive capacity of PCDH10 in medulloblastoma by observing the effects of its re-expression in medulloblastoma cell lines where it has been silenced by promoter CpG methylation.

**Hypothesis**

I hypothesize that PCDH10 is a candidate tumour suppressor gene in medulloblastoma.
Materials and Methods

RT-PCR

RNA extraction: Tissue culture cells in 3.5cm dishes were lysed with 1ml of Trizol. Cells were centrifuged at 12,000g for ten minutes at 4°C. They were incubated for five minutes at 15-30°C, followed by the addition of 200ul chloroform (per 1ml Trizol), cells were shaken vigorously for fifteen seconds, incubated two to three minutes at 15-30°C then centrifuged at 12,000g for 15 minutes at 4°C. The aqueous phase (containing RNA) was then transferred to a new tube and 500ul isopropyl alcohol was added. After ten minutes of incubation at 15-30°C, the tubes were centrifuged at 12,000g for ten minutes at 4°C. The supernatant was then removed and the pellet was washed with 1ml 75% EtOH, vortexed and centrifuged at 7500g for five minutes at 4°C. The pellet was then briefly dried, water was added and then concentration of RNA was measured.

RNA to cDNA: 2ug of RNA was combined with 8ul GIBCO H₂O, on ice. 10ul 2X RT buffer was added to 2ul Superscript III (SSRTIII) and run in the PCR machines (as previously described). The product was then transferred to an Eppendorf tube. 80ul of GIBCO H₂O was added, resulting in 100ul of 20ng/ul cDNA. The cDNA was stored at -20°C.

RealTime PCR: (Refer to Appendix for primers) In each well, 0.5ul of 20ng/ul cDNA was combined with 11.0ul H₂O, and the combined product was added to a mixture of 12.5ul Sybr Green with 0.5ul of each the forward and reverse primer included. Real time PCR is run using
the following conditions. 1. Incubate 50°C for two minutes. 2. Incubate 95°C for two minutes. 3. Incubate 95°C for fifteen seconds. 4. Incubate 59°C for thirty seconds. 5. Plate read. 6. Cycle to step 3 for 39 times. 7. Melting curve from 60-95°C, read every one degree, hold one second. 8. End.

Each of these experiments was performed in triplicate and repeated at least twice.

**Methylated DNA Immunoprecipitation Arrays**

DNA was sheared into fragments of approximately 400-600 base pairs. It was then denatured into single stranded DNA, to improve binding affinity of the antibodies. 5-methylcytidine antibodies which attach to a methyl cytosine were added, and magnetic beads conjugated to anti-mouse-IgG were used to bind the methylcytidine antibodies. The unbound DNA can then be separated out in the supernatant. Proteinase K is then added to digest the antibodies and release the DNA to be used as the methylated fraction. Each fraction, unbound (unmethylated) and bound (methylated) are treated with a difference fluorescent dye (red or green) and unmethylated and methylated fractions are hybridized to an array, and presence and relative quantities of fluorescence is detected.

**Bisulphite treatment**

Preliminary steps include using 4ug of DNA in 20ul (using the 2ml tube provided by the Bisulphite Conversion Kit, Human Genetic Signatures). Add a total volume (5.2ml) of Reagent 1
to Reagent 2, mix by gentle inversion. The NaOH solution was made prior to beginning the protocol, using 1g of NaOH pellets in 8.3ml of H₂O.

2.2ul of 3M NaOH solution was added to 20ul of DNA solution and mixed well by pipetting. Solutions were incubated at 37°C for 15 minutes. 220ul of combined Reagent 1 and 2 was then added and mixed by gentle pipetting, followed by an overlay with 200ul mineral oil. Mixed solutions were incubated at 55°C for sixteen hours, covered with aluminium foil. After removing the mineral oil from the reaction tube, 1-2ul of glycogen was added and mixed by pipetting. Next, 800ul of Reagent 4 was added and mixed well by pipetting up and down at least ten times. After adding 1ml of 100% isopropanol, the mixture was then vortexed for five seconds. After incubating at 4°C for thirty minutes, the mixture was centrifuged for ten minutes at 15,000 rpm at 4°C. Supernatant was then removed very carefully, making sure not to dislodge the pellet, then 500ul of 70% ethanol was added. The mixture was centrifuged for five minutes at 15,000 rpm at 4°C and then all traces of ethanol were removed. The pellet was allowed to dry at room temperature, for fifteen minutes, then was resuspended in 100ul of Reagent 3. The sample was incubated at 72°C for sixty minutes, and centrifuged once during the incubation to reduce condensation. The sample was diluted to 20ng/ul by adding 100ul H₂O. For short term use, DNA was stored at –20°C, and long term aliquots are stored at –80°C.

**Methylation Specific PCR**

The PCR for MSP was mixed as following: 2ul MSP buffer, 2.5ul dNTPs, 0.8ul forward and reverse primers, 0.1ul platinum taq, 4.0ul of 20ng/ul template bisulphite treated DNA, 9.8ul H₂O,
per reaction. The PCR ran under the following protocol: 1. 94°C for three minutes. 2. 94°C for thirty seconds. 3. 60°C for thirty seconds. 4. 72°C for one minute. 5. Cycle to step 2, 29 times. 6. 72°C for five minutes. 7. Store at 4°C.

**Sequenom**

DNA was bisulfite treated as previously described. DNA was then PCR amplified, using tagged primers, designed using Sequenom software. This DNA was then sent off to Sequenom to be processed, where the DNA is transcribed into RNA and the unmethylated cytosines are replaced with uracil. After fragmenting the RNA through a base-specific cleavage, samples are subject to a MALDI-TOF mass spectrometer, which turns the samples into gas, then sent down through a tube and the sensor measures how long the sample takes to reach the sensor, which in turn calculates the weight of the fragment. The spectrum peaks are then used to calculate the percentage of methylated and unmethylated fractions within the sample [104].

**Functional Validation**

The human clone of *PCDH10* in the pCR-BluntII-TOPO vector was purchased from Open Biosystems. The cDNA was then digested with the enzyme EcoRI and buffer 3 for two hours at 37°C. Following this incubation, *PCDH10* was ligated into pcDNA3.1+. Using a five minute ligation with 50ng of the vector and 3-fold the amount of insert, adjusted to 10ul with H₂O, 10ul of 2X QL Buffer was added and mixed in, as well as 1ul of Quick Ligase. This mixture was then
briefly centrifuged and incubated at room temperature for five minutes. Following this, 2ul was added to TOP10 E coli competent cells for transformation.

Transformation:

TOP10 E coli competent cells were incubated on ice for 30 minutes, followed by heat shock for 30 seconds at 42°C, then put immediately on ice. SOC media (250ul) was added at room temperature and mixture was then shaken horizontally at 37°C for one hour. 25-200ul was spread on a prewarmed plate, and incubate overnight at 37°C.

In the morning, plates were taken out of the incubator, and colonies were picked at the end of the day, and shaken overnight in 3ml of 10X LB media. The following day, DNA was extracted using Qiagen Mini Prep kit. Clones were sequenced and multiple clones with the correct sequence were identified. DNA was transiently transfected into hek293 cells to check for expression of the construct. Protein was extracted; cells were lysed using RIPA lysis buffer, with the Complete Mini protease inhibitor tablet. Dishes were washed with 8ml of cold PBS while on ice and then incubated on ice for twenty minutes with RIPA + protease inhibitors. The dishes were then scraped and the cells in media were pipetted into Eppendorf tubes. Protein was then mixed with 2X loading gel at a 1:1ul ratio then boiled at 98°C for five minutes to stabilize the protein. The protein was then stored at -20°C. Western blots to check for expression were carried out using the same protocol as previously described. Unfortunately, expression could not be seen, even within the positive control of normal mouse brain, so we concluded that our antibody was not sufficient to detect expression and went on to tag the construct.

Tagging the construct – primers were designed to include an HA tag on the 3’ end of the gene. PCR was carried out using the following conditions: 1. 94°C for three minutes. 2. 94°C for 30
seconds. 3. 57°C for 30 seconds. 4. 72°C for 1 minute 45 seconds. 5. Cycle to step 2, 39X. 6. 72°C for 5 minutes. 7. Store at 4°C. The following amount of substances were used in the PCR mixture.

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PCR product was then ligated into the pcDNA3.1 vector (Invitrogen) and clones were selected and sequenced as described previously.

DNA from the clone with the correct sequence was then transiently transfected into hek293 cells using the Fugene system, in a SFM:Fugene:DNA, 97:3:1 ratio. The cells were lysed using RIPA lysis buffer as previously described, and the protein was used for western blot. Western blots were carried out according to previous methods, probing for anti-HA to show expression of the construct. GFP was used as a transfection control, and cells were looked at under the fluorescent microscope to confirm a glowing green phenotype.

After showing expression on a western blot, one medulloblastoma cell line, Daoy was grown in tissue culture. 50,000 cells were seeded in a 6 well dish, and 2ug of plasmid and empty vector were transfected using the Fugene system. 48 hours later the media was changed to include G418 selection media. The drug concentration for Daoy was 0.5mg/ml. After one week, complete cell death was seen in the untransfected wells. Cells in other wells were trypsinized and resuspended in 5ml of media. 500ul was taken from each for the cell counter, and cells were then seeded in 96-well plates at a concentration of 1-2 cells/well. Two plates for each the
construct and the empty vector were made. Twice a week, 50ul of fresh media with G418 drug was added to each well, for two weeks. Wells with cells were then trypsinized and plated in 24-well dishes and grown for an additional week in G418 selection media. Following this, cells were expanded into 6-well dishes, and then 10cm dishes, continuing to be expanded in G418 selection media. At this time, one dish of each clone, both empty vector and construct, was lysed for protein analysis. Western blots were carried out accordingly, to test for HA-tagged positive clones. Three positive clones in the Daoy cell line, which were expressing the HA-tag, and thus the protein, were selected for further analysis, along with two empty-vector Daoy clones.

Cell Proliferation Assay

Cells were seeded at 5000 cells/well in a 96-well plate. Each was seeded in triplicate for each time point. At time zero, as well as after 24h, 48h and 72h, proliferation was measured, using the Promega CellTitre 96 Aqueous One Solution Cell Proliferation Assay (MTS) protocol and solution. This assay is based on mitochondrial activity. Dehyrdogenase enzymes which are found in metabolically active cells reduce MTS into into formazan, which can be detected at the 490nm wavelength. This experiment was repeated twice, in triplicate of each sample, with the exception of PCDH10-construct-clone 2, which was performed once. This clone was only tested once due to cell death by bacterial contamination.

Cell Cycle Analysis

DNA staining with propidium iodide for flow cytometry was carried out to assess cell cycle for cells in both the empty vector controls and the construct. To prepare these cells for flow cytometry, 2x10^6 cells were collected by centrifugation at 400xg for five minutes. The
supernatant was aspirated out. The pellet was then centrifuged and re-suspended in 50ul Staining Medium, and added to an Eppendorf containing 1ml of ice cold 80% ethanol. Following this, the cells were vortexed quickly and fixed overnight at 4°C. The following day, fixed cells were collected by centrifugation at 400xg for five minutes, aspirating the supernatant out. The pellet was re-suspended in 500ul 2mg/ml RNAse A solution and incubated for 5 minutes at room temperature. 500ul 0.1mg/ml PI solution was added and vortexed to mix. After incubating for thirty minutes at room temperature, the pellet was re-suspended in solution and filtered through Nitex into FACS tubes.

Reagents:

Staining Medium

1X Hank’s balanced salt solution (HBSS)

10mM HEPES-NaOH, pH 7.2

2% (v/v) calf serum

10mM NaN₃

To 480ml sterile 1xHBSS add 5ml sterile 1M HEPES-NaOH, ph7.2, 10ml sterile FBS and 5ml sterile 1M NaN₃. Store at 4°C.

0.1mg/ml PI solution

0.1mg/ml propidium iodide

HBSS (no serum)

0.6% (w/v) NP-40

Aliquot at 500ul/ aliquot and store, protected from light, at –20°C.
Artificial Wound Healing Assay

To assess the cells’ migratory ability, an artificial wound healing assay was performed. Cell lines were grown to confluency in medium containing 10% FBS. A uniform scratch defect (wound) was created using a p20 sterile pipette tip. Plates were then washed with PBS, followed by the addition of serum-free media. Plates were imaged immediately (t=0) and at 20hr (t=20). This was repeated in two separate plates with two scratch defects in each plate, for each clone. The degree to which the cells had migrated into the initial defect space was then assessed. ImageJ software was used to calculate the area of the original defect and the area of the defect after twenty hours, comparing the percentage of closure by area. The data was then statistically analyzed using MedCalcStats software. This method of quantitatively comparing cell migration has been used throughout the literature [105-107].

Results

Examination of PCDH10 transcript levels in primary medulloblastomas

The RNA expression level of PCDH10 is highly variable across primary medulloblastomas, in comparison to adult cerebellum. As a whole, there is no statistically significant difference between adult cerebellum and primary tumours, with a p-value of 0.1179, but this statistic is clearly affected by the large variability of expression between tumours (Figure 16). We found that a large proportion of tumours (11/26) exhibited at least two-fold decreased RNA expression
of PCDH10, compared to adult cerebellum. Given that genomic deletions were identified in very few tumours, we suspected that epigenetic silencing might account for the remaining cases.

**Re-expression of PCDH10 following cell line treatment with DAC**

Three of five medulloblastoma cell lines (Med8A, ONS76, D283) showed no significant change in expression levels between normal controls and DAC treated cell lines. Daoy cells showed approximately a 2.5 fold increase in the DAC treated cDNA compared to untreated Daoy cells (Figure 17). UW228 showed no expression in the untreated control. However, further experiments using DAC, TSA and both together, showed synergistic effects upon re-expression of PCDH10 (Figure 18). This suggests that in addition to CpG methylation, histone-mediated silencing may play a significant role in the downregulation of PCDH10 transcript.

Figure 16: qRT-PCR for PCDH10 with primary medulloblastoma tumours. Error bars for this and all subsequent graphs were calculated using a calculation of standard error, by dividing the standard deviation by the square root of the number of replicates.
Figure 17: *PCDH10* expression in medulloblastoma cell lines, control versus DAC treated. Error bars for this graph were calculated using a calculation of standard error, by dividing the standard deviation by the square root of the number of replicates. Statistically significant differences are represented with the * symbol.

Figure 18: UW228, a medulloblastoma cell line, showing an additive effect of increased expression with DAC and TSA treatment, compared to no expression in untreated control. Error bars for this graph were calculated using a calculation of standard error, by dividing the standard deviation by the square root of the number of replicates. Statistically significant differences are represented with the * symbol.
Identification of MeDIP-CHIP enrichment at the PCDH10 promoter

Although DAC experiments allow one to examine if DNA methylation has a direct effect on gene expression, sometimes re-expression of transcripts may be due to secondary off target effects, such as the re-expression of other methylated genes that may affect this candidate gene. Hence, candidate genes that are identified from a DAC screen must be validated by any number of bisulphite treatment based techniques. In order to hone in upon the CpG site of methylation responsible for PCDH10 downregulation, we sought to use MeDIP-CHIP as a screening tool. PCDH10 is shown through MeDIP-CHIP experiments done in our lab, to be enriched for the methylated fraction in multiple medulloblastoma cell lines (Figure 19). Not only is there a CpG island in the promoter region of this gene, there is also a CpG island within exon 1 of PCDH10. Both CpG islands showed enrichment within different medulloblastoma cell lines.
Figure 19: MeDIP CHIP profile identifying enrichment at the promoter and exon 1 CpG islands in medulloblastoma cell lines D458 and UW228. This data is from chromosome 4, where the top blue panel represents the CpG sites, the following pink lines titled ‘tiled region’ represent the investigated area and the yellow line represents the transcriptional start site. This data is from medulloblastoma cell lines, where anything above a peak score of 2.0 is considered a significant area of methylation.
Initial validation of methylation of PCDH10 in medulloblastoma

Methylation Specific PCR was carried out on multiple medulloblastoma cell lines, as well as medulloblastoma primary tumours. We found DNA hypermethylation in four of twenty-two samples. Three were primary medulloblastoma tumours and one was a medulloblastoma cell line, UW228 (Figure 20, A-C).

Figure 20: MSP on medulloblastoma cell lines and primary tumours. (A) Adult brain (Ad Cb) and fetal brain (Ft Cb) are unmethylated, and positive control of universally methylated DNA (Uv Me) shows methylation. Cell lines Daoy, D458, D283 and D425. (B) Medulloblastoma cell lines Res262, Med8a, ONS76 and UW228 (Methylated). (C) Primary medulloblastomas.
High-throughput methylation analysis of the PCDH10 promoter locus

To investigate the methylation status of the CpG islands associated with PCDH10, we wanted to analyze a larger cohort of primary medulloblastomas. High-throughput Sequenom analysis allowed us to accomplish this, and although there seems to be no hypermethylation in the CpG in the promoter region within primary tumours compared to normal controls (Figure 21A), this is not the case for the CpG within exon 1. A portion (36%) of the primary medulloblastoma tumours analyzed showed hypermethylation at the CpG island in exon 1, compared to normal controls (Figure 21B).

Figure 21: Sequenom. Targeting both CpG islands associated with PCDH10. (A) CpG island in the promoter region. (B) CpG island within exon one. I found no hypermethylation in the promoter region compared to normal controls and 36% CpG methylation in the CpG island within exon 1.
**Functional validation of PCDH10 as a putative tumour suppressor gene in medulloblastoma tumourigenesis**

*Generation of Daoy stable cell lines with expression of PCDH10*

After the *PCDH10* contract was HA-tagged and sequence verified, we confirmed protein expression in Hek293 cells, by western blot, which has been previously described, and then proceeded to build a stable cell line. We transiently transfected Daoy cells and drug-selected for positively-expressing clones over a four week period. Figure 22 shows re-expression of *PCDH10* in two representative clones, compared to empty vector controls.

![Western blots to confirm expression of PCDH10 in clones. A representative blot showing no HA-expression in Empty Vector Clone 1 or in Water Control. Multiple PCDH10 clones show HA-expression at 80kDa. Positive HA control at 48kDa and BACTIN at 40kDa.](image)

Figure 22: Western blots to confirm expression of *PCDH10* in clones. A representative blot showing no HA-expression in Empty Vector Clone 1 or in Water Control. Multiple *PCDH10* clones show HA-expression at 80kDa. Positive HA control at 48kDa and *BACTIN* at 40kDa.
**Functional assays investigating the tumourigenic capacity of PCDH10**

*Cell Proliferation Assay with a PCDH10 stable cell line*

To assess cell proliferation, a MTS assay was performed (Figure 23). The stable re-expression of *PCDH10* in medulloblastoma cell line Daoy shows no statistically significant changes in proliferation, compared to that of empty vector controls, thus *PCDH10* may not be involved with cell division and growth. We sought to examine these results further with comprehensive cell cycle analysis.

![Cell Proliferation (MTS) Assay](image)

Figure 23: Cell Proliferation (MTS) Assay comparing *PCDH10* reexpressing clones with empty vector controls.
Cell Cycle Analysis

To assess cell cycle distribution between empty vector controls and PCDH10 re-expressing clones, flow cytometry was carried out, using PI staining (Figure 24A). The groups showed no statistical difference, using Welch’s t-test with a p-value of 0.9872 between G0/G1, 0.5694 for the S-phase and p=0.1704 for G2/M (Figure 24B). This demonstrates that cell cycle is not affected by re-expression of PCDH10 and this gene may be involved in other aspects of the tumour formation process.

Figure 24: (A) Representative G1/G2 histograms for PI staining for flow cytometry. (B) Error bars for this graph were calculated using a calculation of standard error, by dividing the standard deviation by the square root of the number of replicates.
Artificial Wound Healing Assay

Despite having no effect on cell proliferation and cell cycle, we expected that *PCDH10* might be involved in cell migration, as it is an adhesion gene. To assess the migratory ability of the cells, wound healing assays were done on both the *PCDH10* re-expressing clones as well as the empty vector controls (Figure 25 A-E). Each experiment was repeated twice on two separate occasions. We found that there was a statistical difference in the area that the cells migrated into (Figure 26), the control empty vectors migrated to a larger degree than the *PCDH10* re-expressing stable clones. This indicates that *PCDH10* re-expression negatively affects the cells’ ability to migrate.

(A)

![Image](image1.png)

(B)

![Image](image2.png)
Figure 25: Wound healing assay. Pictures at X10 magnification. (A) Empty vector (clone 1) at t=0h and t=20h. (B) Empty vector (clone 2) at t=0h and t=20h. (C) PCDH10 (clone 1) at t=0h and t=20hr. (D) PCDH10 (clone 2) at t=0h and t=20h. (E) PCDH10 (clone 3) at t=0h and t=20h.
Figure 26: Graphical results for cell migration assay. Using Welch’s t-test, the p-value was 0.0001, proving that there is a statistically significant difference between the amount of migration in control cells compared to PCDH10 stable cell lines.
**Discussion**

Through genome-wide copy number analysis and confirmatory quantitative PCR, *PCDH10* was seen to be a target of statistically significant homozygous focal loss. Further, at a gene expression level, it was shown that primary tumours have large variability in their expression level of *PCDH10*, compared to adult cerebellum. Through re-expression of *PCDH10* in DAC and TSA treated cell lines, we demonstrated that medulloblastoma cell lines and primary tumours exhibit a correlation between methylation and expression. DNA methylation and histone mediated silencing both play well-documented roles in the silencing of tumour suppressor genes [40, 108-110]. To examine the extent of this methylation further, Methylation Specific PCR (MSP) was utilized and identified methylation in multiple primary tumours as well as one medulloblastoma cell line, in a small region of the proximal promoter CpG island. To obtain a broader range of methylation data, a high throughput DNA methylation analysis technique called Sequenom was used, and a large cohort of primary medulloblastomas was profiled. This was done within both CpG islands, one proximal to the *PCDH10* transcriptional start site and one within coding exon one. Numerous tumours (36%) showed DNA methylation within CpG islands, suggesting that *PCDH10* downregulation may be mediated by DNA hypermethylation events at several CG sites within exon one. In contrast to many other cancers, which exhibit methylation in the promoter region of *PCDH10*, this cohort of medulloblastomas showed predominantly methylation within the CpG island in exon 1. Although the process behind this discrepancy is unclear, methylation of *PCDH10* in exon 1 could be due to CNS specific expression. Through functional studies on the Daoy medulloblastoma cell line, it was demonstrated that *PCDH10* has no effect on cell proliferation, when empty vector controls showed no statistical difference in proliferation, compared to *PCDH10* stable cell lines in a cell proliferation (MTS) assay. This evidence was further supported by flow cytometry analysis on
cell cycle between the two groups of empty vector and PCDH10 stable cell lines. However, when serum-starved cells were tested for migration, the PCDH10 construct exhibited visibly and measurably slower migration than empty vector controls in a scratch-test assay. If extrapolated to a tumour progression model, this may suggest that PCDH10 could slow migration and metastasis within tumours by virtue of its cell adhesive properties. This is significant considering that PCDH10 could be a target for epigenetic therapy within the patients that show loss of expression of this gene. This role in the pathogenesis of medulloblastoma would not be surprising, as it would be a similar role to that of CDH1[74]. Both CDH1 and PCDH10 are deleted and targeted by DNA hypermethylation in other cancers, and conditional inactivation of CDH1 in a murine model did not induce tumour formation alone, but the addition of p53 with this inactivation of CDH1 resulted in invasive carcinoma. This suggests that CDH1 is involved in tumour progression [74].

In the future, studies on a larger cohort of primary tumours could be analyzed by Sequenom, creating a clearer picture of which subgroup of medulloblastomas show methylation at the PCDH10 locus. The subgroup classification of PCDH10 methylation may be important in categorizing risk, predicting clinical outcome, and potentially directing medulloblastoma treatment. Furthering this importance, is the possibility of development of epigenetic related therapies. Other cancer types, including T-cell lymphoma, chronic myelomonocytic leukemia and myelodysplastic syndromes have shown promising clinical response, using epigenetic therapies [111, 112]. Considering that epigenetics may play a significant role in the development and progression of medulloblastoma, new therapies to target these changes are vital to improving treatment [39, 40, 42, 45, 51].
Despite this promising evidence implicating \textit{PCDH10} as an epigenetically silenced tumour suppressor gene, further studies are needed to test the validity of \textit{PCDH10} as a tumour suppressor gene in medulloblastoma. To address this, the \textit{PCDH10} re-expressing stable cell line could be intracranially injected into an immunocompromised mouse model. This would allow us to examine the rate and extent of tumour formation in a medulloblastoma mouse model. Additionally, we can directly test the role of \textit{PCDH10} by breeding a \textit{PCDH10} knockout mouse model to a well characterized medulloblastoma model such as \textit{Ptc}h\textsuperscript{+/-} mice.

Seen through a multitude of literature and experimental results, there is strong evidence that suggests that \textit{PCDH10} is involved in the pathogenesis of medulloblastoma and future studies should further investigate the role it plays.
References
References


Pfister, S., et al., Array-based profiling of reference-independent methylation status (aPRIMES) identifies frequent promoter methylation and consecutive downregulation of ZIC2 in pediatric medulloblastoma. 2007. p. e51-.


104.  
103.  
102.  
101.  
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96.  
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87.  
86.  
85.  


Frequent epigenetic inactivation of the RASSF1A tumour suppressor gene in testicular tumours and distinct methylation profiles of seminoma and nonseminoma testicular germ cell tumours. Oncogene. 22(3): p. 461-466.


Appendix
## Appendix

### RT-PCR Primers

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### PCR primers for constructs

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### MSP primers for PCDH10

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### Sequenom Primers PCDH10

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<td>CagtaatacactataggagagaagctTTTTTAATTTA</td>
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<tr>
<td>CpG in exon, start</td>
<td>aggaagagAGTGTAATTTTGGTTTA</td>
<td>CagtaatacactataggagagaagctTTTTTAATTTA</td>
</tr>
<tr>
<td>CpG in exon, end</td>
<td>aggaagagAGTGTAATTTTGGTTTA</td>
<td>CagtaatacactataggagagaagctTTTTTAATTTA</td>
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