From WOMEN´S AND CHILDREN´S HEALTH
Karolinska Institutet, Stockholm, Sweden

NEW POTENTIAL TARGETS IN MEDULLOBLASTOMA THERAPY - STUDIES ON CELLULAR MECHANISMS AND MEDIATORS

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“There is no love sincerer than the love of food”

(George Bernard Shaw)

ABSTRACT

Medulloblastoma is an embryonal tumour that mainly affects children. It is the most common malignant brain tumour in children and accounts for 15% of all childhood brain tumours. It most often presents in the cerebellum and is considered to be a disorder of normal development. Despite intensive multimodal therapy, survival in high-risk patients is still poor, and long-time survivors suffer from detrimental side effects. To improve outcome, new treatments based on a better understanding of medulloblastoma biology are needed.

Prostaglandin E_2 (PGE_2) is a proinflammatory eicosanoid that is linked to cancer progression and development. It is formed from arachidonic acid through enzymatic conversion catalyzed by cyclooxygenases (COX-1/2). PGE_2 promotes tumour growth by activating signalling pathways that control cell proliferation, invasion, apoptosis, angiogenesis and immunosuppression. We found that COX-2/PGE_2 signalling is activated in medulloblastoma, and that PGE_2 has an important role in medulloblastoma growth. Celecoxib, a selective COX-2 inhibitor, demonstrated promising effects against medulloblastoma tumour growth both alone and when combined with cytostatic drugs. Celecoxib potentiated the effect of the DNA alkylator temozolomide by downregulating MGMT expression and by inhibiting proliferation of CD15/CD133 positive medulloblastoma cells.

Canonical Wnt signalling pathway and phosphoinositide-3-kinase (PI3K)/Akt pathway are crucial for normal cerebellar development. In medulloblastoma, activation of Wnt/β-catenin and PI3K/Akt signalling are commonly observed. Our results show that PI3K/Akt-Wnt/β-catenin cross-talk is important for medulloblastoma tumourigenesis. We demonstrated that OSU03012, a small molecule inhibitor of the PI3K/Akt signalling protein phosphoinositide-dependent protein kinase-1, suppresses medulloblastoma growth both *in vitro* and *in vivo* by interfering with GSK-3β inactivation and β-catenin activity. Furthermore, OSU03012 induced synergistic cytotoxicity when combined with chemotherapeutic drugs and augmented the anti-tumour effect of the mammalian target of rapamycin inhibitor CCI-779 *in vivo*.

Human cytomegalovirus (HCMV) is an oncomodulatory virus that has recently been detected in tumours of different origin. We found a high prevalence of HCMV in medulloblastoma primary tumours and cell lines and showed that infection with HCMV upregulates production of PGE_2 in medulloblastoma. Treatment with the anti-viral drugs ganciclovir/valganciclovir or celecoxib inhibited medulloblastoma tumour growth both *in vitro* and *in vivo*, and the combined therapy demonstrated augmented effects. Based on our observations we suggest that HCMV may, indirectly through the activation of COX-2, be an etiological factor in medulloblastoma development.

In summary, this thesis has identified PGE_2/COX-2, the PI3K/Akt-Wnt/β-catenin cross-talk and HCMV as novel targets in medulloblastoma. Compounds that inhibit these targets demonstrate promising effects in experimental models of medulloblastomas, supporting the rationale for clinical testing as novel adjuvant therapy for children with medulloblastoma.

LIST OF PUBLICATIONS

I. **Tumour growth promoting cyclooxygenase-2 prostaglandin E2 pathway provides medulloblastoma therapeutic targets.**
Ninib Baryawno, Baldur Sveinbjörnsson, Staffan Eksborg, Abiel Orrego, Lova Segerström, Carl-Otto Öqvist, Stefan Holm, Bengt Gustavsson, Bertil Kågedal, Per Kogner & John Inge Johnsen.
Neuro Oncol. 2008;10:661-74

II. **Small molecule inhibitors of PI3K/Akt signaling inhibit Wnt/β-catenin pathway crosstalk and suppress medulloblastoma growth.**
Ninib Baryawno, Baldur Sveinbjörnsson, Staffan Eksborg, Chin-Shih Chen, Per Kogner & John Inge Johnsen.
Cancer Research 2010;1:266-76

III. **Celecoxib reduces MGMT expression and potentiates the effect of temozolomide in childhood medulloblastoma.**
Ninib Baryawno, Jelena Milosevic, Malin Wickström, Baldur Sveinbjörnsson, Staffan Eksborg, Per Kogner & John Inge Johnsen.
Manuscript

IV. **High prevalence of HCMV in medulloblastoma; reduced tumor growth using valganciclovir and celecoxib.**
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Manuscript

* Authors contributed equally to the manuscript and share primary and senior authorship, respectively.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AA	Arachidonic acid
Akt	Protein kinase B
APC	Adenomatous polyposis coli
BBB	Blood brain barrier
CMS	Cerebellar mutism syndrome
CMV	Cytomegalovirus
CNS	Central nervous system
COX	Cyclooxygenase
CSC	Cancer stem cell
CSRT	Craniospinal radiotherapy
EC_{50}	The concentration that inhibits 50% of cell proliferation
EFS	Event-free survival
EGL	External granular layer
FAP	Familial adenomatous polyposis
GSK-3β	Glycogen synthase kinase-3β
GPCR	G-protein coupled receptor
HCMV	Human cytomegalovirus
Hh	Hedgehog
HR	High-risk
Lef	Lymphoid enhancer factor
MGMT	O^6-Methylguanine-DNA methyltransferase
mPGES	Microsomal PGE synthase
MRI	Magnetic resonance imaging
mTOR	Mammalian target of rapamycin
MYC	Myelocytomatosis virus related oncogene
NPC	Neural precursor cell
NSAID	Non-steroidal anti-inflammatory drug
OS	Overall survival
PDK	Phosphoinositide-dependent kinase
PI3K	Phosphoinositide-3-kinase
PGE_2	Prostaglandin E_2
PNET	Primitive neuroectodermal tumour
PTEN	Phosphatase with tensin homology
RTK	Receptor tyrosine kinase
SC	Stem cell
Shh	Sonic hedgehog
SP	Side-population
SR	Standard-risk
SVZ	Subventricular zone
Tcf	T cell factor protein
VEGF	Vascular endothelial growth factor
VZ	Ventricular zone
Wnt	Wingless

1 GENERAL BACKGROUND

1.1 CHILDHOOD CANCER

The annual incidence of childhood cancer in Sweden is 300 cases. The most common childhood malignancies are leukaemias/lymphomas (40%) and primary central nervous system (CNS) tumours [30% (Gustavsson et al., 2007]. Cancer in children differs from adult cancer: these cancers have a more aggressive and proliferating phenotype, exhibit great histological diversity, and can arise in many different sites. Few genetic changes are observed in childhood malignancies (e.g., fewer p53 mutations occur), and these changes often arise in embryonal precursor cells where aberrant signalling in normal development has been implicated [reviewed in (McKinney, 2005)].

In the early 1960s, the survival rate of children with cancer was less than 30%. By combining surgery, radiation and intensified chemotherapy, the survival rate of childhood cancer is now approaching 80% (Steliarova-Foucher et al., 2004). Despite advances in treatment, however, childhood cancer is still the most common cause of childhood death due to disease in western countries (Pritchard-Jones et al., 2006).

1.2 CHILDHOOD BRAIN TUMOURS

Childhood CNS tumours are the most common solid tumours, second only to leukaemia and lymphoma. The annual incidence of CNS tumours in Sweden is 4.2/100,000 children (Lannering et al., 2009). A large variety of CNS tumours exists in children, both in histology and location and in survival outcome. Treatment is often very complex and involves surgery, radiation, and chemotherapy. Complications related to treatment are of significant concern in children with CNS tumours, as long-term side effects are common in surviving patients (Packer, 2008).

1.2.1 Classification

Tumours of the CNS in children are classified according to the World Health Organization (WHO) classification system. The tumours are classified according to histological grading in terms of predicting the biological behaviour of the tumour, which includes a grading scale corresponding to aggressiveness (i.e., a malignancy scale). In a clinical setting, the malignancy scale is a key factor influencing the choice of therapies. Brain tumours in children are graded from Grade I to Grade IV, where Grade I applies to lesions that have low proliferative potential and that are often correlated with a good prognosis. Surgery alone is enough to cure Grade I tumours. Grade IV applies to high-proliferation, mitotically active cells and is associated with a fatal outcome (Louis et al., 2007). Brain tumours in children are further subdivided into smaller groups based on site of origin and type of cells. The most common childhood CNS tumours are located in the posterior fossa: astrocytoma (44%), medulloblastoma (19%) and ependymoma [10%) (Lannering et al., 2009)].

1.2.2 Symptoms and diagnosis

Tumours of the CNS have a clinical presentation that correlates with location of the tumour and age of the child. Children with tumours in the posterior fossa present with nausea, vomiting, headache, and papilledema. These symptoms are caused by obstruction of the cerebrospinal fluid in the fourth ventricle, causing hydrocephalus and increased intracranial pressure (Dhall, 2009). Patients with tumours located supratentorially present with seizures or motor and sensory complaints (Ullrich, 2009).

Discovering exact location and primary metastasis is crucial for surgical assessment and further treatment (Mueller and Chang, 2009). Diagnosis is usually made with a computed tomography (CT) scan or magnetic resonance imaging (MRI), which is by far the most helpful and frequently used technique today. MRI also helps to find leptomeningeal disease in the spinal cord and other parts of the brain. Positron emission tomography (PET) scans and diffusion/perfusion MRI are also used to diagnose brain tumours (Vezina, 2008).

1.2.3 Treatment

1.2.3.1 Surgery

Surgical resection is the initial treatment after a child is diagnosed with a brain tumour. The exact location and histology (type and grade) of the tumour determine if the tumour is surgically accessible. Brain-stem tumours (midbrain, pontine, and cervicomedullary) that were formerly unresectable can today be better managed by using new imaging techniques such as functional imaging, diffusion tensor imaging, and neuro-navigation combined with surgery in several cases. These techniques help the surgeon to map the brain and define tracts and connections, leading to better tumour resection management and less harm to normal tissue (Vezina, 2008).

During surgery, a biopsy is made to define the histology of the tumour. Low-grade gliomas are completely resected and no further treatment is needed. Medulloblastomas and ependymomas, on the other hand, are often only partially resected through surgery. Therefore, radiation and chemotherapy are necessary after the surgery (Mueller and Chang, 2009).

1.2.3.2 Radiation therapy

Radiation therapy can be crucial in the treatment of childhood CNS tumours. However, patients that receive radiation therapy are at risk of developing side effects that negatively affect cognitive, endocrine, and neurological function (Packer, 2008).

The most common radiation technique used today is craniospinal radiotherapy (CSRT). Patients receiving CSRT undergo radiation to the craniospinal axis, with an additional boost of radiation to the tumour. This therapy is difficult to undergo and is accompanied by severe treatment-related toxicity. New techniques that minimize radiation to surrounding normal tissue are available today and have been evaluated in clinical trials. Conformal radiation therapy (CRT) incorporates three-dimensional CT and MRI imaging, allowing more precise planning and delivery and hence helping to identify both the tumour and surrounding critical structures. This helps to reduce the

radiation field and save surrounding tissue from extensive radiation. Proton beam therapy, intensity modulated radiation therapy, and gamma knife radiosurgery are other alternatives that benefit from a higher proportion of tumour versus normal tissue distribution. This increases radiation to tumour mass and hence reduces side effects. One drawback of these techniques is that the tumour cells in the craniospinal axis have a propensity to disseminate and escape treatment [reviewed in (Mueller and Chang, 2009)].

1.2.3.3 Chemotherapy

A large number of chemotherapeutic agents have been shown to be effective in the treatment of CNS tumours, including platinum compounds, nitrosureas, cyclophosphamide, iphosphamide, temozolomide, etoposide and vincristine. Children with CNS tumours who have undergone chemotherapy as well as radiation therapy have significantly higher survival rates. Chemotherapy is today used differently depending on diagnosis and age of the patient. In vulnerable children (e.g. infants and children younger than 4 years) and unresectable tumours, chemotherapy is used to delay radiation therapy or reduce radiation dose while trying to maintain high cure rates. Combination chemotherapy is considered most effective when given as an adjuvant in surgery and radiation treatment to control local and disseminated disease [reviewed in (Mueller and Chang, 2009)].

Chemotherapy resistance and passage of drugs across the blood brain barrier (BBB) are major concerns in chemotherapy. The BBB is an anatomical barrier that prevents compounds, restricted by molecular weight, lipid solubility and pH, from crossing to brain cells (Spector, 2000). Although the BBB is physiologically disrupted in the tumour area, it is uncertain whether small, lipid-soluble molecules at physiological pH can cross the BBB (Muldoon et al., 2007). Other mechanisms to drug resistance have been identified in brain tumours. P-glycoprotein pumps (PGPs), which serve as efflux pumps that transport toxic compounds, have been shown to be overexpressed in gliomas (von Bossanyi et al., 1997). Several paediatric brain tumours also express high levels of the DNA repair protein O^6-Methylguanine-DNA methyltransferase (MGMT), which reverses chemotherapy-induced damages to DNA by drugs such as alkylnitrosoureas and temozolomide (Hongeng et al., 1997).

1.2.4 Late effects

Improvements in survival have resulted in an increased emphasis on the quality of the long-term survival. Late effects are likely the result of a complex interaction between the tumour, the different treatment modalities, and individual characteristics in a growing and developing child (Laughton et al., 2008). Although it is difficult to distinguish which factors contribute most to long-term side effects, it is becoming increasingly clear that radiation may cause neuroendocrine abnormalities, personality change and neurocognitive sequelae for children who survive brain tumours (Packer, 2008). Studies of children receiving whole brain radiation show a major drop in intelligence quotient (IQ) score and white matter loss with a significant correlation to radiation dose (Mulhern et al., 2004). Endocrine sequele after radiation depend on total dose and age. For instance, children radiated with doses of >12 Gray (Gy) in the brain acquire damages in the hypothalamic-pituitary axis with abnormalities in production of

growth hormone (GH), resulting in short stature, bone loss, and precocious puberty. High-dose radiation (>40Gy) to the brain results in endocrine and reproductive dysfunction [e.g. decreased production of thyroid stimulating hormone, adrenocorticotrophic hormone, follicle stimulating hormone, and luteinizing hormone (Laughton et al., 2008)].

1.2.5 Survival

Survival for children with CNS tumours has improved during the last decades, with a current 10 year overall survival (OS) rate of 70%. However, the 10 year OS of children differs considerably according to cancer type. For instance, children with brain-stem tumours, high-grade astrocytoma, medulloblastomas and ependymomas have a poor prognosis, with survival rates of 17%, 26%, 53% and 60% respectively. In contrast, children diagnosed with low-grade astrocytomas, plexus choroideus tumours, and optic nerve/chiasma gliomas have survival rates of over 80% (Lannering et al., 2009).

Although improvements have been made in the treatment of medulloblastoma and ependymoma, little progress has been made in the management of brain-stem gliomas, high-grade gliomas, and infant/disseminated malignant tumours (Lannering et al., 2009; Packer, 2008). To improve survival, understanding of the biology, genetics, and cause of childhood brain tumours must increase significantly. Thus, a new understanding of the biology of childhood brain tumours must be integrated into the treatment before safer and more effective therapies can be developed.

1.3 MEDULLOBLASTOMA

Medulloblastoma is the most common malignant brain tumour in children, accounting for 15% of all brain tumours in children (Lannering et al., 2009). Medulloblastoma is defined as a densely cellular, midline cerebellar tumour that arises over the roof of the fourth ventricle and occurs mainly in children (Rorke, 1983). The origin as well as nomenclature of medulloblastoma has been debated. Historically, medulloblastoma was grouped under the name of primitive neuroectodermal tumour (PNET). It was thought that all embryonic tumours originated from a common precursor cell of the subependymal matrix in the CNS (Rorke, 1983). However, with the introduction of gene-expression profiling, Pomeroy and colleagues have demonstrated that embryonic brain tumours are a heterogeneous group of neoplasms and that these tumours should be classified based on tumour location, histology and patterns of differentiation (Pomeroy et al., 2002). Indeed, it is today accepted that medulloblastoma is composed of multiple histologically and biologically diverse subtypes (Pomeroy et al., 2002; Rood et al., 2004a; Thompson et al., 2006). Increasing evidence also indicate that medulloblastoma may originate from precursor cells (e.g. granule cells) in the external granular layer (EGL) of the cerebellum (Fan and Eberhart, 2008; Pomeroy et al., 2002).

Most medulloblastomas appear sporadically. However, the aetiology of medulloblastoma is unclear except in a small fraction of patients that harbour germ-line mutations in tumour suppressor genes, such as those seen in Gorlin syndrome (Gorlin, 1987) and Turcot syndrome (Hamilton et al., 1995).

1.3.1 Medulloblastoma aetiology

1.3.1.1 A change in normal development?

It is generally accepted that there is a fundamental link between embryonic development and the biology of embryonic tumours (Gilbertson and Ellison, 2008). Many genes that were initially identified as oncogenes and tumour suppressor genes have been identified as key regulators of normal development events. These genes have been shown to play a direct role in tumour aetiology, where processes of proliferation, differentiation, and tumourigenesis have been interrelated. This is particularly pronounced in embryonic tumours of the CNS (Gilbertson and Ellison, 2008). Among genes regulating development are those that control segment polarity in embryos. The majority of these genes regulate components of wingless (Wnt) and the hedgehog (Hh) signalling pathways. It is also clear that many of the pathways required in cerebellum development are activated in medulloblastoma (Fan and Eberhart, 2008; Pomeroy et al., 2002)

1.3.1.1.1 Development of the cerebellum

Maintaining the proliferative state of granule cells and formation of the cerebellum requires activity of several molecules, genes, and developmental programs. Cerebellum forms early in embryonic development but is one of the last brain structures that achieves maturity (e.g. several months after birth). This protracted formation period leads to an increased vulnerability to developing abnormalities and neoplasms (Wang and Zoghbi, 2001).

The cerebellum develops from the dorsal neural tube of the fourth ventricle and is thought to arise from both the mesencephalon territory (midbrain) and the metencephalic rhombic lip (hindbrain). The cells that colonize the cerebellum arise either from the ventricular zone (VZ) or the EGL. The ventricular neuroepithelium lies beneath the developing cerebellar plate (e.g. between the isthmus and choroid plexus) and is responsible for the generation of neuronal populations including deep cerebellar nuclei, Golgi neurons and Purkinje cells (Wang and Zoghbi, 2001). The EGL is formed from derived cells migrating exclusively from the metencephalic rhombic lip. Before birth, the EGL consists of a thin layer of granule precursor cells covering the entire surface of the cerebellum. Cells on the outer layer will continue to proliferate after birth, while cells that move inwards to the inner granule layer (IGL) become post-mitotic granule neurons [**Figure 1** (Wang and Zoghbi, 2001; Goldowitz and Hamre, 1998)].

Otx2 and *Gbx2* are two central genes in cerebellar development. *Otx2* is expressed in the mesencephalon whereas *Gbx2* is expressed in the metencephalon. Both genes regulate the expression of fibroblast growth factor 8 (FGf8), which in turn controls *Wnt1* and *En1*, both of which are important for proper cerebellar development (Wang and Zoghbi, 2001). Sonic hedgehog (Shh) signalling controls cerebellar development at multiple levels through the Shh ligand, which is secreted by Purkinje neurons. Shh regulates cell proliferation of the outer EGL precursors, is also a required mitogen for granule neurons, and induces differentiation of Bergmann glia (Dahmane and Ruiz i Altaba, 1999). Wnt signalling is required for normal development of the cerebellum. Mutants of the proto-oncogene *Wnt-1* show loss of the entire cerebellum (McMahon

and Bradley, 1990; Schuller and Rowitch, 2007; Thomas and Capecchi, 1990), while ablated β-catenin (mediator of Wnt signalling) leads to abnormal cerebellar morphogenesis in mice embryos (McMahon and Bradley, 1990; Schuller and Rowitch, 2007; Thomas and Capecchi, 1990). The final stage of maturation of granule neurons occurs in the IGL. This process is partly controlled by the diffusible factor Wnt-7a, which is released by granule neurons (Hall et al., 2000). The phosphoinositide-3-kinase (PI3K)/Akt signalling pathway has an important role in regulating growth and survival of neuronal precursor cells in the developing cerebellum. Mice homozygous for deletions of phosphatase with tensin homology (PTEN), a negative regulator of PI3K/Akt signalling, show primary granule-cell dysplasia in the cerebellum and abnormalities in cerebellar tissue architecture (Backman et al., 2001).

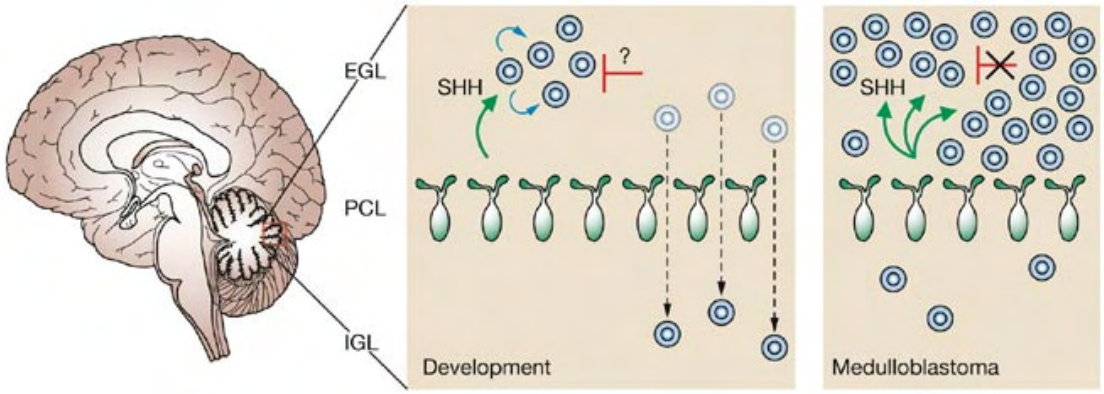

Figure 1. Granule-cell development and tumorigenesis of medulloblastoma. Reprinted, with permission, from Nature Publishing Group. Polkinghorn WR and Tarbell NJ (2007) Medulloblastoma: tumorigenesis, current clinical paradigm, and efforts to improve risk stratification *Nat Clin Pract Oncol* **4**: 295–304. Abbreviations: EGL, external granule layer; IGL, internal granule layer; SHH, sonic hedgehog; PCL, Purkinje cell layer.

1.3.1.1.2 Wnt signalling

The Wnt signalling pathway is a critical regulator of stem cells (SC) and is essential for proper embryonic development. It tightly controls cell-to-cell communication in multiple developmental events and has been implicated in several diseases, particularly in cancer (Logan and Nusse, 2004; Reya and Clevers, 2005). The Wnt cascade regulates cell proliferation, cell fate specification, and cell differentiation through the key mediator β-catenin, and is often called the β-catenin dependent pathway or canonical Wnt pathway (Logan and Nusse, 2004). This section focuses only on well-established core components of the β-catenin dependent pathway and does not cover the non-canonical pathway, or β-catenin independent pathway, which regulates convergent extension during vertebrate gastrulation, the polarity of hairs, and neuronal migration (Veeman et al., 2003).

1.3.1.1.2.1 Overview of canonical Wnt signalling

The Wnt pathway consists of more than 30 extracellular Wnt ligands, which interact with receptors of the Frizzled (Fz) family (**Figure 2**). A major effector of canonical Wnt signalling is the transcription factor β-catenin. The stability of β-catenin is

regulated by the destruction complex that includes adenomatous polyposis coli (APC), axin, glycogen synthase kinase-3β (GSK-3β) and casein kinase Iα (CKIα). In the absence of Wnt ligands, the two scaffolding proteins APC and axin bind newly synthesized β-catenin. The two kinases GSK-3β and CKIα then sequentially phosphorylate a set of conserved Ser and Thr residues on β-catenin. This results in ubiquitination and degradation of β-catenin by the proteosome [reviewed in (Barker and Clevers, 2006; Logan and Nusse, 2004)].

When Wnt signalling is initiated by secreted Wnt ligands, the family of seven transmembrane Fz receptors are engaged with the low-density lipoprotein (LDL) receptor-related protein (LRP) complex, Lrp5/6. Binding of Wnt to the Fz-LRP receptor complex facilitates reconfiguration of the Fz transmembrane domains. Subsequently, the intracellular proteins Dishevelled (Dsh) and Axin are recruited to the cell membrane, and phosphorylation of the cytoplasmic compartment of the LRP will occur. In addition, relocation of Axin to the membrane leads to inhibition of β-catenin phosphorylation and subsequent inactivation of the destruction complex. This elevates cytoplasmic β-catenin protein levels, causing β-catenin to enter the cell nucleus, where it interacts with members of the T cell factor/lymphoid enhancer factor (Tcf/Lef) family of transcription factors. In the absence of a Wnt signal, Tcf/Lef forms a complex with Groucho and histone acetylases to act as repressors of Wnt target genes. The binding of β-catenin to Tcf/Lef relieves the repressive activity of Groucho, and thus activates Tcf target genes such as cellular myelocytomatosis virus related oncogene (*c-Myc*) and *cyclin D1* [reviewed in (Barker and Clevers, 2006; Logan and Nusse, 2004)].

1.3.1.1.2.2 Wnt activation in medulloblastoma

Mutations in key components of the Wnt pathway that promote constitutive activation (e.g. β-catenin stabilization) of Wnt signalling have been found in many different cancers, including colorectal cancer and medulloblastoma (Huang et al., 2000; Reya and Clevers, 2005). The best-known examples of diseases involving Wnt aberrations are cases of hereditary familial adenomatous polyposis (FAP). Individuals diagnosed with FAP have mutations in the *APC* gene and display a predisposition to colorectal adenomas and medulloblastomas (Hamilton et al., 1995). FAP is an autosomal-dominant inherited disease characterized by the development of large numbers of benign adenomatous polyps (adenomas) of the colorectal epithelium. In most cases, these polyps progress to malignancy if not treated (Kinzler and Vogelstein, 1996). Turcot syndrome, which is a subclass of FAP, is an inherited autosomal recessive disease that can either result from mutations in the *APC* gene or a mismatch of repair genes (Hamilton et al., 1995).

Activation of the Wnt signalling pathway is also a feature of up to 25% of sporadic medulloblastomas (Ellison et al., 2005). For instance, mutations in components of the Wnt pathway, including *CTNNB1* (encoding β-catenin), *APC* and *Axin1*, have been demonstrated in approximately 15% of sporadic medulloblastomas (Dahmen et al., 2001; Eberhart et al., 2000; Huang et al., 2000). Several studies using gene expression arrays show that Wnt signalling is activated in a distinct molecular subset of sporadic medulloblastoma (Kool et al., 2008; Pomeroy et al., 2002; Thompson et al., 2006).

1.3.1.1.3 Sonic hedgehog signalling

Hh signalling has long been known to regulate growth and patterning during embryonal development. Recent data also indicate that Hh signalling is essential for SC maintenance and regeneration (Varjosalo and Taipale, 2008). Three Hh genes have been identified in mammals: Shh, Indian hedgehog (Ihh), and desert hedgehog (Dhh). Germ-line mutations that affect Hh signalling activity are associated with developmental disorders, whereas sporadic mutations activating the pathway are linked to multiple forms of cancer, including medulloblastoma. Since Shh is crucial in normal cerebellar development, it has also been studied extensively in medulloblastoma (Varjosalo and Taipale, 2008; Beachy et al., 2004).

1.3.1.1.3.1 Overview of sonic hedgehog signalling

Similar to Wnt, Shh ligands are lipid-modified and have the ability to act over a long range and to control cell function in a time and concentration-dependent manner. In the absence of Shh, the 12-span transmembrane cell-surface receptor Patched acts to inhibit the activity of the 7-span transmembrane receptor-like protein Smoothened (Smo). Lack of Smo activity results in phosphorylation of Gli2/Gli3, which is further sequestered to the cytoplasm, where Gli2/Gli3 forms a complex with Fused and Costal-2. Subsequently, Gli3 is recognized by β-TrCP and proteolytically processed to generate the truncated repressor form GliR. Truncated Gli3 represses a subset of Shh target genes [**Figure 2**, reviewed in (Varjosalo and Taipale, 2008)].

The binding of Shh to Patched releases inhibitory activity on Smo, which triggers the activation of Gli-2 (GliA) by inhibiting the major intracellular inhibitor of Gli, Suppressor of fused (SuFu). Free GliA can translocate to the nucleus and activate expression of target genes including *Gli-1*, *Patched*, *Myc* and *cyclin D1* (Varjosalo and Taipale, 2008).

1.3.1.1.3.2 Shh signalling activation in medulloblastoma

Abnormal Shh activation is implicated in the development of medulloblastoma. The involvement of Shh in cancer was first identified in patients with nevoid basal cell carcinoma syndrome (NBCCS), also known as Gorlin syndrome, who showed mutations in the Shh receptor *PTCH* (Hahn et al., 1996). Gorlin syndrome is an autosomal dominant disorder that predisposes individuals to developmental effects and cancer, including medulloblastoma. Approximately 1-2% of medulloblastomas are attributable to the syndrome (Hahn et al., 1996). Also, individuals with germ-line and somatic mutations in *SUFU* are predisposed to develop medulloblastoma (Taylor et al., 2002). Abnormal Shh signalling activity occurs in approximately 20% of sporadic medulloblastomas (Kool et al., 2008; Thompson et al., 2006). These observations are consistent with data demonstrating that mice with heterozygous germ-line mutations in the *PTCH* gene (e.g. $Ptch^{+/-}$) develop medulloblastoma tumours (Goodrich et al., 1997) and that drugs that specifically target the Shh pathway have profound effects in preclinical models of medulloblastoma (Berman et al., 2002; Romer et al., 2004; Rudin et al., 2009).

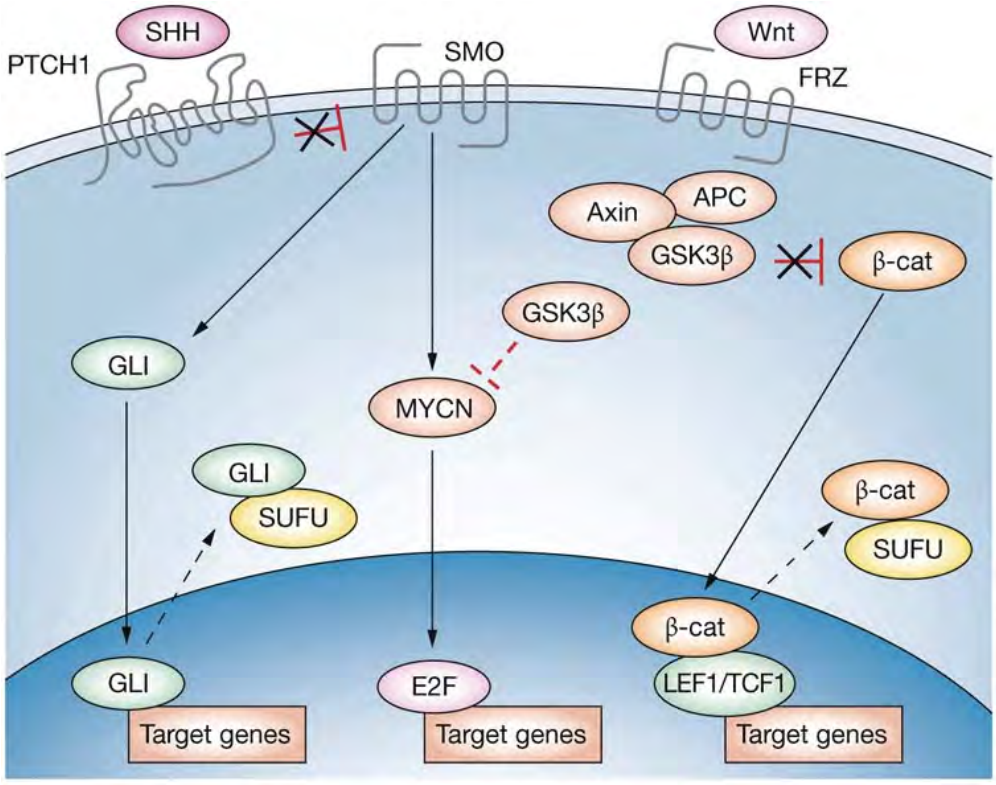

Figure 2. Sonic hedgehog and Wingless signalling pathways implicated in the formation of medulloblastoma. Reprinted, with permission, from Nature Publishing Group. Polkinghorn WR and Tarbell NJ (2007) Medulloblastoma: tumorigenesis, current clinical paradigm, and efforts to improve risk stratification *Nat Clin Pract Oncol* **4**: 295–304.

1.3.1.1.4 PI3K/Akt signalling

PI3K activation is one of the most important signalling nodes of human physiology and disease. It initiates a signal transduction cascade that regulates many essential functions such as promoting cell proliferation, differentiation, angiogenesis and metabolism (Manning and Cantley, 2007). The role of PI3K signalling has also been implicated in development. For instance, proliferation, survival and maintenance of pluripotency in mouse embryonic SCs is regulated by the PI3K/Akt pathway (Takahashi et al., 2005). The master regulator of PI3K is the serine-threonine protein kinase Akt (also known as protein kinase B, PKB), which regulates multiple downstream effectors including GSK-3β and mammalian target of rapamycin (mTOR). Inappropriate activation of the PI3K/Akt pathway has been directly implicated in many diseases, including type-2 diabetes and cancer (Manning and Cantley, 2007).

1.3.1.1.4.1 Overview of PI3K/Akt signalling

Activation of PI3K signalling is mediated through receptor tyrosine kinases (RTKs) with a subsequent activation of Akt, which in turn activates different signalling routes [**Figure 3** (Manning and Cantley, 2007)]. Activation of RTKs is mediated by growth factors or cytokines. PI3Ks are heterodimers that consist of a p85 regulatory and a p110 catalytic subunit. The p85 regulatory subunit is crucial in mediating class IA PI3K activation by RTKs. The Src-homology 2 (SH2) domains of p85 bind to active RTKs.

This binding of SH2 domains serves both to recruit the p85–p110 heterodimer to the plasma membrane, where phosphatidylinositol-4,5-bisphosphate (PIP_2) is converted to phosphatidylinositol-3,4,5-trisphosphate (PIP_3), and to relieve basal inhibition of p110 by p85 [reviewed in (Engelman, 2009; Fan et al., 2009; Fan et al., 2006)].

PI3K activates Akt through two different routes. First, RTKs activate class I PI3K by either direct binding to RTKs or through phosphorylation by the scaffolding adaptor protein IRS1. PI3K then phosphorylates PIP_2 to generate PIP_3 in a reaction that can be reversed by PTEN. PIP_3 then binds to Akt and phosphoinositide-dependent protein kinase 1 (PDK1) at the plasma membrane. This makes PDK1 phosphorylate the activation loop of Akt at motif Threonine308 (Thr308), and thus activates Akt. Second, RTKs can also, through a currently unknown mechanism, activate mTOR complex 2 (mTORC2), which phosphorylates Akt on motif Serine473 (Ser473) to the fully active Akt. Akt can then utilize several downstream substrates such as mTORC1 and GSK-3β to mediate different cellular processes [reviewed in (Cantley, 2002; Engelman, 2009; Manning and Cantley, 2007)].

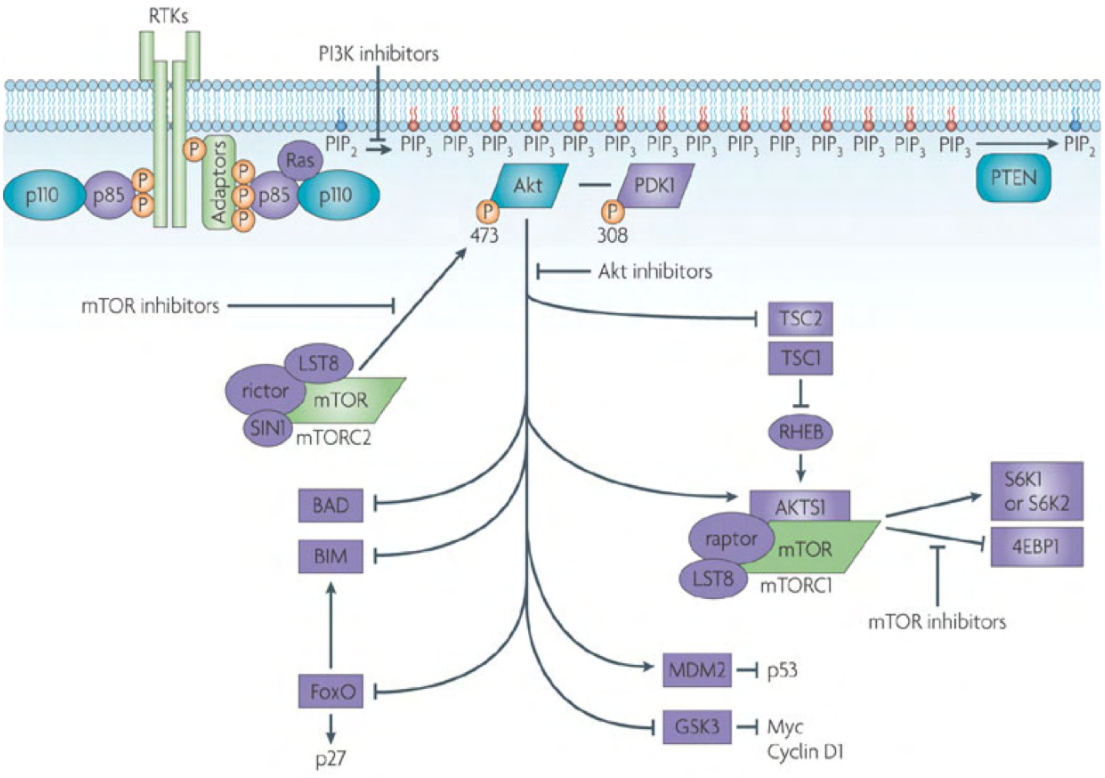

Figure 3. Schematic illustration PI3K/Akt/mTOR signalling pathway. Reprinted, with permission, from Nature Publishing Group. Engelman JA (2009) Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nature Reviews Cancer **9**: 550-62.

1.3.1.1.4.1.1 PI3K/Akt-mTOR signalling

One of the best-conserved functions of Akt is its role in promoting cell growth and survival. The predominant mechanism is mediated by one of the major effectors of Akt, mTORC1. mTORC1 is regulated by the signalling pathways that respond to growth factor stimulation and to changes in energy levels, controlling a wide range of growth-related cellular processes, including translation initiation, ribosome biogenesis, autophagy and hypoxic adaptation. mTORC1 consists of mTOR (responsible for the catalytic activity), a regulatory associated protein of mTOR (raptor), and mLST8 (mammalian lethal with sec13 protein 8, also known as GbL). The main target proteins of mTORC1 are the 4EBP1 family of proteins (translational repressor) and the S6 protein kinases [S6K, reviewed in (Wullschleger et al., 2006)].

mTOR signalling is activated by Akt through two parallel routes (**Figure 3**). Akt can activate mTORC1 indirectly by acting as a negative regulator on the TSC1-TSC2 complex or through PRAS40. Akt phosphorylates TSC2 on multiple sites, which prevents TSC2 from acting as a GTPase-activating protein for Rheb, which in turn allows Rheb-GTP to accumulate in the cytoplasm and thereby phosphorylate 4EBP1 and S6K. Akt can also activate mTORC1 independently of Rheb-GTP through direct phosphorylation of PRAS40, thereby reliving inhibition on mTORC1. Once 4EBP1 is phosphorylated, PDK1 (independently of PIP_3) is recruited and S6K is phosphorylated to be fully active (Bai and Jiang, 2009). Active S6K phosphorylates GSK-3β and ribosomal protein S6, a protein required for translation of 50 terminal oligopyrimidine (TOP) mRNAs encoding ribosomal proteins and elongation factors. Active 4E-BP1 binds and inhibits the translation initiation factor 4E (eIF-4E), a key factor regulating *Myc* and *cyclin D1* expression [reviewed in (Bai and Jiang, 2009; De Benedetti and Graff, 2004; Wullschleger et al., 2006)].

1.3.1.1.4.2 PI3K/Akt activation in medulloblastoma

Activation of the PI3K/Akt pathway is perhaps the most commonly observed activation in human cancer (Engelman, 2009). PI3K/Akt can be aberrantly activated in human cancers either by RTKs or by somatic mutations in specific components of the signalling pathway (Engelman, 2009). The most important negative regulator of PI3K/Akt is the tumour suppressor PTEN. Somatic mutations in PTEN have been discovered in several forms of cancers, such as brain, breast and prostate cancer (Li et al., 1997) as well as in medulloblastoma, where 16% of medulloblastomas display allelic loss of PTEN (Hartmann et al., 2006). Somatic activating mutations in *PIK3CA* (encoding p110α) and *PIK3R1* (encoding p85α) occur in several forms of cancer (Engelman, 2009; Fan et al., 2009; Fan et al., 2006). Activation of the RTK's insulin-like growth factor 1 receptor [IGF-1R (Del Valle et al., 2002)], neurotrophin-3 receptor TRKC (Grotzer et al., 2000) and the ERBB2 receptor (Gilbertson et al., 1995) are also commonly observed in medulloblastoma, indicating activity of PI3K/Akt signalling (**Figure 6**). Furthermore, PI3K/Akt has recently been shown to regulate survival of medulloblastoma cancer stem cells (CSCs) following radiation (Hambardzumyan et al., 2008).

1.3.2 Clinical features of medulloblastoma

1.3.2.1 Histopathology

Medulloblastoma belongs to the family of embryonal brain tumours and is classified as a grade IV tumour. Four distinct histopathological subtypes of medulloblastoma have been described: classical medulloblastoma, desmoplastic/nodular medulloblastoma, medulloblastoma with extensive nodularity (MBEN), and large cell/anaplastic medulloblastoma [LA/C, **Figure 4** (Louis et al., 2007; Polkinghorn and Tarbell, 2007)].

1.3.2.1.1 Classical medulloblastoma

Classical medulloblastoma (65%) is the most common subtype. This type consists of uniform sheets of dense small round blue cells and displays neuronal differentiation (Louis et al., 2007; Polkinghorn and Tarbell, 2007).

1.3.2.1.2 Desmoplastic/nodular medulloblastoma

Desmoplastic medulloblastoma (25%) is characterized by a tissue pattern consisting of reticulin-free nodules surrounded by proliferating cells that produce a reticulin-rich extracellular matrix. This subtype has been linked to inactivating mutations of *PTCH* and displays better prognosis than classical and LC/A subtypes (Louis et al., 2007; Polkinghorn and Tarbell, 2007).

1.3.2.1.3 Medulloblastoma with extensive nodularity

This subtype (5%) is similar to desmoplastic/nodular medulloblastoma but differs from the desmoplastic nodular variant by exhibiting a markedly expanded lobular architecture and advanced neuronal differentiation. MBEN occurs almost exclusively in infants and shows dysregulation in Shh signalling. These patients have a surprisingly good prognosis, considering the fact that infants with medulloblastoma display poor survival rates (Louis et al., 2007; Polkinghorn and Tarbell, 2007).

1.3.2.1.4 Large cell/anaplastic medulloblastoma

The LC/A subtype (5%) is the most undifferentiated subtype. These medulloblastomas display characteristic cells with spherical cells that have large nuclei, open chromatin and prominent nucleoli (Louis et al., 2007; Polkinghorn and Tarbell, 2007). Prognosis is poor depending on the grade of anaplasia (none, slight, moderate and severe) in the tumour. Patients with moderate and severe anaplasia show worse outcomes (Eberhart et al., 2002).

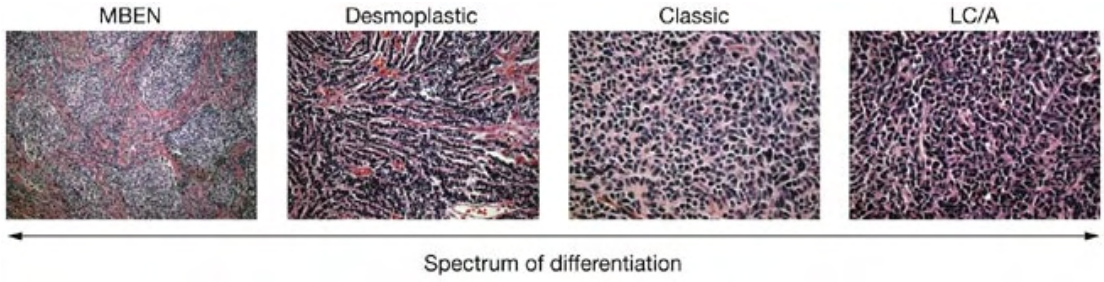

Figure 4. Histopathologic subtypes of medulloblastoma. Reprinted, with permission, from Nature Publishing Group. Polkinghorn WR and Tarbell NJ (2007) Medulloblastoma: tumorigenesis, current clinical paradigm, and efforts to improve risk stratification *Nat Clin Pract Oncol* **4**: 295–304.

1.3.2.2 Risk stratification

Despite recent advances in molecular biology, medulloblastoma risk assessment is solely determined by clinical parameters (Gilbertson, 2004). Since clinical outcome varies according to age, extent of metastatic disease and residual tumour size following surgery, risk-adapted treatment is implemented (**Figure 5**). Patients that are diagnosed at age older than 3 years, with no metastatic disease and with post residual disease <1.5 cm^2, are stratified in the standard-risk (SR) group. Patients who don't meet these criteria are classified as high-risk (HR) patients. Current survival rate for SR patients is 80%, whereas almost half of patients categorized in the HR group will succumb to the disease (Polkinghorn and Tarbell, 2007; Louis et al., 2007; Dhall, 2009). Recent advances in molecular biology have identified new genetic and biological markers with significant implications in survival. These will therefore also be covered in detail.

1.3.2.2.1 Age

Children younger than 3 years have significantly poorer outcomes than older patients with medulloblastoma. Since younger children are excessively vulnerable to and thus restricted from receiving CSRT, survival rates are drastically lower in this group. The probability that children will die within 5 years is twice as high in children younger than 3 years when compared with older patients (Zeltzer et al., 1999). Other contributing factors to poorer survival rates may be related to the biology of tumours or the extent of post residual disease. Tumours that arise in younger children may be associated with aggressiveness and metastasis. It is also more difficult to resect tumours from young children since they are smaller and more vulnerable to surgery (Deutsch, 1988).

1.3.2.2.2 Disseminated disease

Clinically, medulloblastoma patients are staged according to Chang's criteria. In short, M0 patients show no evidence of metastatic disease; M1 patients have cells in the cerebrospinal fluid; M2-M4 patients are presented with metastatic disease in the CNS or outside the CNS (Gilbertson, 2004). Approximately one third of patients have metastatic disease at time of diagnosis (Zeltzer et al., 1999) and metastatic disease is one of the most robust indicators of outcome in medulloblastoma. The current 5-year event-free survival (EFS) rates in patients with metastatic disease compared to patients without metastases are 50-60% *v* 80-85% (Crawford et al., 2007; Packer, 2008).

1.3.2.2.3 Post residual disease

The prognostic power of residual disease is questioned. Data supporting the significance of post residual disease have only been shown in children older than 3 years with non-disseminated disease. In a study enrolling 188 children with medulloblastoma, patients with less than 1.5 cm^2 post residual disease had a 5-year progression-free survival (PFS) of 78 ± 6%, compared to 54 ± 11% for those patients with greater than 1.5 cm^2 of residual disease (Zeltzer et al., 1999).

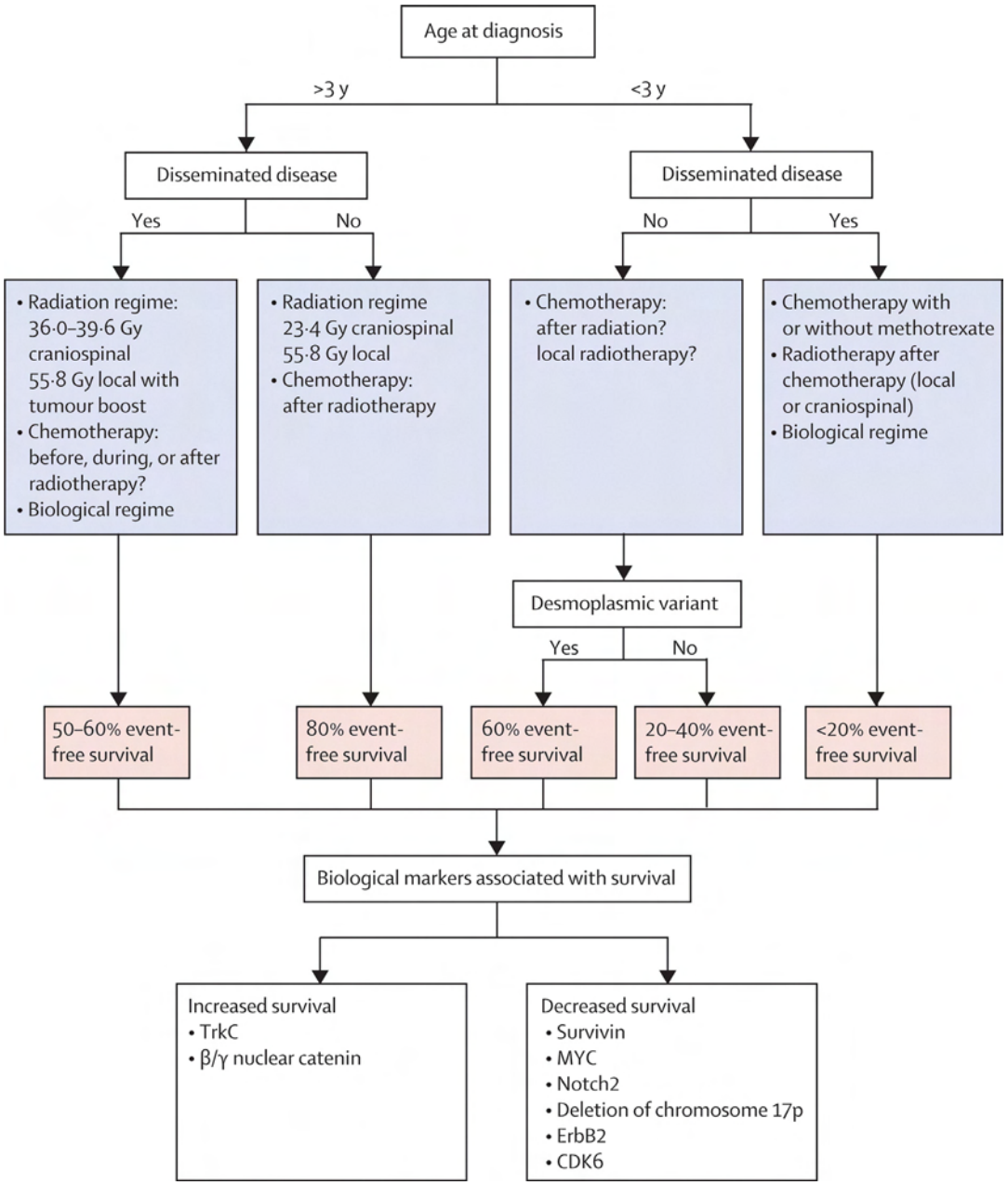

Figure 5. Treatment scheme for medulloblastoma. Reprinted, with permission, from Elsevier. Crawford JR, MacDonald TJ and Packer RJ (2007) Medulloblastoma in childhood: new biological advances. Lancet Neurology 6: 1073-85.

1.3.2.2.4 Biological and genetic markers

1.3.2.2.4.1 Receptor tyrosine kinases

Several RTKs have been correlated with survival in medulloblastoma patients (**Figure 6**). One of the first markers predicting clinical outcome was the neurotrophin-3 receptor TRKC. Neurotrophin-3 (NT-3) activates TRKC and regulates proliferation, differentiation and cell death of the granule cells of the developing cerebellum. High TRKC mRNA expression is today accepted as a powerful independent predictor of favourable outcome in medulloblastoma [5-year cumulative survival rate: 89% *v* 46% (Grotzer et al., 2000)].

The ERBB2 receptor is one of the best-known markers of survival, and has been recognized as a marker for poor prognosis. ERBB2 is activated by epidermal growth factor (EGF) with subsequent activation of Ras and Akt. A study by Gajjar and co-workers revealed that 40% of medulloblastoma patients have ERBB2 receptor expression and that SR patients positive for ERBB2 have a significantly worse PFS when compared to SR patients negative for ERBB2 [5 year survival rate: 54% *v* 100% (Gajjar, et al., 2004)].

1.3.2.2.4.2 Wnt/β-catenin activation

Compelling evidence demonstrates that patients with dysregulated Wnt/β-catenin signalling belong to a distinct molecular sub-group of medulloblastomas (Clifford et al., 2006; Ellison et al., 2005; Thompson et al., 2006). Cases displaying Wnt/β-catenin activation are also exclusively associated with a chromosomal loss of 6p (Clifford et al., 2006). Approximately 25% of all medulloblastoma patients have an active Wnt/β-catenin pathway, indicated by the localisation of β-catenin in the nucleus. Patients displaying β-catenin nucleopositivity have better overall survival (OS) rates compared to patients without Wnt/β-catenin activation [92% *v* 65% (Clifford et al., 2006; Ellison et al., 2005)].

1.3.2.2.4.3 MYC and MYCN

Genomic amplification of *MYC*/v-myc myelocytomatosis viral related oncogene, neuroblastoma derived *(MYCN)* is strongly correlated to poor prognosis [5-year OS: $\text{amplified}_{13\%}$ *v* $\text{not amplified}_{73\%}$ (Pfister et al., 2009; Grotzer et al., 2001)]. High-level amplification of *MYC* (6%) and *MYCN* (4%) is found in approximately 10% of medulloblastomas and occurs predominantly in the more aggressive variant LC/A (Pfister et al., 2009). Low expression of MYC mRNA predicts a good prognosis, particularly when found in combination with increased TRKC expression (Grotzer et al., 2001).

1.3.2.2.4.4 Chromosomal abnormalities

The most common cytogenetic abnormalities found in medulloblastoma are 17q gain (46%) and 17p deletion (37%), and approximately 30% of the patients harbour a combined gain of 17q and loss of 17p [e.g. isochromosome 17q (Pfister et al., 2009)]. The 5-year OS for 17q gain (55%) and isochromosome 17q (35%) are better predictors of outcome than 17p deletions [78% (Pfister et al., 2009)]. Gain of 6q is also correlated with poor prognosis [5-year OS: 15% (Pfister et al., 2009; Grotzer et al., 2001)]. These findings suggest that one or several centrally genes important for the prognosis of medulloblastoma exist on 6q. Surprisingly, all patients (12%) with loss of 6p survive their disease (Pfister et al., 2009). These findings coincide with data showing that loss of 6p is associated with Wnt/β-catenin activation, with a strong correspondence of favourable outcome (Clifford et al., 2006; Ellison et al., 2005).

1.3.3 Treatment

Medulloblastoma is best treated with multimodal therapy, including surgery, radiation therapy and chemotherapy. Currently, treatment regimen is based on risk assessment. Infants (<3 years) and relapsed patients are included in the HR group and are treated according to modified protocols (Dhall, 2009).

1.3.3.1 Surgery

The main objective of the surgeon is to resect as much as possible of the tumour mass (gross total resection) without harming surrounding tissue, hence maintaining acceptable postoperative morbidity. Surgeons are guided by microscopes and preoperative imaging to visualize the tumour and its anatomic relationships to surrounding structures. This helps the surgeon to better perform total resection and avoid damage to adjacent neural tissue (Packer et al., 1999a). Common complications seen after medulloblastoma surgery include cerebellar mutism syndrome (CMS), nerve palsies, brainstem dysfunction and aseptic meningitis (Pollack et al., 1995). CMS is characterized by paucity of speech leading to mutism, hypotonia, ataxia, and emotional instability. Brainstem involvement and hydrocephalus have been linked to the aetiology of CMS (Robertson et al., 2006). Patients with medulloblastoma mostly have tumours located in the midline of posterior fossa, either within the fourth ventricle or the cerebellar vermis. Prior to surgery, depending on location and tumour mass, a ventricular shunt or third ventriculostomy might be needed to relieve the patient from hydrocephalus. Surgery in patients with posterior fossa tumours is often performed as an open craniotomy, patients facing down, through a partial resection of the cerebellar vermis (Packer et al., 1999a).

1.3.3.2 Radiation therapy and chemotherapy

Radiation therapy has been recognized as a cornerstone in medulloblastoma treatment (Mueller and Chang, 2009).
Following surgical resection, medulloblastoma patients aged over 3 years are radiated with a standard dose of 36 Gy to the craniospinal axis with an extra boost to the posterior fossa, giving a total dose of 54-56 Gy. The aim of CSRT is to eliminate potential microscopic disease. However, children under 3 years of age usually do not receive radiation therapy, due to detrimental effects on the developing brain. This has resulted in poorer survival rates in younger children [reviewed in (Dhall, 2009)].

The use of chemotherapy in the treatment of medulloblastoma is now deemed standard care for children in all risk groups. The aim of chemotherapy is to either augment or delay/avoid radiation therapy. The most common used cytostatics are cisplatin, vincristine, lomustine, cyclophosphamide, CCNU and oral etoposide, either alone or in combination (Gajjar et al., 2006; Packer et al., 2006; Polkinghorn and Tarbell, 2007). The oral DNA alkylator temozolomide has also been tested against medulloblastoma with promising results (Wang et al., 2009). Several studies using radiation-avoiding strategies in younger children receiving postoperative chemotherapy alone demonstrate improved survival rates (Grill et al., 2005; Rutkowski et al., 2005). Chemotherapy is therefore frequently used as a course to delay, avoid or reduce radiation therapy (Dhall, 2009).

Radiation induced morbidity is also a problem in SR patients. Identifying the most effective radiation dose, radiation technique and adjuvant chemotherapy is very important. Several studies have addressed whether reduced radiation volume in combination with adjuvant chemotherapy or postoperative chemotherapy alone is sufficient to cure SR patients. This has led to a reduction in the dose of CSRT from 36 Gy to 23.4 Gy given to SR patients, but not to HR patients. However, although

chemotherapy has been important in reducing the dose of CRST, it has not altered survival rates (Gajjar et al., 2006; Packer et al., 2006; Packer et al., 1999b). In addition, postoperative chemotherapy alone can be sufficient in localized medulloblastoma patients that undergo gross total resection, but not enough for treatment of patients with partially resected tumours or those diagnosed with metastasis (Grill et al., 2005).

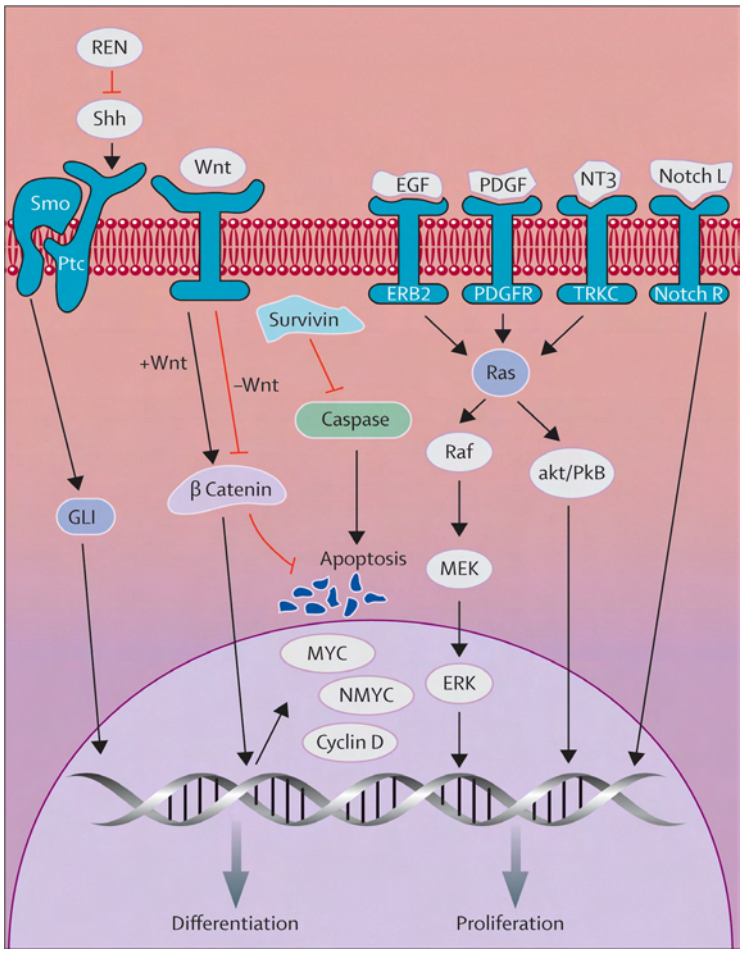

Figure 6. Schematic illustration of signalling pathways common activated in medulloblastoma. Reprinted, with permission, from Elsevier. Crawford JR, MacDonald TJ and Packer RJ (2007) Medulloblastoma in childhood: new biological advances. Lancet Neurology 6: 1073-85.

1.3.3.3 Resistance to treatment

Despite intensive treatment and improved survival rates, still almost half of medulloblastoma patients will relapse and have a dismal prognosis (Dhall, 2009; Lannering et al., 2009). Resistance to treatment is a major drawback in radiation therapy and chemotherapy. As described above, resistance to chemotherapy can be mediated by BBB, efflux pumps and upregulation of DNA repair proteins including MGMT. Increasing evidence also proposes the role of CSCs in the cellular resistance mechanism to post-surgical treatment (Visvader and Lindeman, 2008).

1.3.3.3.1 O^6-Methylguanine-DNA methyltransferase

DNA repair proteins have been known for a long time to be involved in the resistance mechanism to radiation and cytostatics. DNA alkylators, which cause DNA damage by adding groups to DNA and hence inducing apoptosis, are commonly used to treat malignant brain tumours (Mueller and Chang, 2009). One of the most used DNA alkylators against glioblastoma, and also sometimes used in recurrent medulloblastoma,

is the methylating agent temozolomide (Hegi et al., 2008; Nicholson et al., 2007; Wang et al., 2009).

The effect of temozolomide is to modify DNA at several sites, where the most toxic effect is to yield O^6-methylguanine adducts (Newlands et al., 1997). However, these adducts are efficiently repaired by the cellular DNA repair gene *MGMT* [(the gene that encodes the DNA repair protein O^6-alkylguanine (O^6-AG) DNA alkyltransferase, AGT)], which reverses alkylation and methylation at the O^6-methylguanine adducts and thereby neutralizes the cytotoxic effect. The relative expression of MGMT protein has been demonstrated to convey resistance in a variety of tumours and experimental models (Gerson, 2004). Consistent with this, epigenetic silencing of the *MGMT* gene, correlates with improved responsiveness to therapy (Esteller et al., 2000; Hegi et al., 2005).

Several MGMT inhibitors have been shown to inactivate MGMT enzyme activity. The most promising compounds tested in clinical trails are O^6-benzylguanine, O^6-(4-bromothenyl)guanine and 2-amino-O^4-benzylpteridine (Hegi et al., 2008). Another approach to evade resistance is to deplete MGMT by prolonged exposure to low doses of alkylating agents. This is possible since alkyl groups (for instance O^6-methylguanine) can irreversibly inactivate MGMT and thus require *de novo* protein synthesis to maintain enzyme activity. This process is saturable, making an excess of alkyl groups in the DNA and resulting in MGMT depletion. However, since MGMT inhibitors and depletion also lower basal levels of MGMT in normal cells, treatment-induced toxicity is seen in blood cells, often leading to myelosuppression. To overcome these limitations, discovery of new agents that specifically modulate MGMT in tumour cells and thus avoid resistance is highly warranted [reviewed in (Hegi et al., 2008)].

1.3.3.3.2 Cancer stem cells

CSCs, also called tumour initiating cells (TICs), are defined as a distinct subpopulation of cells, hierarchically organized with the self-renewing capacity to generate the diverse cells that comprise the tumour. These cells share important properties with normal stem cells (SCs), including self-renewal (by symmetrical and asymmetrical division) and differentiation capacity. The most convincing demonstration of CSC identity comes from serial transplantation of CSCs into animal models. The CSC should re-establish the phenotypic heterogeneity of the primary tumour and exhibit self-renewal capability on serial passaging [reviewed in (Visvader and Lindeman, 2008)].

The origin and existence of CSCs has been widely debated. The cell of origin specifically refers to the cell type that receives the first oncogenic hit(s). Whether CSCs come from a normal SC, a restricted progenitor or a differentiated cell (that is transformed and has acquired self-renewing capacity) is widely debated (Visvader and Lindeman, 2008).
The first CSCs to be identified came from acute myeloid leukaemia (AML) and were discovered by John Dick's lab in 1994. A rare subset (0.01-1%) of $CD34^+/CD38^-$ cells, with unlimited proliferative capacity, was identified to induce leukaemia in transplanted severe combined immune-deficient mice [SCID (Lapidot et al., 1994)].

CSCs have also been implicated in metastatic disease. Not all cells in a tumour have the ability to metastasize to other organs. In epithelial malignancies, the epithelial mesenchymal transition (EMT) is considered to be a crucial event in the metastatic process, which involves disruption of epithelial cell homeostasis and the acquisition of a migratory mesenchymal phenotype (Thiery, 2002).

One of the first solid tumours from which CSCs were identified was in medulloblastoma (Singh et al., 2003; Singh et al., 2004), and there is compelling evidence today linking medulloblastoma development to CSCs (Fan and Eberhart, 2008). Studies on gene expression and murine models have revealed that many of the pathways (Wnt, Shh, PI3K and Notch) required in neural stem cells (NSC), multipotent cerebellar SC, and lineage-restricted progenitors of the EGL are also aberrantly activated in medulloblastoma [**Figure 6** (Fan and Eberhart, 2008; Hambardzumyan et al., 2008)]. It seems also that developmental SC hierarchies similar to those in foetal brains are maintained in medulloblastoma (Ward et al., 2009). Consistent with this, oncogenic activation of *PTCH* in lineage-restricted granule cell progenitors and NSC has been shown to form medulloblastoma tumours (Schuller et al., 2008; Yang et al., 2008).

In the context of surface markers in medulloblastoma, CD133 (marker for several different SCs) and CD15 (NSC marker/stage-specific embryonic antigen 1) have been instrumental in identifying CSCs in medulloblastoma. The first studies to report CSCs in medulloblastoma showed that primary medulloblastoma tumours consist of a heterogeneous subpopulation of CD133 positive cells with a stem-like phenotype (Singh et al., 2003; Singh et al., 2004). Furthermore, it appears that CD133 positive cells alone can be maintained as multipotent neurospheres in the same culturing conditions as a normal NSC (e.g. neurosphere medium) and form the same heterogeneous phenotype of the original tumour in transplanted animals (Singh et al., 2004). Cells expressing CD15/SSEA-1 and Math-1, but not CD133, are cancer propagating in medulloblastomas derived from *PTCH* heterozygous mice. A subset of human medulloblastomas is CD15 positive; these patients have a poorer prognosis (Ward et al., 2009; Read et al., 2009).

The role of CSCs in resistance to therapy has been of great interest. Quiescent CSCs are resistant to radiation therapy and chemotherapy. For instance, medulloblastoma CSCs residing in the perivascular niche are resistant to radiation through PI3K/Akt pathway regulation, and inhibition of Akt sensitises cells to radiation (Hambardzumyan et al., 2008). In addition, CD133 positive cells in glioblastoma and medulloblastoma confer radioresistance and could therefore be the source of tumour recurrence after radiation (Blazek et al., 2007; Bao et al., 2006). Wnt pathway activation has also been demonstrated to mediate radioresistance in mammary mouse progenitor cells (Woodward et al., 2007). SCs also often express higher levels of drug-resistance proteins such as ATP-binding cassette half-transporter proteins 2 [ABCG2 (Bleau et al., 2009)].

1.4 EICOSANOIDS: INVOLVED IN CANCER AETIOLOGY?

Eicosanoids are lipid mediators that regulate inflammation and control immunity. They also act as second messengers in the CNS and regulate homeostasis. In fact, the eicosanoid cascade is one of the most complex networks in the human body (Funk, 2001). Increasing evidence indicates that the eicosanoid prostaglandin E_2 (PGE_2) has an important role in cancer, notably in colorectal cancer (Wang and Dubois, 2006).

Eicosanoids are generated from either omega-3 (ω-3) fatty acids, which are generally anti-inflammatory, or from omega-6 (ω-6) fatty acids, which promote inflammation. Four family members of eicosanoids have been identified: prostaglandins, leukotrienes, prostacyclins and thromboxanes. Conversion of ω-3/6 fatty acids to eicosanoids is mediated by cyclooxygenases (COX) or lipooxygenases (LOX), where the COX enzymes generate prostanoids (e.g. prostaglandins, prostacyclins and thromboxanes) and the LOX enzymes generate leukotrienes [reviewed in (Funk, 2001)].

1.4.1 Biosynthesis of prostaglandin E_2

The main precursor of PGE_2 is the ω-6 polyunsaturated fatty acid, arachidonic acid (AA). AA is enzymatically converted to PGE_2 in a series of enzymatic steps (**Figure 7)**. In a non-neoplastic setting, PGE_2 is formed when biosynthesis is activated by stimuli such as cytokines, growth factors, stress and mechanical trauma. Activation mobilizes cytosolic phospholipase A_2 ($cPLA_2$), which triggers the release of AA from the cell membrane and the nuclear membrane. Free AA is then presented to COX enzymes (also called prostaglandin H synthase, PGHS) and is further metabolized to an intermediate prostaglandin, PGH_2. Two isoforms of COX are known, COX-1 and COX-2, and they differ in many aspects. COX-1 is responsible for basal, constitutive synthesis of prostaglandins, thromboxanes, and prostacyclines, and appears to regulate normal physiological functions such as regulation of renal blood flow and maintenance of the gastric mucosa. By contrast, COX-2 is inducible by mitogenic and inflammatory stimuli, produces mainly PGE_2 and is frequently expressed in cancerous cells. The conversion of PGH_2 to PGE_2 is catalyzed by microsomal PGE synthase (mPGES) [reviewed in (Funk, 2001; Wang and Dubois, 2006)].

1.4.2 Prostaglandin E_2 signalling

PGE_2 exerts versatile actions in the human body by acting through a group of four different G-protein coupled receptors (GPCRs), designated EP1, EP2, EP3 (three isoforms) and EP4 (Chell et al., 2006; Sugimoto and Narumiya, 2007). GPCRs are seven-transmembrane domain receptors that are coupled to heterodimeric guanine nucleotide-binding proteins (G proteins), consisting of α, β and γ subunits. Ligand interaction with GPCR leads to the release of guanosine diphosphate (GDP) from the α subunit (Gα) and its replacement with guanosine triphosphate (GTP). The binding of GTP to the Gα leads to the dissociation of Gα from Gβγ dimer, triggering Gα-specific and/or Gβγ downstream pathways (Oldham and Hamm, 2008).

Prostanoid receptors show distinct downstream signalling effects and exert different processes depending on tissue/cell distribution and specificity. For instance, activation

of the EP1 receptor produces a transient rise in intracellular calcium, with subsequent PIP_3 formation. EP1 is a contractile receptor that is particularly abundant in kidneys and the smooth muscle cells that are associated with vessels in other organs. Activation of EP1 results in atrial contractility with renal vasoconstriction in the kidney and contraction in pulmonary venous smooth muscle with airway constriction in the lung [reviewed in (Chell et al., 2006; Sugimoto and Narumiya, 2007)].

Activation of the signalling cascade downstream of EP2, EP3 and EP4 is controlled by cAMP. EP2 and EP4 are coupled to $G\alpha_s$, which stimulates cAMP production (relaxant receptors) and is mostly expressed in vascular smooth muscles, in the eye and kidneys. On the contrary, EP3, which is mostly abundant in the kidney tubules, gastrointestinal tract neurons and uterus, is $G\alpha_i$-linked and acts to inhibit cAMP production (inhibitory receptor). cAMP regulates protein kinase A (PKA), which acts to regulate different target proteins and the downstream signalling response [reviewed in (Chell et al., 2006; Sugimoto and Narumiya, 2007)]. Recent evidence shows that EP2 and EP4 can activate the PI3K/Akt pathway with subsequent stimulation of extracellular signal-regulated kinases (ERKs) or Wnt/β-catenin signalling (Castellone et al., 2005; Fujino and Regan, 2003; Fujino et al., 2002).

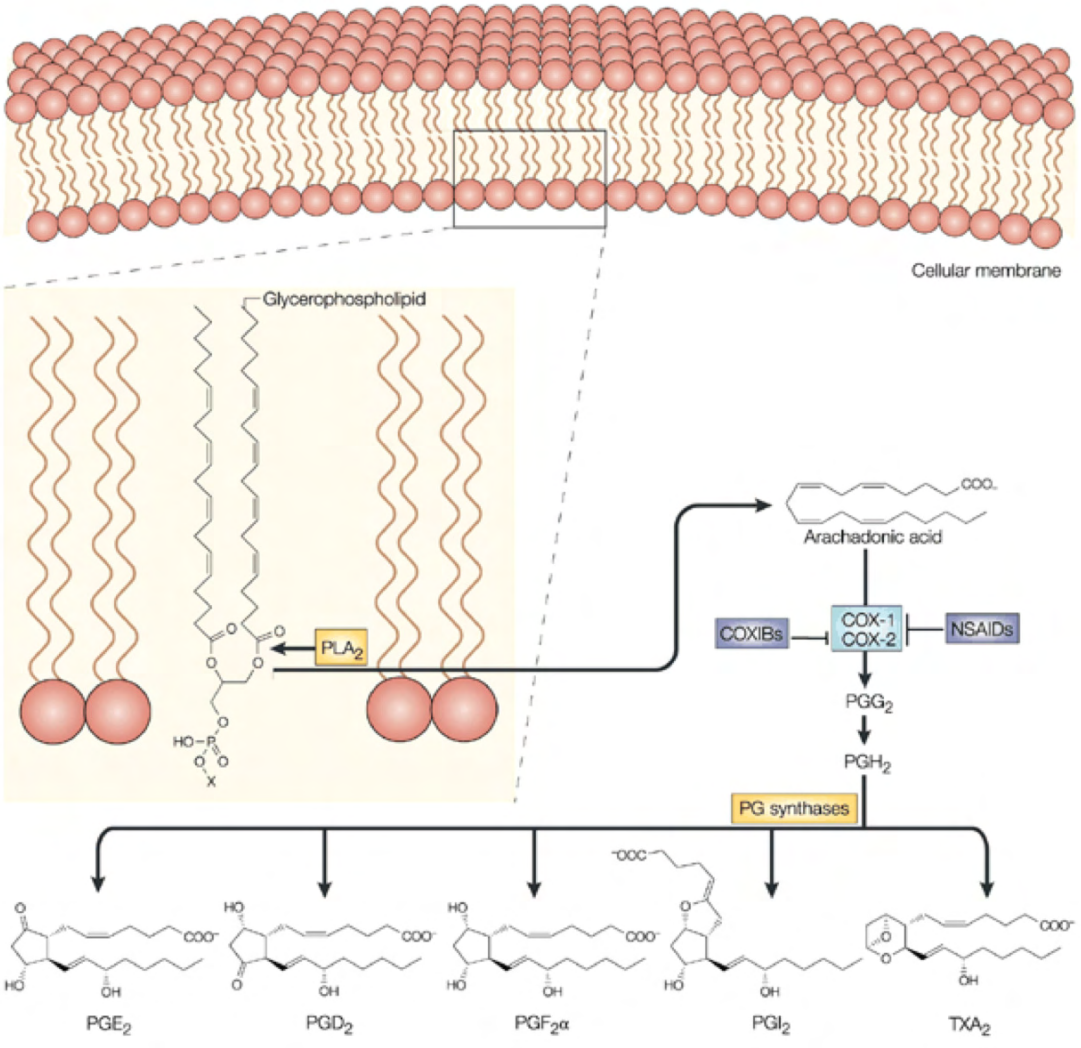

Figure 7. COX enzymes in prostaglandin synthesis. Reprinted, with permission, from Nature Publishing Group. Gupta RA and DuBois RN (2001) Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. Nature Reviews Cancer **1**: 11-21.

1.4.3 Prostaglandin E_2 in cancer

The link between cancer and inflammation was first made in the nineteenth century when it was discovered that tumours often arose at sites of chronic inflammation [reviewed in (Balkwill and Mantovani, 2001)]. Several epidemiological studies and clinical trials demonstrate that inflammation may have an essential role in the aetiology of cancer. For instance, chronic inflammation predisposes individuals to gastric cancer [e.g. Helicobacter pylori infection (Uemura et al., 2001)] and colorectal cancer [e.g. ulcerative colitis (Ekbom et al., 1990)]. Striking evidence also demonstrates that long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) decreases the incidence and mortality of certain malignancies including colorectal, breast, lung and bladder cancer (Wang and Dubois, 2006; Thun et al., 1991; Steinbach et al., 2000; Oshima et al., 1996; Chan et al., 2007; Arber et al., 2006). In fact, it has been recently suggested that inflammation should be added to the six hallmarks of cancer (Colotta et al., 2009; Greenhough et al., 2009), attributes defined by Hanahan and Weinberg as being requisite for tumourigenesis [e.g. self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis (Hanahan and Weinberg, 2000)].

Since the discovery that the NSAID aspirin reduces the risk of fatal colorectal cancer, a new research field has emerged investigating the role of PGE_2 in cancer (Thun et al., 1991). Indeed, PGE_2 has been shown to play a predominant role in promoting cancer progression, where it appears to affect most, if not all, of the hallmarks of cancer (Greenhough et al., 2009).

PGE_2 has been widely studied in colorectal cancer and the majority of colorectal tumours show aberrant expression of COX-2 (Wang and Dubois, 2006). PGE_2 has key roles in influencing the development of colorectal cancer. Accumulating evidence from animal models and clinical studies has further substantiated the evidence and also provided insights into the mechanisms underlying the oncogenic role of PGE_2 (Wang and Dubois, 2006; Thun et al., 1991; Steinbach et al., 2000; Oshima et al., 1996; Chan et al., 2007; Arber et al., 2006). Indeed, PGE_2 has been reported to activate pro-survival apoptotic pathways including canonical Wnt signalling, the PI3K/Akt pathway, ERK signalling, cAMP/PKA signalling and activation of EGF receptor signalling. Furthermore, PGE_2 is also pro-angiogenic and induces release of angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) [reviewed in (Greenhough et al., 2009)].

A large body of evidence from *in vitro* and *in vivo* studies indicates that canonical Wnt signalling participates in early colorectal tumour initiation (Bienz and Clevers, 2000; Fearon and Vogelstein, 1990; Phelps et al., 2009; van de Wetering et al., 2002). Furthermore, PGE_2 is known to act in concert with Wnt signalling to promote growth of colorectal cancer (Bienz and Clevers, 2000; Castellone et al., 2006; Castellone et al., 2005; Wang et al., 2004) and regulate haematopoietic SC homeostasis (North et al., 2007). Recent findings also demonstrate that PGE_2/Wnt interaction is a master regulator of vertebrate regeneration and recovery (Goessling et al., 2009). Taken together, the data show that the role of PGE_2-Wnt activation in tumourigenesis may not

only serve as a plausible explanation to the aetiology of colorectal cancer, but also provide insights to the aetiology of other cancers.

1.4.4 NSAIDs in cancer chemoprevention

The ability of PGE_2 to affect multiple aspects of tumourigenesis may also offer an explanation for the effectiveness of NSAIDs at reducing incidence and mortality of colorectal cancer.

As noted earlier, since the observation that the long-term use of NSAIDs prevents polyp formation in FAP patients, an interest to investigate these agents thoroughly as potential anti-cancer drugs has emerged during last two decades. The first epidemiological data describing NSAIDs as cancer protective agents came from a prospective mortality study showing that the risk of developing fatal colorectal cancer decreased with frequent use of low-dose aspirin (Thun et al., 1991). The effectiveness of NSAIDs as cancer preventing agents was further substantiated by clinical trials demonstrating that twice-daily treatment with 400 mg of the COX-2 specific inhibitor celecoxib leads to a significant reduction in colorectal polyps (Steinbach et al., 2000). Furthermore, the observed cancer preventive effects of aspirin and celecoxib were recently reported to correlate to moderate or strong expression of COX-2 in colorectal tumours (Arber et al., 2006; Chan et al., 2007). Consisting with these data, an animal study addressing the effectiveness of NSAIDs on polyp formation in Apc delta716 knockout mice (a model of human FAP) showed that treatment with celecoxib was more efficient against polyp formation when compared to the dual COX inhibitor sulindac. The same study provided further evidence of COX-2 significance in colorectal tumourigenesis. By knocking the COX-2 gene (Ptgs2) in Apc delta716 knockout mice, the authors demonstrated that the number and size of the intestinal polyps were reduced dramatically (Oshima et al., 1996).

1.4.4.1 Celecoxib and non-COX-2 binding analogs

Celecoxib is a highly selective COX inhibitor that primarily inhibits the COX-2 isoform and hence mainly inhibits prostaglandin synthesis (Grosch et al., 2006). It is a drug that is used to treat rheumatoid arthritis and acute pain and is the only NSAID that has been approved by the food and drug administration (FDA) for adjuvant treatment of patients with FAP (Steinbach et al., 2000). Celecoxib is one of the most widely studied coxibs/COX-2 selective inhibitors in cancer and displays the most potent anti-tumour functions of any NSAID (Schonthal et al., 2008).

Since COX-2 is found to modulate multiple pro-tumourigenic mechanisms, the rationale for combining celecoxib with conventional treatment modalities is of high interest. For instance, overexpression of COX-2 in tumours is associated with an increase in the production of the multidrug resistance protein P-glycoprotein, and this upregulation can be prevented by celecoxib (Patel et al., 2002). Celecoxib also enhances the response to chemotherapy (Altorki et al., 2003; Blanke et al., 2005) and acts as a radiosensitizer in tumour cells (Choy and Milas, 2003).

However, celecoxib has demonstrated anti-tumour effects independent of the involvement of COX-2 at concentrations between 10-100 μM. It is today clear that

celecoxib possess additional pharmacological activities where it is able to target several other intracellular components, including PDK1, β-catenin and survivin, that are critically involved in the regulation of cell growth and survival [reviewed in (Grosch et al., 2006)]. This has been attributed to different structural aspects of the celecoxib molecule, which led to the development of derivatives of celecoxib that harbour only one of these functions. One of the best examples is OSU03012, which is a structural analogue of celecoxib that lacks COX-2 binding inhibitory properties but maintains the anti-tumour effect (Zhu et al., 2004). Interestingly, OSU03012, which is an inhibitor of PDK1, has been proven to be more potent than celecoxib in terms of tumour growth inhibition (Schonthal et al., 2008).

1.4.4.2 Adverse effects with NSAID treatment

Treatment of inflammation and pain with dual COX inhibitors such as sulindac and diclofenac are associated with upper gastrointestinal (GI) events, including perforation, bleeding, obstruction, and ulcer. Side effects observed in patients treated with dual COX inhibitors are due to the inhibition of the COX-1 enzyme [e.g. inhibition of prostaglandins, prostacyclines and thromboxanes (Blandizzi et al., 2009)]. The search for NSAIDs that target COX-2 and minimize side effects led to the development of the new selective COX-2 inhibitors (Blandizzi et al., 2009). However, the long-term use of selective COX-2 inhibitors is associated with severe cardiovascular events (myocardial infarction, stroke, or heart failure). For instance, the COX-2 selective inhibitor rofecoxib (Vioxx) was shown to increase the risk of serious cardiovascular events and was therefore withdrawn from the market by the FDA (Bresalier et al., 2005). Unfortunately, other NSAIDs, such as celecoxib and diclofenac, are also associated with increased risk of cardiovascular events (Cannon et al., 2006; Solomon et al., 2005).

1.5 VIRUSES AND CANCER

A large number of infectious agents such as viruses, bacteria and parasites can be linked to the aetiology of cancer and are accepted as important human carcinogens. It is estimated that 20% of all cancers are caused or promoted by infectious agents. The agents most frequently linked to cancer are Helicobacter pylori (gastric cancer), Epstein-Barr virus (Burkitts lymphoma), human papilloma virus (cervical cancer) and hepatitis B and C viruses [hepatocellular carcinoma, reviewed in (Zur Hausen, 2009)].

Other viruses have also been linked to cancer. Kaposis sarcoma herpesvirus is associated with the aetiology of Kaposis sarcoma and is predominantly found in immunocompromised individuals. Human T-cell lymphotropic virus type 1 (HTLV-1) is a retrovirus found in T-cell leukaemia and lymphoma. People infected with human immunodeficiency virus (HIV) have a 100-200 fold increase in developing Kaposi sarcoma or non-Hodgkin's lymphoma (Pagano et al., 2004; Zur Hausen, 2009). Also, members of the Polyomaviridae family have been linked to several different cancers, but a causative role for these viruses has been difficult to prove. The exception in this case is MCPyV, which may be associated with Merkel cell carcinoma, a rare but aggressive neuroendocrine skin cancer (Moens et al., 2008).

1.5.1 Problems identifying viruses in the aetiology of cancer

To identify viruses as causative factors in human cancers is difficult for several reasons. First of all, although most of the infections are common in human populations, only small fractions of these populations develop the respective cancer type. Secondly, viruses induce malignant transformations very slowly. The time from infection to cancer development is 15-40 years in the adult population. This problem is even more difficult in childhood cancers (McKinney, 2005; Grimmer and Weiss, 2006; Gilbertson and Ellison, 2008). Another intriguing problem is that even though the viral genome and proteins are found in tumour cells, synthesis of the virus occurs in cancer cells extremely rarely [reviewed in (Pagano et al., 2004; Zur Hausen, 2009)].

Additionally, a true oncovirus must fulfil Koch's postulate of correlation between pathogen and disease. In experimental settings, three requirements must be met to show that a virus causes cancer. First, the virus should be able to transform human cells *in vitro*. Second, the viral genome or DNA should be found in tumour cells and not in normal cells. Last, the virus should be able to induce the tumour in an *in vivo* experimental setting, and neutralization of the virus prior to injection should also prevent tumour formation. However, even if all these criteria are met, infections alone are still not enough to induce cancer. Additional modifications or *hits* within the host cell are necessary for a full malignant cell to develop [reviewed in [(Pagano et al., 2004; Zur Hausen, 2009)].

1.5.2 Cytomegalovirus

The role of cytomegalovirus (CMV) in cancer has been widely debated. So far there is no evidence that human CMV (HCMV) is causative in human cancers (Soderberg-Naucler, 2006). Several studies demonstrate that HCMV is found in tumour tissue, but the link to aetiology has not been made (Soderberg-Naucler, 2006). Therefore, this thesis addresses the issue of HCMV and its role in cancer aetiology.

CMV is a member of the Herpesviridae family of DNA viruses that infects 70-100% of the adult human population and shares the ability to establish latency after primary infection. CMV works in close relation with the immune response and sustains a balance in healthy individuals. CMV can be reactivated under conditions of stress, inflammation or immunosuppression [reviewed in (Soderberg-Naucler, 2006)]. HCMV is transmitted from close contact through bodily fluids and can be transmitted to an infant during pregnancy (through the placenta), during delivery or via breast milk. The average incidence of HCMV infection in live births is 1% and approximately 10% of children born with HCMV will display symptoms by the age of six months, including growth reduction, hepatosplenomegaly and neurological symptoms such as seizures, lethargy and microcephaly [reviewed in (Landolfo et al., 2003)].

The brain has been shown to be the principal target organ for HCMV in infants with congenital infection and in immunocompromised patients. In healthy patients (e.g. latent state), HCMV is predominantly found in cells of the myeloid lineage. There is to date no data showing that other cells can be sites of latency in healthy individuals [reviewed in (Landolfo et al., 2003)]. However, recent experimental data on mice

demonstrate that most cells of the brain are susceptible to active murine CMV (MCMV) infection and that neural precursor cells (NPC) residing in the VZ and subventricular zone (SVZ) are the primary targets for MCMV during congenital infection. Furthermore, it has been lately been demonstrated that latent MCMV infection can occur in NPCs as well [reviewed in (Tsutsui et al., 2008)].

1.5.2.1 Cytomegalovirus in the host cell

It usually takes 48-72 hours for CMV to bind, produce and release new particles. The CMV particle consists of an outer envelope and an inner capsid that harbours the DNA. The HCMV genome is 235 kb with 250 open reading frames, and is expressed in immediate early (IE), early (E) and late (L) phase genes. The tegument layer, which is similar to eukaryotic cell cytoplasm, lies between the capsid and the envelope. The tegument layer is believed to be important in the first step of infection. CMV infects cells by binding to cell surface heparan sulphate proteoglycans, which are assumed to be mediated by the viral glycoprotein gB. The IE and E proteins are used to access the cellular replication and translational machinery, avoid immune recognition, and stimulate cellular proliferation and metabolism. When IE proteins are activated, the transcription of E and L genes is further activated. The IE genes are manipulative in host cells and thought to be oncomodulatory [reviewed in (Landolfo et al., 2003)].

1.5.2.2 Human cytomegalovirus in cancer

The relationship between HCMV and cancer has been discussed for decades. Several lines of evidence demonstrate that HCMV gene products can have oncomodulatory effects in human cancer cells, meaning that HCMV is not causative but can influence tumour growth and promote progression (Soderberg-Naucler, 2006). For instance, IE1 has been demonstrated to inhibit apoptosis by blocking p53 (Michaelis et al., 2009), dysregulating PI3K/Akt signalling (Cobbs et al., 2008) and activating the telomerase associated promotor, hTERT (Straat et al., 2009). Recent data also demonstrate that the CMV-encoded chemokine receptor US28 (GPCR homolog) promotes angiogenesis and transforms fibroblasts both *in vitro* and *in vivo* via formation of COX-2 (Maussang et al., 2009). Furthermore, PGE_2 is required for efficient replication of HCMV, which can be prevented by COX inhibitors (Hooks et al., 2006; Zhu et al., 2002).

Studies on patient material have demonstrated expression of multiple HCMV gene products in several forms of cancer, predominantly in malignant gliomas, colorectal and prostate cancer (Cobbs et al., 2002; Harkins et al., 2002; Samanta et al., 2003). Additionally, higher levels of HCMV appear to be found in more malignant tumours (Scheurer et al., 2008). For instance, glioblastoma patients with low levels of HCMV in their tumours have better survival rates compared to patients with high HCMV levels [42 months *v* 12 months (Rahbar et al., 2010)].

2 AIMS OF THE THESIS

The general aim of this thesis was to provide a biological framework to better understand medulloblastoma disease. The knowledge of the biology of medulloblastoma may provide novel targets and better therapies.

The specific aims of the thesis were to:

1. Study the role of the prostaglandin E_2 in medulloblastoma tumourigenesis, and investigate the effects of NSAIDs on medulloblastoma tumour growth.

2. Investigate molecular links between crucial signalling pathways in medulloblastoma with the hope of finding new therapies.

3. Study resistance mechanisms/factors in medulloblastoma and find new therapies that eradicate resistant cells.

4. Investigate new biological factors implicated in medulloblastoma aetiology.

3 MATERIAL AND METHODS

3.1 TUMOUR MATERIAL AND PATIENT CHARACTERISTICS

All tumour tissue samples used in this study were collected at the Department of Oncology and Pathology, Karolinska University Hospital (Stockholm, Sweden), between 1994 and 2005. The diagnoses were confirmed by histological assessment of specimens according to WHO classification criteria (Kleihues et al., 2002). Tumour and patient characteristics are summarized in **paper I**. Ethical approval was obtained by the Karolinska University Hospital Research Ethics Committee (approval no. 03-708, 2008/628-31 & 2009/1608-31/4).

3.2 IN VITRO

3.2.1 Human tumour cell lines

All medulloblastoma cell lines used were cultured in growth medium supplemented with heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin at 37 ^{o}C in a humidified 5% CO2 atmosphere. Cells were grown to 90% confluency before they were harvested and subjected to experimental procedures. Origin and culturing conditions are detailed in **paper I**.

3.2.2 Fluorescent microculture cytotoxicity assay and viability assay

The effects of drugs and substances on medulloblastoma cell growth were determined using either a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoleum (MTT) assay (Johnsen et al., 2004) or a fluorescent microculture cytotoxicity assay [FMCA (Ponthan et al., 2007)].

3.2.3 Clonogenic assay

3.2.3.1 Adherent cells

To determine colony formation, cells were seeded in 50 mm^2 Cell+ Petri dishes at a concentration of 150 cells/dish in triplicates. Cells were allowed to attach to the surface for five hours before treatment with drugs. After 8-12 days of incubation in drug-free medium, cell cultures were rinsed with PBS, fixed in formaldehyde and stained with Giemsa. Colonies (> 75 cells) with 50% plate efficiency (PE) were counted manually using a colony counter. For each treatment combination, the surviving fraction was calculated as the ratio of the mean PE of treated cells over the PE of untreated control cells.

3.2.3.2 Suspension cells

Soft agar assay was used to determine colony formation for suspension cells. Briefly, base agar was made by boiling 0.7% agar in MEM (2 mM L-glutamine, 100 IU/mL penicillin G, and 100 µg/mL streptomycin). The agar was cooled to 40 °C in a water bath before it was plated in Petri dishes. Top agar was made by boiling 0.35% agar in MEM (2 mM L-glutamine, 100 IU/mL penicillin G, and 100 µg/mL streptomycin). Heat-inactivated fetal bovine serum (15%) was added to the mixture before pre-treated

cells (48h treatment) were added to the base agar. Cells were incubated for 20 days before colonies were manually counted.

3.2.4 Fluorescent-activated cell sorting (FACS)

3.2.4.1 Cell cycle analysis

DNA content was assessed as previously described (Ponthan et al., 2003). Cells were stained with 4',6-diamidino-2-phenylindole (DAPI) and further subjected to cell cycle analysis using single parameter DNA flow cytometry. The multicycle program for cell cycle analysis was used for histogram analysis (Phoenix Flow Systems, San Diego, CA).

3.2.4.2 Mitochondrial transmembrane potential

The mitochondrial transmembrane potential was assessed as previously described (Johnsen et al., 2004). Cells were labelled using 25 nmol/L tetramethylrhodamine ethyl ester (TMRE) for 30 minutes. After labelling, cells were harvested, rinsed, resuspended in PBS, and analyzed on the FL2 channel on a FACSCalibur flow cytometer, using Cell Quest Software (Becton Dickinson, San Jose, CA).

3.2.4.3 Expression of surface molecules

The expression of surface molecules in **appendix** was assessed as previously described (De Geer et al., 2008). Briefly, CD15 and CD133 expression was measured by incubating cells with either a directly fluorochrome-conjugated primary antibody or a relevant isotype control antibody (Miltenyi Biotec, Bergisch Gladbach, Germany). After cells were washed in PBS containing 0.1% BSA, expression of relevant molecules was analyzed on a FACScan Xow cytometer.

3.2.5 Immunohistochemistry & Immunofluorescence

Origin of all antibodies and materials used for immunohistochemistry and immunofluorescence are described in detail in **papers I-IV**.

3.2.5.1 Immunohistochemistry

Immunohistochemistry was assessed as previously described (Johnsen et al., 2004). Tissue sections were incubated with the primary antibody overnight at 4 ^{o}C. A SuperPicture TM polymer detection kit with appropriate secondary antibodies was used together with a DAB substrate chromogen system to visualize immunopositivity. As a control for non-specific background staining, corresponding sections were incubated with isotype controls.

Detection of HCMV IE and L proteins in primary medulloblastoma tumours was done as previously described (Samanta et al., 2003).

3.2.5.2 Immunofluorecence

For immunofluorescence analysis, medulloblastoma cells were incubated overnight at 4 ^{o}C with primary antibody. Detection was performed using a secondary biotinylated antibody and a FITC–conjugated streptavidin.

3.2.6 Immunoblotting & Immunoprecipitation

Origins of all antibodies and materials used for immunoblotting (Western blotting) and immunoprecipitation are described in detail in **papers I-IV**.

3.2.6.1 Protein preparation

Proteins from cell cultures were extracted from cells in RIPA buffer [25 mM Tris (pH 7.8), 2 mM EDTA, 20% glycerol, 0.1% **nonyl** phenoxylpolyethoxylethanol (NP-40), 1 mM dithiothreitol, and protease/phosphatase inhibitors.

Fractionated nuclear and cyctosolic protein lysates were obtained using a nuclear extraction buffer (20 mM Tris-HCl[pH 7.5], 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF) or hypotonic lysis buffer (10 mM HEPES [pH 7.9], 60 mM KCl, 0.3% NP40, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF), respectively.

Frozen tumours were disrupted with a rotor-stator homogenizer in a TNEN buffer (50 mM Tris-HCl [7.5], 250 mM NaCl, 0.1% NP-40, 5 mM EDTA) and subjected to five freeze-thaw cycles (liquid nitrogen, 37 °C before clearing by centrifugation). All protein extraction buffers were supplemented with a MiniComplete protease inhibitor cocktail and 1mmol/L NaF, 1 mmol/L NaO_3V_4.

3.2.6.2 Immunoblotting

Immunoblotting was performed by loading equal quantities of proteins, separating the proteins by SDS-PAGE, and then transferring the proteins to nylon membranes. Primary antibodies were used to probe against the target protein. β-actin was used to ensure equal loading. Secondary antibodies conjugated with HRP were used for chemiluminescent detection.

3.2.6.3 Immunoprecipitation

Proteins were immunoprecipitated using a primary antibody coupled to agarose-A beads following the manufacturer's instructions (Sigma-Aldrich).

3.2.7 Transfection experiments

Transfections in **paper I** were performed using lentivirus particles. Briefly, medulloblastoma cells were seeded in 6 well plates at a confluency of 50%. Cells were then cotransfected with $1x10^6$ lentivirus particles containing either short hairpin RNA (shRNA) against the target gene, scrambled shRNA, or enhanced green fluoresent protein plasmid (peGFP).

All transfections in **paper II** were performed using Lipofectamin 2000, according to the manufacturer's instructions (Invitrogen).

3.2.7.1 Reporter experiments

Cells were transfected with an LEF/TCF reporter kit, including negative and positive controls (SA Biosciences, Frederick, MD) and/or the full-length cDNA of β-catenin (pSPORT β -cat; Open Biosystems, Huntsville, AL) for 48 hours. Cells were then

treated with drugs for 6 hours and reporter gene activity was assessed using a Dual-Luciferase reporter assay system (Promega Biotech AB, Stockholm, Sweden).

3.2.8 HCMV infections

Medulloblastoma cells were grown until a confluence of approximately 50-60% and were infected in a low serum medium for an indicated time with HCMV clinical isolate like strain VR1814 at a multiplicity of infection (MOI) of 10. As a comparison, cells were either not exposed to virus or were exposed to UV inactivated virus (UV Stratalinker 1800, Stratagene, La Jolla, CA, USA). HCMV VR1814 (kindly provided by G. Gerna, University of Pavia, Pavia, Italy) was prepared from supernatants from *in vitro* infected human umbilical endothelial cell cultures, grown in EGM2 medium (Clonetics, Cambrex Bio Science, Walkersville) and frozen and stored at -70 ℃ until use. Viral titer was determined by viral plaque assay (Wentworth and French, 1970).

3.2.9 Polymerase chain reaction (PCR)

3.2.9.1 DNA extraction and HCMV PCR

To determine if any viral DNA was present in human medulloblastoma cell lines, PCRs for HCMV DNA were performed. DNA was extracted from cell pellets with an QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. HCMV IE and pp150 genes was detected by RealTime PCR (Invitrogen Life Science Technologies, Carlsbad, CA) with specific primers (IE: GTGACCCATGTGCTTATGACTCTAT, CTCAACATAGTCTGCAGGAACGT, FAM-TTGGTCACGGGTGTCTC and pp150: GGCGCGGGAACCTCTT, CCGTGGGCGACAAAACG, FAM-CAGCCGTCAGCCTCG) and with RnasP as a housekeeping control (Applied Biosystems. Branchburg, NJ).

3.2.9.2 Quantitive PCR

To investigate how HCMV superinfection affects mRNA expression of HCMV IE and pp150, $1x10^6$ meduloblastoma cells were seeded per well in 6 well plates and superinfected with HCMV VR1814 at MOI 10 in a low serum medium. Cells were collected at 6, 12, 24, 48 and 72h after infection and frozen at -80 ℃ before RNA extraction with QIAGEN Rneasy RNA Kit (QIAGEN, Hilden, Germany). cDNA was synthesized from equal amounts of RNA using the SuperScript III First-strand synthesis system (Invitrogen Life Technologies, Carlsbad, CA). cDNA was then used for quantitive PCR for HCMV IE and pp150. RnasP or β-actin (Applied Biosystems, Branchburg, NJ) was used as a housekeeping control.

3.2.10 Enzyme-linked immunosorbent assay (ELISA)

PGE_2 measurement and COX-2 activity in **paper I** was measured using ELISA (Cayman Chemicals), according to the manufacturer's instructions.

3.2.11 Mass spectrometry

In **paper IV**, PGE_2 production was measured using mass spectrometry. Briefly, medulloblastoma cells were harvested, counted, sonicated (5 seconds, three cycles, on

ice) and further incubated with 80 µM arachidonic acid for ten minutes at 37 °C before PGE_2 levels were measured as described below.

100 µl of samples, standards and controls were extracted as previously described by Kempen et al (Kempen et al., 2001) in 4.5 ml polypropylene tubes (Sarstedt, Germany). The residue was then reconstituted in 80 µl mobile phase and analyzed on a Waters Acquity UPLC (Waters Corp., Milford, MA, USA) interfaced to a Waters Quattro Premier XE tandem quadrupole mass spectrometer (Waters, Manchester, UK). The system was controlled by MassLynx version 4.1. The chromatography was performed on a 2.1 x 100 mm Waters Acquity HSS T3 (C18) UPLC column maintained at 50 °C with a programmed gradient from solvent A (methanol/water/ammonium acetate of 10/90/2 mM, v/v/concentration) to B (methanol/water/ammonium acetate 90/10/2 mM, v/v/concentration) at 0.4 ml/min to resolve PGE_2 from PGD_2. The flow was diverted to waste before and after analytes of interest. The mass spectrometer was operated in negative electrospray ion mode, and spray voltage was 3 kV. Sample injection volume was 15 µl and the injection interval was 7 min. Autosampler temperature, 5 °C; desolvation gas temperature, 340 °C; source temperature 120 °C; desolvation gas flow 900 L/h; cone gas flow, 40 L/h; collision gas pressure 3.5 x 10-3 mBar (argon); ion energies, 0.9 V for both quadrupoles. For quantitative analysis of PGE_2, the following MRM transitions were used: m/z 351→333 (quantification ion), m/z 351→271 (qualifier ion) and 351→315 (qualifier ion). MRM transition 355→319 was used for the internal standard. The dwell time was set to 20 ms for each transition. The method showed good linearity and reproducibility with a correlation coefficient (r2) of >0.99 and a coefficient of variation of < 10 %.

3.3 XENOGRAFTING OF MEDULLOBLASTOMA CELLS IN MICE

In **papers I-IV**, we used four- to eight-week-old female NMRI nu/nu mice (Taconic Laboratories, Ejby, Denmark). Animals were maintained at five of each per cage and were given sterile water and food *ad libitium*. Each NMRI nu/nu mouse was subcutaneously injected with either 20 x 10^6 (**paper I**) or 7 x 10^6 (**papers II-IV**) D283 MED medulloblastoma cells. Treatment was started on the appearance of palpable tumours reaching the volume of 0.20 mL (**paper I**) or 0.1 mL (**papers II-IV**). Tumours were measured every day and tumour volume was calculated as (width)2 x length x 0.44. Tumour volume index was calculated using the measured volume at the end of treatment divided by the measured volume at the start of treatment. All control animals received vehicle treatment. Tumour weight was recorded at autopsy, after which tumours were either fixed in formaldehyde or frozen for subsequent immunohistochemical analysis or immunoblotting. All animal experiments were approved by the regional ethics committee for animal research (e.g. N234/05, N304/08) in accordance with the Animal Protection Law (SFS1988:534), the Animal Protection Regulation (SFS 1988:539), and the Regulation for the Swedish National Board for Laboratory Animals (SFS1988:541).

3.4 STATISTICAL ANALYSIS

Calculation of EC_{50} values and testing for synergistic or additive effects of combination therapy was performed as previously described (Johnsen et al., 2008; Ponthan et al., 2007).

To determine statistically significant differences, two independent populations were analyzed by the Mann-Whitney U-test and several independent populations were analyzed by the Kruskal-Wallis test (non-parametric ANOVA). All populations were then measured using Dunn's multiple comparison test. Calculation of median values and their non-parametric approximate 95% confidence intervals was based on the Wilcoxon sign-rank test as outlined by Tukey (Tukey and Daniel, 1978). The one sample t test was used to test whether the mean of a single sample differed significantly from the control. All statistical tests were two-sided.

4 RESULTS AND DISCUSSION

4.1 PROSTAGLANDIN E_2: AN IMPORTANT MEDIATOR OF TUMOURIGENESIS IN MEDULLOBLASTOMA

It is today widely accepted that PGE_2 (Wang and Dubois, 2006) and Wnt signalling (Bienz and Clevers, 2000; Fearon and Vogelstein, 1990; Phelps et al., 2009; van de Wetering et al., 2002) are important aetiological factors in colorectal cancer. It is also known that the PGE_2/Wnt interaction controls colorectal cancer tumour growth and haematopoietic SC development in vertebrates (Castellone et al., 2005; Goessling et al., 2009; North et al., 2007). A large body of evidence from gene expression studies indicates that a subset of medulloblastomas harbour mutations in key genes in Wnt signalling (Kool et al., 2008; Pomeroy et al., 2002; Thompson et al., 2006). Furthermore, germ-line mutations in the *APC* gene (e.g. Turcot syndrome) predispose individuals to both colorectal cancer and medulloblastoma (Hamilton et al., 1995), suggesting aetiological similarities. We therefore suggest that PGE_2 could have a causative role in medulloblastoma as well. Moreover, the possibility that PGE_2 could be important in medulloblastoma was further substantiated when our group demonstrated that neuroblastoma, an embryonal tumour similar to medulloblastoma in many aspects (Grimmer and Weiss, 2006), expresses COX-2 and that COX inhibitors inhibit tumour growth both *in vitro* and *in vivo* (Johnsen et al., 2004). This led us to investigate the role of COX-2/PGE_2 in medulloblastoma.

4.1.1 Prostaglandin E_2 has a significant impact on medulloblastoma tumour growth

For **paper I**, the role of PGE_2 in medulloblastoma was investigated both as a means of controlling tumour growth and also in the context of being an important target in medulloblastoma treatment. For this purpose, 39 primary medulloblastoma tumours and a panel of 9 human medulloblastoma cell lines were investigated for the expression of key enzymes involved in PGE_2 production and the four PGE_2 receptors. Constitutive expression of COX-2, mPGES-1 and EP1-EP4 receptors was shown in all primary tumours and cell lines investigated, but not in stroma and surrounding non-malignant cerebellar tissue. Interestingly, COX-2 was also expressed in the Purkinje cells of the cerebellum, cells also known to secrete Shh ligand and hence regulate cerebellar development (Dahmane and Ruiz i Altaba, 1999).

To assess the role of COX-2 in medulloblastoma, we determined the effect of PGE_2 on medulloblastoma tumour growth by stimulating cells with either PGE_2 or the EP2 agonist butaprost. The EP2 receptor has been demonstrated to play an important role in promoting colorectal cancer cell growth through activation of Wnt signalling pathway, either through a signalling route that involves the activation of PI3K/Akt or directly through axin interaction (Castellone et al., 2005). An increase in cell proliferation was observed with PGE_2 and butaprost stimulation in all cell lines investigated. To substantiate this finding, we next examined whether EP1, EP3 or EP4 receptor antagonists could impede medulloblastoma cell growth. Suppression of EP1 and EP3 receptor activity demonstrated a profound effect on cell viability. In contrast, EP4

inhibition demonstrated a poor effect on cell growth. These results indicate that EP1, EP2 and EP3 may be more important to sustain medulloblastoma cell viability than EP4.

To determine whether we could inhibit the effects of PGE_2 on tumour growth, we next targeted COX-2 with either lentiviral COX-2 small interfering RNA (siRNA) or two commercial available NSAIDs, diclofenac and celecoxib. Suppression of COX-2 with siRNA significantly reduced cell proliferation in both cell lines investigated, further demonstrating that COX-2 is important to sustain tumour growth in medulloblastoma. To further elucidate the significance of COX-2 in medulloblastoma, we next tested two commercially COX inhibitors, the dual COX inhibitor diclofenac and the COX-2 specific inhibitor celecoxib. We first measured the effect of diclofenac and celecoxib on PGE_2 production in medulloblastoma cells with ELISA and observed that the IC_{50} (the inhibitory concentration at 50%) was 35-45 μM for diclofenac and 10-12 μM for celecoxib. Similar data were observed on cell viability. Median EC_{50} (the concentration that inhibits 50% of cell proliferation) was 47 μM with diclofenac treatment, whereas celecoxib was more effective and demonstrated mean EC_{50} at 9.3 μM. These data indicate that medulloblastoma tumour growth is inhibited in a COX-2/PGE_2 dependent manner. *In vivo*, both diclofenac (250 mg/L) and celecoxib (2 mg/day = 90 mg/kg/day) did inhibit tumour growth significantly when compared to untreated animals, but no differences were observed when compared in between. In **papers III** and **IV**, it was further demonstrated that celecoxib inhibits tumour growth both *in vitro* and *in vivo*. Taken together, these results indicate that COX-2/PGE_2 plays an important role in medulloblastoma tumour growth.

4.1.1.1 NSAIDs induce apoptosis in medulloblastoma cells

We demonstrated in **paper I** that treatment with diclofenac and celecoxib leads to depolarization of the mitochondrial membrane with subsequent activation of caspase-9, caspase-3, and PARP, but no Bid cleavage. These data suggest that NSAIDs induce mitochondrial dependent apoptosis in medulloblastoma cells. How COX-2 inhibition induces apoptosis in cancer cells is not fully understood. Some studies have suggested that increased levels of intracellular AA, which is mediated by PGE_2 inhibition, induce apoptosis through alteration of the mitochondrial membrane permeability, causing cytochrome c release and thus apoptosis (Cao et al., 2000). Increased levels of AA can also upregulate the production of ceramide, a potent inducer of apoptosis (Hannun, 1996).

However, apoptosis cannot fully be explained by COX-2 inhibition. Other mechanisms to NSAID-induced apoptosis in cancer cells could not be ruled out. Similar effects on proliferation and apoptosis have been reported in cancer cells, irrespective of their levels of COX-2 expression (Jana, 2008). Also, the doses of NSAIDs required to induce apoptosis are relatively higher than what is needed to inhibit PGE_2 synthesis, further suggesting that NSAIDs can induce COX-2 independent apoptosis. Several mechanisms for COX-2 independent apoptosis have been proposed, including downregulation of NF-kB activity, alterations of pro-and anti-apoptotic proteins, and stress response [reviewed in (Jana, 2008)]. In **paper II**, for instance, it was demonstrated that OSU03012, which is an analog of celecoxib without any COX-2 binding properties, induces apoptosis in a similar way as was shown in **paper I**.

Nevertheless, since we demonstrate that the doses that are required to inhibit PGE_2 production (diclofenac 35-45 μM; celecoxib 10-12 μM) correspond to concentrations that inhibit 50% of cell viability (diclofenac 47 μM; celecoxib 9.7 μM), we suggest that diclofenac and celecoxib induce COX-2 dependent apoptosis in medulloblastoma cells.

4.1.2 Celecoxib as a novel adjuvant in medulloblastoma treatment

To address if celecoxib could be useful as an adjuvant in the treatment of medulloblastoma, we studied (**paper III**) whether celecoxib could potentiate the effect of chemotherapeutic drugs used in medulloblastoma therapy. We also tested celcoxib in combination with two mTOR inhibitors, rapamycin and temsirolimus (CCI-779), currently undergoing phase I-III trials against several forms of cancers (Liu et al., 2009).

Here we found that cyclophosphamide, doxorubicin, irinotecan, vincristine temozolomide, rapamycin and CCI-779 were all additive or synergistic when combined with celecoxib *in vitro*. The observed synergistic/additive effects may occur through different mechanisms. One plausible mechanism is that chemotherapeutic drugs such as taxanes can promote transcription of the COX-2 gene and stabilize COX-2 mRNA transcripts, which in turn increases the production of PGE_2. Blocking COX-2 at the same time as chemotherapy is administered may therefore sensitise cells to COX-2 inhibition (Olsen 2005). This could theoretically improve the clinical efficacy of other cytostatics as well. Interestingly, combination treatment with cisplatin was antagonistic. This observation may be explained by a recent study demonstrating that cisplatin treatment upregulates VEGF expression, which leads to expansion of a "stemness" like side-population (SP) in neuroblastoma, osteosarcoma and rhabdomyosarcoma cells (Tsuchida et al., 2008).

Since temozolomide has been promising in the treatment against recurrent embryonal tumours in children (Wang et al., 2009) we next tested the combination of celecoxib and temozolomide *in vivo*. Here we found that animals treated with temozolomide did not respond to therapy when compared with untreated animals. However, when celecoxib was added, we observed that the effect of temozolomide was potentiated significantly.

4.2 TARGETING THE PI3K/AKT PATHWAY

Emerging evidence has revealed that the PI3K/Akt pathway is activated in several forms of cancers, including medulloblastoma (Grotzer et al., 2000; Hartmann et al., 2006; Del Valle et al., 2002; Gilbertson et al., 1995), and it has also been suggested that key proteins in PI3K/Akt could be potential targets in cancer (Liu et al., 2009).
In **paper II**, we therefore investigated the effects of the specific PDK1 inhibitor OSU03012 in medulloblastoma tumour growth both *in vitro* and *in vivo*. OSU03012 is a celecoxib analogue that lacks COX-2 binding properties, specifically targeting PDK1 at concentrations of 2 μM in with promising anti-cancer effects *in vitro* (Schonthal et al., 2008; Zhu et al., 2004).

In **paper II**, we first demonstrated that PI3K/Akt signalling is constitutively activated in all primary medulloblastoma tumours investigated through phosphorylation of Akt and PDK1, making PDK1 an interesting target. We next tested OSU03012 in a panel of medulloblastoma cell lines and demonstrated that OSU03012 inhibits tumour growth profoundly (median EC_{50}: 2.57 μM). Similar effects were also observed *in vivo*, where we demonstrated that daily treatment with 7 mg/kg OSU03012 significantly inhibited tumour growth. Interestingly, our data show that OSU0312 is more effective on medulloblastoma tumour growth when compared to treatment in neuroblastoma, where significant tumour growth reduction was observed with daily treatment of 100 mg/kg OSU3012 (Lova Perup-Segerström et al, manuscript submitted).

By using Western blotting on protein extracts from cell lines and xenografts treated with OSU03012, we demonstrated that the anti-tumour growth was mediated through PDK1 inhibition with subsequent inhibition of Akt, mTOR and activation of GSK-3β. Interestingly, when comparing data in **paper I** and **II**, it appears that OSU03012 is more potent than celecoxib both *in vitro* (median EC_{50}: OSU03012$_{2.57\ \mu M}$ *v* celecoxib$_{9.3\ \mu M}$) and *in vivo* (significant inhibition OSU03012$_{7\ mg/kg/day}$ *v* celecoxib$_{90\ mg/kg/day}$). OSU03012 has also been tested in a large variety of other human cancer cell lines and was found to stimulate apoptosis with an IC_{50} of around 3 μm, which is an order of magnitude more effective than its parental compound celecoxib (Schonthal et al., 2008; Zhu et al., 2004).

4.2.1 Targeting medulloblastoma from two sides of the same chain

Since our aim is to discover novel adjuvant therapies that can be used in future medulloblastoma therapy, we next investigated whether OSU03012 could potentiate the effect of cytostatics and mTOR inhibitors. Screening data from **paper II** showed that OSU03012 was synergistic in combination with rapamycin, CCI-779 and cyclophosphamide, while it was additive in combination with doxorubicin and antagonistic with temozolomide (discussed below).

PI3K/Akt is a central node in multiple signalling routes that regulates vital cellular processes. Therefore, inhibitors that target most isoforms of PI3K or Akt directly have a poor therapeutic index (Grimmer and Weiss, 2006). Furthermore, feedback loops between pathways can also have dramatic effects on drug responses. For instance, when mTOR is activated, PI3K/Akt activity becomes downregulated through S6K1. If only mTOR is targeted, the net effect can be an increase in PI3K/Akt activity through a positive feedback loop, which can enhance tumour growth (O'Reilly et al., 2006; Liu et al., 2009; Johnsen et al., 2008; Fan et al., 2006).

Inhibitors of mTOR are in many clinical studies as single agents. Results from recent trials with CCI-779 and everolimus showed that these drugs improved survival in patients with advanced renal cell carcinoma (Faivre et al., 2006). However, preliminary results with mTOR inhibitors in many other tumour types, including advanced breast cancer and glioma, indicated low response rate. This somewhat failed monotherapy in cancer suggests that the utility of these agents may be improved through combination therapy (Faivre et al., 2006; Fan et al., 2006; Liu et al., 2009).

We therefore next investigated dual inhibition of PI3K/Akt and mTOR by testing both OSU03012 and the mTOR inhibitor CCI-779 in combination. CCI-779 is an analog of rapamycin that is highly lipophilic (thus able to penetrate the BBB) and that has shown profound effects in medulloblastoma xenografts (Geoerger et al., 2001). In accordance with other studies, we demonstrated that treatment with CCI-779 led to marked activation of Akt (Fan et al., 2006; Johnsen et al., 2008; O'Reilly et al., 2006). Concomitant treatment with OSU03012 blocked CCI-779-induced upregulation of Akt, which may explain the synergistic effects observed on cell cytotoxicity *in vitro* as well as the observed augmentation *in vivo*. This is in line with other reports demonstrating that inhibition of mTOR induces a positive feedback loop on Akt activation and that, by abrogating this induction, cells are sensitized to PI3K/Akt drug inhibition (Fan et al., 2006; Johnsen et al., 2008; O'Reilly et al., 2006). Interestingly, PI3K/Akt inhibition has also been demonstrated to reciprocally augment the efficacy of mTOR inhibitors (Fan et al., 2006). Taken together, these data suggest that dual blockade of PI3K/Akt and mTOR may be an effective strategy against medulloblastoma.

4.2.2 PI3K/Akt-Wnt/β-catenin pathway cross-talk identified in medulloblastoma

Cross-talks between different signalling pathways are important in normal development and cancer. Studies on human cancers have identified cross-talks between Wnt, Shh and PI3K/Akt (Ulloa et al., 2007; Jiang, 2006; Gilbertson, 2004), and these cross-talks may occur in medulloblastoma as well. Studies on breast cancer, for instance, have demonstrated that ERBB1/ERBB2 can interact directly with β-catenin and deregulate Wnt activity (Schroeder et al., 2002). Cross-talks might take place between Wnt and Shh. For instance, SUFU and GLI3 negatively regulate Wnt/β-catenin signalling through the export of β-catenin from the nucleus (Meng et al., 2001; Ulloa et al., 2007). Furthermore, it is also clear that GSK-3β functions similarly in the Shh signalling pathway (Jiang and Hui, 2008). Additionally, upstream signals, such as the PI3K/Akt pathway, regulate GSK-3β activity through induction of phosphorylation (Xu et al., 2009).

GSK-3β is unusual in a way that it is normally active in cells and is primarily regulated through inhibition of its activity. It is mostly known to inactivate the Wnt pathway through phosphorylation of β-catenin. However, GSK-3β is also known to be critical for protein synthesis and is a key regulator of both differentiation and cellular proliferation. GSK-3β has several substrates in which the consequence is to inhibit translocations of transcription factors to the cell nucleus. Interestingly, GSK-3β is unusual among the kinases. Before it can phosphorylate its substrate, it usually requires a "priming kinase" that first phosphorylates the substrate. In this way, GSK-3β has the ability to act as a regulator in several signalling pathways, mediating different cellular processes and cross-talks [reviewed in (Cohen and Frame, 2001)].

In **paper II**, we investigated the importance of GSK-3β in medulloblastoma tumour growth. We further studied whether GSK-3β could act as a cross-talk between PI3K/Akt and Wnt/β-catenin signalling in medulloblastoma.

We first demonstrated that GSK-3β was functionally inactive (e.g. phosphorylated on serine 9) in all primary medulloblastomas analyzed. We also showed that 27% of the primary tumours were nucleopositive for β-catenin and that 31% of the samples had mixed cytoplasmic/nuclear β-catenin staining. As noted earlier, approximately 25% of medulloblastomas are nucleopositive for β-catenin and associated with good prognosis (Ellison et al., 2005). However, our findings and previous reports are in contrast to studies reporting that 15% of all sporadic medulloblastomas harbour mutations in the Wnt pathway (Baeza et al., 2003; Huang et al., 2000; Zurawel et al., 1998). These findings indicate that there are other mechanisms involved in regulating Wnt/β-catenin activation. Indeed, it has been demonstrated that Wnt/β-catenin can be activated by PI3K/Akt through stimulation of EGF, IGF-I and IGF-II (Desbois-Mouthon et al., 2001; Lu et al., 2003; Morali et al., 2001).

To test whether Wnt/β-catenin can be activated through PI3K/Akt, we next investigated the effect of OSU03012 on Wnt/β-catenin pathway activity. Cells treated with OSU03012 reduced nuclear β-catenin translocation with subsequent inhibition of c-Myc and cyclin D1 expression - two proteins that are target genes in Wnt/βcatenin signaling and are important in medulloblastoma tumourigenesis (Gilbertson, 2004; Grimmer and Weiss, 2006). Further on, reporter experiments demonstrated that OSU03012 inhibits β-catenin-TCF/LEF transactivation through forcing overexpression of β-catenin by cotransfection with a β-catenin expression plasmid.

Wnt/β-catenin signalling can be activated by PI3K through different mechanisms. For instance, IGF-I has been shown to stimulate β-catenin activation in hepatocellular carcinoma involving GSK-3β inhibition and Ras activation (Desbois-Mouthon et al., 2001). In contrast, Lu and co-workers demonstrated that the EGF receptor activates β-catenin in a GSK-3β-independent manner. Instead, activation is mediated by caveolin-1 downregulation (Lu et al., 2003).

To investigate whether β-catenin activation was mediated through GSK-3β inhibition, we next treated cells with the GSK-3β activator sodium nitroprusside dihydrate (SNP), two specific inhibitors of GSK-3β, CHIR99021 or SB-216763, and siRNA against GSK-3β. We demonstrated that GSK-3β activation increased the cytotoxicity effects of OSU03012, whereas OSU03012-mediated cytotoxicity was inhibited by GSK-3β inactivation. These findings are in accordance with data demonstrating similar effects with the IGF-I receptor inhibitor NVP-AEW541 (Urbanska et al., 2007). Thus, our data demonstrate that GSK-3β acts as a central node in the cross-talk between PI3K/Akt and Wnt/β-catenin. Furthermore, we also demonstrate that GSK-3β activity has an important role in medulloblastoma tumour growth.

4.3 TARGETING RESISTANCE IN MEDULLOBLASTOMA

The resistance of medulloblastomas to current therapies may be related to the existence of CSCs (Fan and Eberhart, 2008) and expression of MGMT (Rood et al., 2004b). Studies on glioblastoma have shown that CD133 positive glioblastoma cells are resistant to temozolomide (Murat et al., 2008; Pallini et al., 2008). In contrast to previous studies, one recent study demonstrated that temozolomide depletes CD133 positive glioma CSCs (Beier et al., 2008). Interestingly, later work from Eric Holland's lab demonstrated elevated MGMT levels in CD133 positive cells (Bleau et al., 2009).

Since glioblastoma is a heterogeneous disease, differences observed in these studies may be explained by MGMT status in glioblastoma CSCs.

In **paper III** and the **appendix**, we therefore addressed the potential role of celecoxib as an anti-resistant drug in medulloblastoma. Here we found that the combination of celecoxib and temozolomide was synergistic *in vitro*. This is in contrast to data from **paper II**, where OSU0312 was demonstrated to be antagonistic in combination with temozolomide. Since MGMT has been shown to be upregulated in medulloblastoma (Rood et al., 2004b), we considered further investigating the effect of celecoxib on MGMT expression. One major difference between celecoxib and OSU03012 is that OSU03012 lacks the COX-2 inhibitory function. This led us to hypothesise that PGE_2 may be involved in the synergistic effects observed.

To investigate the significance of PGE_2 on MGMT expression, we treated two medulloblastoma cell lines with elevated MGMT expression (He et al., 1992) with either 10 or 20 μM celecoxib and found that celecoxib decreased MGMT expression after 48 and 72 hours. Similar results have been reported in other cancers. For instance, it was demonstrated that interferon-β (IFN-β) sensitises glioma (Natsume et al., 2005) and neuroblastoma cells (Rosati et al., 2008) to the cytotoxic effects of temozolomide through downregulation of MGMT expression. To test if PGE_2 is the mediator of the observed celecoxib effect, we next stimulated medulloblastoma cells with PGE_2 and demonstrated that PGE_2 upregulates MGMT expression. Whether this observed effect is a direct mechanism mediated by PGE_2 or through other signalling routes remains to be determined.

Accumulating evidence suggests that PGE_2 is an important regulator of proliferation in cancer cells and SCs (North et al., 2007; Oshima et al., 1996; Goessling et al., 2009; Castellone et al., 2009). Our findings on COX-2/PGE_2 in **papers I**, **III** and **IV** let us hypothesise that PGE_2 might have an important role in controlling the maintenance of SP in medulloblastoma as well. Both CD15 and CD133 have been proposed as CSC markers in medulloblastoma (Singh et al., 2004; Fan and Eberhart, 2008; Blazek et al., 2007; Ward et al., 2009; Read et al., 2009). In the **appendix**, we therefore investigated the effect of celecoxib on medulloblastoma SP (e.g. $CD15^+/CD133^+$). We first determined the composition of CD15 and CD133 positive cells in D324 MED using FACS analysis. By growing cells in different culturing conditions we noted that cells cultured in RPMI, without serum, were enriched for CD15 (~80%) and CD133 (~25%) positive cells (**appendix**). However, cells that were cultured in RPMI or MEM with 10% serum showed only a small fraction of CD15 (<1%) and CD133 (<10%) positive cells (**data not shown**). We therefore performed our experiments on cells growing in RPMI medium without serum.

Medulloblastoma cells were treated with celecoxib (10 or 20 μM), temozolomide (50 or 100 μM), or the combination of both drugs (10 μM celecoxib + 50 μM temozolomide or 20 μM celecoxib + 100 μM temozolomide) and subjected to FACS analysis. As shown in the **appendix**, cells treated with 20 μM celecoxib demonstrated a decrease from 21% to 11% in the SP. Cells treated with 100 μM temozolomide were unaffected, while the combination of 20 μM celecoxib and 100 μM temozolomide resulted in a reduction to 4%. This indicates that celecoxib can target medulloblastoma

SPs and may also explain the effects we demonstrated in **paper III**. However, these data are preliminary and further studies are needed to determine the mechanism of celecoxib on medulloblastoma SPs.

4.4 HCMV AND MEDULLOBLASTOMA

The full aetiology of medulloblastoma is today unknown. As described earlier, medulloblastomas originate from NPCs originating in the EGL of the cerebellum and retain many features that resemble the progenitor cells of the embryonic brain. It has been suggested that dysfunctional activation of Wnt and Shh is involved in the aetiology of medulloblastoma (Fan and Eberhart, 2008; Wang and Zoghbi, 2001). Infectious agents have also been proposed as aetiological factors. Several studies have suggested that infectious agents may influence the risk of medulloblastomas in children. For instance, children with three or more younger siblings were recently shown to have a higher risk of developing medulloblastoma when compared to children who had no siblings (Altieri et al., 2006). Large families and the number of siblings are possible indicators of early life exposure to infections. This may therefore indicate that infectious agents can be involved in medulloblastoma development (Altieri et al., 2006). There are some contradictory studies showing viral presence of Simian vacuolating virus 40 (SV40) and JC virus (JCV) in medulloblastoma. However, there is still no consistent evidence that explains why children are diseased with medulloblastoma [reviewed in (White et al., 2005)].

Studies in mice harbouring heterozygous *PTCH* mutations have shown that a small fraction of the mice develop medulloblastoma (Goodrich et al., 1997). Mice with both heterozygous *PTCH* mutations and *p53* mutations develop tumours more frequently (Goodrich et al., 1997; Marino et al., 2000). Nevertheless, since *p53* mutations are uncommon in medulloblastoma, it is not likely that p53 is involved in medulloblastoma development, and it has been suggested that a so-called “second hit” is required for medulloblastoma to develop. Several attempts have been made to identify this second hit, but evidence is so far inconsistent (Gilbertson and Ellison, 2008; McKinney, 2005).

HCMV has been implicated in several forms of cancer, including brain, prostate, breast and colon cancer (Soderberg-Naucler, 2008). Recently it was demonstrated that HCMV infection in NPCs blocks differentiation into neurons (Odeberg et al., 2006; Odeberg et al., 2007). This process has been identified to be an important step in early development of brain tumours (Sanai et al., 2005). Indeed, HCMV has been shown to have preference for immature cells in the VZ and SVZ, and *in vitro* and *in vivo* data argue that CMV targets stem cell-rich areas in the brain during development (Cheeran et al., 2009). Several lines of evidence suggest that CSCs exist in medulloblastoma and that they are important in medulloblastoma tumourigenesis (Fan and Eberhart, 2008). In **paper IV**, we therefore hypothesised that random infection on precursor cells, through COX-2 activation, may be an aetiological factor in medulloblastoma development.

4.4.1 HCMV prevalence in medulloblastoma

In **paper IV**, we found that the prevalence of HCMV in medulloblastoma primary tumours was 87% and that all human medulloblastoma cell lines were positive for HCMV DNA. To confirm this finding, we performed *in situ* hybridisation on primary tumours and found that all tumours investigated were positive. Interestingly, we could not detect HCMV IE and L proteins in cell lines positive for HCMV DNA. However, when cells were transplanted into mice, both IE and L proteins were detected. Remarkably, IE proteins were evenly distributed throughout the tumour while L antigens were localised around blood vessels. This finding suggests that HCMV expression may depend on factors in the microenvironment. Furthermore, our finding is highly interesting since CSCs are implied to be located in close proximity to blood vessels (Gilbertson and Rich, 2007). Even though we have shown that HCMV DNA, RNA, and proteins are present in medulloblastoma, we do not know if the whole viral DNA is intact in medulloblastoma cells.

4.4.2 HCMV: a novel target in medulloblastoma?

The evidence that HCMV is present in medulloblastoma makes it an interesting target. Several anti-CMV drugs are today available on the market. The most interesting of these is ganciclovir, which is the first line of treatment for CMV disease therapy. Ganciclovir targets the viral DNA polymerase UL45 and prevents the formation of late genes and production of new particles. It is activated when it is phosphorylated by the CMV kinase UL97 with subsequent phosphorylation by cellular kinases. The active form of ganciclovir, which is a nucleoside analogue, binds to newly formed viral DNA and slows down polymerase activity. Since ganciclovir has poor bioavailability, a valylester form (valganciclovir) derivative that has improved uptake is available and is administered orally [reviewed in (Mercorelli et al., 2008)].

Recently it was shown that the HCMV protein US28 upregulates COX-2 expression and increases VEGF production (Maussang et al., 2009). Additionally, PGE_2 is important for CMV replication, making COX-inhibitors potentially useful in the treatment of CMV (Hooks et al., 2006; Zhu et al., 2002). These data makes our findings on the anti-tumourgenic effects of NSAIDs in medulloblastoma (**paper I** and **III**) even more exciting.

We therefore tested ganciclovir alone *in vitro* using clonogenic assay on three medulloblastoma cell lines and found that the colony forming ability was significantly inhibited with 75-150 μM ganciclovir treatment (approximately 20-50%). Interestingly, a significant augmentation was observed when 10 μM celecoxib was added to the treatment, resulting in a reduction of 75-97%. Medulloblastoma xenografts treated with either 14 mg/kg twice daily valganciclovir or 90 mg/kg/day celecoxib alone showed a significant inhibition (40%) when compared with untreated controls. When both drugs were combined, a reduction of 70% was observed. Since it was shown in **paper I** that PGE_2 is important for medulloblastoma tumour growth, and as noted above it is important for CMV replication, we next investigated whether the mechanism behind the observed effect could be mediated by PGE_2 inhibition. Super-infection of medulloblastoma cells with HCMV showed an increase in PGE_2 production,

demonstrating that HCMV can induce COX-2 expression. Furthermore, treatment with ganciclovir inhibited the production of PGE_2 in a dose-dependent manner. Interestingly, cells that were super-infected with HCMV were more sensitive to the combination treatment when compared with non-super-infected cells. These data further support that COX-2/PGE_2 is the target.

5 GENERAL DISCUSSION AND FUTURE PROSPECTS

5.1 NOVEL THERAPIES IDENTIFIED AGAINST MEDULLOBLASTOMA

Medulloblastoma survival rates have improved during the last two decades. However, despite these better outcomes, half of the patients in the HR group will succumb to disease (Dhall, 2009). In addition, surviving children suffer from long-term side effects with a consequent negative impact on quality of life (Rood et al., 2004a). A greater understanding of medulloblastoma biology is needed to identify new treatments and improvements in the way existing therapies are currently used and to further improve the outcome for children with medulloblastoma (Gilbertson, 2004).

In this thesis, we show that COX-2/PGE_2 signalling is important for medulloblastoma tumour growth and that celecoxib has therapeutic effects on medulloblastoma. We also present data demonstrating that celecoxib enhances the cytotoxic effect of certain chemotherapeutic drugs that are used in the treatment of medulloblastoma. Celecoxib was also shown to target resistance mechanisms such as MGMT expression and SP propagation. From a clinical perspective, these findings are of high interest since resistant cells need to be eradicated to provide long-term disease-free survival. Therefore, adding celecoxib to existing conventional postoperative therapy could prove beneficial.

An important aspect to consider with celecoxib treatment is the association with severe cardiovascular events found in adults after long-term therapy. Currently available evidence of an increase in cardiovascular risk with celecoxib is inconsistent (Arber et al., 2006; Bertagnolli et al., 2006; Frampton and Keating, 2007). Even if a risk exists, I believe that the risk must be weighed against the potential benefits in each individual. The benefit of curing children with cancer is of greater significance than the overhanging risk of side effects in later life. The fact that conventional medulloblastoma treatment is already associated with severe side effects makes celecoxib harmless in comparison (Packer, 2008). Furthermore, the treatment of cancer is relatively short, and thus long-term treatment with celecoxib would not be necessary.

Other drawbacks of celecoxib treatment were also considered. The *in vitro* concentrations to obtain anticarcinogenic effects in this thesis (Mean EC_{50}: 9.7 μM) are higher than that achieved in plasma of patients (~3 μM) or experimental animals [5 μM (Stempak et al., 2002; Grosch et al., 2006)]. However, Pyrko et al. demonstrated that the *in vitro* concentrations needed to inhibit the anti-apoptotic protein survivin is at a minimum of 30-50 μM. Interestingly, this effect could be recapitulated *in vivo* at doses <5 μM (Pyrko et al., 2006). This discrepancy may be explained by the fact that tumour regression in animals and patients requires weeks or months of drug treatment, whereas antiproliferative effects in cultured cells are observed after only a few hours (Maier et al., 2005). This possibility is supported by our finding that celecoxib has an antiproliferative effect in medulloblastoma cells at clinically achievable concentrations (EC_{50} values of 2.5-7 μM) when the treatment period was extended to 96 hours. Thus celecoxib can mediate its anti-tumourigenic effects even though it never exceeds 5 μM

in vivo, showing that celecoxib could be administered to children in doses that are required to obtain therapeutic effects.

Our finding that HCMV is prevalent in medulloblastoma and that HCMV replication is somehow dependent on PGE_2 production may be important in the search for novel therapies against medulloblastoma. The anti-viral drug ganciclovir/valganciclovir showed significant inhibition of tumour growth both *in vitro* and *in vivo.* By adding celecoxib to valganciclovir treatment, tumour growth inhibition was further augmented. Due to the small number of patients, we could not determine the prognostic significance of HCMV in medulloblastoma. However, a recent published study by our collaborators demonstrates that the grade of HCMV infection in glioblastoma multiforme patients was significant for clinical outcome (Rahbar et al., 2010). In this study, the authors show that glioblastoma multiforme patients with low-grade HCMV infections (e.g. 0% to <25% infected tumour cells) lived three times longer when compared to patients with high-grade HCMV infections (>25% infected tumour cells; median survival: 28 months longer). Thus, here we propose that antiviral treatment for HCMV in combination with celecoxib may have promise as an adjuvant treatment strategy for medulloblastoma.

Numerous studies show that celecoxib has COX-2 independent effects such as inhibition of PDK-1 (Grosch et al., 2006). Since we found that celecoxib inhibited tumour growth in medulloblastoma, and PI3K/Akt is activated in medulloblastoma, we further investigated the action of the PDK1 inhibitor OSU03012 in medulloblastoma. We identified that OSU03012 has profound effects on medulloblastoma growth through interference with a molecular cross-talk between PI3K/Akt and Wnt/β-catenin signalling. This new finding will provide new insights into medulloblastoma pathology and may shed new light on the development of novel therapies.
We further demonstrated that OSU03012 enhanced the cytotoxic effects of chemotherapeutic drugs and mTOR inhibitors in a synergistic or additive manner. Moreover, we found that OSU03012 augmented the *in vivo* effect of the mTOR inhibitor CCI-779. Thus, the dual blockade of PI3K/Akt and mTOR may therefore be an effective strategy against medulloblastoma.

Currently, OSU03012 is enrolled under Arno Therapeutics as AR-12, and its appropriate dose and toxicity profile in adult humans are evaluated in a phase I study. There are also plans to start phase II in early 2011. Preclinical data (Arno Therapeutics, personal communication) demonstrates that OSU03012 crosses the BBB within five minutes and accumulates in brain tissue (mean: 2 μM) after 4 hours of 10 mg/kg i.v. bolus injection. Interestingly, OSU03012 accumulates in tumour tissue following 28 days of 100 mg/kg (mean: 15.8 μM) or 200 mg/kg (mean: 177 μM) oral dosing. As noted earlier, in this thesis we have demonstrated that OSU03012 inhibits tumour growth *in vivo* at dosing starting from 7 mg/kg/day and that EC_{50} is <2 μM *in vitro*. Taken together, OSU03012 could be used for therapeutic purposes in the clinic.

5.2 HCMV ACTIVATES COX-2: POTENTIAL ROLE IN MEDULLOBLASTOMA AETIOLOGY

The aetiology of medulloblastoma has been linked to dysfunctional activation of embryonic developmental pathways in the cerebellum (Johnsen et al., 2009; Grimmer and Weiss, 2006; Gilbertson, 2004). To generate a functional mature cerebellum, multiple pathways are required to interact and tightly regulate cell number, cell proliferation and differentiation. The steps when a cell should stop dividing and start differentiating are crucial for correct cerebellum genesis (Goldowitz and Hamre, 1998; Wang and Zoghbi, 2001). Perturbations in these events may provide clues about how a fully transformed medulloblastoma cell is formed (Gilbertson, 2004). Despite intensive efforts, though, the question of why children develop medulloblastoma remains.

To make a normal cell transform to a malignant cell, several incidents (e.g. oncogenic hits) that make the genome unstable are required during a long time period (e.g. 15-40 years). This is especially pronounced in adult cancers (Pagano et al., 2004; Zur Hausen, 2009). Embryonic tumours develop in a far shorter time frame, making oncogenic hits even more crucial for neoplastic formation (McKinney, 2005; Grimmer and Weiss, 2006; Gilbertson and Ellison, 2008). Oncogenic hits can occur in a mix of genetic and environmental events. Germ-line mutations in *PTCH* (e.g Shh activation) or *APC* (e.g Wnt activation), for instance, are highly associated with Gorlin syndrome (Hahn et al., 1996) and Turcot syndrome (Hamilton et al., 1995) respectively. Patients with these disorders are predisposed to develop medulloblastoma (Hahn et al., 1996; Hamilton et al., 1995). However, only a small fraction of medulloblastoma patients harbour these mutations, and aberrant activation in Shh and Wnt are more common in sporadic medulloblastomas (Dhall, 2009). This implies that other events are involved in the aetiology of medulloblastoma, and the influence of environmental factors may therefore be important to fully explain why children are diseased with medulloblastoma. In this regard, the recent finding of $Ptch^{+/-}$ mice overexpressing COX-2 shows an increase in basal cell carcinoma (BCC) burden, whereas genetic deletion of COX-1 or COX-2 resulted in a robust decrease in BCC burden in patch heterozygote mice (Tang et al., 2010). Moreover, pharmacologic COX inhibition in patients with loss of *PTCH* trended toward lower BCC burden compared to the control group (Tang et al., 2010).

The development of colorectal cancer is closely associated with COX-2/PGE_2 pathway activation (Oshima et al., 2009; Oshima et al., 1996; Wang and Dubois, 2006). Several other oncogenic pathways also trigger gastric tumourigenesis. For instance, Wnt/β-catenin signalling is found in 30-50% of gastric cancers (Clements et al., 2002; Oshima et al., 2006). Recent studies have indicated that COX-2 activation is required for the development of adenocarcinoma in the Wnt activated mucosa (Oshima et al., 2009). Indeed, it is widely accepted that PGE_2 and Wnt/β-catenin are causal in colorectal cancer (Oshima et al., 2009). The pathophysiology of medulloblastoma and colorectal cancer is similar in many ways. For instance, patients with Turcot syndrome are predisposed to both colorectal cancer and medulloblastoma (Hamilton et al., 1995). COX-2 expression and Wnt activation have also been reported in medulloblastoma [**Paper I** and (Dahmen et al., 2001; Eberhart et al., 2000; Ellison et al., 2005; Kool et al., 2008; Pomeroy et al., 2002)].

Several reports have implicated COX-2/PGE_2 in HCMV replication (Speir et al., 1998; Maussang et al., 2009; Hooks et al., 2006). HCMV is a virus that has been detected in several forms of cancer, including brain tumours (Soderberg-Naucler, 2008). It was first implied as an oncovirus in the 1970s, when it was observed in prostate tumours (Geder and Rapp, 1977; Geder et al., 1977). This virus may be a perfect candidate for oncogenesis, since it can persist in its host for long time, modulate immune responses, and manipulate cellular machinery (Soderberg-Naucler, 2008). However, only one research group has been able to isolate CMV particles from tumours, transform fibroblasts *in vitro*, and demonstrate oncogenic effects *in vivo* (Geder et al., 1977). Since this experiment could not be repeated, and no one has so far managed to fulfil Kock's postulate, the idea of HCMV as an oncogenic virus was abandoned. Instead, HCMV has been suggested to be oncomodulatory [reviewed in (Soderberg-Naucler, 2006)].

Data from **paper IV** demonstrate that HCMV is detected in medulloblastoma and that HCMV infection *in vitro* upregulates COX-2 and PGE_2. Work from our collaborators shows that HCMV has a preference to infect immature cells such as NPCs (Odeberg et al., 2007; Odeberg et al., 2006). Furthermore, in **paper IV** we show that HCMV late antigens are associated with blood vessels. Since CSCs are implied to be located in close proximity to vessels (Gilbertson and Rich, 2007), our results indicate that HCMV may be expressed in medulloblastoma CSCs. It is also of interest to note that medulloblastoma CSCs are considered to be the origin of disease (Fan and Eberhart, 2008). Moreover, CMV has the ability to transmit to the foetus during pregnancy. The presence of HCMV in the brain could hence be a congenital infection, which can cause severe sequele in the CNS (Soderberg-Naucler, 2006). Recently it was proposed that HCMV may play a causative role in glioblastoma multiforme development (Rahbar et al., 2010). Taking these data together, we suggest that HCMV may be important in the aetiology of medulloblastoma and perhaps other cancers as well. We thus propose that sometime during cerebellar development, HCMV infection in SCs and/or progenitor cells activates PGE_2 signalling, thus inducing malignant transformation that results in medulloblastoma development.

6 SUMMARY AND CONCLUSIONS

In this thesis, new targets implicated in **medulloblastoma** tumourigenesis were identified. The eicosanoid **prostaglandin E_2** was demonstrated to be important in controlling **tumour growth** and **resistance** to treatment in medulloblastoma. Moreover, high prevalence of **human cytomegalovirus** was identified in medulloblastoma and its role as a potential **aetiological factor** is proposed in medulloblastoma development. Novel therapies such as **celcoxib** and **valganciclovir** targeting prostaglandin E_2 and human cytomegalovirus demonstrated promising *in vivo* effects. In addition, it was found that prostaglandin E_2 regulates maintenance of medulloblastoma **side-populations** and expression of **MGMT**, and that both are implicated in resistance to therapy. Furthermore, **GSK-3β** was found to control medulloblastoma tumour growth through **cross-talk** interaction between **PI3K/Akt** and **Wnt/β-catenin**. Substances targeting PI3K/Akt, upstream through **PDK1** and downstream on **mTOR,** were shown to efficiently prevent tumour growth.

In conclusion, **new targets** have been identified in medulloblastoma. These findings have provided us with **new therapies** that appear to be effective against medulloblastoma. From a clinical perspective, we hope that the work presented in this thesis will provide a new framework for the treatment of childhood medulloblastoma.

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