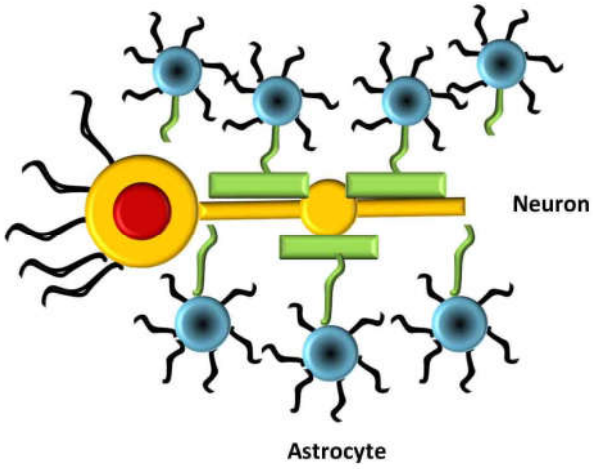

ASTROCYTOMAS: FIBROMAS OF THE BRAIN

TGL 194

Abstract

Astrocytomas, especially pilocytic astrocytomas (PAs), are proliferating lesions whose metastatic potential is low but whose proliferation can be destructive. Astrocytes are the fibroblasts of the brain, providing pathways between the neurons and the vasculature. The challenge of many PAs is total suppression and debulking. We examine this challenge in the context of several current therapeutics and possible new ones.

Terrence McGarty

November, 2022

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Contents

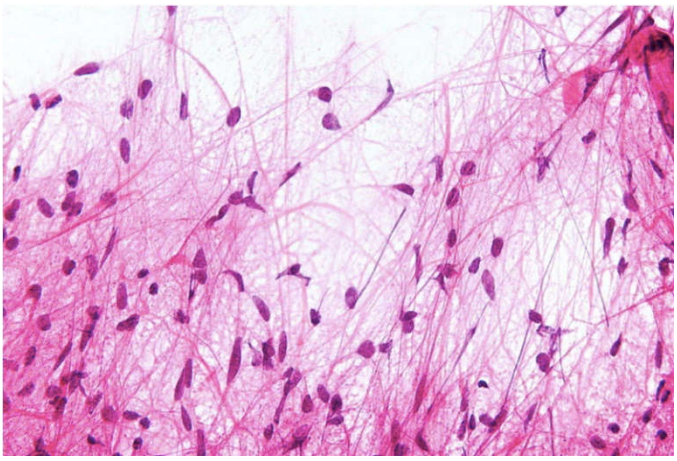
1 INTRODUCTION

Brain tumors have been a challenge to treat. First surgery is complicated and second the blood brain barrier makes it difficult to use some of the more recent therapeutics. We consider here the example of a low grade astrocytoma, the most common childhood tumor. We are just beginning to understand how to deal with these lesions but their general genetic simplicity and their direct connection to the vascular network in the brain make them an interesting lesion.

The objective of this note is to examine the multiplicity of elements related to PA. It allows for the development of a methodological process which we believe can be carried over to more complex lesions.

1.1 PILOCYTIC ASTROCYTOMA

Pilocytic astrocytoma (PA) is a lesion wherein there is an ever expanding proliferation of the astrocytes. A sample is shown below[1]:

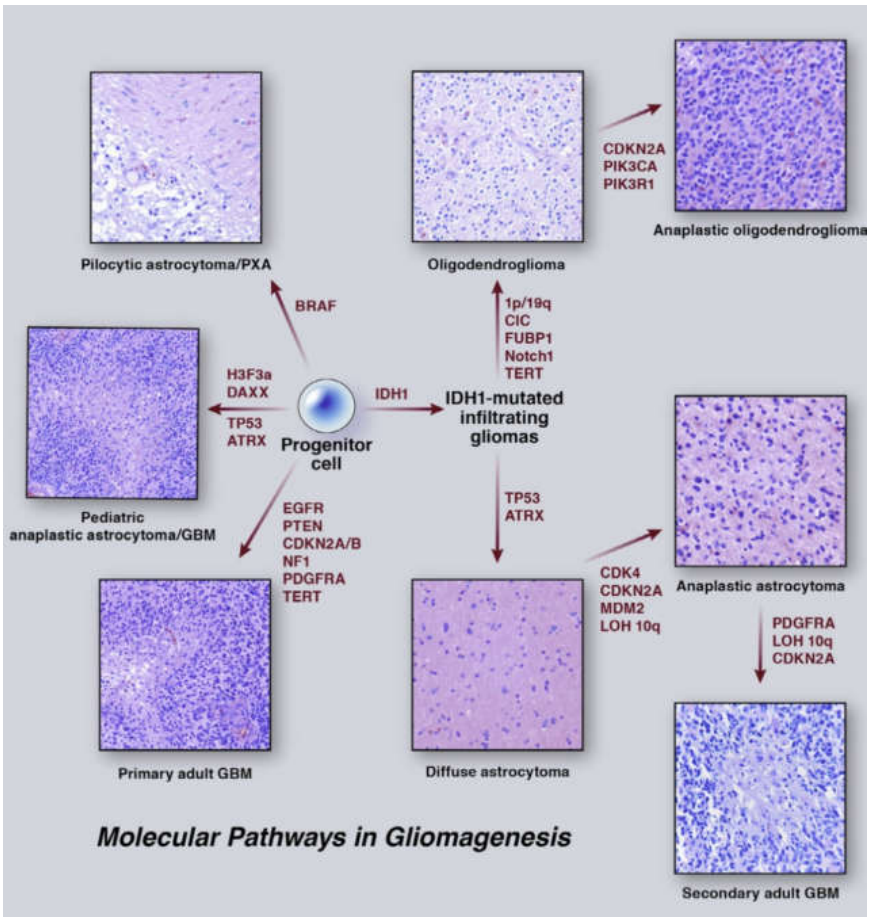


The astrocytes have proliferated and the arms of the astrocytes are hair like in form. In effect the PA lesion appears as a dense clump of astrocytes dominated by cell proliferation. It is somewhat analogous to a lipoma wherein the fat cells have likewise proliferated. However the PA cells often proliferate along significant parts of the CNS.

From Appin and Brat, we have the interesting table below detailing a multiplicity of brain lesions and their dominant genetic drivers for a variety of various CNS lesions[2]:

[1] Contributed from Wikimedia User: Nephron (CC BY-SA 3.0 https://creativecommons.org/licenses/by-sa/3.0

[2] See Appin and Brat, Molecular pathways in gliomagenesis and their relevance to neuropathologic diagnosis, Adv Anat Pathol, . 2015 Jan;22(1):50-8. From Suva lecture at HMS Session on Tumor Microenvironment October 26, 2022.



One can note the PA lesion is driven by a BRAF lesion, typically a BRAF V600(…) type substitution lesion which enhances cell proliferation. One can see the genetic drivers for the other lesions.

1.2 TREATMENTS

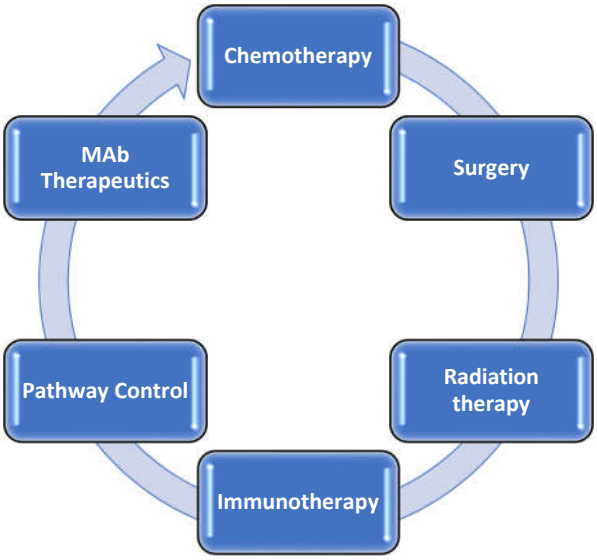
There are a set of treatments that are somewhat established for PA[3]. Yet PA treatments depend heavily on location and putative damage to proximate cells in the CNS[4]. Also there are differences between adult and juvenile[5]. In a sense the surgery is akin to a Mohs type surgery in that the elimination of all proliferating astrocytes which must be accomplished. This means that morphological or other marker oriented approaches must be employed[6].

[3] https://www.cancer.gov/types/brain/hp/child-astrocytoma-treament-pdq#_306

[4] https://www.nccn.org/guidelines/guidelines-detail?category=1&id=1425

[5] https://www.nccn.org/guidelines/guidelines-detail?category=1&id=1509

[6] See the video https://journals.lww.com/onsonline/Fulltext/2021/05000/The_Resection_of_a_Thalamic_Pilocytic_Astrocytoma.17.aspx and



As NCI notes[7]:

Targeted therapy is a type of treatment that uses drugs or other substances to identify and attack specific cancer cells. Targeted therapies usually cause less harm to normal cells than chemotherapy or radiation therapy do.

There are different types of targeted therapy:

Monoclonal antibodies: Monoclonal antibodies are immune system proteins made in the laboratory to treat many diseases, including cancer. As a cancer treatment, these antibodies can attach to a specific target on cancer cells or other cells that may help cancer cells grow. The antibodies are able to then kill the cancer cells, block their growth, or keep them from spreading. Monoclonal antibodies are given by infusion. They may be used alone or to carry drugs, toxins, or radioactive material directly to cancer cells. Bevacizumab is a monoclonal antibody and vascular endothelial growth factor inhibitor that binds to a protein called vascular endothelial growth factor (VEGF) and may prevent the growth of new blood vessels that tumors need to grow. Bevacizumab is used to treat childhood astrocytoma.

Protein kinase inhibitors work in different ways. There are several kinds of protein kinase inhibitors.

https://journals.lww.com/onsonline/Fulltext/2021/05000/The_Resection_of_a_Thalamic_Pilocytic_Astrocytoma.17.aspx# also https://link.springer.com/article/10.1007/s10143-020-01293-4

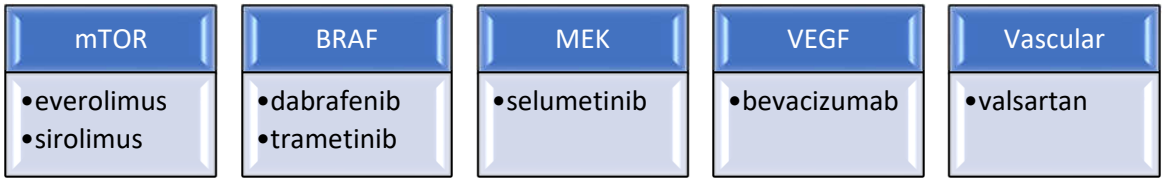
[7] https://www.cancer.gov/types/brain/patient/child-astrocytoma-treament-pdq

mTOR inhibitors: This treatment stops the protein that helps cells divide and survive. Everolimus and sirolimus are mTOR inhibitors used to treat childhood subependymal giant cell astrocytomas.

BRAF inhibitors: This treatment blocks the activity of proteins needed for cell growth and may kill cancer cells. The BRAF gene is found in a mutated (changed) form in some gliomas and blocking it may help keep cancer cells from growing. The combination of the BRAF inhibitors dabrafenib and trametinib are being studied to treat newly diagnosed low-grade astrocytomas or high-grade astrocytomas that have recurred or stopped responding to treatment. Dabrafenib and trametinib given after radiation are also being studied to treat newly diagnosed high-grade gliomas.

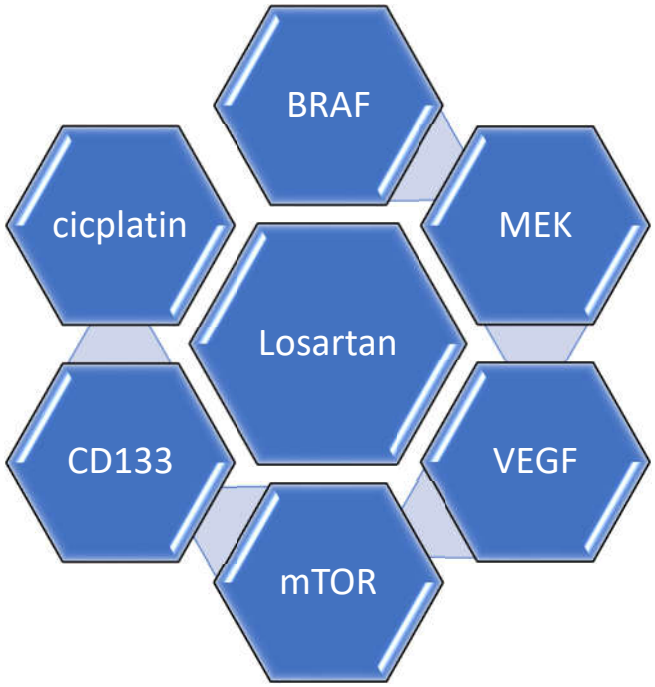
MEK inhibitors: This treatment blocks proteins needed for cell growth and may kill cancer cells. The MEK inhibitor is being studied to treat low-grade astrocytoma that has recurred or stopped responding to treatment. It is also being studied in combination with chemotherapy to treat newly diagnosed NF1-associated low-grade gliomas.

Thus the current targeted treatments seem to be as shown graphically below:



The above are all approved. However no combination techniques have been experienced. Furthermore, even if the BRAF is not mutated or any of the other alterations some results seem to indicate efficacy. It is not a simple and clear cut case. If surgery and radiation are not possible, then perhaps a neo-adjuvant chemo regime followed by some combination of the above may be of benefit. As noted there is no factual data yet available.

The graphic below demonstrates a putative mix of currently approved single targets. The issue is; can one mix and match these approaches and if so to what degree. We have indirectly assumed that some highly targeted radiation therapy can be useful but that is still a work in progress. One must remember that in Hodgkin's the radiation has long term oncogenic effects especially in young patients. However MAb targeted radiation therapy may be useful but it demands a target. One may be available with CD133 for PA but there is no clinical evidence as of yet.



Immunotherapy

Immunotherapy is a treatment that uses the patient's immune system to fight cancer. Substances made by the body or made in a laboratory are used to boost, direct, or restore the body's natural defenses against cancer. This cancer treatment is a type of biologic therapy.

Immune checkpoint inhibitor therapy: Some types of immune cells, such as T cells, and some cancer cells have certain proteins, called checkpoint proteins, on their surface that keep immune responses in check. When cancer cells have large amounts of these proteins, they will not be attacked and killed by T cells. Immune checkpoint inhibitors block these proteins and the ability of T cells to kill cancer cells is increased.

PD-1 and PD-L1 inhibitor therapy: PD-1 is a protein on the surface of T cells that helps keep the body's immune responses in check. PD-L1 is a protein found on some types of cancer cells. When PD-1 attaches to PD-L1, it stops the T cell from killing the cancer cell. PD-1 and PD-L1 inhibitors keep PD-1 and PD-L1 proteins from attaching to each other. This allows the T cells to kill cancer cells. PD-1 inhibitors are being studied to treat high-grade astrocytoma that has recurred.

1.3 OVERVIEW

This Note is not intended to delve deeper into any one of the many elements that we present. It is, however, and attempt to examine PA from a systems perspective. Namely to look at the various elements known to the research community and to attempt to integrate them into a holistic presentation, understanding the gaps, but highlighting the linkages.

For example, we discuss MEK inhibitors, yet we attempt to focus on alternative pathway elements such as BRAF, mTOR, and VEGF. One can suspect that each has some impact but that together a targeted therapeutic approach may be elucidated.

It is noted herein that PA is of interest because it oftentimes presents with few genetic changes. This allows for single cell examination and hopefully precise targeting.

Now our approach proceeds as follows:

1. We first begin by selecting the areas that must be examined to assemble a systems model for PA. This starts with the basis histology and genomic basics. Then we consider the astrocytoma change to a PA and what that involves both histologically and genetically. It is critical to consider the full tumor micro environment, TME, which is the other cells, proteins and supporting vasculature. Then we focus on the putative genes, non-coding RNA, epigenetic factors, and their related products and effects. This is followed by an analysis of the classes of therapeutics. At this point we must also consider multiple therapeutic applications as we try to deal with the complexity of the lesion. We then follow up with current trials and finally present a list of observations. This list is critical since it builds on what have been assembled to create a full system view and what possible productive next steps should be.

2.We have examined the literature in details as well as communicated with principals in the field. The challenge is to elicit key factors to make up a systems model while not diving oo deeply into the specific ongoing lines of research.

3. I is critical to understand that what we assess as of this writing will evolve at a rapid rate. The evolution must be taken into account as one examines therapeutic options.

1.4 SUMMARY

In this note we present the following:

Astrocytes: The astrocytes are large supportive cells in the brain and amongst other functions they interface with the circulatory system as well as the neurons.

Mutations: Mutations in astrocytes happen in the BRAF, MEK, mTOR and other genes resulting in loss of homeostasis and in excess proliferation. There may also be epigenetic changes as well as miRNA type interference that initiates and enhances the mutation effects.

Astrocytoma: The astrocytomas, especially pilocytic astrocytoma (PA) are low level malignancies that for the most part are proliferations of the astrocytes in a cystic type manner. The proliferation is uncontrolled and thus damage results from this effect.

Blood Brain Barrier: The classic blood brain barrier often makes for difficult therapeutic approaches. However since the astrocytes are directly interfacing with the circulatory system this seems to be a lesser issue.

Therapeutics: Therapeutic approaches, other than surgery, chemotherapy and radiation, target know genes. Immunotherapy appears to be more problematic since the immune cells are less frequent in the brain, albeit T cells do enter but last 24 hours or less. Single target therapeutics do work but not for all patients. Perhaps personalized multi therapeutics may be more efficacious. We discuss this at some length. Monoclonal and polyclonal antibodies do not at this time seem workable due to the lack of adequate surface targets and limited immune responses.

Trials: There are a few trials for PA. We have summarized them.

2 ASTROCYTES

The central nervous system is composed of a multiplicity of cells. The operative cells are the neurons. They are generally large cells with large nuclei and a large nucleoli.

From Maida et al:

Astrocytes provide structural isolation of neurons and their synapses and provide ionic (K+) sequestration, trophic support, and support for growth and signaling functions to neurons.

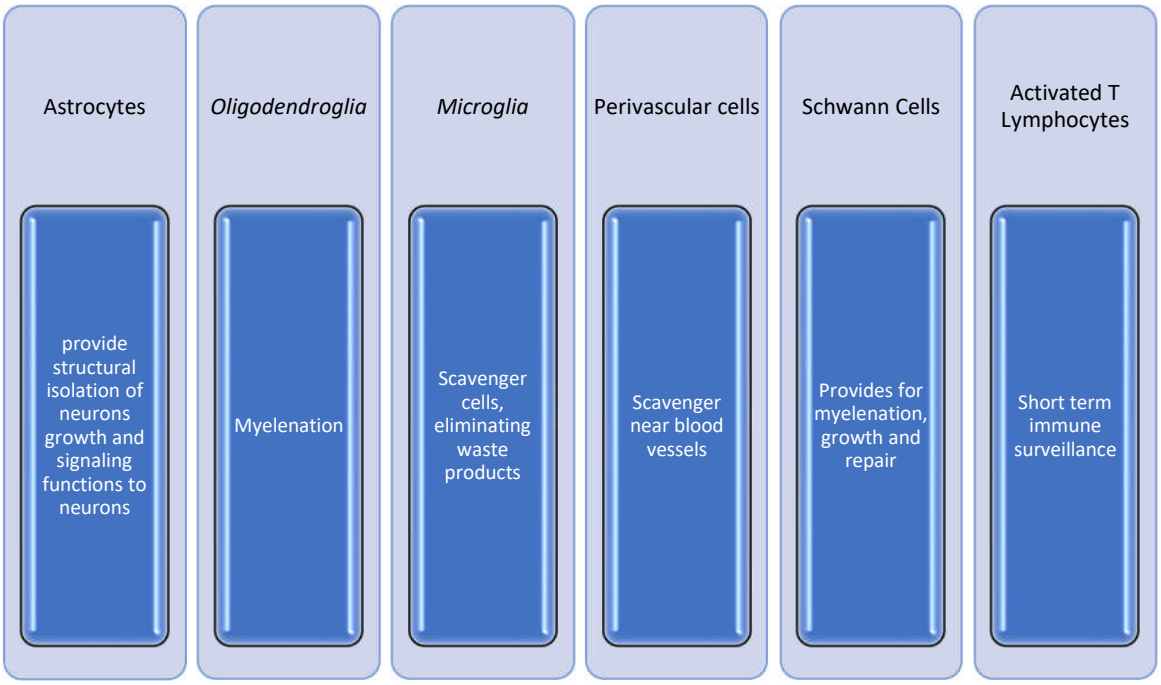
Oligodendroglia (oligodendrocytes) provide ***myelination*** *of axons in the CNS.*

Microglia are ***scavenger cells*** *that participate in phagocytosis, inflammatory responses,* ***cytokine and growth factor secretion****, and some immune reactivity in the CNS.*

Perivascular cells participate in similar activities at sites ***near the blood vessels****.*

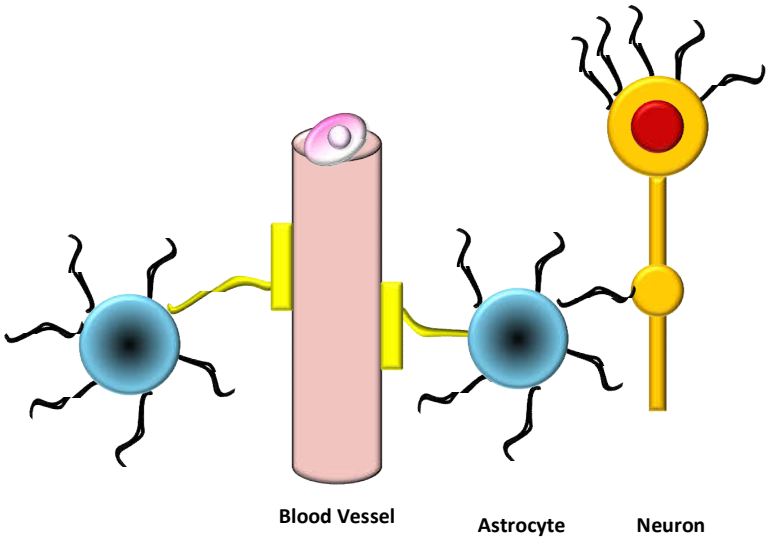
Schwann cells provide ***myelination,*** *ensheathment, trophic support, and actions that contribute to the growth and repair of peripheral neurons.*

Activated T lymphocytes normally can enter and traverse the CNS for immune surveillance for a period of approximately 24 hours.

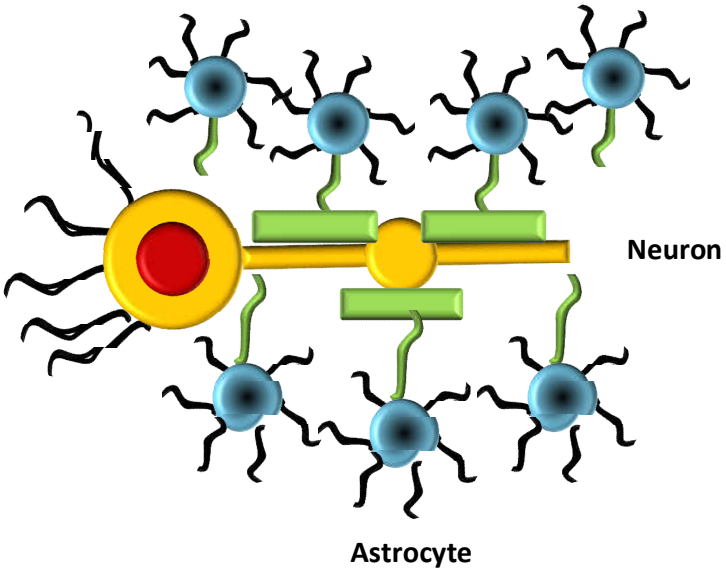


Graphically the astrocytes are a bridge between the vascular system and the neurons which make up the CNS. The astrocytes are prevalent throughout the CNS from the grey matter to the white

matter to the collection of nerves that go beyond this area. The graphic is useful for a simple understanding of the relationship between the astrocytes and their key role in interface a transfer.



Now we can view this in a more focused manner which leads to understanding astrocytomas. Astrocytes are often called the “fibroblasts of the CNS”. Like fibroblasts the astrocytes have a significant role in filling in between other key cells, in this case the neurons. However if the astrocytes lose control of their growth and proliferation they can then surround and overtake cells for which they usually just nourish. We give such a simplified example below.



From Mills:

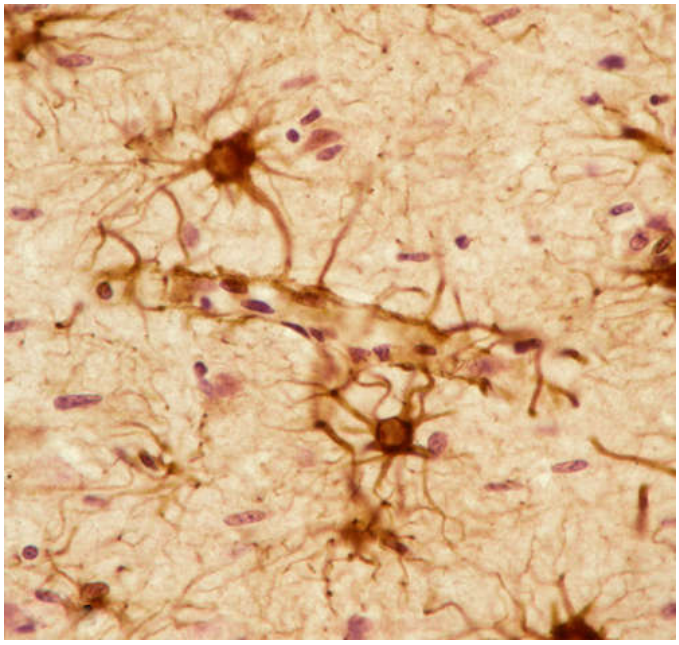
Gray matter and white matter: Gray (G) and white (W) matter can be distinguished with the naked eye on cross section of the brain.

Gray matter contains abundant neuropil surrounding large neurons and smaller astrocytes and oligodendroglia.

Neuropil is the term used for the fine amorphous eosinophilic background matrix of the CNS that fills the space between the cell bodies of the various cellular constituents as seen on H&E stains .
Ultrastructural examination shows the neuropil to be composed of myriad intimately intermingling processes of the cellular constituents (D). White matter, in contrast, is composed primarily of oligodendroglia and the axons that they myelinate, and displays a much more uniform, homogeneous appearance.

2.1 HISTOLOGY

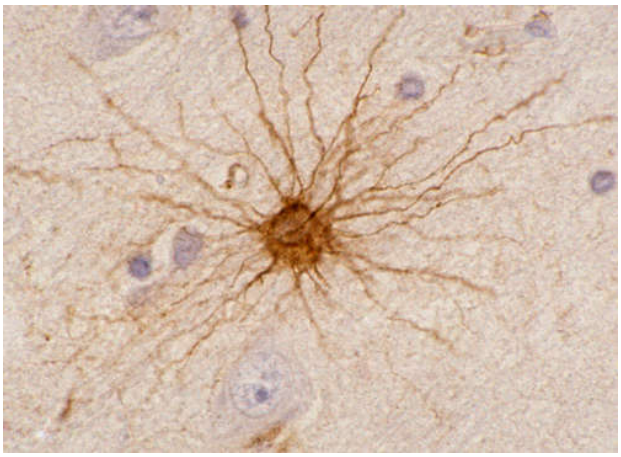
Astrocytes[8] are histologically identifiable. They are largest of the glial cells and they present with multiple processes that come from the cell body. The astrocytes have a tendency to create compartments and in fact they have feet that close of the surface of the brain and spinal cord. We show a typical astrocyte stained below:



One can see the astrocyte and its arms going towards a blood vessel and the attaching foot process is clearly visible.

[8] https://neuropathology-web.org/chapter1/chapter1bAstrocytes.html

With GFAP stain, we can see more detail on an astrocyte as shown below:



2.2 GENETICS

The basic genetics of an astrocyte is a bit complex. Like most cells there are internal mechanisms that deal with growth, proliferation, apoptosis and the like. Again like many cells the surface growth factors are significant players in this process.

As Borodinova et al note:

Astroglia play numerous essential roles in the brain. Decades of research demonstrated that astrocytes regulate neuronal excitability and metabolism, establish and regulate the blood–brain barrier, locally control microcirculation, contribute to neuroinflammation, regulate neurogenesis, support brain tissue clearance, maintain water homeostasis, mediate the exchange between cerebrospinal fluid and interstitial fluid in the brain tissue, and control edema formation in an AQP4-dependent manner.

The aberrant functioning of astroglia was observed in neurodevelopmental and neurodegenerative diseases, brain injury, and neuroinflammation. Given these observations, the modulation of astrocytes affects the progression of brain diseases and is considered a promising neuroprotective and neuroregenerative therapy.

However, targeted modulation of astroglial activity faces numerous problems.

First, various subpopulations of astrocytes are involved in the functions mentioned above, making it difficult to specifically target the relevant astroglia population.

Second, astrocytes are very sensitive to neuronal activity and vice versa. This interdependence means that the activation of neurons and astrocytes influences each other, making it challenging to characterize and observe an independent glial response.

Astrocytes are a heterogenous group of brain cells with diverse molecular markers. This diversity of molecular markers makes it challenging to identify astrocytes by detecting a single marker such as glial fibrillary acidic protein (GFAP) or s100β protein expression alone. There are several subgroups of astrocytes in rodent and primate central nervous systems, such as:

(i) radial astroglia … originating from neuroepithelial cells that are involved in embryonic and adult neurogenesis;

(ii) protoplasmic astrocytes … which are the principal glial constituents of the neurovascular unit; they stay close to neurons due to direct contacts made by their perisynaptic processes and control neuronal excitability, plasticity, metabolic status, and close to brain microvessel endothelial cells due to their end-feet contacts to adjust local microcirculation to the actual needs of neurons;

(iii) fibrous astrocytes… surrounding myelinated fibers and controlling myelinization;

(iv) reactive astrocytes with upregulated expression of GFAP … that take part in the progression of local inflammation and gliosis

2.3 GROWTH FACTORS

Growth factors abound in the cellular environments. Growth Factors abound in cells. They activate, via Growth Factor Receptors (GFR), a variety of internal pathways which in turn control the cells status. They become initiators and promoters of proliferation. The object of this note is to present the collection of growth factors into a somewhat holistic collection. There still is a looseness in these significant drivers of cell behavior. The GFs float around in a manner ranging from endocrine to paracrine, and some say even exocrine and autocrine. They can have a significant effect on the extracellular matrix as well as the mesenchymal to cell interfaces and characteristics. This is not a definitive study, but merely a benchmark to attempt a focus back on the GF elements.

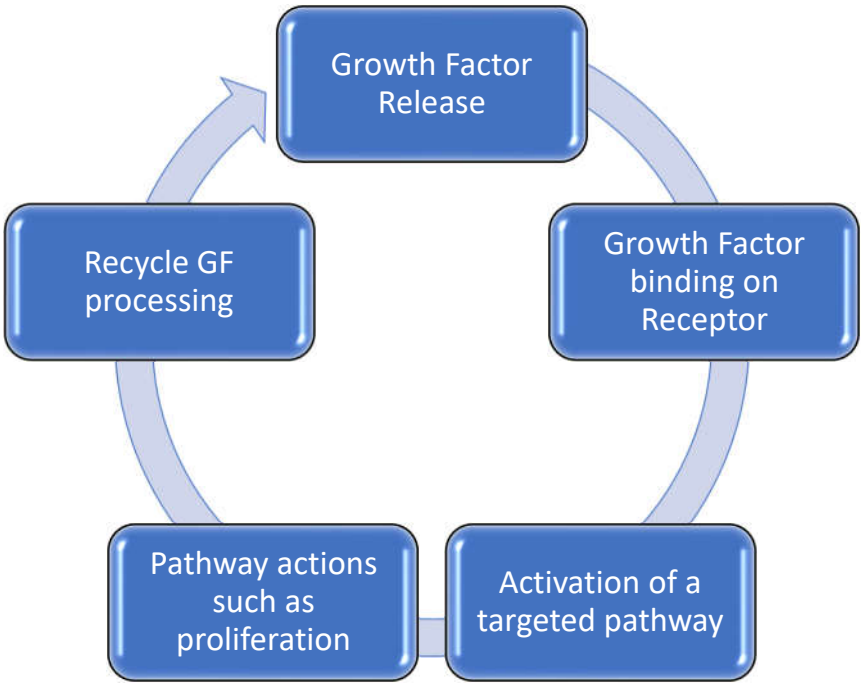
As Tekeuchi and Ito note:

The majority of growth factor receptors are composed of extracellular, transmembrane, and cytoplasmic tyrosine kinase (TK) domains. Receptor tyrosine kinase (RTK) activation regulates many key processes including cell growth and survival. However, dysregulation of RTK has been found in a wide range of cancers, and it has been shown to correlate with the development and progression of numerous cancers.

Therefore, RTK has become an attractive therapeutic target. One way to effectively block signaling from RTK is inhibition of its catalytic activity with small-molecule inhibitors. Low-molecular-weight TK inhibitors (TKIs), such as imatinib, targeting tumors with mutant c-Kit, and gefitinib, targeting non-small cell lung cancer with mutant epidermal growth factor receptor (EGFR), have received marketing approval in Japan. MET, fibroblast growth factor receptor (FGFR), and insulin-like growth factor-I receptor (IGF-IR) are frequently genetically altered in advanced cancers.

TKIs of these receptors have not yet appeared on the market, but many anticancer drug candidates are currently undergoing clinical trials. Most of these TKIs were designed to compete with ATP at the ATP-binding site within the TK domain. … Targeting agents specifically inhibiting the target kinase were previously searched for based on the hypothesis that a narrow target window might reduce unexpected side effects, but agents with multiple targets have been recently developed to overcome tumors resistant against a single-targeting agent.

This is just a simple summary of the possible options available with the multiplicity of GF. We may view the GF actions in the figure below. The end point may be proliferation, apoptosis, and/or sending out GF to other cells.



There are several key questions regarding the collection of GF. Specifically:

1. Are there GF/GFR which are specific for specific cancers and if so what are the details associated with their involvement.

2. If GF/GFR are drivers of certain malignancies what are the drivers of these GF/GFR expressions.

3. If GF/GFR can be such drivers than can we approach mitigating their effects by use of blockers such as mAbs, blocking them by Ab attachments?

4. GF/GFR are used by a variety of cells for normal homeostasis. Blocking them may result in a variety of effects that are detrimental. Can we ascertain what blockages would result in such detrimental effects.

5. GF/GFR are a complex inter-cellular signalling network. What can we say about such a network in general and then in specific cases?

These are just a few of the general questions we try to examine. This is a working paper and as such there is no attempt at completeness and no representation of innovation or therapeutic interpretation.

From Cabezas et al we have:

Astrocytes exert multiple functions in the brain such as

1. *the development of blood–brain barrier characteristics,*
2. *the promotion of neurovascular coupling,*
3. *attraction of cells through the release of chemokines,*
4. *clearance of toxic substances and generation of antioxidant molecules and*
5. *growth factors.*

In this aspect, astrocytes secrete several growth factors (BDNF, GDNF, NGF, and others) that are fundamental for cell viability, oxidant protection, genetic expression and modulation of metabolic functions.

The platelet derived growth factor (PDGF), which is expressed by many SNC cells including astrocytes, is an important molecule that has shown neuroprotective potential, improvement of wound healing, regulation of calcium metabolism and mitochondrial function.

Here we explore some of these astrocyte-driven functions of growth factors and their possible therapeutic uses during neurodegeneration.

Although growth factors in the brain are produced by neurons, oligodendrocytes or microglia, their release by astrocytes is of primordial importance for the maintenance of neuronal functions.

Astrocytes are an important source of BDNF (Brain derived neurotrophic factor), GDNF (Glial cell line derived neurotrophic factor), NGF (nerve growth factor), PDGF and others with prospective neuroprotective functions in the brain.

In the present review, we explore the importance of growth factors in astrocytic metabolic regulation, and highlight the effect of PDGF as a potential therapeutic approach….

Astrocytes are part of the glial cells along with oligodendrocytes and microglia. These cells have a great range of functions that include Blood Brain Barrier (BBB) maintenance, uptake of

glutamate and g-aminobutyric acid (GABA) by specific transporters, production of antioxidant compounds like glutathione (GSH) and superoxide dismutases (SOD) and growth factors that enhance neuronal viability, both in normal and in pathological conditions. This cell type is characterized by a stellate morphology with various processes and ramifications, and by the expression of the intermediate filaments vimentin (Vim) and glial fibrillary acidic protein (GFAP).

There are two main types of astrocytes depending on their location and metabolic functions: protoplasmic astrocytes of the grey matter, which envelope neuronal bodies and synapses, and fibrous astrocytes from the white matter that interact with the nodes of Ranvier and oligodendroglia.

Protoplasmic astrocytes have been associated with increased accumulation of a-synuclein during Parkinson Disease (PD), Alzheimer disease (AD), and Epilepsy, and these cells the main targets during neurodegeneration.

Astrocytic terminal processes, known as endfeet, contact the brain vasculature surface facing ECs (endothelial cells) and pericytes and enwrap neuronal synapses, thus enabling the modulation of both neuronal activity and cerebral blood flow following an elevation in intracellular Ca2+ levels in the endfeet.

Importantly, astrocytic endfeet express specialized molecules such as Kir4.1 K+ channels and aquaporin 4 that regulate ionic concentrations in BBB, and protein transporters like glucose transporter-1 and Pglycoprotein, suggesting the importance of the endfeet in astrocyte polarization. Astrocytes respond to all types of cerebral insults (infection, trauma, ischemia, oxidative stress, neurodegenerative diseases) by a process called reactive astrogliosis, which involves both molecular and morphological changes including hypertrophy of cell bodies and processes, increased expression of GFAP, vimentin, nestin and chondroitin sulfate proteoglycans (CSPGs).

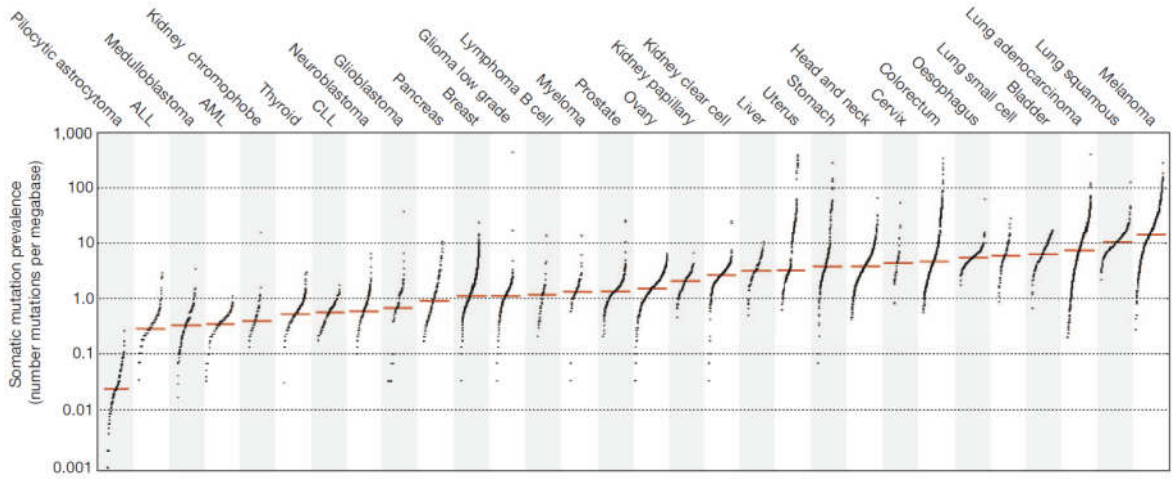
Additional features of astrogliosis include changes in glutamate uptake rate, protection against oxidative stress by the production of glutathione, neuroprotection by the release of adenosine, degradation of beta-amyloid peptides (Aβ), glial scarring, and, in some cases, release of inflammatory cytokines, including tumor necrosis factor (TNF) and ROS production.

From Seifert et al we have a table of key astrocytoma activated genes:

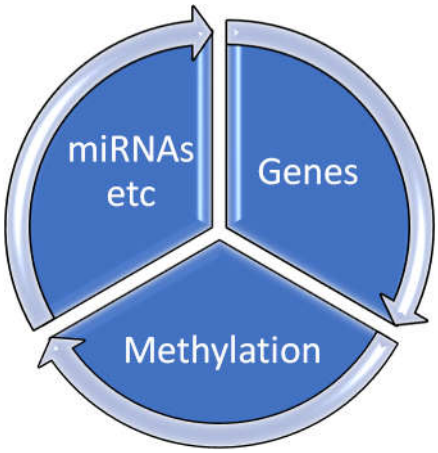
Gene	Chromosome	Band	Tumor	Annotation
H3F3A	1	q42.12	PA I	H3 histone, family 3A
MEIS1	2	p14	PA I	Meis homeobox 1
NEUROD1	2	q31.3	PA I	neuronal differentiation 1
EOMES	3	p24.1	PA I	eomesodermin
ZIC1	3	q24	PA I	Zic family member 1
ZIC4	3	q24	PA I	Zic family member 4
EGR1	5	q31.2	PA I	early growth response 1
EN2	7	q36.3	PA I	engrailed homeobox 2
EGR3	8	p21.3	PA I	early growth response 3
CDKN2B	9	p21.3	PA I	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)
NTRK2	9	q21.33	PA I	neurotrophic tyrosine kinase, receptor, type 2
HIF1AN	10	q24.31	PA I	hypoxia inducible factor 1, alpha subunit inhibitor
SUV420H1	11	q13.2	PA I	suppressor of variegation 4-20 homolog 1 (Drosophila)
KRAS	12	p12.1	PA I	Kirsten rat sarcoma viral oncogene homolog
ZIC2	13	q32.3	PA I	Zic family member 2
SUZ12	17	q11.2	PA I	SUZ12 polycomb repressive complex 2 subunit
SUV420H2	19	q13.42	PA I	suppressor of variegation 4-20 homolog 2 (Drosophila)
OLIG1	21	q22.11	PA I	oligodendrocyte transcription factor 1
OLIG2	21	q22.11	PA I	oligodendrocyte lineage transcription factor 2
ATRX	X	q21.1	PA I	alpha thalassemia/mental retardation syndrome X-linked
ZIC3	X	q26.3	PA I	Zic family member 3

3 PA AND GENETIC MUTATIONS, METHYLATION AND RNAS

From Alexandrov et al we have the following graphic. This graphic is quite compelling. It shows many malignancies as the sorts them by increasing numbers of somatic mutations found in each. At the low end is PA, the case in question, whereas at the top end is melanoma. Ironically multiple therapeutic approaches for melanoma have been found. In the middle is prostate cancer, PCa, for which little has yet been determined, and PA at the lowest with yet limited approaches.



What then are the possible approaches. We graphically present them below.

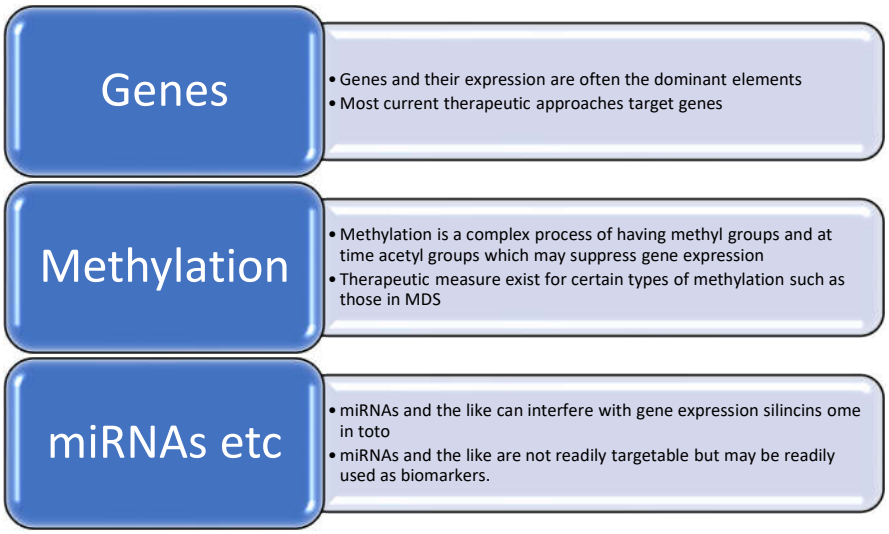


Specifically:

Genes: If we know the mutated target genes then perhaps we can the find a therapeutic that targets them and reduces their effect. An example is the classic BRAF V600 mutation.

Methylation: Broadly speaking this is any epigenetic modification. Here the approaches may be limited.

miRNAs: These are actually all non-coding RNAs. They interfere with normal expression and can arguably be targeted if known. This thus demands a fuller understanding of the genetic profiling of the cells, one at a time.



As Zhang et al have noted:

Optic nerve astrocytomas (ONAs) *are neurological neoplasms in the central nervous system (CNS), and they have the highest incidence rate among all the tumor types in the visual pathway. In this study, we conducted a Surveillance, Epidemiology, and End Results (SEER) -based research to explore the demographic, survival, and prognostic factors of patients diagnosed with ONAs …*

In the central nervous system (CNS), there are three types of tumors: astrocytoma, oligodendroglioma, and ependymoma.

Of all these tumors, astrocytoma is the most common type. ONAs are rare astrocytic tumors that occur in the optic nerve and reach out to the chiasm and the frontal lobe frequently. The classification of ONAs is based upon the World Health Organization (WHO) criteria, and it include Grade I (pilocytic), Grade II (diffuse), Grade III (anaplastic), and Grade IV (glioblastoma) astrocytoma.

Pilocytic astrocytoma has the highest incidence rate in people and it has excellent prognosis and survival rate.

As 50–60% of patients with ONAs have neurofibromatosis type 1 (NF-1)[9], the mutation in the NF-1 suppressor gene is considered to be a predictor for developing ONAs. Most patients are in the pediatric population between the ages of 0 and 14 years. The 5-year survival rate of optic nerve astrocytoma is over 95.0%, while more than 3 quarters of patients' vision is greatly impaired. For a long period, ONAs were regarded as indolent diseases and did not require therapy.

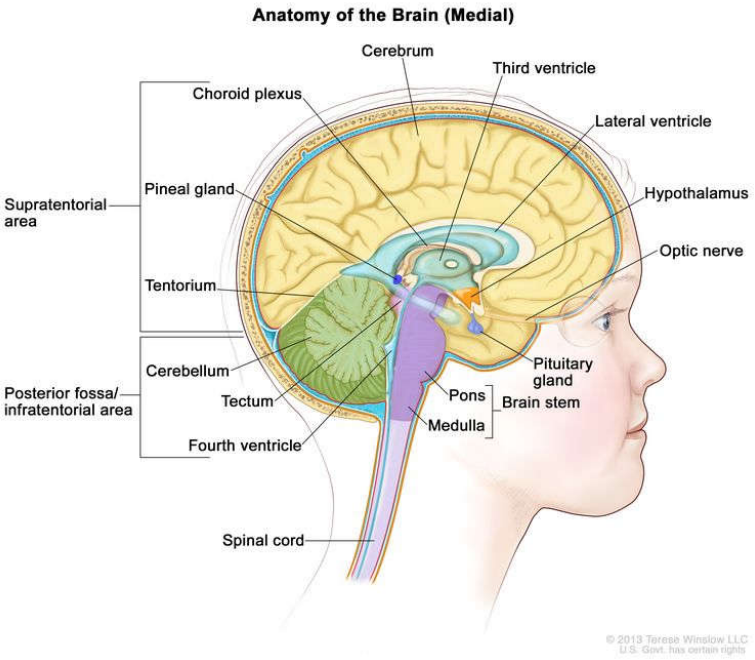
However, the latest research suggests that ONAs have an unpredictable clinical process, ranging from rapid progression to spontaneous regression. Some physicians had a preference for surgical treatment, while some tended to utilize radiotherapy treatment for ONAs patients.

Recently, chemotherapy and observation is believed to be an effective therapeutic method for ONAs. ONAs have unpredictable progression, and the consequences are highly associated with treatment modalities, thus these facts lead to ONAs' controversial treatment choices.

3.1 PILOCYTIC ASTROCYTOMAS

Pilocytic astrocytoma is a low grade tumor of the astrocytes. In simple terms the astrocytes start proliferating to excess and may form a cystic like mass is select areas of the brain. They favor the cerebellum, optic and hypothalamic pathways, brainstem and spinal cord. The astrocytes proliferate slowly be insidiously in a progressing manner placing pressure of the local nerves, such as the optic, and increasing intracerebral pressure.

[9] See Gross et al, *Neurofibromatosis type 1, (NF-1), an autosomal dominant genetic disorder characterized by multiple progressive tumor- -and nontumor manifestations, has limited treatment options. In patients with the disorder, dysfunction of the guanosine triphosphatase– activating protein neurofibromin leads to overactivation of the RAS pathway.* ***Therefore, targeted inhibition of the RAS pathway with mitogenactivated protein kinase (MAPK) kinase (MEK) inhibition is a logical treatment approach3 that has been successful in a preclinical model of neurofibromatosis type 1****. Plexiform neurofibromas are histologically benign peripheral-nerve sheath tumors that occur in up to 50% of persons with neurofibromatosis type 1 and can cause substantial complications*.



3.2 PATHOLOGY

As Collins et al note :

Macroscopically, PAs are generally relatively soft in texture and gray in sections of fixed specimens.

They appear to be well-defined.

Cysts are common both within the tumor tissue as well as around the tumor, the latter resulting in a cyst with a tumor nodule.

Calcium deposits and hemosiderin may be present, the latter secondary to small bleeds into tumor tissue.

Very rarely, PA can present with extensive leptomeningeal involvement without parenchymal involvement, the so-called "primary leptomeningeal gliomatosis".

Histopathologically, PA is a tumor of low to moderate cellularity with compact, densely fibrillated areas rich in Rosenthal fibers, consisting of cells with long bipolar (hairlike) processes and elongated cytologically bland nuclei, as well as loosely textured areas, composed of multipolar cells (protoplasmic astrocyte-like), with bland, round-to-oval nuclei, and multiple, relatively short cytoplasmic extensions. These areas have varying degrees of mucoid background material with micro-cyst development being common, as are also eosinophilic granular bodies or hyaline droplets. The bipolar tumor cells are generally strongly GFAP immunoreactive, while the protoplasmic astrocyte-like tumor cells are less so. In some cases, areas morphologically

similar to oligodendrogliomas may be found, but only rarely is the oligodendroglial like component predominant (see "Differential diagnostic issues").

Cells with pleomorphic nuclei, often multinucleated, may also occur and generally are found in the loose microcystic regions. Rare mitoses are acceptable, but any notable mitotic activity should warrant the consideration of other glioma diagnoses.

Ki67/MIB-1 indices of up to 4 % are common. Microvascular proliferation, resulting in relatively thick-walled, hyalinized, and/or glomeruloid vessels, is often seen, and infarct-like necrosis can occur in some cases (no pseudopalisading). While these findings are all compatible with a diagnosis of PA, they sometimes make the distinction from other gliomas difficult, particularly when examining small biopsies.

While macroscopically appearing relatively well-defined, microscopically, varying degrees of invasion into the adjacent brain are observed. Rare cerebellar tumors show a diffuse pattern of growth, and molecular analysis may be of some help in identifying these tumors as PAs. Consequently, both normal astrocytes and neurons may become trapped in the tumor tissue.

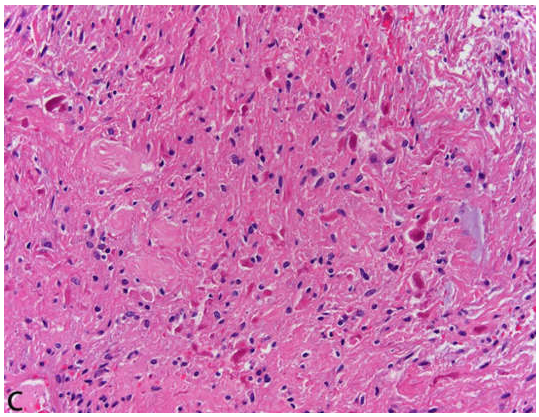
Microscopic infiltration of the leptomeninges frequently occurs, especially in the cerebellum and optic nerve tumors, and is not an ominous finding. Today, it is rare to see surgical resection specimens from optic nerve gliomas in NF1 patients, given the often benign and indolent natural history of these tumors, which may, at times, regress.

On cross section, the optic nerve outline is often visible near the center of the specimen, while the tumor characteristically grows in the subarachnoid space between the nerve and the dural sheath that is markedly expanded. Meningothelial hyperplasia may occur and represent a potential pitfall in the differential diagnosis between optic nerve PA and optic nerve meningioma when only a small and superficial biopsy is obtained.

The authors present a typical demonstrative of a PA lesion. They note:

The tumor has classic PA features with a densely fibrillated appearance and numerous Rosenthal fibers[10]

[10] A Rosenthal fiber is a thick, elongated, worm-like or "corkscrew" eosinophilic (pink) bundle that is found on staining of brain tissue...



3.3 GENES

As Seifert et al have noted:

Astrocytomas are the most common primary brain tumors in the course of life. Molecular origins of astrocytomas are not fully understood. Different studies have identified tumorigenic cells with stem-cell-like properties suggesting that astrocytomas originate from neural stem cells. Astrocytomas are classified by the World Health Organization (WHO) grading system into four histological grades of increasing malignancy.

Here, we focus on a comparative analysis of the most frequently occurring astrocytomas (pilocytic astrocytoma, diffuse astrocytoma, anaplastic astrocytoma, glioblastoma) of different degrees of aggressiveness to assess for similarities and differences at the level of individual genes, signaling pathways, molecular subtypes and regulatory networks. This is highly important to better understand the development of specific astrocytomas. The pilocytic astrocytoma WHO grade I (PA I) is a very slowly growing benign astrocytoma. PA I is the most commonly diagnosed brain tumor in childhood and adolescence. The ten-year overall survival rate of PA I patients is greater than 95 % .

The treatment of choice for PA I is gross total resection, but PA I tumors that are inoperable or only partly accessible by surgery represent a therapeutic challenge often showing a serve clinical course. Recent studies have indicated that PA I is predominantly a single-pathway disease driven by mutations affecting the MAPK pathway. In addition, PA I can also display histological features of glioblastoma (GBM IV) including microvascular proliferation and necrosis, but in contrast to GBM IV, these features are not directly associated with increased malignancy of PA I. In rare cases, progression of PA I to more malignant astrocytomas has been observed. ...

We considered raw gene expression data of 49 PA I and 9 normal cerebellum reference samples (5 fetal and 4 adult samples) available from Gene Expression Omnibus. We performed stringent quality controls of all expression arrays by reconstructing the hybridization images. We removed three arrays with slight hybridization artifacts. All corresponding microarrays were normalized

using GCRMA with a design file from BrainArray. The resulting PA I gene expression data set comprised 47 PA I samples and 8 corresponding normal cerebellum references for which expression levels were measured for 16,973 genes.

We further also downloaded processed DNA methylation profiles available for 38 of the considered PA I samples analyzed in. Tumor-specific DNA methylation profiles were compared to DNA methylation profiles of normal cerebellum samples from four fetal and two adult probes. We refer to for more details. All PA I tumors were diagnosed in children or young adults and fulfill all editorial policies…

The authors then summarize the genes of interest in PA.

Gene	Chromo-some	Band	Expression	Annotation
H3F3A	1	q42.12	-	H3 histone, family 3A
MEIS1	2	p14	-	Meis homeobox 1
NEUROD1	2	q31.3	-	neuronal differentiation 1
EOMES	3	p24.1	-	eomesodermin
ZIC1	3	q24	-	Zic family member 1
ZIC4	3	q24	-	Zic family member 4
EGR1	5	q31.2	+	early growth response 1
EN2	7	q36.3	-	engrailed homeobox 2
EGR3	8	p21.3	+	early growth response 3
CDKN2B	9	p21.3	+	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)
NTRK2	9	q21.33	+	neurotrophic tyrosine kinase, receptor, type 2
HIF1AN	10	q24.31	+	hypoxia inducible factor 1, alpha subunit inhibitor
SUV420H1	11	q13.2	-	suppressor of variegation 4-20 homolog 1 (Drosophila)
KRAS	12	p12.1	-	Kirsten rat sarcoma viral oncogene homolog
ZIC2	13	q32.3	-	Zic family member 2
SUZ12	17	q11.2	-	SUZ12 polycomb repressive complex 2 subunit
SUV420H2	19	q13.42	-	suppressor of variegation 4-20 homolog 2 (Drosophila)
OLIG1	21	q22.11	+	oligodendrocyte transcription factor 1
OLIG2	21	q22.11	+	oligodendrocyte lineage transcription factor 2
ATRX	X	q21.1	-	alpha thalassemia/mental retardation syndrome X-linked
ZIC3	X	q26.3	-	Zinc family member 3

We briefly summarize some of the key genes in astrocytes. We defer until later the specific targets related to astrocytomas. Now from Abraham and Gulley:

Name	Grade	Main Molecular Marker/Molecular Profile
Adult-type diffuse gliomas		
Oligodendroglioma	2 or 3	IDH 1 or 2-mutant; 1p/19q codeleted, TERT promoter mutation, CIC, FUBP1, NOTCH1
Astrocytoma	2, 3, or 4	IDH 1 or 2-mutant, ATRX, TP53, CDKN2A/B
Glioblastoma	4	IDH-wildtype, TERT promoter, chromosome
−7/+10, EGFR		
Circumscribed astrocytic glioma		
Pilocytic astrocytoma	1	KIAA 1549-BRAF, BRAF, NF1
Ependymal tumors		
Supratentorial	2 or 3	ZFTA, RELA, YAP1, MAML2
Supratentorial, ZFTA fusion-positive	2 or 3	ZFTA fusion-positive
Supratentorial, YAP1-fusion positive	2 or 3	YAP1 fusion-positive
Posterior fossa	2 or 3	H3 K27me3, EZHIP (methylome)
Posterior fossa, group PFA	2 or 3	
Posterior fossa, group PFB	2 or 3	
Spinal	2 or 3	NF2, MYNC
Spinal, MYCN-amplified	2 or 3	MYCN-amplified
Myxopapillary	2	
Subependymoma	1	
Meningiomas		
Meningioma	1, 2, or 3	NF2, AKT1, TRAF7, SMO, PIK3CA, KLF4, SMARCE1, BAP1 in subtypes, H3K27me3, TERT promoter, CDKN2A/B in grade 3
Embryonal tumors		
Medulloblastoma		
Medulloblastomas, molecularly defined		
Medulloblastoma, WNT activated	4	CTNNB1, APC

Name	Grade	Main Molecular Marker/Molecular Profile
Medulloblastoma, SHH activated, and TP53-wildtype	4	TP53, PTCH1, SUFU, SMO, MYCN, GLI2 (methylome)
Medulloblastoma, SHH activated, and TP53-mutant	4	TP53, PTCH1, SUFU, SMO, MYCN, GLI2 (methylome)
Medulloblastoma, non-WNT/non-SHH	4	MYC, MYCN, PRDM6, KDM6A (methylome)
Hematolymphoid tumors		
Lymphomas		
CNS lymphomas		
Primary diffuse large B-cell lymphoma of the CNS		BCL6 gene rearrangement, (14;18) translocation, MYC
Pineal tumors		
Pineocytoma	1	
Pineal parenchymal tumor of intermediate differentiation	2 or 3	
Pineoblastoma	4	RB1, MYC
Papillary tumor of the pineal region	2 or 3	loss of chromosomes 10, 3, and 22q and gains of 8p and 12, PTEN
Desmoplastic myxoid tumor of the pineal region, SMARCB1-mutant		
Metastasis to the CNS		
Metastasis to the brain and spinal cord	NA	
Metastasis to the meninges	NA	

From NCI:

Genomic Alterations, Molecular features of low-grade gliomas, Pilocytic and diffuse astrocytomas

Genomic alterations involving activation of BRAF and the ERK/MAPK pathway are very common in sporadic cases of pilocytic astrocytoma, a type of low-grade glioma.

3.3.1 BRAF-KIAA1549 alterations

BRAF activation in pilocytic astrocytoma occurs most commonly through a BRAF-KIAA1549 gene fusion, producing a fusion protein that lacks the BRAF regulatory domain. This fusion is

seen in most infratentorial and midline pilocytic astrocytomas, but is present at lower frequency in supratentorial (hemispheric) tumors.

Presence of the BRAF-KIAA1549 fusion predicted a better clinical outcome (progression-free survival [PFS] and overall survival [OS]) in one report that described children with incompletely resected low-grade gliomas.[27] However, other factors such as CDKN2A deletion, whole chromosome 7 gain, and tumor location may modify the impact of the BRAF mutation on outcome.[29]; [30][Level of evidence: 3iiiDiii] Progression to high-grade glioma is rare for pediatric low-grade glioma with the BRAF-KIAA1549 fusion.[31]

BRAF activation through the BRAF-KIAA1549 fusion has also been described in other pediatric low-grade gliomas (e.g., pilomyxoid astrocytoma).[26,27] Other genomic alterations in pilocytic astrocytomas that can activate the ERK/MAPK pathway (e.g., alternative BRAF gene fusions, RAF1 rearrangements, RAS mutations, and BRAF V600E point mutations) are less commonly observed.[19,21,22,32]

***BRAF V600E mutations**: BRAF V600E point mutations are occasionally observed in pilocytic astrocytoma; the mutations are also observed in nonpilocytic pediatric low-grade gliomas, including ganglioglioma, desmoplastic infantile ganglioglioma, and approximately two-thirds of pleomorphic xanthoastrocytomas.*

Studies have observed the following:

In a retrospective series of more than 400 children with low-grade gliomas, 17% of tumors were BRAF V600E mutant. The 10-year PFS rate was 27% for BRAF V600E–mutant cases, compared with 60% for cases whose tumors did not harbor that mutation. Additional factors associated with this poor prognosis included subtotal resection and CDKN2A deletion.[37] Even in patients who underwent a gross-total resection, recurrence was noted in one-third of these cases, suggesting that BRAF V600E tumors have a more invasive phenotype than do other low-grade glioma variants.

In a similar analysis, children with diencephalic low-grade astrocytomas with a BRAF V600E mutation had a 5-year PFS rate of 22%, compared with a PFS rate of 52% in children who were BRAF wild-type.

The frequency of the BRAF V600E mutation was significantly higher in pediatric low-grade glioma that transformed to high-grade glioma (8 of 18 cases) than was the frequency of the mutation in cases that did not transform to high-grade glioma (10 of 167 cases).[31]

Other mutations: Activating mutations in FGFR1, PTPN11, and NTRK2 fusion genes have also been identified in noncerebellar pilocytic astrocytomas. In pediatric grade II diffuse astrocytomas, the most common alterations reported (up to 53% of tumors) are rearrangements in the MYB family of transcription factors.

3.3.2 GFAP

From NCBI[11]:

This gene encodes (GFAP) one of the major intermediate filament proteins of mature astrocytes. It is used as a marker to distinguish astrocytes from other glial cells during development. Mutations in this gene cause Alexander disease, a rare disorder of astrocytes in the central nervous system.

3.3.3 RTK[12]

Receptor tyrosine kinases (RTKs) are the high-affinity cell surface receptors for many polypeptide growth factors, cytokines, and hormones. Of the 90 unique tyrosine kinase genes identified in the human genome, 58 encode receptor tyrosine kinase proteins. Receptor tyrosine kinases have been shown not only to be key regulators of normal cellular processes but also to have a critical role in the development and progression of many types of cancer. Mutat*ions in receptor tyrosine kinases lead to activation of a series of signalling cascades which have numerous effects on protein expression. Receptor tyrosine kinases are part of the larger family of protein tyrosine kinases, encompassing the receptor tyrosine kinase proteins which contain a transmembrane domain, as well as the non-receptor tyrosine kinases which do not possess transmembrane domains*

Hong et al report on PA in the optic tract and note:

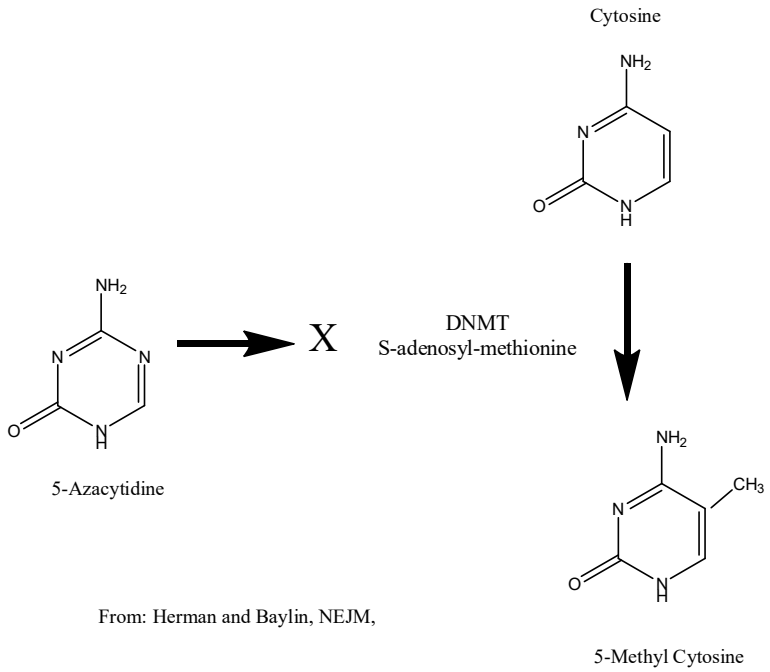
Whole-exome sequencing (WES) was performed on the biopsy in accordance with an institutional review boardapproved protocol (Table 1). Somatic mutations were identified in NF1 (NM_000267:c.T4839G:p.Y1613X), a lossof-function mutation, FGFR1 (NM_023110:c.C1638G:p. N546K), an activating mutation previously reported in glial tumors [5], and PTPN11 (NM_001330437:c.G214A:p. A72T), an activating pathogenic variant on the SH2 domain that may drive oncogenic signaling in cancers, including gliomas [6] No large-scale somatic copy number alterations were identified. BRAF/KIAA1549 fusion was absent by FISH. Additional WES of germline DNA was only notable for a somatic mutation in RYR1 (NM_000540:c.G7300A:p. G2434R), a variant as a well-established susceptibility allele for malignant hyperthermia.

3.4 METHYLATION

Methylation can be a significant driver of cell instability. What is methylation? Simply, the attachment of a methyl group to the cytosine molecule creates a methylated C. This is not a complicated process but one which happens frequently and may have significant effects. Cytosine gets methylated and is converted to 5-methyl cytosine. This is accomplished by means of two enzymes as depicted below. This occurs when we have a C and G adjacent. It occurs to the C in that pair. We depict that transition below. Note also that by using 5-Azacytadine we can block that transition.

[11] https://www.ncbi.nlm.nih.gov/gene/2670

[12] https://en.wikipedia.org/wiki/Receptor_tyrosine_kinase



Now there are the CpG islands. These are C, cytosine, and G, guanine, adjacent nucleotides which are connected via a phosphodiester bone between the two, and multiple collections of these paired nucleotides. The CpG island is then an area dense in these CG pairs connected by the phosphodiester bond, but the "island" may contain nucleotides other than the CG pairs, but generally are high in CG pair concentration, usually more than 50%.

One should note that the statistical probability of such large CG pairings would normally be quite low. One would anticipate equal probability for any nucleotide and any nucleotide pairing. Furthermore such a high concentration is statistically extremely rare but if often existentially quite common.

The CpG islands may be from 300 to over 3,000 base pairs in total length, and are frequently found in gene promoter regions. Thus when the CpG islands are methylated, namely the C is methylated, then the island gets silenced as does the corresponding gene. Namely methylation of CpG islands can result in gene silencing. This then becomes a critical issue if the gene is a control gene such as PTEN, p53, or many of the critical pathway control genes. The CpG islands are also propagated to cell progeny during mitosis, thus a methylated island remains so in the cells progeny.

However understanding methylation of islands, and having a means to demethylate the islands may present a reasonable way to develop therapeutics for cancers resulting from methylated regions. We shall examine that shortly.

As Laird and Jaenisch state:

The normal pattern of 5-methylcytosine distribution DNA methylation in mammals is found as a covalent modification at the fifth carbon position of cytosine residues within CpG dinucleotides. Most of the CpG dinucleotides in the human genome are methylated.

However, 5-methylcytosine makes up less than 1% of all nucleotides, since CpG dinucleotides are under-represented about five-fold in the mammalian genome. The paucity of CpG dinucleotides in the mammalian genome is attributed to a higher mutation rate of methylated versus unmethylated cytosine residues.

CpG dinucleotides and 5-methylcytosine are unevenly distributed in the genome. Most of the genome is heavily methylated with a corresponding deficit in CpG dinucleotides. About 1 to 2% of the genome consists of islands of non-methylated DNA and these sequences show the expected frequency of CpG dinucleotides.

CpG islands are about 1 kb long and are not only CpG-rich, but generally G/C-rich as well and are found at the 5' end of genes. All known housekeeping genes and some tissue-specific genes have associated CpG islands.

3.4.1 Methylation and Gene Expression

We now want to discuss methylation and gene expression. Reference will be made to the work of Herman and Baylin, Jones and Takai, McCabe et al, Allis et al, and Issa and Kantarjian.

We begin with Herman and Baylin and their description of the diagram below:

In most of the mammalian genome, which is depicted here as exons 1, 2, and 3 of a sample gene, introns of the gene (line between the exons), and regions outside the gene, the CpG dinucleotide has been depleted during evolution, as shown by the small number of such sites (circles).

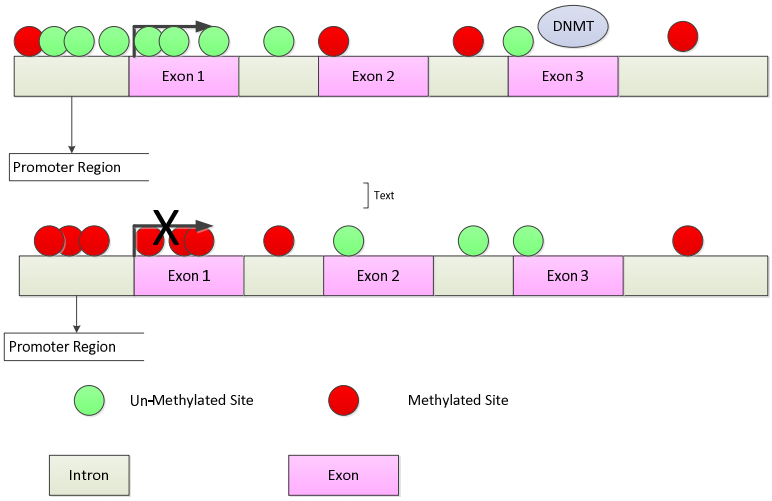
Small regions of DNA, approximately 0.5 to 4.0 kb in size, harbor the expected number of CpG sites and are termed CpG islands. Most of these are associated with promoter regions of approximately half the genes in the genome (numerous circles surrounding and within exon 1 of the sample gene). In normal cells, most CpG sites outside of CpG islands are methylated (black circles), whereas most CpG-island sites in gene promoters are unmethylated (white circles).

This methylated state in the bulk of the genome may help suppress unwanted transcription, whereas the unmethylated state of the CpG islands in gene promoters permits active gene transcription. In cancer cells, the DNA-methylation and chromatin patterns are shifted.

Many CpG sites in the bulk of the genome and in coding regions of genes, which should be methylated, become unmethylated, and a growing list of genes have been identified as having abnormal methylation of promoters containing CpG islands, with associated transcriptional silencing (red X at the transcription start site).

Although there are possible explanations and findings from ongoing investigations, it is not known why the DNA-methylating enzymes fail to methylate where they normally would and which of these enzymes are mediating the abnormal methylation of CpG islands in promoters.

We depict a modified version of their Figure below:



Thus methylation in this case blocks the expression of the targeted gene.

3.4.2 Methylation and Deamination (C to T)

Methylation may also progress to more dramatic changes. We discuss here the change of C to T, a serious change in a DNA base pair which can result in dramatic changes in gene expression.

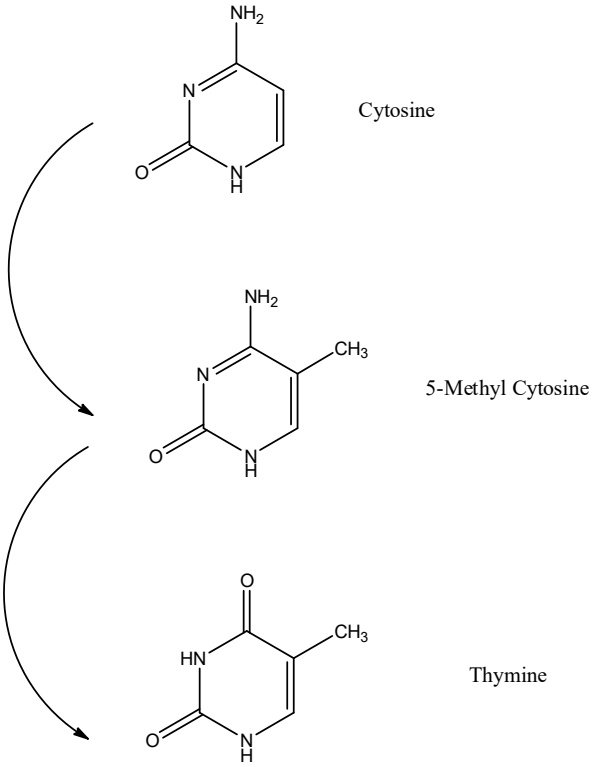
As Herman and Baylin state:

Although only four bases — adenine, guanine, cytosine, and thymine — spell out the primary sequence of DNA, there is a covalent modification of postreplicative DNA (i.e., DNA that has replicated itself in a dividing cell) that produces a "fifth base." Reactions using S -adenosyl-methionine as a methyl donor and catalyzed by enzymes called DNA methyltransferases (DNMTs) add a methyl group to the cytosine ring to form methyl cytosine.

In humans and other mammals, this modification is imposed only on cytosines that precede a guanosine in the DNA sequence (the CpG dinucleotide). The overall frequency of CpGs in the genome is substantially less than what would be mathematically predicted , probably because DNA methylation has progressively depleted the genome of CpG dinucleotides over the course of time.

The mechanism of the depletion is related to the propensity of methylated cytosine to deaminate, thereby forming thymidine. If this mutation is not repaired, a cytosine-to-thymidine change remains.

The depletion of CpG dinucleotides in the genome corresponds directly to sites of such nucleotide transitions, and this change is the most common type of genetic polymorphism (variation) in human populations.



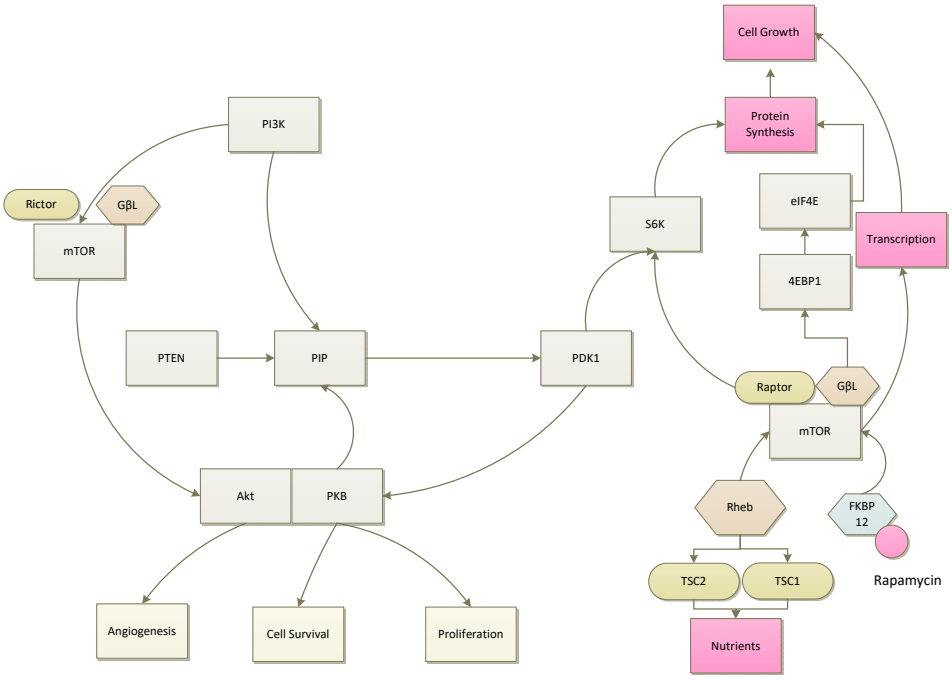
From Robertson (2001) we have some of the genes influenced by methylation or as he states:

CpG-island-associated genes involved in cell growth control or metastasis that can become hypermethylated and silenced in tumors.

We depict the Table below from Robertson on some of the genes impacted by this type of methylation. Most of these are significant regulatory genes.

Gene	*Function*
pRb	Regulator of G1/S phase transition
$p16^{INK4a}$	Cyclin-dependent kinase inhibitor
$p15^{INK4b}$	Cyclin-dependent kinase inhibitor
ARF	Regulator of p53 levels
hMLH1	DNA mismatch repair
APC	Binds β-catenin, Regulation of actin cytoskeleton?
VHL	Stimulates angiogenesis
BRCA1	DNA repair
LKB1	Serine/threonine protein kinase
E-cadherin	Cell-cell adhesion
ER	Transcriptional activation of estrogen-responsive genes
GSTPI	Protects DNA from oxygen radical damage
0^6-MGMT	Repair/removal of bulky adducts from guanine
TIMP3	Matrix metalloproteinase inhibitor
DAPK1	Kinase required for induction of apoptosis by y interferon
p73	Apoptosis structurally similar to p53

For example we show below some typical pathways and the above genes are seen targeted by methylation.



Methylation may then interfere with many of the genes in the above pathways.

3.4.3 Causes of Methylation

The major question which is often asked is what causes methylation. In Allis et al on p 460 the authors discuss some of the putative cause of methylation and methylation related cancers. Although not confirmative it is consistent with clinical correlations as well.

As Issa and Kartarjian state:

Much remains to be learned about the causes of DNA methylation abnormalities in cancer; for the most part, methylation seems to be gene specific. In some cases, a rare methylation event appears in cancer because of selection , while in others methylation anomalies are downstream of an oncogenic event …

As McCabe et al state:

DNA methylation patterns in human cancer cells are considerably distorted. Typically, cancer cells exhibit hypomethylation of intergenic regions that normally comprise the majority of a cell's methyl-cytosine content . Consequently, transposable elements may become active and contribute to the genomic instability observed in cancer cells.

Simultaneously, cancer cells exhibit hypermethylation within the promoter regions of many CpG island-associated tumor suppressor genes, such as the retinoblastoma gene (RB1), glutatione S-transferase pi (GSTP1), and E-cadherin (CDH1). As a result, these regulatory genes are transcriptionally silenced resulting in a loss-of-function. Thus, through the effects of both hypo- and hyper-methylation, DNA methylation significantly affects the genomic landscape of cancer cells, potentially to an even greater extent than coding region mutations, which are relatively rare

McCabe et al continue:

Although the precise molecular mechanisms underlying the establishment of aberrant DNA hypermethylation remain elusive, recent studies have identified some contributing etiologic factors.

For example, chronic exposure of human bronchial epithelial cells to ***tobacco-derived carcinogens drives hypermethylation*** *of several tumor suppressor genes including CDH1 and RASSF2A.*

Stable knockdown of DNMT1 prior to carcinogen exposure prevented methylation of several of these genes indicating a necessary role for this enzyme in the molecular mechanism underlying hypermethylation.

The reactive oxygen species (ROS) associated with chronic inflammation is another source of DNA damage with the potential to affect DNA methylation as halogenated pyrimidines, one form

of ROS-induced damage, mimic 5-methylcytosine and stimulate DNMT1-mediated CpG methylation in vitro and in vivo.

Indeed, study of the glutatione peroxidase 1 and 2 double knockout model of inflammatory bowel disease found that 60% of genes that are hypermethylated in colon cancers also exhibit aberrant methylation in the inflamed noncancerous precursor tissues. Although the mechanisms by which DNA damage mediates DNA methylation are not fully understood, O'Hagan and colleagues have examined the process with an engineered cell culture model in which a unique restriction site was incorporated into the CpG island of the E-cadherin promoter.

Thus the actual molecular mechanics leading to methylation are not fully understood but like most cancers inflammation appears to be a driving factor. What the cause of that inflammation may be is not yet clear.

3.4.4 Methylation Effects on DNA

As is stated in the paper by Miranda and Jones:

DNA methylation is a covalent modification in which the 5₀position of cytosine is methylated in a reaction catalyzed by DNA methyltransferases (DNMTs) with S-adenosyl-methionine as the methyl donor.

In mammals, this modification occurs at CpG dinucleotides and can be catalyzed by three different enzymes, DNMT1, DMNT3a, and DNMT3b.DNAmethylation plays a role in the long-term silencing of transcription and in heterochromatin formation.

As an epigenetic modification, DNA methylation permits these silenced states to be inherited throughout cellular divisions.

We continue with the discussion in Mirand and Jones as follows:

Silencing of genetic elements can be successfully initiated and retained by histone modifications and chromatin structure. However, these modifications are easily reversible making them make poor gatekeepers for long-term silencing. Therefore, mammalian cells must possess an additional mechanism for prolong silencing of these sequences. An important component of this process is DNA methylation. DNA methylation is a stable modification that is inherited throughout cellular divisions.

When found within promoters, DNA methylation prevents the reactivation of silent genes, even when the repressive histone marks are reversed. This allows the daughter cells to retain the same expression pattern as the precursor cells and is important for many cellular processes including the silencing of repetitive elements, X-inactivation, imprinting, and development.

We now present a key Figure from Miranda and Joner regarding the methylated reading of DNA. They state regarding the Figure below:

Chromatin structure of CpG islands and CpG poor regions in healthy cells and during cancer. In healthy cells, CpG islands are generally hypomethylated. This allows for an open chromatin structure. However, the CpG poor regions found in repetitive elements within the intergenic and intronic regions of the genome are methylated and thereby maintain a closed chromatin structure. In cancer and on the inactive X chromosome many CpG islands become methylated, forcing these regions into a closed chromatin structure.

When CpG islands located within promoters are methylated, the corresponding genes are persistently silenced. In contrast, the CpG poor regions become hypomethylated allowing for an open chromatin structure.

As Robertson states:

It is now clear that the genome contains information in two forms, genetic and epigenetic. The genetic information provides the blueprint for the manufacture of all the proteins necessary to create a living thing while the epigenetic information provides instructions on how, where, and when the genetic information should be used.

Ensuring that genes are turned on at the proper time is as important as ensuring that they are turned off when not needed.

The major form of epigenetic information in mammalian cells is DNA methylation, or the covalent addition of a methyl group to the 5-position of cytosine predominantly within the CpG dinucleotide. DNA methylation has profound effects on the mammalian genome.

Some of these effects include transcriptional repression, chromatin structure modulation, X chromosome inactivation, genomic imprinting, and the suppression of the detrimental effects of repetitive and parasitic DNA sequences on genome integrity.

Robertson then proceeds to detail the genes impacted by hypermethylation. We summarize them below:

Gene	*Function*
pRb	Regulator of G1/S phase transition
p16 INK4a	Cyclin-dependent kinase inhibitor
p15 INK4b	Cyclin-dependent kinase inhibitor
ARF	Regulator of p53 levels
hMLH1	DNA mismatch repair
APC	Binds b-catenin, Regulation of actin cyto-skeleton?
VHL	Stimulates angiogenesis
BRCA1	DNA repair
LKB1	Serine/threonine protein kinase
E-cadherin	Cell ± cell adhesion
ER	Transcriptional activation of estrogen-responsive genes
GSTP1	Protects DNA from oxygen radical damage
O6-MGMT	Repair/removal of bulky adducts from guanine
TIMP3	Matrix metallo proteinase inhibitor
DAPK1	Kinase required for induction of apoptosis by g interferon
p73	Apoptosis?, structurally similar to p53

Regarding PIN, the one which is most concern is the GSTP1 gene and its suppression allowing for DNA damage from inflammation and oxygenation damage.

In the context of cancer generation and progression, the epigenetic effect of hyper and hypo methylation is best described by Esteller:

The low level of DNA methylation in tumors as compared with the level of DNA methylation in their normal-tissue counterparts was one of the first epigenetic alterations to be found in human cancer.

The loss of methylation is mainly due to hypomethylation of repetitive DNA sequences and demethylation of coding regions and introns — regions of DNA that allow alternative versions of the messenger RNA (mRNA) that are transcribed from a gene. A recent large-scale study of DNA methylation with the use of genomic microarrays has detected extensive hypo-methylated genomic regions in gene-poor areas.

During the development of a neoplasm, the degree of hypomethylation of genomic DNA increases as the lesion progresses from a benign proliferation of cells to an invasive cancer.

Three mechanisms have been proposed to ex-plain the contribution of DNA hypomethylation to the development of a cancer cell:

(i) generation of chromosomal instability,

(ii) reactivation of transposable elements, and

(iii) loss of imprinting.

Under methylation of DNA can favor mitotic recombination, leading to deletions and translocations, and it can also promote chromosomal rearrangements. This mechanism was seen in experiments in which the depletion of DNA methylation by the disruption of DNMTs caused

aneuploidy. Hypomethylation of DNA in malignant cells can reactivate intra-genomic endo-parasitic DNA.

3.4.5 Hypomethylation

As Laird and Jaenisch state:

Hypomethylation: Reduced levels of global DNA methylation have been reported for a variety of malignancies in the past decade. Gama Sosa and coworkers found that in a wide variety of tumors, hypomethylation not only correlated with transformation, but also with tumor progression . In their analysis, only 7% of 43 normal tissues had a 5-methylcytosine content below 0.8 mol%, whereas 10% of 21 benign tumors, 27% of 62 primary malignancies and 60% of 20 secondary malignancies had a 5-methylcytosine content below 0.8 mol%.

On the other hand, Feinberg and coworkers did not find a further reduction in DNA methylation levels in the progression from benign to malignant colonic neoplasia, suggesting an early role for DNA hypomethylation in colorectal cancer

3.4.6 Hypermethylation

As again with Laird and Jaenisch we have:

Hypermethylation: There have also been many reports of regional increases in DNA methylation levels. Baylin and coworkers have found regional hotspots for hypermethylation on chromosomes 3p, 11p and 17p in a variety of human tumors. These include CpG island areas that are normally never methylated in vivo, but are found to be methylated in tumor tissues. This is reminiscent of the changes that occur at CpG islands at non-essential genes in tissue culture. Baylin's group has dissected the sequential order of hypermethylation events in an in vitro model for lung tumor progression.

There is evidence for inactivation of tumor-suppressor gene function through hypermethylation of the Rb gene in sporadic retinoblastoma. Transient transfection experiments showed that specific hypermethylation in the promoter region of Rb could reduce expression to 8% of an unmethylated control. It is possible, therefore, that hypermethylation of tumor-suppressor genes leading to gene inactivation results in a selective growth advantage of the transformed cells.

3.5 MIRNAS

miRNAs can play a dramatic role in the expression of a variety of genes. In examining PA we have found that many miRNAs and similar non coding RNAs can have similar drastic effects. Targeting these is often problematic but understanding them is critical. We now provides several current examples.

From Chen et al:

Brain tumors are common solid pediatric malignancies and the main reason for cancer-related death in the pediatric setting. Recently, evidence has revealed that noncoding RNAs (ncRNAs), including microRNAs (miRNAs), long ncRNAs (lncRNAs), and circular RNAs (circRNAs), play a critical role in brain tumor development and progression.

Therefore, in this review article, we describe the functions and molecular mechanisms of ncRNAs in multiple types of cancer, including medulloblastoma, pilocytic astrocytoma, ependymoma, atypical teratoid/rhabdoid tumor, glioblastoma, diffuse intrinsic pontine glioma, and craniopharyngioma.

We also mention the limitations of using ncRNAs as therapeutic targets because of the nonspecificity of ncRNA targets and the delivery methods of ncRNAs. Due to the critical role of ncRNAs in brain oncogenesis, targeting aberrantly expressed ncRNAs might be an effective strategy to improve the outcomes of pediatric patients with brain tumors …

Compelling evidence has identified a number of aberrantly expressed miRNAs in PAs, which has aided the discovery of novel diagnostic methods and effective treatments for this type of tumor. A survey of miRNA expression demonstrated that miR-142-5p and miR-25 were significantly upregulated in PAs compared to normal tissue, while miR-129 was strongly downregulated. Compared to those in other CNS pediatric tumors (ATRT, EPN, MB, and glioblastoma), multiple miRNAs, including miR-93 and miR-106b, were observed to be downregulated, whereas several miRNAs, such as miR-432 and miR-34a, were found to be upregulated in PSs.71

Dysregulated expression levels of a subset of miRNAs, including decreased expression of miR-129 and miR-124 and overexpression of miR-21, were also observed in PAs. In addition, miR-650 and miR-1276 levels were increased, while miR-744 and miR-187* levels were decreased, in NF1-associated tumors among the PA subgroups.*

Similarly, miR-15 and miR-24-1 levels were reported to be decreased in PAs.73 Jones et al.74 also found that the Xq26.3 cluster, miR-224, miR-146a, miR-34a, and the miR-106a, miR-363 cluster were upregulated, while miR-124, miR-129, and miR-218 were downregulated.

Predicted targets of differentially regulated miRNAs frequently include components of the extracellular signal-regulated kinase (ERK)/ MAPK and nuclear factor kB (NF-kB) signaling pathways.

Another study identified 88 miRNAs that were expressed to different degrees between PA and cerebral white matter samples.

PA samples had the most downregulated miRNAs regulating classical pathways of tumorigenesis, while the most overexpressed miRNAs were associated with pathways such as focal adhesion, the p53 signaling pathway, and gliomagenesis.

High expression of miR-34a-5p and miR-144-3p and low expression of miR-630 and miR-139-3p were further confirmed by qRT-PCR.75 … also demonstrated that miR-125 family members were downregulated in PAs compared to nonneoplastic brain, and overexpression of

miR-125b in pediatric low-grade glioma decreased cell growth and invasion and induced apoptosis.

Furthermore, one study analyzed the expression of lncRNA HOTAIR in five pediatric tumor types and found higher expression of this gene in juvenile PAs. MBs, as embryonal tumors of the cerebellum, account for approximately 20% of the total brain tumors in this patient population.

Advances in treatment with neurosurgery, radiation therapy, and high-dose chemotherapy have significantly improved the survival rate of these patients. However, long-term sequelae, including neurocognitive, neuroendocrine, and psychosocial deficits caused by intensive therapies administered to the developing brain, remain challenging. Therefore, more effective molecular-targeted strategies with less toxicity are urgently needed to be developed for this disease

From Jones et al:

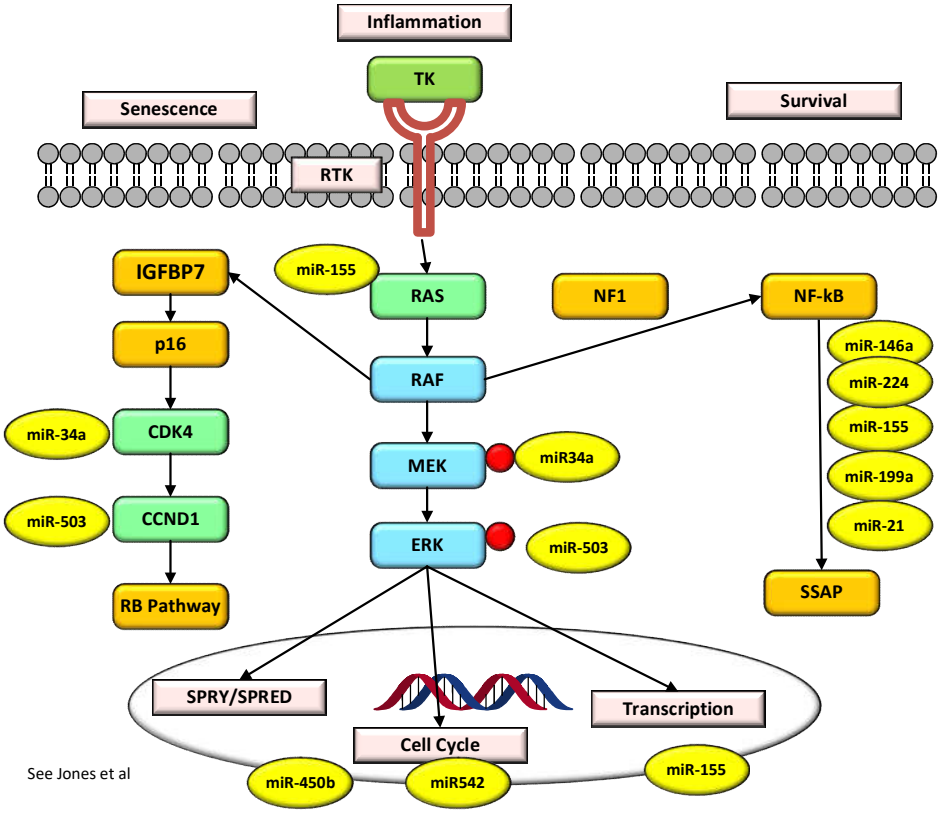
Pilocytic astrocytomas (WHO grade I) are the most common central nervous system tumors in the 5 to 19 year age group.

They are cystic, well-circumscribed tumors, which rarely progress and can usually be removed using surgery. This accounts for their having a more favourable prognosis than diffuse and other infiltrative astrocytomas. Molecular studies on pilocytic astrocytomas have identified recurrent BRAF gene fusions and other alterations that activate the ERK/ MAPK signal transduction pathway. Activated ERK/MAPK signaling is believed to drive cellular proliferation and then to trigger senescence, giving rise to the indolent phenotype of pilocytic astrocytomas.

MicroRNAs are short RNA molecules that modulate gene expression post-transcriptionally by repressing translation or degrading the mRNA transcript. They regulate numerous biological processes including cell proliferation and differentiation, and have major roles in embryogenesis, including brain and spinal cord development and neurogenesis. Many microRNAs are involved in the initiation and progression of cancer, and recent studies of pediatric brain tumors have identified tumor-specific microRNA signatures. To further our knowledge of the molecular drivers of pilocytic astrocytomas, we conducted a detailed investigation of microRNA and gene expression in these tumors, and characterized the findings by pathway analysis.

Our results revealed a distinctive microRNA and gene expression profile in pilocytic astrocytoma compared to other pediatric brain tumors.

We found that many predicted targets of up-regulated microRNAs in pilocytic astrocytomas are known regulators of the ERK/MAPK and NF-κB pathways. These findings suggest an important modulatory role for microRNAs in critical pathways involved in pilocytic astrocytomas.



Yuan et al note:

High resolution platforms have uncovered recurrent alterations in these tumors, i.e. ***BRAF-KIAA1549 fusions as the most frequent recurrent alteration in pilocytic astrocytoma (PA)****, which represents the most frequent subtype of* ***PLGG. BRAF-KIAA1549 fusions lead to mitogen-activated protein kinase (MAPK)****.*

Interestingly, comprehensive sequencing studies reported genetic alterations in MAPK components in essentially all (100%) of PA. PLGG also have heterogeneous histologies, including neoplasms with infiltrating astrocytic or oligodendroglial components, which may have a variety of alterations in alternative drivers (FGFR1, MYB, MYBL1).

The role of non-coding RNAs, particularly microRNAs, is an active field of study in normal physiology and a variety of disorders, including cancer.

MicroRNA upregulation may target tumor suppressors, while downregulation of microRNAs may result in increased levels of oncoproteins.

In the field of glioma, alterations in the expression of numerous microRNAs have been described, and they may play a role in every known process associated with glioma biology (reviewed in9). PLGG in particular are an attractive subject for microRNA profiling, given their relative lack of genomic instability in contrast to high grade tumors.

Most microRNA profiling studies in gliomas have been performed in adults, particularly high grade and diffuse subsets such as glioblastoma, where they appear to mediate a variety of important tumor properties (reviewed in9).

Conversely, studies of microRNA expression in circumscribed and low grade pediatric gliomas are relatively scant.

When focusing on pediatric glioblastoma, and comparing expression levels with non-neoplastic brains, studies have documented relative under-expression of miR-1224, miR-204, miR-874, miR-1296, miR-889, miR-495, miR-34c, miR-10b, miR-125a, miR-10a, while miR-617, miR-200a, miR769-3, miR-584, and miR-527 are overexpressed.

3.6 GROWTH FACTORS

We discussed general growth factors earlier. Here we focus on those related to PA. As Li et al note :

Astrocytes are critical for the development and function of the central nervous system. In developing brains, immature astrocytes undergo morphological, molecular, cellular, and functional changes as they mature.

Although the mechanisms that regulate the maturation of other major cell types in the central nervous system such as neurons and oligodendrocytes have been extensively studied, little is known about the cellular and molecular mechanisms that control astrocyte maturation.

*Here, we **identified molecular markers of astrocyte maturation and established** an in vitro assay for studying the mechanisms of astrocyte maturation. Maturing astrocytes in vitro exhibit similar molecular changes and represent multiple molecular subtypes of astrocytes found in vivo. Using this system, we found that astrocyte-to-astrocyte contact strongly promotes astrocyte maturation.*

***In addition, secreted signals from microglia, oligodendrocyte precursor cells, or endothelial cells affect a small subset of astrocyte genes but do not consistently change astrocyte maturation. To identify molecular mechanisms underlying astrocyte maturation, we treated maturing astrocytes with molecules that affect the function of tumor-associated genes**.*

We found that a positive feedback loop of heparinbinding epidermal growth factor-like growth factor (HBEGF) and epidermal growth factor receptor (EGFR) signaling regulates astrocytes maturation. Furthermore, HBEGF, EGFR, and tumor protein 53 (TP53) affect the expression of genes …

The tumor-associated genes EGFR and TP53 promote and inhibit the proliferation and/or survival of immature astrocytes, respectively As astrocytes mature, they slow down and eventually stop proliferating in.

Tumor-associated genes that affect the cell cycle may therefore affect astrocyte maturation. Mouse, rat, and human astrocytes all highly express the tumor-associated genes EGFR and TP53. We therefore tested the roles of EGFR and TP53 in the proliferation of immature astrocytes. We infected cultured mouse astrocytes with lentiviruses encoding Cas9-EGFP and sgRNA-mCherry targeting EGFR and TP53. EGFP and mCherry double positive cells were knockout cells and other cells were wild type cells. The changes of the percentage of knockout cells in the entire population over time reflect the proliferation and/or survival of knockout cells compared to wild type cells.

We analyzed the percentage of knockout cells at 7, 14, and 21 days after infection. We found that EGFR sgRNA/Cas9 double positive knockout cells decreased over time whereas TP53 sgRNA/Cas9 double positive knockout cells increased over time in the astrocyte population.

Therefore, EGFR promotes and TP53 inhibits the proliferation and/or survival of immature astrocytes, respectively

4 BLOOD BRAIN BARRIER

Chabner et al note the impact of the blood brain barrier in CNS chemotherapy:

Three main factors influence the extent to which a systemically administered anticancer agent distributes into the brain and brain tumors:

(a) the plasma concentration-time profile of the drug,

(b) regional blood flow, and

(c) transport of the agent through the BBB and blood-tumor barrier (BTB).

The two former considerations are common to all solid tumors, whereas the latter is specific to brain tumors. Ehrlich was the first to propose the concept of the BBB at the end of the nineteenth century. On administering the dye trypan blue to rats by intravenous injection, he observed that all body organs were stained except for the brain and spinal cord.

The anatomic basis of the BBB was determined decades ago with the introduction of the electron microscope. It results from modification of the normal vascular endothelium whereby a sheet of cells is connected by a network of proteins (tight junctions) on a basement membrane.

The BBB has a number of important roles, including maintaining a constant biochemical content of the interstitial milieu and protecting the brain from toxic molecules (including xenobiotics). Low hydraulic conductance, low ionic permeability, and high electrical resistance make it difficult for hydrophilic nonelectrolytes to penetrate the BBB in the absence of a membrane carrier. These properties, together with the lack of intracellular fenestrations and pinocytotic vesicles and the presence of a thicker basal lamina, create a physiologic barrier that is relatively impermeable to many water-soluble compounds…

Some drugs utilize specific transport mechanisms present in the endothelial cell to traverse the BBB. However, most cytotoxic drugs that gain access to the CNS cross the BBB by passive diffusion. Aside from pharmacokinetic properties, the main factors that influence the extent to which these compounds distribute into the CNS include lipid solubility, molecular mass, charge, and plasma protein binding. Specifically, small organic compounds with a molecular weight less than 200 that are lipid soluble, neutral at physiologic pH, and not highly bound to plasma proteins readily cross the BBB.

4.1 BLOOD PATHS

From Daneman and Prat:

There are three main structural classes of capillaries.

1. *Continuous nonfenestrated capillaries of the skin and lung are joined together by cellular junctions, have a complete basement membrane (BM), and lack fenestra (pores) in their plasma membrane.*

2. *Continuous fenestrated vessels of the intestinal villi and endocrine glands have a similar continuous structure but contain diaphragmed fenestra throughout their membrane.*

3. *Discontinuous capillaries in the liver have large gaps throughout the cell and have an incomplete BM.*

These classes of capillaries differ greatly in their regulation of movement of solutes between the blood and the tissues, with continuous fenestrated capillaries being the most restrictive, and discontinuous being the least restrictive

4.2 ASTROCYTES

From Daneman and Prat:

Astrocytes are a major glial cell type, which extend polarized cellular processes that ensheath either neuronal processes or blood vessels.

The endfeet of the basal process almost completely ensheath the vascular tube, and contain a discrete array of proteins including dystroclycan, dystrophin, and aquaporin 4. The dysroglycan–dystrophin complex is important to link the endfeet cytoskeleton to the BM by binding agrin.

This linkage coordinates aquaporin 4 into orthogonal arrays of particles, which is critical for regulating water homesostasis in the CNS. Astrocytes provide a cellular link between the neuronal circuitry and blood vessels. This neurovascular coupling enables astrocytes to relay signals that regulate blood flow in response to neuronal. This includes regulating the contraction/dilation of vascular smooth muscle cells surrounding arterioles as well as PCs surrounding capillaries.

Astrocytes have been identified as important mediators of BBB formation and function because of the ability of purified astrocytes to induce barrier properties in non-CNS blood vessels in transplantation studies, as well as induce barrier properties in cultured ECs in in vitro coculture paradigms.

One issue with these studies is that the astrocytes are often cultured from neonatal rodent brains and go through many rounds of cell division, suggesting that these studies are analyzing progenitor cells as opposed to mature astrocytes.

Recent data analyzing the BBB in dissected rodent embryos suggest that the BBB is formed before astrocyte generation and ensheathment of the vasculature, and, thus, these cells do not play a role in the initial induction of the BBB. The identification of astrocytesecreted factors that

do regulate BBB function suggests that mature astrocytes modulate and maintain the barrier once it is formed.

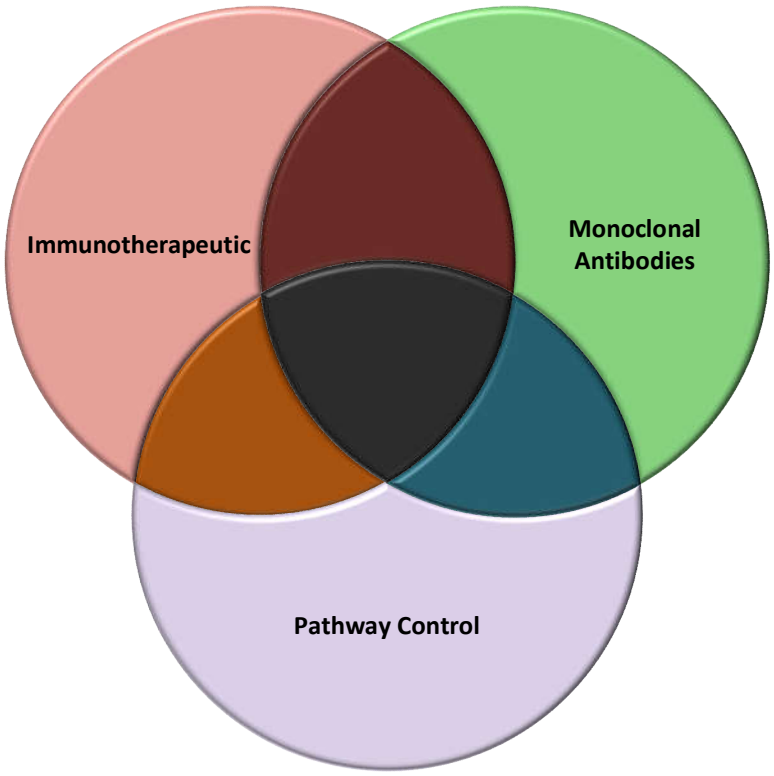
5 THERAPEUTICS

The therapeutic options can be characterized in three classes:

Immunotherapeutic: These are the type whereby the patient's immune system is activated and allowed to target the malignant cells. Fundamentally this requires two steps. First a cell target which can be activated in a T cell, or sometimes in an NK cell. Second a suppression mechanism that allows for suppressing "self" recognition on the surface of the targeted malignant cell. Thus suppression the self-protection allowing the immune system to attack the malignant cell.

Monoclonal antibody (MAb) targeting: This approach uses MAb that target unique malignant cell surface proteins and then can be used to deliver a chemotherapeutic molecule to kill the targeted cell. A second mechanism is to target growth factors which may be over activated thus blocking activation. In either case the MAb requires a known surface protein that can be targeted by a MAb.

Pathway control: Many malignant cells have aberrant internal pathways. The pathways may result in loss of apoptosis or the excess growth and proliferation or both. Blocking a key step in an overactivated pathway may reduce or eliminate proliferation and thus spread.



5.1 STANDARD

Standard therapeutics is either chemotherapy or radiation therapy or even both. However there is the question of overall survival.

As Parsons et al note :

The aim of this study was to understand the use of chemotherapy (CMT) and radiotherapy (RT) in pilocytic astrocytoma (PA) and their impact on overall survival (OS).

Data from the National Cancer Database (NCDB) for patients with non-metastatic WHO grade I PA from 2004 to 2014 were analyzed. Pearson's chi-squared test and multivariate logistic regression analyses were performed to assess the distribution of demographic, clinical, and treatment factors. Inverse probability of treatment weighting (IPTW) was used to account for differences in baseline characteristics. Kaplan–Meier analyses and doubly-robust estimation with multivariate Cox proportional hazards modeling were used to analyze OS.

Of 3865 patients analyzed, 294 received CMT (7.6%), 233 received RT (6.0%), and 42 (1.1%) received both.

On multivariate analyses, decreasing extent of surgical resection was associated with receipt of both CMT and RT.

Brainstem tumors were associated with RT, optic nerve tumors were associated with CMT. Cerebellar tumors were inversely associated with both CMT and RT. Younger age was associated with receipt of CMT; conversely, older age was associated with receipt of RT. After IPTW, receipt of CMT and/or RT were associated with an OS decrement compared with matched patients treated with surgery alone or observation (HR 3.29, $p < 0.01$).

This is the largest study to date to examine patterns of care and resultant OS outcomes in PA. We identified patient characteristics associated with receipt of CMT and RT.

After propensity score matching, receipt of CMT and/or RT was associated with decreased OS.

5.1.1 Chemotherapy

Chemotherapy is one of the oldest forms of cancer treatment. It simply uses toxic drugs to stop cell proliferation and to kill off certain cells. It is generally highly toxic with adverse reactions such as hair loss and similar ancillary effects.

5.1.2 Radiation Therapy

As NCI notes[13]:

[13] https://www.cancer.gov/types/brain/patient/child-astrocytoma-treament-pdq

Radiation therapy is a cancer treatment that uses high-energy x-rays or other types of radiation to kill cancer cells or keep them from growing. External radiation therapy uses a machine outside the body to send radiation toward the area of the body with cancer.

Certain ways of giving radiation therapy can help keep radiation from damaging nearby healthy tissue. These types of radiation therapy include the following:

Conformal radiation therapy: Conformal radiation therapy is a type of external radiation therapy that uses a computer to make a 3-dimensional (3-D) picture of the tumor and shapes the radiation beams to fit the tumor.

Intensity-modulated radiation therapy (IMRT): IMRT is a type of 3-dimensional (3-D) external radiation therapy that uses a computer to make pictures of the size and shape of the tumor. Thin beams of radiation of different intensities (strengths) are aimed at the tumor from many angles.

Stereotactic radiation therapy: Stereotactic radiation therapy is a type of external radiation therapy. A rigid head frame is attached to the skull to keep the head still during the radiation treatment. A machine aims radiation directly at the tumor. The total dose of radiation is divided into several smaller doses given over several days. This procedure is also called stereotactic external-beam radiation therapy and stereotaxic radiation therapy.

Proton beam radiation therapy: Proton beam radiation therapy is a type of high-energy, external radiation therapy that uses streams of protons (tiny particles with a positive charge) to kill tumor cells. This type of treatment can lower the amount of radiation damage to healthy tissue near a tumor.

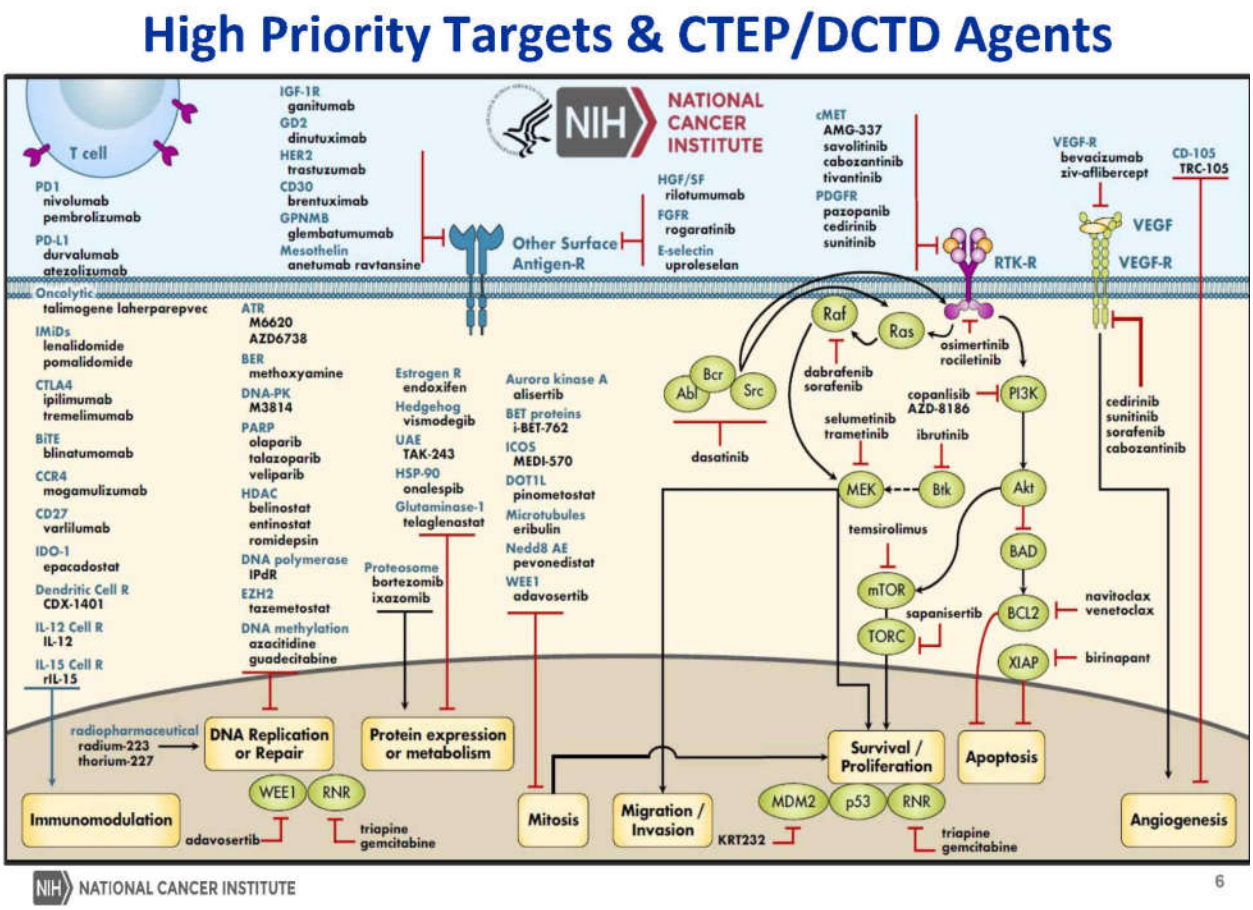
The way the radiation therapy is given depends on the type of tumor and where the tumor formed in the brain or spinal cord. External radiation therapy is used to treat childhood astrocytomas.

From Chmielowski:

PAs are not infiltrative or histologically progressive and, therefore, can be cured by surgical excision. Subtotally resected tumors may be observed or rarely require immediate focal irradiation. Nonresectable tumors (e.g., optic gliomas) may also be followed or can be treated with RT (5,400 cGy, focal fields) or, in very young patients, with chemotherapy if symptoms dictate the need for immediate treatment.

PAs respond to nitrosoureas, procarbazine, cyclophosphamide, vincristine, platinum compounds, and etoposide.

A BRAF inhibitor, such as vemurafenib, can be used to treat PAs with BRAF V600E mutation; however, it should be avoided in patients with BRAF fusion mutation.



From Gross et al:

Neurofibromatosis type 1, an auto somal dominant genetic disorder char acterized by multiple progressive tumor- - and nontumor manifestations, has limited treatment options.1 In patients with the disorder, dysfunction of the guanosine triphosphatase– activating protein neurofibromin leads to overactivation of the RAS pathway.

Therefore, targeted inhibition of the RAS pathway with mitogenactivated protein kinase (MAPK) kinase (MEK) inhibition is a logical treatment approach3 that has been successful in a preclinical model of neurofibromatosis type Plexiform neurofibromas are histologically benign peripheral-nerve sheath tumors that occur in up to 50% of persons with neurofibromatosis type 15,6 and can cause substantial complications

From NCI[14]:

Children with NF1 have an increased propensity to develop WHO grade I and grade II astrocytomas in the visual (optic) pathway; up to 20% of patients with NF1 will develop an optic

[14] https://www.cancer.gov/types/brain/hp/child-astrocytoma-treament-pdq

pathway glioma. In these patients, the tumor may be found on screening evaluations when the child is asymptomatic or has apparent static neurologic and/or visual deficits. Pathological confirmation is frequently not obtained in asymptomatic patients; when biopsies have been performed, these tumors have been found to be predominantly pilocytic (grade I) rather than diffuse astrocytic tumors.[2,5,15] In general, treatment is not required for incidental tumors found with surveillance neuroimaging. Symptomatic lesions, often causing vision impairment, or those that have radiographically progressed may require treatment.[16]

From NCBI[15], NF1 is described as follows::

This gene product appears to function as a negative regulator of the ras signal transduction pathway. Mutations in this gene have been linked to neurofibromatosis type 1, juvenile myelomonocytic leukemia and Watson syndrome. The mRNA for this gene is subject to RNA editing (CGA>UGA->Arg1306Term) resulting in premature translation termination. Alternatively spliced transcript variants encoding different isoforms have also been described for this gene.

5.2 IMMUNOTHERAPEUTICS

Immunotherapy consists of activating the individuals own immune system to attack and eliminate specific cells identified by unique surface markers.

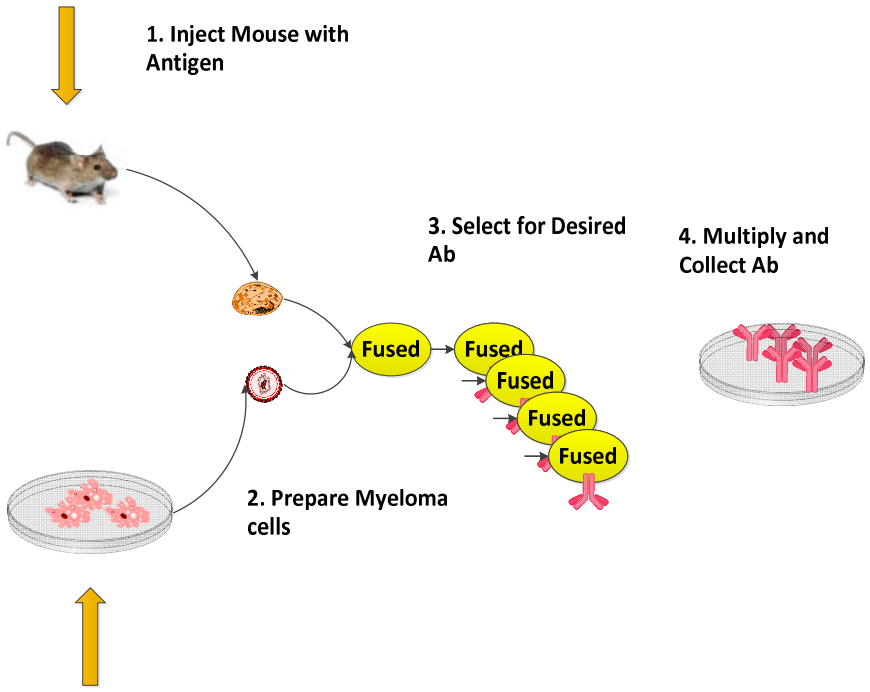
5.3 MONOCLONAL ANTIBODIES

Antibodies are a set of molecules produced by a B cell in response to antigens. They are the principal element of the adaptive immune system. When activated, they are produced in numbers and proceed to attack cells which are perceived a threat and establish multiple paths to the invaders destruction.

Antibodies can be made for specific antigens and antibodies have flexibility in their own lifetimes. We briefly discuss them here and leave detailed analysis to the literature. However, we use this as a way to introduce monoclonal antibodies, specifically because of their use in many immunotherapeutic applications.

Monoclonal Ab can be made in what is comparatively a simple process. Namely we take a mouse, inject it with an antigen, then fuse the B cells from the mouse with Myeloma cells and then allow them to grow. They will produce Ab and one of these will be the one from the antigen, Ag. Then select that and we have a collection of these Ab. It may sound a bit simple but it is quite complex. We will detail this in the next chapter. But recall this is a murine Ab, not a human.

[15] https://www.ncbi.nlm.nih.gov/gene/4763



We then have to convert a murine to all human.

Monoclonal antibodies, Mabs, have been available for decades[16]. Initially they were murine in development but over the past decade we have seen the development of hundreds of new Mabs for a variety of disorders. We will examine them here but we will do so in a manner which constructs another approach to the engineering of immune systems.

Mab development has progressed from mouse models to genetically engineered human analog. It is now possible to accurately design a fully human Ab for use in therapeutic applications. Details are provided in such works as those by Steinitz.

From Steinitz we have:

Human antibodies are elicited in response to invading substances (antigens) by B cells. The antigen(s) could be a part of an invading microbe, nonself-cells, or mutated/altered self-cells such as cancer cells.

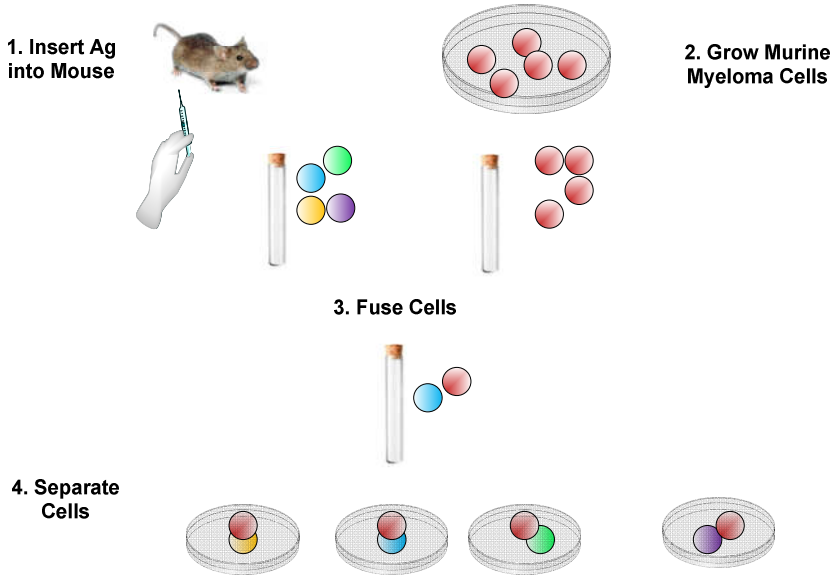
For a complete immune response various immune cells, in addition to B cells, function together to activate the overall immune system. As a result of the immune response B cells produce antibodies that are specific to an antigen or part (epitope) of an antigen. Antibodies by

[16] See Nature Immunology for some historical context. http://www.nature.com/milestones/mileantibodies/Milestones_Poster.pdf and http://www.nature.com/milestones/mileantibodies/collection/index.html Also see Marks, L, The Lock and Key of Medicine: Monoclonal Antibodies and the Transformation of Healthcare, Yale University Press; 1 edition (June 30, 2015).

themselves can destroy or inactivate cells and neutralize substances via a number of mechanisms mediated by nonbinding regions of the antibody.

These mechanisms may require complement and other immune cells, such as NK cells. Because of therapeutic and diagnostic applications of antibodies in human health (control of infectious diseases, autoimmunity, cancer, and other human ailments), they have played a central role in investigative efforts to exploit them to their fullest extent. The first mAbs, of murine origin, were developed more than 35 years ago, as an unlimited source of a single specificity.

However, once in the clinic the xenogeneic nature of the murine mAb resulted in a human anti-murine antibody (HAMA) response in patients that negated the effects of the therapy. Due to these unwanted HAMA responses, various modifications of mAbs to reduce or eliminate the undesired side effects in human were developed which led to the development of chimerized, humanized, and totally human versions. In addition, innovative in vivo diagnostic and therapeutic applications led to modifications of antibody size and enhancement of their biological activities



Monoclonal antibodies are created by injecting human cancer cells, or proteins from cancer cells, into mice. The mouse immune systems respond by creating antibodies against these foreign antigens. The murine cells producing the antibodies are then removed and fused with laboratory-grown cells to create hybrid cells called hybridomas. Hybridomas can indefinitely produce large quantities of these pure antibodies. Monoclonal antibodies can be developed to act against cell growth factors, thus blocking cancer cell growth. Monoclonal antibodies can be conjugated or linked to anticancer drugs, radioisotopes, other biologic response modifiers, or other toxins. When the antibodies bind with antigen-bearing cells, they deliver their load of toxin directly to the tumor. Monoclonal antibodies may also be used to preferentially select normal stem cells from bone marrow or blood in preparation for a hematopoietic stem cell transplant in patients with cancer. Monoclonal antibodies achieve their therapeutic effect through multiple direct and indirect mechanisms

1. Can have direct effects in producing apoptosis or programmed cell death.

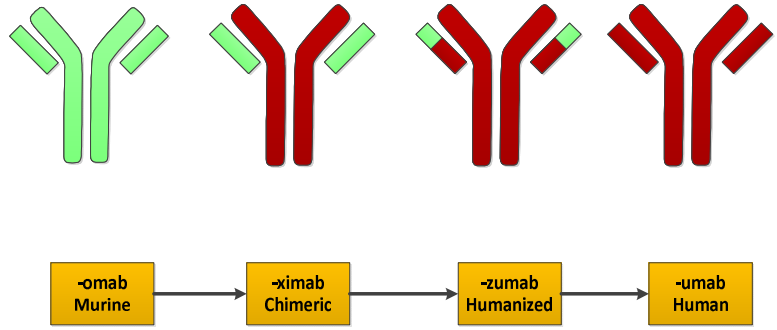
2. Can block growth factor receptors, effectively arresting proliferation of tumor cells.

3. Can bring about anti-idiotype antibody formation in cells that express monoclonal antibodies.

4. Recruiting cells that have cytotoxicity, such as monocytes and macrophages. This type of antibody-mediated cell kill is called antibody-dependent cell mediated cytotoxicity (ADCC),

5. Also bind complement, leading to direct cell toxicity, known as complement dependent cytotoxicity (CDC).

There is an evolution of Mab applications from those which were fully mouse generated which are murine to those fully human. The collection is shown below. Namely we have a murine, chimeric, humanized and human. Recall that the binding to the Ab occurs at the epitope site on one of the two arms.



In the current therapeutic market, most if not all are human genetically engineered and referred to as -umab.

Some typical and recent MAbs are detailed below.

International Nonproprietary Name (INN)	Trade Name	Target and Type	Applications
Inotuzumab ozogamicin	Besponsa	CD22; humanized lgG4, ADC	Hematological malignancy
Durvalumab	Imfinzi	PD-L1; human lgG1	Bladder cancer
Ocrelizumab	Ocrevus	CD20; humanized lgG1	Multiple sclerosis
Avelumab	Bavencio	PD-L1; human lgG1	Merkel cell carcinoma
Atezolizumab	Tecentriq	PD-L1; humanized lgG1	Bladder cancer
Olaratumab	Lartruvo	PDGFRct; human lgG1	Soft-tissue sarcoma
Daratumumab	Darzalex	CD38; human lgG1	Multiple myeloma
Elotuzumab	Empliciti	SLAMF7; humanized lgG1	Multiple myeloma
Necitumumab	Portrazza	EGFR; human lgG1	Non-small cell lung cancer
Dinutuximab	Unituxin	GD2; chimeric lgG1	Neuroblastoma
Nivolumab	Opdivo	PD-1; human lgG4	Melanoma, non-small cell lung cancer
Blinatumomab	Blincyto	CD19, CD3; murine bispecific tandem scFv	Acute lymphoblastic leukemia

International Nonproprietary Name (INN)	Trade Name	Target and Type	Applications
Pembrolizumab	Keytruda	PD-1; humanized lgG4	Melanoma
Ramucirumab	Cyramza	VEGFR-2; human lgG1	Gastric cancer
Siltuximab	Sylvant	IL-6; chimeric lgG1	Castleman disease
Obinutuzumab	Gazyva	CD20; humanized lgG1; glycoengineered	Chronic lymphocytic leukemia
Ado-trastuzumab emtansine	Kadcyla	HER2; humanized lgG1, ADC	Breast cancer
Pertuzumab	Perjeta	HER2; humanized lgG1	Breast cancer
Brentuximab vedotin	Adcetris	CD30; chimeric lgG1, ADC	Hodgkin's lymphoma, systemic anaplastic large cell lymphoma
Ipilimumab	Yervoy	CTLA-4; human lgG1	Metastatic melanoma
Ofatumumab	Arzerra	CD20; human lgG1	Chronic lymphocytic leukemia
Catumaxomab	Removab	EPCAM/CD3; rat/mouse bispecific mAb	Malignant ascites
Panitumumab	Vectibix	EGFR; human lgG2	Colorectal cancer
Bevacizumab	Avastin	VEGF; humanized lgG1	Colorectal cancer
Cetuximab	Erbitux	EGFR; chimeric lgG1	Colorectal cancer
Tositumomab 1-131	Bexxar	CD20; murine lgG2a	Non-Hodgkin's lymphoma
Ibritumomab tiuxetan	Zevalin	CD20; murine lgG1	Non-Hodgkin's lymphoma
Alemtuzumab	MabCampath, Campath-1H; Lemtrada	CD52; humanized lgG1	Chronic myeloid leukemia; multiple sclerosis
Gemtuzumab ozogamicin	Mylotarg	CD33; humanized lgG4, ADC	Acute myeloid leukemia
Trastuzumab	Herceptin	HER2; humanized lgG1	Breast cancer
Rituximab	MabThera, Rituxan	CD20; chimeric lgG1	Non-Hodgkin's lymphoma

5.4 Pathway Controls

Pathway controls are now quite common. However it is essential to know what pathway, or gene, is aberrant. Reis and Phillips have noted specifics in the context of the optic tract. Namely they state:

Pilocytic astrocytoma is a low-grade glioma that affects mostly children and young adults and can occur anywhere in the central nervous system.

Pilocytic astrocytoma of the optic nerve is an equally indolent subtype that is occasionally associated with neurofibromatosis type 1.

In earlier studies, this subtype was considered within the larger category of 'optic pathway glioma,' which included infiltrating astrocytomas and other hypothalamic tumors. However, there have been suggestions that gliomas in the optic nerve, and especially pilocytic astrocytoma of the optic nerve, are biologically different from tumors within the hypothalamus and other parts of the optic tract.

Furthermore, the recent discovery of BRAF duplication and fusion with the KIAA1549 gene is reported to be more typical for posterior fossa tumors, and the rate of this aberration is not well known in pilocytic astrocytoma of the optic nerve.

To determine the distinction of pilocytic astrocytoma of the optic nerve from pilocytic astrocytoma of the posterior fossa and to investigate the prevalence of BRAF aberrations, we reviewed the clinicopathological and molecular features of all such patients in our institution.

Our study demonstrates that BRAF duplication is more frequent in posterior fossa tumors compared with pilocytic astrocytoma of the optic nerve (P ¼ 0.011).

However, the rates of phospho-MAPK1 and CDKN2A expression were high in both pilocytic astrocytoma of the optic nerve and posterior fossa pilocytic astrocytoma, suggesting that the MAPK pathway is active in these tumors.

Our study supports the notion that BRAF duplication is more typical of posterior fossa pilocytic astrocytoma and ***that molecular alterations other than KIAA1549 fusion may underlie MAPK pathway activation in pilocytic astrocytoma of the optic nerve.***

Collins et al have noted:

The use of high-throughput sequencing techniques interrogating the whole genome has shown that single abnormalities of the mitogen-activating protein kinase (MAPK) pathway are exclusively found in almost all cases, indicating that PA represents a one-pathway disease. The most common mechanism is a tandem duplication of a ≈2 Mb-fragment of #7q, giving rise to a fusion between two genes, resulting in a transforming fusion protein, consisting of the N-terminus of KIAA1549 and the kinase domain of BRAF.

Additional infrequent fusion partners have been identified, along with other abnormalities of the MAP-K pathway, affecting tyrosine kinase growth factor receptors at the cell surface (e.g., FGFR1) as well as BRAF V600E, KRAS, and NF1 mutations among others. However, while the KIAA1549-BRAF fusion occurs in all areas, the incidence of the various other mutations identified differs in PAs that develop in different regions of the brain. Unfortunately, from a diagnostic standpoint, almost all mutations found have been reported in other brain tumor types, although some retain considerable utility.

These molecular abnormalities will be reviewed, and the difficulties in their potential use in supporting a diagnosis of PA, when the histopathological findings are equivocal or in the choice of individualized therapy

Actionable Target	Abnormality	Prevalence (%)	Clinical Experience with Targeted Agent
VEGFR	Expression	100	Bevacizumab (VEGF inhibitor) with irinotecan. Sorafenib
EGFR	Amplification or overexpression	40	Erlotinib (EGFR inhibitor) with temozolamide Gefitinib (EGFR inhibitor Vandetanib (VEGFR, EGFR, RET inhibitor)
	Mutation	6	
PDGFR	Overexpression	Unknown	Imatinib (BCR-Abl/cKIT.'PDGFR inhibitor

Actionable Target	Abnormality	Prevalence (%)	Clinical Experience with Targeted Agent
			Sunitinib (VEGFR/PDGFR/RET/KIT/FLT3 inhibitor):
PTEN	Mutation/deletions	17	Temsirolimus (mTOR inhibitor Everolimus (mTOR inhibitor with temozolomide
PIK3CA	Mutation	5	
PIK3R1	Mutation	4	
MGMT	Methylation	45	Methylation of promoter region of gene in tumors associated with superior responses to temozolomide.
TGF-p	Overexpression	Unknown	LY2157299 (TGF-ji inhibitor)
Cytosine deaminase	N/A	N/A	Vocimagene amiretrorepvec
POLE	Mutation	Unknown	Pembrolizumab (PD-1 inhibitor
Biallelic mismatch repair deficiency (bMMRD)	Mutation	Unknown	Nivolumab (PD-1 inhibitor)
IL13Ro2	Expression	Unknown	Chimeric antigen receptor (CAR)-engineered T cells targeting the tumor-associated antigen interleukin-13 receptor alpha 2 (IL13Ro2)
IDH1	Mutation	Unknown	AG-120 (IDH1 inhibitor)
EGFR	Amplification and/or mutation	57.4 %	ABT-414 (EGFR antibody drug conjugate with anti-microtubule agent monomethyl auristatin F):

5.4.1 MEK

MEK is a gene key to the proliferation pathway. It can be over-activated by genes above it in the pathway control mechanism. It is also a putative target for control of PA. Asa McCubrey have noted:

The Ras/Raf/MEK/ERK cascade couples signals from cell surface receptors to transcription factors, which regulate gene expression. Furthermore, this cascade also regulates the activity of many proteins involved in apoptosis. …

This pathway is often activated in certain tumors by chromosomal translocations such as BCR-ABL, mutations in cytokine receptors such as Flt-3, Kit, Fms or overexpression of wild type or mutated receptors, e.g., EGFR.

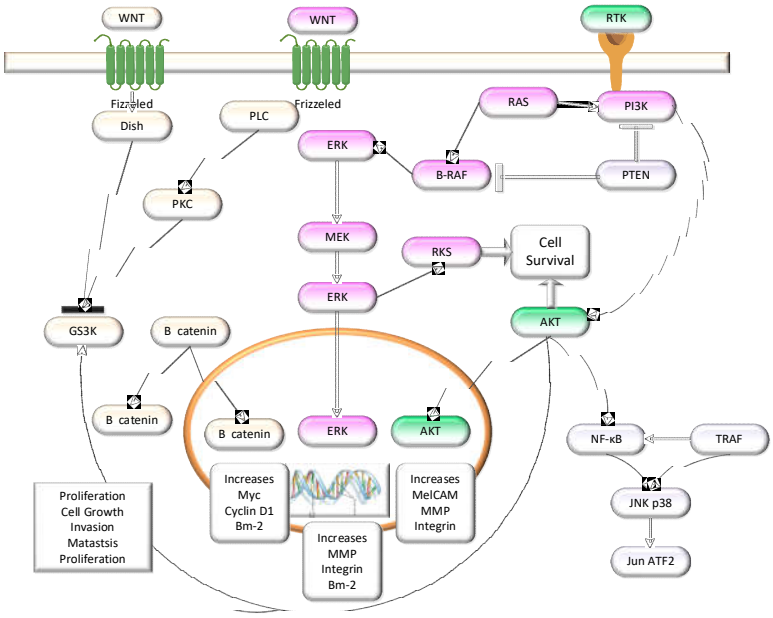
The Raf/MEK/ERK pathway also has profound effects on the regulation of apoptosis by the post-translational phosphorylation of apoptotic regulatory molecules including Bad, Bim, Mcl-1, caspase 9 and more controversially Bcl-2. This pathway has diverse effects which can regulate cell cycle progression, apoptosis or differentiation.

A survey of the literature documents the daily increase in the complexity of this pathway, as there are multiple members of the kinase, transcription factor, apoptotic regulator and caspase executioner families, which can be activated or inactivated by protein phosphorylation. Furthermore, this pathway can induce the transcription of certain genes. Raf, either through downstream MEK and ERK, or independently of MEK and ERK, can induce the phosphorylation of proteins, which control apoptosis.

Additional signal transduction pathways interact with the Raf/MEK/ERK pathway to positively or negatively regulate its activity. Abnormal activation of this pathway occurs in human cancer due to mutations at upstream membrane receptors and Ras and B-Raf as well as genes in other pathways (e.g., PI3K, PTEN, Akt), which serve to regulate Raf activity. The Raf/MEK/ERK pathway also influences chemotherapeutic drug resistance as ectopic activation of Raf induces resistance to doxorubicin and paclitaxel in breast cancer cells.

Mutations at B-Raf have been frequently detected in some malignancies including melanoma and thyroid cancers. For all the above reasons, the Raf/MEK/ERK pathway is an important pathway to target for therapeutic intervention. Inhibitors of Ras, Raf, and MEK and some downstream targets have been developed and many are currently in clinical trials. Naturally, some inhibitors are better than others and certain "specific" inhibitors may inhibit multiple kinases.

We present below the pathways related to the genes in question.

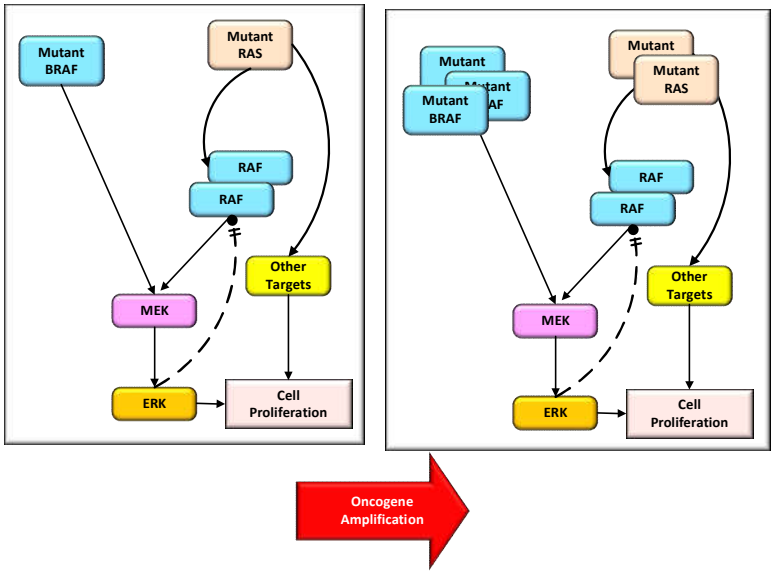


Now Paulikakos and Solit have noted as follows:

*Aberrant activation of the ERK pathway is common in human tumors. This pathway consists of a three-tiered kinase module [comprising the kinases RAF, **mitogen-activated protein kinase (MAPK) kinase (MEK)**, and extracellular signal–regulated kinase (ERK)] which functions as a negative feedback amplifier to confer robustness and stabilization of pathway output.*

Because this pathway is frequently dysregulated in human cancers, intense efforts are under way to develop selective inhibitors of the ERK pathway as anticancer drugs. Although promising results have been reported in early trials for inhibitors of RAF or MEK, resistance invariably occurs.

Amplification of the upstream oncogenic driver of ERK signaling has been identified as a mechanism for MEK inhibitor resistance in cells with mutant BRAF or KRAS. Increased abundance of the oncogenic driver (either KRAS or BRAF in the appropriate cellular context) in response to prolonged drug treatment results in increased flux through the ERK pathway and restoration of ERK activity above the threshold required for cell growth. For patients with BRAF mutant tumors, the results suggest that the addition of a RAF inhibitor to a MEK inhibitor may delay or overcome drug resistance. The data thus provide a mechanistic basis for ongoing trials testing concurrent treatment with RAF and MEK inhibitors.



The authors then note regarding the above:

Amplification of the oncogenic driver of ERK pathway activity confers resistance to MEK inhibitors. The RAF-MEK-ERK pathway is a three-tiered kinase cascade with a central role in regulating cell proliferation and survival. In cells with wild-type BRAF, RAS proteins activate the RAF kinases [ARAF, BRAF, and CRAF (RAF1)] in part by facilitating their dimerization. Activated RAF phosphorylates MEK1 and MEK2 (MEK), which in turn phosphorylate ERK1 and ERK2 (ERK). ERK pathway activation in BRAF(V600E) mutant cells is RAS-independent and does not require the formation of RAF dimers. The ERK pathway contains a classical feedback loop in which the abundance of feedback elements, such as Sprouty and dual-specificity phosphatase family proteins, is determined by ERK activity

As Reitman et al note:

Pilocytic astrocytomas (PAs) are the most common brain tumors in children. These are WHO grade I tumors that can potentially be cured. However, PAs can be associated with considerable treatment-related morbidity from surgical resection, chemotherapy, or radiotherapy. PAs that are incompletely resected tend to recur during childhood, but childhood PA patients usually do not succumb to their disease.

In contrast, higher-grade gliomas are nearly always fatal.

PAs are also distinguished by the simplicity of their genome.

Unlike higher-grade gliomas, which usually exhibit multiple driver mutations, most PAs exhibit a single driver somatic genetic alteration.

These almost always activate the MAPK pathway, with rearrangements generating the KIAA1549[17]-BRAF fusion oncogene accounting for ~70% of PAs.

Targeted therapies directed at the MAPK pathway are undergoing clinical testing for recurrent or incompletely resected PAs16,17. However, whether the MAPK pathway is uniformly activated in PA cancer cells remains incompletely understood. …

These data indicate that PA cancer cells resemble a developmental spectrum ranging from OPCs to mature glia. While the cells overall resembled OPCs, we also observed subpopulations of cells expressing a MAPK signaling signature and signatures reminiscent of mature glia.

In addition, we observed subpopulations that exhibited intermediate expression of both programme. Intriguingly, F-IHC and RNA ish studies of top marker genes for either gene programme indicate that these gene programme may underlie the long-observed biphasic histopathologic features of PA42.

These results indicate that the AC-like gene programme is more highly expressed in the piloid, fibrillary component of the tumors, and that expression of the MAPK gene programme is biased towards the loose, microcystic component of the tumors. It stands to reason that one of these populations may give rise to the other, reflecting a developmental process.

The fact that the cells expressing the MAPK signaling programme exhibits a higher proportion of cycling cells and expresses progenitor cell-associated transcription factors such as SOX2 indicates that cells expressing the MAPK signaling signature may represent an OPC-like progenitor population in PA, which gives rise to an AC-like population. This observation reveals parallels to normal oligodendrocyte differentiation, for which timing of progenitor expansion is tightly regulated by MAPK signaling43.

Identification of a cellular developmental process in PA raises several therapeutic considerations. We found that a MAPK signaling gene programme was expressed in only a subpopulation of cancer cells, which would suggest that this subpopulation would exhibit

[17] https://www.ncbi.nlm.nih.gov/gene/57670

differential responses to MEK inhibition compared to the more AC-like cells. Clinically, MEK inhibitors have shown great promise, but complete responses have been rare17.

The present study raises several testable clinical hypotheses that could explain the heterogeneity of responses to investigational MEK inhibitors and that could guide ongoing clinical investigations. First, MEK inhibition may be inadequate to overcome MAPK signaling in cells with very high levels of MAPK signaling.

If this is the case, we predict that tumors with high MAPK gene programme expression may have poor responses to therapy and poor longterm disease control. Second, cells without active MAPK signaling, such as the AC-like+ cells, may be unaffected by MEK inhibition. If so, we predict that tumors with mostly AC-like+ cells would exhibit a poor initial response to MEK inhibition.

However, such tumors would exhibit good long-term disease control as the AC-like+ cells are not proliferative. Third, on the basis of the tumor cell differentiation processes inferred in this study, MAPK+ cells may be able to transition into AC-like+ cells. If so, we predict that tumors that exhibit poor initial responses may exhibit a shift towards higher AC-like+ tumor cell composition between pre-treatment and post-treatment biopsies.

If such a process is clinically relevant, it will be critical to determine whether this process is reversible to determine whether further MEK inhibition could be of clinical benefit for these patients. Future correlative and experimental studies informed by the gene programme identified here may provide clarity on these issues and guide the selection of the most efficacious treatment strategies for PA.

Expression of activated BRAF can paradoxically lead to oncogene-induced senescence in vitro, which has been speculated to underlie the relatively indolent biology of BRAF-rearranged PA.

We found that the highest expression of senescence related genes was confined to PA cancer cells that highly expressed the MAPK gene programme.

Intriguingly, cells highly expressing the MAPK signaling gene programme were also most likely to express a proliferative gene programme, but expression of the senescence and of the proliferative programme occured in mutually exclusive sets of MAPK-activated cells (see Fig. 5e).

Future experimental work will be needed to determine whether the dosage of MAPK signaling and/or other cellular factors contribute to proliferative vs. senescent cell fate decisions in this context. We speculate that such work could inform therapeutic opportunities to modulate MAPK signaling or other cellular processes to exploit this biology.

As Kahn et al have noted regarding MEK and putative inhibitors[18]:

[18] Also see Cheng and Tian for a summary.

The MAPK/ERK kinase MEK is a shared effector of the frequent cancer drivers KRAS and BRAF that has long been pursued as a drug target in oncology, and more recently in immunotherapy and ageing

However, many MEK inhibitors are limited owing to on-target toxicities and drug resistance. Accordingly, a molecular understanding of the structure and function of MEK within physiological complexes could provide a template for the design of safer and more effective therapies.

Here we report X-ray crystal structures of MEK bound to the scaffold KSR (kinase suppressor of RAS) with various MEK inhibitors, including the clinical drug trametinib.

The structures reveal an unexpected mode of binding in which trametinib directly engages KSR at the MEK interface.

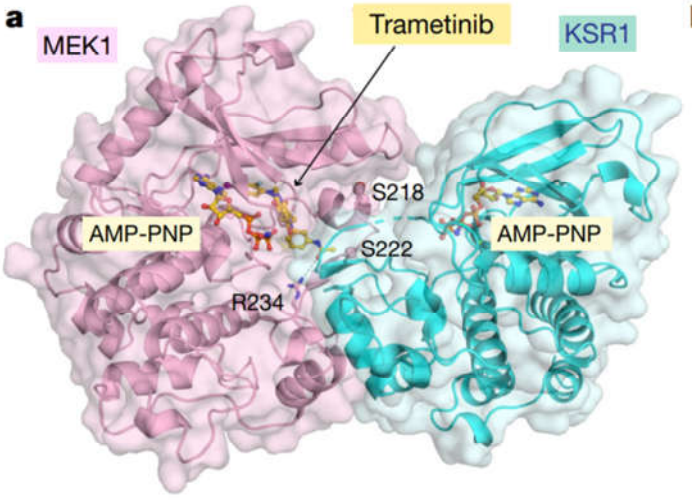
In the bound complex, KSR remodels the prototypical allosteric pocket of the MEK inhibitor, thereby affecting binding and kinetics, including the drug-residence time.

Moreover, trametinib binds KSR–MEK but disrupts the related RAF–MEK complex through a mechanism that exploits evolutionarily conserved interface residues that distinguish these sub-complexes.

On the basis of these insights, we created trametiglue, which limits adaptive resistance to MEK inhibition by enhancing interfacial binding.

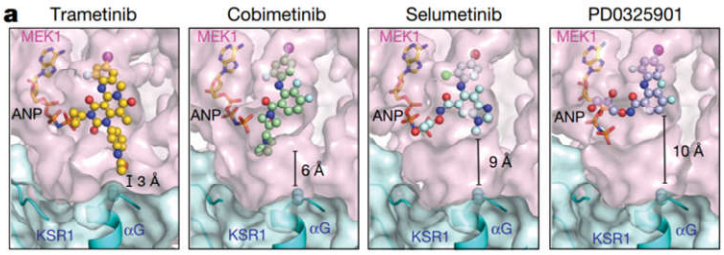
Our results reveal the plasticity of an interface pocket within MEK sub-complexes and have implications for the design of next-generation drugs that target the RAS pathway

Now Kahn et al show the specific binding sites based upon measurements. We show this below:



Trametinib bound to KSR1–MEK1–AMP-PNP. See Kahn et al

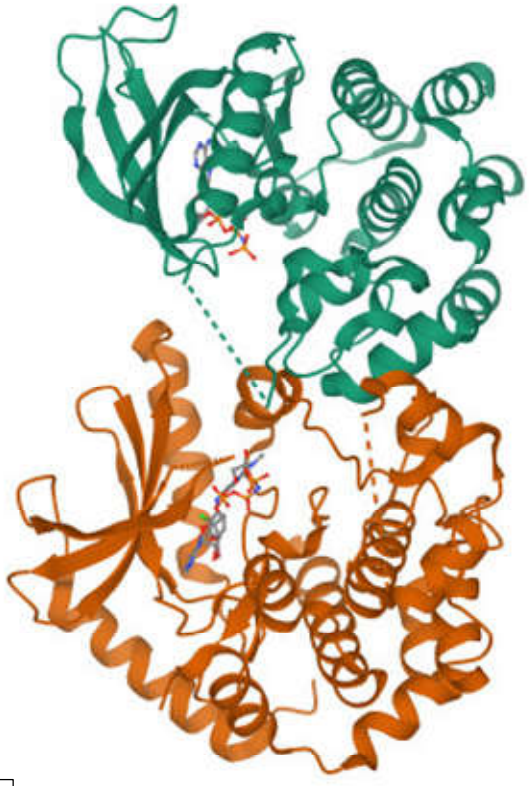
Then the authors also demonstrated the various binding sites for other therapeutics as shown below:



See Kahn et al, Fig 2a

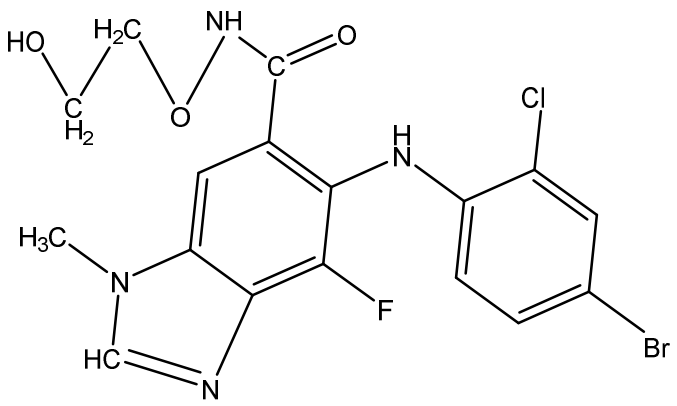
Now it is also useful to see the details of the protein MEK and the target for the most used therapeutic, selumetinib, as shown below:

Crystal Structure of KSR1:MEK1 in complex with AMP-PNP, and allosteric MEK inhibitor Selumetinib



https://www.rcsb.org/structure/7JUZ

To be clearer, selumetinib has the molecular structure as shown below:



The binding site can be alters by a variety of exogeneous factors. Thus this therapeutic must first pass through the blood-brain barrier (BBB) and then find the appropriate binding site to effectively turn off the aberrant MEK in overdrive.

5.4.2 BRAF

From Groves[19]:

*The BRAF gene found on chromosome 7q34 is a **2949 base pair sequence** of 18 exons encoding a **766 amino acid peptide**.*

The B-Raf protein is a key signaling molecule in the mitogen activated protein kinase (MAPK) signaling pathway involved in cell growth, proliferation and survival.

BRAF gene mutations are oncogenic and mutated in ~8% of all malignancies….

Mutations in BRAF can render the RAS-RAF-MEK-ERK pathway constitutively activate and lead to uncontrolled cell proliferation and survival.

BRAF mutations can be categorized into V600E and non-V600E mutations.

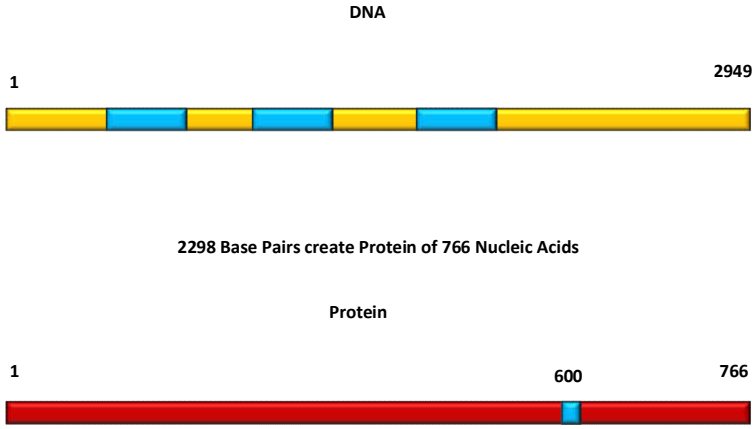
Within NSCLC, BRAF mutations are present in 1.5%–4% of cases; approximately half of these are V600E mutations.

This mutation in BRAF is oncogenic and leads to a 500-fold increase in the kinase domain activity of B-Raf as compared with its wild type, that continuously activates ERK, irrespective of RAS activation and ERK-dependent negative feedback.

[19] https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/braf-gene

Although a relatively small proportion of patients, a precision medicine approach to treatment for patients with BRAF V600E mutations has led to meaningful improvements in patient outcomes and quality of life when compared to traditional chemotherapy.

We show a graphic of BRAF below with the exons in yellow and introns in blue and the resulting protein. The nucleic acid on location 600 of the protein is the operative target. Normally it is valine.



From Jones et al:

Brain tumors are the most common solid tumors of childhood, and pilocytic astrocytomas (PA) are the most common central nervous system tumor in 5 to 19 year olds. Little is known about the genetic alterations underlying their development. Here, we describe a tandem duplication of f2 Mbat 7q34 occurring in 66% of PAs. This rearrangement, which was not observed in a series of 244 higher-grade astrocytomas, results in an in-frame fusion gene incorporating the kinase domain of the BRAF oncogene.

We further show that the resulting fusion protein has constitutive BRAF kinase activity and is able to transform NIH3T3 cells. This is the first report of BRAF activation through rearrangement as a frequent feature in a sporadic tumor. The frequency and specificity of this change underline its potential both as a therapeutic target and as a diagnostic tool. …

Recent reports have shown that constitutive activation of BRAF can lead to oncogene-induced senescence (OIS) in benign tumors . Thus, OIS may contribute to the benign course and slow growth of PAs. The role of activated BRAF in regulating the mitotic spindle checkpoint is also of interest in light of reports of aneuploidy in PAs.

The importance of RAS/RAF signaling in PAs is further supported by the increased incidence of PAs in neurofibromatosis type 1 (NF1). Mutations of the NF1 gene in this syndrome lead to hyperactive RAS signaling and RAF activation. Three tumors in our series (PA19, PA41, and PA42) were diagnosed as having clinical features of NF1. None of these possessed the KIAA1549:BRAF fusion, suggesting that only one ‘‘hit’’ on the mitogen-activated protein kinase pathway is required for PA development. The total number of cases with an identified alteration

in this pathway in our series is 34 of 44 . The identification of a recurrent, transforming genetic event in the majority of PAs represents a significant increase in our understanding of this entity.

The KIAA1549:BRAF fusion is seen across all ages and in various locations in our series, including the cerebellum, ventricles, hypothalamus, and optic nerve. This is in contrast with previous reports suggesting differences in genomic and/or expression signatures with respect to age and tumor location and may suggest a precursor cell common to most cases of PA that displays a degree of oncogene tropism.

In conclusion, due to the frequency and transforming activity of the recurrent fusion event presented here, we consider it likely that this is the initiating lesion in the majority of PAs. The prevalence and specificity of this change strongly indicate potential uses both as a diagnostic marker and in a targeted therapeutic setting.

As Ascierto et al noted :

BRAF is a serine/threonine protein kinase, encoded on chromosome 7q34, that activates the MAP kinase/ERKsignaling pathway.

BRAF is the family member most easily activated by Ras [1,2].

In addition, the basal kinase activity of BRAF is higher than that of other family members [3,4]. This provides a potential rationale for the frequent mutational activation of BRAF observed in human tumors A major advance of the past few years was the discovery that RAF kinases can homo- and heterodimerize, and that, in fact, the structure of an active RAF kinase is that of a side-to-side dimer in which only one partner must have catalytic activity [11].

Dimerization is enhanced by Ras and is subject to negative feedback regulation by ERK. Several RAF mutations have been implicated in the induction of genomic instability, driving the proliferation of cancer cells with the highest frequency in melanoma. For instance, mutated BRAF signals as a monomer, independent of upstream growth stimuli.

The most frequent BRAF mutation, BRAFV600E, causes constitutive activation of the kinase as well as insensitivity to negative feedback mechanisms. BRAFV600E has been implicated in different mechanisms of melanoma progression, and principally the activation of the downstream MEK/ERK pathway, evasion of senescence and apoptosis, unchecked replicative potential, angiogenesis (through MEK-dependent activation of HIF-1α and VEGF), tissue invasion and metastasis (via upregulation of several proteins involved in migration, integrin signaling, cell contractility, tumor- and microenvironment-derived interleukin-8), as well as the evasion of immune response.

Faulkner et al note:

Pilocytic astrocytomas (PAs) are increasingly tested for KIAA1549- BRAF fusions.

We used reverse transcription polymerase chain reaction for the 3 most common KIAA1549-BRAF fusions, together with BRAF V600E and histone H3.3 K27M analyses to identify relationships of these molecular characteristics with clinical features in a cohort of 32 PA patients.

In this group, the overall BRAF fusion detection rate was 24 (75%).

Ten (42%) of the 24 had the 16-9 fusion, 8 (33%) had only the 15-9 fusion, and 1 (4%) of the patients had only the 16-11 fusion. In the PAs with only the 15-9 fusion, 1 PA was in the cerebellum and 7 were centered in the midline outside of the cerebellum, that is, in the hypothalamus (n = 4), optic pathways (n = 2), and brainstem (n = 1).

Tumors within the cerebellum were negatively associated with fusion 15-9. Seven (22%) of the 32 patients had tumor-related deaths and 25 of the patients (78%) were alive between 2 and 14 years after initial biopsy.

Age, sex, tumor location, 16-9 fusion, and 15-9 fusion were not associated with overall survival.

Thus, in this small cohort, 15-9 KIAA1549-BRAF fusion was associated with midline PAs located outside of the cerebellum; these tumors, which are generally difficult to resect, are prone to recurrence.

5.4.2.1 Pathway

The most recent one is the control of a mutated BRAF, a variant of the RAF pathway. It was observed that there was a mutation in the BRAF gene so that what was produced was a different BRAF called V600E which had excessive up-regulation in almost 50-60% of all metastatic melanomas. The identification of this product then allowed for its targeting and suppression as a means to reduce cell proliferation. The results have been reported recently by the work of Chapman et al (2011) and Flaherty et al (2010).

A review by Smalley and Flaherty (2009) had made suggestions on controlling both the BRAF as well as the AKT pathway. We will discuss that later. Recent work by Poulikakos and Solit (2011) has also presented both BRAF and MEK control, trying to avoid the loss of efficacy we discuss here.

As Chabner notes:

Mutations in BRAF are also common, occurring in melanoma and thyroid cancer (both approximately 50%), colorectal adenocarcinoma (10%), lung adenocarcinoma (5% to 10%), and a low frequency of numerous other malignancies.

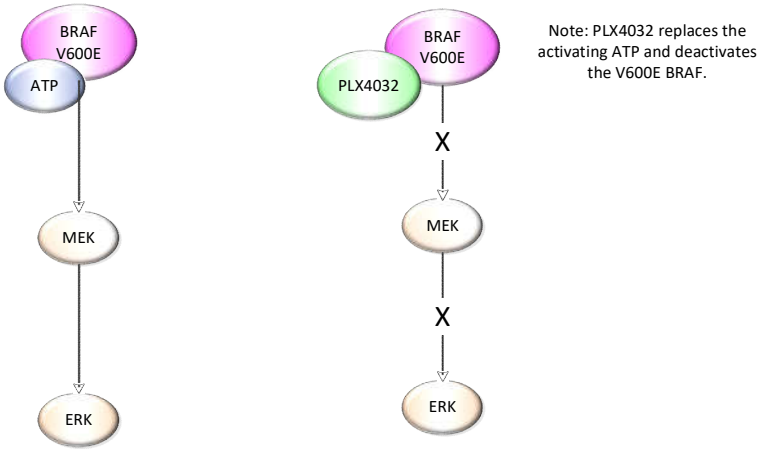
The most common site of BRAF mutations is at the 600th codon, with an amino acid substitution of valine to glutamine (V600E) or lysine (V600K)[20].

[20] Note that the notation 600 is the location, V is valine and K is glutamine.

The V600 mutation eliminates the need for upstream activation by RAS and mitigates the need for dimerization, thus producing constitutive MAPK signaling.

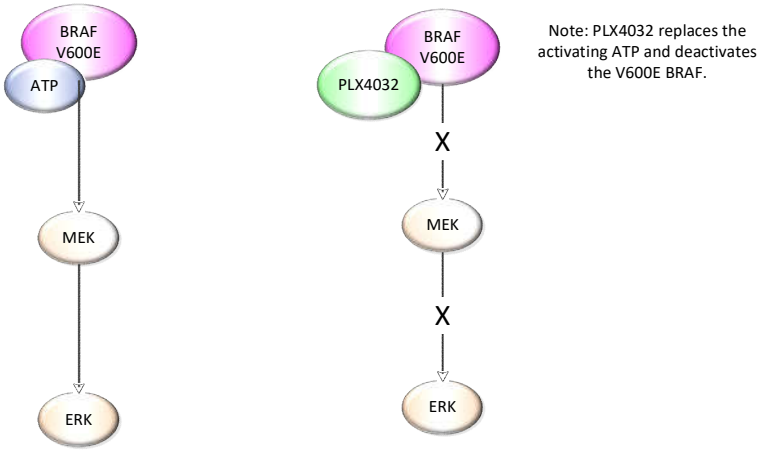
Other non-V600 mutations have also been identified at lower frequencies that also activate MAPK signaling to varying degrees. Finally, fusions and intragenic deletions may activate BRAF by removing the regulatory RAS-binding domain, allowing for unopposed kinase activity. More so than RAS-mutated tumors, V600 BRAF–mutated tumors appear to be the most MAP kinase pathway dependent among the genetically defined subpopulations of cancer.

Specifically, a drug now called Vemurafenib, or PLX4032, binds to the ATP activation site on the B-RAF mutation V600E and as such it blocks the overexpression of this protein and reduces the flow downward which we have shown causes ultimately an up-regulation of proliferation.



Now we can also see that Vemurafenib can lose its effectiveness and there are several proposals for why this happens. We discuss a few here. From Solit and Rosen (2011) we show one of the possible ways in which resistance can occur. We discuss several of their conjectures in detail.

Below we depict the supposition from Solit and Rosen. Arguably this is what accounts for the mortality in the Kaplan Meir data they have from their trials.



The paper by Solit and Rosen propose three reasons for loss of action of PLX4032:

(i) In melanomas with the BRAF V600E mutation, levels of activated RAS are too low to promote adequate formation of RAF dimers, and PLX4032 inhibits RAF activity and ERK signaling … This model is consistent with our observation that the introduction of mutant (activated) RAS into cells with mutant BRAF causes insensitivity of the ERK pathway to the drug. This model suggests that increases in RAF dimerization (because of RAS activation or increased RAF expression) will cause ERK signaling to become insensitive to PLX4032 …

(ii) The findings of Johannessen et al. suggest another mechanism for the resistance of ERK signaling to RAF inhibition in cells driven by the BRAF V600E mutations. These investigators used a new technique — the introduction of a library of DNA constructs, each of which encodes a different kinase — into tumor cells with the BRAF V600E mutation to screen for kinases that confer resistance to RAF inhibition. Using this screen, they confirmed a previous finding: that overexpression of RAF1 confers resistance to RAF inhibition. [8] They further showed that the overexpression of mitogen-activated protein kinase kinase kinase 8 (MAP3K8, or COT), which phosphorylates MEK in a RAF-independent manner, can also mediate resistance to RAF inhibitors…

(iii) a third basis for acquired resistance, one in which the activation of other pathways causes the tumor cell to become less dependent on ERK signaling. In these tumors, ERK activation remains sensitive to the RAF inhibitor. Specifically, they report that platelet derived growth factor receptor β (PDGFRβ), a receptor tyrosine kinase, is overexpressed in cellular models selected for RAF-inhibitor resistance in cell culture and in a subgroup of biopsy samples obtained from patients with progressing tumors. In the cell lines, PDGFRβ overexpression was associated with resistance to the anti-proliferative effects of the RAF inhibitor, despite continued inhibition of ERK signaling in the presence of the drug.

As Khanter et al have noted:

Pilocytic astrocytoma (PA) is emerging as a tumor entity ***with dysregulated RAS/RAF/MEK/ERK signaling****.*

In this study, we report the identification of a novel recurrent BRAF insertion (p.V504_R506dup) in five PA cases harboring exclusively this somatic tandem duplication.

This recurrent alteration leads to an addition of three amino acids in the kinase domain of BRAF and has functional impact on activating MAPK phosphorylation. Importantly, we show that this mutation confers resistance to RAF inhibitors without changing effectiveness while downstream MEK inhibitors remain effective. Our results further emphasize the importance of BRAF alterations in PA and the need to characterize them in a given tumor as this can affect therapeutic strategies and their potential use as tumor marker in molecular diagnostics. ...

The identification of additional patient-specific mutations could provide crucial information regarding molecular pathways underlying non-responding PA and thus point to new therapeutic avenues. In this study, we identified a novel recurrent BRAF tandem insertion (p.V504_R506dup), ***which has an impact on MAPK phosphorylation and confers resistance to RAF inhibitors.***

We provided further evidence of the importance of this recurrent somatic mutation as a tumor-specific driver in PA development and its potential role as a guide for treatment strategies. ... The knowledge about molecular genetics behind development of PA has increased tremendously in recent years. The recurrent BRAF p.V504_R506dup reported in this paper further emphasizes the central role of BRAF in PA tumorigenesis.

The occurrence of an increasing number of BRAF alterations and possibility for MAPK pathway targeted therapy highlights the importance of robust methods for fast and cost-effective detection of BRAF deregulations to guide diagnosis, prognosis, and accurately targeted therapy.

As Appay et al note:

Pilocytic astrocytomas are characterized by alterations in the RAS–MAP kinase pathway, the most frequent being a tandem duplication on chromosome 7q34 involving the BRAF gene, resulting in oncogenic BRAF fusion proteins [4].

The most common partner is the KIAA1549 gene. In this case, a fragment of approximately 2 Mb involving parts of both genes is duplicated and inserted at the breakpoint such that the 5'-end of the KIAA1549 gene becomes fused with the 3'-end of the BRAF gene (Fig. 1).

To date, nine different combinations of KIAA1549 and BRAF gene fusion have been described in the literature. The most common is a fusion between exon 16 of KIAA1549 and exon 9 of BRAF (KIAA1549-BRAF 16;9 fusion, 78%). Less frequent variants include fusion between KIAA1549 exon 15 and BRAF exon 9 (KIAA1549-BRAF 15;9 fusion, 13%) and KIAA1549 exon 16 and BRAF exon 11 (KIAA1549-BRAF 16;11 fusion, 7%) ...

In our study, KIAA1549-BRAF fusion was recorded in 20/40 pilocytic astrocytomas (50%), 3/18 gangliogliomas (16.7%), and in 4/27 (14.8%) of neuroepithelial tumors that remained difficult to

classify (one pilocytic astrocytoma versus ganglioglioma and three pilocytic astrocytomas versus diffuse gliomas), but in none of the dysembryoplastic neuroepithelial tumors. Whether true gangliogliomas display KIAA1549-BRAF fusion or whether these tumors represent misclassified pilocytic astrocytomas is still a matter of debate.

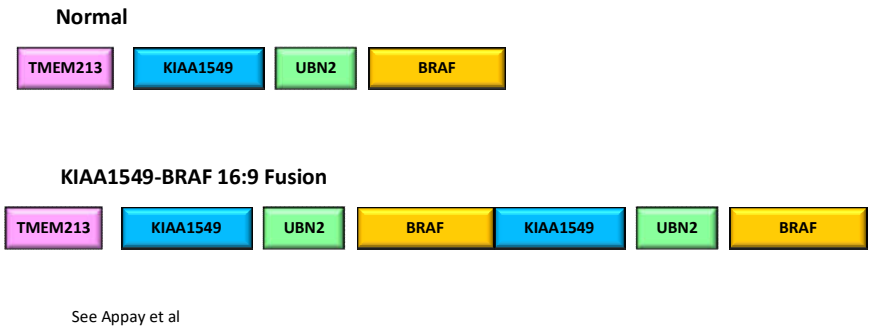
Nevertheless, because KIAA1549-BRAF fusion constitutively activates the MAP kinase pathway, this alteration represents a target for drugs such as MEK inhibitors, and therefore, the detection of this genetic abnormality is of utmost importance in these tumors in the context of clinical trials.

Indeed, searching for KIAA1549- BRAF fusion will be done in all samples from patients that will be included in the incoming SIOP-LOGGIC phase III clinical trial. In the context of KIAA1549-BRAF fusion gene, the most reliable KIAA1549 exon to target to detect KIAA1549-BRAF fusion was exon 4.

Importantly, we also showed that appropriate combinations of primers might be used to define which exon (15 or 16) of the KIAA1549 gene is involved in the KIAA1549-BRAF fusion.

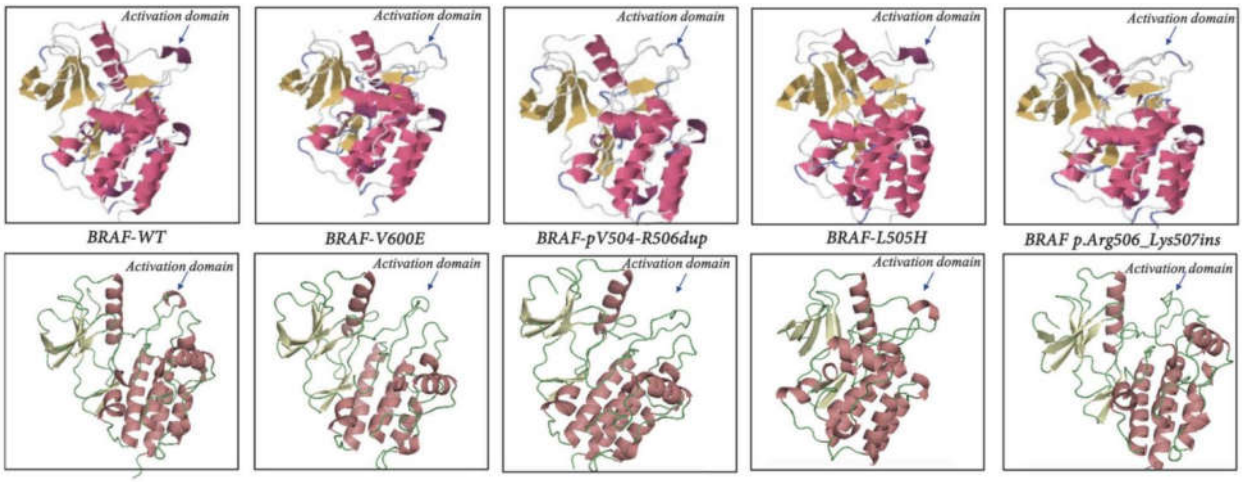
However, the correlation with RNAseq results was not 100% and in routine practice it is not currently necessary to decipher the different combinations of KIAA1549-BRAF fusion transcripts. We also learnt from our study that the highest mean copy number variation value of the ratio between two exons analyzed was never above 3, as expected for a duplication involving one allele in almost all cells.

The fusion is shown below:



Now when we consider therapeutics we see selumetinib targeting MEK but recent research has also noted BRAF targeting as well. Khater et al present a list of BRAFs in their 3D images as shown below[21]:

[21] From Khater et al: *Predicted molecular structures proposed by raptorX (top panel) and PyMol (bottom panel) for different alterations found in BRAF.* https://pymol.org/2/ *and* http://raptorx.uchicago.edu/



The first is the wild type normally found. The second is the 600 location valine replacement. Khater et al further note:

Most PAs have alterations in the mitogen-activated protein kinase (MAPK) signalling pathway. The most common genetic alteration found in PAs is the activating KIAA1549-BRAF fusion found in more than 60% of the cases.

Although less frequent, at least 8 additional partners

(FAM131B, RNF130, CLCN6, MKRN1, GNA11, QK1, FZRI, and MACF1)

have been found in BRAF fusions.

All of them resulted in the loss of the regulatory domain with consequent activation of the MAPK pathway.

Point mutations in BRAF have also been reported, including V600E that is found in 2–9% of PAs. In addition to BRAF-related alterations, other mutations are reported, including FGFR1, NF1, KRAS as well as NTRK-family fusions.

All these mutations are mutually exclusive and lead to the activation of the MAPK pathway, which is detected in nearly 100% of cases, thus making PA a single pathway disease [15, 16]. The identification of additional patient-specific mutations could provide crucial information regarding molecular pathways underlying non-responding PA and thus point to new therapeutic avenues. In this study, we identified a novel recurrent BRAF tandem insertion (p.V504_R506dup), which has an impact on MAPK phosphorylation and confers resistance to RAF inhibitors.

We provided further evidence of the importance of this recurrent somatic mutation as a tumor-specific driver in PA development and its potential role as a guide for treatment strategies.

As Fangusarro et al have noted:

Selumetinib is active against recurrent, refractory or progressive PA harboring common BRAF aberrations and NF1-associated pLGG.

To our knowledge, this is one of the first prospectively tested and successful molecularly-targeted agents in pLGG.

These data not only provide an alternative to standard chemotherapy for these subgroups of patients, but this success has led to an interest in exploring efficacy in patients as a first-line therapy. In fact, these data have directly led to the development of two Children's Oncology Group phase III studies in newly diagnosed pLGG patients both with and without NF1 comparing standard chemotherapy to selumetinib. …

Selumetinib is active against recurrent, refractory or progressive PA with common BRAF aberrations and NF1-associated pLGG. Both strata surpassed their statistical predetermined success thresholds based on RR.

In stratum 1, the percentage of PRs in tumors harboring KIAA1549-BRAF fusions was slightly higher than those with BRAFV600E mutations (38.9% versus 28.6%), but specific BRAF aberration was not statistically predictive of response.

It was, however, predictive for PFS whereby patients with pLGG that harbored a BRAFV600E mutation had worse PFS.

This finding is similar to a recent report showing pLGG with BRAFV600E mutations have worse PFS as compared to both pLGG with wild-type BRAF and KIAA1549-BRAF fusion when treated with chemotherapy and radiotherapy.(25) Interestingly, theses data would be the first to our knowledge showing the same negative prognostic value of BRAFV600E mutation in a homogenous group of pLGG tumors treated prospectively with a MEK inhibitor. For patients with NF1-associated pLGG, 40% showed PR, and only one patient progressed while on therapy.

Numerous children in both strata achieved between 1–49% tumor shrinkage and these observed prolonged "stable disease" outcomes are clinically beneficial. Since the vast majority of patients will not succumb to their disease, current opinions are that PFS and functional outcomes are as important as radiographic response.(15, 19) The 2-year PFS for Stratum 1 and 3 were 70% (95% CI: 47%−85%) and 96% (95% CI: 74% −99.4%), respectively.

These PFS and response rates compare favorably to previous trials in children with recurrent pLGG. A phase II study of weekly vinblastine monotherapy in 50 evaluable patients with recurrent and refractory pLGG found a 36% response rate; however, the designation of "minor response" was included as a response for patients with 25–50% reduction in 2-dimensional tumor measurements which would have been categorized as stable disease in our study.

If this designation were included on the current study, the overall response rate would increase even further. Five-year OS and EFS with vinblastine were 93.2% ± 3.8%, and 42.3% ± 7.2%, respectively.(26) A PBTC phase II study of bevacizumab and irinotecan in 35 evaluable children

with recurrent pLGG showed 2-year PFS of 47.8% (SE ± 9.27%). Two of 35 patients (5.7%) had a documented response and patients received a median of 12 courses of therapy.(27)

Our results for patients with NF1-associated pLGG align with results published for NF1 patients with plexiform neurofibromas. In the phase I plexiform neurofibroma trial, MRI volumetric imaging evaluated response. Approximately 70% of patients were considered partial responders, defined as a ≥20% tumor volume reduction, and the vast majority of patients showed some degree of tumor shrinkage.(22),(28) These volumetric response definitions differ from the classic bi-dimensional 50% shrinkage classically utilized to define a PR in pLGG and used on the current study, perhaps explaining the response variation between the two trials.

This common denominator of NF1 MAPK dysregulation seems to harbor these lesions more responsive to selumetinib therapy; however, the exact mechanisms of this increased susceptibility has yet to be elucidated. As noted, there are patients who did not respond or even progressed while on selumetinib. If additional molecular aberrations were found consistently among these less responsive tumors, combination therapies may be a potential strategy to overcoming this resistance.

A limitation of the current study was the lack of tissue available for more advanced molecular testing. Encouragingly, a significant proportion of patients on both arms remain progression free at a median follow-up of 20.28 months (range=0–35.01; IQR=10.33–26.68) since stopping selumetinib, demonstrating that many pLGG can have prolonged stability even after therapy cessation.

A recent study by Mustansir et al has noted:

Pilocytic astrocytomas (PAs) are the most common pediatric central nervous system tumors. They constitute around 30% of all primary central nervous tumors in the pediatric age group. Their clinical behavior may vary but most of them are indolent and do not undergo malignant transformations compared with their adult counterparts.

PAs are primarily treated with surgery and in cases of progression; chemotherapy may be needed.

They usually carry a good prognosis, with a 10-year survival rate of 90%. BRAFV600E mutations have been identified in approximately 9–15% of patients with PA.

These relatively high mutation frequencies in PA open avenues for treatment using targeted therapies such as BRAFV600E inhibitors (e.g., dabrafenib). There have been a few published case reports and case series showing clinical benefits with BRAF inhibitors in BRAF-positive tumors. We report a case of successful treatment of BRAFV600E immunopositive optic pathway PA in a child with dabrafenib.

Yasri and Wiwanitkit responded in kind:

We read the report on "Dabrafenib in BRAFV600E mutant pilocytic astrocytoma (PA) in a pediatric patient" with a great interest [1]. Mustansir et al. concluded that "We report a case of successful treatment of BRAFV600E immunopositive optic pathway PA in a child with dabrafenib." In fact, the good clinical advantage of dabrafenib for treatment of BRAFV600E immunopositive optic pathway PA is mentioned in many reports in clinical oncology. The basic molecular change due to BRAFV600E mutation can result in altered phenotypic expression including the response to treatment.

Based on standard molecular calculation technique as used in referencing publications, the molecular weight in BRAFV600E mutation is equal to − 28 g/Mol (from to 117.1 to 89.1 g/Mol in V to A variant).

Nevertheless, there are also other genetic factors that can determine the response to dabrafenib. In addition to studied genetic background of the tumor, some genetic polymorphisms of the patient might be associated with PA treatment. The good examples are TP53 codon 72 polymorphisms. Further studies to clarify possible confounding effects from other genetic factors are recommended.

Sigaud et al noted:

Pilocytic astrocytomas (PA) are low-grade gliomas (pLGG) and are the most frequent childhood brain tumors. They are characterized by oncogeneinduced senescence (OIS) initiated and sustained by senescence-associated secretory phenotype (SASP) factors. OIS and SASP in PA are thought to be driven by aberrations of the mitogen-activated protein kinase (MAPK) pathway (e.g. KIAA1549:BRAF fusion, BRAFV600E mutation, for the most common MAPK alterations occurring in PA), leading to its sustained activation. The MAPK pathway cascade is activated in a sequential manner:

1) ERK activation, which phosphorylates downstream partners in both cytoplasm and nucleus.

2) ERK-mediated induction of immediate early genes encoding transcription factors.

3) Induction of MAPK target genes expression.

4) Activation of downstream pathways.

Our aim is to unravel the molecular partners involved at each level of the sustained MAPK pathway activation in pLGG with different genetic backgrounds (KIAA1549:BRAF fusion and BRAFV600E mutation), and leading to the induction of OIS and SASP factors expression. pLGG cell lines DKFZ-BT66 (KIAA1549:BRAF) and BT-40 (BRAFV600E) were treated with the MEK inhibitor trametinib at key time points, and gene expression profile analysis was performed, allowing transcriptome analysis at each step of the MAPK cascade.

This will be combined with a whole proteomic and phospho-proteomic analysis. Combination of the transcriptome and proteome data layers will allow the identification of a) downstream

targetable partners activated by the MAPK pathway involved in PA senescence, b) new putative targets that might bring benefit in combination with MAPK inhibitors.

5.4.3 mTOR

Understanding rapamycin and its adjuncts is essential to understanding the actions of mTOR. We present a few summaries herein[22]. As Li et al noted:

Increased activation of mTORC1 is observed in numerous human cancers due to gain-offunction mutations in oncogenes (i.e., PI3K, AKT, or Ras) and/or loss-of-function mutations in tumor suppressors (i.e., PTEN, LKB1 or TSC1/2), upstream regulators of mTORC1. These mutations provide cancer cells with a selective growth advantage in comparison to normal cells. In order to meet the high demands of proliferation, cancer cells often have fundamental alterations in nutrient uptake and energy metabolism, processes that are directly controlled by the mTORC1 pathway.

Accordingly, in addition to driving protein synthesis, oncogenic activation of mTORC1 promotes a gene expression program that is involved in cancer cell metabolic reprogramming. Activation of mTORC1 promotes glycolysis via upregulation of Hypoxia-inducible factor alpha (HIF1α) and c-Myc; stimulates lipid biosynthesis and the pentose phosphate pathway through sterol regulatory element binding protein 1 (SREBP-1); and positively controls glutamine metabolism by SIRT4 repression.

Thus, drugs that selectively target mTORC1, like rapamycin, are expected to impair cancer metabolism and are considered promising anti-cancer therapies. The poor solubility and pharmacokinetics of rapamycin triggered the development of several rapamycin analogs (rapalogs).

Two water-soluble derivatives of rapamycin, temsirolimus and everolimus, were approved by the Food and Drug Administration (FDA) in 2007 and 2009, respectively, for the treatment of advanced renal cancer carcinoma (RCC). In 2011, the FDA approved the use of everolimus for patients with progressive neuroendocrine tumors of pancreatic origin (PNET). Additionally, temsirolimus was evaluated in several clinical trials for the treatment of advanced neuroendocrine carcinoma (NEC), advanced or recurrent endometrial cancer, and relapsed or refractory mantle cell lymphoma.

Moreover, a few trials of everolimus were conducted in patients with advanced gastric cancer, advanced non-small cell lung cancer (NSCLC), and advanced hepatocellular carcinoma. Ridaforolimus, a rapamycin analog, was also examined in clinical trials for advanced bone and soft-tissue sarcomas as well as a variety of advanced solid tumors.

Overall, however, ***rapalogs have only achieved modest effects in major solid tumors in the clinic. The reasons for the limited clinical success of rapalogs have not been established, but are likely related to the large number of mTORC1-regulated negative feedback loops that***

[22] https://www.researchgate.net/publication/338412510_mTOR_Target_of_Opportunity

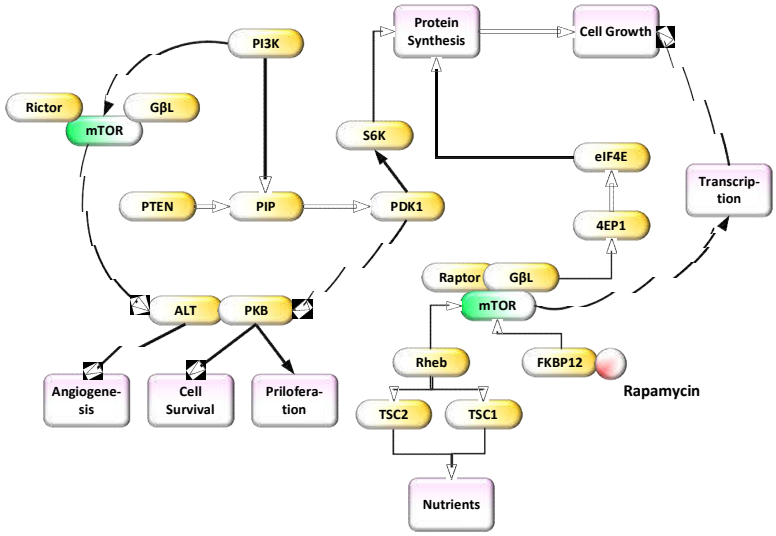
suppress upstream signaling systems such as activation of receptor tyrosine kinases*, PI3K-Akt signaling and Ras-ERK pathway and which can be re-activated with rapamycin (discussed more extensively below). In order to overcome these limitations, alternative strategies have been explored in the past few years.*

The feedback loops are partially displayed herein. The mTOR complexes are indeed in complex loops and one suspects they may be even more complex than we have currently displayed them.

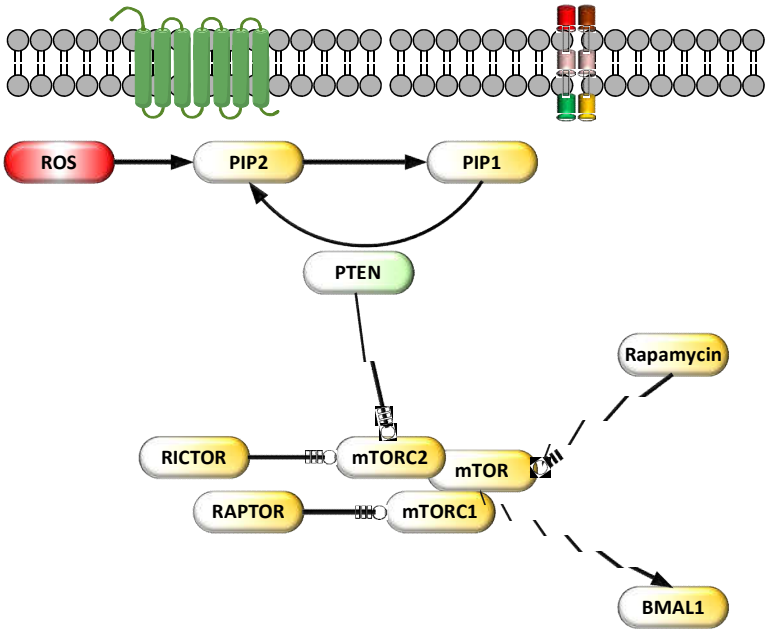
For instance, a number of ATP-competitive mTOR inhibitors have been developed, blocking both mTORC1 and mTORC2 activity. *Due to high sequence homology shared between mTOR and PI3K, some compounds that were originally identified as PI3K inhibitors were later shown to inhibit mTOR. Unlike rapamycin, which is a specific allosteric inhibitor of mTORC1, these ATP-competitive inhibitors target the catalytic site and prevent the feedback-mediated PI3K/Akt activation (described below), and therefore can potentially offer broader, more potent and sustained mTOR inhibition.*

5.4.3.1 mTOR Pathways

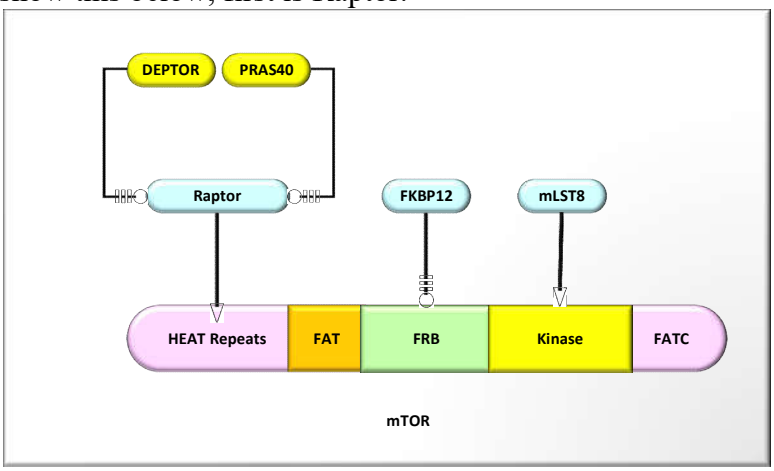
We now consider the mTOR pathways. These are the effecting paths, namely those for which mTOR has influence on cell behavior. We show mTOR with separate Raptor and Rictor conjugates. As noted above they are the fundamental elements of the mTORC1 and mTORC2 conjugates. We shall examine these pathways for several malignancies. The full pathway exposition is shown below:



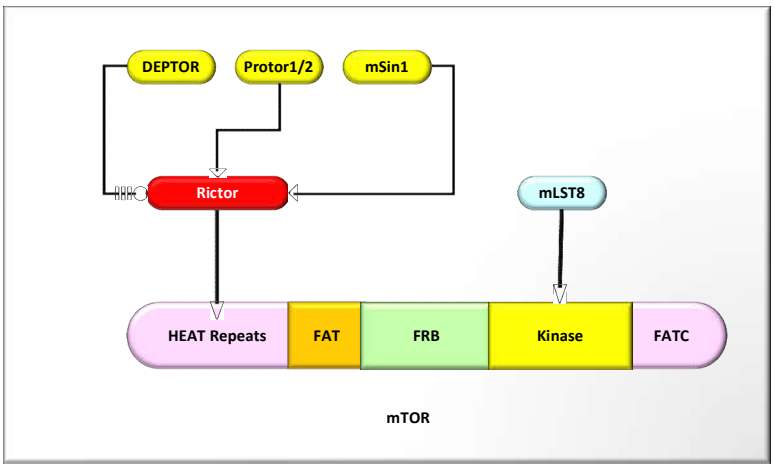
Activation by a reactive oxygen species, ROS, is shown below:



The gene products Rictor and Raptor combine the separate mTOR elements to the combined mTOR result. We show this below, first is Raptor:



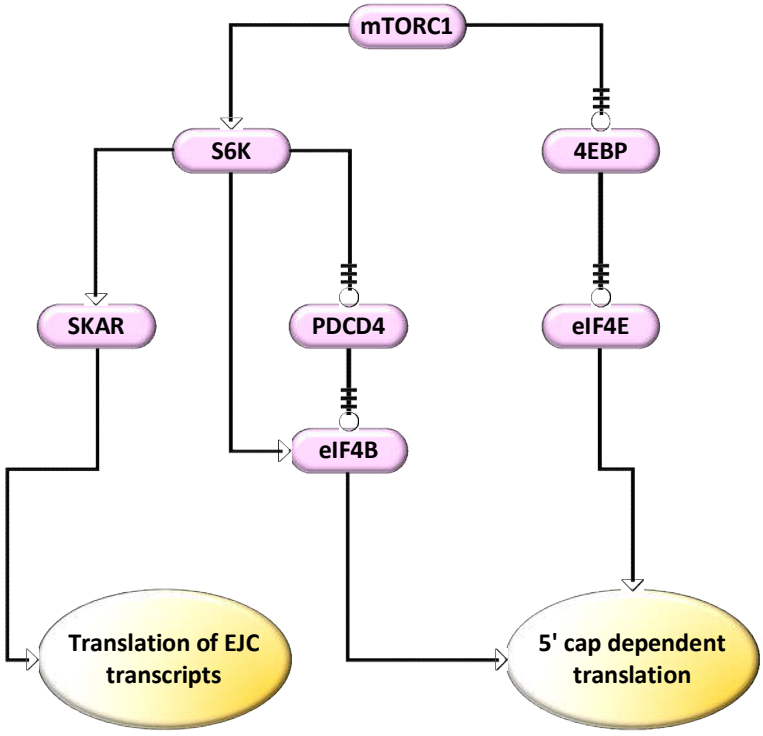
Then we show Rictor:



We have briefly stated the drivers of the mTOR complexes and their resultant impact processes. The specific details are generally understood but like many of these processes there are specifics which are yet to be detailed. We now provide some of the specifics as currently understood for three major functions.

5.4.3.2 RNA Translation

Let us first consider the impact on RNA translation. The figure below demonstrates several of the steps in this process.



Saxton and Sabatini remark on the above as follows:

mTORC1 promotes protein synthesis largely through the phosphorylation of two key effectors, p70S6 Kinase 1 (S6K1) and eIF4E Binding Protein (4EBP. mTORC1 directly phosphorylates S6K1 on its hydrophobic motif site, Thr389, enabling its subsequent phosphorylation and activation by PDK1. S6K1 phosphorylates and activates several substrates that promote mRNA translation initiation, including eIF4B, a positive regulator of the 50cap binding eIF4F complex.

S6K1 also phosphorylates and promotes the degradation of PDCD4, an inhibitor of eIF4B, and enhances the translation efficiency of spliced mRNAs via its interaction with SKAR, a component of exon-junction complexes.

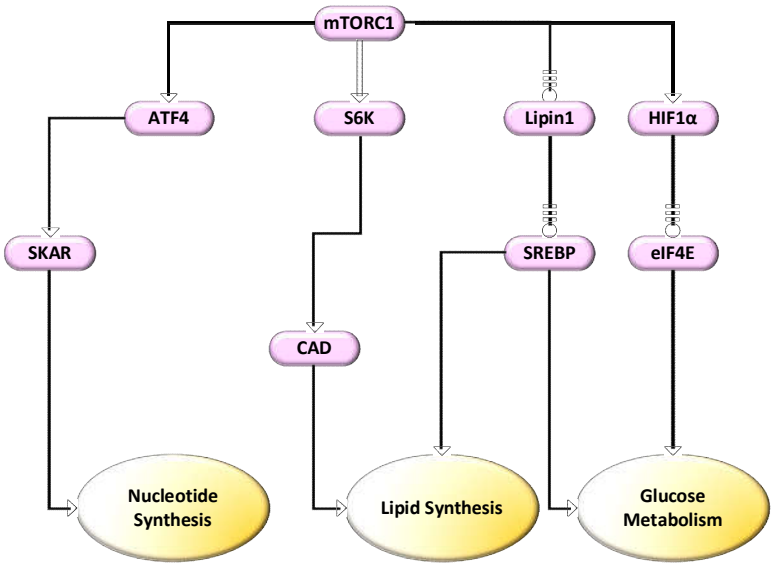
The mTORC1 substrate 4EBP is unrelated to S6K1 and inhibits translation by binding and sequestering eIF4E to prevent assembly of the eIF4F complex. mTORC1 phosphorylates 4EBP at multiple sites to trigger its dissociation from eIF4E, allowing 50cap-dependent mRNA translation to occur.

Although it has long been appreciated that mTORC1 signaling regulates mRNA translation, whether and how it affects specific classes of mRNA transcripts has been debated. Global ribosome footprinting analyses, however, revealed that, while acute mTOR inhibition moderately suppresses general mRNA translation, it most profoundly affects mRNAs containing pyrimidine-rich 50 TOP or ‘‘TOP-like’’ motifs, which includes most genes involved in protein synthesis

The above complex network is significant in that mTORC1 has substantial but intricate controls over the RNA translation.

5.4.3.3 Metabolism

Finally we can show how mTORC1 also plays a significant role in cellular metabolism. These are shown below.



Again, as Saxton and Sabatini note:

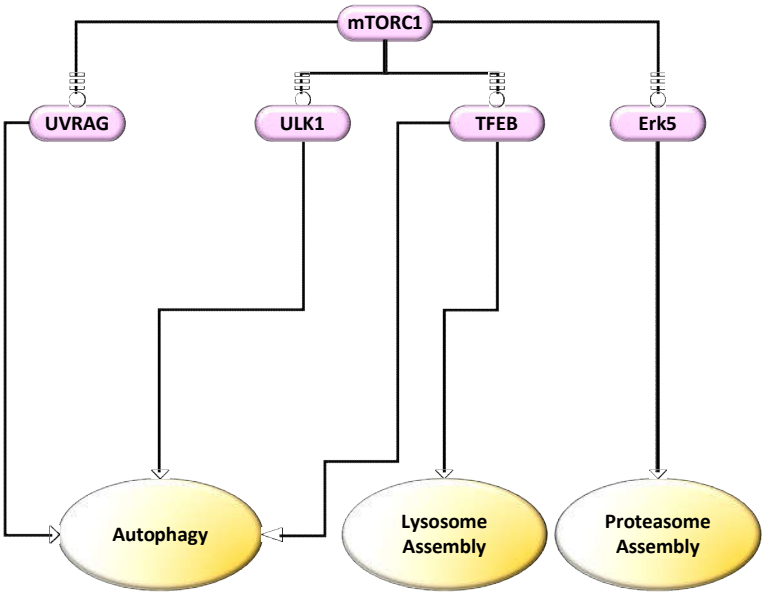
Recent studies established that mTORC1 also promotes the synthesis of nucleotides required for DNA replication and ribosome biogenesis in growing and proliferating cells. mTORC1 increases the ATF4-dependent expression of MTHFD2, a key component of the mitochondrial tetrahydrofolate cycle that provides one carbon units for purine synthesis.

Additionally, S6K1 phosphorylates and activates carbamoyl-phosphate synthetase (CAD), a critical component of the de novo pyrimidine synthesis pathway. mTORC1 also facilitates growth by promoting a shift in glucose metabolism from oxidative phosphorylation to glycolysis, which likely facilitates the incorporation of nutrients into new biomass. mTORC1 increases the translation of the transcription factor HIF1a which drives the expression of several glycolytic enzymes such as phospho-fructo kinase (PFK).

Furthermore, mTORC1- dependent activation of SREBP leads to increased flux through the oxidative pentose phosphate pathway (PPP), which utilizes carbons from glucose to generate NADPH and other intermediary metabolites needed for proliferation and growth.

5.4.3.4 Protein Control

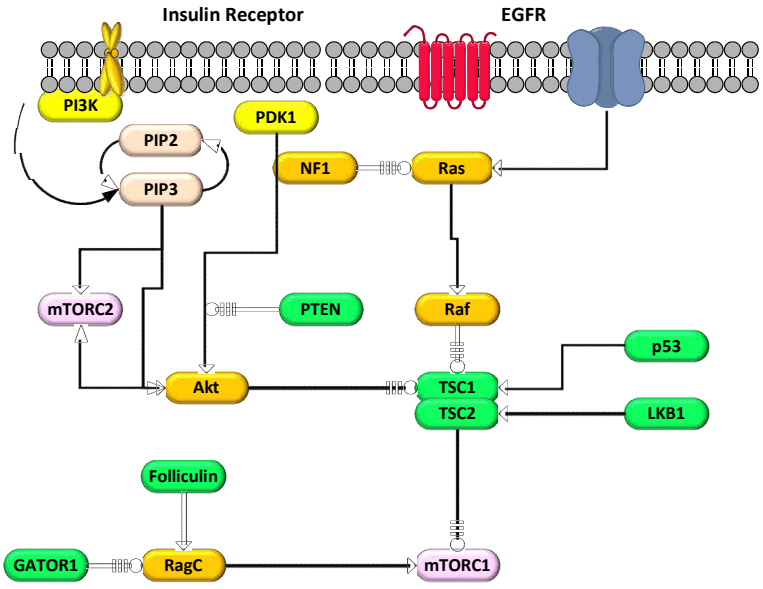
A third major control mechanism is that of proteins. We demonstrate this below:



Autophagy is necessary for homeostasis and loss of that control can be a key element in metastatic growth. Lysosome and proteasome assembly are also key elements in cell progression.

5.4.3.5 mTOR Controlled Cancers

As can be seen from the above, mTOR and its complexes play a variety of roles in cell homeostasis. Any significant disruption of those processes may lead to the development of a variety of cancers. The major mTOR complex pathways are shown below:



We know from the study of many cancers that products like PTEN, RAF/BRAF, p53 and others can control malignant behavior. However we also know from above that activation of the mTOR conjugates have significant downstream effects. Thus one of the issues we are concerned with is what drives the process? As Saxton and Sabatini have noted:

As discussed above, mTORC1 functions as a downstream effector for many frequently mutated oncogenic pathways, including the PI3K/Akt pathway as well as the Ras/Raf/Mek/ Erk (MAPK) pathway, resulting in mTORC1 hyperactivation in a high percentage of human cancers.

Namely mTOR per se does not have to be mutated but that the genes preceding it upstream when mutated can make mTOR over activated.

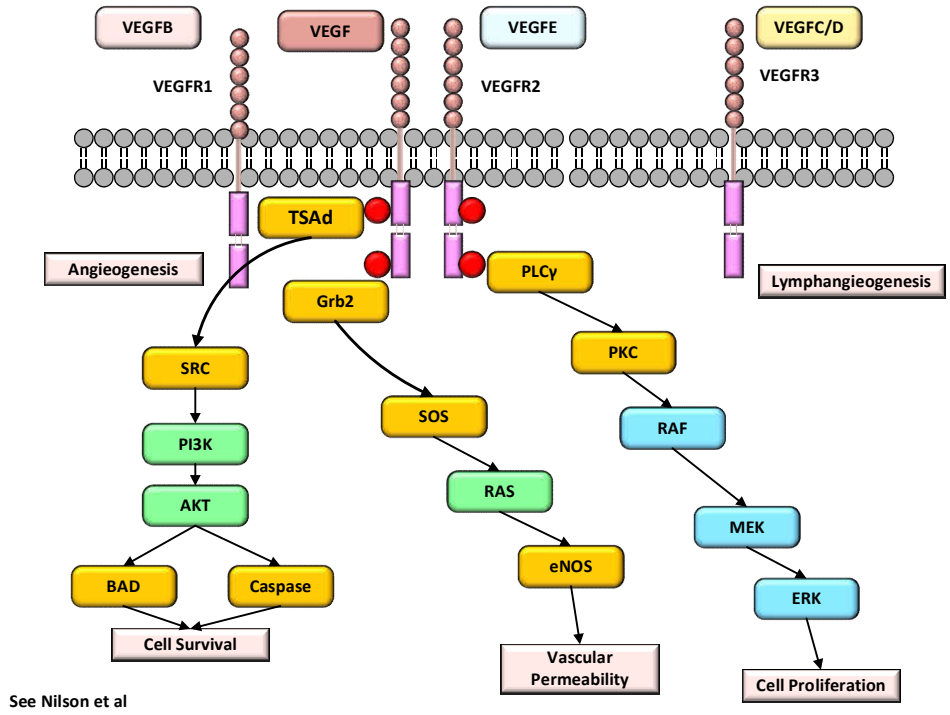
5.4.4 VEGF

From Nilson and Heymach:

VEGF is now known to be the prototypic member of a family of structurally related dimeric proteins including VEGF-B, VEGF-C, VEGF-D, and VEGF-E, as well as placental-growth factor (PlGF) -1 and -2. VEGF is essential for development because homozygous or heterozygous deletion of the VEGF gene is embryonically lethal. Indeed, VEGF family members are important in physiological angiogenic processes in the adult including wound healing, ovulation, and pregnancy, as well as pathological conditions such as cancer.

VEGF ligands activate angiogenic programs through binding of several receptors. VEGFR-1 (Flt-1) binds VEGF, VEGF-B, and PlGF -1,2 and promotes recruitment of endothelial progenitors and monocyte migration. VEGFR-2 (Flk-1/KDR) is expressed on nearly all endothelial cells and binds VEGF, VEGF-C, VEGF-D, and VEGF-E.

Signal transduction through VEGFR2 has been shown to regulate endothelial cell proliferation, migration, and survival. In healthy adults, expression of VEGFR-3 is limited to lymphatic endothelium, although VEGFR-3 may also be expressed on tumor-associated blood vessels. Through binding to VEGF-C and VEGF-D, VEGFR-3 is thought to facilitate the outgrowth of lymphatic vessels. Neuropilin (NRP)-1,2 have been demonstrated to be coreceptors for VEGF. NRP-1 binds VEGF165 and PlGF, and NRP-2 binds VEGF165 and VEGF-C.4 Unlike other VEGFRs, NRP-1,2 lack intracellular signaling domains. Although the specific role of NRP-1,2 in angiogenesis is not fully known, NRP-1,2 bind VEGF ligands and enhance their affinity to other VEGFRs.



From Nilson and Keymach:

Agent	Target within VEGF pathway	Type	Company
Bevacizumab	VEGF	mAB	Genentech
VEGF trap	VEGF	EC receptor domain	Regeneron
IMC-1121B	VEGFR-2	mAb	Imclone
Sunitinib	VEGFR-1,2,3	RTKI	Pfizer
Sorafenib	VEGFR-2,3	RTKI	Bayer
ZD6474,	VEGFR-2,3	RTKI	AstraZeneca
AZD2171	VEGFR-1,2,3	RTKI	AstraZeneca
Vatalinib	VEGFR-2	RTKI	Novartis
AMG706	VEGFR-1,2,3	RTKI	Amgen
Ag-013736	VEGFR-1,2,3	RTKI	Pfizer

As Duffy et al note :

Vascular Endothelial Growth Factor (VEGF) is a major contributor to the growth of malignant tumors of the central nervous system. It stimulates tumor angiogenesis and vascular proliferation characteristic of high grade gliomas.

Elevated expression of VEGF is one the factors responsible for the virulent nature of these tumors. The production of VEGF by malignant glial cells in response to ionizing radiation contributes to treatment failure. The rat C6 glioma is similar to human gliomas with respect to VEGF pathophysiology. Interruption of VEGF-Receptor signaling in preclinical models effectively suppresses tumor growth and demonstrates the potential for anti-angiogenic therapy….

Intact VEGF-Receptor signaling is required for maturation of the central nervous system (CNS). Mutant mice heterozygous for VEGF die in utero and develop multiple anomalies including failure of vascularization of the neuroepithelium, disorganization of neuroepithelial cells, and underdevelopment of the forebrain.1 A single mutant allele can bring this about.

In mature brain tissue VEGF is distributed in areas surrounding the microvasculature where it may assist in maintaining the differentiated state.2 VEGF is also produced in response to CNS trauma. In response to cold thermal injury, VEGF isoform A is upregulated in astrocytes, inflammatory cells, and neovascular endothelium in the rat brain. Increased production of VEGF mRNA was demonstrated as early as six hours after injury by in situ hybridization …

High grade gliomas are incurable by current methods of treatment. They possess the ability to regenerate by mounting a vigorous angiogenic response. VEGF is central to the process. It is the main "accelerant" which fuels tumor growth before and after conventional treatment. In preclinical models, blockade of VEGF-receptor signaling disrupts angiogenesis which causes tumor shrinkage and growth delay. The magnitude of the effect is impressive. However, secondary growth factors (FGF's, PDGF, TGF etc) capable of stimulating angiogenesis are operative in high grade glioma. They can drive the angiogenic engine in the face of VEGF blockade.

Now Melincovici et al note:

Angiogenesis is an extremely complex process, influenced by multiple factors, some of them acting as proangiogenic agents, others as inhibitors of angiogenesis.

An extremely potent pro-angiogenic factor is vascular endothelial growth factor (VEGF) and, for this reason, there are numerous studies that demonstrated its implication in angiogenesis. During the embryonic period, the formation of new vessels occurs by the differentiation of endothelial cells from hemangioblasts (vasculogenesis).

Later, after birth, in certain physiological processes (menstrual cycle, pregnancy, wound healing and repair, etc.), new vascular networks are formed by angiogenesis, based on preexisting vessels (neoangiogenesis).

At the same time, data suggests that VEGF plays an important role in pathological angiogenesis, inducing the development and progression of certain pathological conditions in the postnatal period, such as: tumor growth and metastasis, macular degeneration, diabetic retinopathy, inflammatory processes (e.g., rheumatoid arthritis), ischemic processes (myocardial ischemia), preeclampsia, etc..

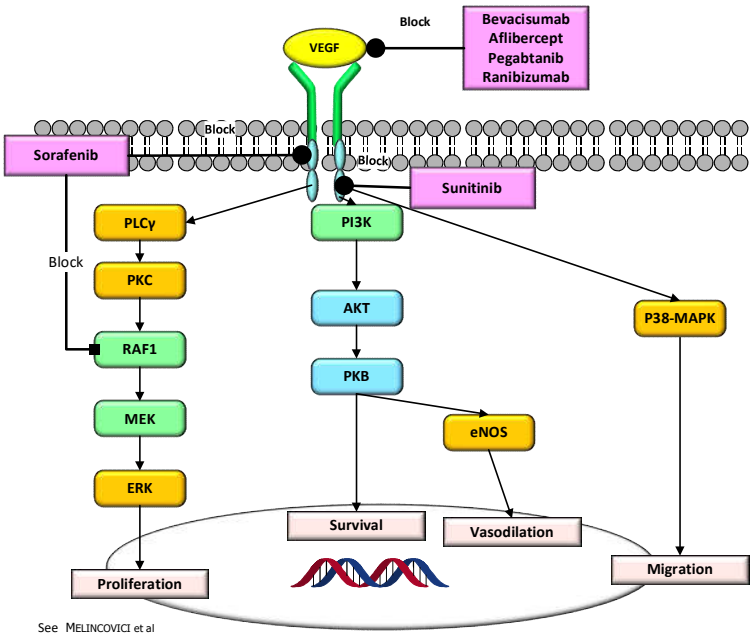
At present, increased attention is focused on the process of formation and development of certain new lymphatic vessels (lymphangiogenesis) The human VEGF gene, located on the 6p21.3 chromosome, is part of the VEGF/platelet-derived growth factor (PDGF) gene family, also called the cystine-knot superfamily of growth factors. From a structural point of view, VEGF is a 40-kDa heterodimeric glycoprotein, which contains the cystine-knot motif, characterized by the disposition of certain bisulfidic bridges in the protein structure.

Alongside VEGF, there are additional growth factors from the cystineknot motif family: PDGF, nerve growth factor (NGF) and transforming growth factor-beta (TGF-β). In humans, the VEGF family includes several members that perform various functions:

VEGF-A (which presents several isoforms), VEGF-B, VEGF-C, VEGF-D, VEGF-E (viral VEGF, in parapoxvirus 1), VEGF-F (snake venom VEGF) and the placenta growth factor (PlGF).

More recently, a new member has been added to this family, named the endocrine gland-derived vascular endothelial growth factor (EG-VEGF)

The authors then present putative therapeutics and targets:



5.5 METHYLATION

We discussed methylation earlier. Here we focus on methylation and PA. Now from Sexton-Oates et al:

Childhood pilocytic astrocytomas (PA) are low-grade tumours with an excellent prognosis. However, a minority, particularly those in surgically inaccessible locations, have poorer long-term outcome.

At present, it is unclear whether anatomical location in isolation, or in combination with underlying biological variation, determines clinical behaviour. Here, we have tested the utility of DNA methylation profiling to inform tumour biology and to predict behaviour in pediatric PA.

Genome-wide DNA methylation profiles were generated for 117 pediatric PAs. Using a combination of analyses, we identified DNA methylation variants specific to tumour location and predictive of behaviour. Receiver-operating characteristic analysis was used to test the predictive utility of clinical and/or DNA methylation features to classify tumour behaviour at diagnosis.

Unsupervised analysis distinguished three methylation clusters associated with tumour location (cortical, midline and infratentorial). Differential methylation of 5404 sites identified enrichment of genes involved in 'embryonic nervous system development'.

Specific hypermethylation of NEUROG1 and NR2E1 was identified as a feature of cortical tumours. A highly accurate method to classify tumours according to behaviour, which combined three clinical features (age, location and extent of resection) and methylation level at a single site, was identified. Our findings show location-specific epigenetic profiles for PAs, potentially reflecting their cell type of origin. This may account for differences in clinical behaviour according to location independent of histopathology.

As Antonelli et al note :

Pilocytic astrocytoma (PA) is a pediatric low-grade glioma (pLGG) and the most common pediatric brain tumor, accounting about for 18% of all pediatric brain tumors and mostly affecting children between 5–15 years of age.

It can arise anywhere in the CNS, but is most commonly localized in the cerebellum followed by the optic pathway/hypothalamic region. It is classified as grade I by the World Health Organization (WHO), reflecting their slow growth and typically non-invasive behavior.

Pilocytic astrocytomas typically contain a BRAF fusion but occasionally a BRAF V600E mutation, RAF1 fusion, intragenic duplication of FGFR1, or other rarer alterations are present. Childhood pilocytic astrocytomas (PA) are low grade tumours with an excellent prognosis.

However, PA can cause extensive morbidity due to local tumor expansion or therapy-related side effects and recurrence or progressive disease (PD), which occurs in up to 80% of patients, depending on location and extent of initial resection.

Therefore, new therapies are needed in order to specifically target the disease and improve the clinical course of these patients.

Epigenetic biomarkers represent a promising area of research, with DNA methylation having the potential to provide information regarding physiological and pathological status.

Methylation signatures can be useful as specific and accurate biomarkers to assist with prognosis.

The aims of the present work were to define biologically distinct groups of PA and possible relevant biomarkers through a global DNA methylation analysis over 27K CpG loci, and to

assess the impact of methylation alterations on gene expression by qRT-PCR. We identified distinct methylation profiles characterizing PAs from different locations (infratentorial vs supratentorial) and tumors with onset before (≤ 3 yrs.) and after (> 3 yrs.) 3 years of age. Our study also identified IRX2 as possible topographical biomarker and TOX2 as tumoral biomarker.

As Sippl et al note :

The pathognomonic molecular characteristic of PAs in pediatric patients is a KIAA1549-BRAF fusion transcript, resulting from a somatic duplication of 7q34. Mutations of the proto-oncogene B-Raf (BRAF V600E mutation) are found in less than 10% of tumors (10,11). However, additional genetic alterations can be present in the relatively uncommon case of PAs in adult patients.

1. ***The main genetic alterations in PAs in adult patients is a***
2. ***KIAA1549-BRAF fusion transcript, found in 20-32% of cases;***
3. ***FGFR1 mutation; and***
4. ***the absence of BRAF V600E mutation.***
5. ***Moreover, IDH1 R132H mutation might play a more important role in adult PAs.***

In case of NF1 mutation, PAs may involve the optic pathways, optic nerve, and chiasm. A review of the literature on adult PAs has shown that most cases remain genetically uncharacterized. Therefore, the question remains whether additional molecular markers can be found at an epigenetic level to help predict the clinical course of the disease. The best studied epigenetic modification is DNA methylation.

In this process, methyl groups are covalently attached to CpG islands in the promoter regions of genes by DNA methyltransferase, resulting in the suppression of transcription. These CpG islands exist in approximately 40% of the promoter regions found in humans. However, not all CP dinucleotides are CpG islands that can be methylated. The methylation status of P15, P16, RB1, and MGMT has been shown to be important in the oncogenesis of WHO grade II-IV gliomas.

P15, P16, and RB1 play a crucial role in the cell cycle as tumor suppressors and influence progression and prognosis in glial tumors.

P15 and p16 can bind and therefore inhibit CDK4 and CDK6. Inactive CDK4 and CDK6 are responsible for the hypophosphorylated status of RB1, resulting in cell arrest.

Therefore, p15 and p16 act as tumor suppressors in the late G1 phase (22).

Mutations of and deletions in RB1, P15, and P16 are among the most frequently observed genetic alterations in glial tumors and can result in a more aggressive biological behavior of the

tumor (23-26). MGMT is a DNA repair protein that removes alkyl groups and adducts at the O6 position of guanine.

It protects healthy cells against mutagenic effects, and loss of expression due to MGMT promoter hypermethylation has been proposed as a predisposing factor for the acquisition of TP53 transition mutations in oncogenesis. MGMT hypermethylation is associated with a significantly shorter progression-free survival (PFS) in patients with breast cancer and low-grade astrocytomas. MGMT can also protect cells with high-grade astrocytomas against the cytotoxic effects of alkylating chemotherapeutic agents. The question arises whether specific methylation patterns of these genes also correlate with the clinical course of PAs as WHO grade I neoplasias.

We hypothesize that in PAs, promoter methylation of P15, P16, RB1, and MGMT results in a higher frequency of relapses with a reduced PFS and overall survival (OS). Furthermore, we expect to find different specific methylation patterns in adult and pediatric Pas.

Jayapalan et al note:

Low-grade gliomas (LGGs) account for about a third of all brain tumours in children. We conducted a detailed study of DNA methylation and gene expression to improve our understanding of the biology of pilocytic and diffuse astrocytomas.

Pilocytic astrocytomas were found to have a distinctive signature at 315 CpG sites, of which 312 were hypomethylated and 3 were hypermethylated. Genomic analysis revealed that 182 of these sites are within annotated enhancers. The signature was not present in diffuse astrocytomas, or in published profiles of other brain tumours and normal brain tissue. The AP-1 transcription factor was predicted to bind within 200 bp of a subset of the 315 differentially methylated CpG sites; the AP-1 factors, FOS and FOSL1 were found to be up-regulated in pilocytic astrocytomas.

We also analysed splice variants of the AP-1 target gene, CCND1, which encodes cell cycle regulator cyclin D1. CCND1a was found to be highly expressed in both pilocytic and diffuse astrocytomas, but diffuse astrocytomas have far higher expression of the oncogenic variant, CCND1b. These findings highlight novel genetic and epigenetic differences between pilocytic and diffuse astrocytoma, in addition to well-described alterations involving BRAF, MYB and FGFR1. ...

In summary, pilocytic astrocytomas contain a hypomethylation signature characterised by CpG sites which are located predominantly in annotated enhancers.

This signature is specific to pilocytic astrocytomas and is not present in diffuse astrocytomas, other brain tumours or normal brain tissue.

The AP-1 transcription factor complex, activated by the MAPK pathway, is predicted to bind at a number of these CpG sites, and FOS transcription factors are up-regulated in pilocytic

astrocytomas. Our findings highlight epigenetic differences between pilocytic and diffuse astrocytoma, in addition to the well-documented genomic alterations.

5.6 CELL CYCLE

Cancer is basically uncontrolled cell growth, replication, and failure for cells to die off, normal apoptosis. It may also include loss of location stability and metabolic enhancement, but let us start with the key issue, replication. PA is initially a proliferation disorder. It is in a sense an uncontrolled cell cycle problem. We examine the cell cycle and understand points of therapeutic address. However there does not seem to be any direct approach to PA mitigation via this path.

Cancer in many ways is a loss of the three factors:

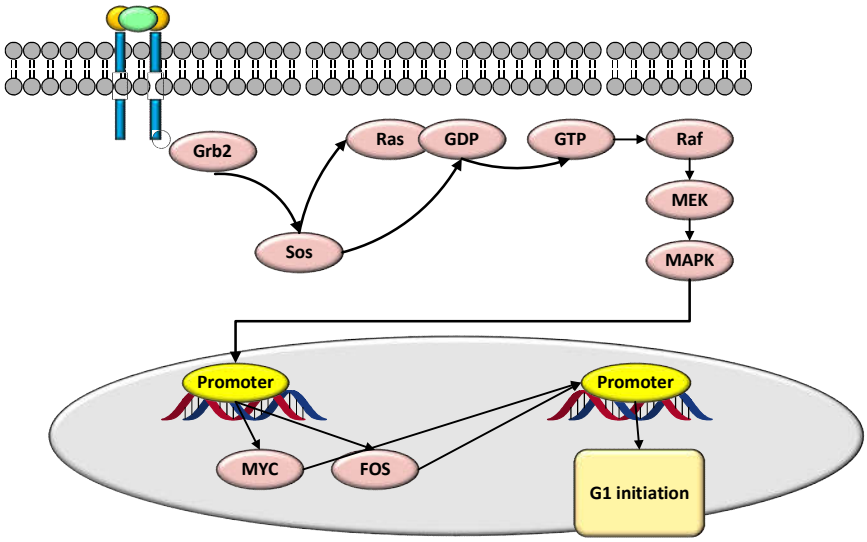
1. Cell Replication: This is the normal or abnormal cell cycle.

2. Cell Death: This is normal cell death or apoptosis.

3. Cell Localization: The establishment and maintenance of a cells relative position and function.

We address cell replication. First we examine the cell cycle from a generic perspective. We then examine the details on the pathways which may result in unstable cell reproduction.

The classic cell replication cycle goes through 4 stages. The dormant stage, G0, is not part of this process. The drivers of the cell cycle are also what we have been discussing. As Morgan notes, many mitogenic signalling pathways begin with the activation of Ras at the cell membrane. We have demonstrated several of these above. We show the totality of the process below based upon Morgan (p. 209).



The above present some of the basic details in the cell cycle. We again refer to Morgan for the specifics. But simply:

1. A receptor is activated by some ligand.

2. It is then phosphorylated thus activating Grb2

4. Sos then is activated which activates the Ras GDP proteins.

5. GDP is transformed to GTP

6. GTP activates the Raf/Raf/MEK kinase pathway

7. A promoter is activated in the nucleus which activates MYC and FOS

8. MYC/FOS activate another promoter which in turn activates the G1 process as we shall describe.

It is this set of sequences we see in PA that results in proliferation, the continue mitotic growth. The therapeutic approaches to stop proliferation than can possibly attack a wide set of targets as we have discussed.

The stages in cell reproduction are:

G0: This is the resting phase. It is during this phase that the cell is producing proteins via normal transcription processes. G0 may be resting related to the reproductive mitotic activities but the cell is quite active as a protein generating factory.

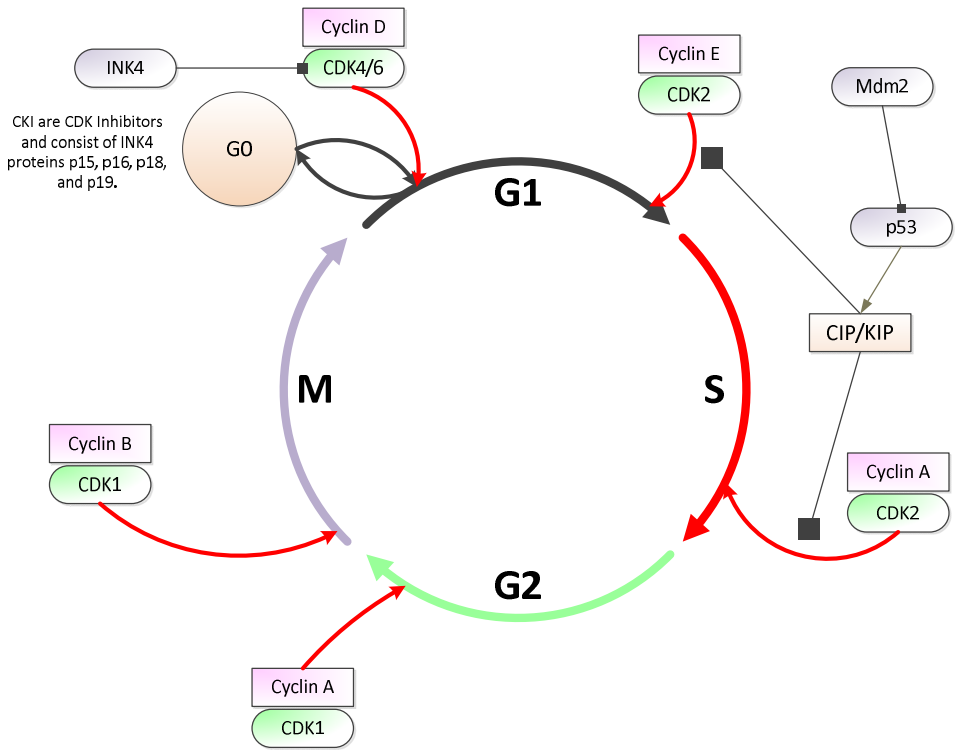
G1: Once the cell begins the G1 phase it is on its way to reproducing via mitosis.

S: The S phase is the phase where the DNA is duplicated. This is a sensitive stage; any error here can be propagated forward albeit there may still be checks available.

G2: This is the second gap phase.

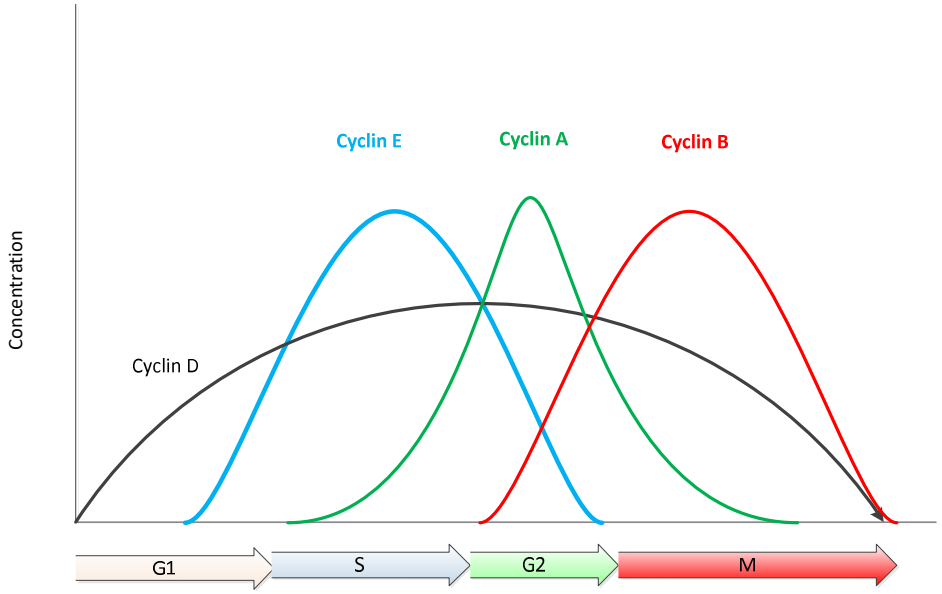
M: M phase includes mitosis and cytokinesis, namely the creation of two identical new cells.

Now the cell starts G1 by being instigated by a bound pair of a cyclin and a CDK, a cyclin dependent kinase. In this specific case we start with a binding of cyclin D and CDK4/6. This is the initiating event moving into G1 from senescence in G0. We depict these processes below:



The cyclins in each stage grow in concentration and as such move the cell along in each of its reproductive stages.

The following shows the phases and the relevant concentrations of cyclin bound to CDKs. Note the increase in concentration activates a change or movement along the mitotic path.



Note in the above the concentration of a specific cyclin above a level of a previous cyclin initiates the next step in mitosis.

Protein[23]	***Gene***	***Function***[24]
Cyclin A (also CCN1; CCNA, CCNA2, Cyclin A2)	4q25-q31	The protein encoded by this gene belongs to the highly conserved cyclin family, whose members are characterized by a dramatic periodicity in protein abundance through the cell cycle. Cyclins function as regulators of CDK kinases.
Cyclin B1 (CCNB1)	5q12	The protein encoded by this gene is a regulatory protein involved in mitosis. The gene product complexes with p34(cdc2) to form the maturation-promoting factor (MPF).
Cyclin B2 (CCNB2)	15q22.2	Cyclin B2 is a member of the cyclin family, specifically the B-type cyclins. The B-type cyclins, B1 and B2, associate with p34cdc2 and are essential components of the cell cycle regulatory machinery.
Cyclin C (CCNC)	6q21	The protein encoded by this gene is a member of the cyclin family of proteins. The encoded protein interacts with cyclin-dependent kinase 8 and induces the phosphorylation of the carboxy-terminal domain of the large subunit of RNA polymerase II.
Cyclin D (Cyclin D1)	11q13	The protein encoded by this gene belongs to the highly conserved cyclin family, whose members are characterized by a dramatic periodicity in protein abundance throughout the cell cycle. Cyclins function as regulators of CDK kinases.
Cyclin E (CCNE1)[25]	19q12	The protein encoded by this gene belongs to the highly conserved cyclin family, whose members are characterized by a dramatic periodicity in protein abundance through the cell cycle. Cyclins function as regulators of CDK kinases. Different cyclins exhibit distinct expression and degradation patterns which contribute to the temporal coordination of each mitotic event. This cyclin forms a complex with and functions as a regulatory subunit of CDK2, whose activity is required for cell cycle G1/S transition.

The CDKs involved are:

Protein[26]	***Gene***	***Function***[27]
CDK 1 (also known as CDC2; CDC28A; P34CDC2)	10q21.1	This protein is a catalytic subunit of the highly conserved protein kinase complex known as M-phase promoting factor (MPF), which is essential for G1/S and G2/M phase transitions of eukaryotic cell cycle. Mitotic cyclins stably associate with this protein and function as regulatory subunits.

[23] http://www.ncbi.nlm.nih.gov/gene/983

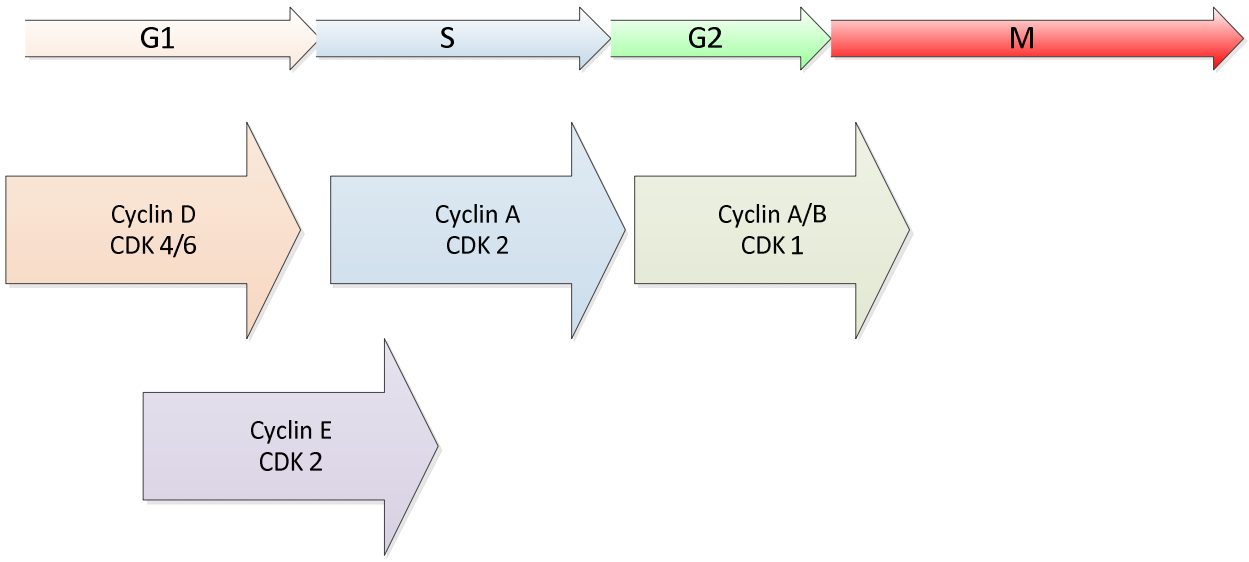
[24] From http://www.ncbi.nlm.nih.gov/gene/595 data bases as a source.

[25] http://www.ncbi.nlm.nih.gov/gene/898
[26] http://www.ncbi.nlm.nih.gov/gene/983

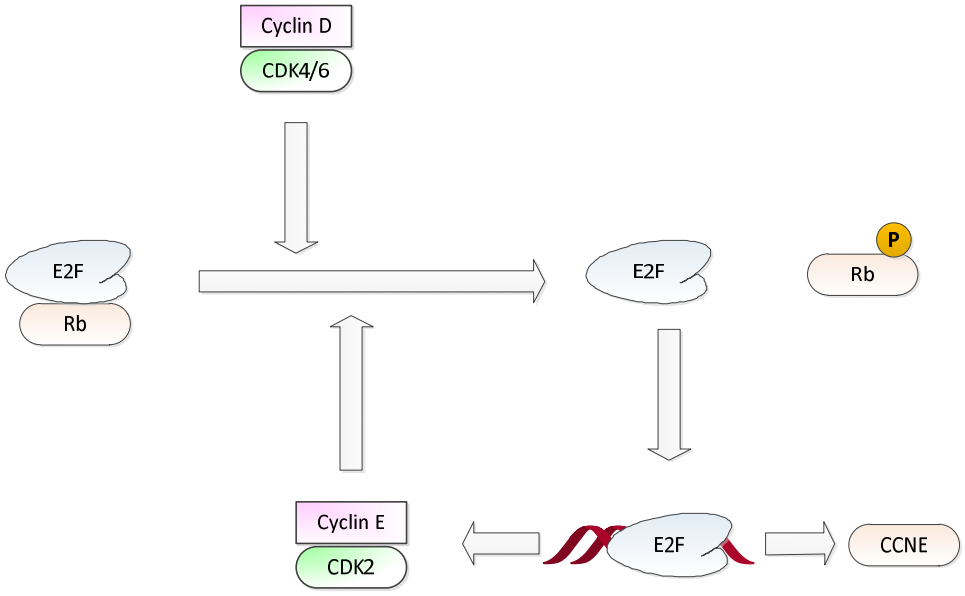
[27] From http://www.ncbi.nlm.nih.gov/gene/595 data bases as a source.

Protein[26]	*Gene*	*Function*[27]
CDK 2 (also called p33)	12q13	It is a catalytic subunit of the cyclin-dependent protein kinase complex, whose activity is restricted to the G1-S phase, and essential for cell cycle G1/S phase transition.
CDK 3	17q22	This gene encodes a member of the cyclin-dependent protein kinase family. The protein promotes entry into S phase, in part by activating members of the E2F family of transcription factors.
CDK 4 (also CMM3; PSK-J3)	12q14	This protein is a catalytic subunit of the protein kinase complex that is important for cell cycle G1 phase progression. The activity of this kinase is restricted to the G1-S phase, which is controlled by the regulatory subunits D-type cyclins and CDK inhibitor p16(INK4a). This kinase was shown to be responsible for the phosphorylation of retinoblastoma gene product (Rb). Mutations in this gene as well as in its related proteins including D-type cyclins, p16(INK4a) and Rb were all found to be associated with tumorigenesis of a variety of cancers.
CDK 6 (also PLSTIRE)	7q21-22	The protein encoded by this gene is a member of the cyclin-dependent protein kinase (CDK) family. CDK family members are known to be important regulators of cell cycle progression. … Expression of this gene is up-regulated in some types of cancer.

Now the question is what activates these proteins, the cyclins and the CDKs, to make the cell cycle progress. This begins the creep upward in this pathway concern. We can redraw this process as follows and it will help to focus:



Now we ask what activates these proteins. We look at the activation of Cyclin E as shown by Bunz (p 219) below:



This is a feedback type reaction initiated by Rb the retinoblastoma gene protein. This feedback generates cyclin E which drives the cell through G1 and into the S cycle.

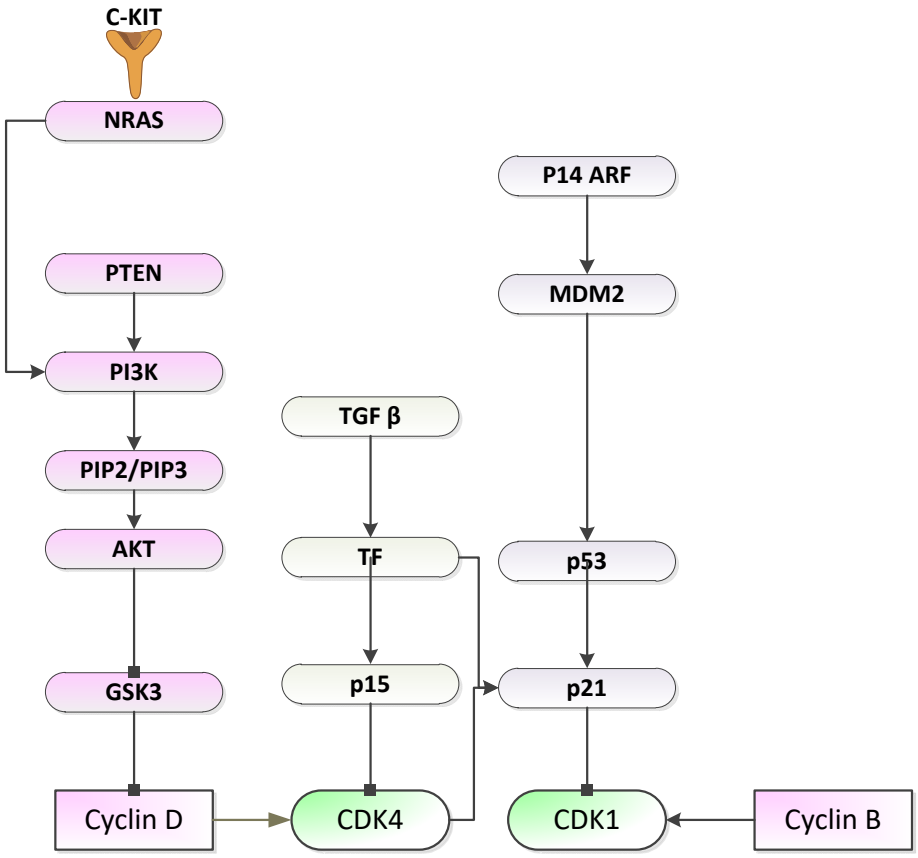
Gene	*Location*	*Function*
E2F1[28] (also RBP3; E2F-1; RBAP1; RBBP3)	20q11.2	The protein encoded by this gene is a member of the E2F family of transcription factors. The E2F family plays a crucial role in the control of cell cycle and action of tumor suppressor proteins and is also a target of the transforming proteins of small DNA tumor viruses.
RB 1[29] (also RB; pRb; OSRC; pp110; p105-Rb)	13q14.2	The protein encoded by this gene is a negative regulator of the cell cycle and was the first tumor suppressor gene found. The encoded protein also stabilizes constitutive heterochromatin to maintain the overall chromatin structure.
CCNE1[30]	19q12	The protein encoded by this gene belongs to the highly conserved cyclin family, whose members are characterized by a dramatic periodicity in protein abundance through the cell cycle. Cyclins function as regulators of CDK kinases. Different cyclins exhibit distinct expression and degradation patterns which contribute to the temporal coordination of each mitotic event. This cyclin forms a complex with and functions as a regulatory subunit of CDK2, whose activity is required for cell cycle G1/S transition.

Now this establishes one base line for understanding cancer at the base of cell reproduction. Namely what can cause this process to continue unabated?

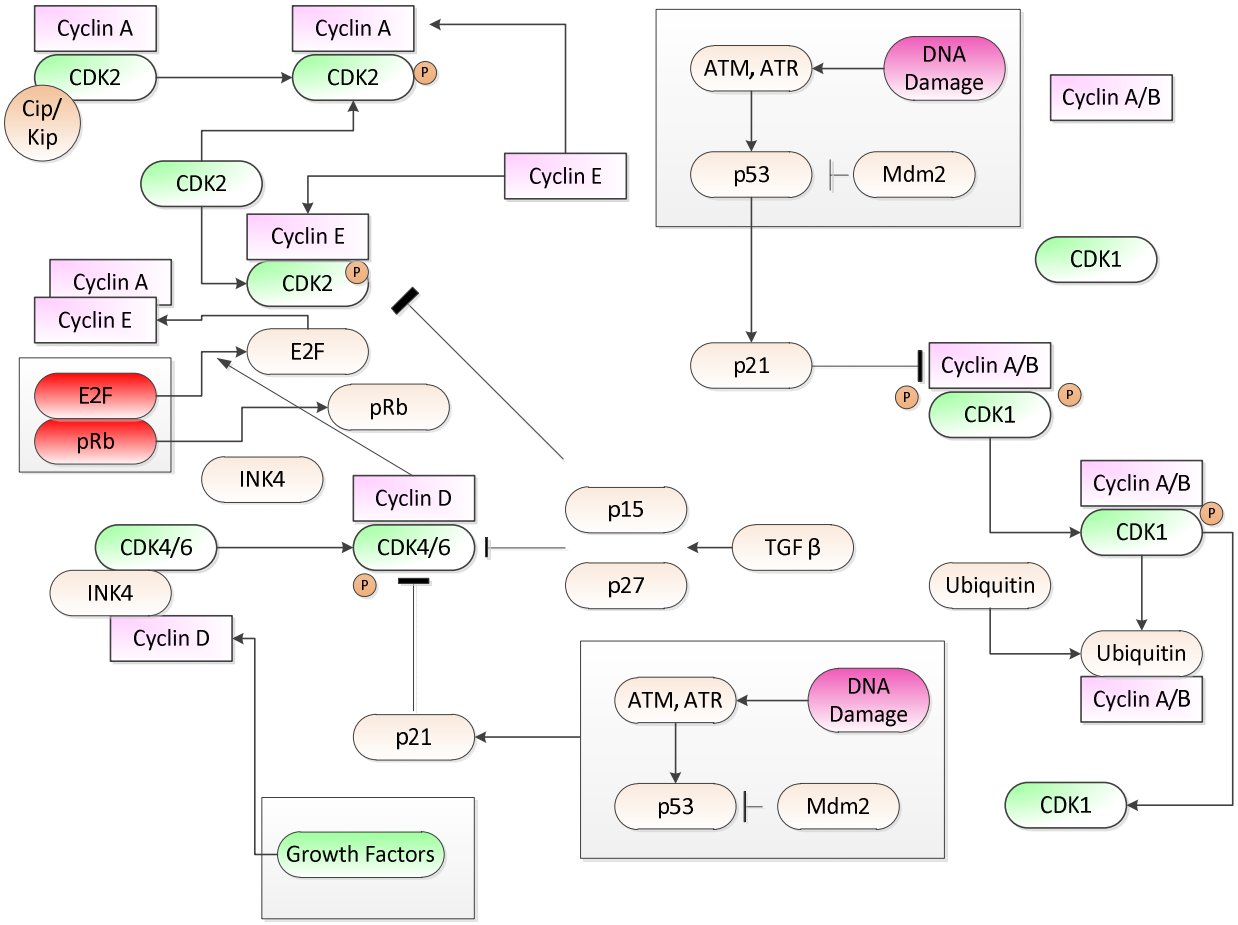
[28] http://www.ncbi.nlm.nih.gov/gene/1869

[29] http://www.ncbi.nlm.nih.gov/gene/5925

[30] http://www.ncbi.nlm.nih.gov/gene/898



A more details analysis has been well developed. We shall use this as a baseline and then add to what we have learned in that period. The network is shown as follows:



Now in the configuration we have the following elements:

1. CDKs: These are the cyclin dependent kinases we have been discussing.

2. Cyclins:

3. CDK Activating Enzymes:

4. CKI or CK Inhibitors

The following is a detailed list of some major CKIs or Cyclin Kinase Inhibitors. We have discussed them briefly before but they play a critical role in managing cell reproduction.

CKI Family	*Member Name*	*Alternative Name*	*Gene*	*Function*
INK4 Family	p15[31] (also P15; MTS2; TP15; CDK4I; INK4B; p15INK4b)	INK-4b	9p21	This gene lies adjacent to the tumor suppressor gene CDKN2A in a region that is frequently mutated and deleted in a wide variety of tumors. This gene encodes a cyclin-dependent kinase inhibitor, which forms a complex with CDK4 or CDK6, and prevents the activation of the CDK kinases, thus the encoded protein functions as a cell growth regulator that controls cell cycle G1 progression.
	p16[32] (also ARF; MLM)	INK-4a	9p21	This gene generates several transcript variants which differ in their first exons. At least three alternatively spliced variants encoding distinct proteins have been reported, two of which encode structurally related isoforms known to function as inhibitors of CDK4 kinase.
	p18[33]	INK-4c	1p32	The protein encoded by this gene is a member of the INK4 family of cyclin-dependent kinase inhibitors. This protein has been shown to interact with CDK4 or CDK6, and prevent the activation of the CDK kinases, thus function as a cell growth regulator that controls cell cycle G1 progression.
	p19[34]	INK-4d	19p13	The protein encoded by this gene is a member of the INK4 family of cyclin-dependent kinase inhibitors. This protein has been shown to form a stable complex with CDK4 or CDK6, and prevent the activation of the CDK kinases, thus function as a cell growth regulator that controls cell cycle G1 progression.
Cip-Kip Family	p21[35] also P21; CIP1; SDI1	Waf1, Cip1	6p21.2	This gene encodes a potent cyclin-dependent kinase inhibitor. The encoded protein binds to and inhibits the activity of cyclin-CDK2 or -CDK4 complexes, and thus functions as a regulator of cell cycle progression at G1. The expression of this gene is tightly controlled by the tumor suppressor protein p53, through which this protein mediates the p53-dependent cell cycle G1 phase arrest in response to a variety of stress stimuli.

[31] http://www.ncbi.nlm.nih.gov/gene/1030

[32] http://www.ncbi.nlm.nih.gov/gene/1029

[33] http://www.ncbi.nlm.nih.gov/gene/1031

CKI Family	*Member Name*	*Alternative Name*	*Gene*	*Function*
	p27[36] also p27; Rpn4	Cip2	12q24.31-q24.32	The 26S proteasome is a multicatalytic proteinase complex with a highly ordered structure composed of 2 complexes, a 20S core and a 19S regulator. An essential function of a modified proteasome, the immunoproteasome, is the processing of class I MHC peptides. This gene encodes a non-ATPase subunit of the 19S regulator.
	p57[37] also BWS; WBS;	Kip2	11p15.5	This gene is imprinted, with preferential expression of the maternal allele. The encoded protein is a tight-binding, strong inhibitor of several G1 cyclin/Cdk complexes and a negative regulator of cell proliferation.

The following depicts cell cycle control:

Gene	*Location*	*Function*
Jun[38]	1p32-p31	This gene is the putative transforming gene of avian sarcoma virus 17. It encodes a protein which is highly similar to the viral protein, and which interacts directly with specific target DNA sequences to regulate gene expression. This gene is intronless and is mapped to 1p32-p31, a chromosomal region involved in both translocations and deletions in human malignancies.
Fos[39]	14q24.3	The Fos gene family consists of 4 members: FOS, FOSB, FOSL1, and FOSL2. These genes encode leucine zipper proteins that can dimerize with proteins of the JUN family, thereby forming the transcription factor complex AP-1. As such, the FOS proteins have been implicated as regulators of cell proliferation, differentiation, and transformation. In some cases, expression of the FOS gene has also been associated with apoptotic cell death.

[34] http://www.ncbi.nlm.nih.gov/gene/1032

[35] http://www.ncbi.nlm.nih.gov/gene/1026

[36] http://www.ncbi.nlm.nih.gov/gene/5715

[37] http://www.ncbi.nlm.nih.gov/gene/1028

[38] http://www.ncbi.nlm.nih.gov/gene/3725

[39] http://www.ncbi.nlm.nih.gov/gene/2353

Gene	*Location*	*Function*
Myc[40]	8q24.21	The protein encoded by this gene is a multifunctional, nuclear phosphoprotein that plays a role in cell cycle progression, apoptosis and cellular transformation. It functions as a transcription factor that regulates transcription of specific target genes. Mutations, overexpression, rearrangement and translocation of this gene have been associated with a variety of hematopoietic tumors,

One of the questions we may ask is related to the kinetics of these processes. For example in many cancers the cell doubling time is highly variable at different locations and at different times and with different cells.

In a recent paper by Solimini et al the authors discuss the concepts of STOP and GO genes and carcinogenesis[41]. The paper reports on some extensive experimental results focusing on the issue of proliferation and the loss of certain sets of gene sites, the STP and GO sites.

The authors begin by discussing the current concepts of changes in oncogenes and tumor suppressor genes, some of the key pathway elements that we examine in analyzing intracellular pathway dynamics. They state:

Cancer progression is directed by alterations in oncogenes and tumor suppressor genes (TSGs) that provide a competitive advantage to increase proliferation, survival, and metastasis. The cancer genome is riddled with amplifications, deletions, rearrangements, point mutations, loss of heterozygosity (LOH), and epigenetic changes that collectively result in tumorigenesis.

How these changes contribute to the disease is a central question in cancer biology. In his "two-hit hypothesis," Knudson proposed that two mutations in the same gene are required for tumorigenesis, indicating a recessive disease. In addition, there are now several examples of haploinsufficient TSGs .

Current models do not explain the recent observation that hemizygous recurrent deletions are found in most tumors. Whether multiple genes within such regions contribute to the tumorigenic phenotype remains to be elucidated…

The last sentence regarding the inability to explain the presence of hemizygous deletions under the current model is the main driver for this effort. Thus they argue and demonstrate experimentally that:

[40] http://www.ncbi.nlm.nih.gov/gene/4609

[41] Solimini, N., et al, Recurrent Hemizygous Deletions in Cancers May Optimize Proliferative Potential, Science, 6 JULY 2012 VOL 337, p 104.

Tumors exhibit numerous recurrent hemizygous focal deletions that contain no known tumor suppressors and are poorly understood. To investigate whether these regions contribute to tumorigenesis, we searched genetically for genes with cancer-relevant properties within these hemizygous deletions.

We identified STOP and GO genes, which negatively and positively regulate proliferation, respectively.

STOP genes include many known tumor suppressors, whereas GO genes are enriched for essential genes.

Analysis of their chromosomal distribution revealed that recurring deletions preferentially over-represent STOP genes and under-represent GO genes.

*We propose a hypothesis called the **cancer gene island model**, whereby gene islands encompassing high densities of STOP genes and low densities of GO genes are hemizygously deleted to maximize proliferative fitness through cumulative haploinsufficiencies.*

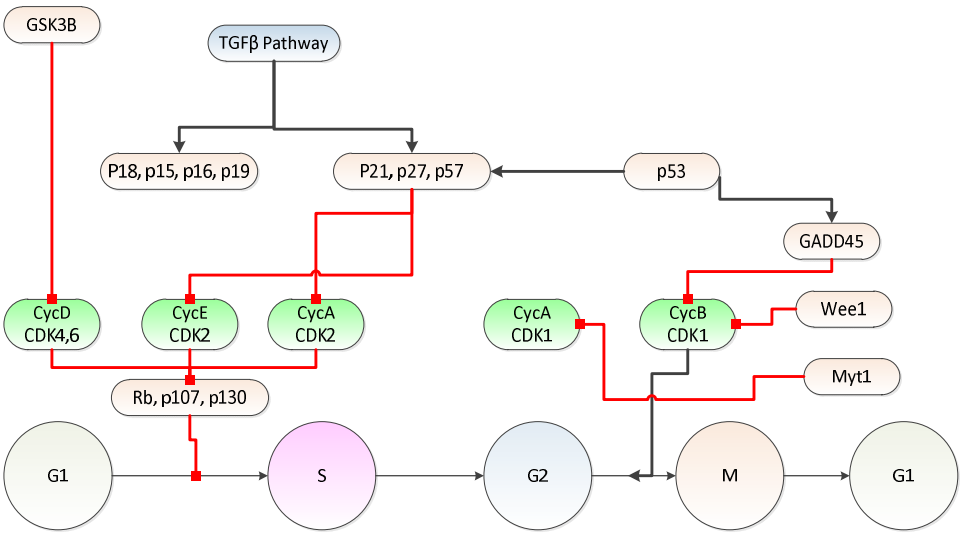
Because hundreds to thousands of genes are hemizygously deleted per tumor, this mechanism may help to drive tumorigenesis across many cancer types.

This is an intriguing hypothesis. It adds more pieces to an already complex puzzle. The Cancer Gene Island, CGI, hypothesis seems to indicate the complex changes in multiple gene sites. In particular there was a deletion of the STOP genes in preference to the GO genes. Unfortunately there did not seem to be a mechanism for these deletions, however the experimental evidence does indicate the phenomenon.

In their experimental analysis they have observed certain in vitro results which compel their hypothesis. They state:

This in silico analysis suggests that the loss of a single copy of GO genes has a negative impact on cellular fitness. To independently test this hypothesis, we turned to the other arm of our screen that identified candidate GO genes whose depletion limits proliferation and survival. Because both normal and cancer cells are dependent on these essential GO genes, we analyzed data from proliferation screens on HMECs, one normal prostate epithelial cell line, and seven breast or prostate cancer cell lines

They provide an interesting pathway model as shown below (as modified, and also not that they have short hairpin RNAs (shRNAs)).



From Solimini et al: "*Known pathways with multiple shRNAs in the validation screen. Genes that validated in the secondary screen by a factor of ≥4 with multiple shRNAs are denoted with circles corresponding to three to five shRNAs (orange) and six or more shRNAs (red).*"

They conclude as follows:

The enrichment for genes localized to deletions suggests that we have identified dozens of new TSGs in recurrent deletions. We have also likely identified more TSGs outside of these regions because the STOP gene set is (i) enriched for known TSGs, many of which are not found in recurrent deletions, and (ii) enriched for genes that undergo somatic loss-of-function mutation.

Finally, this work suggests that cells possess a substantial number of genes that restrain proliferation in vitro, which could be inactivated to promote clonal expansion during tumorigenesis in addition to the traditional driver genes currently known. Given the prevalence of multiple, large, recurring hemizygous deletions encompassing skewed distributions of growth control genes in tumors, we propose that the elimination of cancer gene islands that optimize fitness through cumulative haplo-insufficiencies may play an important role in driving tumorigenesis, with implications for the way in which we think about cancer evolution.

As with many such works this raises as many questions as it seems to answer. However the control or lack thereof of proliferation and the cell cycle is a critical issue in carcinogenesis.

As we have indicated, cell stability also includes the ability of a cell to recognize where it is and remaining in that location relative to other cells. One of the first signs of malignancy is a cell failing to understand its place and setting out on its own.

5.7 HEDGEHOG

The hedgehog pathway is of interest in many malignancies and recently in PA[42]. As Rush et al note:

Hedgehog (Hh) signaling is one mechanism implicated in the growth of two other types of primary brain tumors, medulloblastoma and malignant glioma. Additionally, recent limited clinical data indicate that targeted inhibition of the Hh pathway may be therapeutically beneficial for certain patients with medulloblastoma.

In a prior survey of WHO grade I–IV glioma specimens that included a small collection of pilocytic astrocytomas from adult patients, protein and transcript expression profiles for the Hh receptor Patched (PTCH) suggested that the Hh pathway might be activated to a small extent in this astrocytoma entity.

Therefore, in this study, we have investigated the Hh pathway in a larger panel of pilocytic astrocytoma specimens from pediatric patients.

We demonstrate that the Hh pathway is operational in sporadic pilocytic astrocytomas and activated to a greater extent in tumors from younger patients.

Furthermore, expression of the Hh pathway signal transduction components correlates with expression of the cellular proliferation marker Ki67. …

Recent findings implicate aberrant activation of the MAPK pathway, due to BRAF gene rearrangements or mutations, in 66%–85% of sporadic pilocytic astrocytomas. Integration of MAPK and Hh signaling, by ERK-mediated control of the GLI function, has been implicated in the regulation of cellular proliferation in basal cell and gastric carcinomas. Whether the MAPK and the Hh pathways function synergistically to regulate the growth of pilocytic astrocytomas warrants further study. In this respect, there remains an important need for animal models of sporadic pilocytic astrocytomas.

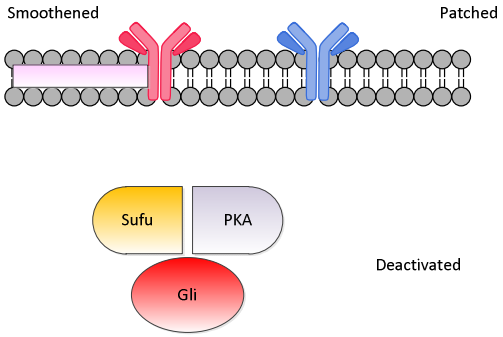
Hedgehog is a ligand which activates receptors and then pathways. The Hedgehog pathway is also a key element characterized as follows:

- In the absence of Hh a cell-surface transmembrane protein called Patched (PTCH) acts to prevent high expression and activity of a 7 membrane spanning receptor called Smoothened (SMO).

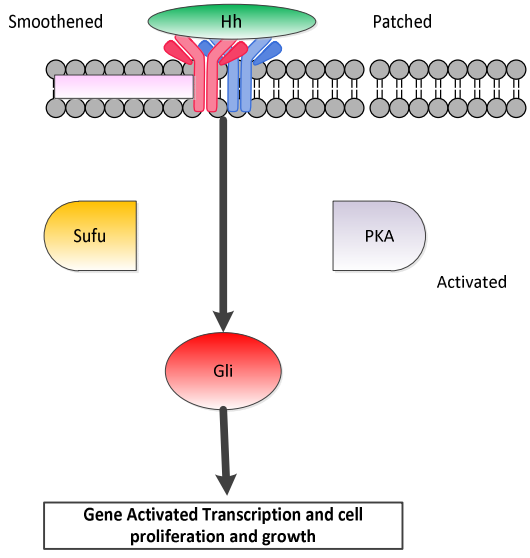
[42] See Cantley et al pp 410-411 for the focus on cancers and pp 107-112 for an overview of the pathway.

- Patched has sequence similarity to known membrane transport proteins. When extracellular Hh is present, it binds to and inhibits Patched, allowing Smoothened to accumulate and inhibit the proteolytic cleavage of the Ci protein.
- In cells with Hh-activated Patched, the intact Ci protein accumulates in the cell cytoplasm and levels of CiR decrease, allowing transcription of some genes such as decapentaplegic (dpp, a member of the BMP growth factor family).
- For other Hh-regulated genes, expression requires not only loss of CiR but also the positive action of uncleaved Ci acting as a transcriptional activator.

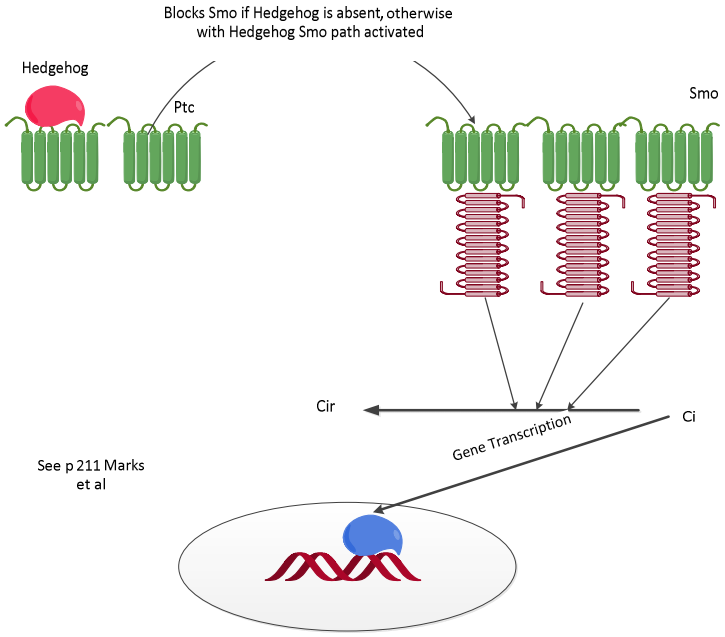
First we show it inactivated state as below. Note we have two separate receptors, Patched and Smoothened, which are separate and non-functional. Sufu and PKA are bound and Gli is also bound. Gli is the encoded transcription factor is activated by the sonic hedgehog signal transduction cascade and regulates stem cell proliferation. The activity and nuclear localization of this protein is negatively regulated by p53 in an inhibitory loop. Thus by activating Hh and combining the two receptors, Smo (Smoothened) and Patched (Ptch) we then activate Gli by unbinding it from Sufu and PKA. This is an example where we have three type at once; Wht the ligand, Smo and Ptch as receptors and Gli as a transcription factor.



Then the activated pathway as follows:

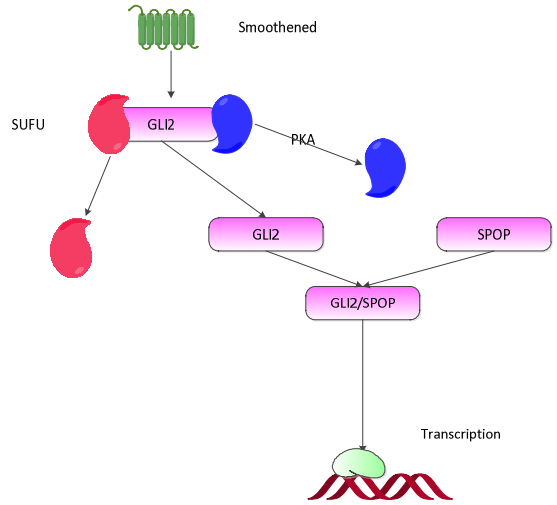


We demonstrate in more detail below the Hh binding. This graphically demonstrates the activation of the transcription factor and its movement into the nucleus and transcribing.



This is a first example. It demonstrates very simplistic terms of operation. Let us examine a bit more in detail. First, there may very well be many sets of receptors. The proximity demonstrated in the above raises the question of having Wnt being able to draw the two receptors together. It is not at all clear how that works. However from a systems perspective we shall assume it a fait accompli. Yet we cannot assume that we may very well have multiple sets, and thus multiple Gli released. That could then raise the rate of transcription. Modeling this level of complexity is essential. Also we have the issue of having an increase in transcription, so what? Having more proteins may or may not be a problem, it depends upon what proteins. These issues in detail are not readily examined at the bench level.

We demonstrate below the Smo, Smoothened, activation in some further detail.



Note that we have shown additional detail on the pathway elements resulting in transcription. It should be noted that there is considerably more detail available but we shall try to keep this at a level adequate for a model.

5.8 NOTCHED

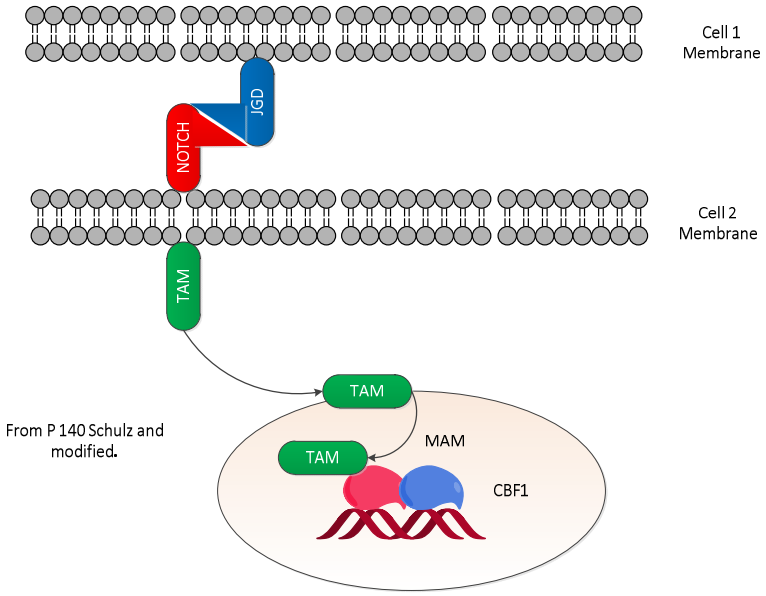
Notched is a bit of an amalgam of the above discussion. The notched pathway is characterized as follows.

The notch protein sits like a trigger spanning the cell membrane, with part of it inside and part outside. Ligand proteins binding to the extracellular domain induce proteolytic cleavage and release of the intracellular domain, which enters the cell nucleus to alter gene expression. The notch signaling pathway is important for cell-cell communication, which involves gene regulation mechanisms that control multiple cell differentiation processes during embryonic and adult life. Notch signaling also has a role in the following processes:

1. neuronal function and development
2. stabilization of arterial endothelial fate and angiogenesis
3. regulation of crucial cell communication events between endocardium and myocardium during both the formation of the valve primordial and ventricular development and differentiation
4. cardiac valve homeostasis, as well as implications in other human disorders involving the cardiovascular system
5. timely cell lineage specification of both endocrine and exocrine pancreas
6. influencing of binary fate decisions of cells that must choose between the secretory and absorptive lineages in the gut
7. expansion of the hematopoietic stem cell compartment during bone development and participation in commitment to the osteoblastic lineage, suggesting a potential therapeutic role for notch in bone regeneration and osteoporosis

8. T cell lineage commitment from common lymphoid precursor
9. regulation of cell-fate decision in mammary glands at several distinct development stages
10. possibly some non-nuclear mechanisms, such as control of the actin cytoskeleton through the tyrosine kinase Ab

We demonstrate Notched and its counterpart Jagged in the following Figure. On the cell surface we have Notched and on the other cell surface we have Jagged. When they bond, in a sense as surface proteins but with a communicating capability, Notched releases or activates Tam which is a transcription factor facilitator.



Notch signaling is dysregulated in many cancers.

6 TRIALS

We now consider some of the current trials as noted by NCI.

6.1 TRIAL 1

The following is a description[43]:

This phase 3 trial compares the effect of selumetinib versus the standard of care treatment with carboplatin and vincristine (CV) in treating patients with newly diagnosed or previously untreated ***low-grade glioma (LGG) that does not have a genetic abnormality called BRAFV600E*** *mutation and is not associated with systemic neurofibromatosis type.*

Selumetinib works by blocking some of the enzymes needed for cell growth and may kill tumor cells*.*

Carboplatin and vincristine are chemotherapy drugs that work in different ways to stop the growth of tumor cells, either by killing the cells or by stopping them from dividing.

The overall goal of this study is to see if selumetinib works just as well as the standard treatment of CV for patients with LGG. Another goal of this study is to compare the effects of selumetinib versus CV in subjects with LGG to find out which is better. Additionally, this trial will also examine if treatment with selumetinib improves the quality of life for subjects who take it.

6.2 TRIAL 2

As described[44]:

This phase III trial investigates the best dose of ***vinblastine in combination with selumetinib*** *and the benefit of adding vinblastine to selumetinib compared to selumetinib alone in treating children and young adults with low-grade glioma (a common type of brain cancer) that has come back after prior treatment (recurrent) or does not respond to therapy (progressive).*

Selumetinib is a drug that works by blocking a protein that lets tumor cells grow without stopping.

Vinblastine blocks cell growth by stopping cell division and may kill cancer cells.

[43] https://www.cancer.gov/about-cancer/treatment/clinical-trials/search/v?a=14&id=NCI-2019-07600&loc=1&rl=1&t=C60781&z=10020

[44] https://www.cancer.gov/about-cancer/treatment/clinical-trials/search/v?a=14&id=NCI-2020-07549&loc=1&rl=1&t=C60781&z=10020

Giving selumetinib in combination with vinblastine may work better than selumetinib alone in treating recurrent or progressive low-grade glioma.

6.3 TRIAL 3

As described[45]:

*This randomized, phase III trial compares **carboplatin with or without vincristine sulfate** in treating younger patients with previously untreated low grade glioma.*

Drugs used in chemotherapy, such as carboplatin and vincristine sulfate, work in different ways to stop the growth of tumor cells, either by killing the cells, by stopping them from dividing, or by stopping them from spreading.

It is not yet known whether carboplatin is more effective with or without vincristine sulfate in treating low grade glioma.

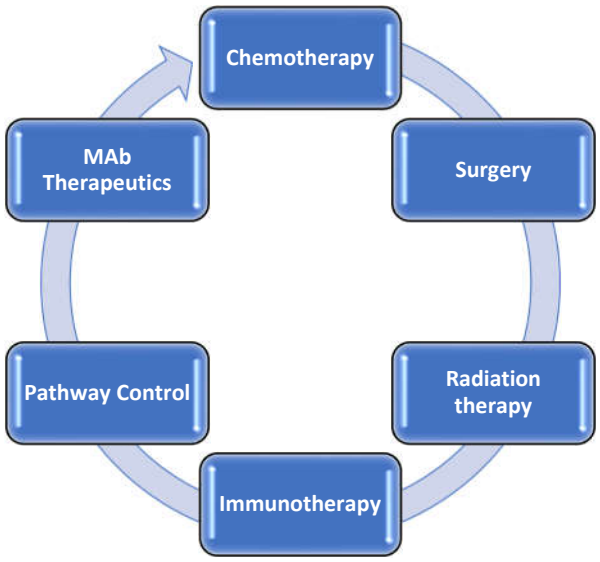
[45] https://www.cancer.gov/about-cancer/treatment/clinical-trials/search/v?a=14&id=NCI-2015-01146&loc=1&rl=1&t=C60781&z=10020

7 OBSERVATIONS

We can now make several observations regarding the compilation as above. Recall that our approach is to attempt a systems analysis of PA, not an in depth summary of any specific area. We use what is known and attempt to create a more holistic picture. This section develops a set of follow on issues which may be useful in addressing the control of PA. What we are examining are not speculations but results from early to mid-stage research efforts.

7.1 THERAPEUTIC OPTIONS ARE COMPLEX.

We have focused on cellular targetable therapeutics. The possibilities shown below are all available depending on extent and location. Surgery is always the first choice depending on location. However as we have noted a complete clear resection is demanded. Chemotherapy has some advantages but likewise significant morbidity is associated with use, and radiation therapy is likewise. Targeting of lesions and surrounding CNS areas is critical since destruction of PA lesions may have concomitant destruction of critical CNS elements.



Pathway controls such as MEK and BRAF inhibitors are being developed as well as those targeting other steps in the proliferation pathway. They are not without their downsides but clearly combination therapy has advantages. MAbs have advantages if we can determine unique surface markers. Not only can they mitigate proliferation but can eliminate existing proliferation. Immunotherapy is still a work in progress.

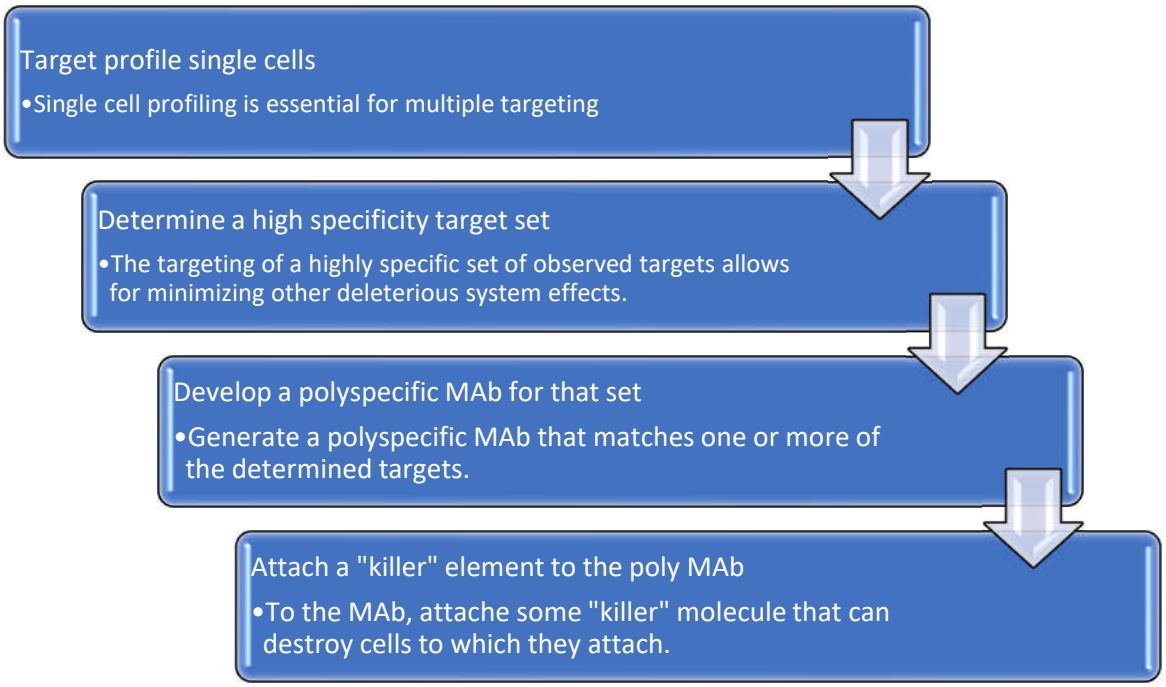
7.2 FUTURE PROSPECTS

Lessons can be learned from the above. The old line chemo techniques just systemically blast away at any rapidly reproducing cells, not only a tumor but hair and even hematopoietic cells. Surgery just cuts what the surgeon can see or is informed of by some marker. Radiation therapy is the "carpet bombing" approach that kills everything in sight and oftentimes induces new malignancies.

In contrast targeted and to some degree immunotherapy "kind of" is focused. Targeted therapies kind of assume all tumors are in a class and then use Mabs targeted for that class. However we know that each patient has a profile that is unique. If we knew the profile we could match a set of targeted therapies for that profile. In fact if we could assess the targets on a cell by cell basis we could create a mix that would be exceptionally beneficial. There are companies doing just that[46].

Now we can take this a step further. If we can do a cell by cell targeting and see a set of cell markers that are unique for that individual malignancy then we could create a polyspecific MAb with targeted chemotherapeutic payloads to destroy just the specific malignant cells of the patient. That in essence is the ideal of personal cancer therapy.

We demonstrate this below:



7.3 WHAT ARE THE PROTEIN-PROTEIN INTERACTIONS AND HOW DO MUTATIONS IMPACT THEM?

[46] https://www.travera.com/

Proteins bind to create reactions. For example the BRAF MEK reaction is detailed in Park. The authors note:

RAF family kinases are RAS-activated switches that initiate signalling through the MAP kinase cascade to control cellular proliferation, differentiation and survival.

RAF activity is tightly regulated and inappropriate activation is a frequent cause of cancer4–6; however, the structural basis for RAF regulation is poorly understood at present. Here we use cryo-electron microscopy to determine autoinhibited and active-state structures of full-length BRAF in complexes with MEK1 and a 14-3-3 dimer.

The reconstruction reveals an inactive BRAF–MEK1 complex restrained in a cradle formed by the 14-3-3 dimer, which binds the phosphorylated S365 and S729 sites that flank the BRAF kinase domain. ***The BRAF cysteine-rich domain occupies a central position that stabilizes this assembly, but the adjacent RAS-binding domain is poorly ordered and peripher****al. The 14-3-3 cradle maintains autoinhibition by sequestering the membrane-binding cysteine-rich domain and blocking dimerization of the BRAF kinase domain.*

In the active state, these inhibitory interactions are released and a single 14-3-3 dimer rearranges to bridge the C-terminal pS729 binding sites of two BRAFs, which drives the formation of an active, back-to-back BRAF dimer. Our structural snapshots provide a foundation for understanding normal RAF regulation and its mutational disruption in cancer and developmental syndromes …

The integral nature of the RAF–MEK–14-3-3 switch has important pharmacologic implications. It is well established that certain MEK and RAF inhibitors can stabilize or destabilize their interaction14,32–34. However, the notion that the RAF–MEK–14-3-3 complex—which is distinct from the isolated RAF and MEK kinases—may represent a relevant pharmacologic receptor for a broader range of inhibitors has not, to our knowledge, been systematically explored.

Perhaps the most perplexing aspect of RAF-inhibitor pharmacology is the paradoxical activation of the MAP kinase pathway by certain RAF kinase inhibitors4,35,36. Diverse RAF inhibitors disrupt autoinhibitory interactions of the BRAF kinase with its N-terminal region37, and some promote dimerization of the isolated BRAF kinase domain23. Considering the extensive interactions of BRAF with ATP in the autoinhibited state, we speculate that RAF inhibitors may promote conformational activation by displacing ATP from quiescent RAF.

Whether this leads to observed paradoxical pathway activation will in turn depend upon ensuing cellular events—potentially including changes in RAF phosphorylation state, RAS-binding, membrane localization and 14-3-3 rearrangements—and on the potency of a particular agent as an inhibitor of activated RAF dimers.

Many questions regarding RAF regulation remain. The structures described here and the ability to prepare full-length autoregulated and active BRAF will inform and enable detailed mechanistic studies of RAF activation and RAF-inhibitor pharmacology. In the long term, a

deeper understanding of RAF regulation should aid in the development of more effective and better-tolerated therapeutics for RAF-driven cancers.

7.4 How do the therapeutics really get into target cells?

In the above set of discussions and especially those on therapeutics there is the lingering question often unasked as to how the therapeutics target the cells in question and in turn how the therapeutic enters the targeted cell. One theory if a diffusive entry and a second is a facilitated entry. The answer is still not well understood. From Lowe:

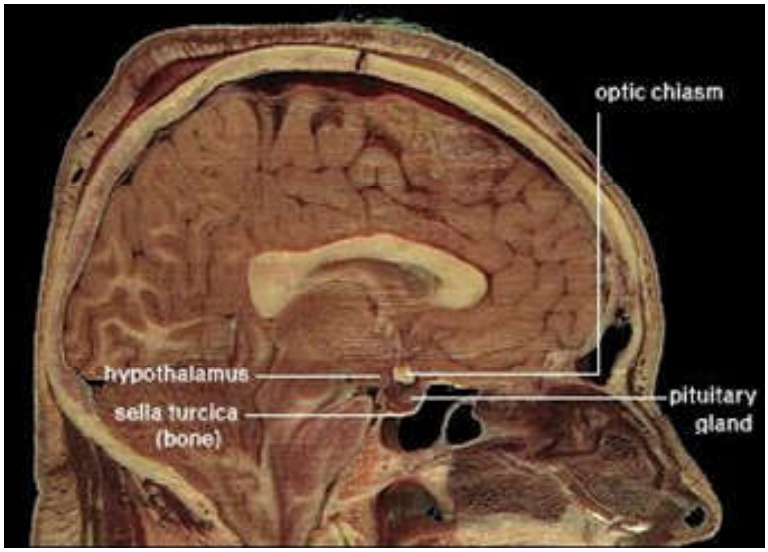
So how do drug molecules (and others) get into cells, anyway? There are two broad answers: they just sort of slide in through the membranes on their own (passive diffusion), or they're taken up by pores and proteins built for bringing things in (active transport). I've always been taught (and believed) that both processes can be operating in most situations. If the properties of your drug molecule stray too far out of the usual range, for example, your cell activity tends to drop, presumably because it's no longer diffusing past the cell membranes. There are other situations where you can prove that you're hitching a ride on active transport proteins, by administering a known inhibitor of one of these systems to cells and watching your compound suddenly become inactive, or by simply overloading and saturating the transporter.

There's another opinion, though, that's been advanced by Paul Dobson and Douglas Kell at Manchester, and coworkers. Their take is that ***carrier-mediated transport is the norm****, and that* ***passive diffusion is hardly important at all****. This has been received with varying degrees of belief. Some people seem to find it a compelling idea, while others regard it as eccentric at best. The case was made a few years ago in Nature Reviews Drug Discovery, and again more recently in Drug Discovery Today:*

"All cells necessarily contain tens, if not hundreds, of carriers for nutrients and intermediary metabolites, and the human genome codes for more than 7000 carriers of various kinds. Here, we illustrate using a typical literature example the widespread but erroneous nature of the assumption that the 'background 1 or 'passive' permeability to drugs occurs in the absence of carriers. Comparison of the rate of drug transport in natural versus artificial membranes shows discrepancies in absolute magnitudes of 100-fold or more, with the carrier containing cells showing the greater permeability.

Expression profiling data show exactly which carriers are expressed in which tissues.

The recognition that drugs necessarily require carriers for uptake into cells provides many opportunities for improving the effectiveness of the drug discovery process."



7.5 CAN ONE OBTAIN MORE EFFECTIVE THERAPEUTIC RESULTS WITH COMPOUND THERAPEUTICS RATHER THAN JUST SINGLE TARGET ONES? IF SO, THEN WHAT AND WHY?

If we have a BRAF mutation then can one use dabrafenib and trametinib plus a MEK inhibitor selumetinib in some combination. The intent should be twofold; block further proliferation as well as eliminating existing aberrant cells. The latter may be a much more complex issue. However with the CD133 surface marker, which must be validated, then one has a possible target for a polyspecific antibody approach or even a CAR-T approach. Needless to say all of these demand FDA approval.

7.6 IS THERE A POSSIBLE USED FOR POLYSPECIFIC ANTIBODIES FOR BETTER TARGETING AND DELIVERY OF CELL ELIMINATION THERAPEUTICS?

Polyspecific antibodies are complex combinations of multi receptor Ig elements[47]. We have examined these previously and the development is in progress but it is too early to speculate.

7.7 KNOWING THAT T CELLS HAVE A PRESENCE IN THE BRAIN, CAN SOME IMMUNOTHERAPY BE AN OPTION AND IF SO HOW?

The brain has limited immune cells. In fact T cells that do make it to the brain last no more than 24 hours. Thus immunotherapy may be problematic but not impossible. If the vasculature can remain open through the lesion perhaps some targeted immunotherapeutic approach is viable.

7.8 WHAT IS THE IMPACT OF METHYLATION ON ASTROCYTOMAS AND THEIR GROWTH?

[47] https://www.researchgate.net/publication/346245151_Poly-specific_Antibodies

Epigenetic changes to the DNA can result in obvious instable conditions. Methylation is one of those factors. However the methylated state of lesions is generally not measured or determined. Furthermore there is a lack of knowledge and understanding at this stage.

7.9 ARE THEIR NON-INVASIVE LIQUID BIOPSY TECHNIQUES AVAILABLE?

Liquid biopsies have become quite significant measuring RNAs, circulating tumor cells, Ct, proteins, and the like[48]. As Volpentesta et al note:

Imaging limitations, invasive tissue biopsies and poor information over the course of treatment to evaluate 'real-time' tumor dynamics justify the emerging use of liquid biopsies in the field of brain tumors. Circulating tumor cells (CTCs) from high-grade astrocytomas might reach the circulation by crossing the blood–brain barrier. Here, for the first time, CTCs cytology in a case of pylocitic astrocytoma is described.

An obstructive hydrocephalous due to a lateral mesencephalic tectum mass occluding the Silvio Aqueduct was diagnosed in a young, 18 years old, male. Considering the location of the tumor and the rapid deterioration of the neurological status, it has been decided to urgency treat the patient with ventriculoperitoneal shunting. Magnetic resonance imaging showed a nodular shaped lesion localized within the left lateral mesencephalic tectum. Stereotactic biopsy was not approachable due significant risk of neurological consequences. The diagnosis was performed by blood sampling, a non-invasive procedure for the patient, in order to provide tumor information.

Cytopathological features on detected circulating atypical GFAP positive cells led to pilocytic diagnosis confirmed by the patient's 68 months outcome

7.10 OTHER MARKERS MAY EXIST

As van Bodegraven et have noted:

Gliomas are a heterogenous group of malignant primary brain tumors that arise from glia cells or their progenitors and rely on accurate diagnosis for prognosis and treatment strategies. Although recent developments in the molecular biology of glioma have improved diagnosis, classical histological methods and biomarkers are still being used.

The glial fibrillary acidic protein (GFAP[49]) is a classical marker of astrocytoma, both in clinical and experimental settings. GFAP is used to determine glial differentiation, which is associated with a less malignant tumor.

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[48] https://www.researchgate.net/publication/362724040_Biomarkers_Targets_for_Cancer_Diagnosis

[49] See https://www.ncbi.nlm.nih.gov/gene/2670 *This gene encodes one of the* ***major intermediate filament proteins of mature astrocytes.*** *It is used as a marker to* ***distinguish astrocytes from other glial cells*** *during development. Mutations in this gene cause Alexander disease, a rare disorder of astrocytes in the central nervous system. Alternative splicing results in multiple transcript variants encoding distinct isoforms.*

However, since GFAP is not only expressed by mature astrocytes but also by radial glia during development and neural stem cells in the adult brain, we hypothesized that GFAP expression in astrocytoma might not be a direct indication of glial differentiation and a less malignant phenotype.

Therefore, we here review all existing literature from 1972 up to 2018 on GFAP expression in astrocytoma patient material to revisit GFAP as a marker of lower grade, more differentiated astrocytoma.

We conclude that GFAP is heterogeneously expressed in astrocytoma, which most likely masks a consistent correlation of GFAP expression to astrocytoma malignancy grade.

The GFAP positive cell population contains cells with differences in morphology, function, and differentiation state showing that GFAP is not merely a marker of less malignant and more differentiated astrocytoma. We suggest that discriminating between the GFAP isoforms GFAPδ and GFAPα will improve the accuracy of assessing the differentiation state of astrocytoma in clinical and experimental settings and will benefit glioma classification …

GFAP as a biomarker for astrocytoma that is still used to date. In the healthy human brain, GFAP is mainly expressed in mature astrocytes. Therefore, in clinical as well as fundamental experimental settings, high GFAP expression is believed to mark more differentiated, less malignant tumors. However, more recently GFAP expression was observed in the radial glia of the developing human brain and in adult neural stem cells of the adult brain , showing that GFAP is also expressed in immature, nondifferentiated CNS cells.

Since then, GFAP is often used to mark cells with stem cell characteristics in glioma and to target neural stem cells to induce gliomagenesis in animal models. In addition, GFAP is up-regulated in non-neoplastic astrocytes that become reactive in response to the growth of the tumor and do not reflect the differentiation state of neoplastic.

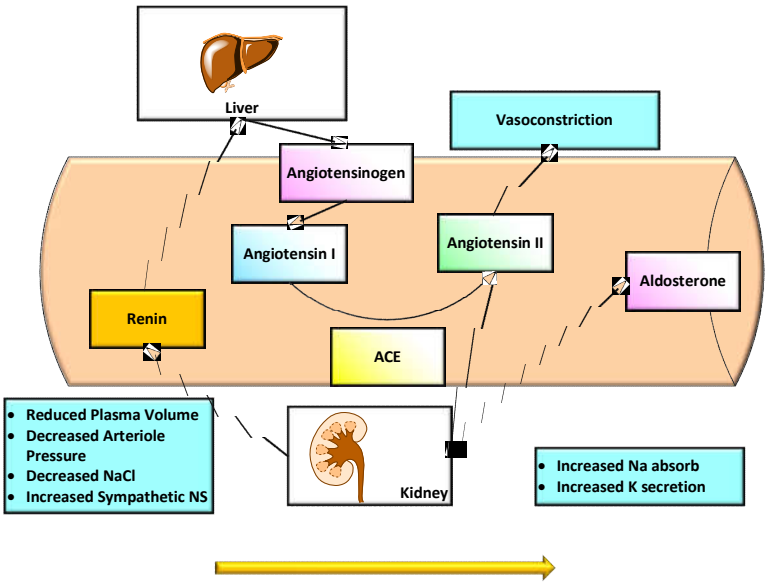
Therefore, high GFAP levels in tumor specimens may not be a direct indication of a less malignant, more differentiated astrocytoma subtype. Indeed, our recent studies in which we determined the expression of different GFAP isoforms show that higher levels of the alternative splice variant GFAPδ relative to the canonical variant GFAPα are associated with a higher malignant and less differentiated astrocytoma subtype.

In vitro studies that show a higher malignant gene expression profile and changes in astrocytoma malignant behavior in cells with higher levels of GFAPδ relative to GFAPα, as observed in neurogenic stem cells of the healthy brain, further support the hypothesis of GFAP as a marker of more than lower malignant astrocytoma.

7.11 WOULD USING THERAPEUTICS THAT INCREASED BLOOD FLOW (I.E. LOSARTAN) IMPROVE RESULTS?

The renin-angiotensin system is depicted below wherein we see that Angiotensin I gets converted to angiotensin II and in turn results in vasoconstriction. This vasoconstriction results in

lower blood flow and higher blood pressure. The lower blood flow putatively inhibits the ability for therapeutics to enter cells. Thus targeting angiotensin II may improve therapeutic efficacy. Losartan plays such a role.



Martin et al have noted:

Abnormal blood and lymphatic vessels create a hostile tumor microenvironment characterized by hypoxia, low pH, and elevated interstitial fluid pressure. These abnormalities fuel tumor progression, immunosuppression, and treatment resistance. …

Blood vessels bring oxygen and nutrients to every cell in the body while removing waste and allowing immune cells to survey. These vessels do the same in cancer and other diseases

In most types of tumors, new vessels produced through angiogenesis have abnormal structure and function, leading to impaired perfusion that paradoxically supports malignancy

Specifically, hypoxia makes cancer cells more aggressive, and leaky vessels give these cells passage to distant sites to metastasize. Additionally, hypoxia prevents immune cells from acting on cancer cells and reduces the efficacy of radio- and chemotherapy. Through hypoxia, cancer cells promote an interlinked cycle of angiogenesis, desmoplasia, and immunosuppression, thereby creating an abnormal tumor microenvironment (TME) that results in disease progression and treatment resistance. Because tumors rely on angiogenesis to grow and metastasize, antiangiogenesis therapies (AATs) initially were developed as a monotherapy that would starve tumors of nutrients.

However, in the initial clinical trials, bevacizumab, an antivascular endothelial growth factor (VEGF) antibody, failed to improve survival as monotherapy but improved the outcome of chemotherapy. This seemed paradoxical: How can an agent that destroys the blood vessels that bring chemotherapeutic drugs to the tumor cells improve the efficacy of chemotherapy? To this end, in 2001 we hypothesized that using AATs with the intent to normalize—not destroy—vessels

would improve their function, thereby enhancing treatment outcomes by increasing oxygen and drug delivery.

We provided preclinical evidence for this hypothesis in a number of models and revealed the molecular mechanisms of vascular normalization. We also demonstrated that cytotoxic therapy given during the window of normalization has a better outcome than the cytotoxic therapy given before or after the window. Moreover, a number of other laboratories have now provided evidence in support of the normalization hypothesis. Although it uncovers new challenges to translation, the clinical use of AATs in the interim supports the normalization hypothesis.

The central lesson of these studies reinforces our prediction that judicious use of AATs is necessary to improve vessel function and thus clinical outcome. Judicious use is complicated by multiple levels of tumor heterogeneities, such as across tumor types, regions in the same lesion, and different lesions in the same patient. Other TME components, such as fibroblasts, reciprocally contribute to these heterogeneities. Thus, overarching questions remain as to how to personalize the use of AATs for patients and how to combine these AATs with other TME modulating therapies that reduce desmoplasia and stimulate antitumor immunity. …

Hypoxia Promotes Tumor Progression, tumor vessel leakiness and compression result in hypoxia and acidity in the TME, thereby promoting immune suppression, which comprises tissue-resident and bloodborne immune cells. To promote immunity against the tumor, these cells must infiltrate the TME by flowing into tumor blood vessels, adhering to the vessel wall, and transmigrating into the interstitial space.

As Pinter and Jain have noted:

The circulating renin-angiotensin system (RAS) is mainly known for its pivotal role in maintaining cardiovascular homeostasis and fluid and electrolyte balance. In addition, a local RAS is expressed in many tissues and mainly acts at the cellular level, where it mediates cell proliferation, growth, and metabolism.

The local RAS works synergistically and independently of the systemic RAS. Angiotensin II (AngII) is the main effector and maintains tissue homeostasis by exerting regulatory and counterregulatory effects through its different receptors. Alternative peptide-receptor axes also assist in maintaining this balance

Dysregulation of the RAS, for example, by overexpression of certain RAS components [such as renin, Ang-converting enzyme (ACE), or AngII type 1 receptor (AT1R)], can be involved in the pathophysiology and progression of a broad range of diseases, such as arterial hypertension, kidney disease, and other cardiovascular conditions. The discoveries of captopril—the first orally active ACE inhibitor (ACEi)—in the mid-1970s and losartan—the first orally active, selective AT1R blocker (ARB)—around a decade later represent milestones in the history of the RAS. Numerous ACEis and ARBs have been developed since then.

Now, ACEis and ARBs are the most common inhibitors of the RAS and are widely used in the management of several diseases, such as arterial hypertension, heart failure, myocardial

infarction, and chronic kidney disease. Direct renin inhibitors (such as aliskiren) represent a third class of RAS-acting agents and have been added to the armamentarium more recently (16). A list of RAS inhibitors (RASi) approved by the U.S. Food and Drug Administration (FDA) is provided in table S1

As Panza et al have then noted:

Patients with high-grade glioma (HGG) such as glioblastoma (GBM) who undergo surgical resection with adjuvant therapy have a mean overall survival of 14.6 months and 100% of recurrence. Thus, these disappointing outcomes in terms of glioblastoma life expectancy require seeking novel pharmacological tools, including drug repurposing.

In the present study, we identify a novel molecular mechanism through which Losartan antagonizes Angiotensin II (Ang II)/Angiotensin II type I receptor (AGTR1) signaling, overexpressed in GBM cells.

For instance, we demonstrate how Losartan drastically inhibits the stimulatory effects of Ang II on aromatase activity and consequently reduces local estrogen production, sustaining cancer progression.

Thus, it is reasonable to repurpose Losartan as an adjuvant pharmacological tool to be implemented prospectively in the novel therapeutic strategies adopted in GBM patients.

New avenues for glioblastoma therapy are required due to the limited mortality benefit of the current treatments. The renin-angiotensin system (RAS) exhibits local actions and works as a paracrine system in different tissues and tumors, including glioma.

The glioblastoma cell lines U-87 MG and T98G overexpresses Angiotensin II (Ang II)/Angiotensin II type I receptor (AGTR1) signaling, which enhances in vitro and in vivo local estrogen production through a direct up-regulation of the aromatase gene promoters p I.f and p I.4. In addition, Ang II/AGTR1 signaling transactivates estrogen receptor-α in a ligand-independent manner through mitogen-activated protein kinase (MAPK) activation.

The higher aromatase mRNA expression in patients with glioblastoma was associated with the worst survival prognostic, according to The Cancer Genome Atlas (TCGA). An intrinsic immunosuppressive glioblastoma tumor milieu has been previously documented.

We demonstrate how Ang II treatment in glioblastoma cells increases programmed death-ligand 1 (PDL1) expression reversed by combined exposure to Losartan (LOS) in vitro and in vivo. Our findings highlight how LOS, in addition, antagonizes the previously documented neoangiogenetic, profibrotic, and immunosuppressive effects of Ang II and drastically inhibits its stimulatory effects on local estrogen production, sustaining glioblastoma cell growth.

Thus, Losartan may represent an adjuvant pharmacological tool to be repurposed prospectively for glioblastoma treatment

7.12 THE TUMOR MICRO ENVIRONMENT PLAYS A DRAMATIC ROLE IN MAY CANCERS. THERE IS THE ISSUE AS TO JUST WHAT ROLE IT PLAYS IN PA?

As Jain has noted:

Our initial work on the tumor microenvironment and drug delivery involved growing tumors in animals, excising them for various measurements, and then using mathematic models to gain insight into the inner workings of tumors.6,7 Although insightful, this approach did not capture dynamic changes at a cellular or subcellular resolution. To overcome this, we developed transparent windows and sophisticated, high-resolution optical imaging techniques that allowed us to visualize events in tumors in real time.8-10 Coupled with molecular probes, image analysis, and mathematic models, this approach has provided unprecedented insights into molecular, cellular, anatomic, and functional changes during tumor progression and in response to treatment

Unlike normal vessels, which are orderly, tumor vessels are tortuous, saccular, and chaotic in their organization and The structure of the vessel wall is also abnormal, with large gaps between endothelial cells, detached pericytes, and abnormally thick or thin basement membranes.13-16 Consequently, tumor vessels are leaky in some places and not in others, with overall leakiness dependent on the host organ. Moreover, these vessels change with tumor growth and treatment …

Clinical experience indicates that a primary tumor may respond to certain therapies, whereas its metastases might not. To understand the role of different host microenvironments in tumor biology or response to treatment, we examined tumors in various organs of mice, such as the brain, mammary fat pad, liver, pancreas, and skin. For instance, when we inoculated the same breast cancer cells in three different sites, the resulting vasculature was abnormal yet vastly different in each site …

In summary, our work has demonstrated that blood and lymphatic vessels as well as the extracellular matrix of tumors are abnormal, and these abnormalities create a hostile microenvironment.

Other stromal cells, such as activated fibroblasts, macrophages, and other immune cells, are also part of the abnormal tumor microenvironment. This microenvironment fuels tumor progression, metastasis, and immunosuppression and induces a stem-cell phenotype—all of which contribute to treatment resistance. Normalization of blood vessels and matrix can alleviate some of these problems, not only in mice but also in patients. Crucially, in two brain tumor trials, patients whose tumor blood perfusion increased survived longer than those whose tumor perfusion did not increase. These exciting discoveries notwithstanding, great challenges remain ahead of us. Perhaps the biggest unmet need is improving antimetastasis therapies. Here, the disparity between preclinical and clinical research in antiangiogenesis remains profound.

Most preclinical studies have been performed on primary tumors; only a handful have occurred in the metastatic or adjuvant setting that recapitulates the clinical situation. Even in these studies, the dose of antiangiogenic agent used is high compared with that in the clinical setting.

Thus, it is not surprising that the resulting hypoxia may enrich for cancer stem cells or increase metastasis.

In our own limited adjuvant studies with two different VEGFR tyrosine kinase inhibitors at lower doses, we have not detected an increase in metastasis—an observation consistent with clinical trials In addition, with improved systemic therapy, there is an alarming increase in incidence of brain metastasis, regarded as the last frontier in the war against cancer.

Our recent work using a VEGFR2-blocking antibody combined with trastuzumab and lapatinib has shown a dramatic effect in an experimental model of brain metastasis of human epidermal growth factor receptor 2–positive breast cancer (Data Supplement Fig S21).164 This finding needs to be tested in the clinic. Although our work has focused on vascular normalization, we are cognizant of other potential mechanisms of benefit from antiangiogenesis alone or when combined with chemotherapy.165,166 These include killing both endothelial and cancer cells by antiangiogenics. Antiangiogenic agents may also sensitize endothelial cells to cytotoxic drugs and impair the recruitment of bone marrow–derived cells that can differentiate to endothelial cells or release proangiogenic molecules.

Finally, cytotoxic agents may kill stromal cells. Although all these mechanisms have been previously examined in preclinical models, their roles in progression-free survival and OS need to be carefully investigated in patients. Specific changes in biomarkers may inform these mechanisms.

7.13 VEGF AND ANGIOGENESIS HAS BEEN DETERMINED TO FACILITATE TUMOR GROWTH. CAN VEGFR BE BLOCKED AND IMPROVE PA CONTROL?

As Pinter and Jain have noted:

Considerable evidence suggests that AngII/AT1R signaling promotes VEGF-mediated angiogenesis in solid tumors. AT1R expression correlates with VEGF and VEGF receptor (VEGFR) expression and microvessel density (MVD) in different human tumors. In experimental studies, AngII promoted VEGF expression in tumor and stromal cells. Treatment with either ACEi or ARB reduced VEGF expression and decreased MVD and neovascularization in vivo (65, 66). VEGF also induces vascular hyperpermeability, one of the main characteristics of the abnormal tumor vasculature. Tumor vessel leakiness promotes tumor hypoxia and acidosis by impairing tumor blood flow. As mentioned above, hypoxia helps to create an immunosuppressive milieu and promotes tumor progression and dissemination. Tumor vessel normalization can alleviate hypoxia, reprogram the immunosuppressive microenvironment, and improve the efficacy of immunotherapy in mice. Glioblastoma patients who show enhanced tumor blood perfusion under antiangiogenic therapy have markedly prolonged survival compared to subjects who experience no change or a decrease in perfusion. RASi also reduces VEGF-mediated vascular leakiness in the dermis and retina of rodents…

As Guo et al have noted:

Unlimited cell proliferation, dedifferentiation and a lack of apoptosis are important biological characteristics of tumours. The activation of the ERK/MAPK signalling pathway promotes proliferation and has an anti-apoptotic effect.

***Hypoxia-induced VEGF can inhibit the apoptosis of serum-starved cells by activating the ERK/MAPK signalling pathway**.*

Inhibiting the expression of this pathway can inhibit the proliferation of and lack of apoptosis in tumour cells, and promote their differentiation

Finally as Martin et al have noted:

Angiogenic signaling promotes immunosuppression through at least four mechanisms.

First, VEGF blocks cytotoxic T lymphocyte trafficking and activity by modulating the inhibitory checkpoints of T cells.

Second, VEGF limits T cell activation by inhibiting dendritic cell maturation and antigen presentation.

Third, VEGF recruits immunosuppressive cells, including Treg cells, myeloid-derived suppressor cells, and protumor M2-like TAMs[50].

Fourth, as discussed above, VEGF-induced vessel leakiness causes hypoxia that results in local and systemic immunosuppression.

It has also been found that entry to PA cells via the circulatory system may be enhanced by many means including ultrasound mechanisms[51]. Will this facilitate PA targeting?

7.14 Is there potential for CAR-T and Targeted Immunotherapy?

The types of therapy mentioned demands unique surface targets like CD19. However among cancer stem cells markers CD133 is significant in solid tumors[52]. As Wang et al have noted:

Among CSC (cancer stem cells) markers, CD133 (also known as Prominin 1, PROM1) is one of the most widely used markers for enrichment and labeling of CSCs in solid tumors [13–17]. Previous studies have focused on whether CD133 is a robust CSC marker; however, its function is still unclear in hESCs. Whether the function of CD133 is conserved in tumors and hESCs and whether CD133 is a potential target to reduce teratoma formation without radical changes to

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[50] https://www.researchgate.net/publication/336116071_Tumor_Associated_Immune_Cells_On_the_one_hand_and_on_the_other_hand

[51] See Aryal

[52] https://www.researchgate.net/publication/309419224_CAR_T_Cells_and_Cancer

differentiation have never been systemically clarified. We found that CD133 is highly expressed in human ESCs, and interestingly, knockout (KO) of CD133 in hESCs significantly attenuates hESC proliferation and teratoma formation but does not affect hESC pluripotency or in vivo differentiation into three germ layers

Now significant work by Xi et al have also noted CD133 as a unique target for PA. They note:

Pilocytic astrocytomas (PAs) are the most common pediatric central nervous system neoplasms. In the majority of cases these tumors are benign and receive favorable prognosis following gross total surgical resection. In patients with progressive or symptomatic tumors, aggressive surgical resection is generally not feasible, thus radiation or chemotherapy are accepted initial or adjuvant interventions. Due to serious long-lasting side effects, radiation is limited in young children; therefore, chemotherapy is widely practiced as an adjuvant treatment for these patients.

However, chemotherapy can promote the emergence of multidrug resistant tumor cells that are more malignant than those of the original tumor*.*

CD133, a putative stem cell marker in normal tissue and malignant brain tumors, enhances multidrug resistant gene 1 (MDR1) expression following chemotherapy in adult malignant glioblastomas. This study examines the relationship between CD133 and MDR1 in pediatric PAs exposed to chemotherapy, with the goal of identifying therapeutic targets that manifest as a result of chemotherapy.

Thus having a unique marker may pave the way for CAR-T cell elimination of the PA cells while leaving the other cells untouched.

7.15 CAN MAB BE USED TO TARGET RADIATION THERAPY IN THE PA LESION?

One can generate an MAb to target the CD133 surface protein. If one can add a radiation target to this MAb as well then this allows for target specific treatment. As Lampson noted:

Monoclonal antibodies (mAbs) are used with increasing success against many tumors, but for brain tumors the blood-brain barrier (BBB) is a special concern.

The BBB prevents antibody entry to the normal brain; however, its role in brain tumor therapy is more complex.

The BBB is closest to normal at micro-tumor sites; its properties and importance change as the tumor grows. In this review, evolving insight into the role of the BBB is balanced against other factors that affect efficacy or interpretation when mAbs are used against brain tumor targets.

As specific examples, glioblastoma multiforme (GBM), primary central nervous system lymphoma (PCNSL) and blood-borne metastases from breast cancer are discussed in the context of treatment, respectively, with the mAbs bevacizumab, rituximab and trastuzumab, each of which is already widely used against tumors outside the brain. It is suggested that success

against brain tumors will require getting past the BBB in two senses: physically, to better attack brain tumor targets, and conceptually, to give equal attention to problems that are shared with other tumor sites.

We have seen the use in such cancers as breast cancer mets to the brain. In addition the use of the PSMA target in prostate cancer has enabled dramatic improvement in both assessment and treatments[53].

As Tepper notes:

Selective delivery of radionuclides to cancer cells using an antibody or other conjugate has been under investigation for more than 30 years. In 2002, the first US Food and Drug Administration (FDA) approval was issued for a radiolabeled antibody. Initially, this form of radionuclide therapy mainly involved the use of antibodies or antibody-derived constructs as carriers of radionuclides; therefore, it is called radioimmunotherapy (RIT). However, because the concept also includes binding to nonantigen receptors, ***targeted radionuclide therapy*** *(TaRT) is a more comprehensive term, and Paul Wallner coined the acronym STaRT for systemic targeting. The development of TaRT has required the cooperation of basic scientists in the areas of radiation biology, chemistry, physics, and immunology with multiple clinical specialists. With the exception of some gene therapy approaches, TaRT differs from external-beam radiation therapy (EBRT) in that selective targeting can be at the cellular rather than target volume level. Among its potential applications, TaRT provides a means of irradiating multiple tumor sites throughout the body with relative sparing of normal tissues. A number of challenges hampering the use of TaRT have been overcome, whereas others are areas of active investigation. Many of these are covered in more detail in other reviews. A more extensive version of this chapter can be found online. The supplemental text contains tables, figures, and references that enhance the print version of this chapter….*

The efficacy of TaRT is dependent on a number of factors, including properties of the targeted antigen or receptor, tumor, and targeting agent. Antigen/receptor variables include affinity, avidity, density, availability, shedding, and heterogeneity of expression. Tumor factors include vascularity, blood flow, and permeability. Antibody features to consider are specificity of the binding site, which affects selective tumor uptake; immunoreactivity, which can affect localization; stability in vivo; and both avidity and affinity. Affinity can be described by an intrinsic association constant K that characterizes binding of a univalent ligand (formation of a stable antibody-antigen complex) and can be calculated from the ratio of the rate constants for association and dissociation. Because intact antibodies and most antigens are multivalent, the tendency to bind depends on the affinity, number of binding sites, and other nonspecific factors involved in aggregation. The term avidity encompasses all of these factors and, therefore, is used to describe the overall tendency of antibody to bind to antigen.

Increased antibody affinity or avidity does not always correlate with increased in vivo efficacy—the optimal level of affinity remains controversial. One might expect that higher-affinity antibodies would result in increased tumor uptake, retention, and improved efficacy. High-

[53] https://www.researchgate.net/publication/352554812_PSMA_A_Prostate_Cancer_Target

affinity antibodies, however, may preferentially bind to perivascular regions in the periphery of tumors, whereas antibodies of lower affinity are able to penetrate deeper into tumors. Hence, the optimal affinity of an antibody is likely to depend on a number of factors, including level of target antigen expression, vascular permeability, and bulkiness of disease.

In many cases, ***targeting of radiolabeled antibodies to tumor can also be improved through preinfusion of unlabeled antibody, which decreases splenic and urinary uptake of radiotracer****. The optimal amount of unlabeled antibody remains undetermined for most agents, but relatively high doses have been as good or better than lower doses in most imaging studies. Efforts by the Seattle transplant team to optimize the amount of unlabeled antibody for individual patients by tracer studies with varying amounts often showed the highest concentration studied to be as good or better for direct RIT with iodine-131 (131I)-antibody conjugates.*

A wide variety of antibodies have been made against tumor-specific and tumor-associated antigens that, although present on some normal cells, are usually expressed at lower levels than on the targeted tumor cells. Most RIT trials have used monoclonal antibodies (Mabs), and many have used intact murine immunoglobulin G (IgG) antibodies. Early on, the immunogenicity of the nonhuman antibodies was recognized as a serious limitation of TaRT.29 With the exception of patients with lymphoma, who are less prone to develop an immune response to murine antibodies (human antimouse antibody [HAMA] response), > 80% of patients usually develop an immune response against a therapeutically administered murine or other species antibody after a single injection of antibody. Such an immune response can occur even after doses as small as 1 mg used for imaging studies or following administration of antibody fragments or smaller constructs (but with less frequency than found following administration of intact IgG).

Clearly targeting is both viable and actionable. The recognition of CD133 may be useful here.

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