Mitochondria and reactive oxygen species in brain development and pediatric brain tumors

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Funding: This work was supported by NINDS R01 NS061070 (AMK), Winship Cancer Institute (P30 Center Grant CA138292)

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Running title: ROS and mitochondria in pediatric brain

Keywords: medulloblastoma, reactive oxygen species, mitochondria, metabolism, cerebellum

Abstract
Pediatric brain tumors, such as medulloblastomas, glioma, and ependymoma, are among the most common causes of cancer death in children, and patients that survive the current treatment paradigm (surgery, chemotherapy, and radiation) face lifelong side effects. Increased understanding of the biological underpinnings of these tumors, which often occur as a result of aberrant regulation of brain developmental signaling pathways, is urgently needed in order to develop treatments that will improve survival and quality of life. Approximately 30% of medulloblastomas are characterized by activation of the Sonic hedgehog (Shh) signaling pathway and its downstream effectors. These tumors are thought to arise from cerebellar granule neuron precursors (CGNPs), whose rapid proliferation during development requires Shh signaling. In recent years there has been a resurgence of interest in how metabolism in cancer could represent a novel therapeutic target. It has been shown that medulloblastomas are heavily dependent upon glycolysis and fatty acid synthesis. More recent studies have shown that Shh induces mitochondrial fragmentation in proliferating medulloblastoma cells. Mitochondria are regulated by and involved in reactive oxygen species (ROS) production and function. ROS are implicated in many processes associated with cancer growth and progression, including inflammation, vascularization, proliferation, and apoptosis. In this review, we trace the evolution of mitochondria from their prokaryotic ancestor up to their present-day role. We highlight the proteins that regulate mitochondrial biogenesis and the concurrent metabolic processes leading to ROS production and we discuss implications for mitochondrial biogenesis and ROS activity for brain development and cancer.
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1. Introduction

Pediatric brain tumors including glioma (high- and low-grade), ependymoma, and medulloblastoma (MB) are the most common malignant solid tumors of childhood. Improvements to current standard therapies involving surgical resection, chemotherapy, and radiation have resulted in an increase in overall survivorship, but some, like the high-grade glioma called diffuse intrinsic pontine glioma, are still universally fatal. Survivors are frequently beset with late effects including cognitive defects, psychiatric illness, developmental problems, major organ damage, infertility, and an increased risk of other cancers, all of which can severely compromise quality of life [1, 2]. A better understanding of the molecular drivers of these cancers will open new avenues of treatment possibilities that could more specifically target cancer cells while sparing normal cells, in contrast to current therapies. Pediatric brain tumors are a very diverse group of diseases which in the past have been grouped based on the location of the tumor, the cell of origin, and histology. Recent advances in research have allowed for better classification of these tumors based on genomic, epigenomic, and proteomic studies. One important discovery has been how frequently components involved in metabolism are dysregulated. The products of this dysregulation are critical to cancer cell survival and proliferation, thereby offering an intriguing area of research.

The observation that metabolism in cancer cells is fundamentally different than in normal cells was first made by Otto Warburg nearly a century ago. It is now accepted that metabolic changes are inherent to cancer and the most recognized phenotype of these changes is termed the Warburg effect, wherein cells favor of aerobic glycolysis over other oxidative means of energy production [3]. While inefficient in terms of ATP synthesis, the Warburg effect does provide ample raw material for biosynthesis of the macromolecules needed for rapid cell division [4]. Additionally, cancer cells must maintain redox homeostasis, balancing the levels of reactive oxygen species (ROS) and antioxidants [5]. There is mounting evidence that disturbances in redox homeostasis and redox signaling are involved in tumor progression and treatment resistance. At the crossroad of these processes are mitochondria, the powerhouses of the cell and major contributors to cellular ROS levels. It is becoming apparent that mitochondrial biogenesis and function are vital to ROS production and together they play important roles in cancer development and maintenance.

Medulloblastoma, which arises in the cerebellum during post-natal development, is the most common malignant brain tumor in children. While advances in treatment have resulted in an approximately ~75% survival rate, tumor recurrence is lethal [1, 6]. Approximately 30% of human medulloblastomas show evidence of aberrant Sonic Hedgehog (Shh) signaling pathway activity [7], which under normal circumstances transiently drives post-natal proliferation of neural precursors in the cerebellum. Importantly, the subgroup of SHH medulloblastoma patients whose tumors feature mutant p53 fare the worst. The remaining 70% of human patient medulloblastomas can be divided into two molecularly, histologically, and demographically distinct subgroups, one marked by Wnt pathway hyperactivation and other comprising less well-understood genetic anomalies[8]. Of all subgroups, the SHH subgroup has been most feasible to study using robust mouse models that closely recapitulate the human tumors. These models are generated using loss of function of the tumor suppressor Patched, a negative
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regulator of Shh signaling, or mutational gain-of-function of Smoothened, the positive transducer of the Shh ligand signal. A significant problem in the field is that mechanisms driving MB recurrence are poorly understood, but it has recently been proposed that recurrent MBs are derived from minor molecular clones that were present at diagnosis but were not the original drivers of tumorigenesis, which are often the proposed therapeutic targets [9].

In the present review, the authors seek to develop a comprehensive understanding of the facts available on the role of mitochondria, redox homeostasis, and ROS signaling in medulloblastoma. While pediatric brain cancers are very different in their genetic drivers, the changes seen in metabolism and mitochondrial function may provide some common ground to improve therapies, resulting in better outcomes for patients, including reduced incidence of tumor recurrence.

2. Mitochondria

2.1 Evolution & Biogenesis

Mitochondria are unique organelles with an interesting evolutionary history as they are thought to have come into existence by endosymbiosis, when a free-living prokaryotic cell (α-proteobacteria) was engulfed by a eukaryotic cell. Somehow, the α-proteobacteria escaped endocytosis by the eukaryotic cell and started leading an endosymbiotic life within the eukaryotic cell. Even though the experimental evidence to prove this hypothesis may have been lost to antiquity, there is substantial evidence in the structure, physiology, and genomics of the present-day mitochondrial DNA that points to the validity of this hypothesis. Mitochondria are characterized by a double membrane and most of their metabolic processes take place at distinct sites on the inner mitochondrial membrane. The inner mitochondrial membrane is extended into finger-like projections called cristae whose structure is critically important for the efficient functioning of the mitochondrion. Another feature that sets mitochondria apart from any other organelle is the presence of its own circular DNA, which resides in the mitochondrial matrix and bears resemblance to the prokaryotic nucleoid. The matrix is also the site for several other important metabolic functions. Mitochondria possess their own transcriptional and translational machinery which bears a striking resemblance to that of a prokaryotic cell. The success of this endosymbiotic relationship may have been a result of the earth’s atmosphere becoming progressively more aerobic and the eukaryotic cell which largely relied on anaerobic respiration needing an aerobic mode of respiration to survive. The endosymbiont, while fulfilling this requirement, could benefit from the nutrients provided by the eukaryotic cell in exchange [10, 11].

Mitochondria have also retained the properties of binary fission and transduction, characteristic of the primitive prokaryotic cell. These are known as the processes of fusion and fission in the modern mitochondrion and confer a highly dynamic nature to the mitochondria. The dynamic nature of mitochondria is critically important for several physiological functions and their fusion and fission activities are maintained even after attaining a highly networked, energetically stable state. Furthermore, it is intriguing that since an equilibrium state can be achieved by simultaneously lowering the fusion and fission rates, the mitochondria still maintain a high rate of fusion and fission activities. Chen and colleagues have suggested an answer to this paradox using their mitofusin deficient cell systems. The fusion and fission processes help compensate for mtDNA mutations, repair minor damage, or flag irreparable mitochondria for
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mitophagy. They observed the presence of specific dysfunctional regions within a normally functioning mitochondrion characterized by either loss of membrane potential or loss of mitochondrial DNA, as well as defective matrix contents. Constant fusion helps mitochondria exchange defective matrix and membrane components, while fission helps get rid of permanently dysfunctional mitochondria. In this way, the detrimental changes are always transient and quickly replaced by effectively functioning organelles [12].

2.2 Mitofusins and Opa1

The proteins responsible for bringing about fusion and fission reactions in mammals are comprised of large GTPases called dynamins. In mammals, Mitofusins (Mfn) 1 and 2 and Opa1 are responsible for fusion of mitochondria. Fusion occurs in a sequential series of steps whereby the outer membranes fuse first due to the action of Mfn1 and 2 followed by inner membrane fusion brought about by Opa1. The energy requirements for this reaction are met by the binding as well as hydrolysis of GTP. The mitochondrial membrane potential also plays important roles in the energetics of the fusion of both inner and outer mitochondrial membranes. Mitofusins reside on the outer mitochondrial membrane and form oligomeric complexes between neighboring mitochondria primarily with the target of fusing together. The oligomeric complexes serve to tether mitochondria together and facilitate the rest of the fusion process. It is suggested that Mfn1 is more efficient than Mfn2 in facilitating fusion as it can form more homo-oligomeric complexes as well as an additional coiled-coil structure which is essential for fusion. A few studies have also reported that Mfn1 is expressed at higher levels in the brain, while Mfn2 is more highly expressed in the testes and heart [13].

Opa1 is the GTPase that is essential for the fusion of the inner mitochondrial membrane. Absence of Opa1 results in a complete loss of fusion and therefore predominantly fragmented mitochondria. It is proteolytically processed by different proteases to generate multiple products, each of which can differentially affect the activity of Opa1. Independent from its role in fusion, Opa1 is also involved in cristae remodeling and one of its proteolytic fragments has been shown to be involved in mitochondrial DNA replication [14]. Loss of Opa1 renders the cells vulnerable to apoptosis along with severely impaired respiratory capacity [15, 16].

It is interesting that even though mammals contain two orthologues for fusion, compared with one GTPase in Drosophila, their functions are not necessarily redundant. In fact, both mitofusins have distinct functions separate from bringing about fusion reactions. In addition to bringing about fragmentation, loss of any one of the mitofusins has been shown to be embryonically lethal. The role of Mfn 2 is particularly highlighted in development during specific events and may suggest a mitochondrial defect as the underlying cause of preeclampsia. Chen and colleagues developed mutant mice models for Mitofusin 1 and 2. They reported that mice which are homozygous mutant for either Mfn1 or Mfn 2 died on embryonic day 10.5, but the heterozygous mutants were able to survive and were fertile. Mouse embryonic fibroblasts developed from mice homozygous for mitofusin knockdown displayed widespread and severely fragmented mitochondria. The unique functions of mitofusins are evident from the fact that while the loss of either or both of them results in fragmentation, the fragmented mitochondria resulting from Mfn1 knockdown are very small and uniform in size compared to those formed after Mfn2
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knockdown. This points to a possible role for Mfn2 in mitochondrial shape maintenance [12].

2.3 Regulation of Mitofusins: The PINK1 & Parkin pathway

The PTEN-induced putative kinase 1 (PINK1) protein is a serine threonine kinase that translocates to the outer mitochondrial membrane directed by its mitochondrial targeting sequence (MTS). It is then imported via the mitochondrial membranes where it undergoes several processing steps, resulting in two cleavage products [17-21]. Import of PINK1 is facilitated by a proton gradient across the two mitochondrial membranes. Accumulation of the cleaved fragment of PINK1 leads to the recruitment of Parkin from the cytoplasm to the mitochondrial membrane. In addition, PINK1 also phosphorylates Parkin at the S65 ubiquitin moiety to activate Parkin at the mitochondrial membrane. Once activated, Parkin brings about ubiquitin mediated degradation of a variety of mitochondrial proteins. Some of the first to be flagged for degradation are the mitofusins, leading to fragmentation of mitochondria. This is a quality control mechanism whereby damaged sections of mitochondria are first separated from the healthy, normally functioning portions, and then flagged using autophagy markers, ultimately leading to their removal via the lysosome [20].

A relatively recent study has highlighted a key role for mitochondria in cognition [22, 23]. Interestingly, this study points to a relationship between mitochondrial morphology and cognition during aging. Younger monkeys used in this study possessed mitochondria spanning a wide range of shapes, characteristic of their dynamic nature. In contrast, aging monkeys had atypical mitochondria consisting of a ring-shaped structure formed by the mitochondrial membranes, surrounding the cytoplasm. Various studies have named these structures differently: toroid, donut-shaped, spheroids etc. Such structures seem to be indicators of oxidative stress and could possibly correspond with cognitive defects. In a separate study, Ding and colleagues demonstrated the formation of such structures and called them mitochondrial spheroids. They have also demonstrated that Parkin and mitofusins are required for their formation, and these structures go back to their normal shapes if total cellular ROS is quenched using antioxidants [24]. Similar structures have also been reported in Huntington’s disease in cells where mitophagy failed to occur due to loss of the PINK1-Parkin pathway function. Similar to the study by Ding and colleagues, formation of these structures required mitofusins and Parkin. Restoration of PINK1 rescued mitophagy indicating a neuroprotective effect of PINK1 [25]. In our recent report, we have highlighted the role of Sonic Hedgehog-induced fission in mitochondria, resulting in disrupted mitochondrial morphology and enhanced proliferation [26]. Considering the perspectives from all of these studies, mitochondrial morphology seems to be important in controlling neuro-cognitive functions. This leads to the question of whether abnormal mitochondrial morphology contributes to the life-long cognitive defects that medulloblastoma patients suffer from as a result of radiation and chemotherapy.

2.4 Drp1 and the fission machinery

Drp1 is a dynamin related GTPase that is primarily responsible for mediating fission in mitochondria. Drp1 is recruited to the outer mitochondrial membrane from the cytosol and it brings about the constriction of the outer membrane followed by scission. The complete process of Drp1 recruitment is still unclear, however it is known that there are a
number of molecules which have specific functions in this process including Fis1, Mff, MiD49, and MiD51. Following recruitment from the cytoplasm, Drp1 accumulates as puncta around the mitochondria at the site where a constriction has been formed. Not all of the puncta that are formed result in a fission event. The recruitment of Drp1 occurs as a result of a receptor mediated interaction between Drp1 and the recruitment protein. It is unknown whether Drp1 directly interacts with the mitochondrial membrane following recruitment or if there are intermediates such as liposomal entities involved in mediating this reaction. Fis1 is believed to be important in mitochondrial fission as the abrogation of Fis1 results in severely defective mitochondrial fission. However, even though it is known that Fis1 can recruit Drp1, complete abrogation of Fis1 does not result in a failure to recruit Drp1. This indicates that other proteins are also involved in the recruitment of Drp1. Mff, MiD49, and MiD51 are all capable of functioning as receptors of Drp1 for its recruitment to the outer mitochondrial membrane, but none of them has been shown to be an absolute requirement for Drp1 mediated fission of mitochondria [27].

Mitochondrial fission results in amplification of mitochondrial numbers. This process is essential for meeting the high metabolic demands of the developing brain, especially during the processes of synapse formation and dendrite development. Mice carrying a Drp1 knockout allele do not survive beyond embryonic day 12.5 and display severe forebrain abnormalities [28, 29]. Most of the developmental deficits start to appear at E8.5, at which time the embryos are similar sized as their wildtype littermates. Between E8.5 and E12.5, reduction in body size, underdeveloped cardiac, liver, and neural tube structures result in lethality. Specifically in the brain, abnormal changes in the size of the forebrain, subdural space, and ventricles are observed. These defects may be a result of hypoxic stress indicating that Drp1 is essential for brain development. Drp1 KO mice also had smooth brain surfaces with nearly absent fissures as compared to control mice. Even with defective growth, proliferation continues in the essential tissues, indicating that Drp1 is essential during embryonic stages for survival but is physiologically dispensable [28, 29].

Cell cultures prepared from Drp1 knockout mice can proliferate for limited periods of time indicating the transient dispensability of Drp1 in cell survival. The mitochondria in these cells were either highly interconnected or, when smaller in size, were clustered around the nucleus. However, mitochondrial function seemed unaffected by Drp1 knockdown. There was minimal expression of the synapse marker, leading to abnormal synapse formation as well as irregular clustering, aggregation, and distribution of mitochondria in the neurites [28, 30, 31].

Abrogation of Drp1 results in hyper-fused, enlarged mitochondria, leading to abnormal neuronal development due to faulty calcium ion signaling, energy redistribution, and synapse formation. Conversely, over-expression of Drp1 leads to excessive fragmentation of mitochondria resulting again in abnormal synapses and dendrites [28, 32-34]. In a report on abnormal brain development in a newborn, Drp1 mutation resulted in lethality [34, 35].

Drp1 has been reported to be selectively activated or upregulated in a number of tumor models, including brain tumors. Not only were mitochondria fragmented in brain tumor initiating cells (BTICs), they also presented mutations at Serine 616, rendering Drp1 constitutively activated. This activating mutation of Drp1...
correlated with poor patient prognosis in the clinic. Downregulation of Drp1 resulted in a rescue of proliferation and tumor formation in vitro and in vivo. Drp1 also potentially downregulates AMPK, a sensor of oxidative stress in cells. Downregulation of Drp1 activated AMPK leading to reduced proliferation and enhanced apoptosis in BTICs [36]. Drp1 knockdown using shRNA in medulloblastoma cells as well as cerebellar granule neuron progenitor cells resulted in rescue of fragmentation as well as proliferation in medulloblastoma cells [26].

2.5 Mitochondria in Brain Development

Mitochondria play important roles in mammalian brain development. The shape of mitochondria governs their functional capacity and various kinds of stress cause their shape to become deformed leading to functional aberrations [22, 23]. Mitochondrial dysfunction in the embryonic stages is embryonically lethal due to placental defects in the developing embryo [27]. Within the brain, differential expression levels of functional mitochondrial genes have been reported. Complex I, for example, is very highly expressed in regions of high neuronal division activity. Events such as synaptogenesis between neurons, which are also high energy demanding events, required intense expressions of complex I evidently due to higher rates of mitochondrial respiration being carried out [37].

Mitochondrial DNA plays important roles in aging and the early onset of aging. Maternal mitochondrial DNA is transmitted with a high rate of mutations, but counteracting processes dilute the amount of mutations passed on by the maternal mtDNA. However, the basal load of mutations transmitted has been shown to lay the foundation for further build-up of somatic mutagenesis so as to result in early onset of ageing [38]. Mitochondria-associated Bax protein causes the release of cytochrome c from mitochondria, driving apoptosis which helps the nervous system get rid of excess neuronal cells and develop efficient synapse connections. This could explain why immature mammalian cells appear more sensitive to apoptosis as compared to mature cells [39].

2.6 Energy supply and demand of the developing brain and tumors

In order to meet the demands of uncontrolled proliferation and cell growth, a transformed cell must make extensive adjustments to its energy metabolism [40]. Energy is a requirement not just for the cell to survive and grow, but also to carry out dedicated functions like signal transduction and synapse formation in neurons. In keeping with Otto Warburg’s hypothesis on aerobic glycolysis in transformed cells, even though energy is being derived by upregulation of the glycolytic pathway, mitochondria are still functional albeit in a deregulated manner. Within a cell, energy is generated in several different compartments. Mitochondria, the traditional “power houses” of the cell, are central players in the cellular metabolic machinery. Several recent discoveries have highlighted the role of mitochondria in brain development and cognition [41]. The last decade has seen an increase in studies evaluating the contribution of mitochondria to metabolic rewiring, but elucidating the nuances of brain tumor metabolism in particular is fast emerging as a promising area of study to develop viable alternatives to traditional therapy in brain tumors.

The highly-evolved vertebrate brain has significant energy requirements which account for utilization of 20% of the body’s total energy. During the course of evolution, with the development of higher degrees of cognition and emotional intelligence, glucose became the molecule of choice for energy
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production and utilization in the developing vertebrate brain. It has been shown that in a normal functioning human brain, glucose is almost completely oxidized to yield high amounts of ATP, the energy currency of the cell [41]. Glucose can be converted into lactic acid via glycolysis, which is considered anaerobic, or further processed into pyruvate which is then used by the tricarboxylic acid cycle leading to oxidative phosphorylation in the mitochondria. About 10% of the total glucose being utilized by the brain is converted to lactic acid by the process of aerobic glycolysis, a phenomenon described by Otto Warburg [42]. In this process, even in the presence of adequate amounts of oxygen, cells utilize glycolysis to yield lactic acid as the final product, along with a mere four molecules of ATP. The adult brain however, shows a differential pattern of energy generation and utilization, based on the region of the brain and the type of cells populating the area. For example, astrocytes in the cerebellum rely primarily on aerobic glycolysis, but the neurons largely use oxidative mechanisms for their energy generation [41, 43-46].

Early development of the mammalian brain includes the proliferation and maturation of billions of cerebellar granule neurons which are formed by the maturation of cerebellar granule neuron precursor cells (CGNPs). The high energy demands presented by the developing brain correspond with rapid proliferation, maturation, and enhanced synaptic development of neuronal cells in general [34, 47-53]. In experiments done on mice at post-natal day 5 (P5), higher expression of glycolytic markers was reported [34]. This is the time when rapid proliferation of CGNPs is taking place inside the cerebellum. In humans, this corresponds to the first few months of post-natal development and this process is under tight regulation by signaling mechanisms so as to prevent uncontrolled proliferation and aberrant metabolism [54].

As development continues in mice between P7 and P21, the neurons start to mature, form synapses and increase their communication with surrounding cells. This period is marked by a pronounced increase in the numbers of mitochondria along with a corresponding elevation in the expression of aerobic respiration related proteins and enzymes in different areas of the developing brain [34, 55-60]. Despite an increase in the mitochondrial mass, the volume of individual mitochondria does not change substantially. Rather, the number of cristae within mitochondria and the density of the mitochondrial matrix increase, which explains the enhanced activity to meet the high energy demands [55, 60, 61]. Through this process, the energy demands of development are met by a program that utilizes aerobic glycolysis during the early, rapid proliferation stages, with mitochondrial activity taking over during the maturation phase.

Developmental signaling pathways such as the Sonic hedgehog pathway are often co-opted in cancer. As in the early stages of development, tumors have a high demand for energy and the Warburg effect meets the needs of growing tumors by providing quick energy and biosynthetic components necessary for cell division. Tumor heterogeneity is another remarkable feature of all cancers including medulloblastoma with the tumor itself composed of several different types of cells at various stages of proliferation and maturation. As an example, cells in the tumor bulk tend to be highly proliferative while stem cells in the perivascular niche are more variable in their proliferation rates [62]. These cell types also differ in their metabolic requirements. In general, MB cells utilize aerobic glycolysis to sustain cell growth and proliferation which may also confer a
selective advantage to transformed cells [4, 54, 63].

3. SHH Medulloblastoma

3.1 SHH Oncogenic pathways and their metabolic connections

Shh signaling is closely coupled with the bioenergetics of MB and metabolic rewiring is one of the emerging hallmarks of cancer. Yes-associated Protein (YAP) is a downstream target of Shh signaling pathway that is required for the mitogenic effects of Shh in CGNPs. Other targets of mitogenic Shh signaling that are conserved in proliferating CGNPs, mouse medulloblastomas, and human medulloblastomas include the oncogenic transcriptional regulators Gli and N-myc. Importantly, we have shown that YAP, a transcriptional co-regulator, and its transcription factor partner TEAD1 are highly expressed in the human SHH subgroup of medulloblastomas. Together, they drive medulloblastoma radiation resistance, and are implicated in tumor recurrence [64, 65]. Under normal growth-inhibitory conditions, YAP is kept in check by Hippo pathway-mediated phosphorylation, which drives its degradation [66, 67]. More recently, it has been found that high levels of YAP predict poor survival in patients, indicating the importance of developing approaches to block the downstream effectors of YAP, which include metabolic regulators [68].

Activation of Yorkie/YAP in Drosophila and mammalian cell lines resulted in hyperfusion of mitochondria and reduction in ROS by the transcriptional upregulation of genes responsible for mitochondrial biogenesis and ROS quenching [69]. This study brings an interesting paradigm to the forefront: Shh-driven MBs exhibit severe fragmentation of mitochondria by suppressing mitofusins [26] while upregulating the glycolytic pathway at the same time [70]. Furthermore, YAP is an established downstream effector of the Shh pathway and is known for inducing proliferation in MBs [64]. In light of the above data, extensive investigation is needed to explore possible compensatory effects of YAP in regulating mitochondrial morphology in Shh driven MBs. Understanding the role played by YAP in medulloblastoma will yield novel candidates for molecularly targeted therapies, improve survival and quality of life, as well as provide increased understanding of how to prevent tumor recurrence.

We have previously reported that concomitant with its effects on cell cycle regulators, Shh also promotes activity of metabolic pathways that appear to contribute to proliferation and tumor cell survival. We demonstrated that Shh signaling is directly coupled to lipogenesis in Shh-dependent medulloblastomas and in proliferating CGNPs. Shh promotes de novo fatty acid synthesis (FAS) by upregulating the fatty acid synthase enzyme and inactivating the Rb/E2F1 tumor repressor complex. Importantly, in vitro inhibition of FAS caused medulloblastoma cell death, while in vivo inhibition of FAS extended the lives of medulloblastoma-bearing mice. We also observed that Shh downregulates fatty acid oxidation or ‘fat burning’ by suppressing key enzymes ACOX1 and MCAD [71]. It is thought that tumor cells become reliant on de novo lipid synthesis in order to support membrane biogenesis, and also that the increase in lipid synthesis causes cancer cell membranes to become more saturated, thus affecting processes such as signal transduction and therapeutic response [72]. A critical building block for FAS is acetyl CoA, a product of glycolysis. Along with featuring high levels of glycolytic enzymes in vivo and in vitro, we have also recently reported that
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Shh induces the production of Acetyl CoA Carboxylase, an enzyme which converts Acetyl CoA into Malonyl CoA, a candidate for FASN [71].

In a separate study, we documented a role for Shh signaling in regulating PPARγ, a nutrient-sensing transcriptional regulator [70]. PPARγ levels were elevated in Shh MBs and its inhibition led to a reduction in proliferation in vitro and extended life-span of tumor-bearing mice in vivo. PPARγ interacts with PGC1α to regulate a number of important mitochondrial functions. In agreement with aberrant mitochondrial activity occurring in medulloblastoma, we have observed increased mitochondrial fission and reduced fusion in the setting of Shh mitogenic/oncogenic signaling, as well as marked loss of structure and presence of abnormal vesicles on mitochondria [26].

4. ROS in Medulloblastoma

4.1 ROS generation and regulation

In addition to producing ATP, an important functional aspect of mitochondrial metabolism is the production of reactive oxygen species (ROS). Mitochondria maintain a membrane potential across their inner and outer membranes to effectively carry out oxidative phosphorylation (OxPhos) and produce ATP. Mitochondrial ROS is a by-product of OxPhos and is formed when oxygen ions that are reduced during OxPhos leak out of the mitochondrial membranes. These superoxides are extremely unstable and quickly combine with hydrogen ions to form hydrogen peroxide either spontaneously or enzymatically via superoxide dismutase. These reactive molecules comprise two of the several different reactive oxygen species that exist in a cell and perform important physiological and pathological functions [73].

In the broadest sense, reactive oxygen species comprise any oxygen-containing molecule with an unbalanced chemical charge on the oxygen atom. Along with closely related reactive nitrogen species (RNS), these include much stronger oxidants than superoxide like singlet oxygen and peroxynitrite, which are more associated with cell damage. These species are generally thought of as too unstable to be involved in signaling the way superoxide and hydrogen peroxide are. The hydroxyl radical is also thought to be involved in signal transduction, but to a lesser extent. Hydrogen peroxide is the most stable species and the species most others are reduced to prior to its own reduction to water. The enzymes and metabolites that contribute to the reduction of ROS are collectively referred to as ROS scavengers and most notably include glutathione, catalase, and superoxide dismutase.

While exogenous diet-derived antioxidants attract much attention, the most important scavengers that effect ROS and ROS signaling are the aforementioned superoxide dismutases (SODs) and the endogenously produced tripeptide glutathione. Glutathione (GSH) is generated via a two-step process from L-glutamate, cysteine, and glycine. Numerous enzymes facilitate glutathione’s neutralization of ROS and general detoxification activities. The most relevant of these for ROS signaling are the glutathione-peroxidases, which along with catalase convert hydrogen peroxide to water to fully detoxify the cell as well as keep the concentration of potentially signaling hydrogen peroxide at an appropriate level. Many of the genes for scavenging enzymes are under the control of the antioxidant response element (ARE) to which the transcription factor Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) binds. The Nrf2 protein is sequestered in the cytoplasm by Kelch like-ECH-associated protein 1 (KEAP1). In normally functioning cells as the
levels of reactive oxygen species begin to rise as a byproduct of metabolism, cysteine residues on KEAP1 become oxidized, changing its conformation and releasing Nrf2 to upregulate the scavengers. This mechanism has been posited to play a role in a number of diseases including cancer [74].

Under normal conditions, ROS serve as intracellular signaling molecules and exist in a homeostatic balance with the scavengers inside a cell [75]. However, under oxidative stress conditions, when this balance is altered, increased glucose catabolism overloads the electron transport chain (ETC) causing excessive release of ROS. This leads to hyperpolarization and fragmentation of mitochondria, followed by first an increase, then a major loss of mitochondrial membrane potential. OxPhos is then rendered dysfunctional due to hyperpolarization of mitochondria. Continued rounds of such insults result in extensive structural damage to the inner mitochondrial membrane, especially the cristae, which are extensions of the inner mitochondrial membrane and harbor the complexes of the ETC. This impairs the ETC, leading to overall mitochondrial depolarization and dysfunction. In this deleterious cycle, impaired ETC continues to produce ROS, and overproduction of ROS leads to damaged and dysfunctional mitochondria [76].

4.2 ROS function

ROS can promote different kinds of signaling events based on their origin, type and quantity generated. For example, overproduction of hydrogen peroxide can lead to apoptosis, while low levels of the same can support p53-mediated cell survival and proliferation [77]. Over expression of catalase or antioxidant enzymes leads to inhibition of proliferation. Progressive elevations in the levels of peroxide, on the other hand, can lead to growth arrest, senescence, and cell death [78]. The role of ROS in cell death is interesting because they are not produced as a result of a dying cell, rather they can initiate signaling events characteristic of all three types of cell death mechanisms e.g. apoptosis, autophagy, and necrosis [79, 80]. Apoptosis can be accomplished by either the intrinsic or the extrinsic mechanism. However, both mechanisms rely on ROS signaling and the source of ROS is immaterial.

The signal transduction can also be more nuanced than the broad results of oxidative stress. Mechanistically speaking, ROS are reported to reversibly oxidize the active sites, often cysteine residues, of enzymes with signaling activity which is modified by the oxidation. One of the classic examples of this are the protein tyrosine phosphatases where a catalytically important cysteine is often oxidized to dampen the activity of the enzymes [81]. Additionally, deubiquitinases are known to have key catalytic cysteines that are modified by reactive oxygen species, leading to the stabilization of proteins that would not be stabilized at lower ROS levels [82]. Phosphatase and tensin homolog (PTEN) can have its phosphatase activity inhibited in this way thus contributing to the upregulation of the PI3K/Akt pathway.

The implications of ROS signaling in medulloblastoma and neural development are striking when the regulation of proliferative pathways are considered. Le Belle and colleagues found higher levels of ROS in multipotent neural progenitor cells with the phenotypic characteristics of neural stem cells. They also reported that these cells not only had self-renewal capacity, but that such effects were dependent on PI3k/Akt signaling [83]. The PI3k/Akt pathway promotes survival of radiation resistant cells in medulloblastoma. It is known that medulloblastomas exhibit high levels of phosphorylated Akt in the areas surrounding the vasculature, also known as the
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The perivascular niche (PVN) [62]. The PVN houses tumor-repopulating cells (TRCs) that play important roles in tumor cell survival and radiation resistance [64, 65]. ROS have an important role in the generation and maintenance of these cells. While low levels of ROS are required for fate determination of normal stem cells and their self-renewal, increases in ROS levels can lead to the proliferation or differentiation of TRCs in a dose-dependent manner [84]. Studies have reported the presence of low levels of ROS in TRCs which makes them less vulnerable to DNA damage during irradiation and helps with tumor recurrence post irradiation [85]. This suggests a tendency for tumor cells’ high ROS levels to actually prime the DNA repair and ROS scavenging responses prior to irradiation.

In addition to mitochondrial production, ROS are also generated by several different “dedicated” producers in the cell. One example is the NADPH oxidase (NOX) family of proteins comprised of seven different enzymes which predominantly regulate ROS signaling and includes two dual oxidases (DUOX) that exhibit peroxidase activity in addition to oxidase activity. These enzymes contribute to ROS signaling through generation of superoxide and its subsequent dismutation to hydrogen peroxide (H₂O₂). Interestingly, these enzymes can form a feed-forward loop which interplays with mitochondrial ROS and augments the production of total ROS [73].

4.3 The NOX family in development and cancer

The NADPH oxidase (NOX) family of enzymes are reported to be the primary producers of ROS in the cytoplasm. However, a few studies have also shown their presence in the mitochondria [86]. Graham and colleagues have demonstrated the presence of a mitochondria localization signal that can also transport proteins into the mitochondria. They also reported higher expression of the NOX4 protein in breast and ovarian tumors [87]. The current literature supports the NOX family of proteins as being a significant source of intracellular ROS production in a multitude of cancers [88-93] More studies are required to examine the role played by the NOX signaling pathway in regulating total cellular ROS and conferring tumor protective and radiation resistance properties to the tumor-repopulating cells in the PVN.

Nox4 is of particular interest and extensive work has been done analyzing the relationship between Nox4 and tumor progression [91, 93, 94], and Nox4-produced ROS has been shown to be protective of vascular function [95]. Interestingly, Nox4 is the only member of the Nox family to display constitutive activation [96-98]. Although Nox4, like other members of the Nox family, does interact with the transmembrane coactivator p22phox, its ability to produce ROS is uniquely not altered by point mutations in p22phox’s proline-rich region that block binding [99]. Given its close cooperativity with ROS production in the endothelial cells and cells of the vasculature [100], as well as the PI-3 Kinase/Akt pathway, it will be interesting for future studies to investigate whether Nox4 or other NOX proteins interact with the PI3K pathway to promote medulloblastoma cell survival and recurrence post-irradiation.

Nox4 knockout mice have been created and studied in myriad contexts including lung fibrosis and oxidative stress [101, 102]. They are also reported to exhibit increased oxidative stress in the kidneys (Jackson Laboratories). However, Jha and colleagues generated a floxed Nox4 mouse expressing Cre recombinase in kidney podocytes that was reported to afford renoprotection when diabetes and thus subsequent nephropathy was induced by streptozotocin. This Nox4(LoxP) mouse could
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be crossed with mice that have Cre recombinase driven by various promoters to investigate Nox4 abrogation in a tissue-specific manner [103]. Currently there is no reported data on brain development or a brain phenotype in the knockout mice although Nox4 has been modulated in wildtype mice with inhibitors and siRNA to study its role post-ischemia [104].

The origin of the ROS species, Nox or mitochondrial, and the quantity generated may lead to differential effects: NOX generated ROS are considered low level species and may have a protective effect while mitochondrial ROS are considered high level species and may lead to apoptosis [105-108]. In development, higher levels of ROS can prove valuable to newly formed neurons which need these molecules to differentiate into neurons from their precursor stages. Inhibition of ROS in these cases has shown to result in smaller neurons while not affecting the total number of neurons [108, 109].

Overproduction of ROS has been consistently documented in the early postnatal stages of the developing mouse brain. Due to the high-energy requirements of the brain, it is understood that some basal level of oxidative stress is a normal occurrence in the developing brain. The oxygen levels in the brain increase drastically following birth, and this stage is also concurrent with the over proliferation and consequent elimination of nerve cells e.g. neurons and glial cells. ROS-induced DNA damage has also been reported by some studies and this observation seems to support the elimination of over abundant cells produced during postnatal development. Specific events occurring postnatally seem to be responsible for a cyclical increase or decrease of ROS production. For example, Wilhelm and colleagues found that immediately after birth, the superoxide production from the neural or glial fraction of the brain does not produce high levels of ROS. Instead, the endothelial vessel walls seem to be producing elevated levels of ROS, indicating a high rate of vascular development. On day two, however, the neuronal cells seem to enhance their production of ROS, while endothelial ROS levels remain low. This period was marked by an increase in the development of these cells of the brain. On day four, converse results were observed with an increase in endothelial ROS and reduction in neural cells. Oxidative stress, therefore, is an inherent phenomenon in early brain development and displays specific time point-related production levels which also correspond to specific developmental and growth events [110].

5. Conclusions

Mitochondria play important roles in normal brain development as well as tumorigenesis. Their role in neural synapse development and cognition makes them vital subjects of studies involving tumor recurrence post-irradiation. Mitochondrial fusion and fission, while reminiscent of the ancient prokaryotic biology, help maintain healthy and functionally relevant mitochondria. In medulloblastoma, the metabolic implications of mitochondrial biogenesis driven by Mitofusions 1 and 2, Opa1 and Drp1 downstream of Sonic hedgehog underlie shifts in the sources and concentrations of ROS that require further study. It is known that varying concentrations of reactive oxygen species are broadly associated with proliferation, differentiation, and senescence and also that the commonly observed species hydrogen peroxide and superoxide have various specific roles in enzyme inhibition, most notably in phosphatases and deubiquitinases, that can impact proliferation in cancers including medulloblastoma. Mitochondria thus represent potential targets of signaling and tumor microenvironment manipulation that could lead to novel treatment options for medulloblastoma.
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References


20. Durcan, T.M. and E.A. Fon, *The three 'P's of mitophagy: PARKIN, PINK1,
Mitochondria and reactive oxygen species in brain development and pediatric brain tumors


Mitochondria and reactive oxygen species in brain development and pediatric brain tumors


57. Land, J.M., et al., *Development of mitochondrial energy metabolism in
Mitochondria and reactive oxygen species in brain development and pediatric brain tumors


Mitochondria and reactive oxygen species in brain development and pediatric brain tumors


Mondol, A.S., N.K. Tonks, and T. Kamata, Nox4 redox regulation of PTP1B contributes to the proliferation and migration of glioblastoma cells by
Mitochondria and reactive oxygen species in brain development and pediatric brain tumors


