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Sonic Hedgehog signaling drives mitochondrial fragmentation by suppressing mitofusins in cerebellar granule neuron precursors and medulloblastoma

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Abstract

Sonic hedgehog (Shh) signaling is closely coupled with bioenergetics of medulloblastoma, the most common malignant pediatric brain tumor. Shh-associated medulloblastoma arises from cerebellar granule neuron precursors (CGNPs), a neural progenitor whose developmental expansion requires signaling by Shh, a ligand secreted by the neighboring Purkinje neurons. Previous observations show that Shh signaling inhibits fatty acid oxidation while driving increased fatty acid synthesis. Proliferating CGNPs and mouse Shh medulloblastomas feature high levels of glycolytic enzymes *in vivo* and *in vitro*. Since both of these metabolic processes are closely linked to mitochondrial bioenergetics, the role of Shh signaling in mitochondrial biogenesis was investigated. This report uncovers a surprising decrease in mitochondrial membrane potential (MMP) and overall ATP production in CGNPs exposed to Shh, consistent with increased glycolysis resulting in high intracellular acidity, leading to mitochondrial fragmentation. Ultrastructural examination of mitochondria revealed a spherical shape in Shh-treated cells, in contrast to the elongated appearance in vehicle-treated post-mitotic cells. Expression of Mitofusin 1 and 2 was reduced in these cells, while their ectopic expression restored the mitochondrial membrane potential to the non-proliferating state and the morphology to a fused, interconnected state. Mouse Shh medulloblastoma cells featured drastically impaired mitochondrial morphology, restoration of which by ectopic mitofusin expression was also associated with a decrease in the expression of Cyclin D2 protein, a marker for proliferation.

Keywords

Sonic hedgehog; metabolism; mitochondria; medulloblastoma; cerebellum

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Author Contributions

A.M. and A.M.K. designed the research. A.M., A.D., and N.P. performed the experiments and analyzed the data. A.M. and A.M.K. wrote the paper. All authors read and commented on the manuscript and approved the final version.

Introduction

Medulloblastoma is the most common malignant pediatric brain tumor, typically arising in children before the age of ten, although infrequently late adolescent or adult patients do present with this tumor. Medulloblastomas arise in the cerebellum, a brain region which regulates posture and coordination as well as some aspects of cognition, and whose development takes place largely post-natally. The current treatment regimen for these tumors includes cranio-spinal radiation, surgical resection and chemotherapy whose cumulative effects on the young brain leaves survivors with long-term cognitive and behavioral defects (1) that severely impact quality of life. Thus, there is a need for understanding tumor-specific processes and characteristics that could in the future be targeted by more specific therapeutic approaches, leaving intact the patients' developing brains.

Concomitant studies by several groups have divided medulloblastoma into 4 genetically and histologically defined subgroups (2–9). The unique sample clusters identified in this study include: WNT, SHH (Sonic hedgehog), group C and group D. The tumors bearing the SHH signature are thought to arise from neuronal progenitors known as Cerebellar Granule Neuron Precursors (CGNPs) (10). Activation of the Shh pathway is required for CGNPs to undergo rapid population expansion in the external granule layer (EGL) of the cerebellum during post-natal development. The Shh ligand, secreted by the Purkinje neurons underlying the EGL, binds to Patched (Ptc), which is a 12-pass transmembrane domain receptor. Ptc inhibits smoothened (Smo), a 7-pass transmembrane protein. Binding of Shh to Ptc relieves the inhibition of Smo, leading to activation of the Shh pathway and translocation of the Gli family transcription factors to the nucleus. This causes activation of target genes that drive proliferation and inhibit differentiation of CGNPs (11–13).

CGNPs can be cultured *in vitro* by harvesting the cells from the cerebellum of post natal day 4/5 mice and can be maintained in a proliferative state for up to 72 hours by adding Shh protein to the medium. We and others have used CGNP cultures to model mitogenic Shh signaling *in vitro*, to study mechanisms driving cell cycle progression, interactions between Shh and other signaling pathways, and proliferation-associated intracellular processes (14). Importantly, robust mouse models for the SHH subclass of medulloblastomas have been generated, such as by deletion of *Ptc* or transgenic expression of activated *Smoothened* (*SmoA1*) (15–17).

Extensive studies have been undertaken to understand the biology of medulloblastoma, but little is known about their metabolic profile. Tumor metabolism was included as an important trait when the hallmarks of cancer were revisited in 2011 (18). Previously, we reported that Shh mitogenic/oncogenic signaling is tightly coupled to the reprogramming of mitochondrial bioenergetics: Shh inhibits fatty acid oxidation (FAO, or β -oxidation) while driving increased fatty acid synthesis (FAS)(19). A critical building block for FAS is acetyl CoA, the final product of glycolysis. We have also shown that proliferating CGNPs and mouse Shh medulloblastomas feature high levels of glycolytic enzymes *in vivo* and *in vitro* (20) consistent with the “Warburg effect” (21). Indeed, the glycolytic enzyme Hexokinase 2 is required for cerebellar neurogenesis and its deletion abrogates Shh-associated mouse

medulloblastoma development and aggressiveness (22), underscoring the importance of this metabolic pathway in rapid Shh-driven CGNP proliferation both in context of normal development and tumorigenesis. Lactate secretion from elevated glycolysis is the main cause of acidic extracellular pH in most tumors (23,24). Additionally, a striking feature of such glycolytic cells is their fragmented mitochondrial profile (25). Mitochondria are dynamic organelles that undergo fusion or fission reactions, collectively termed “biogenesis”, based on their bioenergetic activity. Glycolytic cells present small, rounded mitochondria with expanded matrices whereas mitochondria of respirative cells, characterized by an abundance of substrate, appear interconnected, with enlarged inter-cristae compartment (26). Mitochondrial biogenesis is important in maintaining their physiology, morphology and respiratory capacity (27). Thus, mitochondria are not only central to cellular energetics, but functional mitochondria are also required for a transformed cell (28).

Mitochondrial cristae carry out oxidative phosphorylation (Oxphos) which gives rise to an electro motive force across the two mitochondrial membranes and is known as the mitochondrial membrane potential (MMP). MMP therefore, is a key indicator of cell health (29). Importantly, cancer cells do not shut down energy metabolism completely, rather, they alter the mitochondrial pathways (30). Even with elevated glycolytic levels, mitochondrial oxphos is still functional albeit, in a deregulated manner (21). Dysfunctional oxphos indicates a lower MMP, but most cancer cells exhibit elevated MMP, which can be understood as a phenomenon to resist apoptosis (31) and favor high proliferative rates characteristic of cancer cells. Mitochondria are well known to signal pathways regulating apoptosis (32), and cells undergoing apoptosis characteristically show a decrease in MMP (32). Thus despite being deregulated, mitochondria of cancer cells display a complex balance of metabolic and apoptotic signals which promotes proliferation.

Our present study highlights the effects of Shh pathway activity on mitochondria in proliferating CGNPs and medulloblastoma cells (MBC). We report that Shh causes marked reduction in mitochondrial mass, accompanied by reduced membrane potential, which is not associated with cell death, and reduces mitochondrial fusion by down-regulating the mitofusins 1 and 2. Over expression of the mitofusins in CGNPs and medulloblastoma cells not only restores the mitochondrial morphology but also restores their non-proliferative phenotype. These novel findings suggest that in future studies it will be important to ask whether manipulation of mitochondrial biogenesis can impact function sufficiently to complement current medulloblastoma treatment regimens.

Materials and Methods

Animal Studies

Harvest of neural precursors from neonatal mice and preparation of cerebella and tumor tissue from wild-type and mutant mice for cell culture or histological analysis were carried out in compliance with the Emory University Institutional Animal Care and Use Committee guidelines. NeuroD2-SmoA1 mice were obtained from Jackson Research Laboratories.

Culture of CGNPs and medulloblastoma cells

CGNP cultures were generated as described previously (33). Cells were plated on poly-DL-ornithine (Sigma) precoated plates or precoated glass coverslips. Where indicated, Shh was used at a concentration of 3 µg/mL. Cyclopamine (R&D Systems) was used at 1 µg/mL.

Medulloblastoma cells (MBC) were harvested from SmoA1 mouse medulloblastomas. Briefly, tumors were dissociated and cells were incubated in Papain/DNAse solution for 30 minutes, and then passed through a cell strainer. Cells were subsequently separated on a density step gradient of 35% and 60% Percoll solution (Sigma). Purified MBCs were enriched by pre-plating on uncoated tissue culture dishes to remove adherent fibroblasts and glial cells. Non-adherent cells were plated on tissue culture dishes or glass coverslips pre-coated with poly D-lysine (Sigma) and Matrigel (BD Biosciences), infected with adenoviruses and cultured for 48 hours before western blotting.

Adenovirus production and infection

Replication incompetent adenoviruses were obtained from Vector Biolabs. A GFP-only adenovirus was used as control. For viral expansion, 60–80% confluent HEK293T cells were infected with the respective adenovirus and cultured for 48–72 hours. The cells were harvested, pelleted by centrifugation and subjected to 4 cycles of freeze-thaw in freezing mixture. Viruses were pelleted by ultracentrifugation at 24000 rpm, for 3 hours at 4°C. For infection, CGNPs or MBCs were cultured for 3 hours before addition of viruses at 100 MOI. The cells were allowed to grow for 48 hours post-infection.

Protein preparation and Immunoblotting

Cells were washed once in PBS and protein extracts were prepared as described in (33). Protein estimation was performed using the Bradford method in the Bio-Rad protein assay. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed according to standard procedures. Thirty micrograms of the total cell lysate was loaded on 10% polyacrylamide gels and transferred to activated Immobilon PVDF (Millipore) membrane, which were blocked in 5% milk. Antibodies used for western blotting were Mfn1, Mfn2 (Abcam), Drp1, CyclinD2 (Santa Cruz Biotechnology Inc.), Beta-actin and GFP (Cell Signaling). Donkey anti-mouse HRP-linked secondary was from Jackson Research Laboratories, and goat anti-rabbit was from Thermo-Scientific. Peroxidase activity was detected using ECL reagent (Thermo Scientific) and exposing membranes to GE-Amersham film.

Immunofluorescence

Cells were cultured on pre-coated glass coverslips and fixed with 4% paraformaldehyde for 20 min. For BrdU incorporation, cells were pulsed with BrdU at a final concentration of 10µM for 3 hours prior to fixation. Immunofluorescence was performed by standard methods. Antibodies used for Immunofluorescence were Tom20 (Santa Cruz Biotechnology Inc.), PCNA (Merck) mouse, BrdU (BD), F(ab')₂ Fragment Goat Ant-Mouse IgG (H+L) (Jackson Immunoresearch laboratories Inc.) and rabbit IgG (Life technologies).

Mitotracker staining

100nM mitotracker (MitoTracker® Red CMXRos, Life Technologies) was prepared in requisite medium for CGNPs ($\pm$ Shh) or MBCs, from a 1mM stock solution in DMSO. Cells were incubated for 45 mins at 37°C and then fixed in 4% formaldehyde. After 3 washes in PBS, the coverslips were mounted in ProLong® Gold Antifade Mountant (Life Technologies). Quantification of mitotracker staining was carried out using ImageJ and normalized to number of nuclei stained with DAPI.

Image Capturing

Confocal Images of stained primary cells and MBCs were acquired using an Olympus FV1000 laser confocal microscope and captured with Olympus Fluoview Software (Integrated Cellular Imaging Core, Emory University). Quantification of western blots was carried out using Adobe Photoshop software.

Electron Microscopy

Cells were cultured on pre-coated glass coverslips and fixed overnight in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4°C. For tissue sections, tumors or normal cerebellar tissue was dissected out of a tumor bearing SmoA1 mouse and immediately fixed in the above fixative. Cells/tissues were then washed with the same buffer and post-fixed in 1% buffered osmium tetroxide, dehydrated through a graded ethanol series to 100%, and embedded in Eponate 12 resin (Ted pella Inc., Redding, CA). Ultrathin sections were cut on a Leica UC6rt ultramicrotome (Leica Microsystems, Bannockburn, IL) at 70–80nm, and counter-stained with 5% aqueous uranyl acetate and 2% lead citrate. Sections were examined on a JEOL JEM-1400 transmission electron microscope (Tokyo, Japan) equipped with a 2K $\times$ 2K Gatan US1000 CCD camera Pleasanton, CA).

Aspect ratio Measurement

For each cell treatment, approximately 120 mitochondria were measured to determine aspect ratios using ImageJ software (1.48f, Wayne Rasband, National Institutes of Health, USA).

ATP Assay

Total ATP was extracted from CGNPs $\pm$ Shh in 2.5% trichloroacetic acid (TCA). ATP was measured using the ENLITEN ATP Assay kit (Promega) following the manufacturer's protocol.

RNA extraction and RT-PCR

Total RNA from CGNPs was extracted and purified using the TRIzol® reagent (Invitrogen) according to manufacturer's instructions. cDNA was prepared from 1 μ g of total RNA by using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as per the manufacturer's instructions. Quantitative PCR was performed using SsoAdvanced Universal SYBR Green Supermix (BioRad). RNA expression data were acquired using C1000 Touch Thermal Cycler and CFX96 Real-Time PCR Detection System. The data was analyzed for average results and standard errors are presented. Primers for Mfn1, Mfn2, Drp1, Gli1 and GusB were purchased from BioRad.

Results & Discussion

Mitochondria are the primary site of energy production in the eukaryotic cell. We have shown previously that Acetyl Coenzyme A Carboxylase (ACC), a key enzyme to convert Acetyl-CoA into Malonyl-CoA, is altered by the action of Shh (19). This indicated a central role of mitochondria in medulloblastoma metabolism as ACC not only inhibits mitochondrial β -oxidation, but is also a substrate for FASN. One of the first indicators of dysfunctional mitochondria is the MMP (29). Therefore, to investigate the effect of Shh on MMP, we stained CGNPs with Mitotracker red, a membrane potential based mitochondrial dye. As mitochondria become depolarized, they lose their membrane potential and incorporate less dye into their membrane. Low intensity staining was observed in the Shh treated CGNPs as compared to vehicle-treated non-proliferating cells. To determine whether the canonical Shh pathway caused this effect, we treated the cells with Cyclopamine, a Shh antagonist. The intensity of mitotracker was restored after this treatment indicating re-polarization of mitochondria (Figure 1a, c). Stable variation in MMP has been reported from different tumor cell subpopulations *in vitro*. These differences have been correlated with tumor invasiveness, metastasis and general progression of disease (34). In general, a higher MMP is indicative of a rapidly proliferating cell while lower MMP would indicate initiation of apoptosis (35). Shh treatment of CGNPs induces rapid proliferation in these cells and prevents them from commencing differentiation. This is evident from the cyclin D2 expression in Shh treated CGNPs (33). Thus, our observation of a decrease in MMP in Shh treated CGNPs stands in contrast to previous reports of a higher MMP in proliferating cells.

When mitochondria are subjected to stress, considerable changes in their morphology become visible. While mitochondria in cells undergoing normal respiratory processes are interconnected, long and tubular, those in cells under stress depict a fragmented morphology, with short and rounded mitochondria (26). To investigate whether the reduced MMP we observe in Shh-treated CGNPs is associated with such morphological changes, we carried out immunofluorescence with Tom20, an outer mitochondrial membrane protein which is a common mitochondrial marker (36). While vehicle-treated CGNPs demonstrated long interconnected tubular mitochondria, Shh treated cells presented a fragmented pattern of mitochondria, with reduced Tom20 staining (Figure 1b, S1B). Treatment with the Smoothened inhibitor cyclopamine restored the long filamentous appearance of mitochondria (Figure 1b). Since mitochondria produce most of the cell's ATP, the energy currency of the cell, we wanted to see if Shh treatment and associated reduction in MMP and mitochondrial fusion had an impact on the total ATP production in CGNPs. Indeed, we carried out an ATP quantification assay and observed a two-fold reduction in total ATP in Shh-treated cells, normalized to protein concentration (Figure 1d). Taken together, these results indicate that Shh signaling in CGNPs reduces mitochondrial function, likely through effects on mitochondrial biogenesis as shown in Figure 1b.

Mitochondria carry out the tricarboxylic acid cycle within their lumen and utilize the NADH and succinate thus produced, to drive oxidative phosphorylation, also known as the electron transport chain (ETC). This cycles electrons through five different complexes and releases ATP and water outside the mitochondria into the cytoplasm. As mentioned above, the electron cycling and resulting proton gradient also gives rise to a difference in potential

across the two mitochondrial membranes, known as MMP (29). All the complexes of ETC are located on extensions of the inner mitochondrial membrane called cristae. As our next step, we used transmission electron microscopy to observe the ultrastructure of mitochondria in Shh treated CGNPs. Consistent with the high MMP and filamentous structure shown in Figure 1, the vehicle-only cells demonstrated elongated mitochondria with well-defined cristae structure while the Shh treated cells consisted of rounded, smaller-sized mitochondria with a reduced network of cristae (Figure 2a). To further quantify the morphology of mitochondria, we analyzed aspect ratios (AR) of mitochondria from the electron micrographs. For each cell treatment, about 120 mitochondria were measured from three distinct cell preparations, to determine aspect ratios using ImageJ software (1.48f, Wayne Rasband, National Institutes of Health, USA). The vehicle treated CGNPs had an average aspect ratio of 1.94 (stdev=0.05) while the Shh treated CGNPs were at 1.51 (stdev=0.084), and the difference in aspect ratio was found to be significant (p -value= 0.0013). The AR of mitochondria in vehicle treated CGNPs were therefore significantly higher than those of Shh treated cells.

Changes in cristae structure could result in disruption of ETC, which accounts for the low MMP and ATP observed in Figure 1. The shape and size of mitochondria are largely governed by fusion and fission events. Such events help maintain functionality in mitochondria when the cell undergoes metabolic or environmental stress. Fission plays a role in quality control by removing the non-functional portion of a defective mitochondrion. Fusion, in turn, complements mitochondria by mixing the contents of partly functional mitochondria with those of fully functional ones. The dynamin family of GTPases control the fusion and fission processes of mitochondria. Mitofusins 1 and 2 (Mfn1, 2), which are membrane-anchored dynamins, regulate fusion of outer membranes. Drp1, on the other hand, is recruited from the cytoplasm to the outer mitochondrial membrane to fission off mitochondria (37). To examine how these proteins were affected by the action of Shh, we carried out western blot analysis on lysates prepared from CGNPs cultured for 48hrs $\pm$ Shh. Mfn1 and 2 levels were down regulated in the presence of Shh and were restored after treatment with cyclopamine (Figure 2b and c). Drp1 did not show significant change in the presence of Shh (data not shown). We, therefore, believe that suppression of fusion proteins accounted for the more fragmented appearance of Shh treated mitochondria in Figure 1b and 2a. Of note, gene expression analysis of mitofusins and Drp1 did not show significant change in transcript levels between vehicle and Shh treated CGNPs (Figure S1a), indicating a post-transcriptional mechanism of regulation.

Since mitochondria manifested a fragmented appearance after treatment with Shh, we wanted to see if restoring the expression of mitofusins could rescue the fused mitochondrial morphology. To do this, we carried out a gain-of-function analysis using adenovirus mediated over expression of Mfn1 (Figure 3a). Indeed, the mitochondrial MMP and the long tubular morphology of mitochondria were restored following ectopic expression of Mfn1 (Figure 3b,c, S2a). The ultrastructure analysis also revealed more elongated mitochondria in Mfn1 over-expressing CGNPs (Figure 3d). Similar observations were made in CGNPs over expressing Mfn2 (Figure 3e–g, S2c). Since Drp1 is primarily responsible for mitochondrial fission, we carried out a loss-of-function analysis using adenovirus-mediated down-

regulation of Drp1 (Figure 4a). The MMP was restored after Drp1 knockdown indicating repolarization of mitochondria (Figure 4b, S3a). A reduction in fission of mitochondria was visible after Drp1 knockdown as the mitochondria start to become more fused (Figure 4c). We also noted a decrease in cyclinD2 levels after restoration of mitofusins or knock-down of Drp1, indicating a decrease in proliferation of Shh treated CGNPs (Figure 3a,e and 4a). To further confirm this, we carried out BrdU labeling of proliferating cells after adenovirus infections. Indeed, we observed a rescue in proliferation after restoration of the mitofusins and Drp1 in CGNPs (Figure S2b,d and S3b).

To determine if the abnormal mitochondrial morphology was also present in SmoA1 tumors, we analyzed electron micrographs of tumor or adjacent normal cerebellar tissues. As expected, the mitochondria of SmoA1 tumor sections were small, fragmented with ill-defined cristae while those in the normal tissue displayed elongated mitochondria with well-defined cristae (Figure 5a). To further investigate this, we carried out western blot analysis of mouse cerebellar tumor tissue as well as adjacent normal tumor tissue. The levels of Mfn1 and 2 proteins were remarkably reduced in the tumor tissue as compared to the normal tissue. The levels of Drp1, on the other hand, were strikingly higher in the tumor tissue relative to the normal tissue (Figure 5b). We also examined micrographs of SmoA1 medulloblastoma cells cultured *in vitro*. The mitochondria in these cells were severely affected with swollen cristae and non-existent mitochondrial lumen (Figure 5c). To study the effects of mitofusin over-expression in medulloblastoma cells, we prepared *in vitro* cultures of SmoA1 tumor cells and carried out adenovirus-mediated over-expression of Mfn1 and 2, and down-regulation of Drp1. Similar to our observations in CGNPs, we observed reduced levels of CyclinD2 upon ectopic expression of Mitofusins 1 and 2 (Figure 5d, e) or adenovirus-mediated knock-down of Drp1 (Figure 5f). The MMP of SmoA1 tumor cells *in vitro* appeared to have been rescued by over-expression of Mfn1, 2 or knock-down of Drp1 (Figure 5g,i S4a, b). Immunofluorescence using Tom20 initially revealed a near absence of structurally intact mitochondria in Shh treated CGNPs. This was rescued after overexpression of Mfn1 and 2 and knockdown of Drp1 (Figure 5h, j).

It is noteworthy from our results that restoration of mitochondrial biogenesis could rescue the non-proliferative phenotype of Shh treated CGNPs as well as MBCs. It has been observed that alterations in energy metabolism can lead to cell-cycle exit in *Drosophila* neural stem cells (38). More recently, it has been shown that manipulation of α -ketoglutarate levels in embryonic stem cells can affect self-renewal and differentiation, adding to a growing body of evidence that alteration of metabolic regulation can impact upon cell fate (39). Mitochondria are well known to house and initiate cascades of caspases leading the cell to apoptosis (32). However, under our conditions of cell culture, and in the developing cerebellum, Shh stimulation drives CGNP proliferation, not apoptosis, suggesting a novel role for Shh in affecting cellular metabolism by altering mitochondrial form and biogenesis, resulting in impaired oxphos and reduced MMP.

Previous studies have implicated Shh signaling in heightened activity of FASN and other enzymes contributing to increased fatty acid synthesis (19,40). Here, we report a decrease in MMP coupled with a decrease in the overall ATP production by primary CGNPs treated with exogenous Shh to drive proliferation, and in MBCs bearing constitutive Shh pathway

activity. Due to the site-specific nature of oxphos inside the mitochondria, any changes induced by the action of Shh on mitochondrial structure would lead to altered mitochondrial activity. Therefore, the effect of Shh on mitochondrial biogenesis gains special importance. The mitochondria of Shh treated cells presented a fragmented morphology, which was confirmed in the ultrastructure of cells. The proteins regulating fusion and fission events, primarily responsible for the tubular or fragmented morphology of mitochondria, were also de-regulated under the effect of Shh signaling. Restoration of the mitofusins and knock down of Drp1, however, restored the normal morphology of the mitochondria (Figure 6), and was associated with reduced levels of cyclin D2, an indicator of proliferation. Since we did not observe any changes at the transcript level of the mitofusins or Drp1, we can speculate that the mechanism of Shh effects on these proteins could either be accelerated degradation or reduced translation of the biogenesis proteins. Given the significance of the mTOR pathway in regulation of mitochondrial biogenesis, it is worthwhile also to speculate on these lines, especially since our lab has previously demonstrated that Shh activity associated with enhanced mTOR pathway activation (41,42).

Our findings illustrate the changes incurred in the morphology of mitochondria and the consequent impact on cellular proliferation due to the mitogenic action of Shh signaling. With important roles for mitochondria in apoptosis already established, this study highlights mitochondrial morphology as a potential therapeutic target whose restoration can alter the metabolic profile of tumor cells to that of non-transformed, non-proliferating cells, thus potentially suggesting a novel treatment paradigm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Implications

This report exposes a novel role for Shh in regulating mitochondrial dynamics and rescue of the metabolic profile of tumor cells to that of non-transformed, non-proliferating cells and represents a potential avenue for development of medulloblastoma therapeutics.

Figure 1.

Mitochondrial activity and biogenesis are reduced in CGNPs exposed to Shh. (a) CGNPs were cultured for 48h on glass coverslips pre-coated with poly-DL-ornithine. Sonic Hedgehog and Cyclopamine were used where indicated. CGNPs were stained with mitotracker Red and incubated at 37°C for 45 minutes. Shh treated CGNPs showed a decrease in their fluorescence intensity indicating depolarized mitochondria with a reduced MMP. Treatment with cyclopamine restored the MMP to similar levels as vehicle. (b) CGNPs $\pm$ Shh were stained with Tom 20 and visualized by confocal microscopy. Vehicle treated CGNPs presented dense, long networks of mitochondria while Shh treated CGNPs showed more fragmented mitochondria. Treatment with cyclopamine restored the long

filamentous mitochondria. (c) Automated quantification, using ImageJ software, of CGNPs $\pm$ Shh, cyclopamine stained with mitotracker Red. (d) Measurement of Total ATP produced by CGNPs. Shh treated CGNPs produced less ATP as compared to vehicle treated cells.

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Figure 2.

Shh treatment alters the morphology of mitochondria. (a) CGNPs $\pm$ Shh were cultivated on poly-DL-ornithine pre-coated plates and processed for transmission electron microscopy. Shh treated CGNPs reveal swollen morphology with aberrant cristae structure, whereas, vehicle treated cells show elongated mitochondria with well-defined cristae structure. (b, c) Representative western blot analysis of mitochondrial fusion proteins Mfn1 and 2 respectively in CGNPs $\pm$ Shh. Both show a decrease in Shh treated CGNPs, which is restored after treatment with Cyclopamine, a Shh antagonist. Enhanced Cyclin D2 level depicts enhanced proliferation brought about by the action of Shh. The relative band intensities of Mfn1 and Mfn2 relative to B-actin were measured using Adobe Photoshop and are depicted below each lane.

Figure 3.

Manipulation of mitofusins rescues proliferation. (a) Western Blot showing induction in Mfn1 expression following adenoviral transduction of Mfn1. CGNPs were transduced with either GFP or Mfn1 expressing adenoviruses. Over expression led to reduced cyclin D2 expression indicating reduced proliferation. The relative band intensity of Mfn1 relative to B-actin was measured using Adobe Photoshop and is depicted below each lane. (b) The reduction in MMP brought about by Shh treatment of CGNPs, was restored to near-normal levels by ectopic expression of Mfn1 in CGNPs. (c) The deformed and fragmented morphology of mitochondria in Shh treated CGNPs was restored to their long, filamentous form following ectopic expression of Mfn1, as revealed by staining with Tom20. (d) Electron micrographs showing ultrastructure of mitochondria in CGNPs $\pm$ Shh and ectopically expressing Mfn1. The fragmented mitochondria visible in Shh treated CGNPs, appear to be fusing together in Mfn1 over-expressing CGNPs and are longer in shape with well-defined cristae structure. (e) Western Blot showing induction in Mfn2 expression following adenoviral transduction of Mfn2. The relative band intensity of Mfn2 relative to B-actin was measured using Adobe Photoshop and is depicted below each lane. (f) Ectopic

expression of Mfn2 restores mitochondrial membrane potential, and the long, filamentous form of mitochondria, as revealed by staining with Tom20 (g).

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Figure 4.

Manipulation of Drp1 rescues proliferation (a) Western Blot showing reduction in Drp1 protein expression following adenoviral knockdown. CGNPs were transduced with either scrambled-GFP or Drp1-shRNA expressing adenoviruses. Drp1 knockdown led to reduced cyclin D2 expression indicating reduced proliferation. (b) The reduction in membrane potential brought about by Shh treatment of CGNPs, was restored to near-normal levels by knockdown of Drp1 in CGNPs. (c) The deformed and fragmented morphology of mitochondria in Shh treated CGNPs was restored to their long, filamentous form following knockdown of Drp1, as revealed by staining with Tom20.

Figure 5.

Mitochondrial morphology is altered in SmoA1 mouse medulloblastomas. (a) Scanning electron micrographs of medulloblastoma tissue and normal cerebellum obtained from NeuroD2-SmoA1 transgenic mice. Note the swollen, rounded, smaller-sized mitochondria with aberrant cristae structure in SmoA1 tumor tissue as compared to elongated mitochondria with well-defined cristae in normal SmoA1 mouse cerebellum. (b) Representative western blot analysis of fusion (Mfn1, 2) and fission (Drp1) proteins in NeuroD2-SmoA1 mouse medulloblastoma tissue and adjacent, normal cerebellum. Mfn1 and 2 are reduced while Drp1 is induced, indicating increased fragmentation of mitochondria in medulloblastoma tissue. (c) SmoA1 mouse medulloblastoma cells (MBCs) were cultured in vitro for 48h and processed for Electron Micrography. MBCs displayed gross abnormality in the structure of mitochondria that were swollen with blebbing and

complete disruption of cristae. (d,e) Western Blots showing induction in Mfn1 and 2 protein expression following adenoviral transduction. Over expression of Mfn1 or 2 led to reduced cyclin D2 expression indicating reduced proliferation. The relative band intensities of Mfn1 and Mfn2 relative to B-actin were measured using Adobe Photoshop and are depicted below each lane. (f) Western Blot showing reduction in Drp1 protein expression following adenoviral knockdown. Drp1 knockdown led to reduced cyclin D2 expression indicating reduced proliferation. (g) Since MBCs are derived from a mouse model that constitutively express Shh, we expected the MMP to be lower in untreated cells. In MBCs that were transduced with Mfn1 or Mfn2 adenovirus, however, the MMP was higher compared to GFP transduced MBCs, indicating a restoration of MMP in these cells. (h) Aberrant mitochondrial morphology in MBCs was restored to long, filamentous form following ectopic expression of Mfn1 or 2, as revealed by staining with Tom20. (i,j) Similar results were observed after Drp1 knockdown in MBCs stained with mitotracker or Tom20.

Modified from:
Alirol & Martinou, 2006

Figure 6.

Model for the action of Shh on mitochondrial structure and function. In an untreated cell with a respirative phenotype, the environment is aerobic and the mitochondria are long and tubular with an enlarged cristae compartment. In a Shh treated cell, the cellular environment becomes acidic due to enhanced glycolysis (26,40) and the mitochondria become fragmented with a reduced cristae compartment. This morphology, however, can be rescued by the restoration of mitochondrial biogenesis genes: Mitofusins 1,2 and Drp1. This also rescues the cell to its original non-proliferative phenotype.