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Review

Tumor Suppressor microRNAs as Potential Treatment Therapeutics for Advanced Medulloblastoma

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Abstract

Medulloblastoma (MB) is one of the most prevalent forms of malignant brain cancer observed within pediatric patients and is particularly difficult to diagnose and treat due to the anatomical localization of tumors near the brainstem. Currently, there are four molecular classifications of MBL: *WNT*, *SHH*, *Group 3*, and *Group 4* tumor subgroups. Wingless-type (WNT) mutant tumors are the least common, often caused by mutations in the *CTNNB1* gene that plays a crucial role in the wingless cell signaling pathway, yet associates with the best prognosis as compared to all other MBL subtypes. Sonic hedgehog (SHH) mutant tumors arise due to continued release of Shh from purkinje cells, and an uninhibited proliferation response by granular neuronal precursors (GNPs). *Group 3* and 4 MBL subgroups are still a molecularly heterogeneous class of tumors, with *Group 3* MBL being highly associated with metastasis upon diagnosis, and more prevalently characterized by MYC amplification and activation. *Group 4* MBL tumors comprise approximately 40% of all MBL, and remain remarkably heterogeneous with respect to somatic mutations of genes such as *KDM6A*, *OTX2*, *ZMYM3,* with an approximate 80% of tumors harboring chromosome 17 copy number alterations. While a majority of MBL cases cannot be linked to a single protein coding gene alteration, the role of non-coding RNAs, such as miRNAs, seems quite promising as a genetic marker to further sub-categorize MBL at the molecular level. Furthermore, miRNA-based therapy is proving to be a promising treatment to curb the growth of a number of cancer types within the clinic, with particular miRNAs under investigation including miR-34a, miR-211, and miR-584-5p. These miRNAs are known to induce cell cycle arrest in mouse models and demonstrate anti-tumorigenic properties *in vitro*, meriting further investigation of miRNA-based clinical trials for pediatric MBL patients.

1. Introduction

Accounting for about 20-30% of pediatric CNS cancers [1], medulloblastoma (MBL) is the most common embryonal brain tumor observed in children [2], and because of the proximity to the brainstem and cerebellum, diagnosis and treatment of MBL can be particularly challenging. Occurring in the posterior fossa, at the base of the brain, MBL tumors can block the flow of cerebrospinal fluid if not treated with maximally safe resection, causing elevated intracranial pressure and hypertension [3]. Many times the diagnosis of MBL can be difficult to conclude, especially in infants, due to a lack of localizing symptoms, which are common and include vomiting, headaches, fussiness, decreased appetite, and gait ataxia. Furthermore, the open

fontanelles and sutures of an infant's skull allow the head to expand, which in some cases, masks the presentation of a growing MBL tumor [3,4]. Standard MBL treatment consists of a combination of chemotherapy and craniospinal irradiation (CSI). With this multimodal treatment approach, the long-term survival rate for all patients is approximately 70% [5]. Despite this approach, many patients have significant reductions in their quality of life as well as their activities of daily living. By developing early detection methods for MBL, such as enhanced magnetic resonance imaging (MRI) and biomarker testing of the cerebrospinal fluid (CSF) for cancer-specific antigens such as TGF-β, α-ketoglutarate, and NF-kB, clinicians aim to develop newer and safer medications to abate MBL growth while sparing damage to the surrounding neuronal tissue of the brain [5,6].

MBL was initially coined in 1925 by Harvey Cushing and Percival Bailey, and treatment was limited to resection surgery alone, which yielded a 30% mortality rate amongst children [1]. The introduction of craniospinal irradiation in the 1950s and later cytotoxic chemotherapy in the 1970s greatly improved these survival rates, but led to severe long-term motor and cognitive effects, chronic neuropathy, and endocrine dysfunction [1,3]. Surgery, radiation, and chemotherapy now remain the clinical standard approach despite the inability for these methods to address the inter- and intra-tumoral molecular heterogeneity of MBL [1]. Therefore, 5-year survival rates stubbornly remain around 60-80% in MBL patients [4,7], with approximately 20-25% of cases presenting with advanced metastatic disease [3].

In an effort to vastly improve upon these metrics, the World Health Organization (WHO) transitioned from solely categorizing MBL based on morphology, such as desmoplastic/nodular (DN), large cell/anaplastic (LCA), classic, and MBL with extensive nodularity (MBEN) [1,8], to a more refined system that includes the molecular subgroups mentioned earlier: *MBL-WNT*, *MBL-SHH*, *Group 3*, and *Group 4* [1,9]. These distinct molecular categorizations, further subdivided based on genomic methylation profiles, more accurately reflect both the genetic and epigenetic context of a specific patient's MBL tumor type, (see Figure 1), allowing researchers and doctors to develop patient-specific treatment plans as well as innovative personalized drug therapies.

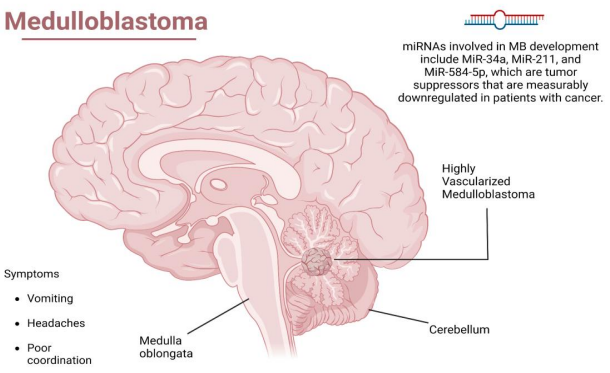

Figure 1. Common symptoms associated with MBL tumors. Overview of medulloblastomas in relation to miRNA research. Group 1 MBs are commonly known to have leaky vessels, making them excellent targets for miRNAs due to the deterioration of the blood-brain barrier. Many of the symptoms in pediatric patients include vomiting, headaches, and deteriorating motor skills. However, due to these symptoms having a multitude of possible causes, it can make timely diagnosis difficult [1-4,10].

2. Development and Molecular Categorization of MBL

Elucidating a conserved developmental pathway underlying MBL formation is key in understanding the prevalence and pathophysiology of MBL in children. Upon closer investigation, MBL typically develops within the embryonic rhombic lip (RL), a region that is separated into two distinct molecular and anatomical structural zones separated only by a vascular plexus [3,5,11]. The ventricular RL is composed mostly of dormant neural stem cells, while the subventricular RL contains the majority of neural precursor and progenitor cells with markedly enhanced levels of proliferation. When cells within the RL fail to differentiate, the development of heterotopias or persistent RLs (PeRLs) represents a premalignant lesion that expands into a *Group 3* or *Group 4* MBL [12]. Together, it is clear that during early cerebellar development, the RL is the location responsible for the majority of normal neuronal activity across the entire human brain, and provides a rational explanation for the paucity of MBL development within the brainstem. The notion that MBL tumors arise from pre-existing genetic lesions within these subventricular RL neurons also provides an opportunity for non-coding RNA biologists to begin a more targeted approach when assessing regulatory pathways that control neural proliferation and differentiation.

Continued efforts to further characterize MBL have resulted in the identification of various subgroups arising from distinct cellular origins within the brainstem region, influencing tumor growth behavior and response to current radio- and chemo-therapeutic treatments. For instance, *MBL-WNT* tends to originate from mossy fiber neurons of the dorsal brainstem, while *MBL-SHH* develops from granule neuronal precursors [3,4], with each tumor subclass conferring a specific overall prognosis and survival rate. We will discuss these molecular subtypes below, and how this categorization process can result in the development of novel treatment paradigms that can significantly extend both survival and quality of life metrics of MBL patients.

2.1 Group 1 MBL - WNT Subtype

The least common MBL subgroup is linked to mutations in the WNT or Wingless signaling pathway, and accounts for 9-10% of all MBL [7]. Within this subclass, MBL presents in both genders equally, at around 6-10 years of age, and carries a favorable 5-year overall survival rate of 90% [13]. Additionally, nearly 90% of tumors within this group have a stabilizing point mutation in the *CTNNB1* gene; encoding for the protein β-catenin, responsible for regulating cell-cell adhesion

and promoting epithelial-like gene transcription patterns within MBL tumors [7]. In support of *Group 1* MBL being a molecularly distinct category of brain tumors, almost 50% of all *MBL-WNT* tumors harbor mutations in the X-linked RNA helicase DDX3X [13]. This particular DEAD-box RNA helicase binds to and activities casein kinase-1 (CK1ε) thereby promoting the phosphorylation of dishevelled and maintaining active WNT signalling within the tumor [14]. While DDX3X mutations are present in a variety of leukemias, mesotheliomas, lung cancers, and lymphomas; DDX3X is ubiquitously expressed within neurons, astrocytes, and ependymal cells, while further being positively correlated with WHO pathological grading of gliomas [15,16]. Therefore it is quite clear that WNT signalling alterations in MBL present an opportunity for clinical intervention.

The WNT signaling pathway is typically viewed as a generally ubiquitous signaling pathway, yet it was recently discovered that hyperactive WNT signaling occurs in a specific spatio-temporal manner within proliferating mossy fiber neurons of the embryonic dorsal brainstem [7,17]. This signalling activity is quite active during early neuronal development and then becomes much more quiescent as the cerebellum ages, which was not originally thought to be the case. Therefore, mutations arising within the WNT signaling pathway during brainstem development could represent the key window of time where a tumor-initiating event results in the formation of *Group 1* or *MBL-WNT* tumors. This is an important finding, given tumors will always present with a vast number of dysregulated gene expression networks, many of which are considered passenger or non-driver mutations. For instance, almost all late-onset adult cases of *Group 1* MBL tumors have monosomy of chromosome 6, yet none of the 16 WNT gene isoforms are located on chr:6, therefore it is not clear as to which sets of genes on this chromosome are initially responsible for, or support the development of, *Group 1* MBL [5,13].

The phenotypic consequence of WNT mutations results in a favorable prognosis for patients with *Group 1* MBL. This is in part, due to the leaky vasculature of the tumor, heavy reliance on angiogenesis, and a subsequent breakdown of the blood-brain barrier [18]. This process in fact facilitates the improved uptake of chemotherapeutic agents being delivered to MBL tumor cells. Given the age of the patient population and the higher remission rates of *Group 1* MBL, a concern arises related to the prolonged use of WNT-based therapeutics. Despite the spatio-temporal WNT-related activity present during embryonic dorsal brainstem development, WNT is also ubiquitously expressed amongst most other cell types and therefore persistent therapeutic reduction of WNT activity is linked with some side effect such as the early onset of osteoporosis [13]. Furthermore, long term use of WNT inhibitors, similar to any other single use small molecular inhibitors, are associated with tumor recurrence and increased chemotherapeutic resistance [10], making the drug discovery process to treat refractory disease an urgent priority. We postulate that some of the next generation therapies targeting patients with relapsed MBL will involve non-coding RNAs. In fact several clinical trials, such as NCT05057702 and NCT05535166 already ongoing, have included RNA sequencing data from both the coding and non-coding RNA genomes of MBL patients [19,20]. The data gleaned from these studies will be necessary to identify alternative or supplementary treatments for *Group 1* MBL.

2.2 Group 2 MBL - SHH Subtype

The most well-studied MBL subgroup is termed ‘sonic hedgehog-activated’ (*MBL-SHH*), and affects 28-30% of the patient population, with a bimodal age distribution of tumors presenting within infants as well as older children [7,21]. *MBL-SHH* typically presents with DN histology, but amongst children, tumors can harbor a classic- or LCA-type morphology. *MBL-SHH* is derived from granule neuron precursors (GNPs) located within the cerebellar hemisphere, or within the cochlear nuclei of the brainstem. Overactive SHH signaling within these GNPs results in the migration of granule neurons into the internal granule layer of the cerebral cortex. Despite this migratory behavior of SHH activated GNPs, the average 5-year survival rate for these patients is around 70%, due to the wild-type status of *TP53*, a protein important in maintaining DNA repair, cell cycle checkpoint, and inducing cell death, thus preventing cancer formation [13].

For patients with *MBL-SHH* harboring *TP53* mutations, the overall survival rate is markedly reduced to 40% [22], indicating *MBL-SHH* tumors may be intrinsically resistant to therapeutic agents targeting the DNA repair pathway [13]. To further support the role of *TP53* in *MBL-SHH*, hereditary *TP53* mutations have been identified in approximately 20% of MBL patients aged 5-16, while certain syndromes such as Li-Fraumeni co-present with a number of *MBL-SHH* patients. Interestingly, *TP53* mutations also occur in the *MBL-WNT* subgroup, but the exact risk to overall survival conferred by these mutations remains unclear [23,24]. Rather, this risk could be due to the elevated amplification of the MYCN locus often found in *TP53* mutant *MBL-SHH* tumors [13].

Given the SHH signaling pathway plays a key role in cell differentiation, axonal growth, and dorsal-ventral development of the entire central nervous system [7,25], it is not surprising that dysregulation of SHH signalling can result in MBL development. In fact, *MBL-SHH* is now further categorized into four distinct subtypes under the 5th edition of the WHO classification system [7], (see Table 1). Much of this categorization focuses on the aberrant activity of particular SHH-signalling target genes such as *GLI1*, *SMO*, and *PTCH1*, whilst including known tumor promoting mutations such as *TP53*, *MYC*, *TERT*, and *PTEN* [26]. Further on in this review we discuss how aberrant activity of certain non-coding RNAs, such as miRNAs, can influence MBL tumor formation.

Table 1. The demographics, patient survival and metastasis rates, gender bias, and the molecular or histological characteristics of the four *MBL-SHH* subtypes

Subtype	**SHH γ**	**SHH β**	**SHH α**		**SHH δ**
Age group	Infant (0-3)	Infant (0-3)	Children (4-10)	Children (4-10); Adolescents (10-17)	Adult (>17)
Survival Rate	~85%	~67%	~70%	~40%	~85%
Gender	Male = Female	Male > Female	Male > Female	Male = Female	Male > Female
Metastasis	~9%	~30%	~9%	~50%	~9%
Genetic anomalies	***PTCH1***, *SUFU,* MBEN histology	*PTCH1, SUFU,* ***PTEN***	*ELP1, PTCH1,* ***MYCN***	***TP53***, *U1* snRNA, *GLI 2*	*U1/U11* snRNA, ***SMO***, *TERTp, DDX3X, XPO1*

In the established SHH pathway, glycoprotein SHH binds and inactivates the receptor PTCH1, located on cellular cilia, which inhibits the transmembrane protein Smoothened (SMO) [7,27]. SMO then initiates an intracellular signaling cascade, leading to the translocation of GLI-1 and -2 into the nucleus, thus activating a series of transcriptional gene targets [7]. SMO also negatively regulates and suppresses the activity of Suppressor of fused (SUFU) given SUFU functions as a gene expression balancer by sequestering GLI-1 and -2 protein away from the nucleus [7,28,29]. Therefore, loss-of- or gain-of-function mutations of genetic elements within the SHH pathway result in a molecularly heterogeneous *SHH-MBL* tumor subgroup. More interestingly, is that mutations within each SHH-related kinase or transcription factor that contributes to the canonical SHH signaling pathway results in various clinical outcomes for MBL patients. This inherently argues for yet another layer of regulatory action, such as those occurring at the epigenetic or RNA level, which plays a crucial role in promoting this dynamic and complex molecular heterogeneity of the *SHH-MBL* tumor subgroup. One example of this, is the finding that key mutations in the SHH pathway include those of the U1 snRNA, which recognizes splicing sites during translation, as well as ELP1, which encodes for the largest subunit of the elongator complex required for proper tRNA modification [7,27].

While clinical scientists determine whether potential SMO inhibitors could prevent SUFU activation and translocation of GLI proteins to the nucleus [30], none have assessed how these compounds alter the cellular non-coding RNA milieu. For instance, Vismodegib (GDC-0449), a synthetic SMO inhibitor based on cyclopamine found in California corn lilies [13], is being investigated in clinical trial NCT01878617, as a potential agent for adult patients harboring the *MBL-SHH-δ* subtype [31]. While early signs suggest relapsed adult *MBL-SHH* respond well to Vismodegib, the long term use of cyclopamine derivatives are known to cause developmental birth defects [32]. Furthermore, patients with SUFU and GLI1 mutations do not respond to Vismodegib [13]. Therefore, additional clinical studies will have to be performed to fully elucidate the effects of Vismodegib, since pre-clinical evidence indicates cyclopamine derivatives can alter the activity of miR-21, miR-27, and ciRS-7, all of which are *bona-fide* non-coding RNA regulators of tumorigenesis [33]. Finally, a number of therapeutic treatments, small molecule inhibitor or otherwise, for SHH-driven MBLs have limited pharmacokinetic efficacy due to a reduced ability to cross the blood-brain barrier [34]. Therefore, it is necessary to find effective delivery methods that facilitate the formulated therapeutic payload required to eradicate MBL tumor cells located in anatomically unreachable brain locations via surgical procedures.

2.3 Group 3 MBL Subtype

Group 3 MBL associates with the lowest overall prognosis, with a 5-year survival rate of 50%, given the lack of specific biomolecules available to augment standard radio- and chemo-therapeutic treatments [7,13]. The histology of these tumors tend to present as classic and LCA type tumors, present in males more frequently than women, and are thought to arise from neural stem cells [35]. This subtype presents in 19-25% of patients, mostly around the ages of 3-5 years, and these patients frequently experience relapse with metastatic disease rather than local recurrence. This highlights that several cellular mechanisms are involved in promoting a more non-differentiated and proliferative *Group 3* MBL tumor subtype.

Group 3 MB is typically characterized by an overexpression of MYC [5], which is driven by MYC loci amplification and PVT1–MYC rearrangement [13]. Abnormal activation of MYC disrupts a multitude of cellular pathways, including mRNA processing and protein translation [5]. Other common genetic features include GFI1 enhancer activation, OTX2 amplification (3%), chromosome 17 imbalances, gain of chromosome 1q, and loss of chromosome 5q [5,13]. While clinical research scientists have elucidated how MYCN promotes tumor initiation, maintenance, and progression; and similarly how OTX2 functions mechanistically as a master transcriptional regulator within developing neuronal stem cells [5], in reality these are difficult genetic elements to convert into deliverable therapeutics. Some investigators are further modeling various MBL subgroups using 3D hydrogels and discovered that these tumor cells can metastasize predominantly through a thin laminar coating [13,36]. Studying the molecular repertoire of non-migratory versus migratory MBL tumor cells will help to develop new experimental therapeutics for these patients.

Other investigators appreciate that current treatment protocols for those with high-risk MBL will place an additional considerable morbidity on these patients, and thus newer therapeutics are required to have reduced side effects on the patients while still targeting metastatic

MBL tumor cells. As an example, some groups have looked at TGF-β in Group 3 MBL tumors with somewhat limited success [37]. We suggest that an assessment of the non-coding RNA expression profile, as well as an elucidation of the activity of these non-coding RNAs might result in the discovery of new molecular pathways that these MBL tumors are oncogenically addicted too.

One example of this methodological approach is miR-1253, which lies in the 17p13.3 locus, and typically regulates bone morphogenic proteins (BMP) [38]. Interestingly, BMP and TGF-β share similar downstream intracellular tyrosine kinases that promote cellular proliferation and tumor de-differentiation. miR-1253 also targets the oncogene CDK6 [39], which encodes a key regulator of the G1-S transition during mitosis [38]. High expression of CDK6 is correlated with a poor prognosis, and the re-expression of miR-1253 inhibits CDK6 activity. Therefore, two new therapeutics could be developed from this particular research study. The first involves regulating CDK6, to which there is already an FDA-approved breast cancer drug palbociclib, designed to inhibit CDK6 activity, and is currently in early phase clinical trials for use in combination with standard chemotherapy [13,38]. The second involves development of a miR-1253 mimic that could be delivered to not only control CDK6 activity, but to regulate other pro-tumorigenic factors in this MBL tumor subtype, such as MYCN, and OTX2.

2.4 Group 4 MBL Subtype

Group 4 MBL is the most common subtype of MBL, and affects males more than females at a 3:1 ratio [5,6,13]. The histology profile is quite similar to *Group 3* MBL tumors, and similarly present with a poor prognosis with high rates of metastatic recurrence. These MBL tumors also are quite common in infants and children, but also present in adults. As an entire subclass, *Group 4* MBL comprises the majority of diagnosed MBL tumors and affects 35-43% of all patients. Similar to *Group 3*, a number of shared mutations have been identified in *Group 4* MBL such as OTX2, MYCN, and CDK6. While *Group 4* tumors display a high rate of chromosomal copy variations [13], gains of isochromosome 17q and losses of chromosome 11 do not correlate with poor outcomes [5,13]. Therefore, the most striking difference of *Group 4* MBL is that these tumors develop in the upper rhombic lip, making these tumors anatomically distinct from all other MBL subgroups.

We suggest that the lack of driver mutations, and the perplexing overlap of gene expression profiles between *Group 3* and *Group 4* MBL is simply due to the notion that a driver mutation occurs with a non-coding RNA gene, or that these MBL tumors are regulated at the epigenetic level (i.e., methylation or chromatin modification). In support of this claim, *Group 4* MBL frequently contains mutations in *KDM6A* and *SCAIP*, both of which are known to be targeted and regulated by miR-145 and miR-150 in other neural tumor models, respectively [40,41]. It would be interesting to determine if these miRNAs, and additional noncoding RNAs were able to fully distinguish *Group 4* MBL from all other subtypes, or even help to further stratify *Group 4* into additional subgroups as was explained earlier with the *MBL-SHH* subtype. In support of this, the most prevalent driver event of *Group 4* MBL involves the overexpression of PRDM6 [5,13,36], which normally regulates transcription within the developing cardiovascular system [42]. However, PRDM6 alone cannot convert neuroepithelial stem cells to a *Group 4* MBL; unknown additional factors are required [42]. We postulate that miRNAs, such as miR-181a, are these 'factors' that could control the expression of its cognate target gene PRDM6 [43], as well as other tissue specific transcription factors within the developing upper rhombic lip, and upon dysregulation of these gene networks, provides an advantage for a pro-tumorigenic process to emerge.

Similar to *Group 3* MBL, the treatment paradigms offer the standard subpar clinical response as compared to *MBL-WNT* or *MBL-SHH* subtypes. The chemotherapeutic agent, vincristine, is not effective against *Group 4* MBL [36], but is still used as a chemotherapeutic alternative during relapsed disease. This indicates that a molecular profiling study on *Group 4* MBL is a top priority for the research community in order to identify noncoding RNA deliverables that are already being developed for other tumor systems. Given the status of advanced nanoparticle technology, and cell specific modified RNA compounds, these studies would offer a gamut of novel therapeutic compounds for clinical scientists to utilize to combat one the most deadly and genetically perplexing MBL tumor subtypes.

3. A Role for Non-Coding RNA in MBL

Large-scale single-cell RNA sequencing, international collaborations, and increased funding has led to a steep increase in MBL research in the past twenty years, allowing scientists to identify new RNAs of interest [2,7]. MicroRNAs are just one type of these non-coding RNAs that are known to regulate thousands of target mRNA genes via post-transcriptional processes. Moreover, aberrant expression of miRNAs result in a variety of pathological illnesses, including cancer [44]. The biogenesis of these miRNAs stem from precursor pri-miRNAs that are transcribed from the human genome. The fully processed mature miRNAs are found to be widely conserved across all species, from human to worm, and have remained virtually unchanged with respect to the primary mechanisms of action in both plants and animals [45]. Additionally, there is new evidence indicating that miRNA sequences are under evolutionary pressures as certain miRNA family members and isoforms are emerging as sequence alterations in closely related cognate mRNA targets genes occur within the genome. Furthermore, many miRNA genes have arisen from duplication and translocation events within the genome, creating new families of miRNAs that share similar sequences and functions [45,46].

In the context of cancer, researchers now understand that miRNAs, and the regulatory networks these entities

control, also become dysregulated and are therefore classified into two categories of action based on the physiological consequence of this dysregulation. Oncogenic miRNAs, also named "oncomiRs" can promote cancer development by downregulating tumor suppressor genes, enhancing cell proliferation, inhibiting apoptosis, and facilitating metastasis, while "tumor suppressor" miRNAs keep tumor formation in check by initiating cellular apoptosis, DNA repair, and cellular differentiation [38,47,48]. As will be explained later in this review, miRNAs regulate gene expression primarily by binding to complementary sequences within the 3' untranslated region of target mRNAs, mediated by a 2-7 nucleotide sequence binding event at the 5' end of the miRNA [49,50]. Once bound, miRNAs inhibit the translation of the mRNA via the RISC complex, or recruit AGO2 to promote mRNA cleavage resulting in mRNA degradation and gene silencing [27,47]. Because of this relative explanation of miRNA function, many clinical scientists have been on a mission to categorize miRNA into oncomiRs or tumor suppressor miRNAs based on abundance in cancerous tissue versus adjacent normal tissue, or through loss of function / gain of function studies *in vitro*.

This line of thought has emerged into the field of MBL genomics as well. Potentially thousands of miRNAs affect MBL development, and research has yet to link the effects of each miRNA on tumor cell function and phenotype. Due to varying miRNA expression patterns and heterogenous genetic signatures within MBL, miRNAs are likely to play an important role in classifying MBL phenotypes. Elucidating how each miRNA becomes dysregulated throughout the tumorigenic process will allow researchers to determine which gene targets become altered in MBL tumor cells, as compared to normal neuronal cells of the CNS [5,51], (see Figure 2). From there, miRNA-based treatments could be developed to help patients recover from refractory disease. In fact, preliminary studies suggest that miRNAs have potential in treating aggressive MBL tumors or those responding poorly to the standard treatments of surgery, chemotherapy, and radiation therapy [51-54]. Here, we outline the subtypes of MBL, the various etiologies of MBL, relevant miRNAs dysregulated in MBL, current clinical trials to effectively deliver miRNA into the brain via nanoparticles, as well as to discuss the ongoing challenges required to overcome delivery across the blood-brain-barrier so as to target drug resist MBL tumor cells.

Summary of the suppressive and oncogenic miRNAs under investigation for medulloblastoma therapeutics. Downregulated miRNAs hold potential for future treatments since tumors suppress mechanisms that block stem cell-like characteristics. Oncogenic miRNAs pose as good molecular targets alongside chemo- and radio-therapy because correction of miRNA expression could reduce the treatment resistance of many of these tumors, making them more susceptible to existing therapies [16,27,29,33,36,38,39].

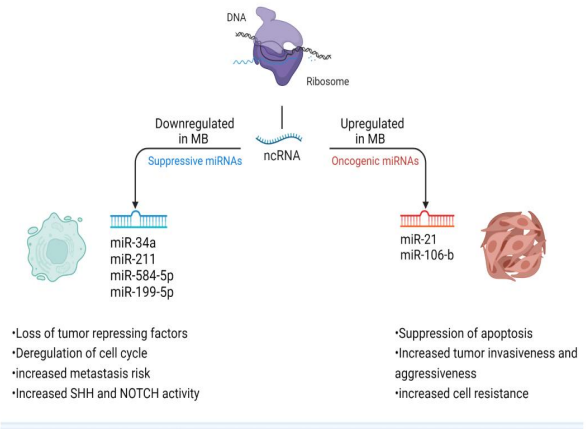

Figure 2. Key miRNAs known to control MBL tumor formation.

4. miRNAs as Informative Noncoding RNAs for MBL

MicroRNAs (miRNAs) are noncoding RNAs that are important in the negative regulation of gene expression through translational repression, and mRNA inhibition by either cleavage or decay [55-64], (see Figure 3). Understanding the biogenesis of miRNAs in more detail may allow for MBL research to assess the dysregulation of not only miRNAs in their research, but the levels and activity of the miNRA biogenesis proteins as well. For instance mutations in Dicer have been linked to Wilm's tumors of the kidneys. Therefore it is possible that mutations in Dicer, or Drosha, could be a tumorigenic initiation event in MBL.

As was mentioned earlier, miRNAs are generated through the transcription of primary miRNA (pri-miRNA) transcripts by RNA polymerase II, which form polycistronic hairpin structures, which could contain up to ten or more miRNAs [53], (see Figure 3). These pri-miRNAs are processed in the nucleus of the cell by the RNAseIII Drosha-DGCR8 complex to generate an approximate 85nt hairpin precursor miRNA (pre-miRNA), which are then exported to the cytoplasm by exportin-5 [54]. It is normally thought that once in the cytoplasm, these miRNAs are further cleaved by Dicer, a second RNase III endonuclease, that recognizes the stem loop of the pre-miRNA, as well as the the top or first bulge of the duplexed region of the pre-miRNA [55,56]. However, this process most likely occurs near the rough endoplasmic reticulum given the proximity to polysome complexes which are the eventual targets of miRNAs. In general, miRNA-protein complexes that are sequestered to the cytoplasm are thought to be stored for later use, or are considered sites of RNA degradation, depending upon which protein complexes the miRNAs are bound [57,58]. Given many researchers are still trying to elucidate a mechanism of action for miRNAs in MBL, it is important to note that, more *in vitro* cell culture work is required of the field so as to properly determine whether there are biases of certain miRNA species or isoforms being sequestered for pro-tumorigenic functions.

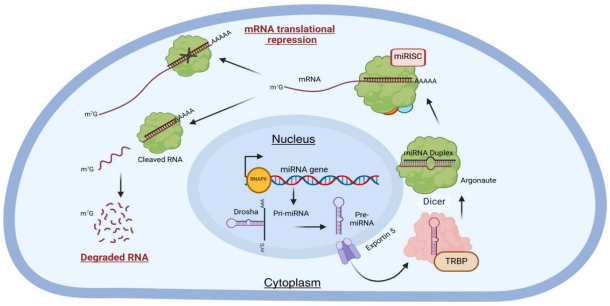

Figure 3. The Canonical miRNA Biogenesis pathway.

The canonical miRNA biogenesis pathway, that depicts primary miRNA transcript being processed through a series of RNaseIII enzymatic reactions that yields a 22-nt duplexed RNA. After strand selection, a miRNA will target and bind a cognate target mRNA, inducing mRNA cleavage, and translational inhibition resulting in loss of function of the target protein [59-63].

Returning to miRNA biogenesis, once Dicer binds the 5'-phosphates of the pre-miRNA and cleaves the stem loop to form a 22-nucleotide mature double stranded miRNA duplex, these miRNAs now inherently, through the enzymatic cleavage process, have overhanging 3' and 5' ends [54]. This is important to mention, because simultaneously after Dicer cleavage, Dicer recognizing the 2-nucleotide 3' overhang on one of the strands and facilitates loading of this one strand of the miRNA duplex strand into the PIWI-AGO-ZWILLIE complex [59,60]. This new understanding of miRNA biogenesis is important to mention, as the stability of the mature miRNA duplex determines which single miRNA strand will be loaded into the AGO effector complex. In many early MBL profiling studies, a particular 5p or 3p strand of a certain miRNA would be more predominant than another without understanding the regulatory implications of this process. Given the complementarity of the 5p and 3p strands of a miRNA, each of these strands would have vastly different gene targets, as miRNAs function by hybridization to the 3'UTR of mRNAs. Given the level of isoform generation, mutation rates, and RNA editing now known to occur in cancer [61,62], it has been observed that even single nucleotide changes in miRNA sequences, occurring within the miRNA duplex region can effect the abundance of either the 5p or 3p miRNA strand within the cell, resulting in a vastly different genetic profile of the tumor.

Despite the vastly heterogeneous nature of miRNA biogenesis, in the canonical biochemical pathway, once processed, a mature miRNA sequence from the the miRNA duplex is loaded onto Argonaute (AGO) protein, which forms the RNA-induced silencing complex (RISC) [54]. There it guides the RISC to specific messenger RNAs (mRNA) through base-pairing in nucleotides 2-7 with complementary sequences within the mRNA 3' untranslated region [13,25,54]. This miRISC complex can suppress gene expression by destabilizing the target mRNA or inhibiting its translation [13,54].

MiRNA precursors have been widely conserved across species, with virtually unchanged primary mechanisms in plants and animals [23]. Additionally, we can also see how miRNA evolution is closely related to that of its target genes, as many miRNA genes have arisen from duplication events of existing miRNAs, creating families of miRNAs that share similar sequences and functions [23,27]. MiRNAs regulate cellular processes such as apoptosis, differentiation, metabolism, and proliferation, and are directly correlated with brain and several other organ cancers in humans [57,58]. Listed below are some examples of important miRNAs that harbor tumorigenic regulatory potential in MBL.

5. Specific miRNAs Involved in MBL Development

5.1 miR-34a

MiR-34a is a tumor suppressing miR that is downregulated in a variety of cancers, including medulloblastomas [65]. MiR-34a targets several genes responsible for cell cycle continuation, tumor invasion promotion proteins, and apoptosis suppressors [56]. When miR-34a is upregulated in triple negative breast cancer cells, they exhibit both reduced invasiveness and decreased growth properties by inhibition of multiple mechanisms, including the FAK/SRC, c-SRC, and NOTCH signaling pathways [66-68]. Medulloblastoma tumors are dependent on upregulation of miR-34a's target gene MYCN, and mouse models have found miR-34a to be significantly suppressed in medulloblastoma tissue versus the standard cerebellum tissue [65]. Overexpression of MYCN in mouse granule cell progenitors caused severe medulloblastomas activated independent of SHH, with histologies matching Group 3's typical large cell anaplastic, or classic pathologies [65,67].

MiR-34a therapy in cell lines has shown to degrade SIRT1, a cell longevity gene, when used with high concentrations of nicotinamide, which is an mitotic inhibitor etoposide treatment [56]. While this shows potential for medulloblastoma treatment development, it likely cannot be used alone effectively [56].

The pathway p53 regulates the cell cycle and sustains gene integrity via target gene transactivation, and p53 activation is relied upon when treating medulloblastomas with chemotherapy [56]. Some chemotherapy resistant medulloblastomas, specifically SHH, are associated with dysfunctional p53 pathways, which transcriptionally target miR-34a and cause upregulation under normal conditions. MiR-34a's cytotoxic effects cause tumor suppression, and miR-34a treatment could potentially replenish miR-34a levels and assist with chemosensitivity in chemoresistant tumors with p53-dependent pathway mutations [56].

5.2 miR-211

MiR-211 is an intronic RNA and a crucial regulator of neural cell descendants that has been found to be a medulloblastoma tumor suppressor in vitro and in vivo. It targets the genes ACSL4, an acyl-CoA synthetase long-chain family member which controls anabolic and catabolic pathway equilibrium, and NUAK1, a kinase

responsible for regulating the cell cycle and metastasis processes in neuronal cells [69].

MiR-211 was found to be particularly downregulated in SHH subgroups cell lines, expressed somewhat in Group 4 cell lines, and normally regulated in Group 3 cell lines. All studied patient derived cell samples had substantially low regulation [69]. In a study of the MAGIC database, it was found that Group 3 medulloblastoma expresses miR-211 in 14% of samples, and the WNT subgroup medulloblastomas expressed the miR-211 in 27% of samples, the highest of any group, however this sample size was limited [69].

MiR-211 is regulated by the TRPM1 (melastatin) promoter, making TRPM1-targeting therapeutics a potential treatment for medulloblastomas in subgroups SHH or *Group 4*. MiR-211 expression has been found to be dose-dependently responsive to TRPM1 mRNA in mice models [69]. Induced ACSL4 expression has shown to reverse miR-211 phenotypes and related effects in cell invasion and survival in medulloblastoma cells expressing miR-211. This seems to promote cancer, and provides insight on treatment possibilities with miR-211, as it downregulates ACSL4 [69]. MiR-211 has potential to be important in medulloblastoma treatment, but at the time of publishing, not many studies have been formed involving miR-211 expression in medulloblastoma.

5.3 miR-584-5p

MiR-584-5p in neuroblastomas, gliomas, and renal cell carcinomas is an effective tumor suppressor, and in mice preclinical tumor models showed inhibition of medulloblastoma tumors. Overexpression MiR-584-5p inhibited the medulloblastoma cell growth by causing DNA damage, spindle defects, and cell cycle arrest [52].

In patients with medulloblastoma tumors, there was a negative correlation between low levels of miR-584-5p and histone deacetylase 1, HDAC1, and eukaryotic translation initiation factor 4e family member 3, eIF4E3 [52]. High expression of HDAC1 promotes medulloblastoma growth, and elF4E3 is a translation initiating protein believed to be a tumor suppressor [70]. However, a few studies have noted that eIF4E3 displays tumor promotion in MB patients [52]. This correlation makes miR-584-5p a valuable candidate for medulloblastoma miR therapy studies. Additionally, miR-584-5p has been shown to sensitize a vincristine, VCR, a chemotherapy drug currently given to medulloblastoma patients, and causes neurotoxicity in the high levels needed to curb medulloblastoma tumor growth [52]. Sensitizing this treatment would reduce the amount of VCR needed, reducing patient harm and side effects [52] (see Table 2).

Table 2. Summarizes current medulloblastoma related miRNAs, their effect in relation to tumor growth, their target genes, and their regulatory elements

miRNA	Effect on MBL	Gene Targets	Regulated By	References
miR-34a	Tumor suppressor	MYCN, SIRT1, SRC1, HMGB1	TP53 (Promoter)	[65-67]
miR-211	Tumor suppressor	EZRIN, NUAK1, ZEB1	TRPM1, PERK (Promoter Region)	[69]
miR-584-5p	Tumor suppressor	eIF4E3, HDAC1	DNMT1 (Promoter Region)	[52,70]
miR-199-5p	Tumor Suppressor	mTOR, HES1, and CD44	DNA Methylation upstream mRNA promoter	[71-73]
miR-21	Oncogene	PTEN, PDCD4, and TP53INP1	NF-kB, circRNAs	[74-76]
miR-106b	Oncogene	E2F1, PTEN, and CDK6	MCM7 (Host gene)	[77]

5.4 miR-199-5p

MiR-199-5p is crucial for cell differentiation, proliferation, and apoptosis. It has been identified as a tumor suppressor in multiple cancers, as it targets mTOR, HES1, and CD44, inhibiting to induce apoptosis [71]. It has been notoriously downregulated in multiple cancers, including medulloblastomas, where it correlates with poor prognosis and aggressive tumor behavior, especially in the development of metastasis [72].

Specifically to medulloblastomas, miR-199-5p inhibits HES1, which is a downstream effector of both the canonical Notch and non-canonical SHH pathway [72]. The expression of miR-199-5p in medulloblastomas is considerably lower than the control, but restored levels could serve as a therapeutic target [73].

5.5 miR-21

MiR-21 is one of the most conserved miRNAs across species, and thus also one of the most studied ones. It regulates multiple tumor suppressor genes, such as PTEN, PDCD4, and TP53INP1, and when upregulated it leads to cell proliferation, migration, and invasion by suppressing apoptosis and increasing tumor invasiveness [74]. It is regulated by a multitude of factors, including NF-kB, circRNAs, and compounds such as curcumin [75]. It is especially characterized in the SHH group [76].

Recent research has focused on the inhibition of miR-21 in medulloblastomas [76]. Among these, antagomirs (chemically modified, single-stranded RNA molecules) complementary to miR-21 have shown promise in preclinical models. Antagomirs bind to miR-21, preventing it from interacting with its target mRNAs,

restoring the expression of tumor suppressor genes, and inhibiting tumor growth [76].

5.6 miR-106b

MiR-106b belongs to the miR-106 family, and it contributes to cell cycle regulation, apoptosis, and differentiation by targeting the genes E2F1, PTEN, and CDK6 [77]. It is upregulated in medulloblastomas, specifically in the group 4 subtype, where it contributes to tumor progression, leading to uncontrolled cell proliferation and tumor aggressiveness [77]. Furthermore, when miR-106b is overexpressed, patients have a poor prognosis and high metastatic potential. More specifically, miR-106b affects the PI3K/AKT signaling pathway, which results in enhanced cell survival and resistance to apoptosis, but it can also modulate the expression of matrix metalloproteinases, particularly MMP-2, which plays a critical role in tumor invasion and metastasis [77].

6. Future Directions

Given the heterogeneity of MBL, the development of new therapeutics to treat the disease is ongoing. The current challenge is to provide curative results yet to not impinge on quality of life metrics. The elucidation of the complex regulatory networks noncoding RNAs play in MBL will allow for the development of such therapies. Given the overall rarity of brain cancer amongst all other tumor types, and smaller number of clinical research scientists working on non-coding RNA research, it has taken quite some time to develop clinical trials specifically focused on non-coding RNAs and MBL. Currently, there are no clinical trials being conducted with a miRNA as the target drug compound in MBL patients [44,78]. However, a number of studies have included RNA sequencing approaches to attain miRNA and other non-coding RNA expression profiles from the MBL tumor and/or patient serum/plasma. One of these trials, NCT03630861, investigated over 700 miRNAs in the plasma of central nervous system (CNS) cancer patients–mainly glioblastomas, lymphomas, and those with secondary metastases to the brain. The study goal was to develop a diagnostic picture of miRNA alterations that are specific to glioblastomas, while also collecting miRNA signatures for related brain tumors [79]. Much of the research findings from the study was not reported to FDA since no therapeutics were developed, nor were any non-coding RNA therapeutic agents involved in the study.

Despite this, two new studies focused efforts on RNA profiling, with one in particular investigating whether atypical levels of miR-10b in glioma patients could function as a biomarker for glioma tumors and aid in diagnosis [80]. Results from study NCT01849952, estimated to close January 2025, will provide valuable information for more informative clinical trial design for MBL patients. The second study, NCT02162732, which was completed in January of 2024, performed genomic profiling (DNA and RNA) to determine if they could accurately diagnose the type of tumor in relapsed pediatric CNS cancer patients [81]. Results indicated that the profiling collection method was both safe and effective; however it is not clear as to which noncoding RNA associated prognosis rates, as the study included over 200 brain tumor patients, with each undergoing therapeutic treatments based on their specific diagnoses. The cancer types investigated included gliomas, neuroblastomas, and medulloblastomas.

As is evident in these clinical trials, the current goal is to continue characterizing miRNAs associated with specific types of cancers so that a diagnostic biomarker panel can be diagnosed quickly and efficiently so as to subcategorize the molecular heterogeneity of MBL even further [82]. To achieve this goal, clinical scientists are currently using diagnostic methods that rely on tissue biopsy, yet is gradually being replaced or supplemented with less invasive methods such as liquid biopsy, and involves analyzing typical tumor protein biomarkers circulating tumor DNA, as well as circulating miRNA and circRNA abundance in cerebrospinal fluid or plasma/serum [83]. This presents a particularly promising benefit for tumors located in regions that are not easily accessible or identifiable by surgeons, including the brainstem and posterior fossa.

With the information provided by RNA sequencing data, researchers are developing techniques to directly deliver noncoding RNAs to brain regions where residual MBL tumor cells may reside post surgery. To aid in this delivery process, several technologies have been developed to allow RNA delivery through the blood brain barrier. Nanoparticle-based drug delivery systems have been modified quite substantially over the past decade and have been shown to deliver therapeutic payloads to specific organ sites separate from the liver [84]. Alternative approaches include intrathecal administration of compounds that reduce both local tumor inflammation as well as local growth factor receptor kinase activity [85]. Additionally, miRNA therapies could be incorporated into conventional treatments such as chemotherapy to improve overall results, which is crucial for high-risk subtypes such as *Group 3* and *Group 4*, both of which are less responsive to traditional therapies [5,13]. Overall, these miRNA-based strategies are proving to be viable options for drug delivery to the brain and have also been effective in abating tumor growth in other cancer types, making miRNA therapeutic a strong alternative approach for targeting MBL tumors.

7. Conclusion

Over the past 20 years, medulloblastoma research has increased vastly due to government funding, but there is still so much work to be done. While advances in RNA sequencing have allowed for scientists to identify upregulated and downregulated miRNAs in MBL tumors, there have been no clinical trials with effective delivery to the brain. Furthermore, most of these studies were done with 2D in-vitro models, while the tumors themselves are 3D and have complex microenvironments that are not considered in the studies. While these clinical trials are developed, other treatment options that could help patients include finding alternatives to the

harsh radiation and chemotherapy that negatively impact the development of the pediatric patients. Another option is to find an early detection method as MB tumors are often detected late when the tumor has grown large. Overall, the future of MB treatment involves a personalized regimen based on tumor type and subgroup.

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Author Contributions

C.L., G.S., L.G., M.A., A.A., A.P., and B.A. performed the literature research and co-wrote the manuscript. P.T., J.I., R.A., M.N., and Y.C. provided conceptual editing support, and developed the figures and tables for the manuscript. C.L., L.G., Y.C., J.I., E.S., and B.A. reviewed and editorially revised the manuscript. B.A. reviewed and provided advice on the content of this manuscript.

Competing Interests

The authors declare no competing interests.

Ethical Approval

Not applicable in this study.

Consent to Publish

Authors give their consent to the publisher to publish this manuscript upon acceptance.

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