MiR-128 controls the activity of Polycomb Repressor Complexes 1 and 2 in Neural Stem Cells: Implications of its loss in gliomagenesis.

DISSERTATION

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Abstract

The Polycomb Repressor Complex (PRC) is an epigenetic regulator of transcription whose action is mediated by two protein complexes, PRC1 and PRC2. PRC activity normally plays a key role in stem cell maintenance, but it is oncogenic in glioblastoma where it is involved in cancer stem cell maintenance and radio-resistance. Here we show that miR-128 directly targets the mRNA of BMI1 and SUZ12, key components of PRC1 and PRC2, respectively. This blocks the partially redundant functions of PRC1/PRC2, thereby significantly reducing PRC activity and its associated histone modifications. MiR-128 and SUZ12/BMI1 show opposite expression in human glioblastomas versus normal brain and in glioma stem-like versus neural stem cells. MiR-128 expression is significantly reduced in the brain of young mice genetically engineered to develop glioblastomas before tumor development, as compared to the elevated expression of miR-128 in the brain of wild-type mice. This suggests that loss of miR-128 expression in brain is an early event in gliomagenesis. Moreover, knock-down of miR-128 expression in non-malignant mouse and human neural stem cells led to elevated expression of PRC components and increased clonogenicity, suggesting that miR-128 is an important suppressor of PRC activity and its absence is an early event in gliomagenesis.
Finally, miR-128 renders glioma initiating cells less radio-resistant by preventing the radiation-induced expression of both PRC components thus impairing the DNA repair after radiation-induced double strand DNA breaks.

Taken together our results suggest an important role for the loss of miR-128 in the pathogenesis of glioblastoma and its potential for a future therapeutic use.
Dedication

to Cinna and what we were;

to Maia and Luna, and what will be
Acknowledgments

Thank you Dr Chiocca for your constant support, help, advice.
And for being an exceptional role model during all these years.

Thanks to Sean Lawler, for bringing me into this adventure

Thanks to my mother Giuly, for giving me some of her ability to dream

Thanks to my wife Irene, for grounding me and standing by
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Publications


9. **Peruzzi PP**, Puente E, Bergese S, Chiocca EA. Use of Intraoperative MRI (ioMRI) in


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**Fields of Study**

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Chapter 1

Introduction and Review of the Literature

1.1 Glioblastoma multiforme and Glioma Initiating Cells (GIC)

The most common primary brain tumor in the adult population, glioblastoma multiforme (GBM) is also associated with the worst prognosis of all primary intracranial neoplasms, with a median overall survival after standard treatment (maximal surgical resection followed by radiation and chemotherapy) of about 15 months (1). Over the past few decades, despite extensive research and countless clinical trials, only minor strides have been made towards achieving a significant improvement in survival. Surgical resection, although the mainstay of treatment and fundamental to attain maximal survival (2), has obvious limitations because of the infiltrative nature of the tumor into surrounding normal brain, and, in fact, never achieves tumor eradication. Of the available adjuvant armamentarium, ionizing irradiation has proven effective, but only in delaying tumor recurrence, which, with exceedingly rare exceptions, happens in all cases (3). Similarly, chemotherapy with Temozolomide, an alkylating agent (1) and, more recently, with antiangiogenic compounds inhibiting the VEGF signaling pathway (4) provide consistent
but moderate benefit, partly because their limited access to the tumor, especially at the interface with normal brain, due to the presence of the Blood Brain Barrier (BBB) (5,6), and partly because of the inherent resistance of a subset of malignant cells, hereafter called Glioma Initiating Cells (GIC), to cytotoxic insults (7-11).

Glioblastoma multiforme owes its name to its non-univocal appearance at histopathologic analysis, which is also paralleled by a wide variety of molecular derangements. A major classification criterium, which has been initially clinically based and consequently corroborated by molecular analysis, relies on the modality of tumor onset: in fact, glioblastoma may arise as a “de novo” tumor (hence called primary GBM), i.e. presenting at diagnosis with all the pathognomonic characteristics of a grade IV glioma (namely nuclear atypia, high mitotic index, and endovascular proliferation with common necrosis); or it can derive as the malignant progression, over several years, of an initially lower grade tumor, usually a grade II glioma (secondary GBM). Primary tumors tend to arise in people older than 50, while patients affected by secondary GBM are usually younger, since lower grade tumors peak in incidence during the 3\textsuperscript{rd}- 4\textsuperscript{th} decade, and the average time to progression is within 5-7 years (12,13). Also, primary GBM is much more common than secondary. Once the tumor has the histopathologic characteristics of GBM, its behavior and its response to therapy is similar, regardless whether it is primary or secondary (14). Importantly, there are specific molecular signatures identifying primary from secondary glioblastoma: in particular, primary GBM is characterized by
Endothelial Growth Factor Receptor (EGFR) amplifications and activating mutations, as well as loss of heterozygosity of Chromosome 10q, deletion of PTEN and deletion of CDKN2 (responsible for the production of p14 and p16, two important tumor suppressors). On the contrary, secondary GBM are usually characterized by p53 mutations and activation of Platelet Derived Growth Factor Receptor A (PDGFRA) as well as frequent mutation of the Isocitrate Dehydrogenases IDH1 and IDH2 (15, 16). The latter have recently become object of intense research, as numerous studies have demonstrated how mutations in cytosolic IDH1 (and, to a lesser extent IDH2, its mitochondrial homologous) represent a “bona fide” signature of secondary glioblastoma, and predictors of significant better prognosis (255, 256). IDHs mutations have been reported in Acute Myeloid Leukemia, Prostate and Colon cancers as well (257). Isocitrate dehydrogenases catalyze the oxidative decarboxylation of isocitrate to α-ketoglutarate and reduce NAD(P)$^+$ to NAD(P)H. The presence of the mutated enzyme determines the production of 2-hydroxyglutarate (2HG) (258) which has been shown to act as an onco-metabolite, as it has been shown to competitively inhibit multiple α-Ketoglutarate-dependent dioxygenases, including histone demethylases (259). Both mutant IDH1 enzymes or direct introduction of 2HG to culture medium have shown to arrest differentiation and to increase global and promoter-specific H3K9 and H3K27 methylation by decreasing the protein levels of KDM4C, a demethylase required for cell differentiation (260). All mutations observed for IDH1 occurred at codon R132 (255).
The majority of mutations observed for IDH2 involved residue R172 (256): in both cases the mutations occur in the enzyme catalytic site, determining a profound reduction of enzymatic activity, which results in impaired metabolism of isocitrate and final production of 2-hydroxyglutarate from α-Ketoglutarate (258).

As a result of whole genome analysis of multiple glioblastoma samples through The Cancer Genome Atlas consortium, a further sub classification has been proposed according to clustering of lineage gene expression and tumor suppressors/oncogenes signatures: accordingly, at least four subgroups have been identified for glioblastoma multiforme, namely Proneural, Neural, Classical and Mesenchymal: two major features of the Proneural class are alterations of PDGFRA and point mutations of IDH1; EGFR amplification and CDKN2A deletion are characteristic of the Classical subgroup; NF1 deletion was mainly observed in the Mesenchymal subgroup, associated with overexpression of MET. Finally, the Neuronal subtype displays the expression of neuron markers, such as NEFL and SYT1 (17). The importance of this classification is that for the first time the molecular signature of the tumor was linked to prognosis and response to treatment (i.e the Proneural subtype appears to have the best course, but no gain from intensive adjuvant treatment, while the classical and mesenchymal subsets are those which respond the best to chemotherapy) (17). Particularly interesting has been the recent finding that there is a specific subgroup of glioblastomas that show a concerted hypermethylation at large number of loci, particularly at CpG islands (and thus called
Glioma-CpG Island Methylator Phenotype, or G-CIMP), and whose transcriptome is strikingly associated to the Proneural group. As high as 87% of CIMP+ tumors were also classified as Proneural, it is currently accepted that CIMP tumors are a subgroup of the Proneural subset; what is more striking though, is the significant longer survival that CIMP+ tumors have in comparison to all other glioblastomas. Finally, IDH1 mutation (discussed above) has been tightly associated to CIMP+ tumors: altogether, IDH1 and CIMP status represent two novel molecular signatures that define a subgroup of glioblastomas that appear to behave much less aggressively than all other forms of the tumor (261).

The existence of Cancer Stem Cells in human GBM was first demonstrated in in vitro experiments showing that cells isolated from freshly resected glioblastomas were able to form spheres when cultured in absence of serum and in presence of EGF and FGF-2. Those cells were also able to undergo some degree of differentiation into astrocytes or neurons upon differentiation stimuli (18, 115). Importantly, when grown under stem cell conditions, these cells commonly expressed markers like CD133, Nestin, the RNA-binding protein Musashi-1, SOX2 and BMI1 (116-119). Further experiments showed that these cells, when implanted into the brain of immunodeficient rodents, not only could originate a tumor, with extremely high oncogenic ability (as low as 100 cells were able to generate an intracranial tumors), but also at histological and genetic evaluation the xenograft appeared much more closely resembling to the parental human tumor than
those originating from classical GBM cell lines (120). Subsequently, the membrane protein CD133 was used to isolate and characterize cancer stem cells from GBM specimen, as experimental evidence suggested that CD133+ but not CD133- cells were able to originate neurospheres in Neural Stem Cell NSC culture media. CD133 cells also have shown capacity to differentiate into astrocytic or neuronal lineage upon exposure to differentiation stimuli (121,122), suggesting that only CD133+ cells could recapitulate the original human GBM when transplanted into animals. CD133 has thus been broadly accepted as a marker identifying “bona fide” Glioblastoma Initiating Cells (GIC). Over the years, this unifying role of CD133 in stemness determination has been challenged, as studies have shown that also CD133- cells retain the capability to generate brain tumors in nude animals and that those cells became CD133+ after serial orthotopic xenotransplants, suggesting that the condition of growth is an essential determinant of this cell marker’s expression and thus of cellular stemness (123).

Although it is still uncertain how GIC originate, increasing evidence suggests that they derive from aberrant neuroprogenitors located in the Subventricular Zone (SVZ) (25-27). Because glioblastomas are histologically heterogeneous, containing cells expressing neural progenitor/stem cell, neuronal, and astroglial markers, it was proposed that these tumors could originate from the transformation of multipotent NSCs. Holland et al. provided one of the first evidences that NSCs within the SVZ may be involved in gliomagenesis. By using viral-mediated transfer of oncogenes AKT and RAS, the authors
showed that only Nestin + SVZ cells, and not differentiated astrocytes became tumorigenic after gene transfer, suggesting that Nestin-positive cells within the SVZ might represent the cell of origin for glioblastoma (124).

Similarly, a recent study revealed that expression of a mutant form of p53 through Cre-mediated recombination in all GFAP+ cells, induced GBM-like tumor formation in mice only from cells located in the SVZ. This study further suggests that sole p53 deficiency in cells located in the SVZ is sufficient to initiate the process of glioma formation (125). These findings are consistent with previous reports showing SVZ hyperplasia in p53 +/- mice (126).

Other investigators reported the development of GBM-like tumors arising from the SVZ following exposure of pregnant rats to N-nitrosourea (ENU), a highly potent mutagen (127). Recently, Alcantara et al. demonstrated using tamoxifen-inducible Nestin-CRE transgenic mice crossed with Nf1, p53, and Pten conditional mutants that only cells located in the SVZ develop into GBM-like tumors (128). These data suggest that only cells within the SVZ can to give rise to GBM-like tumors, while parenchymal brain cells, including astrocytes, do not have such capacity (128). The process of gliomagenesis, however, remains controversial: substantial evidence suggests that, although glioblastoma appears to be supported by a sub-population of primitive, poorly differentiated cells, fully differentiated cells within the central nervous system can undergo a process of dedifferentiation when the expression of certain oncogenes is
enforced: very recently, Friedmann-Morvinski et al. have shown that mature murine neurons or astrocyte can be dedifferentiated to neural precursors by selective knock down of Nf1 and Rtp53, and that such cells are tumorigenic in vivo, suggesting that, while the growth of glioblastoma is supported by cells that are in a primordial, undifferentiated configuration, their origin is not necessarily the Neural Stem Cell reservoir, but can substantially be any cell within the central Nervous System which undergoes a process of dedifferentiation (251).

1.2 Polycomb Repressor Complexes 1 and 2 and epigenetic regulation of gene expression

Polycomb Group (PcG) proteins were initially identified in Drosophila through their role in silencing homeotic (Hox) genes: in PcG-mutant flies, Hox gene expression did not follow the normal segmental cranio-caudal pattern, resulting in characteristic aberrancies of body patterning (28,29). Historically, many PcG mutants were identified as heterozygotes flies that had two or three thorax number 1 segments (T1-T1-T3 or T1-T1-T1) instead of the normal T1-T2-T3 patterns. T2-to-T1 and T3-to-T1 transformations were mostly evident in male flies because mating structures, called sex combs, are normally located in the T1 segment (by the anterior first pair of legs). Since in mutants they are found by the first two, or all three pair of legs, this observation accounted for the
names given to the genes responsible for the mutations: Polycomb, Posterior sex combs, Sex comb Extra, etc (64).

More recently, genome-wide chromatin immunoprecipitation (ChIP) studies have revealed that PcG proteins are not limited at Hox gene loci, but in fact target several hundreds of genes in Drosophila and mammalian cells (30, 31, 32). PcG targets are predominantly transcription factors and the signaling components of most major developmental pathways: it is now accepted that they constitute a global silencing system, regulating major developmental pathways and controlling stem cell biology (33,34).

PcG proteins work by forming complexes that interact with chromatin and, by inducing post-translational histone modifications, silence gene expression.

The two major and better known complexes are named Polycomb Repressor Complex 1 (PRC1) and PRC2.

PRC1 functions by inducing ubiquitylation of histone H2A at Lysine 119 (H2aK119 ubiquityl) (31-34): although there is no consensus on how this histone modification would interfere with gene expression, the most accredited theory proposes that the ubiquityl group would determine a physical impediment to the movement of RNA polymerase along the DNA during transcription (35,36). As a protein complex, in Drosophila, PRC1 is made by stoichiometric amounts of 4 PcG subunits, namely Polycomb, Polyhomeotic, Posterior Sex Combs and RING. The same is true in human
cells, with the sole difference that each subunit has several homologues, resulting in dozens of possible different combinations (table 1). The RING subunit possess the ubiquityl ligase activity of the whole complex, but Posterior Sex Combs (BMI1 in humans) is a necessary cofactor for its functionality. Both Polyhomeotic and Polycomb have scaffolding function and mediate recognition and attachment of the complex to target genomic DNA sequences (33,34).

PRC2 functions by tri-methylating Histone H3 at Lysine 27(H3K27\text{me3})(33). In Drosophila, the complex is made by three fundamental subunits named Enhancer of Zeste, Suppressor of Zeste 12 (SUZ12) and Extra Sex Combs. In human cells, the complex retains the same structure as it has been described in flies, but Enhancer of Zeste exists in two different homologues, EZH1 and EZH2 (table 1). Whereas EZH2 is highly expressed during embryogenesis and in proliferating cells, EZH1 predominates in adult, quiescent tissue (37,38). PRC2 containing EZH1 has much less methyltransferase activity than PRC2-EZH2. EZH2 is responsible for the methyltransferase activity of the complex, but all three subunits are fundamental for its activity (34). Also, there are accessory elements (RBBP4 and RBBP7) that are however not exclusive of PRC2, as they are associated to other nucleosome remodeling complexes, like the NuRD complex, and whose main function is interaction with DNA and scaffolding (34,39)
<table>
<thead>
<tr>
<th>PCG SUBUNITS IN DROSOPHILA</th>
<th>PCG SUBUNITS IN HUMANS</th>
<th>PCG COMPLEX</th>
<th>BIOCHEMICAL ROLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polycomb</td>
<td>CBX2,CBX4,CBX6,CBX7, CBX8</td>
<td>PRC1</td>
<td>Binds trimethylated H3K27</td>
</tr>
<tr>
<td>Polyhomeotic</td>
<td>PHC1, PHC2</td>
<td>PRC1</td>
<td>Higher order interactions</td>
</tr>
<tr>
<td>Sex combs extra (RING)</td>
<td>RING1, RING1B</td>
<td>PRC1</td>
<td>E3 ubiquitin ligase for H2K119</td>
</tr>
<tr>
<td>Posterior sex combs</td>
<td>BMI1, MEL18</td>
<td>PRC1</td>
<td>Co-factor for E3 ubiquitin ligase and compacts nucleosomes</td>
</tr>
<tr>
<td>Enhancer of Zeste</td>
<td>EZH1, EZH2</td>
<td>PRC2</td>
<td>Catalytic subunit of H3K27 histone methyltransferase</td>
</tr>
<tr>
<td>Suppressor of Zeste 12</td>
<td>SUZ12</td>
<td>PRC2</td>
<td>Stimulates H3K27 histone methyltransferase</td>
</tr>
<tr>
<td>Extra sex combs</td>
<td>EED</td>
<td>PRC2</td>
<td>Stimulates H3K27 histone methyltransferase</td>
</tr>
<tr>
<td>Nucleosome remodeling factor 55</td>
<td>RBBP4, RBBP7</td>
<td>PRC2,</td>
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</tr>
<tr>
<td>Polycomb-like</td>
<td>PHF1, MTF2, PHF19</td>
<td>Binds PRC2</td>
<td>Recruits PRC2</td>
</tr>
</tbody>
</table>

Table 1.1: subunits of Polycomb Repressor Complexes in flies and humans

PRC1 and PRC2 are believed to work in concert to silence gene expression, but the mechanism of interaction is still debated. In Drosophila, PRC2 is directed to target genes by docking proteins (known as Polycomb-like proteins) that recognize specific
DNA sequences named Polycomb Response Elements (PREs). The best characterized PREs have been delimited to several hundred base-pairs, but there is no simple consensus sequence that can predict PRE functions. However it is clear that, at least in Drosophila, the DNA-binding sites for the zinc finger protein Pleiohomeotic (PHO) are a common element within PREs, and, in fact, PHO is required for PRE-mediated transgene silencing, as a required “protein bridge” between PRC and DNA (51). Contrarily, in mammalian cells specific PREs have not been identified (40). However, ChIP experiments have shown association of PRC2 complexes to CpG islands located at promoter regions of target genes (41) and, interestingly, many of these regions share the consensus sequence for YY1, a vertebrate homolog of PHO (52), which has also been shown to interact with PRC1 subunits (44). Indeed, YY1 knockdown dislodges the PRC2 subunit EZH2 and removes H3K27me3 from target genes in mouse myoblasts (53).

Recently, a role for long non-coding RNAs (ncRNAs) in regulating chromatin expression has been elucidated: the ncRNAs HOTAIR, KCNQ1OT1 and REPA have been shown to recruit Polycomb Proteins to chromatin through interaction with the PRC2 complex, and their downregulation determined an impaired function of PRC2-mediated silencing (83): it has been proposed that long ncRNAs bind DNA in a sequence-specific manner at the promoter of target genes and recruit Polycomb Proteins through interactions with their SET domains (EZH2 has a SET domain) or chromodomains (CBX proteins have
chromodomains). Both SET domains and chromodomains are protein-nucleic acid interaction modules (84,85), and CBX has been experimentally shown to bind RNA (86). After PRC2 has provided trimethylation to H3, the methyl moiety serves as an anchor for the docking of PRC1, which recognizes the histone modification through its Polycomb (CBX2, CBX4, CBX6, CBX7 or CBX8) component (42,43) and proceeds with H2A ubiquitylation (34). Although H3K27 trimethylation is generally considered a repressive mark, there is no agreement whether the trimethylation by itself is sufficient to induce gene silencing: to date it is believed that the actual “stop” sign is provided by PRC1 activity, while PRC2 has no direct effect on gene transcription (31). In any case, the understanding of the functional relationship between PRC1 and PRC2 is far from being completely dissected as PRC1 and PRC2 have also been shown to function independently from each other. While it is true that disruption of PRC2 activity leads to loss of PRC1 from chromatin targets in Drosophila (44,45) and human cells (46) there is evidence supporting that recruitment of PRC1 might also be independent of methylated H3K27. Genome-wide distribution of PRC1 and PRC2 subunits in fly cultured cells showed discrete peaks of PRC1 and PRC2 at presumptive PREs, whereas H3K27me3 was more broadly distributed (47). Also, PRC1 has been shown to target certain loci without PRC2 or H3K27 methylation, as both CBX8 and BMI1 have been shown to be able to directly interact with chromatin (48,49,50). This implies that PRC1 and PRC2 can silence genes either synergistically or independently of each other (31,60).
Figure 1.1: Mechanism of epigenetic gene silencing by PRCs. PRC2 carries out the specific tri-methylation of H3 at lysine 27 (1), which in turn recruits PRC1 by interaction with its CBX component. PRC1 then transfers one monoubiquitin residue to H2A (2). Both the physical presence of PRC1, as well as the ubiquitil group on H2A keep the chromatin in a silenced, compacted state, and prevent RNA Polymerase 2 to bind to DNA and proceed with gene transcription (3).

1.3 Polycomb proteins and “stemness”

The feature of “stemness” pertains to a particular subset of cells that retain the ability to proliferate, self renew (i.e producing daughter cells which are genetically and functionally identical to parental cells) and, in the presence of appropriate conditions, differentiate into a variegated progeny of specialized cells, usually displaying reduced,
if not abolished, mitotic and self renewal ability. The maintenance of this primordial, undifferentiated, cellular status is achieved by fine-tuned regulation of gene expression, whereby genes responsible for metabolic activity and proliferation are overexpressed, while those associated with differentiation are repressed (54). Over the years this process has been elucidated in its molecular components and described as a concerted “pro-proliferative” action exerted by several fundamental transcription factors, namely NANOG, Oct4 and SOX2, (55), paralleled by an “anti-differentiation” action mainly provided by Polycomb Proteins (56,57,58). After a genome-wide analysis of SUZ12 occupancy, Lee et al found that SUZ12 is distributed across large portions of over 200 genes responsible for developmental regulation in Embryonic Stem Cells (ES), and that most of those PRC2 target genes are preferentially activated during differentiation (59). Importantly, the authors also showed that those PRC2 targets are shared with key Embryonic Stem cell regulators Oct4, SOX2 and NANOG, revealing for the first time a direct synergy among them in repressing genes involved in differentiation. Not surprisingly, SUZ12 -/- ES cells, although viable, are characterized by higher expression of differentiation-specific genes (61) and mice lacking PRC2 function show severe abnormalities and decreased cellular proliferation at the early embryonic stages. EED-knockout ES cells are able to give rise to chimeras, confirming that they are pluripotent in vivo. However, despite the robust occurrence of EED-depleted cells in chimeric embryos at 9.5 days, they are scarce at 12.5 days, due to lethality of
differentiated cells (61) Even though PRC2 is not essential for maintenance of ES cell
self renewal, as its absence does not compromise the expression of NANOG and
Oct4(62), the complex is required for the successful reprogramming of differentiated
cells towards pluripotency and it is also required for maintaining stemness (63).

Similarly, PRC1 has been shown to play a fundamental role in ES cell homeostasis. ES
cells lacking RING1b expression are capable of maintaining the expression of Oct4 and
NANOG, but they display a de-repression of PRC1 target genes and are impaired in
proper differentiation (65, 66). Furthermore, double KO of RING1a and RING1b has
been shown to completely abrogate ES self renewal and to induce preferential de-
repression of genes involved in differentiation (67).

An important concept that pertains to PRCs and their function on stemness maintenance
is their redundancy and partial independence of complex activity: in RING1b/EED
double knockout ES cells, the number of de-repressed genes, which are direct PRC
targets, increased about two times, compared to single EED or RING1b knockout. Half of
these genes were de-repressed only after the loss of both PRC1 and PRC2, suggesting
that the two complex are redundant for repression of an important subset of genes, and
that downregulation of one does not necessarily imply loss of function of the other (31).

PRCs appears to be fundamental in the biology of Neural Stem Cells (NSCs), EZH2 has
been found to target more than 1500 genes in NSCs and its expression decreases upon
induction of neural or astrocytic differentiation, paralleled by derepression of lineage
specific genes. Also, forced EZH2 downregulation in NSCs determines decreased proliferation and induction of differentiation (68). Similarly, overexpression of BMI1 in mouse astrocytes determined cellular de-differentiation to pluripotent NSCs (80). Using a mouse model of conditional activation of BMI1 in the central nervous system, Yadirgi et al have shown that BMI1 upregulation in postnatal NSC is associated with increased self renewal and decreased apoptosis, although it was not associated to tumor onset (69). Similarly, BMI1 overexpression has been shown to restore the impaired proliferative capacity of ATM -/- NSC (70). Furthermore, overexpression of EZH2 in astrocytes was shown to induce dedifferentiation towards neural stem cells, which resulted in decreased expression of differentiation markers S100 and GFAP and increase in stemness markers SOX2, nestin and CD133 (71). In cerebellar granule cell progenitors, BMI1 has been shown to directly downregulate CDKN2A/B and CDKN1c expression (with corresponding decrease in p14^{ARF}, p16^{INK4A} and p21^{WAF/CIP1}), thus stimulating cell growth. In that cellular setting the authors also showed that BMI1 levels are increased by activation of the Sonic Hedgehog pathway (72). Downregulation of PRC1 (by RING1 knockdown) or PRC2 (by either EZH2 or EED knockdown) has been shown to keep NSC in a “neurogenic” status, impeding the transition to the “astrogenic” status by preventing PRC-mediated silencing of Neurogenin1, a proneural transcription factor (73). Another study confirmed the importance of PRC1 in the biology of NSC by showing that in absence of RING1b neural stem/progenitor cell proliferation in vivo and in neurosphere
assays is impaired, and their self-renewal and multipotential abilities, assessed as sphere formation and differentiation from single cells, were severely affected. Also neuronal, but not glial, differentiation of mutant stem/progenitor cells under proliferating conditions was observed. mRNA analysis of mutant cells showed upregulation of some neuronal differentiation-related transcription factors and the cell proliferation inhibitor Cdkn1a/p21, as well as downregulation of effectors of the Notch signaling pathway, a known inhibitor of neuronal differentiation of stem/progenitor cells. These data suggested a role for Ring1B in maintenance of the undifferentiated state of embryonic neural stem/progenitor cells. They also suggested that Ring1B may modulate the differentiation potential of NSCs to neurons and glia (74). In a seminal study, Molofsky et al. observed that KO of BMI1 determined reduced self-renewal of neural stem cells, leading to their postnatal depletion and an 80% reduction in forebrain Subventricular Zone neurosphere formation at 30 days after birth, thus explaining the observed postnatal growth retardation observed in Bmi1 -/- mice, associated to neurological defects. The authors also observed that in the absence of Bmi1, cell cycle repressors p16\textsuperscript{Ink4a} and p19\textsuperscript{Arf} were upregulated, and that concomitant knock out of p16\textsuperscript{ink4a} was able to rescue the phenotype observed with Bmi1 suppression. Importantly they also found that while Bmi1 is essential for the self renewal of adult NSC, it was dispensable for the proliferation of neural progenitors (75). The same authors then demonstrated that BMI1 directly targets the p16\textsuperscript{Ink4a} and p19\textsuperscript{Arf} senescence pathways (76).
Similarly, a study in Drosophila confirmed that BMI1 is essential for NSC survival in postembryonic neurogenesis (77). Fasano et al further showed that Bmi1 is also essential for NSC and neuroprogenitors self renewal at the embryonic stage mainly through a p21-mediated effect. In other words, while Bmi1-mediated downregulation of p16/p19 is essential for maintaining adult NSC self renewal, at the embryonic stage Bmi1 still retains its fundamental “stemness” role by downregulating p21<sup>Waf1</sup>, another cell cycle inhibitor, usually silenced in stem cells (78). In fact, Bmi1 has been shown to localize at, and directly repress the CDKN1c/p21<sup>Waf1</sup> locus (79). BMI1 has also been reported to be essential for multipotency of hematopoietic stem cells, as it controls the B-cell lineage developmental regulators Ebf1 and Pax5 (81), and for maintenance and self renewal of prostate p63-positive stem cells (82).

1.4 Polycomb proteins in cancer and Glioma Initiating Cells

Because of their role in stemness maintenance, Polycomb Proteins have been object of intensive investigation in regards to their possible oncogenic properties. BMI1 and EZH2 are often overexpressed in cancer. The former has been described to be upregulated in squamous cell carcinomas (87), leukemia (88), bladder carcinoma (89), neuroblastoma (90) and medulloblastoma (91). Similarly, EZH2 is upregulated in bladder (92), colon (93), breast (94) and prostate (95).
The role of Polycomb Proteins in the maintenance of the cancer stem cell population has been at least partly attributed to their ability to bind and repress the CDKN2A and CDKN2B loci, which encode the tumor suppressor proteins $p14^{ARF}$ and $p16^{INK4a}$ and $p15^{INK4b}$, respectively (96,97). $p15^{INK4b}$ and $p16^{INK4a}$ function upstream in the RB pathway and $p14^{ARF}$ functions upstream in the p53 pathway. Since these loci are frequently epigenetically silenced by DNA methylation, an association of Polycomb Proteins and DNA Methyltransferases (DNMTs) has been proposed (98). The reports that EZH2 and CBX7 can physically associate with DNMTs suggest a mechanism whereby the Polycomb Proteins contribute to the altered DNA methylation profiles that are observed in multiple cancers (99,100). In fact, polycomb targets are as much as 12 times as likely to be aberrantly silenced by DNA methylation in cancer as non-polycomb target genes, and poorly differentiated and aggressive human tumors show preferential repression of polycomb target genes (101).

What triggers the aberrant silencing of polycomb target genes that is observed in many cancers is still object of debate. One potential reason is that polycomb proteins are upregulated in many cancer, as described above, and that, per se, is likely to determine an increased silencing effect of genes involved in cellular differentiation and senescence (102). An alternative and complementary theory proposes that the function of polycomb proteins in cancer is mediated by factors that are required for their association to target genes, including, as described above, the transcription factors SOX2, Oct4 and NANOG,
as well as long non-coding RNAs. Therefore, it would be the deranged expression of these transcription factors to determine an excessive activation of polycomb-mediated repressive mechanisms, particularly at the expense of genes involved in differentiation and cellular senescence (102). To suggest a sort of feed-forward loop among oncogenic transcription factors and Polycomb proteins, there is growing evidence that polycomb proteins themselves are under the direct control of key oncogenes: Myc, for example, has been found to be a key inducer of the expression of all three genes constituting the PRC2 complex (EZH2, EED and SUZ12), as well as of BMI1 (103, 104).

Growing evidence suggests that Polycomb deregulation is an active driver of oncogenesis. In 1991, BMI1 was originally identified as a proto-oncogene that, in association with c-Myc, was able to induce lymphomagenesis in mice (88). More recent studies have shown that BMI1 is also crucial for lung tumorigenesis, whereby the onset of bronchoalveolar tumor in a K-ras-overexpressing mouse model only happens in the presence of BMI1, while in BMI1 KO mice tumor onset is not observed (105). Both BMI1 and SUZ12 have been found to be the responsible factors driving fibroblast transformation by organic arsenic, a known human carcinogen associated with cancers of the skin, lung, liver and bladder (106); SUZ12 expression has been shown to be fundamental for the formation and maintenance of cancer stem cells in a breast tumor model. Not only has its downregulation been associated to block of formation of mammospheres *in vitro* and tumorigenesis *in vivo*, but also ectopic expression of SUZ12
in transformed cells was sufficient to generate cancer stem cells (107). Finally, several studies have also linked polycomb proteins expression to the activation of major signaling pathways associated to cancer stem cell biology: for example it has been demonstrated that BMI1 is directly downstream of Hedgehog, and that its activation is fundamental for the onset of medulloblastoma (108). Similarly, SUZ12 has been shown to be activated by non-canonical Wnt pathway during the blastic phase of chronic myeloid leukemia, resulting in block of cellular differentiation (109).

Many of the protein constituents of PRCs are highly expressed in glioblastoma compared to normal brain, including BMI1 (110,111), SUZ12 (112) and others (113). In a panel of 305 grade II, III and IV astrocytomas and oligodendroglialomas, BMI1 expression was detected in 302 tumors (99%) (129). Another study revealed that BMI1 is highly expressed in human GBM samples and that Bmi1 deficiency reduces the invasiveness and tumorigenicity of malignant mouse astrocytes carrying a null mutation in the Ink4a/Arf locus (130). Using INK4A/ARF null human GBM specimens, BMI1 was showed to be enriched in CD133+ cells and required to sustain their self-renewal through prevention of CD133+ cells apoptosis and/or differentiation into neurons and astrocytes (131).

Notably, CD133+ GBM cell fractions were enriched for expression of the stem cell markers SOX2, BMI1, and EZH2 (132), again confirming the important role of
polycomb proteins in the biology of Glioma initiating Cells, to the point that some authors have postulated an “addiction” of glioblastoma initiating cells for Polycomb protein expression: importantly, oncogenic addiction distinguishes CSCs from normal stem cells and can be viewed as a survival mechanism to overcome mutations affecting cancer cells viability (133). This situation may render GBM stem cells more sensitive to BMI1 inhibition than normal stem cells present in the brain, and thus could be exploited in a therapeutic context [132, 134].

EZH2 has been shown to play an important role in maintaining the undifferentiated status of GIC by repressing the expression of Bone Morphogenetic Protein Receptor 1B (BMPR1B), whereby, upon EZH2 downregulation by interfering RNA, the authors observed a dramatic decrease of CD133 levels (113). Inhibition of EZH2, either pharmacologically or by RNA interference, resulted in impaired self renewal of GIC and marked decrease of tumorigenicity in vivo; the authors also found that downregulation of EZH2 corresponded to decrease levels of c-Myc in GIC, while forced c-Myc expression was able to rescue the phenotype associated to EZH2 loss (114).

1.5 MicroRNA biology

MicroRNAs are small (~22 nucleotides) non-coding RNAs that were first discovered in C. elegans but have since been found to exist in almost all eukaryotes ranging from plants
MicroRNAs are transcribed by RNA polymerase II as long RNA primary transcripts (pri-miRNAs) which are then processed into ~70 nt hairpin structures known as precursors (pre-miRNAs) by a nuclear enzyme complex known as the microprocessor. The microprocessor contains an endoribonuclease known as Drosha as well as a double-stranded RNA binding protein known as DiGeorge syndrome critical region 8 (DGCR8) in mammals and Partner of Drosha (Pasha) in D. melanogaster and C. elegans. DGCR8/Pasha is required for proper pri-miRNA processing (137, 138, 139). Drosha contains two RNase domains that cleave the 5’ and 3’ ends, releasing the pre-miRNA (140). In some instances, the sequence of the mature pre-miRNA corresponds precisely to the sequence of a spliced intron. These spliced-out pre-miRNAs, known as mirtrons, no longer require microprocessor activity in order to generate mature miRNAs (141, 142). Pre-miRNAs are transported from the nucleus to the cytoplasm by a complex of Exportin5 and Ran-GTP (143), where they are processed into ~22 bp double stranded RNAs by the RISC loading complex. The RISC loading complex consists of the RNase Dicer, the dsRNA binding protein TRBP, PACT (protein activator of PKR), and Argonaute protein (144, 145). Once processed, one of two strands of the miRNA is loaded into a ribonucleoprotein complex, referred to as a miRNA-induced silencing complex (miR-RISC); The miR-RISC complex is the final effector of gene silencing and the specificity of mRNA targeting is determined by the sequence of incorporated microRNAs. Pre-miRNA processing by Dicer and miRISC
complex assembly (loading of the mature miRNA onto Argonaute proteins) are thought to occur simultaneously at the RISC loading complex. In the vast majority of cases, mRNAs are targeted by microRNAs at their 3’ UTR sequences, but miRNAs can also regulate gene expression of mRNAs that contain miRNA target sites in their 5’ UTR (146, 147). A possible explanation for the preferential 3’UTR location of miR target sites has been proposed by Gu et al who showed that when miR target sequences are within the coding sequence, microRNAs can be displaced by actively translating ribosomes upstream of target sites within the coding sequence partially restored miRNA mediated repression. These results suggest that actively translating ribosomes may displace the miRISC complex from target sites positioned within the coding sequence, thus explaining the evolutionary selection of sites located in the 3’UTR, i.e past the stop signal to protein translation (148).

Specificity of miRNA function is controlled through the direct base pairing of a miRNA-loaded RISC to miRNA-complementary target sites on targeted mRNAs (149). miRNA-regulated mRNAs often harbor multiple miRNA target sites within their 3’ UTRs, sites that in many cases are phylogenetically conserved between species (150). Also, the main feature of microRNA-based silencing system is the imperfect complementarity with target mRNA sequences, whereby only the near-perfect binding of the “seed” sequence of 6-8 nucleotides at the 5’ end of the microRNA is required for recognition of its target. As a result, often one single mature microRNA has multiple mRNA targets, making them
rather non-specific. MiRNAs are structurally very similar to small-interfering RNAs (siRNAs) but they act differently: although both miRNAs and siRNAs interact with Argonaute (Ago) proteins, miRNAs are distinct from siRNAs since they imperfectly base pair to target sites and do not lead to endonucleolytic cleavage of targeted mRNAs. Early reports generally suggested that miRNAs inhibit gene expression at the post-transcriptional level, at some stage post-translation initiation. These reports also suggested that miRNA action had little or no effect on the abundance or stability of target mRNAs. More recent results challenge these data as results from both in vitro and in vivo studies have shown that miRNAs can inhibit translation initiation as well as promote decay of target mRNAs. As such, the literature now contains reports favoring three different potential modes of miRNA-mediated repression, and it has been proposed the primary mode of miRNA mediated gene regulation may vary by cell type or developmental stage, possibly controlled by miRNA levels or miRISC complex components. Thus miRNAs may (1) inhibit translation initiation (151, 152), (2) block translation at some stage after initiation (153) or (3) destabilize target mRNAs (156, 157). In fact, although the original discovery of the lin-4 and let-7 miRNAs in C. elegans was accompanied by the demonstration that they inhibit translation without affecting mRNA stability (154, 155), it has recently been observed by many authors that microRNAs also determine mRNA destabilization, suggesting that miRNA-mediated translational repression and mRNA decay act in tandem to facilitate repression of gene expression.
miRNAs elicited a 95% reduction in reporter expression, as well as a 50% decrease in target mRNA levels (156). In addition, Wu et al. reported that miR-125b expression reduced target protein production by 90%, while mRNA levels were reduced by around 70% (157).

In contrast to siRNA-mediated mRNA endonucleolytic cleavage (158), miRNA-mediated enhancement in the rate of mRNA decay appears to be secondary to deadenylation-dependent degradation pathways (159). MiRNAs can induce deadenylation in both Drosophila melanogaster S2 cells and as well as in HeLa and NIH-3T3 cells (157, 160). MiRNA mediated deadenylation appears to be mediated by the Caf1–CCR4–Not1 deadenylation complex, and further studies demonstrated, in D. melanogaster cells, that miRNA-dependent mRNA decay is inhibited by siRNA knockdown of deadenylation factors Not1 and Ccr4 as well as the decapping enzyme Dep1/2 (160). Interestingly, miRNA-mediated mRNA degradation and translational repression are suggested to function as independent mechanisms of action. Several groups have demonstrated that mRNAs that are not actively translating can still undergo miRNA-mediated deadenylation and/or decay (161, 162). Two recent reports have provided a large-scale picture of miRNA mediated control of both target protein and mRNA levels using mass spectrometric proteomic approaches in parallel with microarray-based analysis of mRNA levels. Selbach et al. introduced five different miRNAs (miR-1, miR-155, miR-16, miR30a, and let7b) into HeLa cells by
transfection and also used a locked nucleic acid to knockdown let7b and looked at changes in protein and mRNA levels on a genome-wide scale. They report that most targets are repressed at both the mRNA and protein level, with the relative contributions of mRNA destabilization and translation inhibition varying from miRNA to miRNA and from target to target. Interestingly, they found that proteins translated at the endoplasmic reticulum were overrepresented in the class of targets that were repressed mainly at the protein level. That study demonstrated the ubiquity of the miRNA response, showing that transfection of single miRNAs generally repressed hundreds of genes at the post-transcriptional level, although few targets were repressed by more than three or fourfold (163).

As a confirmation that microRNAs are also involved in mRNA decay, the activity of miRISC has been mainly localized at the cellular processing bodies (P-bodies, subcellular structures involved in mRNA storage and decay). Interestingly, a conserved group of proteins, including GW182, have been found to be the link between RISC and P-bodies: in fact they have been shown to be able to bind both P-bodies as well as the Argonaute protein Ago1, a component of RISC (164, 165). Importantly, it was initially demonstrated in Drosophila S2 cells that knockdown of the GW182 homolog Gawky disrupted miRNA-mediated repression of reporter protein production (166), a result that was reproduced in human cells (167) and in C. elegans (168). Recent studies have implicated the C-terminus of GW182 proteins as the region responsible and sufficient for mediating
gene silencing (169,170). Also, a Gawky mutant that fails to localize to P-bodies, but contains the N-terminal Argonaute binding domain and C-terminal silencing domain is still able to mediate microRNAs-dependent repression while it is not able to localize RISC to P-bodies (171). Taken together these data suggest that GW182 might be the final effector of microRNAs-mediated repression and decay of mRNA transcripts, and that the role of Argonaute proteins is to bring RISC in contact with GW182 (171).

A recent study published by Eiring et al. suggested that microRNAs can also have a non-canonical function by acting as a decoy regulating the activity of RNA-binding proteins: the authors showed that miR-328 and C/EBPa, a master regulator of myeloid differentiation, share a common consensus RNA sequence for hnRNP E2, a poly(rC)-binding protein that controls translation of specific mRNAs, including C/EBPa: when present, miR-328 occupies the RNA binding site of hnRNP E2, thus preventing its inhibitory function on C/EBPa. Vice versa, when miR-328 is downregulated, as in blast crisis of chronic myeloid leukemia, hnRNP E2 suppressive action on C/EBPa remains uninhibited (172). Finally, Khraiwesh et al have shown in plant cells another non-canonical function of microRNAs, according to which microRNAs can also directly play a role in epigenetic control of gene expression: the authors have shown that in situations of high miR/target mRNA rations, in addition to the formation of the RISC, targeting mRNA, microRNAs assemble into RNA-induced Transcriptional Silencing (RITS) Complexes that induce methylation and subsequent downregulation of the corresponding
genes, whereby the target specificity would still be directed by the microRNAs sequence (173).

All known microRNAs are catalogued in miRBase (www.mirbase.org), a database of microRNAs sequences, targets and nomenclature that applies to various species. With the exception of the very first described microRNAs (e.g. let-7 and lin-4) microRNAs are named according to a numerical progression, and in the form XXXX-mir-YYYY, where XXX is the species code (e.g. hsa for humans) and YYY is a progressive number: any newly discovered microRNA, in any species, is given the next numeric identifier, so that, for example, hsa-mir-500 follows mmu-mir-449, and so forth. When the same microRNAs is encoded by multiple genes, each gene is labeled with further numerals (e.g. mir-128-1 and mir-128-2, indicating that the same mature miR-128 derives from two different genes). Finally, when mature miRs are closely related in terms of their sequences, they are given additional alphabetical suffixes (e.g. miR-200a, miR-200b and miR-200c).

Because the biological effect of microRNAs on target mRNAs is based on an imperfect sequence match, multiple combinations of microRNAs-mRNA are possible: in fact, different microRNAs can target the same mRNA and, more importantly, the same microRNA can have more than one mRNA target. For this reason, the prediction of targeting is based on a bioinformatic approach of sequence analysis and free energy of
base-pairing. Although the first targets of microRNAs were discovered by genetic screens, experimental techniques are still too expensive and time consuming to validate targets of microRNAs on larger scale. Currently, a significant fraction of the reported confirmed target genes has been predicted computationally before experimental validation. Initially, each mature microRNA from miRBase is matched against control sets of 3’ UTRs to create an Extreme Value Distribution. Next, that microRNAs is matched against all known 3’ UTRs sequences from the same (or related) species, and the previously derived EVD values are used to create p-values from the scores obtained for each potential site detected. Finally, those sites which appear to be conserved among orthologous 3’UTR for that given microRNAs are assigned modified p-values depending on the amount of conservation across their full length; this approach derives from the evidence that genes have been under selective pressure to conserve microRNAs taget sites across evolution, so conserved 3’UTR sites are likely to be real targets for microRNAs with a matching sequence (223, www.mirbase.org, www.targetscan.org).

MicroRNA have been shown to play an essential role in organogenesis: germline KO mutations of Dicer are lethal at early embryonic stage, resulting in severely impaired organogenesis and greatly reduced pool of pluripotent cells (262). Conditional knockdown of Dicer within the central nervous system showed severely impaired corticogenesis, failed terminal cellular differentiation, increased cell death resulting in microcephaly and enlarged ventricles. MiR-9, miR-34a, miR-124, miR-125b, miR-128
and miR-137 have been those mainly associated with cortical development (263). Mir-1 has been shown to be fundamental for cardiogenesis and muscle development, and its deletion in mice resulted in ventricular septal defects, arrhythmias, and cellular hyperplasia (264).

1.7 microRNAs in cancer

The fact that microRNAs have been described to take part in the regulation of essentially all cellular processes (metabolism, differentiation, motility, proliferation, apoptosis), led investigators to hypothesize a role in cancer biology too. In 2002, Calin et al. were the first to identify that the loss of two microRNAs (miR-15 and miR-16) was a frequent event occurring in Chronic Lymphocytic Leukemia (174) and that it was associated with more aggressive behavior (175). Since then, the number of described microRNAs has increased almost exponentially (according to the most recent version of mirbase.gov (version 19, released August 2012), more than 2,200 microRNAs have been sequenced in human cells to date), and a multitude of studies have shown, for a quantity of them, a role in cancer development and progression. Posed that the main microRNA cellular mechanism is suppression of mRNA translation, microRNAs can be broadly divided into
tumor-suppressors because of their effect of oncogene downregulation, or oncogenic, because of their action on tumor suppressor genes.

Although the expression of some miRNAs is increased in malignant cells, more commonly a widespread underexpression of miRNAs is observed in cancer. Because miRNA expression generally increases as cells differentiate, the apparent underexpression of miRNAs in cancer cells may, in part, reflect their more undifferentiated state (176).

Two main mechanisms have been proposed as the underlying cause of the global downregulation of miRNAs in cancer cells (figure 1.2): one involves transcriptional repression by oncogenic transcription factors, like the oncoprotein MYC, which is overexpressed in many cancers (177), and which has been shown to directly repress let-7, miR-15a/16-1, miR-26a and miR-34 (178) or the loss of key tumor suppressors, like p53 that are also responsible for the activation of microRNAs genes (177). The other mechanism involves changes in miRNA biogenesis and is based on the observation that cancer cells often display reduced levels, or altered activity, of factors in the miRNA biogenesis pathway (179), as in vivo experiments have shown that the repression of miRNA processing by the partial depletion of Dicer1 and Drosha accelerates cellular transformation and tumorigenesis (180).
Figure 1.2: MYC and p53 are master regulators of microRNAs expression in cancer (modified from Lujambio et al. (179))

Among the microRNAs that have consistently been associated with oncogenic properties, the miR17-92 cluster (i.e a group of microRNAs that are grouped in the same genomic locus and are usually transcribed together) has been shown to downregulate suppressors of phosphatidylinositol-3-OH kinase signaling, pro-apoptotic proteins like...
BCL-2 and tumor suppressor PTEN (181). Infection of murine hematopoietic stem cells with a retrovirus carrying the miR-17-92 cluster accelerated the development of lymphomas in Myc transgenic mice (182). Transgenic mice overexpressing miR-17-92 cluster in B cells were discovered to develop lymphoproliferative disease and autoimmunity (183). Interestingly, MYC directly activates the transcription of miR-17-92 polycistronic cluster and, given its oncogenic role, it may contribute to MYC-induced tumorigenesis (184). Similarly, miR-155 has been shown to be able to induce B-cell leukemia when overexpressed in the lymphoid compartment (185). miR-10b, which is highly expressed in metastatic breast cancer cells and positively regulates cell migration and invasion, and showed that it is directly regulated by Twist, a transcription factor critically involved in Epithelial-Mesenchymal Transition (186). An interesting feedback loop has been described in prostate cancer cells whereby EZH2 epigenetically silences the expression of several tumor suppressor microRNAs (miR-181, miR-124) that, in turns, are involved in downregulation of PRC1 components: thus the dysregulation of EZH2 in prostate cancer determines a concomitant upregulation of PRC1 by repressing microRNAs that normally keep PRC1 activation under control (187). To complicate the picture, it is evident that miRNAs can act as oncogenes or tumor suppressors depending on the cellular context in which they are expressed. This apparent paradox is in part explained by the fact that each microRNA targets several mRNAs, and its final effect as
oncogene or tumor suppressor depends on the specific set of mRNAs that it targets in the specific cellular context. MiR29, for example has been shown to target both DNMT3A and DNMT3B in lung tumors, thus exerting a tumor suppressor role, but, by targeting Tristetraprolin in breast cells it behaves as an oncogenic miR, inducing epithelial-mesenchymal transition and metastasis (188, 189).

In glioblastoma multiforme a specific signature of microRNAs de-regulation was initially described by Ciafre and colleagues by microarray analysis. The authors reported miR-125b, miR-128 and miR-181-a, b, c to be significantly downregulated in tumor tissue, while miR-21 and miR-10b to be upregulated (190). These results were successively validated and extended by other independent studies, which added miR-124 and miR-137 among the most consistently downregulated microRNAs in glioblastoma (191, 192). For all these a functional correlation to glioblastoma biology was then verified: miR-21 is an anti-apoptotic miRNA that was first identified as an antagonist of programmed cell death in glioblastoma cells. miR-21 levels were shown to be elevated in glioblastoma tissues, with knockdown of miR-21 resulting in caspase activation and apoptosis in vitro (193). Oligonucleotide inhibitors of miR-21 were also shown to increase apoptosis in a breast cancer xenograft model, an effect that was likely due to down-regulation of Bcl-2 and demonstrated that increased EGFR signaling due to a positive EGFR
mutational status could enhance expression of miR-21, suggesting that miR-21 is a regulatory target of EGFR (194).

miR-10b upregulation in glioblastoma was recently shown to be associated to downregulation of CDKN1A/p21, CDKN2A/p16 and BCL-2, an its downregulation in glioblastoma cells led to decreased growth potential \textit{in vivo} (195). Upregulation of miR-124 and miR-137 has been shown to decrease glioblastoma stem cell proliferation and to induce their differentiation towards a neuronal lineage, with resultant impaired tumor formation \textit{in vivo}. The authors also provided evidence showing promoter methylation as the major mechanism for their downregulation in glioblastoma (192). Interestingly, miR-124 has been directly linked to the repression of REST, a fundamental transcription factor involved in maintenance of undifferentiated neural stem cells and glioblastoma stem cells (196). Similarly, when re-expressed in glioblastoma cells, miR-125b has been shown to decrease the stem cell population and reduce cell proliferation by downregulating the E2F2 transcription factor (197). Finally, miR-128 has been initially shown by our laboratory to be implicated in the regulation of BMI-1, as explained above, a key factor for maintaining the stemness status in glioblastoma cells (191). Further studies have also shown its role in EGFR and PDGFRa downregulation (198) as well as suppression of E2F3a transcription factor (199).
1.8 miR-128

Mature miR-128 is encoded by two different genes, named miR-128-1 and miR-128-2 (www.ensembl.org). Although the DNA sequence of the two genes is different, the 80 nucleotide sequence encoding the final hairpin (i.e pre-miR-128) is the same, yielding the same mature miR-128 after processing by Dicer. In human cells, miR-128-1 maps to the long arm of chromosome 2 (q21.3) and is embedded in the sequence of the gene R3HDM1, within intron 9; miR-128-2 is located in the short arm of chromosome 3 (p22.3), within intron 18 of ARPP-21 gene. Although the expression of intronic miRs (that account for the majority of known microRNAs) has been shown to be linked to the transcription status of their own host genes (200-202), miR-128-2 has been shown to have independent transcriptional ability (203). The function of R3HDM1 has not been elucidated yet, while ARPP-21 has been described to be expressed in striatal dopaminergic neurons (204, 205). An analysis of their expression in GBM samples by TCGA (The Cancer Genome Database, http://tcga-portal.nci.nih.gov), shows that both of them are invariably downregulated in tumor.

Mir-128 was initially described in 2003 as one of few brain-enriched microRNAs, together with miR-124 and miR-9, and its expression was correlated with degree of cortical maturation in mouse embryos analyzed at different gestational ages as well as postnatally (206). Its role in neurogenesis was then confirmed shortly thereafter when it was found that its levels in embryonal carcinoma cells increased significantly after
administration of Retinoic Acid, a known inducer of neural differentiation (207). Smirnova et al. showed that miR-128 is preferentially expressed in primary neurons, while it is downregulated in astrocytes (208). The crucial role of miR-128 to neuronal differentiation has also been recently reported by Bruno et al, demonstrating how miR-128 downregulate expression of UPF1 and MLN51, two key proteins involved in Nonsense-Mediated RNA Decay in Neural Stem Cells, thus favoring expression of neuron-specific genes (209). Concomitantly, miR-128, together with other microRNAs, was found to be downregulated in glioblastomas (190), and several studies have shown its role as an anti-oncogenic microRNAs particularly in glioblastoma (191, 198, 199), but also in neuroblastoma (210). Finally, our laboratory has recently shown that miR-128 targets BMI-1 in glioblastoma cells and that restoring its expression inhibits tumor formation in vivo (191).

1.9 Rationale for this study

It is evident from the literature that Polycomb Proteins play a fundamental role in stemness determination, and also in the biology of cancer stem cells, including glioblastoma. It is also clear that a functional relationship exists among different Polycomb Repressive Complexes, and in particular between PRC1 and PRC2, towards
the final goal of repressing gene expression and keeping an undifferentiated cellular status. However, how the cell regulates the expression and interaction between these two complexes is unknown. The fact that PRCs are constituted by multiple subunits, that each one is essential for the function of the complex they belong to, and that the two complexes have often redundant functions suggests that PRC activity could be regulated by modulating the availability of its components and that, in order to obtain a meaningful effect in gene expression, the activity of both complexes needs to be modified at the same time. There are likely different strategies the cell uses to accomplish this, but a conceptually simple one would entail a regulating factor which is in common with the two complexes. In light of preliminary evidence that miR-128 targets BMI1 (PRC1) in the central nervous system (119), we hypothesize that mir-128 also targets other polycomb proteins associated to PRC2, and that miR-128 is a crucial regulator of the integrated activity of PRCs in the central nervous system, with the important implication that its loss would determine a deregulated activity of PRCs and, consequently, could represent a potential key event in gliomagenesis.

**Specific aim #1:**

**Defining the relationship between miR-128 and PRC regulation in glioblastoma**

- evaluate miR-128 targets among polycomb proteins constituting different PRCs
- characterize the relevance of miR-128-targeted polycomb proteins in glioblastoma
investigate the effect of miR-128 overexpression in glioblastoma cells and its role in regulating PRC regulation. the activity of PRCs

We hypothesize that miR-128 regulates expression of multiple Polycomb Proteins and that its overexpression in glioblastoma cells determines reduced activity of PRC1 and PRC2

Specific aim #2:
Investigating the role of miR-128 loss in the genesis of gliomas

- characterize the temporal pattern of miR-128 downregulation in glioblastoma
- investigate the association of miR-128 loss in Neural Stem Cells with target polycomb protein expression
- describe the proliferative phenotype of neural stem cells deprived of miR-128 expression

We hypothesize that loss of miR-128 at the Neural Stem Cell level is one of the factors leading to cellular transformation and generation of Glioma Initiating Cells, likely through unchecked activity of PRC1 and PRC2.
MATERIALS AND METHODS

**Human Specimens**

All tumor samples were obtained as approved by the Institutional Review Board at The Ohio State University. Surgery was performed by E.A. Chiocca and/or P. Peruzzi. For each patient, a sample of both tumor and brain devoid of gross tumor were resected, aliquotted, and processed for extraction of total RNA (Tryzol; Invitrogen) and whole cell protein lysate (Cell Lysis Buffer; Cell Signaling).

Tumor specimens were also used to isolate and establish patient-derived stem-like glioblastoma cells (Glioblastoma Initiating Cells) used in this study: briefly tissue was minced with forceps and transferred to ultra-low attachment flasks (BD Biosciences) in Stem Cell Medium, constituted with DMEM/F12/Glutamax (Invitrogen) supplemented with 2% B27 (Invitrogen), 1% N2 (Invitrogen) and 20 ng/ml EGF and FGF-2 (Peprotech). After several days at 37 °C, flasks were assessed for the formation of cellular aggregates, which were than isolated, dissociated with Tryple Express (Invitrogen) and further subcultured in Stem Cell Medium. After 3-5 passages cells were sorted for CD133-positivity by flow cytometry, expanded further and either used for experiments or stored in liquid nitrogen.
Cell culture

U87MG and U251MG glioblastoma cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM (Invitrogen) supplemented with 10% Fetal Calf Serum (Sigma). Primary human glioma stem like cell lines GSC84, GSC528, GSC718, GSC816, GSC1123, AC17, AC20 were obtained from freshly resected human malignant glioma specimens as described above (GSC84, 528, 718, 816 are Grade IV glioblastoma and GSC1123, AC17 and 20 are Grade III oligodendrogliomas) and grown in stem cell medium, as previously reported (18). Cells were grown as adherent monolayer in Poly-L-Ornithine and Laminin (Invitrogen)-covered dishes as previously described (218). Human fetal neural stem cells SCP27 and h16WF were a kind gift of Dr Kaspar and Dr Nakano, from Columbus Nationwide Children’s Hospital and The Ohio State University, respectively. They were grown in monolayer as above. Mouse Subventricular Zone (SVZ) cells were obtained by harvesting mouse brain after euthanasia, isolating SVZ region, dissociating tissue with Tryple Express (Invitrogen) and culturing as neurospheres in stem cell medium. Differentiation experiments were carried over by removing EGF and FGF-2 supplements and replacing with either 10% FCS or 2 μM All-Trans Retinoic Acid (ATRA) (Sigma) for 5 days to obtain astrocytic or neuronal differentiation, respectively (226).

Vector Construction:
miR-128 precursor

A DNA fragment containing the hsa-miR-128-1 locus situated at chromosome 2 (2q21.3) plus 500 bp upstream and 500 bp downstream from the 82 bp miR-128-1 precursor was amplified from U87MG genomic DNA and cloned into the pCDH-cGFP lentiviral vector (System Bioscience). Transducing virus particles were obtained by co-transfecting HEK293 cells with pCDH-miR-128-1 plasmid DNA with Lentiviral Plasmid Packaging Mix (System Biosciences) in the presence of Lipofectamine (Invitrogen) following manufacturer’s recommendations.

PCR primers used to amplify the miR-128-1 locus are: forward 5’ TATAGGCGCGCCACTGGAGTCAATGAAAGCAA and reverse 5’ CCCCCGCTAGC TAAGCAATAGCTTTTCACAAATT

Luciferase reporters:

A 468-bp fragment of the SUZ12 3’UTR, amplified from U87 genomic DNA, and containing the 2 target sequences for miR-128 was cloned into pMirTarget control vector (Origene) to obtain pMirTarget-SUZ12wt. The construct was then mutated using the QuickChange kit (Stratagene) in which the predicted miR-128 target sequences ACTGTGG and ACTGTGA were subsequentially substituted with AGTCAGG and AAAGGCT, to obtain pMir-target-SUZ12mut1 and pMir-Target-SUZ12mut1,2 respectively. A luciferase vector containing a 338-bp fragment of the Bmi-1 3’-UTR encompassing the target sequence for miR-128, and its mutated version were already
available from the prior work of Godlewski et al (191). All vectors were sequenced to confirm correspondence with predicted sequences. Primer sets (5’-3’) were as follows:

<table>
<thead>
<tr>
<th>VECTOR</th>
<th>FWD SEQUENCE</th>
<th>REV SEQUENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMir-Target-SUZ12wt</td>
<td>GCAGAAGTCAGAATCTCGACAGGTCAGGCACAGGTTACCTCGCTGTGAATACCAATAGG</td>
<td>CTAGATGGCGCGCCGCTGAAAGC TGCAGTTTCTCTTTCC</td>
</tr>
<tr>
<td>pmiR-Target-SUZ12mut1</td>
<td>GTTTATCTCTCAACCTCGACAGTCAGGTCAGGCACAGGTTACCTCGCTGTGAATACCAATAGG</td>
<td>GAGGTGATGTCTACACTCAACCTG ACTGCTCCGGAGTGGGAATACAAAC</td>
</tr>
<tr>
<td>pmiR-Target-SUZ12mut1,2</td>
<td>CTGATGGAGCTGGACTTTAACTGCTCTGAAAGGCTATTTAAACATCCAGTAAATCAG</td>
<td>ATTCGTTAAATAGCCITTTAATACAAACATCCAGTAAATCAG</td>
</tr>
</tbody>
</table>

Table 1.2 : PCR primers for amplification of wild type and mutated SUZ12 3’UTR sequences

Anti miR-128

A lentiviral vector carrying the inhibitory sequence for miR-128 (miRZip-miR-128-puro) was purchased from System Biosciences. miRZip-puro (negative control) was obtained by removing the anti-miR-128 sequence from the parental construct by restriction digestion, agarose gel DNA electrophoresis and plasmid subcloning. Correct removal of anti-miR-128 sequence was confirmed by DNA sequencing.

Nanostring
Total RNA was extracted using Tryzol (Invitrogen) and treated with RNase-free DNase (Qiagen) as we previously described. In order to identify microRNA that are specifically dysregulated in different subtype of GBM stem cells, we compared the microRNA expression patterns of 10 samples, characterized as two normal neural stem cells, four GBM stem cells mesenchymal type and four GBM stem cells proneural type. Nanostring microRNA microarray was used to search for unique gene signatures linked to GBM stem cells subtypes. Total RNA was used for the nCounter microRNA platform. Sample preparation and hybridization was performed according to the manufacturer’s instructions. All hybridization reactions were incubated at 65 °C for a minimum of 12 h. Hybridized probes were purified and counted on the nCounter Prep Station and Digital Analyzer (NanoString) following the manufacturer’s instructions. For each assay, a high-density scan was performed. For platform validation using synthetic oligonucleotides, NanoString nCounter microRNA raw data was normalized for lane-to-lane variation with a dilution series of six positive controls. The sum of the six positive controls for a given lane was divided by the average sum across lanes to yield a normalization factor, which was then multiplied by the raw counts in each lane to give normalized values. All MicroRNAs (or global microRNA expression data from the three type of cell) were used for visualization in a heat map and was analyzed by Principal component analysis (PCA) (198) using the dChip software with Statistical R package.
The array was perform at the Ohio State University Comprehensive Cancer Center Microarray, Nucleic Acids, Proteomic Shared Facilities with their technical assistance.

**Real-Time PCR**

Mature miR-128 expression analysis was carried out using the miR real-time PCR detection kit (Applied Biosystems) and following manufacturer’s protocol. qRT-PCR was performed using the Applied Biosystems Step One Plus PCR apparatus. U6 snRNA was used as endogenous control. Of note, miR-128 and U6 probe sequences were identical for both human and mouse transcripts. cDNA for RT-PCR was synthesized using iScript (BioRad); mRNA expression analysis was carried out using Power SYBR Green (Applied Biosystems). Human primers used are visualized in Table 1.3 (5’-3’).

<table>
<thead>
<tr>
<th>GENE</th>
<th>FWD SEQUENCE</th>
<th>REV SEQUENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI1</td>
<td>GGAGACCAGCAAGTATTGTCCATTT</td>
<td>CATTGCTGCTGTGGGCATCGTAAG</td>
</tr>
<tr>
<td>PHC2</td>
<td>AGCACTTCAAGCAATGTG</td>
<td>TGCTGCAGAGTTCACTCT</td>
</tr>
<tr>
<td>CBX7</td>
<td>ATGGAGCTGTCAGCCATC</td>
<td>GGCCATCTTTCCACTTC</td>
</tr>
<tr>
<td>SUZ12</td>
<td>GAGATGACCTGCATTGCCCTT</td>
<td>CCAGGTTGGCGATGATATCC</td>
</tr>
<tr>
<td>EZH1</td>
<td>ACAGCCTTAAGCAAATATGG</td>
<td>TGACTGAACAGGTTGGACACG</td>
</tr>
<tr>
<td>EZH2</td>
<td>TACTTGTGGAGCCGCTGAC</td>
<td>CTGCCACGTGAGATGGT</td>
</tr>
<tr>
<td>18s RNA</td>
<td>AACTTTCGATGGTAGTGCGCCG</td>
<td>CCTGGATGTGTTAGCCGTTT</td>
</tr>
</tbody>
</table>

*Table 1.3: primers for quantitative Real Time PCR*
**Cell studies**

MicroRNAs precursors (both miR-128 and negative control oligonucleotides) were purchased from Ambion and transfected into cells at a final concentration of 50 nmol/L using Lipofectamine 2000 (Invitrogen) and following the manufacturer’s instructions. Human BMI1, SUZ12 and negative control siRNAs were purchased from Quiagen and transfected into cells at a final concentration of 50 nmol/L, using Lipofectamine 2000 as above.

Similarly, luciferase reporter plasmid DNA was transfected into HEK 293 cells with Lipofectamine 2000. Five hundred nanograms of purified DNA were used for each 12-well dish.

To create stable GBM cell lines expressing miR-128, U87MG and G528 were infected with pCDH-128-1-cGFP or pCDH-cGFP and allowed to grow for 7 days. Cells stably expressing transgene were then sorted by GFP expression through flow cytometry (FACSaria III cell sorter, Beckton Dickinson).

Western Blot analysis was carried out with whole cell protein lysate. Antibodies used were: Bmi-1, GFAP, Nestin (Millipore), Suz12, p21Waf/Cip1, H2aK119-Ub, H3K27-me3, H3, Tuj-1 (Cell Signaling), α-Tubulin (Sigma), and CD133 (Amersham). Horseradish Peroxidase-conjugated secondary antibodies to rabbit or mouse IgG (Amersham) were then used for blotting.
Cell proliferation after miR-128 expression was quantified either by manual counting after cell dissociation or by the Click-it Edu cell proliferation assay Kit (Invitrogen). Briefly, for cell counting, 150,000 cells were plated on day 0 into 6 cm well-plates, transfected on day 1, and counted 4 days after transfection. For Edu studies, 10,000 cells were seeded into 48-well plates and let recover overnight. The following day, Edu was added to the medium to a final concentration of 10 μM. Cells were returned to the incubator for 3 hours before they were fixed with 4% PFA and Edu incorporation assessed following the manufacturer’s protocol.

For the clonogenic assay cells were dissociated with Tryple Express and plated with complete stem cell medium mixed with 0.4% low gelling temperature agarose (Sigma) pre-warmed at 37 degrees into 6-well dishes over a layer of solidified 0.8% agarose. After allowing cells to settle down to the 0.8%/0.4% agarose interface, the dishes were kept at room temperature for 10 minutes to allow the medium to jellify before placing the plates back at 37 degrees for culturing. Fresh stem cell medium was then used to cover the agarose and changed every 3 days.

For DNA damage and response experiments, cells were irradiated with 2 to 4 gray x-rays from an RS-2000 Biological Irradiator (Rad Source, Suwanee, GA). Quantification of apoptosis and necrosis was carried out with the FITC Annexin V/Dead Cell Apoptosis Kit (Invitrogen) using the FACScalibur machine (Becton Dickinson). Briefly, 150,000 cells were plated into 6 cm dishes and let recover overnight. The following day cells were
transfected with 50 nmol/L miR-128 or negative control oligonucleotides. After 36 hours, cells were irradiated with 2 or 4 Gy (except negative control samples) and returned to the incubator for additional 48 hours, at which time they were collected for analysis following manufacturer’s protocol.

Evaluation of DNA repair was performed using the Comet Assay Kit (Trevigen) according to the manufacturer’s protocol. Briefly, 100,000 cells were plated into 6-well-plate dishes and let recover overnight. The following day cells were transfected with 50 nmol/L miR-128 or negative control oligonucleotides and let grow for 36 hours. Cells were then either left untreated or irradiated with 2 Gy and harvested at 30 minutes and 15 hours after exposure to ionizing radiations. For each sample, 500 dissociated cells were then embedded into agarose and fixed onto glass coverslips for quantification of DNA damage.

**Immunohistochemistry**

Human operative specimens were fixed in formalin and embedded in paraffin before being sectioned at 4 μM thickness. The Ventana Benchmark System (Ventana Medical System) was used for staining. Expression of BMI-1 and SUZ12 were optimized at 1:500 and 1:250 dilution, respectively. Each sample was counterstained with Hematoxylin/Eosin.
**Statistical analysis**

Results are expressed as mean ± s.d., and differences were compared using the 2-tailed Student's *t*-test. Statistical analyses were performed using Microsoft Office Excel 2010 software (Microsoft Corp.). *P* values less than 0.05 were considered statistically significant and indicated with asterisks (*) in the figures.
Introduction: why miR-128?

The decision to study miR-128 in the setting of glioblastoma multiforme was mainly due to the results of a microarray showing a significant downregulation of miR-128 in tumors compared to matched (i.e. from the same patient) adjacent brain devoid of gross tumor. This, together with the known role of miR-128 in neuronal fate determination, suggested an interesting correlation between miR-128 loss and glioblastoma. In the same array, other microRNAs significantly downregulated in tumor were miR-124, miR-137, miR-218, miR-139, miR-323, and miR-483 (Table 2.1).
<table>
<thead>
<tr>
<th>microRNA</th>
<th>Fold change (tumor/brain)</th>
<th>SAM score *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsa-miR-124a</td>
<td>0.04</td>
<td>- 6.05</td>
</tr>
<tr>
<td>Hsa-miR-137</td>
<td>0.06</td>
<td>- 5.34</td>
</tr>
<tr>
<td>Hsa-miR-323</td>
<td>0.09</td>
<td>- 4.11</td>
</tr>
<tr>
<td>Hsa-miR-139</td>
<td>0.04</td>
<td>- 3.46</td>
</tr>
<tr>
<td>Hsa-miR-218</td>
<td>0.09</td>
<td>- 3.25</td>
</tr>
<tr>
<td>Hsa-miR-128-2</td>
<td>0.10</td>
<td>- 3.24</td>
</tr>
<tr>
<td>Hsa-miR-483</td>
<td>0.13</td>
<td>- 2.84</td>
</tr>
<tr>
<td>Hsa-miR-128-1</td>
<td>0.14</td>
<td>- 2.36</td>
</tr>
<tr>
<td>Hsa-miR-299</td>
<td>0.13</td>
<td>- 2.29</td>
</tr>
<tr>
<td>Hsa-miR-511-1</td>
<td>0.20</td>
<td>- 1.51</td>
</tr>
<tr>
<td>Hsa-miR-190</td>
<td>0.25</td>
<td>- 1.14</td>
</tr>
<tr>
<td>Hsa-miR-383</td>
<td>4.53</td>
<td>2.64</td>
</tr>
<tr>
<td>Hsa-miR-519d</td>
<td>5.90</td>
<td>2.59</td>
</tr>
<tr>
<td>Hsa-miR-21</td>
<td>28.01</td>
<td>2.20</td>
</tr>
<tr>
<td>Hsa-miR-26a</td>
<td>4.84</td>
<td>1.22</td>
</tr>
<tr>
<td>Hsa-miR-10b</td>
<td>5.26</td>
<td>1.19</td>
</tr>
<tr>
<td>Hsa-miR-486</td>
<td>12.03</td>
<td>1.12</td>
</tr>
</tbody>
</table>

Table 2.1: MicroRNAs alteration in glioblastoma multiforme. Comparison of miR expression between glioblastoma samples and adjacent brain devoid of tumor from the same patients (n=3). * SAM (Significance Analysis of Microarray) score identifies genes with statistically significant changes in expression: each gene is assigned a score based on its change in gene expression relative to the Standard Deviation of repeated measurements for that gene. Genes with scores greater than 1 (in absolute value) are deemed potentially significant. Modified from Godlewski et al., 2008 (191)
Among these, miR-124, miR-137 and miR-483 have been shown to impair growth of glioblastoma cell lines and to induce differentiation of GIC (192, 211) while miR-218 has been demonstrated to inhibit glioma invasion (212). Loss of miR-139 has been associated to colorectal cancer progression (213). Interestingly, according to in silico analysis (www.targetscan.org, www.microrna.org) each of these microRNAs were predicted to target at least one component of either PRC1 or PRC2, suggesting that regulation of Polycomb Repressor Complexes by microRNAs is crucial in glioblastoma pathology. In fact, our group had previously linked miR-128 to BMI1 downregulation and antineoplastic effect in glioblastoma cells (191). Interestingly, upon further analysis, we noticed that miR-128 and miR-124 were potentially able to target both complexes at the same time: in fact, miR-128 was predicted to target PHC 2 and BMI1 within PRC1 and EZH1 and SUZ12 within PRC2, while miR-124 could potentially suppress CBX7 of PRC1 and EZH2 of PRC2 (Figure 2.1).
Figure 2.1: Predicted miR-124 and miR-128 targets within the components of PRCs: targets of miR-124 are color-coded in violet, while miR-128 targets are in yellow.

With the exception of miR-128 targeting BMI1 in glioblastoma, there were no studies confirming the predicted actions of those two microRNAs on their targets. This observation led to the hypothesis that microRNAs could provide a crucial mechanism controlling and integrating the function of the two Polycomb Complexes, since, as it has been discussed above, the two complex can function both independently and cooperatively to achieve gene silencing. Since there was no evidence in the literature for relevance of these target proteins to glioblastoma biology, with the exception of BMI1
and EZH2, we next analyzed whether each putatively targeted polycomb protein was likely to be relevant in glioblastoma by querying the TCGA database for their differential expression in tumor versus brain. It showed that while the majority of polycomb proteins are overexpressed in glioblastoma, a subset of them, like CBX2, CBX6, CBX7 and EZH1 are actually downregulated (figure 2.2).

Figure 2.2: Differential expression of Polycomb genes in glioblastoma multiforme. Analysis of TCGA-based expression profile of single polycomb genes in glioblastoma multiforme versus normal brain in the 600 available patients. The percentage of patients in whom that gene is at least 0.5 log overexpressed in tumor than in normal brain is shown for each polycomb gene. Consistently with figure 2.1, polycomb genes targeted by miR-124 are in violet, while genes targeted by miR-128 are in yellow.
Evidently, BMI1, PHC2, SUZ12 and EZH2 appeared to be overexpressed in glioblastoma. Contrarily, CBX7 and EZH1 are strongly downregulated. Assuming that both miR-124 and miR-128 would determine a downregulation of their target mRNA, and that both microRNAs have been described as tumor suppressors, we reasoned that most likely, at least in glioblastoma, the role of miR-124 controlling CBX7 and miR-128 controlling EZH1 was irrelevant. With this rationale, we excluded miR-124 as a potential regulator of both PRC1 and PRC2, as the only polycomb gene that seemed to be potentially affected by miR-124 loss in glioblastoma was EZH2. That left us with miR-128 targeting three different polycomb genes, two of which (BMI1 and PHC2) are components of PRC1 and one (SUZ12) component of PRC2, all of which, importantly, appear to be upregulated in glioblastoma.

To further confirm this, we performed quantitative Real Time PCR in 9 human matched specimen to analyze the expression of BMI1, PHC2, CBX7, SUZ12, EZH2 and EZH1 in glioblastoma versus surrounding brain devoid of tumor, and we found a complete correlation with the data obtained from the TCGA database (Figure 2.3).
Figure 2.3: Expression of polycomb genes in matched operative specimens. Relative expression of selected polycomb genes in GBM versus surrounding brain devoid of gross tumor in matched samples obtained from 9 patients. * = p<0.05; ** = p<0.01

Altogether, this was suggestive of a fundamental role for miR-128 in controlling the activity of both PRC1 and PRC2 within the central nervous system, thus providing the basis for our hypothesis that the two complexes could be regulated in a coordinated fashion by a single microRNA.
In fact, during our preliminary search, microRNAs miR-200b and miR-200c also appeared to have a profound action on PRC regulation (by targeting several polycomb transcripts), but their expression was not altered in our microarray (nor in other microarrays performed by other groups (214)) and, overall, there was no evidence in the literature that those two microRNAs are relevant to glioblastoma and CNS physiology. Mir-200b/c have been extensively studied for their role in epithelial-mesenchymal transition in a number of solid tumors (breast, prostate), and they have also been shown to downregulate BMI1 in pancreatic cancer cells (215) and SUZ12 in breast cancer cells (107). Interestingly, in our preliminary experiment we did not observe effects on BMI1 or SUZ12 protein level after miR-200b overexpression in glioblastoma cell lines (data not shown), confirming that these miRs are not relevant to the GBM model and leaving us with miR-128 as the best candidate microRNA supporting our research hypothesis.

**MiR-128 is downregulated in glioblastoma**

To validate the preliminary findings obtained from the microarray, operative specimens from 9 patients who underwent surgery for resection of primarily diagnosed GBM, i.e. with tumors that had never been treated before, and confirmed at histopathological analysis, were collected at the Ohio State University Medical Center and immediately processed for RNA and protein extraction. For each patient, one fragment of tumor and one fragment of grossly normal brain surrounding the resection cavity were obtained.
MiR-128 expression was then analyzed and compared for each pair of matched samples by quantitative Real Time PCR. The average miR-128 Relative Quantification (tumor Vs surrounding brain) analysis for the nine patients is shown in figure 2.4, demonstrating a significant and profound downregulation of miR-128 in GBM.

**Figure 2.4: Relative quantification of miR-128 expression in glioblastoma versus surrounding brain.** The panel on the left show the results for each tested patient, while the graph on the right provides an average of the nine results. ** = P<0.01

TCGA data was then analyzed to compare overall survival of patients whose gbm had higher-than-average levels of miR-128 with those who had tumors with lower-than-
average miR-128 expression: this revealed that higher expression of miR-128 was positively correlated to better survival (figure 2.5)

Figure 2.5: Association of miR-128 expression with patient survival.
Data obtained from The Cancer Genome Atlas. Up-regulated n=21; down-regulated n=20; intermediary n=155. Up vs. down-regulated $P=0.0308$; up vs. intermediary $P=0.0018$; down vs. intermediary $P=0.8322$
BMI1 and SUZ12 are upregulated in glioblastoma and are important in its biology

We have already shown that BMI1 and SUZ12 are overexpressed in glioblastoma at the mRNA level (figures 2.2 and 2.3). To confirm the finding that BMI-1 and SUZ12 are overexpressed in glioblastoma, we analyzed the relative protein expression in six human specimen available to us. Of note, although we had shown that PHC2 expression was also upregulated in glioblastoma, we did not pursue further studies with PHC2, since our primary goal was to evaluate the dual effect of miR-128 on PRC1 and PRC2, so we decided to proceed with one target per each complex. Furthermore, there was no convincing evidence in the literature associating PHC2 with cancer, and no described relationship to glioblastoma or brain development in particular. Finally, since PHC2 can be replaced by PHC1 or PHC3 in different PRC1 complexes, it is likely that its downregulation would not affect the overall function of the complex to a great degree.

Three randomly selected matched specimens were collected and for each of them whole cell lysate was obtained after removal of red blood cells. The protein lysate was then used to perform Western blot analysis (figure 2.6). In all three patients the amount of BMI1 and SUZ12 were convincingly higher in tumor than it was in surrounding brain. α-Tubulin was used as a loading control.
Figure 2.6: BMI1 and SUZ12 are overexpressed in glioblastoma. Western blot analysis for BMI1 and SUZ12 protein levels in three representative operatory specimens.

For further confirmation, another three, formalin-fixed, paraffin-embedded samples of proven GBM were obtained from the Department of Pathology at the Ohio State University and immunohistochemistry was performed, looking for differential expression of BMI1 and SUZ12. Below is shown a representative sample where, in the same tissue slice it was possible to compare the expression level of each protein both in the tumor and in the surrounding, normal-appearing, brain. In this case too, 3/3 specimens demonstrated higher level of BMI1 and SUZ12 expression in tumor (figure 2.7).
Figure 2.7: Overexpression of BMI1 and SUZ12 proteins in operative specimens
Immunostaining for BMI1 and SUZ12 in a representative sample of glioblastoma and matched surrounding brain (upper panel). The number of positive cells/field is rendered in the lower panel (n=3 patients)
While there is already experimental evidence that BMI1 is important for the proliferation and self renewal of glioma initiating cells (191,198), less is known about the role of SUZ12. As described above, SUZ12 upregulation has been linked to the origin of breast cancer stem cells, and there are several studies showing how SUZ12 is an essential factor for the functioning of PRC2 (34,107). For this reason we decided to assess the effect of single knock down of either BMI1 or SUZ12 in glioblastoma initiating cells G528, analyzing the effect of knock down both phenotypically (i.e. cellular proliferation), as well as molecularly, by measuring the expression level of p21, a validated key downstream target of both PRC complexes (72,78,238). Also, the level of each histone modification dependent on the respective PRC was evaluated. To this end, G528 cells were seeded in triplicates onto 6 cm dishes at a plating number of 100,000/plate and let grow for 4 days after transfection of siRNA oligonucleotides. The cells were then dissociated and counted (fig A). Protein lysate was then obtained from the same specimen and analyzed for the amount of p21, as well as for ubiquilated H2A and trimethylated H3 (fig B). As shown, downregulation of both BMI1 and SUZ12 determined a significant decrease in cell proliferation rate, paralleled by overexpression of p21 and the expected decreased level in ubiquilated H2A after BMI1 knock down and trimethylated H3 after SUZ12 knock down. All this suggests that downregulation of either BMI1 or SUZ12 exerts an antitumoral effect, at least partially by de-repressing p21, a
key gene involved in cell cycle control, and confirms that both BMI1 and SUZ12 are plausible targets in the miR-128-mediated anti-neoplastic effect.

Figure 2.8: BMI1 and SUZ12 are pro-tumorigenic proteins in glioblastoma. Panel A shows that proliferation of G528 cells is impaired after isolated knock down of either BMI1 or SUZ12 (n=3). Panel B represents a Western blot analysis from transfected G528 cells (48 hours after transfection), showing the efficacy of siRNA-mediated downregulation of either BMI1 or SUZ12 and the resulting effect on the levels of p21 as well as of histone modifications. The bar-graph on the right side represent densitometric quantification related to the histone modification blot (n=2).
Very interestingly, while downregulation of BMI1 and SUZ12 determined, as expected, decreased levels of H2Ak119-Ubi and H3k27me3, respectively, we observed an increase in BMI1 levels after SUZ12 downregulation (but not vice versa), associated also to increase in H2Ak119Ubi. This suggested the possibility that, indeed, downregulation of PRC2 result in a compensatory upregulation of BMI1 and PRC1 activity at large. This has the important implication that in order to achieve a complete effect on the activity of PRCs, both of them need to be downregulated at the same time.

Indeed, after repeating the experiment in different glioblastoma cell lines, we obtained similar results, showing that BMI1 is consistently upregulated after knock down of SUZ12 (figure 2.9a). A possible explanation for this observation comes from the analysis of Chip-Seq data available at http://genome.ucsc.edu, and showing that there are several SUZ12 binding sites in the close proximities of the BMI1 Transcription Starting Site, justifying how the decreased level of SUZ12 could determine an increase in BMI1 expression (Fig 2.9c).
Figure 2.9: Knock down of SUZ12 results in upregulation of BMI1. Panel A shows Western blot analysis of BMI1 and SUZ12 of three different glioblastoma cell lines after transient transfection with siRNA anti BMI1 and SUZ12, respectively. Panel B represents a densitometric quantification of the blots in A. * represents $p=0.02$; **= $p<0.01$. Panel C shows the location of SUZ12 binding sites at the BMI1 Transcription Starting Site (modified from http://genome.ucsc.edu)
Upon review of the literature, we found that our observation had already been partially documented by Bracken and colleagues, who reported upregulation of BMI-1 at the transcriptional level after knock down of any of the three main components of PRC2 (EZH2, SUZ12 and EED)(30). On the contrary, PRC2 activity appears to be independent from that of PRC1, as it has been described elsewhere (239). In any case, this suggests that not only are PRC1 and PRC2 functionally related for the task of gene silencing (i.e. the fact that PRC1 preferentially targets genes which have already been “marked” by PRC2), but that there are specific cellular mechanisms keeping the two complexes in balance, and one of these possibly being miR-128, with its predicted ability to target both PRC1 and PRC2 at the same time.

**MiR-128 downregulation in glioblastoma happens in the GIC sub-population**

As discussed earlier, miR-128 is expressed at progressively higher levels in CNS during embryo development, concomitantly with brain maturation as it has been demonstrated comparing expression from brain homogenates at different embryonic stages (206). This has been confirmed at the cellular level, whereby miR-128 amounts expressed at the Neural Stem Cell/Neuroprogenitor levels are comparatively lower than in mature neurons. Also, miR-128 expression is negligible in astrocytes (208), strongly suggesting that miR-128 is a “neuronal” microRNA.
This raises the question whether the lower miR-128 expression we observed in GBM is due to an intrinsic inability of tumor cells to produce miR-128 or, more simply, is due to the fact that glioblastoma being a tumor of essentially glial lineage, miR-128 is irrelevant to its physiology, i.e. its expression had already been lost as a result of the glial lineage specification.

To address this question, we performed a microarray for microRNAs expression to compare the baseline expression of miR-128 in normal human fetal neuroprogenitors (SCP27 and h16wf) with the levels expressed in glioma initiating cells obtained from different operative specimens and cultured under the same conditions. The rationale for this approach was to verify that tumor cells have a lower expression of miR-128 independently from their differentiation status. Interestingly, we observed that miR-128 was among the top four most differentially expressed microRNAs when comparing non-malignant NSC to GIC. Furthermore, the signature of these four miRs (including miR-128) distinguished (with one exception) between proneural and mesenchymal GSCs (figure 2.10), whereby proneural cells tend to have higher levels of miR-128 compared to mesenchymal.
Figure 2.10: miR-128 is downregulated at the stem cell level in glioblastoma and distinguishes GIC subtypes. Heat map showing the top 4 down regulated (more than 2 fold) microRNAs based on Nanostring array on 10 cell lines. P <0.05. N=NSC; M=Mesenchymal GIC; PN=Proneural GIC

The screening results were confirmed by qRT-PCR analysis of RNA obtained from the lysate of two cell lines for each group, underlining the significant difference in miR-128 expression between NSCs and GIC (figure 2.11), and suggesting that miR-128 expression, far from being relevant only at the differentiated, neuronal, stage, plays a role also in the maintenance of “healthy” neural stem cells.
Figure 2.11: miR-128 is downregulated in GIC respect to NSC. Quantitative Real Time PCR shows that the relative amount of miR-128 in GIC is consistently less than it is in neural stem cells (N = Neural Stem Cells, PN; PN= GIC of Proneural subtype; M= GIC of Mesenchymal subtype). For each group, two different cell lines were used.

A possible criticism to this approach is that the difference in miR-128 expression could be related to the fact that the “normal” NSC were of fetal origin, while those derived from glioblastoma were derived from adult, postnatal tissue. Another source of possible bias is that the genetic background within patients is not necessarily homogeneous, and
that could also explain, or contribute to, the difference in observed miR-128 expression at the cellular level.

To prove or disprove our hypothesis that miR-128 expression is dysregulated at the stem cell level in Glioma Initiating Cells, we reasoned that we should implement a model where all cells would be derived from the same area of the brain, from age-matched samples, passaged in culture for the same amount of time and number of passages, under the same conditions and with homogeneous genetic background. To accomplish this, we took advantage of a mouse model of spontaneous gliomagenesis (hereafter called mut-3 mice) which was kindly provided by Dr Kwon at the Ohio State University. As previously described, these mice harbor a p53 germline heterozygosity together with somatic heterozygosity of Nf1, a negative regulator of the Ras pathway, driven in neural cells by GFAP-cre. The resulting animals thus only carry one p53 and one NF1 locus and, overtime, due to spontaneous loss of heterozygosity (LOH), they lose expression of both genes, inducing glioma formation by age 25-30 weeks (216).

Subventricular Zone (SVZ) cells from control mice and GIC from intracranial tumors developed in mut-3 mice were then isolated. Cells were cultured as spheroids in stem cell medium, and after three passages they were dissociated and total RNA was obtained for quantification of miR-128 expression. With this strategy we were able to equalize the cells as far as number of passages in vitro, their origin from adult brain and genetic background.
The results were in agreement with those obtained from human cells, confirming that expression of miR-128 is decreased in stem cells that derive from tumor as compared with those obtained from normal brain (figure 2.12).

**Figure 2.12: miR-128 is downregulated at the stem cell level in mouse GBM.** Relative quantification of miR-128 expression by Real Time PCR comparing expression in SVZ cells obtained from wild type animals versus GIC obtained from mut-3 animals after the development of brain tumor. **= p<0.01
In GIC miR-128 expression cannot be induced upon differentiation stimuli

Normal neuroprogenitor cells usually undergo differentiation into neurons, astrocytes or oligodendrocytes when growth factors are removed and upon administration of either serum, retinoic acid or IGF-1(217). To some extent, GIC have been show to undergo similar changes, although the differentiation process is usually incomplete and not completely predictable (218).

To further prove that decreased miR-128 levels in glioblastoma derive from an impaired ability of GIC to express miR-128, we hypothesized that the difference in miR-128 expression among normal neuroprogenitors and GIC would increase after cells are induced to differentiate. SCP27 and G528 cells were grown either under stem cell condition or in presence of serum (astrocytic differentiation) or Retinoic Acid (neuronal differentiation) for 5 day. Then cells were harvested, total RNA was collected and analyzed for miR-128 expression.

As expected, SCP27, upon serum challenge, assumed an astrocytic phenotype, with loss of Nestin expression and induction of GFAP, but no β3-tubulin. Concomitantly, miR-128 expression decreased by half compared to the baseline level of the undifferentiated state. In the presence of Retinoic Acid, however, there was a two-fold induction of miR-128 expression, which went in parallel with expression of neural marker β3-tubulin (figure 2.13). The higher amounts of GFAP observed during retinoic acid-induced neural differentiation indicates that most likely the differentiation process was not
completed by the time of harvesting, since β3-tubulin, although specific, is a marker of relative early stages of neuronal differentiation (240), and GFAP upregulation is common after retinoic acid challenge of neural precursors and before the terminal phase of neuronal specification (241).

Figure 2.13: **miR-128 expression during neural stem cell differentiation.** Human fetal Neural Stem Cells SCP27 are able to undergo differentiation when challenged with the appropriate stimuli: after 5 days in presence of 10% Fetal Bovine Serum (FBS) Nestin disappears and cells produce GFAP but not β3Tubulin, which is a neural marker, and which only appears after culturing with 2 μM Retinoic Acid (panel A). In the same conditions, miR-128 expression is downregulated when NSC are induced to become astrocytes, but upon neuronal differentiation miR-128 becomes overexpressed (panel B). ** = p<0.01

Contrarily, the level of miR-128 in G528 or OG02, another GIC line, did not change significantly in either differentiation conditions (fig 2.14)
Figure 2.14: miR-128 expression during GIC differentiation. Quantitative Real Time PCR showing miR-128 level changes in different "differentiation" conditions for two GIC lines G528 and OG02: Upon the same conditions used for differentiation of NSC SCP27 (shown in figure 2.11), it is evident that Glioma Initiating Cells do not display the physiologic regulation of miR-128 expression upon astrocytic or neuronal differentiation, as the baseline levels of miR-128 of the stem cell condition do not change with differentiation stimuli.

MiR-128 expression is inversely correlated to BMI-1 and SUZ12 at the cellular level

After verifying that miR-128 expression is impaired at the cellular level in glioblastoma, we sought to determine whether this associated to a different expression level for BMI-1 and SUZ12, reasoning that, if miR-128 is important for BMI-1 and SUZ12 regulation, an inverse correlation should be observed, and expecting a result similar to the inverse correlation observed between miR-128 and BMI1/SUZ12 in patient specimens.
As above, in order to limit bias and confounding factors we employed our animal model of spontaneous gliomas and obtained protein lysate from either wild-type SVZ cells or GIC obtained from mut-3 mice. Western Blot analysis for BMI-1 and SUZ12 was then performed and results are shown below in figure 2.15. As anticipated, BMI-1 and SUZ12 resulted to be expressed at a higher level in GIC cells (roughly twice as much) compared to the level seen in normal SVZ cells. This confirmed an inverse correlation between miR-128 expression and BMI-1 and SUZ12 levels.

Figure 2.15: BMI1 and SUZ12 protein levels in mouse SVZ cells versus GIC. The right panel shows the relative quantification of protein amount calculated on the average densitometry of the three cell lines reported in the Western blot on the left. α-Tubulin was used as loading control. * = p<0.05; ** = p<0.01
**MiR-128 downregulates both BMI-1 and SUZ12**

Now that a strong inverse correlation between miR-128 and BMI1/SUZ12 was described, both at the histological and at the cellular levels we next sought to test whether a causal relationship exists between miR-128 and BMI-1/SUZ12, i.e whether artificially manipulating the levels of miR-128 in glioblastoma cells would result in a change of BMI1 and/or SUZ12 protein amount. To accomplish this we proceeded with overexpressing miR-128 in glioma cell lines (U87MG and U251) as well as in primary GIC G528.

As a first approach, we transfected cells with miR-128 precursor oligonucleotides or scrambled negative control oligonucleotides, and, after 48 hours, cells were harvested and whole cell lysate was obtained for Western blot analysis. As shown in figure 2.16, the level of protein expression for both BMI-1 and SUZ12 was significantly reduced after miR-128 overexpression as compared to controls.
Figure 2.16: Transient miR-128 overexpression induces a decrease in BMI1 and SUZ12. Upper panel shows representative Western blots from two glioblastoma cell lines (U251 and U87) as well as from one GIC (G528) after being transfected with either scrambled, non-relevant control microRNAs (NC) or miR-128 oligonucleotides. Cells were harvested 48 hours after transfection. The bottom panel shows densitometric quantification of blots (averaged on three experiments/cell line). *= p<0.05; **=p<0.01
One caveat to results obtained with miR oligonucleotide transfections is that usually they increase the cellular amount of microRNA to the level that is likely above the physiologically relevant range. So, to further validate these results and observe if overexpression of miR-128 closer to its endogenous, physiological, levels would still be able to produce an effect on BMI-1 and SUZ12 expression, we created U87MG and G528 cell lines stably overexpressing miR-128. This was obtained by infecting cells with replication-incompetent lentiviral vectors retrofitted with a 500 bp genomic DNA sequence encompassing miR-128 precursor under the control of CMV promoter, and also constitutively expressing Green Fluorescent Protein (GFP). Control cell lines were created by infection with empty lentiviral vector, i.e only expressing GFP but no miR-128 precursor. Transduced cells were then selected by GFP expression in a flow-cytometer and expanded in culture for three passages before being utilized for experiments. Then cells were harvested and both total RNA and proteins were obtained.

To confirm the functionality of our miR-128 transgene, mature miR-128 expression was quantified by Real Time PCR and compared to baseline expression in control cells: as shown in figure 2.17, mature miR-128 expression was increased by about 10-15 times, close to a level usually observed in normal cells.

The same cells were then analyzed for BMI-1 and SUZ12 expression by Western blot, and, as shown in figure 2.17, in both cases, “physiologic” overexpression of miR-128 consistently reduced the expression of targeted proteins.
Figure 2.17: Stable expression of miR-128 decreases BMI1 and SUZ12. Lentiviral-mediated overexpression of miR-128 determines physiologic increase in mature miR-128 levels and results in downregulation of BMI1 and SUZ12 proteins, both in GBM cell line U87 and in GIC G528. GFP = cells transduced with lentiviral vector only carrying GFP transcript and devoid of miR-128 sequence; GFP-miR-128= cells transduced with lentiviral vector overexpressing both GFP and miR-128. ** = p<0.01

BMI-1 and SUZ12 are direct targets of miR-128

By overexpressing miR-128 to “physiological” levels by lentiviral vectors we have shown that the observed effect on polycomb targets is a genuine cellular response to miR-128 expression, and not the result of nonspecific disruption of cellular biology by
miR-128 “flooding”. However, because of the multitude of predicted miR-128 targets, the question was then raised whether the observed downregulation of BMI1 and SUZ12 was specific or whether it was secondary to the effect of miR-128 on other, unrecognized transcripts, causing a decrease in gene transcription.

To address this question, a luciferase reporter vectors were made, so that the DNA sequence containing the predicted miR-128 target sites within the 3’ UTR of BMI1 or SUZ12 genes were cloned downstream to a firefly luciferase cDNA driven by a constitutively active promoter (SV40). Such vectors, once transfected into cells, produce luciferase at a constant amount, which is assumed to be independent from other cellular process, and whose amount can be quantified by its luminescence. If miR-128 really targets the RNA sequence (either BMI1 or SUZ12 predicted target sequences) cloned downstream to the luciferase gene it will bring about a decrease in luciferase protein and, as a consequence, a decrease in observed luminescence. Contrarily, if the observed decreased amount of BMI1 and SUZ12 proteins after miR-128 overexpression that has been described above, is secondary to mechanisms other than 3’UTR-mediated targeting, the amount of observed luciferase is not expected to change after miR-128 overexpression. Also, as a final proof that miR-128 downregulation is really mediated by its interaction with the 3’ UTR, mutations to the 6-8 nucleotides constituting the “seed” sequence predicted to be recognized by miR-128 should result in abrogation of downregulation. In figure 2.18 we show that, in fact, Human Embryonic Kidney (HEK)
293 cells, that usually express a very low level of endogenous miR-128, upon cotransfection with the luciferase reporter plasmid and either miR-128 or negative control oligonucleotides, show a marked reduction of luciferase expression only when miR-128 is concomitantly overexpressed. Importantly, after mutation of the predicted BMI-1 and SUZ12 target sequence(s), miR-128 is no longer able to downregulate luciferase expression. While BMI1 3’UTR has only one miR-128 binding site, SUZ12 3’UTR has two of them, approximately 200 bp apart (Figure 2.18 A).

Interestingly, the two sites in the SUZ12 UTR seems to have an additive function, as mutation of both sites results in complete abrogation of miR-128 effect on SUZ12, while mutation of only one site exerts only a partial decrease of miR-128 effect (Figure 2.18 B). Altogether, this proves that miR-128 specifically targets the predicted 3’UTR sequences in the BMI-1 and SUZ12 transcripts, and that the observed downregulation of BMI1 and SUZ12 upon miR-128 overexpression is indeed secondary to its direct action on the two transcripts.
**Figure 2.18:** miR-128 directly targets BMI1 and SUZ12 at their 3’ UTRs. Panel A shows the target sites for miR-128 in BMI1 (one site) and SUZ12 (two sites) 3’ UTR sequences and how they were mutated to prevent miR-128 recognition. Panel B shows relative quantification of Luminescence from whole cell lysate of HEK 293 cells, 48 hours after transfection with miR-128 Vs negative control oligonucleotides (NC) and a plasmid encoding either wild type (WT) or mutated (MUT) 3’ UTR sequences cloned downstream of firefly luciferase. For SUZ12, MUT1 indicates that only one of the two sites was mutated (position 1141-1147), while MUT1,2 indicates that both miR-128 sites were mutated. Reported data is averaged from 3 independent experiments. **= p<0.01.
**MiR-128 overexpression impairs PRC1 and PRC2 activity**

Our initial working hypothesis is based on the assumption that since miR-128 targets multiple polycomb proteins, some of which are components of different PRCs, then its overexpression in glioblastoma should cause a functional impairment to the activity of PRC1 and PRC2. Because the enzymatic activity of the two complexes are characterized by specific histone modifications (PRC1 mediates monoubiquitilation of H2A at Lysine 119 and PRC2 is responsible for tri-methylation of H3 at lysine 27, respectively), a simple approach to determine the final molecular effect of miR-128 re-expression in glioblastoma cells, is to quantify the levels of such histone modifications after restoration of miR-128 expression.

Accordingly, after transient transfection of miR-128 or scrambled control oligonucleotides in G528 and U87 cells, Western blot analysis showed consistent downregulation of the two histone markers, suggesting a functional effect of miR-128 in PRCs activity. Similar results were obtained in cells stably expressing miR-128 transgene (fig 2.19). This observation suggests that the final activity of Polycomb Repressor Complexes can be impaired by targeting simple, key components of each complexes, and, more importantly, that miR-128 has the ability to interfere with the functionality of both of them.
Figure 2.19: miR-128 expression impairs PRC-mediated histone modifications. Panel A shows Western blot analysis for Ubiquitilated H2A and tri-methylated H3 in G528 cells after oligonucleotidte transfection. Panel B shows Western blot analysis in U87 and G528 cells stably overexpressing either GFP or GFP-miR-128. The quantification of average reduction of histone modification (n=2) is provided in panel C.

* = p<0.05; ** = p <0.01
As discussed above, one thoroughly validated target of Polycomb Complex mediated silencing, is the gene CDKN1A, coding for the protein p21, an important cell cycle regulator and tumor suppressor: p21 binds to and inhibits the activity of cyclin-CDK2 or -CDK1 complexes, and thus functions as a regulator of cell cycle progression at G1 (219). Various studies have shown CDKN1A promoter occupancy by both PRC1 and PRC2, and that their downregulation was associated with increased levels of p21 (59, 79, 220). For this reason, and since we had demonstrated that p21 levels increase after siRNA mediated downregulation of BMI1 or SUZ12 (fig 2.8) we used p21 as another bona-fide molecular marker to evaluate the functional activity of PRCs after miR-128 overexpression. As shown in figure 2.20, both glioblastoma cell line U87 and Glioma Initiating Cells G528 stably expressing miR-128 showed upregulation of p21 expression in comparison to controls. Quantitative RT-PCR performed from stable U87 cells also showed an increase in CDKN1A/p21 mRNA after miR-128 overexpression, further suggesting that the observed increase in p21 levels is secondary to an increase in CDKN1A mRNA, suggestive of gene activation after miR-128-mediated downregulation of PRC1 and PRC2 activity.
Figure 2.20: miR-128 determines an increase in CDKN1/p21 expression.
Western blot analysis for p21 in U87 and G528 cells stably expressing miR-128 versus cells expressing GFP only. Panel B shows relative quantification of CDKN1A mRNA in U87 cells after stable miR-128 upregulation (n=2). ** = p <0.01

Taken together these experiments show that not only does miR-128 decrease BMI1 and SUZ12 protein level, but also that it reduces the molecular “signature” associated with the function of the complexes they belong to (histone modifications), resulting in their impaired function and subsequent de-repression of otherwise suppressed genes. Also, very importantly, downregulation of PRC components by miR-128 appears to bypass the compensatory mechanism that we have described above (fig 2.8 and 2.9), suggesting that,
Indeed, miR-128 has a prominent role in keeping the overall activity of PRCs under control.

To further prove that miR-128-mediated simultaneous downregulation of both PRC complexes is indeed functionally important, we focused our attention on the expression levels of miR-124 in glioblastoma cells. MiR-124, another microRNA abundantly expressed in the CNS and particularly in neurons, has been already described to be profoundly downregulated in glioblastoma cells, and its re-expression has been shown to decrease cell proliferation and stemness, and to induce neuronal differentiation (192). Analyzing the data from prior ChIP-Seq experiments performed by other groups and available at http://genome.ucsc.edu (Figure 2.21a), we noticed that SUZ12 binding sites were enriched in close proximity to all three genes encoding miR-124, suggesting that indeed miR-124 expression could be regulated by the activity of PRCs. We therefore analyzed the expression of mature miR-124 by quantitative Real Time PCR after inhibition of PRC2 and PRC1 activity by siRNA-mediated knock down of either BMI1, SUZ12, or the combination of the two in G528 cells. Interestingly, we noticed a minor increase in miR-124 expression after single knock-downs (approaching significance for BMI1 and statistically significant for SUZ12), but a more marked upregulation up to 3-fold, after concomitant knock down of both proteins, which was significantly higher than the upregulation observed with single
polycomb knockdown (Figure 2.21b). This suggested that PRCs play a role in the expression levels of miR-124 and that the concomitant downregulation of both resulted in a more marked de-repression. To confirm that PRCs actually have a role in downregulating miR-124, we then incubated G528 cells with 5μM 3-Deazaflanocin A (DZNep), an inhibitor of EZH2 methyltransferase activity which has been shown to also decrease the protein levels of SUZ12 and BMI1 (224, 225) and to have an antineoplastic effect in glioblastoma GCS (227). PCR results showed upregulation of miR-124 expression to levels similar to those obtained after double knock down of SUZ12 and BMI1 (Figure 2.21c). Finally, we measured the expression of miR-124 after either transient or stable upregulation of miR-128 (which, in our hypothesis, would act as a biological equivalent of DZNep), and we observed, as expected, an increase in miR-124 expression (Figure 2.21d).

Together, these data support the role of miR-128 as a key regulator of the combined function of PRC1 and PRC2, and suggests an important role for the regulation of the expression of other genes (p21, miR-124) that are profoundly deranged in glioblastoma.
Figure 2.21: miR-128 regulates miR-124 expression in GIC.
Simultaneous targeting of PRC1 and PRC2 by miR-128 is functionally more effective than single PRC1 or PRC2 downregulation. Panel A shows SUZ12 consensus sites in close proximity to all 3 loci encoding for miR-124 (orange rectangle), suggesting a regulatory role for PRC in miR-124 expression. Panel B shows relative quantification of miR-124 expression level after selective downregulation of BMI1 alone, SUZ12 alone or BMI1 and SUZ12 in combination in G528 cells. Panel C: 3-Deazaneplanocin A (DZNep), a known inhibitor of PRC function, reduces the levels of BMI1 and SUZ12 proteins (left) and results in increase in miR-124 expression (right) in G528 cells. Panel D shows the relative quantification of miR-124 in G528 cells overexpressing miR-128 versus controls. * = p<0.05; ** = p<0.01
**MiR-128 overexpression results in decreased proliferation of glioblastoma cells**

The fact that miR-128 expression resulted in upregulation of p21 and miR-124 and that PRCs have been found to be important in the silencing of other important tumor suppressor genes like CDKN2A/B, suggested that overexpressing miR-128 would result in an antiproliferative effect.

To assess the effect of miR-128 on cellular proliferative capacity, U251 cells were initially transiently transfected with miR-128 or negative control oligonucleotides and then they were incubated for 2 hours with EDU (5-ethynyl-2'-deoxyuridine). EDU is a modified nucleoside that, similarly to BrDU (Bromodeoxyuridine), competes with Thymidine as a DNA Polymerase substrate during DNA replication. The amount of EDU incorporated into DNA during a given time, as well as the amount of EDU-positive cells within the tested cell population is considered a “bona fide” measurement of DNA replication, and thus cell proliferation (Figure 2.22a).

Likewise, U87 cells stably expressing either GFP of GFP-miR-128 transgene were plated and total cell number counted 4 days after seeding (Figure 2.22b). The same cells were also incubated with EDU for 3 hours, and, similarly to U251 cells, decreased cell proliferation was observed, strongly suggesting that miR-128, regardless whether by transient or stable overexpression is able to decrease the rate of cell growth (Figure 2.22c).
Figure 2.22: miR-128 reduces cellular proliferation. Panel A: after incubation with EDU for 3 hours, U251 cells transiently overexpressing miR-128 show a decrease rate of EDU uptake compared to cells transfected with negative control oligonucleotides. Quantification of EDU+ cells/total cells is given in the right side of the panel. Similarly, U87 stably expressing miR-128 show decrease proliferation rate both in terms of cell count after 5 days in vitro (panel B) and EDU uptake after 3 hour incubation (panel C). Data averaged on three experiments. **=p<0.01
MiR-128 expression determines loss of stemness and decreased clonal ability and proliferation of GIC

Since miR-128 expression has been linked to a cellular state that is differentiated towards a neural phenotype, we reasoned that its overexpression in GIC would determine a loss of stemness and a result in impaired capacity for self renewal, a prominent feature of stem cells. The molecular marker CD133 remains a widely accepted indicator of cellular stemness in glioblastoma cells, as it has been described that CD133 cells have a much greater ability than CD133 negative cells to proliferate in suspension and to form tumors in vivo (18,19). We thus investigated the effect of miR-128 overexpression (either transient or stable) on CD133 expression in GIC 528. As a result, we observed that cells overexpressing miR-128 have a 50% reduction in CD133 expression (Figure 2.23a)

We then performed a soft agarose clonogenic assay with GIC 528 stably expressing miR-128 or negative control. As described previously, the agarose clonogenic assay has been specifically developed to assess the capacity for single cells to produce a spheroid, which is a bona fide confirmation of the cell’s ability to self renew and produce progeny (221). The addition of agarose to the medium confers to this culture method the advantage of growing the cells in a solid substrate, keeping them fixed in a particular location of the culture plate and, importantly, preventing cells from aggregating: the formation of cellular aggregates is always observed when these cells are grown in suspension in liquid media, and results from cell-cell interaction and integrin-mediated
adhesion. The formation of aggregates obviously masks the real clonogenic potential of each cell, which is the final endpoint of our experiment.

In our experiments, GIC 528 were seeded at a low density (20 cells/mm²) so that each single cell would remain isolated from others, and the spheroid formation was assessed 14 days after plating, (a time after which no further increase in the number of spheroids was generally observed in pilot experiments). Figures 2.23b and c show that overexpression of miR-128 resulted in a statistically significant decrease in total number of spheroids. Also, when the observed spheroids were separated into three different size categories (small, medium and large), it was evident that the spheroids formed by cells overexpressing miR-128 were significantly smaller than those formed by cells overexpressing the negative control vector (2.23d). Overall, this experiment confirmed that miR-128 was able to reduce the self renewal ability of glioma initiating cells (as a lesser number of spheroids were formed from the same amount of seeded cells, compared to controls). Moreover, the smaller spheroid size confirmed the prior results obtained with glioblastoma cell lines (Figure 2.23b), i.e. that miR-128 also reduces cellular proliferation rate (spheres are smaller because constituted by less cells). That size of sphere in our experiment setting is a direct indicator of total cell number within the sphere and not, for example, the result of a different pattern of cellular compaction (and possible migration) in the agarose substrate between the two different cell samples, was proven by selectively isolating spheres of different sizes from either miR-128 positive or
negative control plates, and plating them directly onto a laminin-coated dish. After 24 hours, in both cases, the cells constituting the spheroids were all spread and attached to the bottom of the dish, while the spheroid itself had disappeared. However, the difference in cell amount was readily evident under the microscope (image not shown), suggesting that, indeed, smaller spheres were the result of decreased cell number per sphere.

Figure 2.23: miR-128 reduces stemness in glioma initiating cells. G528 cells stably expressing either GFP-miR-128 show a marked reduction of CD133 expression (A). Phenotypically, this translates into an about 50% decrease in cell clonal ability and self renewal in a soft agarose clonogenic assay (B, C). Also, cells appear to proliferate less, as spheroid size is statistically smaller in cells overexpressing miR-128 (D). * =p<0.05; ** = p<0.01
MiR-128 downregulation is an early event in gliomagenesis

Our experiments have shown, so far, that glioblastoma is consistently associated to a decreased expression of miR-128 and upregulation of Polycomb Repressor Complex activity. Since we had already shown that miR-128 expression is altered at the GIC level, i.e in cells that are originated from “full blown” glioblastoma, we next sought to determine whether miR-128 deregulation happens before the onset of tumor, or, in other words, at a pretumoral cellular state and if, as a consequence, miR-128 could represent a novel gene involved in gliomagenesis. To address this question, we took advantage of the spontaneous glioma mouse model described in chapter xyz: these mice develop high grade gliomas, including glioblastoma, at 25-30 weeks of age, as a consequence of Trp53 and Nf1 loss of heterozygosity. We have already shown that glioblastoma stem cells originated from these tumors have lower miR-128 levels than the normal subventricular zone cell obtained from control mice. We reasoned that, since these mice are poised to develop gliomas, their cells, particularly subventricular zone cells, should have early genetic cancerous signatures even before the actual onset of tumor. Therefore, we compared the expression levels of miR-128 in 3 different subventricular zone cell lines obtained from young mut-3 mice (at 4 weeks of age, i.e well before the onset of brain tumor) with another three subventricular zone cell lines obtained from control, wt mice, i.e mice that will not develop tumor. As shown in Figure 2.24, miR-128 was consistently downregulated in SVZ cells obtained from mut-3 mice. This suggested that miR-128 is
downregulated at the precancerous stage in cells that are poised to originate tumor. It is important to note that mut-3 SVZ cells are in fact tumorigenic when implanted in nude mice flanks, confirming that these cells, although not derived from tumor, have the intrinsic property to form tumor. With this approach we were able to confirm our hypothesis that miR-128 deregulation, observed in glioblastoma, actually happens before the full onset of glioblastoma, and that it might in fact represent one key step in the formation of the tumor.

**Figure 2.24: miR-128 is downregulated in precancerous mouse SVZ cells.** Relative quantification of miR-128 expression in SVZ cells obtained from young (i.e. 4 week-old) wild type (WT) or mut3 mice and grown in stem cell medium for 3 passages, at which time they were harvested and RNA purified. Each dot/square represents results obtained from one cell line. Average and standard deviation of each group are represented by the horizontal and vertical lines, respectively. **=p<0.01
**BMI1 and SUZ12 are upregulated in precancerous subventricular zone cells**

In our prior experiments we had observed a strong inverse correlation between miR-128 and BMI1 and SUZ12 and a direct effect of miR-128 in the level of those two proteins. We next sought to determine whether this correlation existed also in SVZ cells obtained from young mice, i.e., whether the upregulation of the two proteins was also a pre-tumoral event.

To determine this, whole cell protein lysates from three wild type and three mut-3 SVZ cells, all obtained from mice at 4 weeks of age, were obtained and Western blot was performed. As shown in Figure 2.25, the protein level of BMI1 and SUZ12 is significantly higher in mut-3 SVZ cells than in wild type SVZ cells. This finding confirmed the inverse correlation between miR-128 and BMI1 and SUZ12, confirming a likely causal relationship between their level of expression. Moreover, these data also suggest that BMI1 and SUZ12 upregulation is an early event in gliomagenesis, stressing the importance of the derangement of PRC complex activity in the initial phases of glial tumors formation.
Figure 2.25: **BMI1 and SUZ12 are overexpressed in precancerous mouse SVZ cells.** Western blot analysis for BMI1 and SUZ12 comparing the protein level of SVZ cells obtained from either wild type (WT) or mut3 mice. The graph on the right shows the relative quantification of BMI1 and SUZ12 levels for each tested cell line in respect to the corresponding α Tubulin level. Each dot/square represents results obtained from one cell line. Average and standard deviation of each group are represented by the horizontal and vertical lines, respectively. **=p<0.01

**Induced miR-128 downregulation in Neural Stem Cells determines an increase in BMI1 and SUZ12 levels**

All the data shown so far, suggest a strong correlation between miR-128 expression and BMI1 and SUZ12. To ultimately prove a causal relationship, we next proceeded to downregulate miR-128 in non-malignant Neural Stem Cells, both human and murine.
To achieve this, we took advantage of the miR-Zip technology provided by System Biosciences: briefly, a lentiviral vector was purchased from the vendor endowed with a short (60 bp) DNA sequence encoding for a hairpin structure whose one strand is perfectly complementary to endogenous miR-128. Once expressed, this hairpin eventually functions like a shRNA and targets mature miR-128 by forming a double stranded structure with it, and thus rendering it unavailable for UTR seed sequence recognition and preventing its action on target mRNAs. The fact that mature miR-128 sequence is identical in human and mouse cells made it possible for us to use this strategy in both contexts.

Human fetal Neural Stem Cells SCP27 and one SVZ cell line obtained from our wild type mice were infected with lentiviral particles carrying either miR-Zip-128-puroR (anti-miR-128) or miR-Zip-puroR as negative control in order to obtain stable genomic insertion of the transgene. After selection of transduced cells was obtained by selective culturing in medium containing 1 μg/ml puromycin for 14 days, we first proceeded to determine whether we could detect a measurable difference at QRT-PCR in mature miR-128 levels between miR-Zip-128 and control cells. As shown in fig 2.26 (left panel), expression of mir-Zip-128 resulted in marked decreased detection of miR-128 (SCP27 shown). We then measured the levels of BMI1 and SUZ12 proteins in both SCP27 and mouse SVZ cells by Western blot. As shown in fig 2.26 (right panel), downregulation of miR-128 caused a significant increase in BMI1 and SUZ12 levels in both SCP27 and
SVZ cells. This finally confirmed that miR-128 is a key determinant of the level of these two proteins and that its downregulation in glioblastoma is likely the cause for their aberrant level in the tumor.

Figure 2.26: miR-128 downregulation results in increased BMI1 and SUZ12
Human Neural Stem Cells SCP27 stably expressing anti-miR-128 (ZIP-miR-128) show strong downregulation of mature miR-128 at qRT-PCR in comparison to negative control cells transduced with ZIP-puroR (A). Western blot analysis for BMI1 and SUZ12 for the same cells is shown in panel B. *=p<0.01
Induced miR-128 downregulation in Neural Stem Cells determines an increase in clonal ability and proliferation

Since miR-128 overexpression determines a downregulation of BMI1 and SUZ12 and results in decreased clonal ability of Glioma Initiating Cells, we reasoned that, if miR-128 loss is an important step in gliomagenesis, its downregulation in non-tumoral cells should cause a phenotypic change consistent with neoplastic behavior, or at least increase proliferation. We therefore performed a clonogenic assay using both SCP27 and wild type mouse SVZ cells stably expressing miR-Zip-128 or cells transduced with ZIP-puroR as negative control. As described above, cells were embedded at low density (20 cells/mm$^2$) into stem cell medium containing 0.4% agarose and cultured for 14 days before the sphere formation was analyzed at light microscope. As it is evident on figure 2.27, neural stem cells with decreased miR-128 levels produce more and bigger spheres compared to control. This was evident both in human and murine cells. Mouse cells, because of the very few spheres that were formed in the negative control group, it was impossible to determine the differences in sphere size as it was done for SCP27 cells. These results, that are the exact opposite of those observed in glioma initiating cells after miR-128 overexpression suggests that miR-128 indeed plays an important role in regulating self renewal and proliferation at the stem cell level.
Figure 2.27: miR-128 downregulation increases clonal ability and proliferation in NSC and SVZ cells.
Clonogenic assay with human NSC SCP27 stably transduced with either ZIP or ZIP-miR-128 (A). Cells with repressed miR-128 expression showed increased number of colonies and bigger colony size compared to control cells (B). Likewise, mouse wild type SVZ cells display a greater BMI1 and SUZ12 protein level after miR-128 knock down by lentiviral transduction with ZIP-miR-128 (C) and this results in marked increase in clonogenicity (D and E). **=p<0.01
Figure 2.27 Continued

Mouse WT SVZ #1

C

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DISCUSSION

MicroRNAs are fundamental regulators of cell biology and the disruption of their expression has been convincingly associated to cancer. While the mechanism by which they exert their biological activity has been widely accepted, i.e. by either blocking mRNA translation or inducing mRNA degradation, with a final net result of protein downregulation, their role in cellular physiology is complex and is the result of the concomitant action on multiple targets. Here we report, for the first time, that: 1) miR-128 simultaneously targets important constituents of Polycomb Repressor Complexes 1 and 2, and that its downregulation in glioblastoma contributes to their high level of expression compared to normal brain; 2) specific knock-down of SUZ12 (PRC2) resulted in elevated expression of PRC1 component BMI1 and consequently elevated PRC1 activity, while specific knock-down of BMI1 (PRC1) resulted in increased activity of PRC2, suggesting redundancy between both PRCs as postulated previously; 3) miR-128 expression correlates clinically with improved survivorship in subjects with glioblastoma; 4) miR-128 is one of a foursome of miRs that are sufficient to discriminate neural stem cells and GSCs; 5) miR-128 down-regulation with corresponding SUZ12 and BMI1 up-regulation occurs in SVZ-derived NSCs in 4-week old mice genetically engineered to develop gliomas by week 20-30. Taken in conjunction, these novel
findings provide evidence of the important regulatory role of miR-128 in PRC function and point toward de-regulation of miR-128 as an early event in glioma.

The expression of miR-128 and PRC components SUZ12 and BMI1 is inversely correlated in human glioblastoma patient samples, as we demonstrated on multiple levels (miR/mRNA, proteins, immunohistochemistry), and relatively higher expression of miR-128 in patient samples correlated with better survival. We also demonstrated that miR-128-mediated downregulation of PRC components led to a significant inhibition of PRC-dependent histone modifications, decreased expression of CD133, de-repression of the tumor suppressors p21 and miR-124, decrease of glioma cell proliferation and decreased GSC self-renewal.

A number of microRNAs have been shown to function by repressing the expression of multiple genes in a signaling pathway, or in pathways that are functionally related; however, to our knowledge, this is the first report where a single microRNA is shown to be important for the regulation and proper coupling of two protein complexes that are essential to each other to execute a very particular and specific function, that is silencing of gene expression by histone modification. In this case, PRC1 and PRC2 are both necessary to accomplish gene silencing; however the two complexes appear to be under control of each other, so that when one complex is downregulated, the other becomes upregulated, likely in an attempt to rescue the function of lost “companion” complex. Within this interaction, miR-128 provides a “coupling” mechanism whereby, in its
presence, both complexes are downregulated at the same time, allowing for a meaningful abolition of their effect. As a proof of principle for our hypothesis we showed in our study how the expression of miR-124, a known antioncogenic microRNA, consistently downregulated in glioblastoma, and known to be silenced by both a SUZ12-mediated histone modification (59) as well as by DNA methylation (242,243) was only slightly increased after single knock down of either BMI1 or SUZ12. However, upon simultaneous downregulation of both polycomb proteins miR-124 levels increased three-fold, similarly to what was observed after pharmacological disruption of PRC1 and PRC2 by DZNep. This meant that downregulation of both PRC1 and PRC2 is required to remove the repressive mark on miR-124 gene, and that, as such, the simultaneous downregulation of both PRCs (by the action of miR-128) is functionally relevant at the cellular level. In fact, similarly, miR-128 overexpression resulted in 3-4 fold upregulation of miR-124. Contrarily to SUZ12, there is no evidence of BMI1 (or other PRC1 components) consensus sites at the miR-124 locus: however, since BMI1 has been strongly associated to the induction of DNA methylation (228), and since miR-124 has been shown to be silenced by promoter hypermethylation, we speculate that BMI1 knock down indirectly affects miR-124 expression by means of reducing its hypermethylated status, while SUZ12 knock down directly impedes repressive histone marking at its transcription start, and thus only knockdown of both result in re-expression of miR-124
The fact that miR-mediated regulation of PRC activity is important for central nervous system biology is inferred by the high number of CNS-enriched microRNAs that are shown to potentially target at least one of the many polycomb protein transcripts. Of these microRNAs, mir-128 seemed the only that could directly target both PRC1 and PRC2 by downregulating BMI1 and SUZ12, respectively. Importantly, miR-128 expression in glioma cells appears to be lost (or downregulated) specifically in the stem cell sub-population, and not simply as a consequence of the glial lineage of the tumors: in fact this observation is not trivial, as miR-128 is mainly expressed in neurons, and for this reason, it should not be surprising to observe its downregulation even in normal glia/astrocytes or tumors of astrocytic origin like glioblastoma. However, the fact that glioma stem cells cannot overexpress miR-128 in response to neural differentiation stimuli provided by retinoic acid (as it happens in normal Neural Stem Cells), along with the observation that the baseline level of miR-128 in glioma stem cells is significantly lower than it is in Neural Stem Cells clearly suggests that miR-128 homeostasis is impaired in the glioma stem cells. Moreover, we showed that miR-128 is among 4 of the most consistently and significantly downregulated microRNAs discerning Neural Stem Cells from Glioblastoma Stem Cells. Therapies aimed at inducing differentiation of glioma stem cells have been experimented for long time with mixed results: retinoic acid or cyclic AMP mimics have shown some effect at reducing cellular proliferation and clonal ability in vitro (245, 246), along with induction
of “bona fide” differentiation markers, like neuronal specific beta3 tubulin or GFAP, but their use in clinical trials has failed to yield significant improvements in tumor response to therapy or survival (247). The general thought is that the degree of differentiation is only partial and likely reversible. Also, miR-128 itself has been shown to induce some degree of differentiation in glioma stem cells, both by us and by other authors (198). However it would be too simplistic to assume that restoration of the expression of a single microRNA would be the solution to glioblastoma multiforme treatment. In future experiments it would be interesting to evaluate the degree of differentiation obtained with pharmacologic treatment after restoration of miR-128 expression, which, for what we have shown, should render glioma stem cells more similar to Neural Stem Cells, and thus more responsive to differentiation stimuli.

Recently published findings provided compelling evidence that expression of miR-128 is severely impaired in a mouse genetic model of glioma (H-RasV12, Trp53 +/-), and that its re-expression prevents glioma development (198). Our results confirmed loss of miR-128 in a mouse model of glioma – Mut3 (Nf1^lox/+; Trp53), which is accompanied by elevated levels of SUZ12 and BMI1. Moreover, we demonstrated that loss of miR-128 expression and concomitant increase of PRC activity is observed in young, pre-symptomatic animals, suggesting that it is an early event in gliomagenesis. We also showed that knockdown of miR-128 in mouse and human non-malignant NSC led to their increased clonal ability. Additionally, miR-128 has the ability to target a broad
range of oncogenic targets (EGFR and PDGFR signaling (198), E2F3 (199), Nanog (248) and Musashi-1 (249) among others). Recently published work identified the oncprotein SNAIL as direct negative regulator of miR-128 in breast cancer (248). Interestingly, it was demonstrated that both p53 and NF1 repress SNAIL (250,251), providing a plausible explanation for miR-128 downregulation in Mut3 cells observed in our experiments. In ovarian cancer, SNAIL was shown to induce stem-like phenotype and radio-resistance (252). Finally, SNAIL is targeted by p53-inducible miR-34, which was identified as tumor suppressor in glioma cells (253,254). It remains unclear whether the mechanism of regulation of miR-128 in glioma cells is the same as in breast cancer.

Having shown that miR-128 is among the most differentially expressed microRNAs between Neural Stem Cells and Glioma Initiating Cells certainly warrants further investigation in order to shed some light on the process of miR-128 silencing during gliomagenesis.
A Possible Translational Application for miR-128:
Sensitizing GIC to Ionizing Radiations Through Downregulation
of PRC1 and PRC2

INTRODUCTION AND RATIONALE

Irradiation is one of the mainstay of treatment for glioblastoma multiforme (1). The rationale for its use derives from the ability of ionizing irradiation to cause DNA damage, particularly double strand breaks, which, if unrepaired, eventually determine cell death, either by apoptosis or necrosis (229). It has been shown, though, that a subset of tumor cells, and particularly CD133-positive GIC, have hyperactive mechanisms of DNA repair, which eventually can neutralize the genotoxic effects of radiations (11). This, ultimately, prevents eradication of neoplastic cells, and is at the basis for the almost inevitable tumor recurrence. Also, central nervous system irradiation is not a benign procedure because of the effects that it exerts on normal brain surrounding the
tumor (resulting in radiation necrosis and/or cognitive impairment (230). As a consequence, in clinical practice the amount of given irradiation has to be balanced between the antitumor effect being sought and its side effects. For these reasons, any interventions that would sensitize tumor cells, and particularly GIC, to radiation treatment would increase the therapeutic benefit of irradiation, keeping the associated side effects limited, and would thus have a direct practical application to the clinical setting.

The cellular response to DNA damage consists of a series of complex biochemical protein activation and recruitment, that determine modification in chromatin conformation and, ultimately, make it accessible for repair. The DNA damage response mechanism also ensures activation of cell cycle checkpoints that cause cell cycle arrest. This allows time for repair without propagation of damaged DNA, and directs toward apoptosis or cell senescence in case where DNA repair is unsuccessful. There are two major pathways of DNA double strand break repair: homologous recombination (HR), where an undamaged DNA template is used to “copy” a new, identical DNA sequence which then replaces the damaged sequence. Although error free, this modality is only possible when the cell is post DNA replication (S or G2 phases). In all other situations (G2 phase), another mechanism, called non-homologous end joining (NHEJ) repairs the break, but it is much more prone to making errors, particularly DNA deletions. While these mechanisms are vital for normal cells to maintain their identities and to prevent
dangerous mutations, they are conserved in cancerous cells as well, hence the notion that targeting the efficacy of DNA double strand repair could result in increased efficacy in tumor cell eradication (231).

The role of Polycomb Proteins in the mechanisms of DNA repair has recently become object of intense research: several studies have shown that numerous polycomb proteins accumulate at sites of DNA damage (232, 233, 234). BMI1 has been shown to co-purify with DNA Double Strand Breaks (DBS) response and non-homologous end joining (NHEJ) repair proteins in GBM cells (235). BMI1 was enriched at the chromatin after irradiation, co-localized and was co-purified with ATM and the histone gammaH2AX. ATM (Ataxia Telangectasia Mutated) is a serine/threonine protein kinase that is recruited and activated by DNA double-strand breaks. It phosphorylates several key proteins that initiate activation of the DNA damage checkpoint, leading to cell cycle arrest, DNA repair or apoptosis (265). Histone 2AX is one of the several variants of Histone 2A: H2AX becomes phosphorylated in codon 139 by ATM (then called gammaH2AX) as a consequence of DSB, and that results in a less condensed chromatin state, allowing space for recruitment of repair proteins to the damaged DNA (266). BMI1 deficiency in GBM cells severely impaired DNA DSB response, including recruitment of ATM and RNF8 to site of damage, and resulting in increased sensitivity to radiation (235). BMI1 accumulation at DSB foci occurs very early and, unlike the
recruitment of the majority of other DSB-responsive proteins, it is independent from H2AX phosphorylation, likely representing an independent pathway from that mediated by H2AX activation (236). Similarly, EZH2, EED and SUZ12 appear to have similar functions, as their downregulation caused an increase in radiation sensitivity after UV-induced DNA damage (234). Interestingly, such activity does not seem necessarily related to the main function of PRCs (that is, to turn off the genes) but to their ability to “mark” chromatin, providing a molecular signature that is recognized by the DNA repair machinery as a docking signal, leading to a correct localization and activation of the molecular cascade leading to the DNA repair process (232, 235).

The ability to repair DNA has already been proven to be essential for glioblastoma cell survival, particularly after treatment: in a seminal study, Stupp and colleagues have demonstrated that patients with methylated (thus silenced) MGMT gene (O-6-Methylguanine-DNA-Methyltrasferase), the enzyme that is responsible for removing the methyl group from O-6 Methylguanine, restoring the normal matching of Guanine with Cytidine instead that with Thymine) live significantly longer and respond more profoundly to temozolomide, an alkylating agent, because their cancerous cells couldn't reverse the mutagenic effect of the drug, and, as a consequence, undergo cell death (237). However, as MGMT methylation has been shown to strongly correlate with CIMP+ tumors (267), it is still unclear whether the survival benefit is secondary to MGMT per se or it is just consequence of the more favorable genome-wide methylation profile.
In light of the profound effect miR-128 exerts on BMI1 and SUZ12, as described above, we hypothesized that miR-128 overexpression could potentiate radiation-induced cell death in glioblastoma multiforme.

RESULTS

**BMI1 and SUZ12 expression levels increase after cell irradiation, but concomitant miR-128 overexpression impairs the response**

Since Polycomb Proteins have been linked to cancer cell resistance to ionizing radiation, we hypothesized that, upon irradiation, glioblastoma cells would upregulate BMI1 and SUZ12 expression as a pro-survival response. To evaluate this, two cell lines (U87MG and U251) and one GIC cell line (528) were irradiated with different intensities (0-2-4 Gy) and the expression of BMI1 and SUZ12 was evaluated at different time points by quantitative RT-PCR. For all lines, an increase expression was apparent at 1 hour after irradiation with a peak at 12 hours. This confirmed that upregulation of BMI1 and SUZ12 is a part of cellular response to ionizing insult (Figure 3.1). Using the same RNA we also checked whether irradiation changes the expression levels for miR-128, but we did not
detect any significant differences between irradiated and non irradiated cells (data not shown). This is not surprising considering that in glioblastoma cells miR-128 is already strongly downregulated.

**Figure 3.1: BMI1 and SUZ12 expression increases after ionizing irradiation.** Results from quantitative Real Time PCR from multiple glioblastoma cell lines, including GIC line G528, showing a similar pattern of Polycomb gene upregulation either after 2 or 4 Gy
We next sought to determine whether overexpression of miR-128 in this context would still be capable to downregulate the level of BMI1 and SUZ12 similarly to what we have already shown in normal conditions. To test this, G528 cells were seeded on laminin coated plates at a density of 200 cells/mm$^2$ and let grow for 12 hours. Cells were then transfected with either miR-128 or negative control oligonucleotides and after 24 hours cell were irradiated with either 0, 2 or 4 Gy and let to recover for another 24 hours, at which point cells were collected and whole cell protein lysate was obtained. Western blot was then performed with anti-BMI1 and anti-SUZ12 (Figure 3.2). Similarly to what was observed at the mRNA level, we observed an increase in polycomb proteins in the irradiated cells compared to the non irradiated ones, and in a dose-dependent fashion. However, cells pre-treated with miR-128 oligonucleotides still showed a significant decrease in both BMI1 and SUZ12 levels. Importantly, the protein level attained by the miR-128-overexpressing cells after irradiation (either 2 or 4 Gy) remained lower, or at least not significantly greater than the baseline level expressed by miR-128-negative, non-irradiated cells. This suggests that while the mechanism leading to Polycomb upregulation after irradiation remains functional in cells overexpressing miR-128, the overexpression of miR-128 prevented the cells to upregulate BMI1 and SUZ12 to the level that they would normally do in response to genotoxic stimulus (Figure 3.2), substantially impairing the cellular response to irradiation.
MiR-128 overexpression acts in synergy with irradiation to decrease clonal potential of GICs

As we had already shown, GIC stably overexpressing miR-128, display a reduced clonogenic potential, as well as a decreased proliferation rate. Now, in light of our observation that miR-128 might sensitize glioma cells to genotoxic effect of ionizing radiations by targeting polycomb proteins, we sought to determine whether this
combination would result in a further reduction in clonal ability. A seminal study had previously shown that CD133-positive cells have greater resistance to radiation respect to CD133-negative cells and have greater clonal ability when cultured after irradiation (11). Since we had previously shown that miR-128 overexpression resulted in decreased CD133 expression in GIC, we expected that irradiating cells after miR-128 overexpression would result in higher anti-proliferative effect and marked decrease in self renewal ability.

G528 stably expressing either miR-128 or GFP were plated in 6 well plates, in triplicates, at a density of 20 cells/mm² in agarose substrate and stem cell-supportive medium in order to perform clonogenic assay, as described previously. Twenty-four hours after plating, cells were irradiated with either 0, 2 or 4 Gy and returned to the incubator. After 14 days the number of spheres was counted for each well. As previously observed, for the non-irradiated cells, those expressing miR-128 showed a statistically significant reduction of colony number compared to the negative controls. Irradiation with 2 Gy caused a moderate decrease in colony counts in the negative control, while resulted in a major reduction in number of spheres in the cells expressing miR-128. In comparison, the reduction in sphere number between miR-128-positive and miR-128-negative was significantly lower after irradiation with 2 Gy than without irradiation. When 4 Gy irradiation was used, a similar effect was observed, with virtual eradication of the miR-128-positive cells. However, the amount of energy was high enough to bring about a
marked decrease in sphere formation also in the negative control cells, suggesting that at 4 Gy the impact of radiation likely becomes prominent and determines impairment of cell growth regardless of other experimental conditions, particularly the presence/absence of miR-128. Nevertheless it was evident that overexpression of miR-128 increased cell susceptibility to radiation, particularly at low, clinically relevant doses (Figure 3.3).

**Figure 3.3:** miR-128 impairs clonal capacity of GIC after ionizing irradiation. The reduction in colony number was statistically significant between all experimental conditions versus negative controls, however there was a statistically significant higher decrease of clonogenicity between miR-128+/0Gy and miR-128+/2Gy. =p<0.05
To further confirm that there is a synergy between miR-128 expression and ionizing radiation, G528 cells were plated onto laminin substrate in the stem cell medium, and after 12 hours they were transfected with either miR-128 or negative control oligonucleotides. Twenty-four hours after transfection, cells were irradiated with either 0 or 2 Gy and let recover for further 48 hours, at which time pictures were taken to evaluate cell density and morphology and finally cells were dissociated and counted. The experiment was done in triplicates.

Similarly to what was observed in the clonogenic assay, there was a decrease in cell proliferation observed in cells overexpressing miR-128 compared to negative control (in the absence of irradiation) and in negative control cells irradiated with 2 Gy compared to negative control cells without irradiation. However, irradiated cells overexpressing miR-128 showed a much more marked decrease in proliferation rate, and the final cell number was even inferior to the number of cells plated 48 hours before, suggesting active cell loss. This confirmed that when preceded by miR-128 overexpression, irradiation exerts a much more profound effect on the proliferation/survival of glioblastoma initiating cells (Figure 3.4)
Figure 3.4: miR-128 increases susceptibility of GIC to ionizing irradiation. The reduction in proliferation was statistically significant between all experimental conditions versus negative controls, however there was a statistically significant higher decrease of clonogenicity between miR-128+/0Gy and miR-128+/2Gy. **=p<0.01

The combination of miR-128 overexpression followed by irradiation results in cell death

The marked reduction of both clonal potential and proliferation observed in G528 cells when irradiated after miR-128 overexpression was suggestive of an active mechanism leading to cell death, in addition to the already described antiproliferative effect exerted
by miR-128 (see above). In fact, miR-128 has already been reported not to have major roles in inducing apoptosis or cell death per se (198). On the other hand, ionizing radiations are known to induce apoptosis and/or necrosis, although their effect in glioblastoma cells is dependent on dose and varies from cell to cell (229). To test whether we could detect any measurable molecular differences which would explain this dramatic difference in cell survival after irradiation in the presence or absence of miR-128, G528 cells were seeded onto laminin-coated plates and transfected with either miR-128 or negative control ologonucleotides. After 36 hours cells were irradiated with either 0 or 2 Gy and let grow thereafter for 48 hours. Cells were then collected and analyzed by flowcytometry after Propidium Iodide (PI) and Annexin V stain, in order to detect any changes in cell death (PI) and/or apoptosis rate (Annexin V).

Figure 3.5 shows the results obtained with a pool of three different repeated experiments: compared to negative controls (no miR-128 expression, no irradiation), cells overexpressing miR-128 only, or irradiated only, did not show any significant increases in either apoptosis nor cell death, confirming that miR-128 overexpression only results in reduced cellular proliferation, but no active cell death. In this setting and with these particular cells, irradiation also did not determine significant cell death. However, cells that received irradiation after miR-128 had been overexpressed showed a marked increase in PI positivity (more than twice the baseline level), suggestive of increased cell
death rate. Interestingly, the amount of apoptotic cells was not different from baseline, suggesting that cell death was not due to apoptosis.

**Figure 3.5: MiR-128 potentiates radiation-induced citotoxicity.**
G528 cells were transiently transfected with miR negative control (NC) or miR-128 precursor (128) and after 36h cells were either left untreated or radiated using a 2Gy dose. After further 48h flow cytometry was performed. Data shown as percentage of PI positive (top) and negative (bottom) cell staining.
MiR-128 overexpression impairs DNA repair in glioma initiating cells

Our hypothesis that miR-128 overexpression would potentiate the cytotoxic effect of ionizing radiation in glioma cells derived from our observation that miR-128 downregulates BMI1 and SUZ12, important for DNA repair, particularly after double strand breaks. After demonstrating phenotypic and molecular evidence of the synergy between miR-128 and ionizing radiation, we aimed to prove/disprove that the involved mechanism was indeed an impaired ability to repair DNA after damage; we thus performed a single cell gel electrophoresis assay (also known as comet assay) on G528 cells in a similar setting as that used for the prior experiments. The comet assay is a simple and sensitive test to evaluate the amount of DNA damage at a single cell level (244). Briefly, this method is based on the quantification, in situ, of DNA breaks, by measuring the amount of nuclear DNA migration after a given time under an electrical field; the rationale is based on the fact that the intact DNA contained in each nucleus migrates rather homogeneously and very slowly under an electrical field, while broken DNA (as the result of double strand DNA breaks) produces a “smear” because of the smaller and variably long DNA fragments caused by the DNA breaks. The smears are visualized under the microscope as “comet tails”: the longer and the more intense the tail, the higher the amount of broken DNA. If this test is performed after cells are treated with a genotoxic insult which is known to cause DNA damage (i.e. ionizing radiation), the
presence/absence of comet tails at known temporal intervals is a reliable indicator of the cell ability to repair DNA. In our case, G528 cells were plated onto laminin-coated dishes and, after 12 hours, were transfected with either miR-128 or negative controls oligonucleotides. Cells were then let to recover for 24 hours. At this point, cells were irradiated with 2 Gy and collected at 30 minutes and 15 hours after irradiation. Also, not irradiated cells were used as negative controls. The rationale for this protocol is that 30 minutes after irradiation, the majority of cells, independently from the presence or absence of miR-128, would have double strand DNA breaks (as breaks happen within seconds/minutes after irradiation) and the repair process would have not started yet. On the contrary, 15 hours after irradiation, the DNA repair mechanism would have had time to work and repair DNA, so that, if any difference in DNA repair was to be attributed to the presence of miR-128, a difference in tail would be observed between miR-128 and negative control cells. Figure 3.6 shows that the percentage of cells with a tail was low in the samples that had not been previously irradiated, suggesting that at baseline, the presence of DNA double strand breaks is minimal and, importantly, not significantly different between miR-128-positive or negative control cells. Thirty minutes after irradiation, up to 90% of cells had a tail, indicative of DNA damage. Importantly, no significant differences were noticed between miR-128 or negative control cells, suggesting that miR-128, by itself, does not increase the incidence of radiation-mediated DNA breaks. However, when the cells are observed 15 hours after irradiation, 50% of
cells overexpressing miR-128 had tails compared to only 25% of negative control cells. This suggests that during the 15 hours elapsed from irradiation, cells overexpressing miR-128 had not been able to repair DNA as efficiently as those not expressing miR-128, confirming our hypothesis that DNA repair is actually impaired by miR-128, likely by depleting the cellular pool of available BMI1 and SUZ12.

Figure 3.6: miR-128 overexpression impairs DNA repair
Representative images of cells with fragmented DNA tails are shown (left) under low power and high power magnification. Data expressed as percentage of cells with tail over totality of cells. Number of cells. ** = P<0.01.
It is evident that practical strides in the treatment of glioblastoma multiforme will be possible only with a meaningful combination of different approaches able to provide a synergistic cytotoxic effect to tumor cells. Particularly these efforts should be oriented towards the subset of cancer stem cells that show the greatest degree of resistance to conventional therapies. Radiation therapy, for example, has proven to be a very valuable adjunct to surgical resection to significantly prolonging patient survival. However, the response to ionizing irradiation is not complete, and tumor cells eventually manage to escape death and continue to proliferate.

Here we show that glioblastoma initiating cells, i.e those that are known to be the best equipped with the ability to escape cell death caused by radiotherapy, are prone to a significantly higher degree of radiation-induced cytotoxicity after expression of miR-128 is reintroduced, and PRC function is thus inhibited. The role of PRCs in DNA repair has been recently demonstrated and there is currently widespread interest for its possible therapeutic implications. We postulated that miR-128 inhibiting PRC activity could cause an impairment in DNA repair after radiation-induced double strand break
We have initially showed that glioblastoma cells upregulated both BMI1 and SUZ12 in response to irradiation and we hypothesized that this would represent a pro-survival response in reaction genotoxic insult. This observation was in accordance with prior studies that have described that BMI1 and other PRC2 components take part in the Double Strand Break DNA repair mechanism by relocating at sites of DNA break, and that their downregulation resulted in impaired cell survival after irradiation (234, 235).

Consequentially, we then observed that when irradiation is administered in the presence of miR-128, the response of overexpression of BMI1 and SUZ12 is contained and does not reach the physiological levels that the cells would normally attain in normal circumstances (i.e without miR-128 overexpression), suggesting that miR-128 overexpression can, in fact, interfere with the cellular response to DNA damage.

Evidence that miR-128 expression could be synergistic to irradiation in determining cell death was obtained from both clonogenic and proliferation assays, showing that, while miR-128 or irradiation (administered separately) have an effect on cell proliferation and clonal ability, this effect is much more pronounced when irradiation is administered in a condition of miR-128 overexpression (and thus of decreased availability of BMI1 and SUZ12). This synergy was observed mostly when low energy irradiation (2 Gy) was used, as with more intensive irradiation cells were showing signs of deterioration regardless of the miR-128 status: this observation is interesting because it suggests that
miR-128 overexpression could be a strategy to lower down the irradiation required in a potential clinical setting, thus limiting side effects associated to stronger irradiation.

Flow cytometry analysis of the treated cells showed that the decreased proliferation observed both in those overexpressing miR-128 or those simply treated with low energy irradiation was not secondary to induction of active cell death, but rather more likely resulted from a decreased proliferative capacity: indeed, other authors have shown miR-128 not to induce apoptosis when overexpressed in glioblastoma cells (198). Similarly apoptosis (and necrosis) is a known result of irradiation in glioblastoma cells, that is usually observed at energy levels greater than 2 Gy, particularly in cells that are resistant to irradiation like U87 or CD133-positive glioma stem cells (229). However the amount of dead cells observed when low energy irradiation was applied after miR-128 overexpression was significantly higher, with at least a 2-fold increase compared to the baseline levels observed in other experimental situations.

The final results obtained with the comet assay confirmed the mechanism whereby miR-128 expression is able to impair cell survival after irradiation: cells not overexpressing miR-128 display a certain amount of double strand DNA breaks immediately after irradiation, but overtime, they are able to repair it, and thus to overcome the damage and survive. Contrarily, cells overexpressing miR-128 clearly show a significantly decreased ability to repair DNA, and this likely account for the observed increase in cell death.
At this point, it appears evident that miR-128 has an effect on glioblastoma cells that transcends its antiproliferative capacity and that relates to its ability to impair DNA repair after double strand DNA break. It remains unclear whether this effect is mediated by the downregulating effect of mir-128 on Polycomb Proteins or on other still unrecognized miR-128 targets.
Chapter 4

Conclusions and Future Directions

This study on miR-128 provides evidence of how rather unspecific nature of microRNA targeting is actually fundamental for the regulation of action of cellular complexes that, like PRC1 and PRC2, are interdependent, but otherwise functionally “uncoupled”. The same unspecific and broad nature of action, and thus the ability to regulate multiple pathways, or complexes, at the same time, is a great opportunity for providing new perspectives to the study of the biology of cancer, that results from multiple aberrancies, and that would thus require intervention at multiple levels. Understanding the process that brings to impaired miR-128 expression in glioblastoma cells merits further investigation, as it has the potential to disclose regulatory processes high in hierarchy of processes involved in the transition from Neural Stem Cells to cancerous cells along with shedding some light on the still poorly understood process of gliomagenesis. Of great relevance will also be to study the relationship of miR-128 with the number of other microRNAs whose expression is consistently deregulated in glioblastoma: is it just the case that in most tumors the same microRNAs appear to be deregulated? Or is there a
common factor that controls the biology of a cluster of microRNAs that appear to be so relevant for the correct function of Neural Stem Cells? We suspect the second hypothesis to be true and this hypothesis will be object of our future investigations.

Furthermore, the evidence that miR-128 overexpression is synergistic to irradiation in determining increased cell death is a further demonstration that there are plentiful possibilities for the use of these small biological molecules in practical, therapeutic applications, which will require an improvement in delivery methods for in vivo studies.
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