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MicroRNAs and Cancer Stem Cells in Medulloblastoma

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1. Introduction

In this chapter, we are describing the biology of medulloblastoma influenced by several genes/pathways which concur to its pathogenesis. Of note, several levels of regulation are mediated by miRNAs functions, which we dissect their "state of art" to underline their crucial roles on controlling cancer development.
In brain tumours, literatures data, are supporting the values of Cancer Stem Cells (tumor propagating cells) and their functions for tumour recurrence for future therapeutic treatments. Thus, we link the potential use of miRNAs as "shuttle" to impair Cancer Stem Cells in medulloblastoma.

1.1 Medulloblastoma pathology and implication in medicine.

Medulloblastoma (MB) is an highly invasive embryonal tumor of the cerebellum, the most common malignant brain tumor in children and accounts for more than 25% of childhood cancer-related deaths (Wang et al., 2008). MB occurs bimodally, with peaks of incidences between 3 and 4 years and 8 and 9 years of age, even if can also arise in adults, showing the highest incidence at 20-34 years of age (Crawford et al., 2007). Patients with MB generally show symptoms of obstruction of cerebrospinal fluid flow and cerebellar disfunction including macrocephaly, vomiting and ataxia (Crawford et al., 2007). It is currently classified in several variants: classic, desmoplastic, anaplastic, large-cell and with extensive nodularity (Gilbertson and Ellison, 2008). The classic medulloblastoma is composed of small round or ellipsoid cells with a high nuclear to cytoplasmic ratio and round to oval or triangular hyperchromatic nuclei. The desmoplastic medulloblastoma is defined as having a biphasic architecture that consists of regions with dense intercellular reticulin and nodular reticulin-free zones, in which tumor cells show a neurocytic phenotype (McManamy et al., 2007). The Desmoplastic variant represents 50% of adult cases of MB and 15% of children related cancer. The original description of the large-cell medulloblastoma drew attention to the presence of large round cells with a prominent single nucleolus (Giangaspero et al., 1999). These cells occupy one end of the range of medulloblastoma cell size and have an area 2–3 times greater than the mean nuclear area of small round cells in classic tumors

(McManamy et al., 2003). The anaplastic medulloblastoma phenotype is applied to tumors dominated by combination of marked nuclear pleomorphism and high cell turnover, this phenotype is also present in a proportion of non–large-cell medulloblastomas, and accounting for almost 10%-22% of all MB tumors (McManamy et al., 2003, Brown et al., 2000, Eberhart et al., 2002). Because large-cell and anaplastic medulloblastomas share morphophenotypes and an aggressive biological behavior, are often grouped as large-cell/anaplastic (LCA) medulloblatomas, and represents the most malignant variant (Gilbertson and Ellison, 2008). Most medulloblastomas are confined to the posterior fossa, however all of the variants can metastasize and 11-43% of patients show disseminated disease either along the craniospinal axis, or, more rarely, to extraneural sites (Crawford et al., 2007). Disease dissemination rate, patient age and post-operative residual mass represent the most important prognostic markers for MB tumors. MB patients are indeed divided into risk-stratification groups with patients older than 3 years and gross or near-total surgical tumor resection assigned to the average-risk category, which accounts for 60%-70% of all MB patients; and patients with disseminated disease at presentation or greater than 1.5 cm^2 of residual tumor mass identified as high-risk category. A third stratification scheme is used for patients younger than 3 years old, who generally have worse outcomes mostly due to the increased risk of metastatic disease at presentation, increased rate of subtotal resection, and not receiving craniospinal radiation therapy (Crawford et al., 2007). Risk-adapted treatments are currently adopted in the management of the MB, including surgical tumor resection, radiotherapy and chemotherapy. Surgery represents the first approach and a fundamental part of MB treatment. It's aimed to the maximal tumor resection and has shown clear effects of survival improvement, particularly in patients with localized disease (Rutkowski et al., 2005). Addition of radiation therapy to the surgery, has allowed an overall improvement of MB patients survival. However, craniospinal axis irradiation often results in severe deleterious effects, particularly in infants, thus it is delayed or not given to children younger than 3 years (Gilbertson and Ellison, 2008). MB patients belonging to all risk-groups are also commonly treated with chemotherapeutic drugs, including vincristine, cyclophosphamide, etoposide and methotrexate. For younger patients, chemotherapy is widely used as the initial treatment, aimed to delay or avoid radiation therapy. Indeed, intensive postoperative chemotherapy alone showed promising results for treatment of young children without initial metastases (Rutkowski et al., 2005). Other therapeutic approaches include the use of myeloablative doses of chemotherapy followed by autologous stem cell rescue. Early studies have reported a survival improvement for patients with high-risk medulloblastoma; however, 15% of patients died of treatment-related toxicity (Perez-Martinez et al., 2005). Relapse of MB generally manifest within 2 years from the initial therapy in infant, and upon 5 years in adults. The management of patients with relapsing disease varies and depends on a range of factors including the age of the patient and the dissemination rate of the disease. However, surgery and possible combined use of chemotherapy and radiotherapy represent the leading therapeutic approach (Crawford et al, 2007). The employment of multimodality treatment regimens has significantly improved survival rates for MB. However, although patients belonging to the average-risk category show an overall survival rate approaching 70% to 80%, the high-risk patients are generally associated to a very poor prognosis, and MB results still incurable for more than one third of patients. Moreover, survivors commonly suffer of severe long-term side effects due to the aggressive treatments (Crawford et al., 2007, Gilbertson and Ellison, 2008).

The tumor's cell origin and the cellular pathways active in MB tumorigenesis are believed to play a crucial role in the prognosis and possibly response to therapy of MB (De Bont et al., 2008). Therefore, a better understanding of the pathology and molecular biology of medulloblastoma tumorigenesis is necessary, to identify more efficient therapeutic approaches, thereby improving survival and quality of life of MB patients.

1.2 Cancer stem cells in medulloblastoma

Once upon a time, cancer was viewed as a homogeneous mass of rapidly proliferating cells, and therapeutics were designed to eliminate highly proliferative cells. Recent studies have suggested that tumor cells are heterogeneous respect to proliferation and differentiation, and that a cell's proliferative rate may be a poor indicator of its tumorigenic potential. In several malignancies, the capacity to initiate and maintain tumor growth has been found to reside in a small population of cells called cancer stem cells (CSCs) (Al-Hajj et al.,2004, Reya et al., 2001, Wicha et al.,2006). Like normal stem cells, CSCs have the ability to self-renew and to give rise to the variety of proliferating and differentiated cells that make up the bulk of a tumor. Importantly, CSCs are often relatively quiescent and therefore may not be affected by therapies targeting rapidly dividing cells. Elevated expression of transporters that pump out chemotherapeutic agents (Donnenberg and Donnenberg, 2005) and an increased capacity to repair DNA damage (Bao et al., 2006a) may also contribute to CSCs' ability to survive conventional modes of therapy. The resistance of CSCs to conventional therapies may help explain why such therapies often fail: although they may destroy the bulk of a tumor, they cannot prevent the surviving CSCs from kicking into action and regenerating it again (Al-Hajj et al., 2004; Reya et al., 2001; Wicha et al., 2006). Moreover, the properties of CSCs appear to be influenced by both the specific genetic aberrancies in a given tumor as well as the stage of disease progression and the types of drugs used to challenge tumor growth. In some cases the number of Cancer Stem Cell able to generate a tumorigenic cascade are relatively rare, whereas in others CSCs can constitute a substantial proportion of the tumor mass. Moreover the cancer cells within a single tumor exist naturally in multiple states of differentiation that show distinct tumor-seeding properties. As suggested by Kelly, et al., 2007 and Quintana et al., 2008, CSCs representation may be a function of the cell type of origin, stromal microenvironment, accumulated somatic mutations and stage of malignant progression reached by a tumor, indeed selective pressures associated with neoplastic progression may lead to a higher frequency and to an higher variable properties of functionally defined CSCs in secondary or metastatic stages, as well inter-patient and intra-patient heterogeneity of CSCs (Kelly, et al., 2007, Quintana et al., 2008). The study of CSCs biology is predicated on the ability to accurately assess CSCs representation within cancer cell populations. However, measurements of CSCs representation are complicated by the quality of the host tissue in which tumor-initiating ability is assessed. Thus, animal hosts that offer a hospitable environment to engrafted tumor cells will yield measures of CSCs far higher than hosts that fail to do so. Indeed the use of immune-compromised mice make inaccurate the estimation of CSCs number do not taking into account that the immune system plays a pivotal role in a number of solid tumors (Bonertz et al., 2009). Another aspects of host biology that can affect cancer cell engraftment rate include vascularisation at the site of implantation, extracellular matrix constitution, growth factor availability. In light of these complexities, CSCs numbers cannot presently be stated in absolute terms, but only relative to the animal model used to measure CSC

representation. (Piyush et al., 2009) In this view, the CSCs could be viewed as a parody, an abnormal deviant of tumorigenic cell influenced by environment stimuli.

In the 2004, Singh and colleagues were the first to identify prospectively isolated population of CSCs in medulloblastoma. These cells were isolated by their ability of expressing the 120-kDa 5-transmembrane cell surface protein Prominin (CD133), which marks normal human neural stem cells. Between 1% and 21% of cells in freshly resected medulloblastomas express CD133, but only few as 1000 to 5000 of these CD133+ cells are capable of forming tumors. In their experiments Singh and colleagues after cells dissociation, using magnetic bead cell sorting, separeted the CD133 positive brain tumour cells from their CD133 negative counterparts. Only prominin positive cells when were transplanted into the brains of nonobese diabetic/severe combined immunodeficient (NOD/SCID) of six-week-old mice, are able to generate a newly tumor. Moreover from analysis of mouse brains following CD133+ cells engraftment revealed that as few as 100 CD133+ cells were sufficient for the formation of human brain tumours in NOD-SCID mice that were analysed at 12–24 weeks post-injection. In the same experiment they also showed that tumor formed resembles the original patient tumour, in fact when injected cells derived from classic medulloblastomas showed small round blue cell morphology characteristic histologic structures (Homer–Wright rosettes), while CD133+ cells derived from a different MB variant, desmoplastic medulloblastoma, recapitulated the cytoarchitecture associated with this subtype.

The xenograft and the original tumour both expressed the cytoplasmic primitive intermediate filaments, neural precursor cell marker, Nestin, Vimentin, the neuronal marker βIII-tubulin (TUJI), and show a high proliferative index (MIB-1), which is further increased in the xenograft. The astrocyte cell marker GFAP appared to be also expressed in a small number of cells in the patient tumour and xenograf.

Recently Read and colleagues and Ward and colleagues have identified a rare, phenotypically primitive, multipotent, and tumorigenic population of Ptc $^{+/-}$ cells that can be propagated by espressing the neural progenitor marker Math1 and Carbohydrate antigen (Stage Specific Embrionic Antigen 1, SSEA1) CD15. They have shown that into tumors from Ptc $^{+/-}$/ P53 $^{-/-}$, an acclaimed genetic MB mouse model, CD15 positive cells are able to recapitulate the heterogeneity within the original tumor and in particular are also able to generate both CD15 positive as well as CD15 negative cells, during proliferation, suggesting that these cells may sit at the top of a hierarchy of differentiation within the tumor. Those cells retain activated Hh and Notch signaling and do not necessarily display Ptc1 loss of heterozygosity (LOH) or loss of wild-type (WT) Ptc1 gene expression. Moreover their analysis revealed that CD15 cells have a distinct gene expression profile characterized by increased expression of genes associated with proliferation and self-renewal and decreased expression of genes involved in apoptosis and differentiation. Consistent with this expression profile, they also observed that CD15+ cells are more proliferative than CD15- cells which are thought to be genetically similar to human nodular/desmoplastic medulloblastomas (Read et al., 2009, Ward et al., 2009).

1.3 Pathways involved in MB cancer stem cells maintenance

The origins of MB are intrinsically linked to the cerebellum development and MB tumor initiating cells can originate from progenitor cells and neuronal stem cells of the cerebellum (Yang et al., 2008). Pathways, such as Shh, Wnt, Notch and AKT/PI3K, regulating the normal cerebellum development, play a crucial role in the MB tumorigenesis (Marino et al.,

2005). These pathways are considered to be the master regulators of cerebellum development, and several genes belonging to these pathways are frequently mutated or deregulated in human MBs.

The hedgehog signaling (Shh) pathway is pivotal to the development of most vertebrate organs and tissues, and has been implicated in birth defects and a multitude of tumour types (McMahon et al., 2003, Riobo and Manning, 2007, Wang et al., 2007). Genomic alterations in components of the Shh signalling pathway, specifically inactivating mutations of PTCH1 and SUFU and/or activating mutations of SMO, have been found in ~15% of sporadic medulloblastomas (Pietsch et al., 1997, Raffel et al., 1997, Reifenberger et al., 1998, Taylor et al., 2002). Additionally, germline mutations in PTCH1 cause Gorlin's syndrome, a rare congenital condition that is characterized by an increased incidence of several tumour types, including medulloblastoma (Hahn et al., 1996). Shh signalling is known to drive proliferation in the granule neuron precursors of the cerebellum, and pathway dysregulation resulting from genomic alterations of its components presumably drives medulloblastoma formation through analogous downstream effects (Pomeroy et al., 2003).

Cell signaling cascades activated by Wnt proteins (the Wnt signaling pathways) have been well conserved throughout evolution. In addition to regulating cellular processes including proliferation, differentiation, motility, and survival and/or apoptosis, the Wnt signaling pathways play key roles in embryonic development and maintenance of homeostasis in mature tissues. Dysregulation of the Wnt pathway has also been linked to the development of medulloblastoma. Wnt ligand binds to its receptor frizzled (FZD) leading to the release of its downstream effector β–catenin from an inhibitory complex that includes the tumour suppressor adenomatous polyposis coli (APC) and the axin proteins. Subsequent nuclear accumulation of β–catenin is thought to mediate its tumorigenic functions, presumably through the activation of target genes such as MYC, cyclin D1 (CCND1) and Re1–silencing transcription factor (REST), which have established roles in cellular proliferation, differentiation and inhibition of apoptosis (Eberhart et al., 2000, Rossi et al., 2008). Approximately 20% of sporadic medulloblastomas harbour mutations in ApC, AXIN1, AXIN2 or CTNNB1 (which encodes β–catenin) (Baeza et al., 2003, Huang et al., 2000, Koch et al., 2007, Koch et al., 2001, Zurawe et al., 2008), and a similarly sized fraction (18%) has separately been shown to exhibit nuclear β–catenin immunostaining (Eberhart et al., 2000). Furthermore, Turcot's syndrome, which is caused by mutations in APC, is characterized by an increased incidence of medulloblastoma and other neuroepithelial tumours. Finally, medulloblastomas that are driven by increased Wnt signalling, as shown by nuclear β–catenin staining, may follow a relatively favourable clinical course (Ellison et al., 2005).

The Notch pathway is a short-range communication system in which contact between a cell expressing a membrane associated ligand and a cell expressing a transmembrane receptor sends the receptor-expressing cell (and possibly both cells) a cell fate regulatory signal. The Notch pathway has repeatedly been linked to the biology of normal neural stem cells (Wang, et al., 2009). Ligand binding to the Notch receptor results in its cleavage and the release of the Notch intracellular domain (NICD), the subsequent nuclear translocation of which activates various target genes. The Notch pathway is activated in MB (Shih et al., 2006). Furthermore, increased Notch signalling enhances the efflux of cytotoxic drugs through ABC transporters such as ABCG2, a recognized property of stem-like tumour cells that contributes to their resistance to conventional therapies (Bhattacharya et al., 2007, Fan et al., 2006). Using fluorescent Hoechst dye, which is also an ABCG2 substrate, stem cells can

be effectively sorted by fluorescence-activated cell sorting (FACS) from brain tumours as a 'side population' (SP) that exhibits a lower level of fluorescence than their non-stem cell-like counterparts in the 'main population' (MP) (Fan et al., 2006,Bleau et al., 2009). NICD overexpression increases SP cell number, with Notch pathway inhibition having the opposite effect (Fan et al., 2006). Notch signaling also has been linked to MB progression by promoting a stem-like state (Jason et al., 20010, Eberhart et al., 2007). Several lines of evidence have linked Notch signaling to MB engraftment and progression. Notch pathways are upregulated in MB and increased expression of HES1, a target of both the canonical notch pathway and the non-canonical shh pathway, is associated with poor prognosis in MB patients (Fan et al., 2004, Hallahan et al., 2004, Ingram et al., 2008). Notch2 and Hes5 are overexpressed in the shh-activated SmoA1 mouse, suggesting that activation of the shh pathway is sufficient to induce notch pathway genes (Hallahan et al., 2004). Previous studies using human medulloblastoma cell lines have suggested that Notch signaling is required for maintaining subpopulations of progenitor-like cells potentially capable of re-populating tumors after initial therapy (Fan et al., 2006), and that notch pathway inhibition can limit tumor cell growth (Hallahan et al., 2004, Fan et al., 2006). Recent findings indicats that the Notch pathway is not essential for shh-driven medulloblastoma genesis or maintenance Notch signaling is not essential for the initiation, engraftment, or maintenance of sonic hedgehog pathway driven MB (Hatton et al., 2010). This interpretation is supported by the accompanying article that evaluates MB formation in the absence of RBP-J, which is a convergence point of all notch pathways (Julian et al., 20010).

The AKT/PI3K pathway has also been identified as a major effector of stem-like behaviour in malignant brain tumours. Increasing AKT signalling through PTEN loss increases SP cell number in mouse glioblastomas, at least partially through the direct activation of ABCG2 (Bleau et al., 2009). Furthermore, in mouse medulloblastoma models, activation of the PI3K–AKT–mTOR pathway seems to contribute to the relative resistance of perivascular CSCs to therapeutic irradiation (Hambardzumyan et al., 2008). Combining small molecule AKT pathway inhibitors with radiotherapy significantly decreases the survival of this resistant stem cell-like pool, indicating a promising avenue for future treatment strategies. Supporting these findings, another group has recently reported that PTEN loss and Tp53 deletion are crucial for the maintenance of self-renewal in neural stem cells and seem to mediate these effects at least in part through the induction of MYC (Zheng et al., 2008).

The cross talk among these pathways provides an interpretation for the synergy in the regulation of MB progression and in CSCs maintenance. Thus the low penetrance in medulloblastoma tumor formation in transgenic mice with single pathway deregulation would be explained by the need to target multiple pathways to achieve a high frequency of tumor formation. Shh signaling synergizes with both Notch and Wnt signaling in medulloblastoma development by controlling Notch and Wnt pathway ligand, receptor and/or target gene expression. Notch2 and the Notch target gene, HES5, were found significantly elevated in Smo-induced medulloblastoma in transgenic mice, showing that Shh pathway activation is sufficient to induce Notch pathway signaling (Hallahan et al., 2004). Expression of several components of the Notch and Wnt signaling pathways, and activation of Notch signaling were observed in medulloblastoma from Ptch mice that have elevated Shh signaling. Marked downregulation in the expression of Notch2, Jagged1, Hes1, in cerebella of developing mice with reduced Shh signaling was also observed, suggesting that Shh signaling regulates the expression of these genes (Dakubo et al., 2006). Indeed

neuronal precursor cells in the developing cerebellum require activity of the sonic hedgehog (Shh) and phosphoinositide-3-kinase (PI3K) pathways for growth and survival. Synergy between these signaling pathways are implicated in the neuronal precursor cell cycle progression in MB. Recent studies, also show that molecular cross-talk between the beta-catenin and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways is crucial to sustain medulloblastoma pathophysiology, in fact, constitutive activation of phosphoinositide-dependent protein kinase 1 (PDK1), Akt, and phosphorylation of GSK-3beta was detected by immunohistochemistry in all primary medulloblastomas examined. Small-molecule inhibitors targeting the PI3K/Akt signaling pathway induced beta-catenin signaling by activation of GSK-3beta, resulting in cytoplasmic retention of beta-catenin and reduced expression of its target genes cyclin D1 and c-Myc (Baryawno et al., 2009).

2. MiRNAs functions in cancer

In addition to protein-encoding genes, a second class of genes producing small noncoding RNAs (i.e. microRNAs) has been discovered over the last few years. These short RNAs (18- to 24-nucleotides) bind to cis-regulatory elements mainly present in the 3′ UTR of mRNAs, resulting in translational inhibition or mRNA degradation (Bartel 2004; Hammond 2006). In mammals, miRNAs are predicted to control the activity of ~50% of all protein-coding genes. Functional studies indicate that miRNAs participate in the regulation of almost every cellular process investigated so far and that changes in their expression are associated with many human pathologies (Krol J., et al., 2010; Jeffrey T. DeSano and Liang Xu, 2009). Furthermore, microRNAs have been recently shown to be useful tools to silence cancers, they might be able to fill this gap through their control of multiple target genes. MicroRNAs have been linked to the initiation, progression, and metastasis of human malignancies, with some species displaying oncogenic and others tumor suppressive potential (Calin, Croce 2006; Chen 2005). They are often expressed aberrantly in tumors as compared to normal tissues and are likely to contribute to tumorigenesis by dysregulating critical target genes (Esquela-Kerscher and Slack 2006; Kent and Mendell 2006; Croce 2009). The case for miRNAs as tumor suppressors and oncogenes reflects their loss or gain, respectively, as a function of neoplastic transformation, their dysregulation in different tumors, the relevance of their mRNA targets to mechanisms underlying tumorigenesis and their ability to alter tumorigenesis directly in model cells and organisms (Esquela-Kerscher and Slack 2006; Kent and Mendell 2006; Croce 2009; Garzon 2009). Typically, miRNAs that serve as oncogenes are present at high levels, which inhibit the transcription of genes encoding tumor suppressors. Conversely, tumorsuppressor miRNAs are present at low levels, resulting in the overexpression of transcripts encoded by oncogenes (Esquela-Kerscher and Slack 2006).

The best characterized tumor-suppressor miRNAs are miR-15a and miR-16-1 B-cell chronic lymphocytic leukemia (CLL) (Calin, 2002). Tumor suppression by miR-15a and miR-16-1, in part, reflects inhibition of the expression of the anti-apoptotic oncogenic protein Bcl-2, which is characteristically overexpressed in CLL, promoting the survival of leukemia cells. Expression of these miRNAs inhibits cell proliferation, promotes apoptosis of cancer cells, and suppresses tumorigenicity both in vitro and in vivo. miR-15a and miR-16-1 function by targeting multiple oncogenes, including BCL2, MCL1, CCND1, and WNT3A. Down-regulation of these miRNAs has been also reported in pituitary adenomas, and prostate carcinoma (Aqeilan 2009).

Another important microRNA is the Let-7 that act as tumor suppressor gene targeting the human Ras family of proteins, oncogenes that are commonly mutated in many human tumors. Indeed, K-Ras and N-Ras expression in human cells is regulated by let-7 family members. Moreover, loss of let-7 expression in human tumors correlates with the overexpression of Ras proteins (Johnson 2005). MIR Let-7 its family members are highly conserved across species in sequence and function, and misregulation of let-7 leads to a less differentiated cellular state and the development (Roush and Slack, 2008).

The best characterized microRNA that acts as oncogenes is the cluster of miR 17 comprises a group of six miRNAs (miR-17-5p, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92) that are overexpressed in many types of tumors, including lymphoma, colon, lung, breast, pancreas and prostate (O'Donnell et al., 2005; Mendell 2008). Expression of these miRNAs promotes cell proliferation, suppresses apoptosis of cancer cells, and induces tumor angiogenesis. New work reveals essential functions for these miRNAs not only in tumor formation but also during normal development of the heart, lungs, and immune system. The human genomic locus encoding these miRNAs, 13q31.3, undergoes amplification in several types of lymphoma and solid tumors. The best-characterized target of the miR-17 cluster is the E2F1 transcription factor and furthermore, the transcription of the miR-17-92 cluster is directly transactivated by c-Myc (O'Donnell et al., 2005; Mendell 2008).

Moreover, also miRNA-21, plays a crucial role in cancer, in fact it is overexpressed in many solid tumors, including breast, colon, lung, prostate and stomach, and in endocrine pancreas tumors, glioblastomas and uterine leiomyomas (Si et al., 2007). This miRNA is encoded at chromosome 17q23.2, a genetic locus that is frequently amplified in many tumors. The tumorigenic effects of miR-21 are mediated, in part, by targeting a number of mediators in critical cell-survival pathways. Thus, in glioblastoma cells in vitro, miR-21 modulates the expression of the common tumor suppressor PTEN, a central regulator of cell growth, proliferation and survival, which is mediated by the in phosphatidylinositol 3-kinase / Akt pathway. Also, miR-21 regulates breast cancer cell growth by reciprocally regulating apoptosis and proliferation, in part reflecting regulation of the anti-apoptotic protein, Bcl-2. In vitro studies have demonstrated that miR-21 knockdown in tumor cell lines leads to increased apoptotic cell death (Seike et al., 2009). Furthermore, miR-21 depletion reduces the growth of tumor cell lines xenografts in mice (Si et al., 2007). The transcription of miR-21 primary RNA is controlled by a conserved upstream enhancer, which has been demonstrated to be regulated by gp130-activated Stat3 in myeloma cells, and by AP-1 in promyelocitic differentiation induced by TPA (Loffler et al., 2007; Fujita et al., 2008). AP-1 activity is necessary, but not sufficient, for the induction of miR-21 triggered by Ras (Talotta et al., 2009). The miR 21 is recently been studied also in thyroid and lung tumors where it is upregulated both in vitro and in vivo by oncogenic Ras. MiR-21 regulation by Ras occurs with a delayed kinetic and requires at least two Ras downstream pathways. A screen of human thyroid cancers and non-small-cell lung cancers for the expression of miR-21 reveals that it is overexpressed mainly in anaplastic thyroid carcinomas, the most aggressive form of thyroid cancer, whereas in lung its overexpression appears to be inversely correlated with tumor progression. A LNA directed against miR-21 slows down tumor growth in mice. Consistently, a search for mRNAs downregulated by miR-21 shows an enrichment for mRNAs encoding cell cycle checkpoints regulators, suggesting an important role for miR-21 in oncogenic Ras-induced cell proliferation (Frezzetti et al., Oncogene 2010, in press).

2.1 MiRNA involved in Medulloblastoma and cancer stem cells biology

In MB, up to date, few microRNAs have been studied. MiR-17/92 is best characterized, in 6% of pediatric MBs it's found a recurrent amplification of the miR-17/92 polycistron proto-oncogene (Uziel et al., 2009). Profiling the expression of 427 mature miRNAs in a series of 90 primary human MBs revealed that components of the miR-17/92 polycistron (miR-92, miR-19a, and miR-20) are the most highly up-regulated miRNAs in MB. Expression of miR-17/92 was highest in the subgroup of MBs associated with activation of the sonic hedgehog (Shh) signaling pathway compared with other subgroups of MB. MBs in which miR-17/92 was up-regulated also had elevated levels of MYC/MYCN expression. Consistent with its regulation by Shh, the Shh treatment of primary cerebellar granule neuron precursors (CGNP), proposed cells of origin for the Shh-associated MBs, resulted in increased miR-17/92 expression. In CGNPs, the Shh effector N-myc, but not Gli1, induced miR-17/92 expression. Ectopic miR-17/92 expression in CGNPs synergized with exogenous Shh to increase proliferation and also enabled them to proliferate in the absence of Shh. MiR-17/92 is a positive effector of Shh-mediated proliferation and that aberrant expression/ amplification of this miRNA confers a growth advantage to MBs (Uziel et al., 2009). Northcutt and colleagues identified a high-level, focal amplification on chromosome 13q31.3, which mapped to the same miRNA cluster. The expression of miR-17/92 was most elevated in MBs with activated hedgehog signalling and was also associated with elevated c-Myc and n-Myc. These studies suggest that aberrant expression of miRNAs encoded by the miR-17/92 enhance the growth potential of MB and that miRNA-mediated modulation of hedgehog signaling may be an important contributing factor to MB pathogenesis (Northcutt et al., 2009).

A high throughput microRNA expression profiles was performed in human primary MB specimens to investigate microRNA involvement in MB carcinogenesis. It has been identified specific microRNA expression patterns which distinguish MB differing in histotypes (anaplastic, classic and desmoplastic), in molecular features (ErbB2 or c-Myc overexpressing tumors) and in disease-risk stratification (Ferretti et al., 2008). They proposed a role for miRNAs in modulating hedgehog signaling. In details, they showed that miR-125b, miR-326, and miR-324–5p expression was decreased in MB and that the altered expression of these miRNAs led to tumor cell proliferation through a hedgehog-dependent mechanism. Furthermore, they used high-throughput screening to examine miRNA expression profiles in 34 patients with MB. They identified 78 miRNAs with altered expression in MB, compared with normal adult and fetal cerebellar cells. Several of the identified miRNAs have been implicated in other cancer types including glioblastoma (Ciafrè et al., 2005). The majority of these miRNAs were downregulated in MB, supporting a role for miRNAs as tumor suppressors. Additionally, they found increased expression of miR-9 and miR-125a and that increased expression of these miRNAs was capable of decreasing proliferation, augmenting apoptosis, and ultimately promoting arrest of tumor growth. The proapoptotic effect was mediated by miR-9 and miR- 125a targeting of the t-TrkC receptor, which was found in this study to be upregulated in MB cells. The authors also found that miR-let7g, miR-19a, miR-106b, and miR-191 were significantly upregulated in anaplastic compared with desmoplastic MBs; miR-let7g and miR-106b were differentially expressed in desmoplastic compared with classic MBs. Changes in expression of Her2 (ErbB2) and c-Myc have been demonstrated to impact biological activity and clinical features of MB. (Gilbertson et al., 1997; Grotzer et al., 2001; Herms et al., 2000). Ferretti et al. examined miRNA expression from MBs overexpressing either Her2 or c-Myc and identified

an miRNA signature in each group of MBs. Expression of miR-10b, miR-135a, miR-135b, miR-125b and miR-153 was altered in Her2-overexpressing tumors, whereas c-Myc overexpressing MBs had expression changes in miR-181b, miR-128a, and miR-128b. Additionally, the amount of expression change of 2 miRNAs correlated with disease risk. Though miR-31 and miR-153 were downregulated in all MBs, the group found that the degree of change was directly proportional to disease severity (Ferretti et al., 2009). Although, these data of Ferretti et al., are of interest, miRNAs signature is sicking of validation in a large tumors cohorts analysis.

MiR-124, is another miRNA, preferentially expressed in differentiating and mature neurons and in external granule cells of cerebellum that are thought to be cells of origins of MBs (Pierson et al., 2008). MiR-124 deregulation is common in MBs, and restoration of its function inhibits cell proliferation, suggesting that it may act as a growth suppressor. Two target of miR-124 have been studied: cyclin dependent kinase 6 (CDK6) that was identified as an adverse prognostic marker in MB and SLC16A1 that may represent a novel therapeutic target for treatment of malignant MBs (Pierson J. et al., 2008). SLC16A1, solute carrier family 16, functions to efflux lactic acid during aerobic glycolysis, and its inhibition resulted in a decrease of intracellular pH to a lethal level. This study demonstrates that miR-124 deregulation is common in medulloblastomas, and restoration of its function inhibits cell proliferation, suggesting that miR-124 may act as a growth suppressor, raising the possibility that the miR-124/SLC16A1 pathway may represent a novel therapeutic target for treatment of malignant medulloblastomas (Li et al., 2009).

A recent work, demonstrated that miR-30b and miR-30d are amplified in MB and are putative oncogenic target(s) of a novel recurrent medulloblastoma amplicon at 8q24.22-q24.23 (Lu Y. et al., 2009). Furthermore, miR-128a, inhibits growth of medulloblastoma cells by targeting the Bmi-1 oncogene. This miRNA alters the intracellular redox state of the tumor cells and promotes cellular senescence. MiR-128a has growth suppressive activity in medulloblastoma and this activity is partially mediated by targeting Bmi-1. This data has implications for the modulation of redox states in cancer stem cells, which are thought to be resistant to therapy due to their low ROS states (Venkataraman S, et al., 2010). A complete list of miRNAs implicated in MB biology with their function is showed in table 1.

Our research represents the first work that discovers a microRNA that regulate the Notch pathway and depletes the tumor stem cells compartment by using an in vivo therapeutic approach using an adenovirus type 5 as carrier, indicating the possibility for the targeting of these cells in brain tumors (Garzia et al., 2009). We identified miR199b-5p as targeting HES1, the principal Notch effector. We started our study by an in-silico analysis of the mirBase targets database (Griffiths-Jones, 2004) towards identification of miRNAs potentially targeting HES1. To determine whether HES1 is a target of miR-199b-5p, the HES1 3′ untraslated region (UTR) was cloned downstream of a luciferase reporter gene vector; pre-miR-199b-5p was also cloned in a mammalian expression vector. HEK-293 cells were then transfected with the relative luciferase activity showing that miR-199b-5p co-transfection decreased reporter gene activity, thus indicating binding with the 3′UTR and destabilisation of productive translation of luciferase mRNA. To determine the role of miR-199b-5p in MB cell biology, the miR-199b-5p expression construct was transfected into Daoy cells, and several stable clones over-expressing miR-199b-5p were selected. These effects of miR-199b-5p on HES1 protein expression were not restricted to the stable clones or Daoy cells, as D283MED cells transiently transfected with the expression construct for miR-199b-5p also

showed reduced HES1 levels. Furthermore, we silenced the expression of the miRNA 199b-5p by 2-ossy-methyl antisense ribonucleotide in daoy cell line overexpressing the miRNA 199b-5p and we demonstrated that HES1 levels were restored, suggesting 2-OM block of HES1 repression by miR-199b-5p, providing further confirmation that miR-199b-5p targets HES1 directly. The clones overexpressing the miRNA 199b-5p had reduced proliferation rates under standard culture conditions, when compared to the control clone. The clones also showed a decreased in S-phase fractions, and an increase in cells in G0-G1 as compared to the empty vector clone suggesting that exit from the cell cycle has a role in the reduced proliferation of miRNA 199b-5p stable clones.

We then evaluated the effects of the induction of miR-199b-5p on molecular markers of proliferation and differentiation by a real time approach. MAP2, which is mostly expressed in mature neurons, was up-regulated in the miR 199b-5p stable clones. Similarly, GFAP levels were increased. Among the other genes, GABRA6, a marker of cerebellar granule cell differentiation, was also significantly over-expressed in the stable clones. A fine-tuned cascade of positive and negative bHLH transcription factors is central to neurogenesis, with genes such as MASH1, MATH3 and NGN2 inducing neurogenesis. Both miR-199b-5p stable clones showed increases in expression of pro-neural bHLH. In agreement with their reduction in proliferation rate, the proliferation markers c-Myc and cyclin D1 were decreased demonstrating that these stable clones had an impairment in proliferation rate and on the other hand they acquired markers of cerebellar differentiation. We next examined in a standard clonogenic assay, if the anchorage independent growth was affected by miR-199b-5p. There was a significatively reduction in colony formation potential in miR 199b-5p stable clones, compared to the empty-vector clone.

The Notch pathway has been linked to the fraction of MB tumor cells that harbour precursor stem-cell markers (Fan et al., 2006), and HES1 has a role in self-renewing of multipotent progenitor cells (Nakamura et al., 2000). This side population (SP) of tumor cells has a role in the engrafting of a tumor in animal models (Kondo et al., 2004). We thus examined the influence of miR-199b-5p on the population of tumor cells that exclude the Hoechst 33342 dye, a strategy to identify these SP cells. This was determined by flow cytometry in the Daoy cell line, the SP was decreased in miR 199b-5p overexpressing clones, as compared with the empty vector clones. It is also known that central nervous system tumor stem cells express the CD133 antigen, and that these cells are uniquely capable of tumor formation in NOD-SCID mice (Singh et al., 2004; Singh et al., 2004). Additionally, the Notch pathway has a central role in the self renewing process, with its inhibition leading to depletion of CD133-positive (CD133+) Daoy cells via induction of apoptosis of progenitor-like cells (Fan et al., 2006). Recently it was shown that CD133+ Daoy cells promote tumor growth in the flank of nude mice, while CD133 - cells do not (Eberhart 2007). For these reasons, we evaluated the CD133 positivity of Daoy cells as compared to the miR 199b-5p stable clones showing the significatively reduction of the CD133+ cells. This thus demonstrated a role for miR-199b-5p in negative regulation of this fraction of tumor-initiating cells. We demonstrate that the miR 199b-5p depletes the side population compartment in the Daoy cell line and negatively regulates MB tumor stem-cell populations positive to CD133 antigen, that are the uniquely capable of tumor formation in NOD-SCID mice.

We further study the role of miRNA 199b-5p in an in vivo tumor model stabilizing the previous clones overexpressing miR 199b-5p and control clone with an expression vector carrying luciferase cDNA. These stable obtained, were then injected into the left and right flanks, respectively, of five athymic nude/nude mice. Tumor growth was evaluated by

weekly in-vivo bioluminescence imaging (BLI) of injected mice. Overall, after 8 weeks a significant difference in tumor volumes between control flanks and miR-199b-5p flanks was seen and also the bioluminescence measurements showed significant reductions in emission for the miR-199b-5p sides during tumor growth, as compared with the control sides. MiR-199b-5p thus can impair tumor formation in vivo in athymic nude mice. To further investigate the ability of miR-199b-5p to regulate MB growth, we injected the stable clones orthotopically into the fourth ventricle of nude mice of 5 weeks of age. After four weeks of in vivo non-invasive monitoring tumor growth by BLI, in mice injected with the clone overexpressing the miR 199b-5p, the tumor growth was considerably lower than that observed in the control cells injected side. As further confirmation of these effects, we also injected the daoy cells infected with an adenovirus coding for miR-199b-5p: in agreement with the previous findings, these mice also showed reduced BLI after 4 weeks. Hematoxylin-eosine staining of frozen tissues showed tumor mass in the cerebellum of the injected animals. Serial parallel frozen histological sections were examined by fluorescence microscopy for endogenous green fluorescence protein (GFP) expressed by adenovirus-infected cells. Then, we evaluated HES1 protein expression by immunohistochemistry staining of other paraffin-embedded tissues, using an anti-HES1 antibody. Overall, we assessed the levels of persistence of adenovirus expression in infected cells, as the down-regulation of HES1 expression due to miR199b-5p carrying the adenovirus expression, thus following tumor growth over time by BLI. Then, two additional nude mice underwent PET-CT studies at 12 weeks post-injection, to assess tumor proliferative activity. The BLI data were showed also by 3D reconstruction of the orthotopic engraftment. These analyses showed significant reduction of tumor mass in the animal injected with the cells infected with an adenovirus carrying the miR 199b-5p, as compared to the control mice, with PET-CT analyses also providing tumor volumes (0.024 cm3 versus 0.044 cm3, respectively). Overall, these data indicate a beneficial effect of over-expression of miR199b-5p, as a negative regulator of tumor growth of MB cells in this orthotopic xenograft nude-mouse model.

We focus our attention on the expression of the miRNA 199b-5p in human cerebella tissues and human MB tissues. We used 13 control samples obtained from the NICHD Brain and Tissue Bank for Developmental Disorders, at the University of Maryland, USA. We measured miR-199b-5p expression, comparing five cerebellum samples obtained from 0–1-year-old children with six from 13–16- year-old children by miRNAs PCR real time TaqMan assay. MiR-199b-5p showed greater expression in the explants from the younger healthy controls. To determine whether miR-199b-5p expression has a role in human MB, samples from a cohort of 61 MB patients were analysed. Indeed, it has already been shown that HES1 protein levels correlate with negative outcome in MB patients (Fan et al., 2004). The whole patient population (n= 61) was then divided into two groups, as low versus high miR- 199b-5p expression, based on the overall median. The distribution of miR-199b-5p expression between non-metastatic (M0) and metastatic (M1, M2 and M3) cases showed that miR-199b-5p expression in the non-metastatic cases was significantly higher than in the metastatic cases. In the subset of patients where follow-up information was available (n= 45), the survival curve for the patients who expressed miR-199b-5p at high levels showed a positive trend, with better overall survival than the low-expressing patients. These data showing down-regulation of miR-199b-5p in metastatic MBs indicates a mechanism of silencing through epigenetic or genetic alterations. We thus tested expression of miR-199b-5p by real-time PCR TaqMan assay in a panel of MB cell lines following induction of de-methylation with 5-aza-deoxycytidine. Indeed, two cell lines (Med8a and UW228) showed significant up-

regulation of miR-199b-5p, thus supporting the hypothesis of epigenetic control of miR-199b-5p expression. We can conclude that the expression levels of miR-199b-5p in M0 and M+ patients, might be due to genetic and epigenetic regulation during carcinogenesis. In patients with moderate or high expression of the miRNA 199b-5p, an increase in miR-199b-5p expression represses HES1, which then leads to an increase in pro-neural bHLHs gene expression, driving the cell towards differentiation processes. In patients with moderate or low miR-199b-5p expression the genetic or epigenetic control mechanisms, silenced the miRNA 199b-5p expression and then HES1 is over-expressed, leading to cell proliferation and induction of the SP and hence an increase in CD133+ cancer stem cells. As for many other transcription factors, HES1 is a point of integration between and among different signal transduction pathways, and its balance of expression determines fundamental cell decisions, such as whether or not to start a differentiation program. With this scenario, miR-199b-5p can be seen as part of the complex Notch signal-transduction pathway, as a fine regulator of expression levels of the HES1 bHLH transcription factor. These phenomena could be considered to occur in a variety of tissues and cancers where an activated Notch pathway is involved.

The novelties of our work are the finding of a new mechanism of regulation of Notch pathway (a fundamental signal transduction in MB development) mediated by a miRNA. The miRNA 199b-5p inhibits the Hes1 expression by binding to its 3'UTR, it is known that HES1 plays a crucial role in MB biology because high levels of Hes1 protein expression correlate with negative outcome in MB patients by negatively influence on their survival rate (Fan et al., 2004). Furthermore, the other novelty is the role of the miRNA 199b-5p in the impairing of the MB cancer stem cells by decreasing both SP cells and CD133+ cells. In MB, the expression of the neural stem cell marker CD133 has been associated with both tumour initiation capacity and radioresistance, so it is of fundamental the expression of the miRNA 199b-5p in directly target of these cells.

It is well know that one miRNA can target more than hundred of target genes (Peter, 2010), this enhance the need to study the so called "off target effect" to exclude possible unwanted side effects. We used an *in vivo* xenographt model performed by Daoy cell line, we can further use another cell line of classic histotype to confirm the same data as we evaluated *in vitro* with UW228 and D283 cell lines. Moreover, the miRNA 199b-5p function can be evaluated in MB genetic mouse models as $Ptc1^{+/-}/p53^{-/-}$ through a direct injection of the adenovirus carrying the miRNA 199b-5p in the cerebellum of mouse. Moreover, the survival analysis can be extend in a larger cohort of MB patients with consistent time of follow-up data to verify the implication of this miRNA on survival rate.

2.2 Future research

We are proceeding with the study of miRNA 199b-5p role in MB, particularly we are evaluating the finely regulation mechanism of this miRNA by its target and by other genetic and epigenetic changes. Furthermore, we are studying its possible regulation of the other marker CD15 of MB Cancer Stem Cells. The multiple effects of this miRNA function can be due to the inhibition of multiple pathways involved in MB progression, for this reason, we are studying all the other possible targets.

Finally, other miRNAs involved in medulloblastoma pathogenesis and in the impairing of MB Cancer Stem Cells are under investigation. One of this, is miRNA 34a, another regulator of the Notch pathway (Li et al., 2009) by targeting several pathways/genes of potential interests for its therapeutic application.

3. Conclusion

The study of miRNAs in medulloblastoma and in the other brain tumors is still at the beginning, but there is strong evidence of miRNAs involvement in medulloblastoma tumor development and progression also by regulation of Cancer Stem Cells. We shows our hypotesis by a schematic model pictured in figure 1, in which we underline the crucial role of miRNAs that impaired the CSCs in MB. These miRNAs act by a directly hit of the CSCs that are responsible of the origin and the progression of MB (Fig.1). It is becoming clear that miRNAs are essential regulators of many of the key pathways implicated in tumor pathogenesis. While adding another layer of complexity, the discovery of the role miRNAs in brain tumors has also revealed a new category of therapeutic targets. As miRNA research continues to evolve, novel therapeutic targets for the treatment of brain tumors will continue to emerge in the near future.

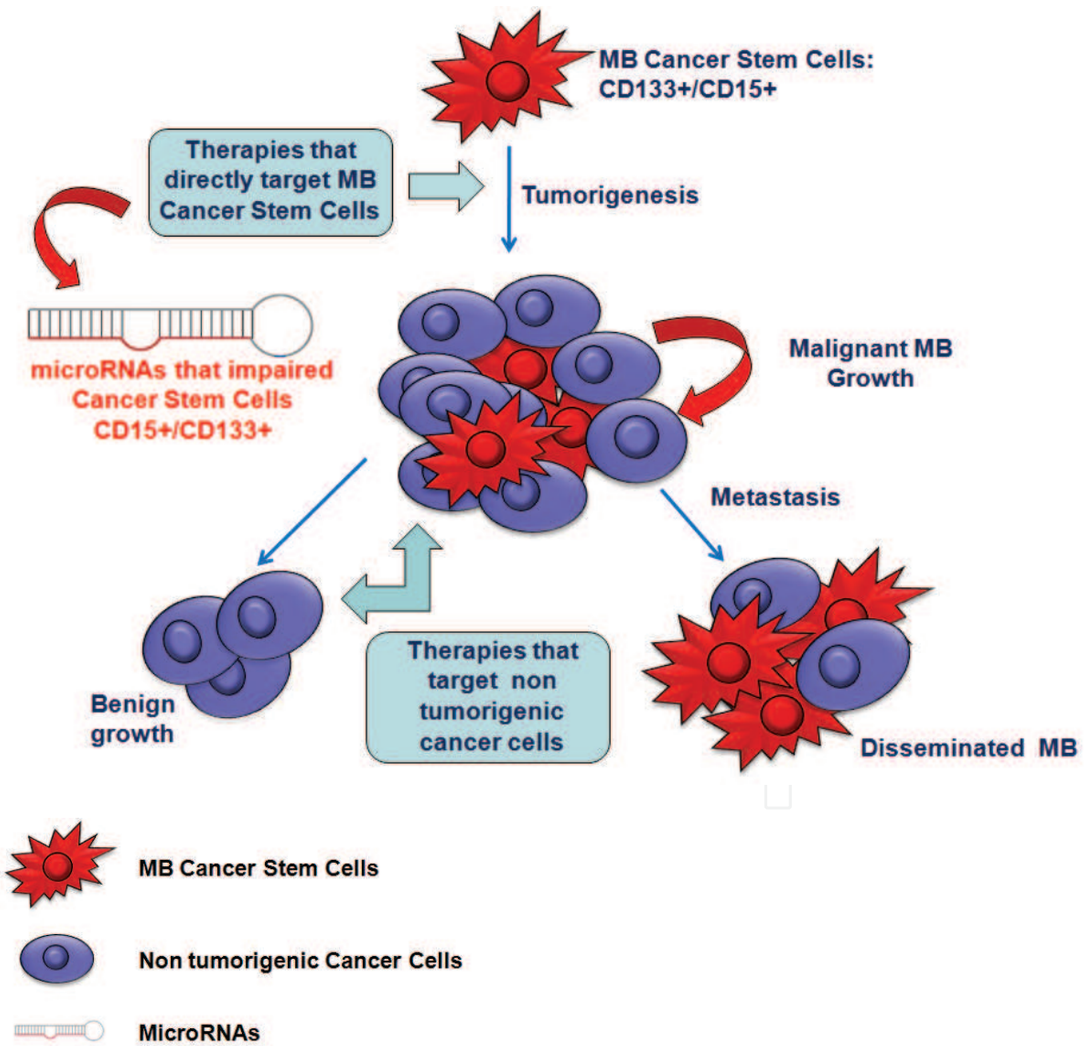

Fig. 1. Schematic model of microRNAs function in MB Cancer Stem Cells.

MiRNAs ID	MIRNAs function in MB	References
miR-124	Regulation of cell cycle by CKD6	Pierson J, et al., 2008
miR-199b-5p	Impairement of Cancer stem cells CD133+ by Notch inhibition	Garzia et al., 2009
miR-let7g, miR-9, miR-106b, miR-125a-b, miR-191	Regulation of proliferation and apoptosis of MB cells	Ferretti et al., 2009
miR-324-5p, miR-326, miR-19a, miR-20, miR-92	Hedgehog dependent proliferation	Ferretti E., et al., 2008
miR-17-92	Over-expressed in the subgroup of MBs associated with activation of the sonic hedgehog (Shh) signaling pathway and elevated levels of MYC/MYCN expression	Uziel T.et al., 2009; Northcutt PA. et al., 2009
miR128	Inhibition of medulloblastoma cells growth by targeting Bmi-1	Venkataraman S, et al., 2010
miR30b, miR30d	Target of a novel recurrent medulloblastoma amplicon at 8q24.22-q24.23	Lu Y. et al., 2009

Table 1. MiRNAs involved in MB biology

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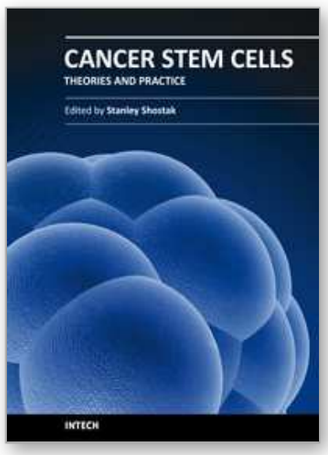

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Cancer Stem Cells Theories and Practice does not 'boldly go where no one has gone before!' Rather, Cancer Stem Cells Theories and Practice boldly goes where the cutting edge of research theory meets the concrete challenges of clinical practice. Cancer Stem Cells Theories and Practice is firmly grounded in the latest results on cancer stem cells (CSCs) from world-class cancer research laboratories, but its twenty-two chapters also tease apart cancer's vulnerabilities and identify opportunities for early detection, targeted therapy, and reducing remission and resistance.

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