Milano-Bicocca University

DIMET PhD-Translational and Molecular Medicine

CONSULTAZIONE TESI DI DOTTORATO DI RICERCA

La sottoscritta **Maria Carmela Speranza** (**matricola. 745157**), nata a Vallo della Lucania (SA) il 2 maggio 1983

Autore della tesi di DOTTORATO dal titolo:

# NEDD9, a novel target of miR-145, increases the invasiveness of glioblastoma

Autorizza

la consultazione della tesi stessa, fatto divieto di riprodurre, in tutto o in parte, quanto in essa contenuto.

Data Firma

“A true adventure starts when imagination collides with reality”. Science is an adventure. We (the researchers) are adventurers and dreamers.

— Karel Capek

## Table of Contents


***Chapter 1*** **7**

**General Introduction** 8

Glioblastoma: "The Terminator" 8

Glioblastoma: Molecular Classification 10

Glioblastoma: Treatments 14

MicroRNAs: the functions 16

MiR-145 and cancer 19

HEF1/CAS-L/NEDD9 23

NEDD9 and cancer 24

**Scope of the Thesis** 27

**References** 29

***Chapter 2*** 43

NEDD9, a novel target of miR-145, increases the invasiveness of glioblastoma.

Oncotarget. 2012 Jul;3(7):723-34. PMID:22869051

***Chapter 3*** 88

**Summary** 89

**Conclusions** 91

**Future Perspectives** 94

**References** 97

**Acknowledgement** 104

# *Chapter 1*

# General introduction

## Glioblastoma: "The terminator"

Glioblastoma (GB) is the highest-grade glioma tumor and one of the most common primary malignant brain tumor (3.5 GB cases per 100,000 people per year) [1]. The GB hallmarks consist in aggressive growth, angiogenesis, resistence to radiation and chemotherapy, infiltrative ability and invasion. The tumor cells are able to invade the surrounding tissue making this tumor a surgically incurable disease and tumor recurrence almost inevitable, with 90% of patients developing new lesions within 2-3 cm of the original site or at distant sites in the brain [1–3]. For these reasons, GB is one of the most lethal human cancers: patients have a median survival of 12–15 months and only the 3% 5-year survival rate, significantly worse than the 60% survival rate seen for other brain tumors such as oligodendroglioma and medulloblastoma [4–6]. In addition, GB is typically characterized by complex chromosome abnormalities and extensive cytogenetic and histological heterogeneity. Indeed, cytogenetically related or unrelated clones coexist in different regions within the same tumor increasing the difficulty in targeting and eradicating the tumor [7].

The current standard-of-care therapies of GB patients is the same of the last decade and consist in surgery resections of as much of the tumor as is safe, followed by radio- and chemotherapy (to induce

DNA damage or inhibits DNA replication); thus, given the poor outcome of this treatment, new therapies and targeted treatments are necessary [8, 9].

The WHO (World Health Organization) classifies gliomas on the basis of their histologial and immunohistochemical features. GBs present a highly malignant behavior associated with several biological characteristics such as necrosis, mitotic features, neo-angiogenesis, nuclear atypia. Clinically GBs are subdivided in primary and secondary. The majority of patients present a primary GB with de novo lesions, while the remaining 5-10% show a secondary GB [10]. Even though these two GBs are histopathologically indistinguishable, they have distinct genetic profiles and distinct treatment implications. Genetic changes associated with de novo GBs include amplification and/or overexpression of Epidermal Growth Factor Receptor (EGFR) and Mouse double minute 2 (Mdm2), inactivating mutations of Phosphatase and Tensin Homolog Deleted on Chromosome 10 (PTEN), deletion mutations of Cyclin-Dependent Kinase inhibitor 2A (CDKN2A). Thus, these alterations result in the activation of proto-oncogenes involved in cancer pathways that arise in older patients with a predominant wild-type isocitrate dehydrogenase 1 (IDH1) genotype and a worse prognosis. While, secondary GBs develop from the low-grade glioma progression by the accumulation of molecular alterations, such as TP53 lesion, retinoblastoma protein 1 (RB1) loss or mutation and EGFR amplification. In this case, the tumor occurs in younger patients with a frequent mutant IDH1 genotype that confers a better prognosis (Figure 1) [1, 6, 11, 12].

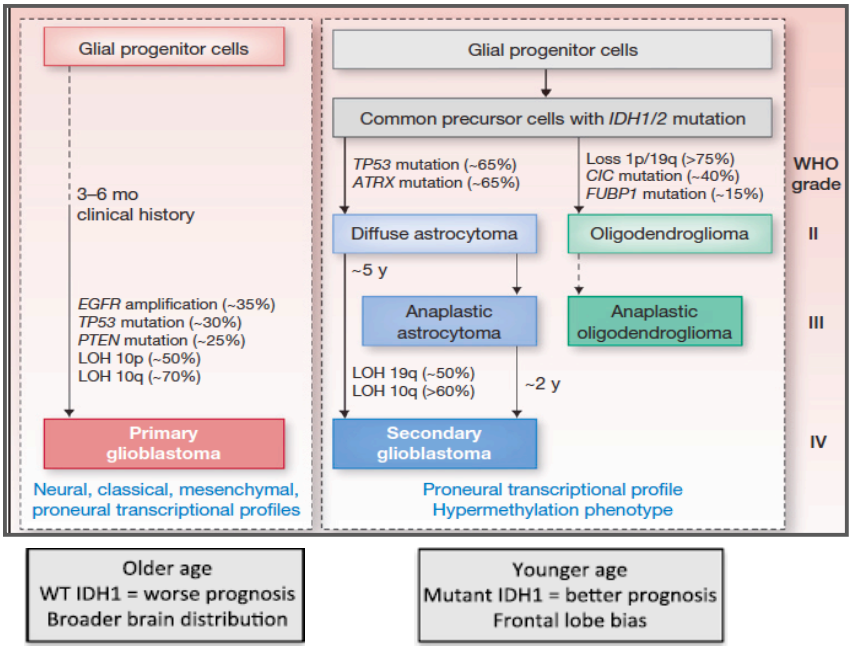



Figure 1 adapted from Dunn G.P. et al. Genes & Development 2012 and Ohgaki H. et al. Clinical Cancer Research 2013.

## Glioblastoma: Molecular Classification

Glioblastoma is a product of accumulated genetic and epigenetic alterations, characterized by genetic instability and complex alterations in chromosome structure and copy number. The most important somatically alterated genes are TP53, PTEN, neurofibromatosis type 1 (NF1), EGFR, RB1, IDH1 PIK3R1 and

PIK3CA (Figure 2). All these genetic lesions lead to alterations especially into three pathways: receptor tyrosine kinase (RTK)/ Ras/ phosphoinositide 3-kinas (PI3K) signaling is altered in 88% of GB; p53 signaling is impaired in 87% of the GB through CDKN2A deletion, MDM2 and MDM4 amplification and mutation and deletion of TP53; Rb signaling is altered in 78% of GB through CDKN2 family deletion, amplification of CDK4/6 and CCND2 and mutation or delation of RB1. Recently, somatic mutations of FAT1, a regulator of Wnt signaling, were found in 20% of GB [1, 6, 13].

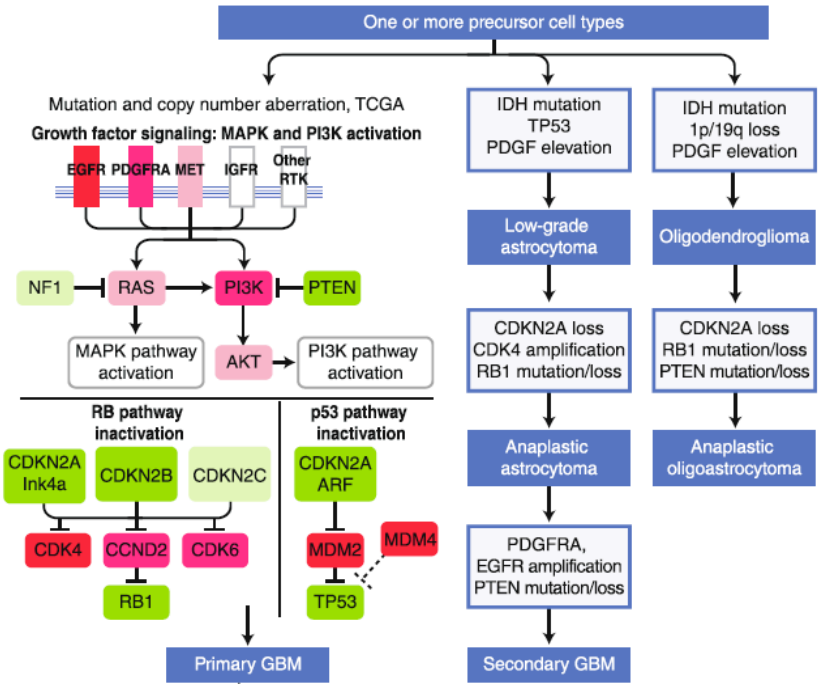



Figure 2 adapted from Breannan C. Curr Neurol Neurosci Rep 2011.

In the last years different groups tried to define the molecular hallmarks of GBs. Phillips et al in 2006 [14] classified GBs in three different subtype based on several genes whose expression was most strongly correlated with survival. This analysis identified three different groups which rappresent the three different subtype of GBs defined as proneural, proliferative and mesenchymal. The proneural subclass (PN) contains a mixture of grade III tumors (AA) and grade IV (GB) with or without necrosis; the patients are younger with a longer median survival rate than the other subtypes. The PN molecular signature is associated with an intact PTEN, normal EGFR and activation of the Notch pathway. The proliferative (Prolif) and mesenchymal (Mes) sublclasses are predominantly or exclusively grade IV tumors characterized by the presence of necrosis, with a worst outcome and common in older patients. These signatures are characterized by PTEN loss, Akt pathway activation and EGFR could be amplified. They are distinguished by a preponderance of either proliferation or angiogenesis, while PN are characterized by a low proliferation rate and express neuroblasts and immature neuronal markers.

In 2010 Verhaak et al. [15] used the TGCA (The Cancer Genome Atlas) data to identify the molecular subclasses of malignant glioma. Starting from 200 tumor specimens and 2 normal brain tissues they defined four subtype of malignant gliomas: proneural, neural, classic and mesenchymal on the basis of prior naming and the expression of signature genes (Figure 3). The proneural subtype includes the alterations of PDGFRA, point mutations in IDH1, is enriched for p53 mutations and is associated with younger patients.

The classical subtype shows a chromosome 7 amplification, loss of the 10, CDKN2A deletions, high-level EGFR amplification and lack of TP53 mutations. The major features of the mesenchymal subtype are the deletion/mutations of the NF1 gene. Both classical and mesenchymal subtypes respond better to the aggressive treatments with radio- and chemotherapy.

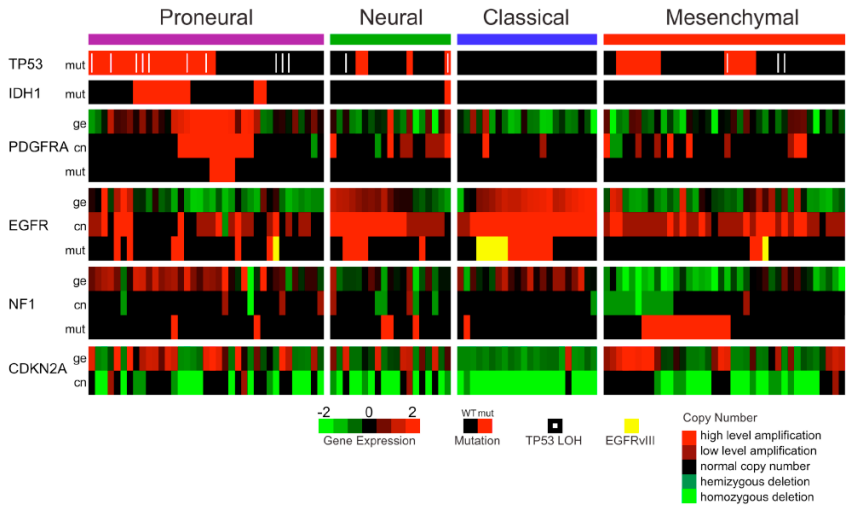



Figure 3 from Verhaak R.G.W. et al. Cancer Cell 2011.

In 2012 De Bacco et al. [16] classified GBs in three different substype according to gene expression profile: classical, mesenchymal and proneural. They show that mesenchymal and proneural subtypes are characterized by the expression of the MET oncogene, while EGFR alteration and transcription is preferentially associated with the classical subtype. Neurospheres (NS), considered as the appropriate

way to grow glioma stem cells in vitro, maintain in vitro many features of their original subclassification, defined in agreement to Verhaak et al. Notably, Met expression was absent from NS expressing the classical signature and expecially was mutually exclusive with amplification and expression of the EGFR gene.

GB is one of the most molecularly profiled of all human tumors; all these genomic analysis are intended to provide new biological knowledges of this tumor for the development of innovative and personalized therapies.

## Glioblastoma: Treatments

The current treatment for glioblastoma patients is radiotheraoy and temozolomide [17] and MGMT (O6 -methylguanine-DNA methyltransferase) promoter methylation has the most powerful molecular prognosticator in malignant gliomas and is predictive for response to alkylating agent chemotherapy in glioblastoma [18].

In the last decades several studies have been made and consequent significant progresses in understanding the biology of GB have been obtained. Therefore, why is so hard to find a new treatments? Glioblastoma is characterized by the alteration of different pathways and different driver mutations contributing to the formation of different subclasses of GBs. In addition, there are numerous mechanisms of resistance due to the formation of new

mutations induced by chemotherapy that allow the tumor to resume growth.

EGFR is amplified in 40% of GB patients, in particular the variant EGFRVIII, but several clinical trials of EGFR kinase inhibitors have been unsuccessful. Another target used in various clinical trials is the platelet-derived growth factor receptor (PDGF), which is overexpressed in some GBs and lower-grade gliomas [19–21].

Anti-angiogenic therapies have been tested in clinical trials. Bevacizumab is a recombinant humanized monoclonal immunoglobulin G1 antibody neutralizes the biologic activity of VEGF by blocking the bind between VEGF and its receptors (Figure 4). A phase III study in addition to standard radiotherapy is ongoing and shows a significantly increased survival of the patients [22, 23].

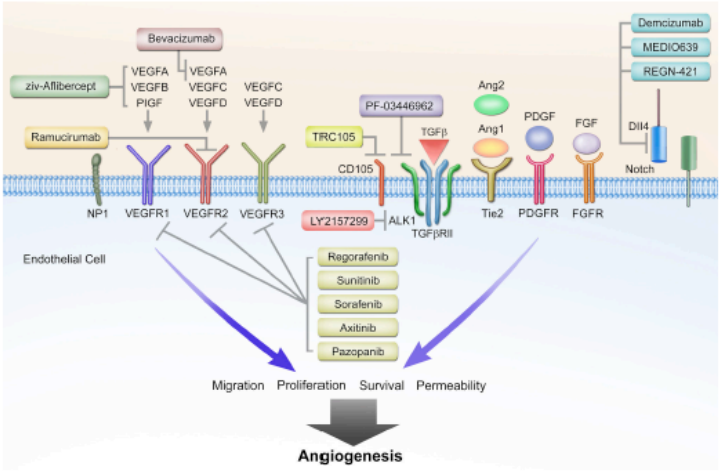



Figure 4 from Clarke J.M. et al. J Gastrointest Oncol 2013.

Hovewer, only about 8% of all investigational cancer drugs that enter phase I clinical testing will ultimately enter the market; the failure rate of cancer drugs in Phase III clinical trials is 50%. [24]. For this reason novel therapeutic approaches are desirable. In recent years there is increasing evidence suggesting that miRNAs are promising agents in cancer therapy also thanks to the success of existing and new delivery technologies [24–26].

## MicroRNAs: the functions

Mammalian microRNA (miRNAs) are short (20-23 nt), higly conserved, non-coding RNA molecules which regulate the expression of more than 30% of protein-coding genes at the post-transcriptional and translational level [27]. There are more than 24,000 miRNA entries in the miRbase database, including 1872 precursors and 2578 mature miRNAs for Homo Sapiens (miRbase release 20, June 2013). On the basis of the function and chromosomal location of miRNAs, miRNAs have been assigned to different families in various biological processes [28].

Most miRNAs are located in intergenic regions, while a sizeable minority was found in the intronic regions of known genes in the sense or antisense orientation. Moreover, ~50% of known miRNAs are found in proximity to other miRNAs, which raised the

possibility that these clustered miRNAs might be transcribed from a single polycistronic transcription unit, althrought different analysis showed that miRNA genes can be transcribed from their own promoters, and that the clustered miRNAs are generated as polycistronic primary transcripts (pri-miRNAs) [29, 30].

The canonical miRNA biogenesis starts into the nucleus where RNA polymerase II transcribes for a primary microRNA (pri-miRNA), a 100 nucleotides RNA precursor with a 5'cap and 3'poly A tails. This pri-miRNA is recognized and processed in a miRNA precursor (pre-miRNA) by a microprocessor complex composed by Drosha (nuclear RNase III), dsRBS (double-stranded RNA binding domain) and DGCR8 (DiGeorge syndrome critical region 8). The pre-miRNA is 70 nucleotide long with a stem-loop structure: it is transported into cytoplasm by Exportin 5 and is further processed into the 19–24 nucleotide double-stranded miRNA miRNA* complex by Dicer (RNase III enzyme) together with its dsRBD (double-stranded RNA binding domain) partner TRBP. Finally, the mature miRNA sequences enter into thr RISC complex (RNA-induced silence complex) and target expression of specific genes while the opposite strand is degraded (Figure 5) [30, 31].

MiRNA-binding sites are mostly located in the 3'UTR of the target mRNA, but can also be located in the 5′ UTR or the gene coding region. Given that binding between miRNAs and their targets is imperfect, a single miRNA can affect a broad range of mRNAs and can potentially regulate thousands of genes. The estimation that approximately one third of the protein-coding genes are controlled by

miRNAs indicates that almost all cellular pathways are directly or indirectly influenced by miRNAs. Perfect complementarity is required only between the "seed" region of the miRNA (nt 2–7 of the mature sequence) and the target mRNA leads to degradation or translational inhibition of the mRNA and consequently silences gene expression. The binding sites are primarily located in the 3′ UTR of the target mRNA, some genes avoid miRNA regulation with the help of very short 3′ UTRs that are specifically depleted of these sites [32–35].

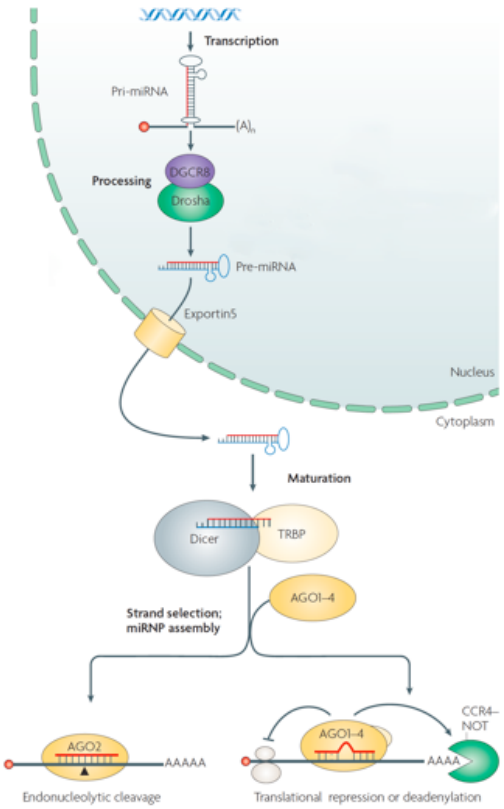



Figure 5 adapted form Filipowicz W. Nat Rev Genet 2008.

Over the past several years, a lot of studies had demonstrated that regulation of gene expression by miRNAs has major implications in various areas of cell biology and that the aberrant expression of miRNAs contributes to a variety of pathological conditions, including carcinogenesis by promoting the expression of proto-oncogenes or by inhibiting the expression of tumor suppressor genes. In addition, more than 50% of microRNA genes are located in cancer-associated genomic regions or in fragile sites, suggesting that miRNAs may play a more important role in the pathogenesis [36].

In GB, various miRNAs have been identified acting as oncogenes such as miR-21, miR- 221/222, miR-125 and miR-10b; or as tumor suppressor genes like miR-34a, miR-326 and miR-145 [37, 38].

## MiR-145 and cancer

MiR-145 was identified for the first time in mice from heart tissue using small RNA cloning techniques. In human miR-145 is located on the long arm of chromosome 5 (5q32-33) within a 4.09 kb region that co-trascribed miR-145 and miR-143 and that is often deleted in cancer. This microRNA is enriched in germline and mesoderm-derived tissues, such as uterus, ovary, testis, prostate, spleen, and heart while its downregulation has been reported in

different kinds of tumors, such as breast cancer, colon cancer, lung cancer, prostate cancer, colon-rectal cancer, bladder cancer and malignant gliomas [39–43]. MiR-145 is regulated by several factor including p53 [44], Foxo [45], RREB1 [46] and C/EBPbeta [47] (Figure 6), but mechanisms of its down-regulation are unclearb.

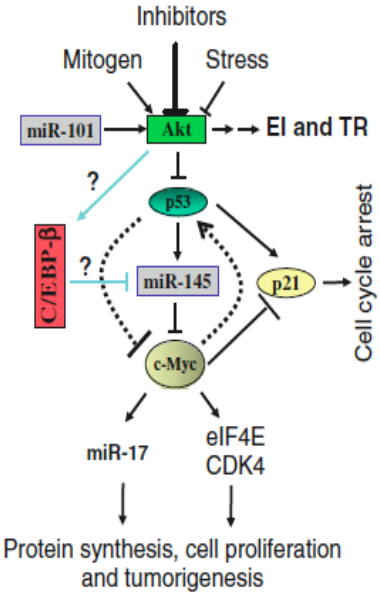



Figure 6 from M. Xu and YY. M, Cell Mol. Life Sci. 2012.

Generally, miR-145 acts as tumor suppressor and is able to inhibit proliferation, invasion and apoptosis by targeting different genes such as Fascin homologue 1 (FSCN1), JAM-A, Rhotekin (RTKN), DNA Fragmentation Factor-45 (DFF-45), ADAM17, c-Myc, EGFR, Neural precursor cell Expressed Developmentally Down-regulated 9 (NEDD9) [38, 39, 44, 47–53]. Moreover, miR-145 is able

to suppress the pluripotency of embryonic stem cells by binding SOX2, OCT4 and KLF4 (Figure 7) [54].

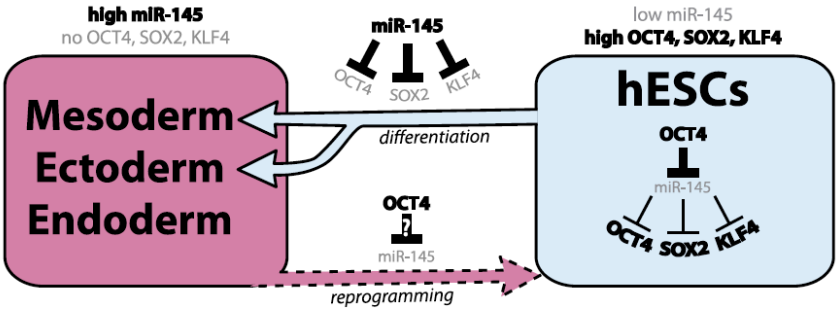



Figure 7 from N. Xu et al. Cell. 2009.

In 2012 Yi-Ping Yang [55] showed that miR-145 is a key regulator of the mechanisms involved in reducing cancer stem-like cells and in chemoradioresistance thanks to down-regulation of downstream targets like Sox2 and Oct4. Sang-Jin Lee [56] demonstrated that the over-expression of miR-145 in combination with HSVtk might enhance anti-tumor activity and therefore they confirmed the roles of miR-145 in glioma invasion, migration and tumor growth in vivo. Similarly, we demostrateted the miR-145 does not exert significant effects on the proliferation of the glioblastoma stem-like cells, but rather suppresses the invasion by inhibiting NEDD9 expression [38].

More recently Lu Y. et al. [53], Rani S.B. et al. [57], Lee H.K. et al. [37] confirmed the miR-145 is downregulated in malignant gliomas and that is able to inhibit invasion, migration and proliferation of glioblastoma stem cells. Lu et al. [53] demostrated that miR-145 functions as an anti-oncogene in glioma cells and that its ectopic expression is correlated with a lower protein expression of ADAM17 and EGFR. Rani et al. [57] showed that down-regulation of miR-145 in GB leads to activation of Sox9 and ADD3, causing a proinvasive behavior of glioblastoma cells. Lee et al. [37] described as a novel target of miR-145 the connective tissue growth factor (CTGF), able to mediate its effects on cell migration via downregulation of SPARC.

## HEF1/CAS-L/NEDD9

NEDD9, Neural precursor cell Expressed, Developmentally Down-regulated 9 also known as HEF1 or Cas-L, is a member of the Cas family and is an adhesion docking protein forming part of a signaling hub at integrin-mediated adhesion sites. Lacking enzymatic activity [58], NEDD9 regulates the formation of signalling complexes via its protein–protein interaction domains: Src homology 3 (SH3), in the N-terminus, SH2 binding sites in a substrate domain, a serine rich domain and a HLH motif in the highly-conserved C terminus [59]. Phosphorylation of serines, threonines and tyrosines in the NEDD9 sequence modulates NEDD9 interaction with down-stream signalling partners and regulates NEDD9 signalling function [60].

NEDD9 was identified for the first time in 1996 for its ability to induce pseudohyphal growth in Saccharomyces cerevisiae [61] and its expression is confined to epithelial tissues [62]. Although part of the HEF1/NEDD9/Cas-L protein pool is cytoplasmic, in response to intrinsic and extrinsic cues HEF1/NEDD9/Cas-L concentrates at focal adhesions, and at the centrosome and mitotic spindle. Cell cycle-regulated processing produces four isoforms of this protein. Isoform p115 arises from p105 phosphorylation, is predominantly cytoplasmic, associated with focal adhesions and appears later in the cell cycle. Isoform p55 arises from p105 as a result of cleavage at a caspase cleavage-related site, is associated with mitotic spindle and appears just during the mitosis, whereas the last isoform, p65, is poorly detected. NEDD9 has 4 isoforms  the most common have 105 and 115 kDa mw, significantly higher than its predicted molecular weight of ~93 kDa, reflecting the extensive phosphorylation of HEF1/NEDD9/Cas-L [58].

The major regulators of NEDD9 phoshorylation are FAK (Focal Adhesion Kinase) and Src family kinases, which phosphorylate NEDD9 on a tyrosine near the C-terminus, creating a binding site for Src. This last protein phosphorylates NEDD9 in the substrate domain, producing the binding of HEF1 to the downstream effectors that promote migration, invasion, and proliferation-related signaling (Figure 8) [62].

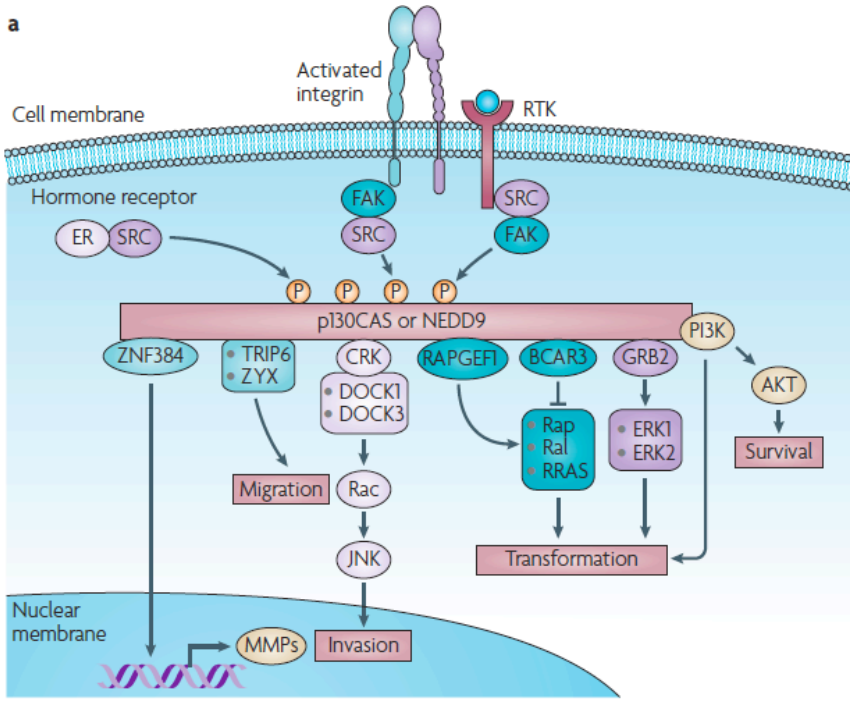



Figure 8 from Cabodi S et al. Nature 2010.

## NEDD9 and cancer

NEDD9 has key roles in the regulation of several biological processes, such as apoptosis, cell cycle, migration and invasion. Law et al. [63] demonstrated that NEDD9 overexpression in MCF-7 or HeLa cells causes extensive apoptosis. The proteolytic cleavage of NEDD9 by caspase 3/7 releases a 65- and 55-kDa fragments and a new isoform of 28 kDa in response to the induction of apoptosis. The production of this new detected form is associated with the death-

promoting activity of over-expressed NEDD9.

The abundance of NEDD9 is regulated by cell cycle, starting with low levels in G1, an increased expression during S phase and a peack in late G2/M. Cells over-expressing NEDD9 accumulate multipolar spindles and centrosomes, while a depletion of this protein causes prematurely separated centrosomes in interphase leading to high levels of monopolar or asymmetric spindles. Cells with aberrant expression of NEDD9 that pass through mitosis commonly arrest in G1 phase of cell cycle, compatible with triggering of mitotic checkpoints, and ultimately are cleared by apoptosis (Figure 9) [58, 64].

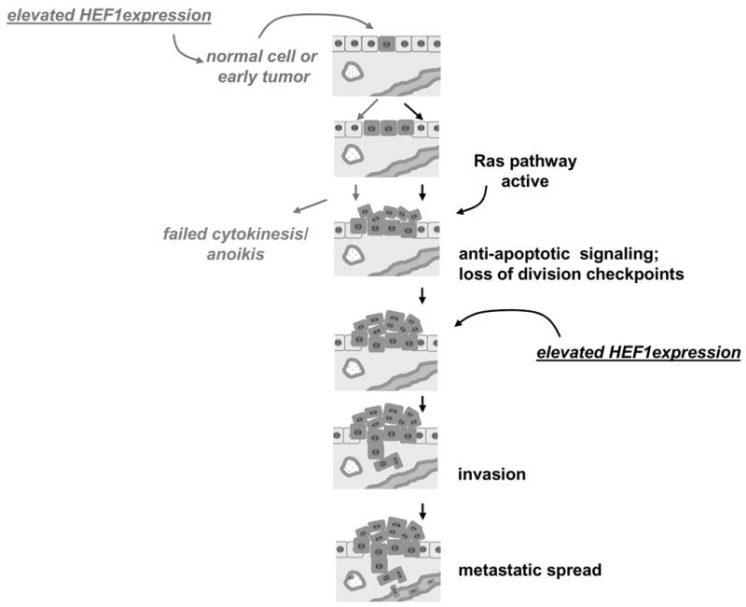



Figure 9 from Singh MK et al. Cell Biochem Biophys. 2007

More important, elevate protein level of NEDD9 is positively correlated to an increase of migration and invasion of tumor cells. NEDD9 has been identified as a metastasis gene in melanoma [65], in head and neck squamous cell carcinoma (HNSCC) [66] and in primary lung carcinomas [67]. In colorectal cancer is associated with increased migration and cancer progression [68]. Natarajan et al [69] demonstrated that NEDD9 is a specific and necessary downstream effector of FAK and is involved in conferring an invasive behaviour of glioblastomas. In 2012, our group showed that NEDD9 is correlated with a lower survival of low-grade glioma patients and that the inhibition of NEDD9 expression in glioblastoma cell-like cells leads to a significant reduction of invasion *in vitro* [38].

In melanoma the role of NEDD9 as a pro-migratory protein has been determined on the basis of its ability to activate RAC1 resulting in a switch from amoeboid to mesenchymal movement. In Jin Y et al in 2013 [70] have demonstrated that in lung cancer NEDD9 promotes metastasis through the epithelial to mesenchymal transition (EMT) potentially via FAK activation. In this way NEDD9 is also responsible for a more migratory and invasive behavior of the cancer cells.

Taken together, all these results show the important role of NEDD9 in cancer and its role as a coordinator of apoptosis, cell cycle and invasion. The key functions of this protein identify NEDD9 as a potential target for potential metastatic therapies.

# Scope of the Thesis

Glioblastoma is one of the most lethal human cancers and despite the advances in the last decades, standard therapies based on neuro-surgery followed by radio- and chemotherapy with the alkylant agent temozolomide, are associated with overall survival shorter than 18 months. The search for new treatments requires a deepened understanding of GB biology.

Given the important role of miR-145 in cancer and stem cell biology, our first aim was to investigate its expression in our GB samples. We found that miR-145 is strongly downregulated in glioblastoma (GB) specimens, in the corresponding glioblastoma-neurospheres (GB-NS) and in low-grade gliomas (LGG) compared to normal brain. More importantly, we demonstrated that a lower expression of miR-145 is associated with a poor prognosis in both LGG and GB patients.

The second step was to find new targets of miR-145 by gene expression profiling comparing GB-NS over-expressing miR-145 with GB-NS transduced with empty vector. Consequently we focused our attention on HEF1/Cas-L/NEDD9, a scaffold protein involved in invasion in several types of cancer. We confirmed a significant down-regulation of NEDD9 in miRover-NS and we also found a higher expression in GB and GB-NS. LGG patients are characterized by a

heterogeneous expression of NEDD9 and patients with higher NEDD9 expression showed a shorter progression free survival than others.

The third aim was to evaluate the effect of miR-145 expression in vivo: mice injected with miRover-NS survived significantly longer than the controls and showed a significant down-regulation of NEDD9. In addition, we demonstrated a significant inhibition of invasion in silencing experiments with shNEDD9, and we observed an up-regulation of miR-145 in shNEDD9, thereby suggesting a double-negative feedback loop between miR-145 and NEDD9.

# References


[1] T. F. Cloughesy, W. K. Cavenee, and P. S. Mischel, "Glioblastoma: From Molecular Pathology to Targeted Treatment.," *Annu. Rev. Pathol.*, no. July 2013, Aug. 2013.

[2] S. W. Lee, B. a Fraass, L. H. Marsh, K. Herbort, S. S. Gebarski, M. K. Martel, E. H. Radany, a S. Lichter, and H. M. Sandler, "Patterns of failure following high-dose 3-D conformal radiotherapy for high-grade astrocytomas: a quantitative dosimetric study.," *Int. J. Radiat. Oncol. Biol. Phys.*, vol. 43, no. 1, pp. 79–88, Jan. 1999.

[3] K. a van Nifterik, P. H. M. Elkhuizen, R. J. van Andel, L. J. a Stalpers, S. Leenstra, M. V. M. Lafleur, W. P. Vandertop, B. J. Slotman, T. J. M. Hulsebos, and P. Sminia, "Genetic profiling of a distant second glioblastoma multiforme after radiotherapy: Recurrence or second primary tumor?," *J. Neurosurg.*, vol. 105, no. 5, pp. 739–44, Nov. 2006.

[4] E. Tracey, D. Baker, W. Chen, E. Stavrou, J. Bishop, C. Cook-yarborough, A. Pelquest-hunt, D. Matthews, H. Byrne, D. Bradfi, and B. Guerin, *Cancer in New South Wales : Incidence , Mortality and Prevalence Report 2005*. 2005.

[5] T. Surawicz, F. Davis, and S. Freels, “Brain Tumor survival: Results from the National Cancer Data Base,” *J. neuro-* ..., no. 40, pp. 151–160, 1998.

[6] G. Dunn and M. Rinne, “Emerging insights into the molecular and cellular basis of glioblastoma,” *Genes* ..., pp. 756–784, 2012.

[7] A. Soeda, M. Park, D. Lee, A. Mintz, A. Androutsellis-Theotokis, R. D. McKay, J. Engh, T. Iwama, T. Kunisada, A. B. Kassam, I. F. Pollack, and D. M. Park, “Hypoxia promotes expansion of the CD133-positive glioma stem cells through activation of HIF-1alpha.,” *Oncogene*, vol. 28, no. 45, pp. 3949–59, Nov. 2009.

[8] R. Stupp, W. P. Mason, M. J. van den Bent, M. Weller, B. Fisher, M. J. B. Taphoorn, K. Belanger, A. a Brandes, C. Marosi, U. Bogdahn, J. Curschmann, R. C. Janzer, S. K. Ludwin, T. Gorlia, A. Allgeier, D. Lacombe, J. G. Cairncross, E. Eisenhauer, and R. O. Mirimanoff, “Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma.,” *N. Engl. J. Med.*, vol. 352, no. 10, pp. 987–96, Mar. 2005.

[9] E. C. Holland, “Glioblastoma multiforme: the terminator.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 97, no. 12, pp. 6242–4, Jun. 2000.

[10] H. Ohgaki and P. Kleihues, “The definition of primary and secondary glioblastoma.,” *Clin. Cancer Res.*, vol. 19, no. 4, pp. 764–72, Feb. 2013.

[11] H. Ohgaki and P. Kleihues, “Genetic pathways to primary and secondary glioblastoma.,” *Am. J. Pathol.*, vol. 170, no. 5, pp. 1445–53, May 2007.

[12] J. Zhong, A. Paul, S. J. Kellie, and G. M. O’Neill, “Mesenchymal migration as a therapeutic target in glioblastoma.,” *J. Oncol.*, vol. 2010, p. 430142, Jan. 2010.

[13] C. Brennan, “Genomic profiles of glioma.,” *Curr. Neurol. Neurosci. Rep.*, vol. 11, no. 3, pp. 291–7, Jun. 2011.

[14] H. S. Phillips, S. Kharbanda, R. Chen, W. F. Forrest, R. H. Soriano, T. D. Wu, A. Misra, J. M. Nigro, H. Colman, L. Soroceanu, P. M. Williams, Z. Modrusan, B. G. Feuerstein, and K. Aldape, “Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis.,” *Cancer Cell*, vol. 9, no. 3, pp. 157–73, Mar. 2006.

[15] R. G. W. Verhaak, K. A. Hoadley, E. Purdom, V. Wang, Y. Qi, M. D. Wilkerson, C. R. Miller, L. Ding, T. Golub, J. P. Mesirov, G. Alexe, M. Lawrence, M. O’Kelly, P. Tamayo, B. A. Weir, S. Gabriel, W. Winckler, S. Gupta, L. Jakkula, H. S. Feiler, J. G. Hodgson, C. D. James, J. N. Sarkaria, C. Brennan, A. Kahn, P. T. Spellman, R. K. Wilson, T. P. Speed, J. W.

Gray, M. Meyerson, G. Getz, C. M. Perou, and D. N. Hayes, “Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1.,” *Cancer Cell*, vol. 17, no. 1, pp. 98–110, Jan. 2010.

[16] F. De Bacco, E. Casanova, E. Medico, S. Pellegatta, F. Orzan, R. Albano, P. Luraghi, G. Reato, A. D’Ambrosio, P. Porrati, M. Patanè, E. Maderna, B. Pollo, P. M. Comoglio, G. Finocchiaro, and C. Boccaccio, “The MET Oncogene Is a Functional Marker of a Glioblastoma Stem Cell Subtype.,” *Cancer Res.*, vol. 72, no. 17, pp. 4537–50, Sep. 2012.

[17] J. D. Hainsworth, T. Ervin, E. Friedman, V. Priego, P. B. Murphy, B. L. Clark, and R. E. Lamar, “Concurrent radiotherapy and temozolomide followed by temozolomide and sorafenib in the first-line treatment of patients with glioblastoma multiforme.,” *Cancer*, vol. 116, no. 15, pp. 3663–9, Aug. 2010.

[18] M. Weller, R. Stupp, G. Reifenberger, A. a Brandes, M. J. van den Bent, W. Wick, and M. E. Hegi, “MGMT promoter methylation in malignant gliomas: ready for personalized medicine?,” *Nat. Rev. Neurol.*, vol. 6, no. 1, pp. 39–51, Jan. 2010.

[19] J. T. Huse, E. Holland, and L. M. DeAngelis, “Glioblastoma: molecular analysis and clinical implications.,” *Annu. Rev. Med.*, vol. 64, pp. 59–70, Jan. 2013.

[20] a J. Wong, J. M. Ruppert, S. H. Bigner, C. H. Grzeschik, P. a Humphrey, D. S. Bigner, and B. Vogelstein, “Structural alterations of the epidermal growth factor receptor gene in human gliomas.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 89, no. 7, pp. 2965–9, Apr. 1992.

[21] D. a Reardon, A. Desjardins, J. J. Vredenburgh, S. Gururangan, A. H. Friedman, J. E. Herndon, J. Marcello, J. a Norfleet, R. E. McLendon, J. H. Sampson, and H. S. Friedman, “Phase 2 trial of erlotinib plus sirolimus in adults with recurrent glioblastoma.,” *J. Neurooncol.*, vol. 96, no. 2, pp. 219–30, Jan. 2010.

[22] M. J. Gil-Gil, C. Mesia, M. Rey, and J. Bruna, “Bevacizumab for the treatment of glioblastoma.,” *Clin. Med. Insights. Oncol.*, vol. 7, pp. 123–35, Jan. 2013.

[23] A. D. Norden, G. S. Young, K. Setayesh, A. Muzikansky, R. Klufas, G. L. Ross, A. S. Ciampa, L. G. Ebbeling, B. Levy, J. Drappatz, S. Kesari, and P. Y. Wen, “Bevacizumab for recurrent malignant gliomas: efficacy, toxicity, and patterns of recurrence.,” *Neurology*, vol. 70, no. 10, pp. 779–87, Mar. 2008.

[24] a G. Bader, D. Brown, J. Stoudemire, and P. Lammers, “Developing therapeutic microRNAs for cancer.,” *Gene Ther.*, vol. 18, no. 12, pp. 1121–6, Dec. 2011.

[25] M. E. Davis, J. E. Zuckerman, C. H. J. Choi, D. Seligson, A. Tolcher, C. a Alabi, Y. Yen, J. D. Heidel, and A. Ribas, “Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles.,” *Nature*, vol. 464, no. 7291, pp. 1067–70, Apr. 2010.

[26] Y. Wu, M. Crawford, Y. Mao, R. J. Lee, I. C. Davis, T. S. Elton, L. J. Lee, and S. P. Nana-Sinkam, “Therapeutic Delivery of MicroRNA-29b by Cationic Lipoplexes for Lung Cancer.,” *Mol. Ther. Nucleic Acids*, vol. 2, no. April, p. e84, Jan. 2013.

[27] R. W. Carthew, “Gene regulation by microRNAs.,” *Curr. Opin. Genet. Dev.*, vol. 16, no. 2, pp. 203–8, Apr. 2006.

[28] S. Baranwal and S. K. Alahari, “miRNA control of tumor cell invasion and metastasis.,” *Int. J. Cancer*, vol. 126, no. 6, pp. 1283–90, Mar. 2010.

[29] V. N. Kim, “MicroRNA biogenesis: coordinated cropping and dicing.,” *Nat. Rev. Mol. Cell Biol.*, vol. 6, no. 5, pp. 376–85, May 2005.

[30] V. Kim, “Small RNAs : Classification , Biogenesis , and Function,” *Mol Cells*, vol. 19, no. 1, pp. 1–15, 2005.

[31] D. Bartel, “MicroRNAs: genomics, biogenesis, mechanism, and function,” *Cell*, vol. 116, pp. 281–297, 2004.

[32] M. Biogenesis, J. Winter, and S. Diederichs, “MicroRNA and Cancer,” *Change*, vol. 676, 2011.

[33] W. Filipowicz, S. N. Bhattacharyya, and N. Sonenberg, “Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight?,” *Nat. Rev. Genet.*, vol. 9, no. 2, pp. 102–14, Feb. 2008.

[34] D. Baek, J. Villén, C. Shin, F. D. Camargo, S. P. Gygi, and D. P. Bartel, “The impact of microRNAs on protein output.,” *Nature*, vol. 455, no. 7209, pp. 64–71, Sep. 2008.

[35] M. Selbach, B. Schwanhäusser, N. Thierfelder, Z. Fang, R. Khanin, and N. Rajewsky, “Widespread changes in protein synthesis induced by microRNAs.,” *Nature*, vol. 455, no. 7209, pp. 58–63, Sep. 2008.

[36] S. Bandyopadhyay, R. Mitra, U. Maulik, and M. Q. Zhang, “Development of the human cancer microRNA network.,” *Silence*, vol. 1, no. 1, p. 6, Jan. 2010.

[37] H. Lee, S. Finniss, S. Cazacu, and E. Bucris, “Mesenchymal stem cells deliver synthetic microRNA mimics to glioma cells and glioma stem cells and inhibit their cell migration and self-renewal,” *Oncotarget*, vol. 4, no. 2, pp. 346–361, 2013.

[38] M. C. Speranza, V. Frattini, F. Pisati, D. Kapetis, P. Porrati, M. Eoli, S. Pellegatta, and G. Finocchiaro, “NEDD9, a novel target

of miR-145, increases the invasiveness of glioblastoma.," *Oncotarget*, vol. 3, no. 7, pp. 723–34, Jul. 2012.

[39] M. Sachdeva and Y.-Y. Mo, "MicroRNA-145 suppresses cell invasion and metastasis by directly targeting mucin 1.," *Cancer Res.*, vol. 70, no. 1, pp. 378–87, Jan. 2010.

[40] J. Zhang, H. Guo, H. Zhang, H. Wang, G. Qian, X. Fan, A. R. Hoffman, J.-F. Hu, and S. Ge, "Putative tumor suppressor miR-145 inhibits colon cancer cell growth by targeting oncogene Friend leukemia virus integration 1 gene.," *Cancer*, vol. 117, no. 1, pp. 86–95, Jan. 2011.

[41] M. Fuse, N. Nohata, S. Kojima, S. Sakamoto, T. Chiyomaru, K. Kawakami, H. Enokida, M. Nakagawa, Y. Naya, T. Ichikawa, and N. Seki, "Restoration of miR-145 expression suppresses cell proliferation, migration and invasion in prostate cancer by targeting FSCN1.," *Int. J. Oncol.*, vol. 38, no. 4, pp. 1093–101, Apr. 2011.

[42] Q. Xu, L.-Z. Liu, X. Qian, Q. Chen, Y. Jiang, D. Li, L. Lai, and B.-H. Jiang, "MiR-145 directly targets p70S6K1 in cancer cells to inhibit tumor growth and angiogenesis.," *Nucleic Acids Res.*, pp. 1–14, Sep. 2011.

[43] T. Chiyomaru, H. Enokida, S. Tatarano, K. Kawahara, Y. Uchida, K. Nishiyama, L. Fujimura, N. Kikkawa, N. Seki, and M. Nakagawa, "miR-145 and miR-133a function as tumour

suppressors and directly regulate FSCN1 expression in bladder cancer.," *Br. J. Cancer*, vol. 102, no. 5, pp. 883–91, Mar. 2010.

[44] M. Sachdeva, S. Zhu, F. Wu, H. Wu, V. Walia, S. Kumar, R. Elble, K. Watabe, and Y.-Y. Mo, "p53 represses c-Myc through induction of the tumor suppressor miR-145.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 106, no. 9, pp. 3207–12, Mar. 2009.

[45] B. Gan, C. Lim, G. Chu, S. Hua, Z. Ding, M. Collins, J. Hu, S. Jiang, E. Fletcher-Sananikone, L. Zhuang, M. Chang, H. Zheng, Y. A. Wang, D. J. Kwiatkowski, W. G. Kaelin, S. Signoretti, and R. A. DePinho, "FoxOs enforce a progression checkpoint to constrain mTORC1-activated renal tumorigenesis.," *Cancer Cell*, vol. 18, no. 5, pp. 472–84, Nov. 2010.

[46] O. A. Kent, R. R. Chivukula, M. Mullendore, E. A. Wentzel, G. Feldmann, K. H. Lee, S. Liu, S. D. Leach, A. Maitra, and J. T. Mendell, "Repression of the miR-143 / 145 cluster by oncogenic Ras initiates a tumor-promoting feed-forward pathway," no. 410, pp. 2754–2759, 2010.

[47] M. Sachdeva, Q. Liu, J. Cao, Z. Lu, and Y.-Y. Mo, "Negative regulation of miR-145 by C/EBP-β through the Akt pathway in cancer cells.," *Nucleic Acids Res.*, vol. 40, no. 14, pp. 6683–92, Aug. 2012.

[48] S.-J. Kim, J.-S. Oh, J.-Y. Shin, K.-D. Lee, K. W. Sung, S. J. Nam, and K.-H. Chun, "Development of microRNA-145 for

therapeutic application in breast cancer.," *J. Control. Release*, vol. 155, no. 3, pp. 427–34, Nov. 2011.

[49] M. Götte, C. Mohr, C.-Y. Koo, C. Stock, a-K. Vaske, M. Viola, S. a Ibrahim, S. Peddibhotla, Y. H.-F. Teng, J.-Y. Low, K. Ebnet, L. Kiesel, and G. W. Yip, "miR-145-dependent targeting of junctional adhesion molecule A and modulation of fascin expression are associated with reduced breast cancer cell motility and invasiveness.," *Oncogene*, vol. 29, no. 50, pp. 6569–80, Dec. 2010.

[50] X. Zhang, S. Liu, T. Hu, S. Liu, Y. He, and S. Sun, "Up-regulated microRNA-143 transcribed by nuclear factor kappa B enhances hepatocarcinoma metastasis by repressing fibronectin expression.," *Hepatology*, vol. 50, no. 2, pp. 490–9, Aug. 2009.

[51] S. Wang, C. Bian, Z. Yang, and Y. Bo, "miR-145 inhibits breast cancer cell growth through RTKN.," ... *J. Oncol.*, pp. 1461–1466, 2009.

[52] W. Cho, A. Chow, and J. Au, "MiR-145 inhibits cell proliferation of human lung adenocarcinoma by targeting EGFR and NUDT1," *RNA Biol.*, vol. 145, no. February, pp. 125–131, 2011.

[53] Y. Lu, M. Chopp, X. Zheng, M. Katakowski, B. Buller, and F. Jiang, "MiR-145 reduces ADAM17 expression and inhibits in vitro migration and invasion of glioma cells.," *Oncol. Rep.*, vol. 29, no. 1, pp. 67–72, Jan. 2013.

[54] N. Xu, T. Papagiannakopoulos, G. Pan, J. a Thomson, and K. S. Kosik, “MicroRNA-145 regulates OCT4, SOX2, and KLF4 and represses pluripotency in human embryonic stem cells.,” *Cell*, vol. 137, no. 4, pp. 647–58, May 2009.

[55] Y.-P. Yang, Y. Chien, G.-Y. Chiou, J.-Y. Cherng, M.-L. Wang, W.-L. Lo, Y.-L. Chang, P.-I. Huang, Y.-W. Chen, Y.-H. Shih, M.-T. Chen, and S.-H. Chiou, “Inhibition of cancer stem cell-like properties and reduced chemoradioresistance of glioblastoma using microRNA145 with cationic polyurethane-short branch PEI.,” *Biomaterials*, vol. 33, no. 5, pp. 1462–76, Mar. 2012.

[56] S.-J. Lee, S.-J. Kim, H.-H. Seo, S.-P. Shin, D. Kim, C.-S. Park, K.-T. Kim, Y.-H. Kim, J.-S. Jeong, and I.-H. Kim, “Over-expression of miR-145 enhances the effectiveness of HSVtk gene therapy for malignant glioma.,” *Cancer Lett.*, pp. 1–9, Jan. 2012.

[57] S. B. Rani, S. S. Rathod, S. Karthik, N. Kaur, D. Muzumdar, and A. S. Shiras, “MiR-145 functions as a tumor-suppressive RNA by targeting Sox9 and adducin 3 in human glioma cells.,” *Neuro. Oncol.*, vol. 15, no. 10, pp. 1302–16, Oct. 2013.

[58] M. K. Singh, L. Cowell, S. Seo, G. M. O’Neill, and E. A. Golemis, “Molecular basis for HEF1/NEDD9/Cas-L action as a multifunctional coordinator of invasion, apoptosis and cell cycle,” *Cell Biochem Biophys*, vol. 48, no. 1, pp. 54–72, Jul. 2007.

[59] V. Hivert, J. Pierre, and J. Raingeaud, “Phosphorylation of human enhancer of filamentation (HEF1) on serine 369 induces its proteasomal degradation.,” *Biochem. Pharmacol.*, vol. 78, no. 8, pp. 1017–25, Oct. 2009.

[60] P. Bradbury, M. Mahmassani, J. Zhong, K. Turner, A. Paul, N. M. Verrills, and G. M. O’Neill, “PP2A phosphatase suppresses function of the mesenchymal invasion regulator NEDD9.,” *Biochim. Biophys. Acta*, vol. 1823, no. 2, pp. 290–7, Feb. 2012.

[61] S. F. Law, J. Estojak, B. Wang, T. Mysliwiec, G. Kruh, E. A. Golemis, L. A. W. E. T. Al, and M. O. L. C. E. L. L. B. Iol, “Human Enhancer of Filamentation 1 , a Novel p130 cas -Like Docking Protein , Associates with Focal Adhesion Kinase and Induces Pseudohyphal Growth in Saccharomyces cerevisiae,” vol. 16, no. 7, pp. 3327–3337, 1996.

[62] S. Cabodi, M. del Pilar Camacho-Leal, P. Di Stefano, and P. Defilippi, “Integrin signalling adaptors: not only figurants in the cancer story.,” *Nat. Rev. Cancer*, vol. 10, no. 12, pp. 858–70, Dec. 2010.

[63] S. F. Law, G. M. O’Neill, S. J. Fashena, M. B. Einarson, and E. a Golemis, “The docking protein HEF1 is an apoptotic mediator at focal adhesion sites.,” *Mol. Cell. Biol.*, vol. 20, no. 14, pp. 5184–95, Jul. 2000.

[64] E. N. Pugacheva and E. A. Golemis, “HEF1-aurora A interactions: points of dialog between the cell cycle and cell

attachment signaling networks.," *Cell Cycle*, vol. 5, no. 4, pp. 384–91, Feb. 2006.

[65] M. Kim, J. D. Gans, C. Nogueira, A. Wang, J.-H. Paik, B. Feng, C. Brennan, W. C. Hahn, C. Cordon-Cardo, S. N. Wagner, T. J. Flotte, L. M. Duncan, S. R. Granter, and L. Chin, "Comparative oncogenomics identifies NEDD9 as a melanoma metastasis gene.," *Cell*, vol. 125, no. 7, pp. 1269–81, Jun. 2006.

[66] J. Lucas, B. Salimath, M. Slomiany, and S. Rosenzweig, "Regulation of invasive behavior by vascular endothelial growth factor is HEF1-dependent," *Oncogene*, vol. 29, no. 31, pp. 4449–4459, 2010.

[67] A. J. Minn, G. P. Gupta, P. M. Siegel, P. D. Bos, W. Shu, D. D. Giri, A. Viale, A. B. Olshen, W. L. Gerald, and J. Massagué, "Genes that mediate breast cancer metastasis to lung.," *Nature*, vol. 436, no. 7050, pp. 518–24, Jul. 2005.

[68] Y. Li, J. H. Bavarva, Z. Wang, J. Guo, C. Qian, S. N. Thibodeau, E. A. Golemis, and W. Liu, "HEF1, a novel target of Wnt signaling, promotes colonic cell migration and cancer progression.," *Oncogene*, vol. 30, no. 23, pp. 2633–43, Jun. 2011.

[69] M. Natarajan, J. E. Stewart, E. a Golemis, E. N. Pugacheva, K. Alexandropoulos, B. D. Cox, W. Wang, J. R. Grammer, and C. L. Gladson, "HEF1 is a necessary and specific downstream

effector of FAK that promotes the migration of glioblastoma cells.," *Oncogene*, vol. 25, no. 12, pp. 1721–32, Mar. 2006.

[70] Y. Jin, F. Li, C. Zheng, Y. Wang, Z. Fang, C. Guo, X. Wang, H. Liu, L. Deng, C. Li, H. Wang, H. Chen, Y. Feng, and H. Ji, "NEDD9 promotes lung cancer metastasis through Epithelial-Mesenchymal Transition.," *Int. J. Cancer*, pp. 1–11, Oct. 2013.

# *Chapter 2*

# NEDD9, a novel target of miR-145, increases the inasiveness of glioblastoma

Maria Carmela Speranza [1,3], Véronique Frattini[1,3], Federica Pisati[1,3], Dimos Kapetis[2], Paola Porrati[1], Marica Eoli[1], Serena Pellegatta [1, 3] and Gaetano Finocchiaro[1, 3]

***Published**: Oncotarget. 2012 Jul;3(7):723-34.*

**ABSTRACT**

miR-145 is an important repressor of pluripotency in embryonic stem cells and a tumor suppressor in different cancers. Here, we found that miR-145 is strongly down-regulated in glioblastoma (GB) specimens and corresponding glioblastoma-neurospheres (GB-NS, containing GB stem-like cells) compared to normal brain (NB) and to low-grade gliomas (LGG). We observed a direct correlation between miR-145 expression and the progression-free survival (PFS) in LGG patients and overall survival (OS) in GB patients. Using microarray analysis, we identified relevant differences in gene expression profiles between GB-NS over-expressing miR-145 (miRover-NS) and GB-NS Empty (Empty-NS). We focused our attention on HEF1/Cas-L/NEDD9, a scaffold protein involved in invasion in several types of cancer. We confirmed a significant down-regulation of NEDD9 in miRover-NS and we found a higher expression in GB and GB-NS compared to NB. Approximately 50% of LGG patients expressed higher levels of NEDD9 than NB, and the PFS of such patients was shorter than in patients expressing lower levels of NEDD9. We observed that intracranial injection of GB-NS over-expressing miR-145 delays significantly tumor development :deriving tumors showed a significant down-regulation of NEDD9. In addition, we demonstrated a significant inhibition of invasion in silencing experiments with GB-NS shNEDD9 (shNEDD9), and an up-regulation of miR-145 in shNEDD9, suggesting a double-negative feedback loop between miR-145 and NEDD9. Our results demonstrate the critical role of miR-145 and NEDD9 in regulating glioblastoma invasion and suggest a potential role of NEDD9 as a biomarker for glioma progression.

Keywords: miR-145, NEDD9, invasion, progression, glioma, glioblastoma

## INTRODUCTION

Glioblastomas (GB) and other cancers may contain a populations of cells expressing stem cell programs and sharing expression patterns with embryonic stem cells [1]. Oct-4, Sox-2 and Nanog are core genes in embryonic stem cell maintenance and they are all up-regulated in GB and malignant gliomas [2]. Recent data suggest that their expression is tightly regulated by one microRNA, miR-145 [3]. MicroRNAs (miRNAs) are small non-coding RNA molecules with length of 20-22 nucleotides and are generated by the cleavage of 70-100 nucleotide hairpin pre-miRNA precursors [4]. miRNAs are able to regulate the expression of more than 30% of human genes via specific base pairing to the 3'-UTRs of messenger RNAs, which either blocks translation or promotes the degradation of the mRNA target. miRNAs are post-transcriptional modulators of gene expression and are involved in the regulation of several cellular processes, such as the cell cycle, apoptosis, proliferation and development. In particular, the abnormal expression of miRNAs is associated with several examples of human tumorigenesis: as such miRNAs may represent a novel, important class of oncogenes or tumor suppressor genes [5], [6]. MiR-145 is induced by the tumor suppressor gene TP53 through a p53 responsive element in its promoter and contributes to the silencing of the c-Myc oncogene [7], [8]. MiR-145 may also down-regulate the expression of MDM2, an E3 ubiquitin ligase promoting p53 degradation, creating a feedback loop with TP53 [9]. In mice, EGFR plays a negative role on miR-145 expression [10]. MiR-145 may also target VEGF-A expression in breast cancer [11].

This information and recent data indicate that miR-145 is a tumor suppressor capable of inhibiting proliferation in colon cancer and lung adenocarcinomas by targeting EGFR and NUDT1 [12–14]. Moreover, miR-145 is able to down-regulate several genes implicated in cell invasion, such as JAM-A, MUC1 and FSCN1, in breast, bladder and prostate cancer [15–18].

We have investigated the expression of miR-145 in gliomas. Our results show that its expression is down-regulated in these tumors and particularly in malignant subtypes, is associated to survival. We also identified a novel target of miR-145, NEDD9 and found that its regulation modulates the invasion potential of gliomas. Interestingly, NEDD9 and miR-145 expression appear reciprocally inter-connected.

# RESULT

### miR-145 is strongly down-regulated in malignant gliomas

In previous unpublished studies, we attempted an in-depth characterization of the microRNA expression profiles of glioblastoma (GB) specimens, primary cell lines derived from GB growing as neurospheres (GB-NS) in the presence of b-fibroblast growth factor (b-FGF) and epidermal growth factor (EGF).

We evaluated the expression levels of different microRNAs previously known to be down-regulated in both tumors and stem cells: miR128a [15], let7a [16], miR181a [17], miR101 [18], and miR-145. miR128a is a typical brain-enriched miRNA that is usually up-regulated during differentiation and development; we found miR128a down-regulated in GB-NS compared to normal brain tissue. miRNA let7a is typically down-regulated in many cancers [19–21], whereas miR181 is mainly up-regulated during differentiation [22]. Both of these miRNAs are weakly expressed or down-regulated in GB [23]. miR101 is a well-known negative regulator of EZH2 [18], a histone methyltransferase of polycomb repressive complex 2 (PRC2) that catalyzes the trimethylation of histone H3 at lysine 27. H3K27 methylation causes gene silencing and is important for stem cell maintenance and proliferation [24], and miR101 was found to be moderately down-regulated in GB-NS. The most interesting result was found for miR-145; this miRNA, which is usually down-regulated in human embryonic stem cells and acts as a negative regulator of stemness [25–27], was expressed in low-grade gliomas but strongly down-regulated in both GB specimens and GB-NS.

To investigate miR-145 expression in gliomas we studied 27 low-grade gliomas (LGG), 18 glioblastomas (GB) and 18 GB primary cell lines growing in culture as neurospheres (GB-NS). We found that miR-145 is expressed at much lower levels in GB compared to normal brain tissue and LGG ($P < 10\text{-}10$; $p < 0.001$, respectively). We also observed that miR-145 expression was very low or undetectable in GB-NS ($P < 10\text{-}10$ vs. normal brain; $P < 10\text{-}6$ vs. GB) (Figure 1A).

The expression of miR-145 was correlated with the progression-free survival (PFS) of LGG patients and with overall survival (OS) of GB patients. We compared LGG with higher and lower expression of miR-145 (LGG-miR-H, n = 10, relative expression vs. normal brain expressed as 2-DDCt ≥ 0.3; LGG-miR-L, n = 10, 2-DDCt < 0.3 vs. normal brain). LGG-miR-H patients showed significantly greater survival than LGG-miR-L (median PFS: 85.5 months in LGG-miR-H, 41 months in LGG-miR-L; P < 0.008) (Figure 1B). OS in LGG could not be evaluated, as the number of deceased patients was insufficient for the analysis. MiR-145 expression could be correlated with OS in a subgroup of 15 GB: GB with higher expression of miR-145 (GB-miR-H, 2-DDCt ≥ 0.08, n = 8) had significantly longer survival than GB with lower expression of miR-145 (GB-miR-L, 2-DDCt < 0.08, n = 7, P = 0.0183); median OS was 6.5 months in GB-miR-L and 10.4 months in GB-miR-H, (P = 0.018; Figure 1C). A trend for longer PFS in GB-miR-H was present (P < 0.09). These data indicate that low expression of miR-145 in gliomas is associated with a more aggressive phenotype.

**miR-145 affects glioblastoma stemness and migration**

To verify the involvement of miR-145 in glioblastoma stemness and migration, we over-expressed miR-145 in seven GB-NS cell lines (Figure 2A, left panel), subsequently defined as miRover-NS. MiR-145 mRNA levels were up-regulated 3 days only after infection, and the level of expression significantly increased after 15 days.

First, we observed that in all GB-NS SOX2 was up-regulated (P = 0.0038), whereas the astrocyte marker GFAP was absent (P = 1.7x10-10). To assess the inverse relationship between expression of SOX2 and miR-145, we analyzed SOX2 levels in GB-NS following miR-145 over-expression. We found a significant decrease of SOX2 expression and a concomitant increase of GFAP in miRover-NS compared to cells infected with the empty vector (Empty-NS; P = 0.0001 and P = 0.04, respectively).

We also observed an increased level of miR-145 expression in GB-NS exposed to 3% serum. The expression pattern of SOX2 was symmetric, as it decreased after one day of exposure to serum (P = 0.005). Differentiation was also associated with an increased expression of GFAP (P = 0.0003) (Figure 2A, central panel).

Next, the effects of miR-145 over-expression on migration and proliferation were evaluated in miRover-NS and Empty-NS. MiRover-NS had a significantly lower migration capacity compared to Empty-NS (P = 6.4x10-6; Figure 2B).

An analysis of the proliferation kinetics revealed some decrease in cell proliferation in miRover-NS relative to Empty-NS but not significant (P = 0.6, data not shown) [28].

These data show that in miR-145 over-expression affects migration but not proliferation of GB-NS.

**miR-145 and NEDD9 expression are negatively correlated in GB-NS**

To further characterize the effect of miR-145 on GB-NS, we compared gene expression profiles of miRover-NS and Empty-NS using the GeneChip Human Genome U133Plus Array, which includes over 47,000 human transcripts. We used a filter based on an expression value threshold of 100 and eliminated 32,438 of the 54,675 probe sets. The remaining 22,237 probe sets were used for the identification of DEGs using a fold change (FC) threshold. A total of 249 DEGs passed the FC cut-off in all the sample comparisons that were considered (Table S1). Functional classifications based on Gene Ontology annotations are listed in Table S2. We focused our validation experiments on NEDD9, as this gene is a putative target of miR-145 (mirSVR score of -0.766 on ww.microRNA.org). The potential for regulation of the 3' UTR of NEDD9 by miR-145 has been demonstrated in a bioinformatic study [29]. Moreover, the 3' UTR of NEDD9 exactly matches positions 2-8 of the mature miR-145 (8-mer, probability of conserved targeting – PCT > 0.75, www.TargetScanHuman.org).

Using real-time PCR, we observed that NEDD9 is down-regulated in miRover-NS compared to Empty-NS six days after infection (P = 0.0001) (Figure 2C). Down-regulation of NEDD9 expression was also demonstrated in GB-NS after serum exposure (P = 4.8x10-5) (Figure 2D).

The down-regulation of NEDD9 was also confirmed by Western blot analysis performed on seven samples of GB-NS over-expressing miR-145 relative to Empty-NS (Figure 2E).

Overall we found a strong inverse correlation between NEDD9 and miR-145 expression. These data support the assertion that miR-145 is responsible for NEDD9 regulation in GB-NS.

**miR-145 affects glioblastoma invasion by NEDD9 modulation in vivo**

To elucidate the involvement of miR-145 in tumorigenesis, we stereotaxically implanted miRover-NS and Empty-NS into the nucleus caudatum of immunodeficient mice. The overexpression of miR-145 reduced the tumorigenicity of GB-NS, and mice injected with miRover-NS had a significantly higher survival rate (P = 0.003 vs. Empty-NS) (Figures 3A and S2). Histological and immunohistochemical analyses of the tumors derived from miRover-NS and Empty-NS showed a similar proliferation index measured by Ki67+ cells, in agreement with in vitro data. miRover tumors showed a significant down-regulation of the neural stem cell marker nestin, a strong up-regulation of GFAP and a significant decrease in the number of migrating cells evaluated as doublecortin (DCX) positive cells [30], [31] (P = 8.7x10-8, P = 4.9x10-7, and P = 0.0001, respectively, compared to Empty tumors). Notably, a significant reduction in tumor-invading cells, as measured in NEDD9+ cells, was detected in miRover tumors (P = 2.5x10-8 vs. Empty tumors) (Figure

3B). These data suggest that miR-145 overexpression reduces the invasion potential of GB-NS in vivo. A significantly higher expression of miR-145 was observed by real-time PCR performed on paraffin-embedded, serial sections derived from the miRover tumors (P = 1.59x10-6 vs. Empty tumors), indicating that overexpression of miR-145 was maintained during tumor formation. Concurrently, NEDD9 was significantly down-regulated (P = 10-4 vs. Empty tumors) (Figure 3C).

The over-expression of miR-145 affects the aggressive and invasive features of GB-NS, as confirmed by a greater differentiation of the tumor phenotype and a highly significant decrease in NEDD9, supporting the concept that miR-145 plays an important role in glioblastoma invasiveness by regulating NEDD9.

**NEDD9 expression affects low-grade glioma progression and glioblastoma invasiveness**

We studied the contribution of NEDD9 to glioma progression and invasiveness using real-time PCR to analyze mRNA expression levels in 18 GB specimens, 18 GB-NS cell lines and 27 LGG specimens previously investigated for expression of miR-145. NEDD9 was differentially expressed in GB (mean ± SD: 1.3 ± 2.5; P = 0.03 vs. normal brain) and in GB-NS (mean ± SD: 1.9 ± 4.5; P = 0.02 vs. normal brain) (Figure 4A).

LGG are characterized by the heterogeneous expression of NEDD9 (mean value 1.5 ± 0.8, median 1.3; P = 0.04 vs. normal brain). We distinguished two groups of LGG, identified by higher (LGG-NEDD9-H, 2-DDCt ≥ 1.3, n = 11) and lower (LGG-NEDD9-L, 2-DDCt < 1.3, n = 11) levels of NEDD9 expression (mean ± SD: 2.56 ± 0.07 and 0.88 ± 0.04, respectively, P = 9.9x10-6).

We then compared the level of NEDD9 expression with the PFS in a subgroup of 22 LGG patients and found that LGG-NEDD9-H patients had a significantly lower survival than LGG-NEDD9-L patients (median OS: 41 months for LGG-NEDD9-L, 82 months for LGG-NEDD9-H; P < 0.05) (Figure 4B).

To confirm the role of NEDD9 in glioblastoma invasion, we performed silencing experiments using the same cell lines used for miR-145 over-expression. We observed a strong inhibition of NEDD9, as measured based on protein levels (Figure 4C), and real-time PCR (data not shown). We further evaluated in shNEDD9-NS and scrambled-NS the effect of NEDD9 inhibition on invasion. We observed a significant inhibition of invasion in shNEDD9-NS compared to scrambled-NS (P = 0.008) (Figure 4D). The proliferation kinetics based on three time points (48, 72 and 96 h) showed no significant differences between shNEDD9-NS and scrambled-NS (data not shown).

Notably, we found that shNEDD9-NS expressed miR-145 at levels comparable to that of normal brain and 1000-fold higher than in GB-NS (Figure 4E).

## DISCUSSION

MiR-145 is down-regulated in several types of cancers, such as bladder, urothelial, breast, prostate and colon carcinoma [32–35].

In our study, we found down-regulated expression of miR-145 in gliomas, confirming recent reports suggesting a tumor-suppressing role for this microRNA [27], [36]. We found that the expression levels of miR-145 are greatly reduced or absent in GB and in glioblastoma stem-like cells compared to normal brain, suggesting tumor suppressive functions for this microRNA. In particular, miR-145 is moderately down-regulated in low-grade gliomas but almost absent in GB and GB-NS. Interestingly, higher expression of this microRNA is correlated with better survival in LGG and GB patients, thereby confirming the involvement of miR-145 in glioma progression.

MiR-145 has been implicated in stemness maintenance. Fang et al [25] proposed a bistable system involving reciprocal interactions of SOX2 and miR-145 and suggested the involvement of miR-145 in GB stemness. Yang et al [27] demonstrated that miR-145 suppresses the self-renewal and tumor-initiating properties of GB stem-like cells both in vitro and in vivo. These studies support the concept that the miR-145-controlled pathways are important for reducing GB stem-like cells and their chemoradioresistance, partly via the downstream targets of miR-145 SOX2 and OCT4.

We confirmed this observation in primary cell lines derived from glioblastoma specimens growing in culture as neurospheres [37]. We found that the over-expression of miR-145 is correlated with a reduction in the expression of stem cell marker SOX2 and with increased expression of the astrocyte marker GFAP.

The over-expression of miR-145 in GB-NS also affects migration in vitro and in vivo, thus supporting a key role of miR-145 in GB invasion, as observed by Lee [36]. Because the invasive ability is one of the most important features of GB and one of the causes of poor prognosis, miR-145 appears to be an important factor for GB aggressiveness.

As a small, regulatory RNA, miR-145 has the potential to regulate several genes implicated in cell proliferation, apoptosis, stemness and invasion [6], [25], [26], [32], [33], [38], [39].

Gene expression profiling was used to identify three main signatures involved in stemness, apoptosis and invasion in GB-NS over-expressing miR-145.

We focused our validation experiments on the gene NEDD9 to test the relationship of miR-145 with invasion ability.

HEF1/CAS-L/NEDD9 is a non-catalytic scaffolding protein implicated in the invasion ability of several types of cancer [40–42]. NEDD9 has been proposed as a biomarker of invasiveness in lung cancer [43] and melanoma due to its role in the regulation and activation of transcriptional pathways relevant for metastasis and cancer progression, including FAK and Src [44]. The interactions of

NEDD9 with FAK and Src lead to the tyrosine phosphorylation of NEDD9 to create binding sites for effector proteins such as Rac and the Cas-Crk complex, which have been previously studied in the context of cell migration [45]. In the past several years, NEDD9 has been studied in breast cancer, as a cancer cell-intrinsic protein with a pro-oncogenic role and as a candidate biomarker of tumor aggressiveness [40]. In GB, NEDD9 is a downstream effector of FAK that causes an increase in migration capacity [46]: in HNSCC, NEDD9 functions as an invasion regulator via VEGF activation [42].

We found that NEDD9 is up-regulated in malignant gliomas and in GB-NS, where the over-expression of miR-145 leads to the down-regulation of NEDD9.

NEDD9 is also expressed in a subgroup of low-grade glioma specimens and was show to be correlated with lower patient survival, indicating a relevant role of NEDD9 in glioma progression.

In vitro NEDD9 silencing in GB-NS is responsible for the significant inhibition of invasion ability, thereby confirming the involvement of NEDD9 in glioblastoma invasiveness. Finally, we demonstrated that NEDD9 silencing leads to the up-regulation of miR-145. Together, these results suggest that miR-145 down-regulates NEDD9, and NEDD9 down-regulates miR-145, forming a double-negative feedback loop in GB-NS. This hypothesis is supported by the presence of NEDD9-binding regions in the miR-145 locus, which would allow the direct binding of the NEDD9 3'UTR to the genomic region of miR-145 ([29] www.targetscan.org).

Recent studies [47] [48] support the idea that microRNAs can be regulated by their target interactions. This mechanism has led to the concept that “regulation in miRNA pathways is a two-way street”.

In our study, further investigations will be required to characterize the double-negative feedback loop between NEDD9 and miR-145.

The high levels of NEDD9 expression in GB and in LGG with lower PFS, along with the absence of NEDD9 in normal brain tissue, support the concept that NEDD9 expression is an important requisite for glioma invasion and progression.

**Acknowledgements**

This project was supported by a grant from the Istituto Superiore di Sanità and by funds from Associazione Italiana per la Ricerca sul Cancro (AIRC) to GF.

## MATERIALS AND METHODS

### Tumor specimens and cell cultures

Primary glioblastomas (GB) and grade II gliomas, including fibrillary and gemistocytic astrocytomas (low-grade gliomas, LGG), were obtained from the Department of Neurosurgery of the "Istituto Neurologico Carlo Besta" with informed consent from the patients. Human glioma samples were frozen in liquid nitrogen and/or placed in saline solution immediately after surgery. GB cell lines (GB-NS) were obtained following the dissociation of GB specimens in collagenase type I (Invitrogen-Life Technologies, Carlsbad, California, USA) and were grown in DMEM/F12 (GIBCO- Life Technologies, Carlsbad, California/USA) with penicillin-streptomycin (1:100, EuroClone-Milan, Italy), B-27 supplement (1:50, GIBCO- Life Technologies, Carlsbad, California, USA), human recombinant fibroblast growth factor 2 (bFGF; 20 ng/mL; Tebu-bio, Milan, Italy), epidermal growth factor (EGF; 20 ng/mL; Tebu-bio, Milan, Italy) and heparin (5 microg/ml; Sigma Aldrich, St Louis, Missouri, USA).

### RNA extraction and reverse transcription

Total RNA was extracted from GB-NS, human frozen GB and LGG specimens using TRIzol® Reagent (Invitrogen) according to the manufacturer's instructions. Total RNA for miRNA analysis was reverse-transcribed using the TaqMan® microRNA Reverse

Transcription Kit (Applied Biosystems-Life Technologies, Carlsbad, California, USA) with miRNA-specific primers. Total RNA for NEDD9 analysis was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems-Life Technologies, Carlsbad, California, USA).

The extraction of total RNA from FFPE tissue sections was performed using the miRNeasy FFPE kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions; the samples used for these extractions were obtained from the brains of mice injected with GB-NS over-expressing miR-145 (miRover-NS) or GB-NS Empty (Empty-NS).

**Real-time polymerase chain reaction analyses**

Real-time PCR for the quantification of miR-145 was performed on an ABI PRISM 7900 real-time PCR system (Applied Biosystems, Foster City, CA, USA) with TaqMan chemistry (Applied Biosystems, Foster City, CA, USA) using 2.5 ng of cDNA from the RT-PCR solution in a final volume of 20 microL To quantify the mature miRNA and detect miR-145, we used the TaqMan® MicroRNA Assay kits (Applied Biosystems, Foster City, CA, USA) and a customized Assay on Demand (assay ID TM: 002278). We normalized miR-145 with respect to RNU6B (assay ID TM: 001093). The expression of NEDD9 was detected by SybrGreen chemistry (Forward: GAGCTGGATGGATGACTACGA; Reverse: AGCTCTTTCTGTTGCCTCTCA), and normalized relative to beta-2

microglobulin (Forward: GTGCTCGCGCTACTCTCTCT; Reverse: CCCAGACACATAGCAATTCAG). Commercial RNA from human normal brain (Ambion, AB) was used as the calibrator for the calculation of expression levels using the ΔΔCt method [49].

**Prediction of microRNA targets**

To identify potential target genes and their conserved sites, we used the TargetScanHuman (release 5.2, http://www.targetscan.org), microRNA (www.microrna.org), MicroCosm Targets (www.ebi.ac.uk/enright.svr/microcosm/), PicTar (www.pictar.mdc-berlin.de), miRDB (www.mirdb.orf), and miRECORDS (www.mirecords.biolead.org) databases.

**Proliferation and Invasion Assay**

Proliferation kinetics were measured by plating 8,000 cells/25 cm2. The cell count was obtained using the trypan blue exclusion test performed every 3 days (days 0, 3, 6, 9, and 12).

Invasion was assayed in vitro using the Transwell-96 system (Becton Dickinson, USA) as recommended by the manufacturer. After 24 h and 48 h, migrating cells were stained with crystal violet solubilized with 10% acetic acid, and the absorbance was determined at 570 nm.

**Western blot and antibodies**

Protein samples were pelleted in RIPA lysis buffer with phosphatase and protease inhibitors, resolved using 10% SDS-PAGE and electroblotted onto nitrocellulose membranes. Membranes with transferred proteins were incubated with the primary antibody anti-NEDD9 (1:2000, Acris, Herford, Germany) or anti-GAPDH (1:5000, Abcam, Cambridge, UK). The primary antibody incubation was followed by incubation with the secondary antibody anti-mouse (1:10000). A chemiluminescence reaction using the ECL (enhanced chemiluminescence) Plus kit (Amersham, GE Healthcare, Buckinghamshire, UK) was detected using film.

**Plasmids and Infection**

pCLNSX-miR-145 and pCLNSX-Empty were used to transfect packaging cells using Phoenix Ampho. After 48 h of transfection, we infected GB-NS with supernatant containing virions with miR-145 cDNA or the empty vector (miRover-NS or empty-NS, respectively). The infection was repeated twice, and cells were selected for resistance to Neomycin (0.4 mg/ml).

shNEDD9 plasmid DNA (Mission RNAi, TRCN000004967, Sigma-Aldrich, St Louis, Missouri, USA) for NEDD9 silencing was used for transient transfection according to the manufacturer's instructions. The transfection was performed using 5 microgrammi of shNEED9/Scrambled plasmids for every 200000 primary GB-NS cells. The cell pellets for RNA and protein analyses were collected at

48 h after transfection.

**Microarray analysis**

Microarray analysis was performed on five different NS cell lines overexpressing miR-145, and Empty-NS cells were used as controls.

Fragmented cRNAs were hybridized to the HG-U133 Plus 2.0 GeneChip (Affymetrix, Santa Clara, USA) following standard procedures. Data processing was mainly performed using Bioconductor 2.10 and R.15 [50]. The Robust Multichip Average [51] algorithm was applied to normalize using the quantile method, and normalized probeset intensities were calculated. A signal-based filtering was applied to the expression level (>100) of each probeset for all of the different groups that were considered. Differentially expressed genes were identified using a fold change (FC) threshold of 1.2 for all sample comparisons. The functional annotation of genes that passed the FC and expression signal cut-offs was performed using the Gene Ontology (GO) Biological Process category and the hypergeometric test (hyperGTest function) [50] for gene over-representation.

**In vivo experiments**

A total of 45 immune-deficient CD1-nude mice received brain injections of 105 miRover-NS or Empty-NS cells (n = 10/group for survival, n = 5/group for histological studies) from four GB-NS cell lines (BT165NS, BT168NS, BT273NS, BT275NS) infected with retroviral vectors (pCLNSX-miR-145 and pCLNSX-Empty).

**Immunohistochemistry and immunofluorescence analyses**

Immunohistochemical analyses for Ki67 (1:100, BD, New Jersey, USA), Nestin (1:100, R&D System, Minnesota, USA), GFAP (1:100, DAKO, Glostrup, Denmark), DXC (Abcam, 1:100) and NEDD9 (Acris, 1:50) were performed on paraffin-embedded sections.

Tumor sections were blocked with 5% goat serum in PBS for 60 min, incubated overnight with primary antibodies, and incubated with biotinylated secondary antibodies (1:200 Vector Lab) for 1 h. Antibody binding was detected using the Vectastain Elite Avidin–Biotin Complex-Peroxidase kit according to the manufacturer's instructions.

All sections were counterstained with Mayer's hematoxylin and visualized using a LEICA MDLB light microscope.

The percentages of Ki67-, GFAP-, and NEDD9-positive cells were calculated in 5 independent high-magnification fields. Positive cells were counted only within the tumor area. The results are expressed as percentages.

The positive rates were counted manually in triplicate by two observers (F.P. and MC.S.) using the photomicrographs.

**Statistical analysis**

Statistical comparisons of the data sets were performed using a two-tailed Student's T-test, and the results were considered significant at $p<0.05$.

Cumulative survival curves were constructed using the Kaplan-Meier method (MedCalc 9.3).

## REFERENCES


[1] I. Ben-Porath et al., “An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors.,” Nature genetics, vol. 40, no. 5, pp. 499-507, May 2008.

[2] Y. Guo et al., “Expression profile of embryonic stem cell-associated genes Oct4 , Sox2 and Nanog in human gliomas,” pp. 763-775, 2011.

[3] R. R. Chivukula and J. T. Mendell, “Abate and switch: miR-145 in stem cell differentiation.,” Cell, vol. 137, no. 4, pp. 606-8, May 2009.

[4] D. Bartel, “MicroRNAs : Genomics , Biogenesis , Mechanism , and Function Genomics.,” Cell, vol. 116, pp. 281-297, 2004.

[5] W. Zhang, J. E. Dahlberg, and W. Tam, “MicroRNAs in tumorigenesis: a primer.,” The American journal of pathology, vol. 171, no. 3, pp. 728-38, Sep. 2007.

[6] S.-J. Kim et al., “Development of microRNA-145 for therapeutic application in breast cancer.,” Journal of controlled release : official journal of the Controlled Release Society, vol. 155, no. 3, pp. 427-34, Nov. 2011.

[7] M. Sachdeva et al., “p53 represses c-Myc through induction of the tumor suppressor miR-145.,” Proceedings of the National Academy of Sciences of the United States of America, vol. 106, no. 9, pp. 3207-12, Mar. 2009.

[8] H. I. Suzuki, K. Yamagata, K. Sugimoto, T. Iwamoto, S. Kato, and K. Miyazono, “Modulation of microRNA processing by p53.,” Nature, vol. 460, no. 7254, pp. 529-33, Jul. 2009.

[9] J. Zhang, Q. Sun, Z. Zhang, S. Ge, Z.-g Han, and W.-t Chen, “Loss of microRNA-143 / 145 disturbs cellular growth and apoptosis of human epithelial cancers by impairing the MDM2-p53 feedback loop,” no. January, pp. 1-9, 2012.

[10] H. M. Zhu et al., “EGFR signals down-regulate tumor suppressors miR-143 and miR-145 in Western diet-promoted murine colon cancer: Role of G1 regulators.,” Molecular cancer research : MCR, Jun. 2011.

[11] C. Zou et al., “MiR-145 inhibits tumor angiogenesis and growth by N-RAS and VEGF © 2012 Landes Bioscience . Do not distribute . © 2012 Landes Bioscience . Do not distribute .,” pp. 2137-2145, 2012.

[12] H. Zhu et al., “EGFR signals downregulate tumor suppressors miR-143 and miR-145 in Western diet-promoted murine colon cancer: role of G1 regulators.,” Molecular cancer research : MCR, vol. 9, no. 7, pp. 960-75, Jul. 2011.

[13] W. C. S. Cho, “miR-145 inhibits cell proliferation of human lung adenocarcinoma by targeting EGFR and NUDT1,” Rna Biology, vol. 145, no. February, pp. 125-131, 2011.

[14] Q. Xu et al., “MiR-145 directly targets p70S6K1 in cancer cells to inhibit tumor growth and angiogenesis.,” Nucleic acids research, pp. 1-14, Sep. 2011.

[15] T. Papagiannakopoulos et al., “Pro-neural miR-128 is a glioma tumor suppressor that targets mitogenic kinases.,” Oncogene, vol. 31, no. 15, pp. 1884-95, Apr. 2012.

[16] B. Boyerinas, S.-M. Park, A. Hau, A. E. Murmann, and M. E. Peter, “The role of let-7 in cell differentiation and cancer.,” Endocrine-related cancer, vol. 17, no. 1, pp. F19-36, Mar. 2010.

[17] G. Chen et al., “MicroRNA-181a sensitizes human malignant glioma U87MG cells to radiation by targeting Bcl-2,” BMC Cancer, pp. 997-1003, 2010.

[18] M. Smits et al., “miR-101 is down-regulated in glioblastoma resulting in EZH2- induced proliferation , migration , and angiogenesis AbstrAct : Background : Glioblastoma ( GBM ) is a malignant brain tumor with dismal,” vol. 1, no. 8, pp. 710-720, 2010.

[19] Y. Akao, Y. Nakagawa, and T. Naoe, “let-7 microRNA functions as a potential growth suppressor in human colon cancer cells,” Biological and Pharmaceutical Bulletin, vol. 29, no. May, pp. 903-906, 2006.

[20] S. M. Johnson et al., “RAS is regulated by the let-7 microRNA family.,” Cell, vol. 120, no. 5, pp. 635-47, Mar. 2005.

[21] Q. Dong et al., “MicroRNA let-7a inhibits proliferation of human prostate cancer cells in vitro and in vivo by targeting E2F2 and CCND2.,” PloS one, vol. 5, no. 4, p. e10147, Jan. 2010.

[22] X. Li et al., “MiR-181 mediates cell differentiation by interrupting the Lin28 and let-7 feedback circuit.,” Cell death and differentiation, vol. 19, no. 3, pp. 378-86, Mar. 2012.

[23] S. a Ciafrè et al., “Extensive modulation of a set of microRNAs in primary glioblastoma.,” Biochemical and biophysical research communications, vol. 334, no. 4, pp. 1351-8, Sep. 2005.

[24] F. Orzan et al., “Enhancer of Zeste 2 (EZH2) is up-regulated in malignant gliomas and in glioma stem-like cells.,” Neuropathology and applied neurobiology, vol. 37, no. 4, pp. 381-94, Jun. 2011.

[25] X. Fang et al., “The SOX2 response program in glioblastoma multiforme: an integrated ChIP-seq, expression microarray, and microRNA analysis.,” BMC genomics, vol. 12, no. 1, p. 11, Jan. 2011.

[26] G. La Rocca et al., “Regulation of microRNA-145 by growth arrest and differentiation.,” Experimental cell research, vol. 317, no. 4, pp. 488-95, Feb. 2011.

[27] Y.-P. Yang et al., “Inhibition of cancer stem cell-like properties and reduced chemoradioresistance of glioblastoma using microRNA145 with cationic polyurethane-short branch PEI.,” Biomaterials, vol. 33, no. 5, pp. 1462-76, Mar. 2012.

[28] S.-J. Lee et al., “Over-expression of miR-145 enhances the effectiveness of HSVtk gene therapy for malignant glioma.,” Cancer letters, pp. 1-9, Jan. 2012.

[29] D. H. Tran, K. Satou, and T. B. Ho, “Finding microRNA regulatory modules in human genome using rule induction,” BMC Bioinformatics, vol. 10, pp. 1-10, 2008.

[30] M.-C. Daou, T. W. Smith, N. S. Litofsky, C. C. Hsieh, and A. H. Ross, “Doublecortin is preferentially expressed in invasive human brain tumors.,” Acta neuropathologica, vol. 110, no. 5, pp. 472-80, Nov. 2005.

[31] K. Masui, S.-ya Mawatari, S. O. Suzuki, and T. Iwaki, “Evaluation of sensitivity and specificity of doublecortin immunostatining for the detection of infiltrating glioma cells.,” Brain tumor pathology, vol. 25, no. 1, pp. 1-7, Jan. 2008.

[32] M. S. Ostenfeld et al., “miR-145 induces caspase-dependent and -independent cell death in urothelial cancer cell lines with targeting of an expression signature present in Ta bladder tumors,” Oncogene, pp. 1073-1084, 2010.

[33] S. Wang, C. Bian, Z. Yang, Y. Bo, and J. Li, “miR-145 inhibits breast cancer cell growth through RTKN,” International journal of, pp. 1461-1466, 2009.

[34] M. Fuse et al., “Restoration of miR-145 expression suppresses cell proliferation, migration and invasion in prostate cancer by targeting FSCN1.,” International journal of oncology, vol. 38, no. 4, pp. 1093-101, Apr. 2011.

[35] A. F. Ibrahim, U. Weirauch, M. Thomas, A. Grünweller, R. K. Hartmann, and A. Aigner, “MicroRNA Replacement Therapy for miR-145 and miR-33a Is Efficacious in a Model of Colon Carcinoma.,” Cancer research, Jul. 2011.

[36] S.-jin Lee et al., “Over-expression of miR-145 enhances the effectiveness of HSVtk gene therapy for malignant glioma,” Cancer Letters, vol. 320, no. 1, pp. 72-80, 2012.

[37] P. Tunici et al., “Genetic alterations and in vivo tumorigenicity of neurospheres derived from an adult glioblastoma,” Molecular Cancer, vol. 2, pp. 1-5, 2004.

[38] J. Zhang et al., “MiR-145, a new regulator of the DNA fragmentation factor-45 (DFF45)-mediated apoptotic network.,” Molecular cancer, vol. 9, p. 211, Jan. 2010.

[39] M. Sachdeva and Y.-Y. Mo, “miR-145-mediated suppression of cell growth, invasion and metastasis.,” American journal of translational research, vol. 2, no. 2, pp. 170-80, Jan. 2010.

[40] E. Izumchenko et al., “NEDD9 promotes oncogenic signaling in mammary tumor development.,” Cancer research, vol. 69, no. 18, pp. 7198-206, Sep. 2009.

[41] M. K. Singh, E. Izumchenko, A. J. Klein-Szanto, B. L. Egleston, M. Wolfson, and E. a Golemis, “Enhanced genetic instability and dasatinib sensitivity in mammary tumor cells lacking NEDD9.,” Cancer research, vol. 70, no. 21, pp. 8907-16, Nov. 2010.

[42] J. T. L. Jr, B. P. Salimath, M. G. Slomiany, and S. A. Rosenzweig, “Regulation of invasive behavior by vascular endothelial growth factor is HEF1-dependent,” Oncogene, vol. 29, no. 31, pp. 4449-4459, 2010.

[43] J.-X. Chang, F. Gao, G.-Q. Zhao, and G.-J. Zhang, “Expression and clinical significance of NEDD9 in lung tissues.,” Medical oncology (Northwood, London, England), Mar. 2012.

[44] M. Kim et al., “Comparative oncogenomics identifies NEDD9 as a melanoma metastasis gene.,” Cell, vol. 125, no. 7, pp. 1269-81, Jun. 2006.

[45] G. M. O’Neill, S. Seo, I. G. Serebriiskii, S. R. Lessin, and E. a Golemis, “A new central scaffold for metastasis: parsing HEF1/Cas-L/NEDD9.,” Cancer research, vol. 67, no. 19, pp. 8975-9, Oct. 2007.

[46] M. Natarajan et al., “HEF1 is a necessary and specific downstream effector of FAK that promotes the migration of glioblastoma cells.,” Oncogene, vol. 25, no. 12, pp. 1721-32, Mar. 2006.

[47] A. Baccarini, H. Chauhan, T. J. Gardner, A. D. Jayaprakash, R. Sachidanandam, and B. D. Brown, “Kinetic analysis reveals the fate of a microRNA following target regulation in mammalian cells.,” Current biology : CB, vol. 21, no. 5, pp. 369-76, Mar. 2011.

[48] A. E. Pasquinelli, “MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship,” Nature Reviews Genetics, vol. 13, no. 4, pp. 271-282, Mar. 2012.

[49] K. J. Livak and T. D. Schmittgen, “Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.,” Methods (San Diego, Calif.), vol. 25, no. 4, pp. 402-8, Dec. 2001.

[50] R. C. Gentleman et al., “Bioconductor : open software development for computational biology and bioinformatics,” Genome Biology, no. 10, 2004.

[51] R. A. Irizarry, B. M. Bolstad, F. Collin, L. M. Cope, B. Hobbs, and T. P. Speed, “Summaries of Affymetrix GeneChip probe level data,” World Wide Web Internet And Web Information Systems, vol. 31, no. 4, pp. 1-8, 2003.

**FIGURES**

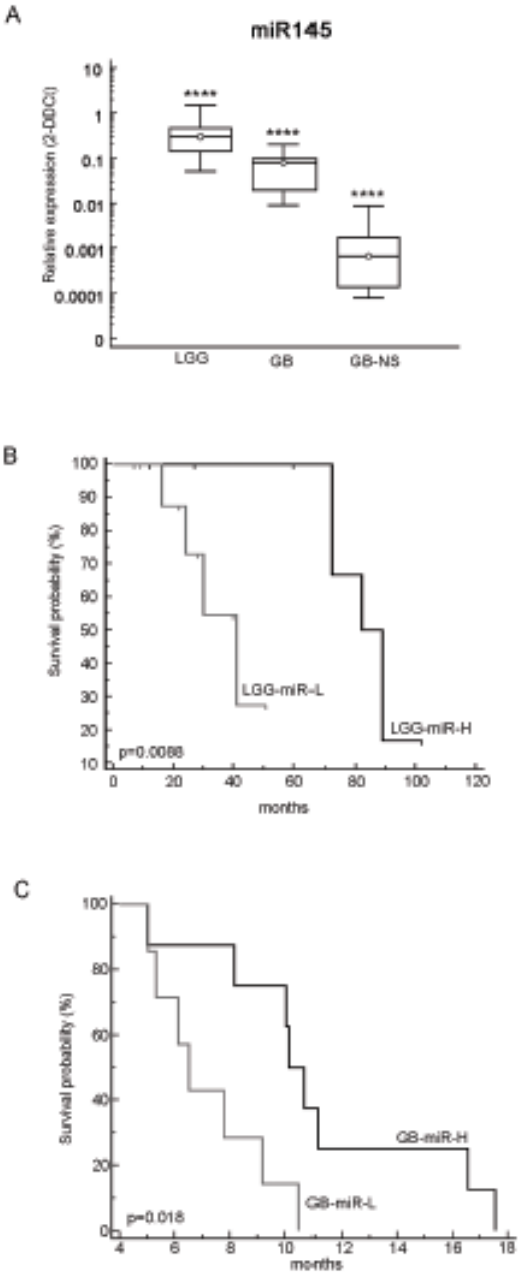

A
miR145
Relative expression (2-DDCt)
10
1
0.1
0.01
0.001
0.0001
0
****
****
****
LGG
GB
GB-NS
B
Survival probability (%)
100
90
80
70
60
50
40
30
20
10
LGG-miR-L
LGG-miR-H
p=0.0088
0
20
40
60
80
100
120
months
C
Survival probability (%)
100
80
60
40
20
0
GB-miR-H
GB-miR-L
p=0.018
4
6
8
10
12
14
16
18
months

**Figure 1**. Characterization of miR-145 in malignant gliomas.

A) A real-time PCR analysis was performed on 27 LGG samples, 18 GB samples and 18 corresponding GB-NS cell lines. The RNA data were normalized to the housekeeping gene RNU6B. miR-145 expression in LGG (mean ± SD: 0.41 ± 0.39, median: 0.3; P < 10-9) was significantly higher than in GB (mean ± SD: 0.07 ± 0.049, median: 0.08; P < 10-10) and in GB-NS (mean ± SD: 0.0019 ± 0.0029, median: 0.0007; P < 10-10) compared to normal brain samples (**** P < 0.0001).

B) Kaplan-Meier survival analysis showing the progression-free survival of 20 LGG patients with low miR-145 expression compared to other patients.

C) Kaplan-Meier survival analysis showing the OS of GB patients expressing high levels of miR-145 (n = 8) compared to GB with a lower expression of miR-145 (n = 7).

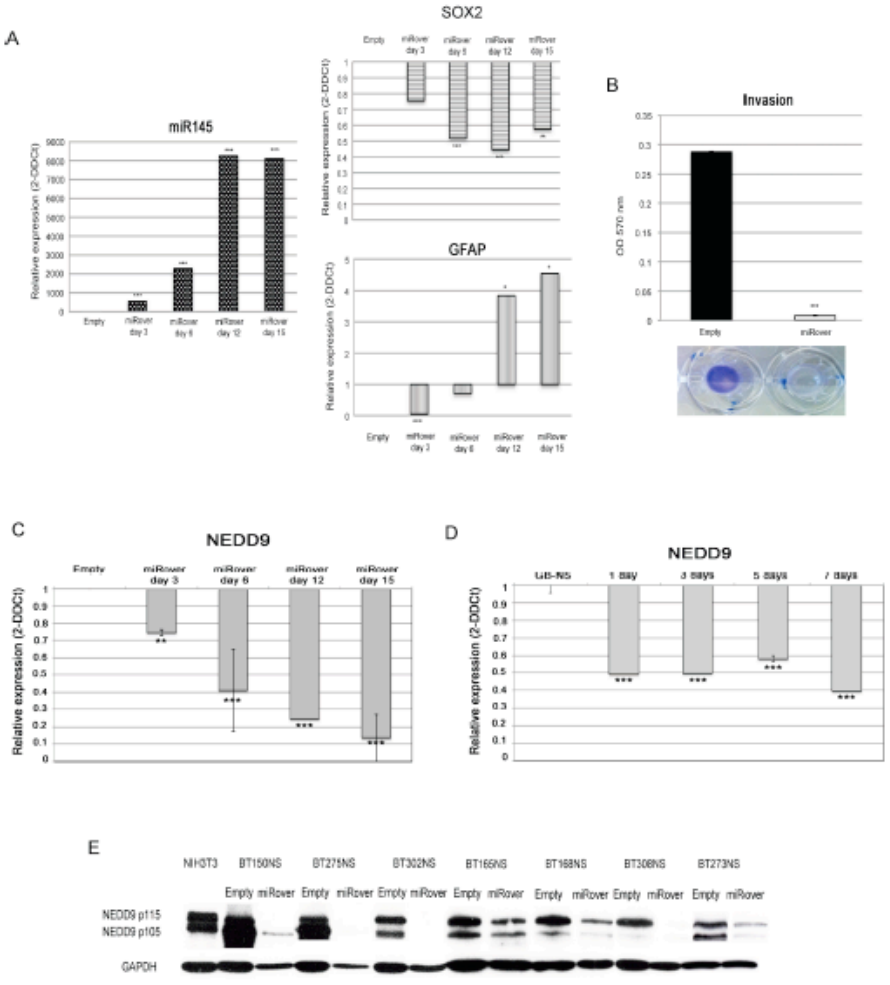



**Figure 2**. MiR-145 in GB stem-like cells.

A) Real-time PCR analysis showed, as expected, that miR-145 expression was significantly higher in miRover-NS (miRover) than in Empty-NS (Empty) (*** $P < 0.001$). A strong decrease in SOX2 (*** $P < 0.001$) and a concomitant increase of GFAP were found in miRover-NS vs. Empty-NS (* $P < 0.05$).

B) Significantly decreased invasion was observed in miRover-NS compared to Empty-NS in seven GB stem-like cell lines analyzed (*** $P < 0.001$).

C) Real-time PCR analysis demonstrated down-regulation of NEDD9 in miRover-NS 3- 15 days after infection and in GB-NS during differentiation taking place 1-7 days after addition of serum. The histogram is based on a representative cell line; the experiment was performed on a total of seven different miRover-NS, and NEDD9 showed the same behavior in all lines (** $P < 0.01$; *** $P < 0.001$).

D) Real-time PCR analysis demonstrated down-regulation of NEDD9 in GB-NS during differentiation taking place 1-7 days after addition of serum. The histogram represents the average of three different cell lines compared to normal brain.

E) Western blot analysis of NEDD9 expression in 7 GB-NS (BT150NS, BT275NS, BT302NS, BT165NS, BT168NS, BT308NS, and BT273NS) after infection with pCLNSX-miR-145 retroviral vector. Compared to Empty, the levels of NEDD9 protein are significantly reduced. GAPDH was used as housekeeping protein.

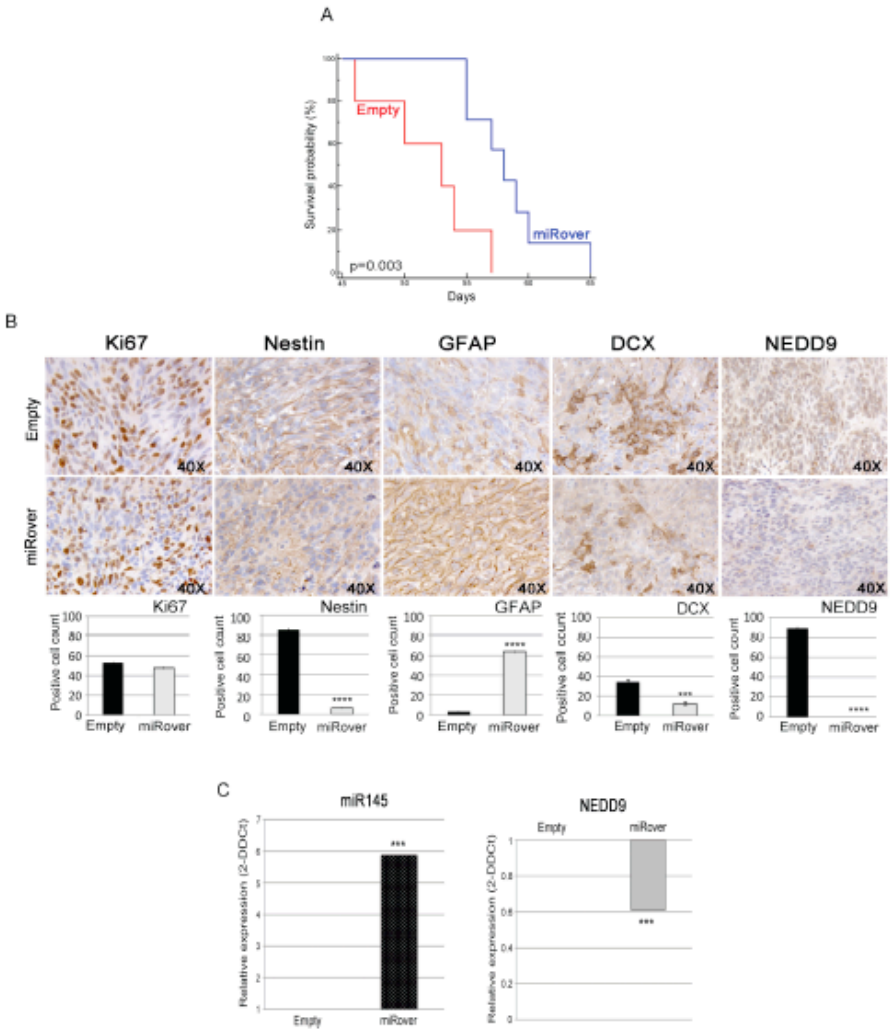



**Figure 3**. Effects of miR-145 overexpression in vivo.

A) Kaplan-Meier survival analysis shows that mice injected with miRover-NS survive longer than mice injected with Empty-NS (P = 0.003).

B) Immunohistochemistry of miRover and Empty tumors (40X). Ki67-, Nestin-, GFAP, DCX- and NEDD9-positive cell were counted on three to five independent 40X fields per tumor in 2-3 animals per group. miRover tumors present a higher amount of GFAP-positive cells and a lower amount of cells positive for Ki67, Nestin, DCX and NEDD9 compared to Empty. Histograms represent the quantification of positive cells: Ki67 (48 ± 1.2 % in miRover vs. 53.2 ± 0.6 in empty), Nestin (6.2 ± 0.7 % in miRover vs. 85.7 ± 1.3 in empty), GFAP (64.4 ± 1.1 % in miRover vs. 3.5 ± 0.5 in empty), DCX (12.5 ± 1.7 % in miRover vs. 34.5 ± 1.9 in empty), and NEDD9 (0 ± 0 % in miRover vs. 89.3 ± 1.2 in empty). A representative image for each tumor is displayed (* $P < 0.05$; *** $P < 0.001$; ****$P < 0.0001$).

C) Real-time PCR analysis of the expression of miR-145 and NEDD9 in tumors derived from miRover-NS relative to Empty-NS paraffin sections. The RNA inputs were normalized to the housekeeping gene RNU6B for miR-145 and beta-2 microglobulin for NEDD9. The over-expression of miR-145 and down-regulation of NEDD9 were maintained in vivo (*** $P < 0.0001$).

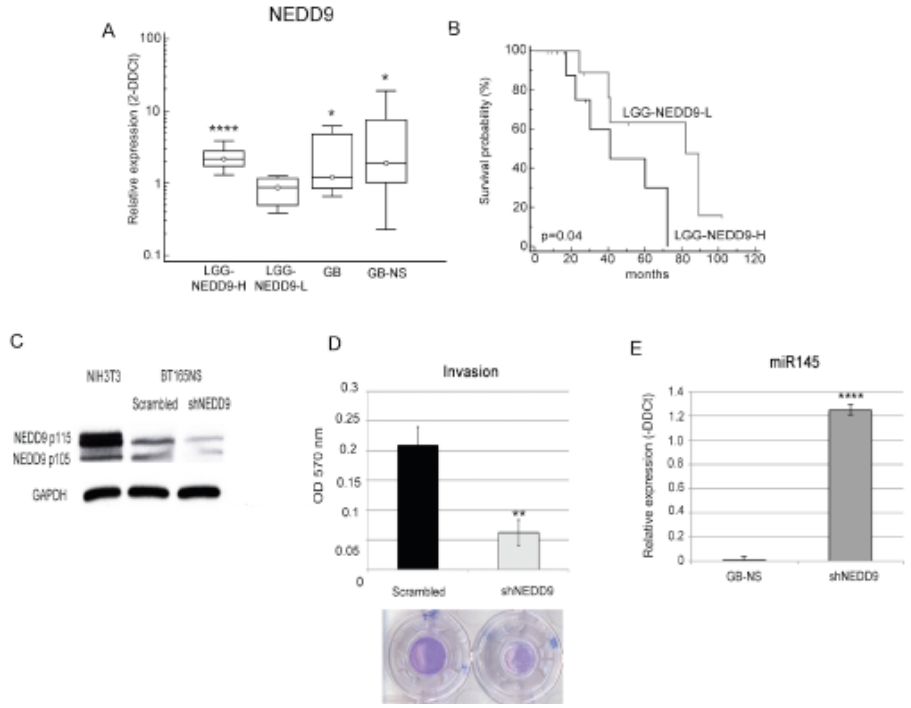



**Figure 4**. NEDD9 expression is associated to glioma survival and invasion.

A) Real-time PCR analysis performed on 27 LGG and 18 GB and 18 GB-NS compared to normal brain: LGG-NEDD9-L, mean ± SD: 0.85 ± 0.33, p < 0.5; LGG-NEDD9-H, mean ± SD: 2.18 ± 0.85, P < 10-4; GB 2.55 ± 2.17 folds, P < 0.05; GB-NS mean ± SD: 4.45 ± 5.37, P < 0.05. Data were normalized against the housekeeping gene beta-2 microglobulin. (* P < 0.05; **** P < 0.0001).

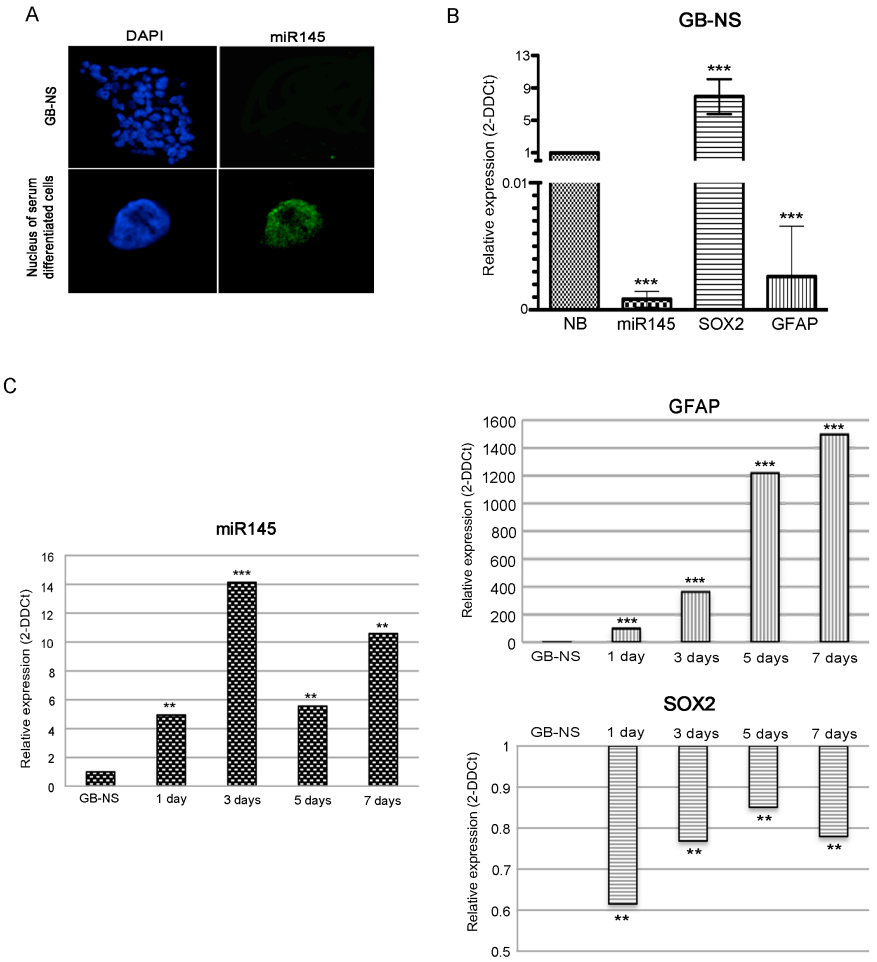
B) Kaplan-Meier survival analysis shows a significant correlation between the expression of NEDD9 and the PFS of LGG patients. LGG patients expressing lower levels of NEDD9 survived longer than LGG patients expressing higher levels of NEDD9 (median survival 41 months for LGG-NEDD9-L, 82 months for LGG-NEDD9-H, $p<0.05$).

C) Western blot analysis performed on a representative cell lines shows a significant reduction of NEDD9 expression in shNEDD9 cells compared to control cells (scrambled). GAPDH was used as a loading control.

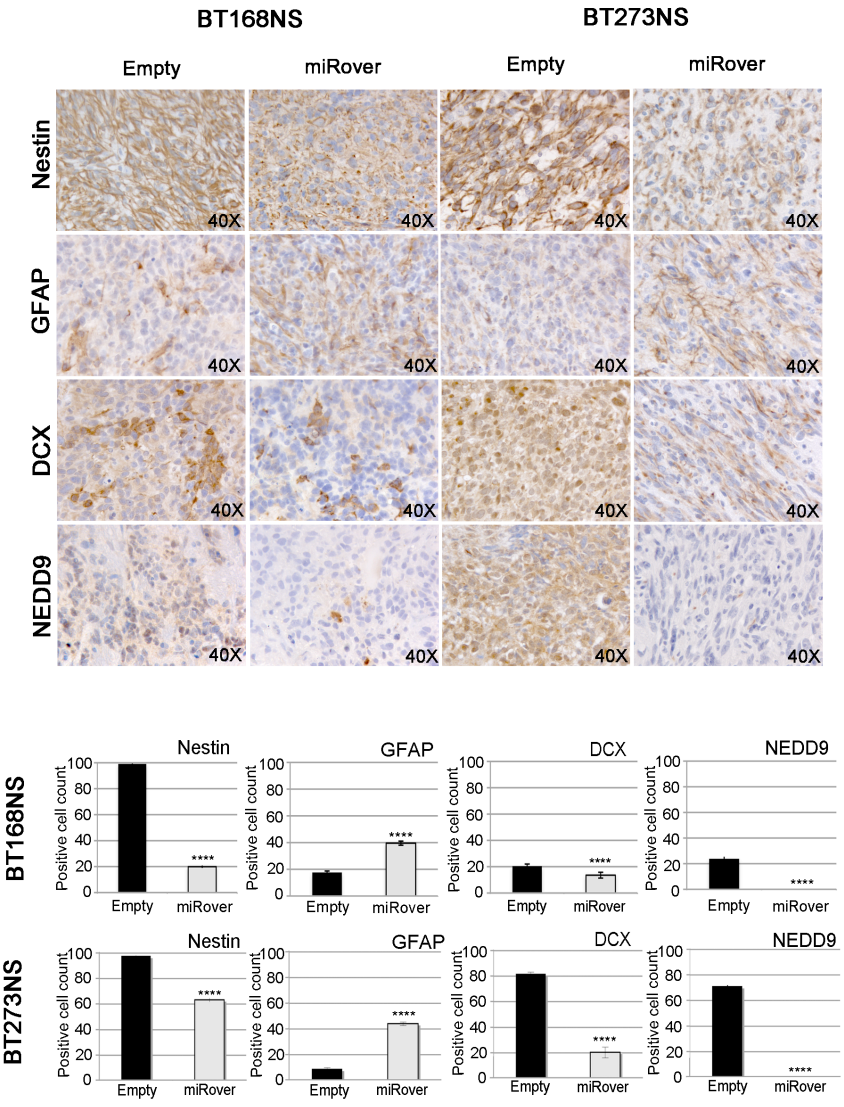
D) The migration assay confirms a significant decrease in the migration ability in shNEDD9 relative to scrambled (** $P < 0.01$).

E) Real-time PCR on BT165NS-shNEDD9 (shNEDD9) shows an up-regulation of miR-145, which is expressed as in normal brain tissue, with a fold change > 1000 compared to BT165NS (GB-NS) (*** $P < 0.001$).

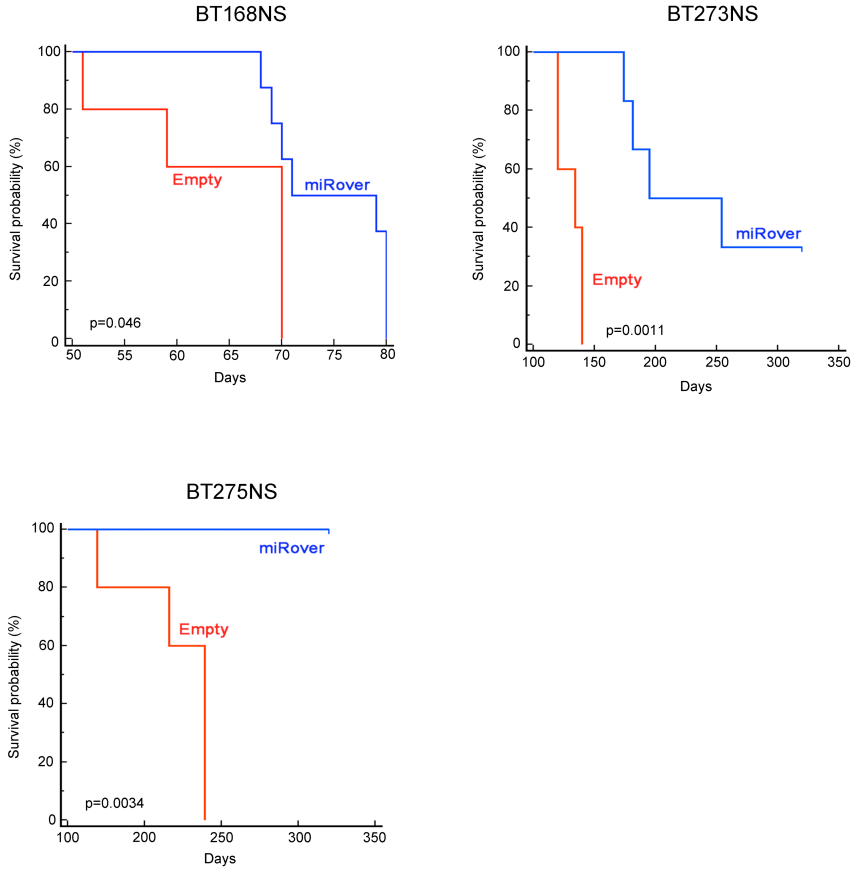
# SUPPLEMENTARY



**Supplementary Figure S1.** A) FISH analysis confirmed the absence of miR145 expression in GB-NS as well as the increased levels of miR145 after differentiation in the presence of serum (GB-NS 6 days after 3% serum differentiation). B) Real-time PCR analysis was used to assess the correlations between the levels of miR145, SOX2 and GFAP expression in GB-NS. We observed a high level of SOX2 expression and very low levels of miR145 and GFAP expression compared to normal brain (** $P < 0.01$; *** $P < 0.001$). The histograms represent the mean ± SD of three different GB-NS cell lines compared to normal brain (miR145, mean ± SD: 0.00083 ± 0.00062; SOX2, mean ± SD: 7.94 ± 2.15, GFAP, mean ± SD: 0.0026 ± 0.0039). SOX2 and GFAP were normalized to the housekeeping gene beta-2 microglobulin, and miR145 expression was normalized to RNU6B. C) Real-time PCR analysis was performed using GB-NS cell lines before and after differentiation (1, 3, 5 and 7 days), and the results showed that miR145 and GFAP were up-regulated and that SOX2 was down-regulated. The histograms represent the mean ± SD of three different GB-NS cell lines (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).


BT168NS
BT273NS
Empty
miRover
Empty
miRover
Nestin
GFAP
DCX
NEDD9
40X
BT168NS
BT273NS
Nestin
GFAP
DCX
NEDD9
Positive cell count
0
20
40
60
80
100
****
Empty
miRover

**Supplementary Figure S2.** Histological analysis performed on miRover and Empty tumors (40X) of two different cell lines (BT168NS and BT273NS). miRover tumors had a greater amount of GFAP-positive cells and lower amounts of cells positive for Nestin, DCX and NEDD9 compared to Empty tumors. The histograms represent the immunohistochemical quantification of Nestin, GFAP, DCX, and NEDD9 in miRover tumors compared to empty tumors. Positive cell counting was performed on five independent 40X fields. Three mice from each group were analyzed, and representative images for each type of tumor are displayed. Staining was performed on adjacent sections of paraffin-embedded brain samples (* $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$).




**Supplementary Figure S3.** Kaplan-Meier survival analysis of immunodeficient mice injected with $10^5$ NS cells (BT165, BT168, BT273 and BT275) and infected with the pCLNSX-miR145 retroviral vector (n = 10 mice) or pCLNSX-Empty retroviral vector (n = 5 mice). Mice injected with miRover survived significantly longer than mice injected with Empty.

**Supplementary Methods.**

FISH: Non-radioactive in situ hybridization for miR145 was performed using the FITC-labeled miRCURY$^{TM}$ LNA microRNA detection probe (Exiqon, Vedbaek, Denmark).

Deparaffinized and rehydrated tumor sections (5 microm) were treated with proteinase K (10 microg/ml; Qiagen) at 37°C for 5 min and then fixed with 4% paraformaldehyde for 10 min. All slides were placed in a Hybrite unit (Vysis) and incubated in a pre-hybridization solution at 60°C for 30 min followed by incubation in a hybridization solution with miR145 FITC-labeled probe at 60°C for 60 min. Stringent washes were performed in pre-heated 0.1X SSC buffer at 65°C, and the slides were placed in 0.2X SSC buffer at room temperature for 5 min. Tyramide signal amplification (PerkinElmer Life and Analytic Sciences Inc., Shelton, USA) was used to enhance the fluorescent signal. After quenching the endogenous peroxidase activity, slides were blocked for 30 min in blocking buffer solution and then incubated with anti FITC-HRP (1:400, DAKO) for 30 min. After washing in TNT buffer, tumor sections were incubated in a Fluorophore Tyramide Amplification Reagent working solution for 10 min. The slides were counterstained with DAPI (4′,6-diamidino-2-phenylindole, Sigma) and examined using a LEICA SP2 confocal microscope.

# *Chapter 3*

# Summary

MiR-145 is a small non-coding RNA molecule that is down-regulated in several kind of tumors and plays an important role as tumor suppressor.

In the present study we demonstrated that miR-145 is strongly down-regulated in primary glioblastoma specimens and in corresponding cell lines growing in culture in the presence of mitogenic factors (Epidermal Growth Factor - EGF and b-Fibroblast Growth Factor – bFGF) as neurospheres (NS) compared to normal brain.

In addition, we found that the expression of this microRNA was higher in low-grade glioma than in normal brain or glioblastoma tumors. Importantly, the expression of miR-145 is also significantly associated with a better prognosis in low-grade gliomas and in glioblastoma specimens, suggesting a key role in the development and manteinance of this tumor.

Our results suggest that miR-145 is mainly implicated in glioblastoma stemness and invasion, both in vitro and in vivo. The over-expression of this microRNA is correlated with an increased expression of the astrocytic marker GFAP and with reduction of expression of the stem cell marker SOX2. Moreover, microarray analyses showed that over-expression of miR-145 leads to a down-regulation of HEF1/CAS-L/NEDD9, a non-catalytic scaffolding protein implicated in the invasion ability of several types of cancers.

NEDD9 has been proposed as an invasive biomarker in lung cancer and melanoma and is a downstream effector of FAK in GB. In addition, we found that NEDD9 is also correlated with lower patient survival in a subgroup of low-grade gliomas and that in vitro silencing causes a significant inhibition of invasion ability. We also demonstrated that NEDD9 silencing leads to an up-regulation of miR-145, forming a double-negative feedback loop in GB-NS.

Finally, intracranial injection in nude mouse brains of GB-NS overexpressing miR-145 prolonged significantly survival and immunohystochemical analysis showed a less invasive and more differentiated tumors.

These findings demostrated the critical role of miR-145 in regulating glioblastoma stemness and invasion. We also proposed for the first time a potential role of NEDD9 as biomarker for glioma progression.

# Conclusions

Malignant gliomas are the most common type of primary malignant brain tumor and account for 82% of malignant gliomas. GB is a grade IV glioma histologically characterized by mitotic activity, vascular proliferation and necrosis. GB is also one of the most invasive tumors even if remains confined to the central nervous system (CNS) and very rarely metastasizes outside the central nervous system [1–2]. In the past decades, several papers have demonstrated that a small subpopulation of tumor cells, called glioma stem cells or glioma initiating cells (GSCs/GIC), have the ability to drive tumor recurrence [3–4].

In our lab we isolate and culture GSC from fresh GB specimens, growing as neurospheres in the presence of EGF and bFGF. We demonstrated that cells derived from GB specimens, if growing without serum, in the presence of mitogenic factors, are more similar to the actual biology of GB. In addition, these cells are always tumorigenic and the tumors are particularly aggressive and invasive, thus representing a better model of human GB [5].

Glioblastoma is one of the first solid tumors in which was defined a well characterized genomic landscape and a cell hierarchy including tumorigenic (stem) and non-tumorigenic (non-stem) [6–7]. The knowledge of glioblastoma-driver genes is increasing and the key network connections are under active scritiny [8–10]. Nevertheless the

molecular pathways underlying its heterogeneity remain unclear, making its therapeutic targeting very challenging. In fact, compared to the advances in treatment of other types of tumors, GB treatment remains poorly effective and essentially palliative.

GB GSCs seem to have most important characteristics of normal neural stem cells (NSC) like expression of CD133 and Nestin markers, and ability to make neurospheres, aggregates of clonally derived cells that are able to self-renew and differentiate into neuronal, astroglial or oligodendroglial cells. Moreover, GSCs seem to resist to radiation and chemotherapy and stimulate angiogenesis [11–15]. Recent data show that tumor microenvironment can favor the amplification of cancer cells exploiting stem cell programs for survival [16]. Thus, the hierarchic model proposed for different types of cancers, may not work for all, and could be substituted by a more flexible concept of GSC that are able to evolve and adapt, depending on external and internal stimuli.

MicroRNA could have an important role in tumor progression: the dysregulated expression of these small non-cording RNAs supports their identification as a novel class of oncogenes or tumor suppressor genes [17]. MiR-145 was found down-regulated in several kind of tumors where act as tumor suppressor. In head and neck cancer its targeting of SOX9 and ADAM17 resulted in the inhibition of self-renewal, tumor initiation, and metastatic properties of HNC-TIC cells [18]. Lee H at al. [19] found that microRNA-145 is able to regulated the migration of glioma cell by targeting the Connective Tissue Growth Factor. We found that miR-145 is strongly down-

regulated in glioblastoma specimens and in GB cell lines playing a critical role in regulating invasion and differentiation of glioma stem-like cells. Lee S et al. [20] showed that the over-expression of this miRNA causes a down-regulation of metastasis-related genes leading to a block of glioma cell migration and invasion. Yang YP et al. [21] confirmed that expression levels of miR-145 are inversely correlated with levels of Oct4 and Sox2 in GB-CD133+ cells. More importantly, by using polyurethane-short branch polyethylenimine (PU-PEI) as a therapeutic-delivery vehicle, they demonstrated that PU-PEI-mediated miR-145 delivery to GB-CD133+ significantly inhibited their tumorigenity, suppressed the expression of drug-resistance and anti-apoptotic genes and increased the sensitivity of the cells to radiation and temozolomide.

These results support the idea that miR-145 is an important microRNA in glioblastoma progression, especially in invasion, and that its delivery could represent a novel therapeutic tool for malignant gliomas.

# Future Perspectives

Presently, the field of miRNA in neurological disorders ranging from neuro-degenerative disease to cancer [22] is booming. This is due to the realization of the crucial role that these small, non-coding transcripts may play in a multitude of molecular and biological processes in normal and pathological tissues. The dysregulated expression of miRNAs is associated with several examples of human tumorigenesis, supporting their identification as a novel class of oncogenes or tumor suppressor genes [23–24].

In glioblastoma there are more than 30 deregulated microRNAs, which act as oncomiR or tumor suppressor miRNA and that are involved in several pathways such as proliferation, apoptosis, invasion, angiogenesis, stemness. In the last decades, several studies were done in order to understand the role of microRNA in cancer and the impact on patient prognosis and survival [25–29]. For examle, Mu Y et al. [30] demonstrated that down-regulation of miR-183 in human osteosarcoma is significantly associated with a high tumor grade, positive metastasis and recurrence and poor prognosis. Moreover, Shapira et al. [31] showed that there are significant differences in microRNA profiles in pre-surgical plasma from women affected by ovarian cancer and with a short overall survival when compared with long overall survival. In particular circulating plasma miRNA profiles were found useful in distinguishing women with ovarian cancer and in identifying women who benefited from treatments. In this scenario we

have contributed describing a direct correlation between miR-145 expression, the progression free survival in LGG patients and overall survival in GB patients [32].

Overall these observations stimulate to investigate the potential role of microRNA as cancer therapeutics. However currently their clinical application is limited mainly due to inefficient delivery systems [19]. Notably, recent data described an in vivo delivery of miR145 using polyurethane-short branch polyethylenimine (PU-PEI) as a therapeutic-delivery vehicle. PU-PEI-miR145 significantly suppressed tumorigenesis in glioblastoma-transplanted immune-compromised mice [21]. We believe that this information provides an important background for further studies.

First, we plan to test the efficacy of intracranial and peripheral injection of commercial miR-145 mimic (miRVana) in xenograft model of human glioblastoma. For the future we are going to develop nanocarriers as tracking and miR-145 delivery vectors.
Nanomedicine is a tool fwith great potential in clinical reserach because the endogenous transport mechanisms governing entry and exit cellular processes operate on a scale of tens of nanometers, allowing the access of engineered nanoparticles (NPs) to organs and their sub-cellular location. In the last decade, many efforts have been made to design multifunctional NPs that can improve drug payload and targeting to diseased cells [33]. In addition, correct and functional engineered NPs are able to pass complex biological barriers such as the blood brain barrier (BBB) [34] working as possible vectors for the

delivery of CNS therapeutics. Davis et al. [35] have recently demonstrated that nanocarriers are not limited to the delivery of small-molecules, but can be used to deliver large bioactive molecules such as proteins and nucleic acids, including small RNAs. These results suggest that NPs are the promising candidates for the delivery of miRNA into the brain.

In order to steer this research translationally we propose to design nanocarriers for the therapeutic delivery of microRNA into the brain, followed by a characterization of the biocompatibility and cytotoxicity of the nanocarriers and nanoplexes in in vitro model systems. After that, we would evaluate in xenograft models the therapeutic efficacy of in vivo delivery of miR-145 via traceable nanocarriers and, finally, observe the impact of miR-145 on tumor progression.

The main challenge is to develop and characterize nanoparticles as an innovative solution for in vivo delivery of miRNA as a potential novel therapeutic approach for glioblastoma.

# References


[1] A. Omuro and L. M. DeAngelis, “Glioblastoma and other malignant gliomas: a clinical review.,” *JAMA*, vol. 310, no. 17, pp. 1842–50, Nov. 2013.

[2] T. a Dolecek, J. M. Propp, N. E. Stroup, and C. Kruchko, “CBTRUS statistical report: primary brain and central nervous system tumors diagnosed in the United States in 2005-2009.,” *Neuro. Oncol.*, vol. 14 Suppl 5, pp. v1–49, Nov. 2012.

[3] S. Das, M. Srikanth, and J. A. Kessler, “Cancer stem cells and glioma.,” *Nat. Clin. Pract. Neurol.*, vol. 4, pp. 427–435, 2008.

[4] H. A. Fine, “Glioma stem cells: not all created equal.,” *Cancer Cell*, vol. 15, pp. 247–249, 2009.

[5] P. Tunici, L. Bissola, E. Lualdi, and B. Pollo, “Genetic alterations and in vivo tumorigenicity of neurospheres derived from an adult glioblastoma,” *Mol ...*, vol. 2, pp. 1–5, 2004.

[6] C. Boccaccio and P. M. Comoglio, “The MET oncogene in glioblastoma stem cells: implications as a diagnostic marker and a therapeutic target.,” *Cancer Res.*, vol. 73, no. 11, pp. 3193–9, Jun. 2013.

[7] R. Galli, E. Binda, U. Orfanelli, B. Cipelletti, A. Gritti, S. De Vitis, R. Fiocco, C. Foroni, F. Dimeco, and A. Vescovi,

"Isolation and Characterization of Tumorigenic , Stem-like Neural Precursors from Human Glioblastoma Isolation and Characterization of Tumorigenic , Stem-like Neural Precursors from Human Glioblastoma," pp. 7011–7021, 2004.

[8] C. W. Brennan, R. G. W. Verhaak, A. McKenna, B. Campos, H. Noushmehr, S. R. Salama, S. Zheng, D. Chakravarty, J. Z. Sanborn, S. H. Berman, R. Beroukhim, B. Bernard, C.-J. Wu, G. Genovese, I. Shmulevich, J. Barnholtz-Sloan, L. Zou, R. Vegesna, S. a Shukla, G. Ciriello, W. K. Yung, W. Zhang, C. Sougnez, T. Mikkelsen, K. Aldape, D. D. Bigner, E. G. Van Meir, M. Prados, A. Sloan, K. L. Black, J. Eschbacher, G. Finocchiaro, W. Friedman, D. W. Andrews, A. Guha, M. Iacocca, B. P. O'Neill, G. Foltz, J. Myers, D. J. Weisenberger, R. Penny, R. Kucherlapati, C. M. Perou, D. N. Hayes, R. Gibbs, M. Marra, G. B. Mills, E. Lander, P. Spellman, R. Wilson, C. Sander, J. Weinstein, M. Meyerson, S. Gabriel, P. W. Laird, D. Haussler, G. Getz, and L. Chin, "The somatic genomic landscape of glioblastoma.," *Cell*, vol. 155, no. 2, pp. 462–77, Oct. 2013.

[9] R. Chen, M. C. Nishimura, S. M. Bumbaca, S. Kharbanda, W. F. Forrest, I. M. Kasman, J. M. Greve, R. H. Soriano, L. L. Gilmour, C. S. Rivers, Z. Modrusan, S. Nacu, S. Guerrero, K. a Edgar, J. J. Wallin, K. Lamszus, M. Westphal, S. Heim, C. D. James, S. R. VandenBerg, J. F. Costello, S. Moorefield, C. J. Cowdrey, M. Prados, and H. S. Phillips, "A hierarchy of self-

renewing tumor-initiating cell types in glioblastoma.," *Cancer Cell*, vol. 17, no. 4, pp. 362–75, Apr. 2010.

[10] F. De Bacco, E. Casanova, E. Medico, S. Pellegatta, F. Orzan, R. Albano, P. Luraghi, G. Reato, A. D'Ambrosio, P. Porrati, M. Patanè, E. Maderna, B. Pollo, P. M. Comoglio, G. Finocchiaro, and C. Boccaccio, "The MET Oncogene Is a Functional Marker of a Glioblastoma Stem Cell Subtype.," *Cancer Res.*, vol. 72, no. 17, pp. 4537–50, Sep. 2012.

[11] T. Denysenko, L. Gennero, C. Juenemann, I. Morra, P. Masperi, V. Ceroni, A. Pragliola, A. Ponzetto, and A. Melcarne, "Heterogeneous phenotype of human glioblastoma. In vitro study.," *Cell Biochem. Funct.*, no. April, Jul. 2013.

[12] L. Cheng, S. Bao, and J. N. Rich, "Potential therapeutic implications of cancer stem cells in glioblastoma.," *Biochem. Pharmacol.*, vol. 80, no. 5, pp. 654–65, Sep. 2010.

[13] C. Richichi, P. Brescia, V. Alberizzi, L. Fornasari, and G. Pelicci, "Marker-independent Method for Isolating Slow-Dividing Cancer Stem Cells in," vol. 15, no. 7, pp. 840–847, 2013.

[14] X. Yuan, J. Curtin, Y. Xiong, G. Liu, S. Waschsmann-Hogiu, D. L. Farkas, K. L. Black, and J. S. Yu, "Isolation of cancer stem cells from adult glioblastoma multiforme.," *Oncogene*, vol. 23, no. 58, pp. 9392–400, Dec. 2004.

[15] O. Sampetrean and H. Saya, “Characteristics of glioma stem cells.,” *Brain Tumor Pathol.*, 2013.

[16] A. Soeda, M. Park, D. Lee, A. Mintz, A. Androutsellis-Theotokis, R. D. McKay, J. Engh, T. Iwama, T. Kunisada, A. B. Kassam, I. F. Pollack, and D. M. Park, “Hypoxia promotes expansion of the CD133-positive glioma stem cells through activation of HIF-1alpha.,” *Oncogene*, vol. 28, no. 45, pp. 3949–59, Nov. 2009.

[17] W. J. Kim, C.-W. Chang, M. Lee, and S. W. Kim, “Efficient siRNA delivery using water soluble lipopolymer for anti-angiogenic gene therapy.,” *J. Control. Release*, vol. 118, no. 3, pp. 357–63, Apr. 2007.

[18] C.-C. Yu, L.-L. Tsai, M.-L. Wang, C.-H. Yu, W.-L. Lo, Y.-C. Chang, G.-Y. Chiou, M.-Y. Chou, and S.-H. Chiou, “miR145 targets the SOX9/ADAM17 axis to inhibit tumor-initiating cells and IL-6-mediated paracrine effects in head and neck cancer.,” *Cancer Res.*, vol. 73, no. 11, pp. 3425–40, Jun. 2013.

[19] H. Lee, S. Finniss, S. Cazacu, and E. Bucris, “Mesenchymal stem cells deliver synthetic microRNA mimics to glioma cells and glioma stem cells and inhibit their cell migration and self-renewal,” *Oncotarget*, vol. 4, no. 2, pp. 346–361, 2013.

[20] S.-J. Lee, S.-J. Kim, H.-H. Seo, S.-P. Shin, D. Kim, C.-S. Park, K.-T. Kim, Y.-H. Kim, J.-S. Jeong, and I.-H. Kim, “Over-expression of miR-145 enhances the effectiveness of HSVtk

gene therapy for malignant glioma.," *Cancer Lett.*, pp. 1–9, Jan. 2012.

[21] Y.-P. Yang, Y. Chien, G.-Y. Chiou, J.-Y. Cherng, M.-L. Wang, W.-L. Lo, Y.-L. Chang, P.-I. Huang, Y.-W. Chen, Y.-H. Shih, M.-T. Chen, and S.-H. Chiou, "Inhibition of cancer stem cell-like properties and reduced chemoradioresistance of glioblastoma using microRNA145 with cationic polyurethane-short branch PEI.," *Biomaterials*, vol. 33, no. 5, pp. 1462–76, Mar. 2012.

[22] P. T. Nelson and J. N. Keller, "R EVIEW A RTICLE RNA in Brain Disease : No Longer Just B The Messenger in the Middle ^," vol. 66, no. 6, pp. 461–468, 2007.

[23] W. Zhang, J. E. Dahlberg, and W. Tam, "MicroRNAs in tumorigenesis: a primer.," *Am. J. Pathol.*, vol. 171, no. 3, pp. 728–38, Sep. 2007.

[24] S.-J. Kim, J.-S. Oh, J.-Y. Shin, K.-D. Lee, K. W. Sung, S. J. Nam, and K.-H. Chun, "Development of microRNA-145 for therapeutic application in breast cancer.," *J. Control. Release*, vol. 155, no. 3, pp. 427–34, Nov. 2011.

[25] G. G. Gomez, J. Wykosky, C. Zanca, F. B. Furnari, and W. K. Cavenee, "Therapeutic resistance in cancer: microRNA regulation of EGFR signaling networks.," *Cancer Biol. Med.*, vol. 10, no. 4, pp. 192–205, Dec. 2013.

[26] K. K. W. To, “MicroRNA: a prognostic biomarker and a possible druggable target for circumventing multidrug resistance in cancer chemotherapy.,” *J. Biomed. Sci.*, vol. 20, p. 99, Jan. 2013.

[27] Y. Saito, H. Saito, G. Liang, and J. M. Friedman, “Epigenetic Alterations and MicroRNA Misexpression in Cancer and Autoimmune Diseases: a Critical Review.,” *Clin. Rev. Allergy Immunol.*, Dec. 2013.

[28] X. Zhu, M. Lv, H. Wang, and W. Guan, “Identification of Circulating MicroRNAs as Novel Potential Biomarkers for Gastric Cancer Detection: A Systematic Review and Meta-Analysis.,” *Dig. Dis. Sci.*, no. 321, Dec. 2013.

[29] N. H. Farina, M. E. Wood, S. D. Perrapato, C. S. Francklyn, G. S. Stein, J. L. Stein, and J. B. Lian, “Standardizing Analysis of Circulating MicroRNA: Clinical and Biological Relevance.,” *J. Cell. Biochem.*, no. December, pp. 1–23, Dec. 2013.

[30] Y. Mu, H. Zhang, L. Che, and K. Li, “Clinical significance of microRNA-183/Ezrin axis in judging the prognosis of patients with osteosarcoma.,” *Med. Oncol.*, vol. 31, no. 2, p. 821, Feb. 2014.

[31] I. Shapira, M. Oswald, J. Lovecchio, H. Khalili, a Menzin, J. Whyte, L. Dos Santos, S. Liang, T. Bhuiya, M. Keogh, C. Mason, K. Sultan, D. Budman, P. K. Gregersen, and a T. Lee, “Circulating biomarkers for detection of ovarian cancer and

predicting cancer outcomes.," *Br. J. Cancer*, no. August, pp. 1–8, Dec. 2013.

[32] M. Speranza, V. Frattini, F. Pisati, and D. Kapetis, "NEDD9, a novel target of miR-145, increases the invasiveness of glioblastoma," *Oncotarget*, vol. 3, no. 7, 2012.

[33] R. a Petros and J. M. DeSimone, "Strategies in the design of nanoparticles for therapeutic applications.," *Nat. Rev. Drug Discov.*, vol. 9, no. 8, pp. 615–27, Aug. 2010.

[34] H. L. Wong, X. Y. Wu, and R. Bendayan, "Nanotechnological advances for the delivery of CNS therapeutics.," *Adv. Drug Deliv. Rev.*, vol. 64, no. 7, pp. 686–700, May 2012.

[35] M. E. Davis, J. E. Zuckerman, C. H. J. Choi, D. Seligson, A. Tolcher, C. a Alabi, Y. Yen, J. D. Heidel, and A. Ribas, "Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles.," *Nature*, vol. 464, no. 7291, pp. 1067–70, Apr. 2010.

# Acknowledgements

Thanks to GF for welcoming me in his laboratory and for being my tutor.

Thanks to Serena, for making me a better researcher, for being at my side always during this long journey together and for becoming a friend.

Thanks to Dr. d'Adda di Fagagna for being my mentor.

Thanks to Véronique to be a great friend before being a great co - worker.

Thanks to all the GF 's lab: double P for teaching me how treat cells, Lori and Rosi for the suggestions in molecular biology field, Sara Senior and Junior with whom I shared so many coffee…we had a great time together. Also thanks to Cristina, Donata, Federica, Dimos, Monica and Gabriele.

Grazie ai miei amici per aver ascoltato i miei sfoghi e per aver gioito con me delle mie vittorie, per aver ascoltato le mie presentazioni e i miei sproloqui, anche se non capivano nulla di quello che stavo dicendo! Guys…that's friendship!

Grazie ai miei genitori, sono stati e saranno sempre i migliori genitori del mondo!.... really, they are amazing.

Thanks to my bro, Vincenzo, you’re awesome!!

Grazie ai miei zii e cugini di vari gradi, milanesi e non, a cui voglio tanto tanto bene.

E soprattutto grazie a Francesco, ormai mio marito, che mi ha sopportato, supportato, sgridato e ascoltato. Grazie perchè credi in me anche quando io mollo. Grazie per amarmi incondizionatamente e per essere qui a Boston con me. Thanks hub!

And thanks to Boston, my new house, my new lab and my new friends for adopting me.