Epigenetic silencing of miR-145-5p contributes to brain metastasis

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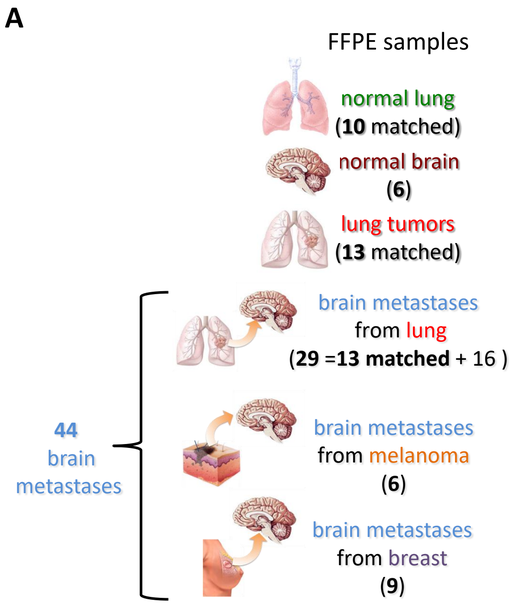
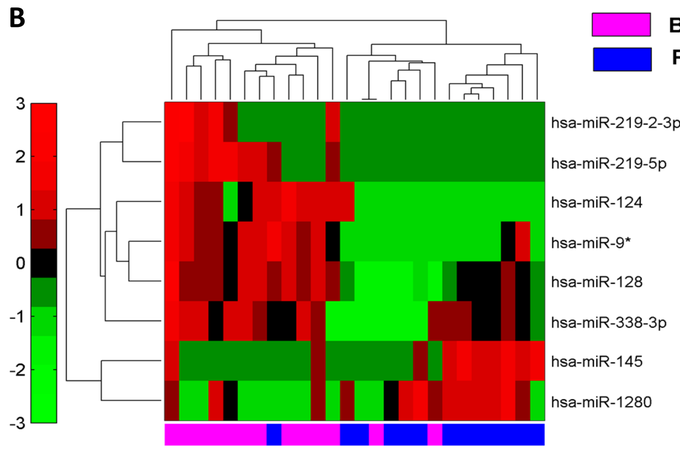
Brain metastasis is a major cause of morbidity and mortality of lung cancer patients. We assessed whether aberrant expression of specific microRNAs could contribute to brain metastasis. Comparison of primary lung tumors and their matched metastatic brain disseminations identified shared patterns of several microRNAs, including common down-regulation of miR-145-5p. Down-regulation was attributed to methylation of miR-145's promoter and affiliated elevation of several protein targets, such as EGFR, OCT-4, MUC-1, c-MYC and, interestingly, tumor protein D52 (TPD52). In line with these observations, restored expression of miR-145-5p and selective depletion of individual targets markedly reduced in vitro and in vivo cancer cell migration. In aggregate, our results attribute to miR-145-5p and its direct targets pivotal roles in malignancy progression and in metastasis.

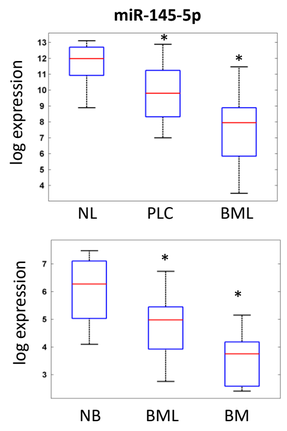
1 miR-145-5p expression is down-regulated in brain metastasis



To explore the involvement of miRNAs in brain metastases we collected FFPE (Formalin-Fixed, Paraffin-Embedded) samples from patients affected by one of the 3 main types of tumors exhibiting the highest incidence of brain metastases, namely melanoma, breast and lung cancer (**A**).

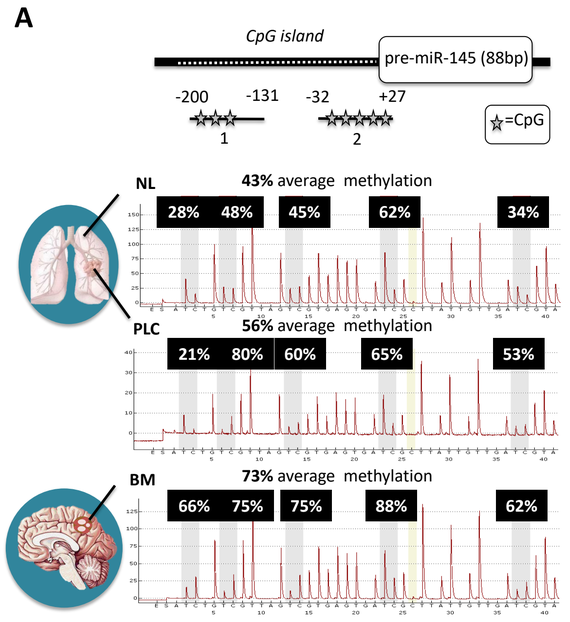


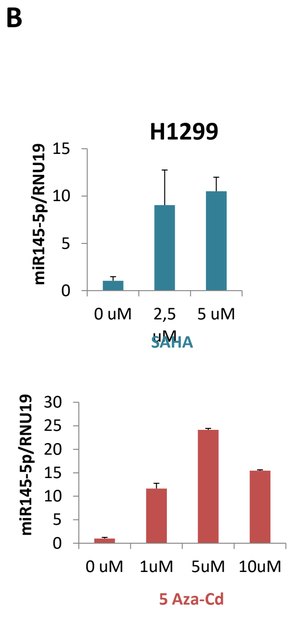
Expression profile of 906 human miRNAs in 13 primary lung cancers (PLC) and their matched brain metastases (BM) ,identified 8 differentially expressed miRNAs (**B**).



MiR-145-5p down-regulation, evidenced when compared non-tumoral lung tissues (NL) and matched primary lung tumors (PLC), was significantly more pronounced when compared to brain metastases (BML). MiR-145-5p expression was downregulated also in brain metastases originated from melanoma and breast tumors (BM) when compared to normal brain (NB) (**C**).

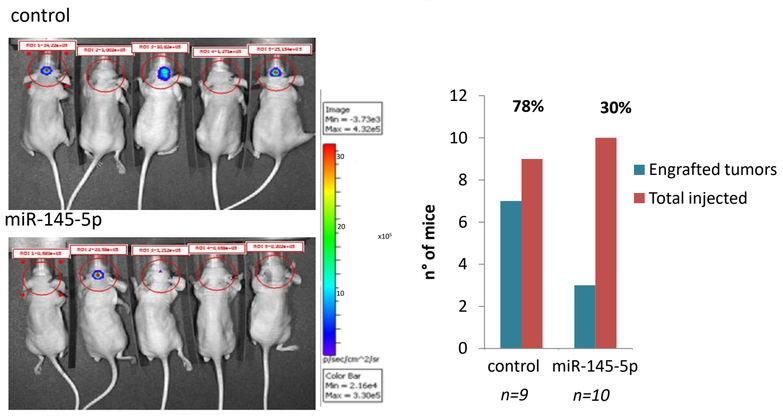
2 CpG island methylation restrains the expression of miR-145-5p





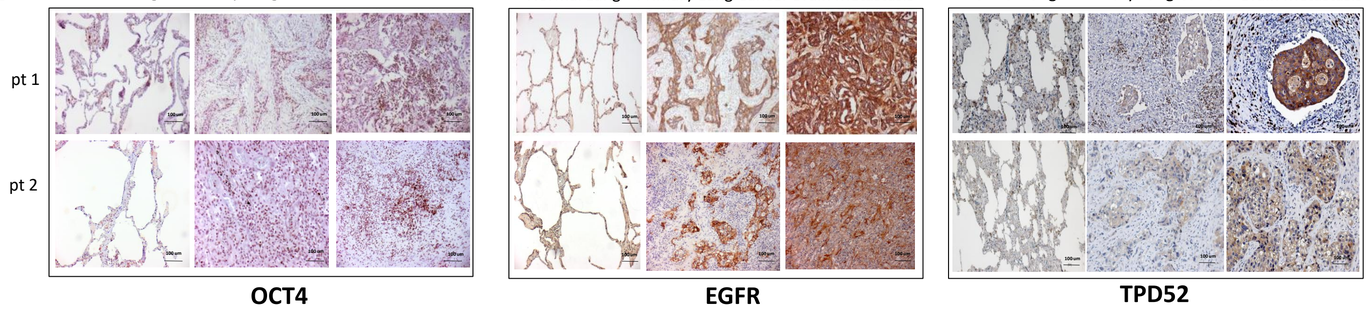
By pyrosequencing analysis we assessed the methylation status of a CpG island located upstream 200bp from the pre-miR-145-5p sequence in the normal lung tissue (NL), the primitive lung cancer (PLC) and brain metastases (BM) tissues (**A**). These analyses uncovered a significant increase in the methylation status of miR-145 CpG island when normal lung tissues were compared to primitive lung cancer and brain metastases (**A**). We also assessed that treatment with two chemical epigenetic modifiers, namely 5-azacytidine (5-aza-Cd) and vorinostat (SAHA) restored miR-145-5p expression in H1299 cells (**B**).

3 Ectopic expression of miR-145-5p restrains brain orthotopic tumor engraftment

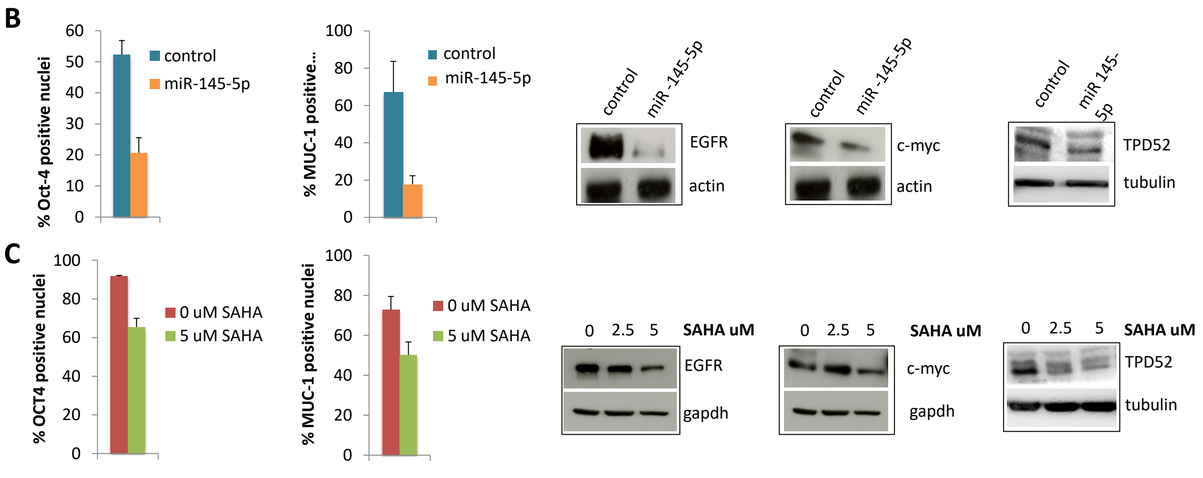


To investigate the role of miR-145-5p on brain metastasis we performed intracranial orthotopically injection of A549-luc/miR- 145-5p and A549-luc/control cells in nu/nu athymic mice. Bioluminescence images collected at day 8 and at day 20 revealed that A549-luc/miR-145-5p engrafted in 3/10 mice (30%) while A549-luc/control cells in 7/9 (78%) mice (**A-B**).
These findings strongly support an *in vivo* evidence of the role of miR-145-5p in brain metastasis.

4 Downregulation of miR-145-5p releases the expression of OCT4,MUC-1, EGFR, c-MYC, TPD52



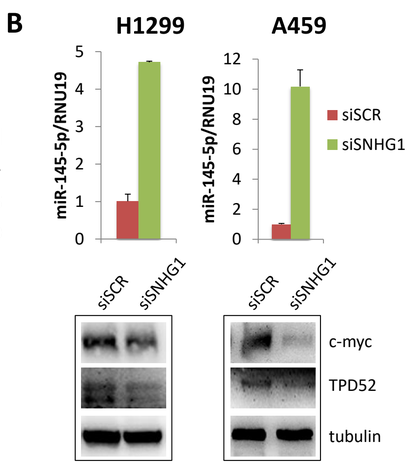
The expression of OCT-4, EGFR and TPD52 proteins, whose mRNAs are targets of miR-145-5p, was increased in both primary lung cancers and matched brain metastases when compared to matched normal lung tissues (**A**).



Ectopic expression of miR-145-5p in H1299 cells (**B**) or SAHA treatment (**C**), that induces miR-145-5p expression, reduces the protein levels of miR-145-5p targets (OCT4, MUC-1, EGFR, c-MYC, TPD52)

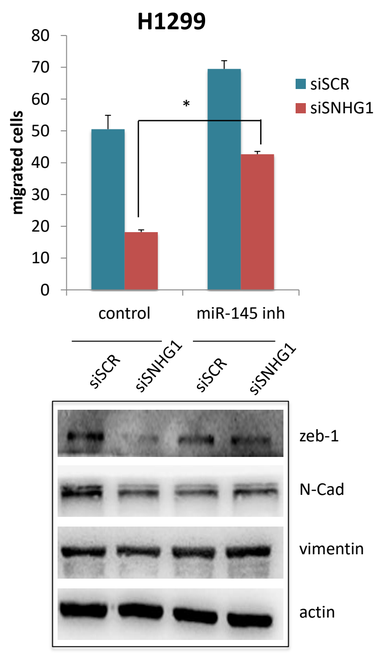
5 The long non-coding RNA SNHG1 targets miR-145-5p

The long non-coding RNA SNHG1 is predicted to hold two binding sites (BS) for miR-145-5p on its sequence. We validated SNHG1-miR-145-5p interaction by performing luciferase assays with two reporter constructs, containing the two BS of SNHG1 (wild-type or mutated) in H1299 cells transiently transfected with miR-145-5p (**A**).



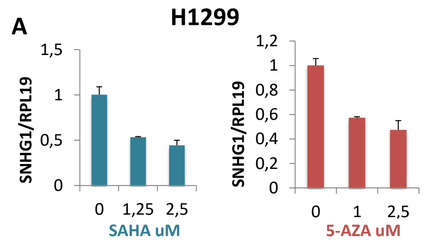
SNHG1 depletion in two lung cancer cell lines (H1299 and A459) restores miR-145-5p expression and consequently impairs the protein levels of two miR-145-5p targets (c-myc and TPD52) (**B**).

6 SNHG1 counteracts the antimigratory effects of miR-145-5p



Depletion of SNHG1 expression impairs the migratory ability of H1299 cells, as reflected by the reduction in the protein levels of Zeb-1, N-Cadherin and Vimentin. The inhibition of miR-145-5p expression in this cellular context, rescues the migratory ability of the cells by promoting the expression of pro-metastatic proteins.

7 Epigenetic drugs inhibit SNHG1 expression



Treatment with vorinostat (SAHA) and 5-azacytidine (5-aza-Cd) impinge on SNHG1 expression in H1299 cells

Conclusions

Mir-145-5p expression in highly metastatic lung cancer cells is finely regulated both at transcriptional and post-transcriptional levels: epigenetic modifications on its promoter inhibit miR-145-5p transcription, while the long non coding RNA SNHG1, that is expressed at high levels in the cells, binds to mature miR-145-5p by preventing its inhibitory activity on pro-metastatic proteins.
Epigenetic drugs, such as vorinostat and 5-azacytidine, are able to restore miR-145-5p expression by changing the epigenetic landscape on its promoter and by inhibiting the expression of SNHG1. This kind of treatment could represent an effective choice for lung cancer patients with metastases.

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