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N6-adenosine methylation of miRNA-200b-3p influences its functionality and is a theranostic tool.

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Running title: Adenosine methylation miR-200b-3p as a prodrug?

Keywords: miRNA methylation, glioblastoma, Adenosine methylation, prodrug, biomarker

ABSTRACT

Micro-RNAs (miRNAs or miR) play crucial roles in biological and pathological processes. Some miRNAs also appear as promising biomarkers and therapeutic tools. However, the epitranscriptomic regulation of miRNAs is not yet fully elucidated in all their fields of application.

We report that adenosine methylation of miR-200b-3p inhibits its repressive function toward its mRNA targets such as XIAP by blocking the formation of the miRNA/3'UTR^{mRNA} duplex.

Our data indicate that the adenosine methylation of miR-200b-3p is associated with the survival of glioblastoma patients.

Altogether, our data support the idea that the adenosine methylation of miR-200b-3p can be used as a prodrug having a selective cytotoxicity against cancer cells (while being harmless to PBMC, astrocytes, neuron and hepatocytes).

INTRODUCTION

MicroRNA (miRNA) are short non-coding RNAs that regulate protein expression towards their function of translational repressor. Thus, miRNA are crucial regulators of many cellular processes including proliferation, apoptosis, immunogenicity, development and differentiation. miRNA biogenesis can be epigenetically regulated in both physiological and pathological conditions toward the DNA methylation of miRNA genes. Wang *et al.* report that the expression of approximately 50 % of miRNA genes is putatively regulated by DNA methylation since they are associated with CpG islands ¹. A variety of DNA methylation-specific methyl-CpG-binding domain proteins (MBD) were also found to transcriptionally regulate miRNA genes ². Finally, Malumbres *et al.* also report that the miRNA genes expression is also regulated through histone modifications, such as lysine methylation and acetylation ³.

Several publications report that chemical modifications can occur in miRNA and that these modifications regulate the miRNA processing or functionality. Among these modifications, some can affect the phosphate at the 5'-end of miRNA. Thus, Xhemalce *et al.* (2012) report that the BCDIN3D-mediated phospho-dimethylation of miRNAs (such as pre-miR-145) negatively regulates the miRNA maturation and impacts on the tumorigenic phenotype ⁴. Other chemical modifications of miRNA affect the internal bases of miRNA. Alarcón *et al.* (2015) and Berulava *et al.* (2015) report that miRNA can be adenosine methylated and that the presence of this methylation promotes the initiation of miRNA biogenesis and increases the stability of adenosine methylated miRNAs, respectively ^{5,6}. Konno *et al.* (2019) also report that miRNA can be adenosine methylated ⁷. Besides, in this report, authors introduced the idea that the adenosine methylation of miRNA can be used as biomarker for the diagnosis of early-stage cancer. Pandolfini *et al.* (2019) report that miRNA can be guanosine methylated and that this methylation inhibits the miRNA maturation ⁸. Recently, our laboratory published that miRNAs can be cytosine methylated and that the presence of this methylation represses the miRNA function ⁹.

Several enzymes catalyze these base modifications: METTL1 (Methyltransferase-like protein 1, Uniprot Q9UBP6) and DNMT3A (DNA (cytosine-5)-methyltransferase 3A, Uniprot Q9Y6K1) are defined to promote the guanosine and cytosine methylation of miRNAs, respectively ^{8,9}. The complex METTL3-WTAP-METTL14 is described as a miRNA adenosine methylase or

writer, while FTO (Fat mass and obesity-associated protein, Uniprot Q9C0B1) and ALKBH5 (Alkylated DNA repair protein alkB homolog 5, Uniprot Q6P6C2) are described as miRNA adenosine demethylases or erasers^{6,10,11,12,5,13}. Interestingly, these two enzymes are *alpha*-KetoGlutarate-dependent (α KG) suggesting that the adenosine methylation of miRNA can be regulated by the intracellular level of α KG. α KG is a Krebs cycle metabolite. It is formed from isocitrate by oxidative decarboxylation catalyzed by IDH proteins and plays a key role in multiple metabolic and cellular pathways *via* its co-substrate role of several enzymes such as FTO and ALKBH5¹⁴. Thus, in theory, high level of α KG should increase the FTO activity and should promote a decrease of adenosine methylation of miRNA.

Despite these undeniable advances, further studies of the molecular mechanisms governing the chemical modifications of miRNA in a tumor context is required in order to increase the understanding of the role played by these modifications in tumors.

We here focus our study on the impact of presence of N6-adenosine methylation in miRNA-200b-3p in samples of patients suffering from glioblastoma multiforme (GBM). Our study is particularly focused on the impact of miRNA-200b-3p and its adenosine methylation on the expression of XIAP (X-linked inhibitor of apoptosis protein, Uniprot P98170). XIAP acts as an anti-apoptotic protein *via* the inhibition of caspase-3 and -7 activation and high XIAP expression is associated with a poor survival in several solid tumors^{15,16}. Thus, the miR-200b-3p-mediated repression of XIAP mRNA expression appears as a mechanism governing the caspase-3 and -7 activity and the apoptosis. In theory, in the presence of miR-200b-3p, XIAP mRNA expression is repressed and caspase-3 and -7 can be activated to promote apoptosis. However, in absence or inactivation of the miR-200b-3p, XIAP is expressed, blocks caspase-3 and -7 activation and therefore inhibits apoptosis. This is why it is important to combine miR-200b-3p expression study to its action capacity.

RESULTS

The m6A methyltransferase METTL3, the m6A demethylase FTO and alpha-ketoglutarate regulate the N6-adenosine methylation of miR-200b-3p

Literature reports that miR-200 and particularly miR-200b-3p play a role in GBM^{17,18,19,20}. Berulava *et al.* (2015) have identified the presence of m6A in certain miRNAs such as miR-200b-3p⁶. In agreement with these findings, we have investigated the miR-200b-3p level

expression (miR-200b-3p^{exp}) and the percentage of miRNA-200b-3p containing m6A (miR-200b-3p^{%m6A}) in a collection of 32 GBM samples (**Table S1**). RT-qPCR experiments indicated a high level of heterogeneity in miR-200b-3p^{exp} with a max/min ratio equal to 37.6 (**Figure 1A**). RNA immunoprecipitation performed with an anti-m6A antibody followed by qPCR analysis (miRIP^{m6A}-qPCR) indicated that 10/32 tumors contained a miR-200b-3p^{%m6A}>10% (**Figure 1B**). In addition, we observed a correlation between miR-200b-3p^{%m6A} and miR-200b-3p^{exp} ($p=0.0022$) (**Figure 1B**).

In order to identify the molecular mechanisms governing the N6-adenosine methylation of miR-200b-3p in GBM patients, we first focused our analyses on FTO and α KG, since FTO is an adenosine demethylase that requires alpha-ketoglutarate (α KG) to catalyze the adenosine demethylation¹¹. In our collection of 32 GBMs, Pearson's correlation tests show an absence of significant correlation FTO expression level with miR-200b-3p^{%m6A} ($p=0.0824$) (**Figure 1C**) and between α KG and miR-200b-3p^{%m6A} ($p=0.0668$) (**Figure 1D**). To consider these two parameters, we isolated GBM samples harboring a low FTO expression level (lower than median) and a low α KG level (lower than median) (FTO^{Low}/ α KG^{Low}) from the other GBM samples (**Figure S1**). Based on this subdivision, we noted that GBM samples harboring FTO^{Low}/ α KG^{Low} were more m6A-methylated than other GBM samples ($p=0.0042$) (**Figure 1E**). Thus, we conclude that both FTO and α KG affect the m6A-methylation level of miR-200b-3p: the N6-adenosine methylation level of miR-200b-3p is elevated when FTO and α KG levels are lower. The involvement of FTO and α KG in the N6-adenosine methylation of miR was also supported by the fact that siRNA directed against FTO increased miR-200b-3p^{%m6A} (**Figure 1F and Figure S2**), α KG treatment decreased miR-200b-3p^{%m6A} (**Figure 1G**), Meclofenamic Acid (MA, a selective FTO inhibitor²¹) increased the miR-200b-3p^{%m6A} (**Figure 1H**). In addition, we noted that the knock-down of ALKBH5 (a RNA adenosine demethylase¹⁰) did not change the miR-200b-3p^{%m6A} (**Figure S3**). Thus, all these results support the idea that FTO and α KG act in concert to decrease the adenosine methylation of miR-200b-3p.

Alarcón *et al.* (2015) having identified that methyltransferase-like 3 (METTL3) methylates pri-miRNA in mammalian cells⁵, we hypothesized that METTL3 could be implicated in the adenosine methylation of miR-200b-3p. To support this hypothesis, we first observed a significant correlation between miR-200b-3p^{%m6A} and the METTL3 expression level

($p=0.0010$) (**Figure 1I**). Secondly, acellular experiments indicated that the immunoprecipitate of METTL3 (*i.e.* METTL3-including complexes) methylates miRNA-200b-3p *in vitro* (**Figure 1J**). Thirdly, METTL3 knock-down (siRNA method) decreased the level of m6A in miR-200b-3p (**Figures 1K et S4**). To conclude, these three distinct experiments implicate METTL3 as a writer of N6-adenosine methylation of miR-200b-3p.

All the above results suggest that α KG, FTO and METTL3 collectively influence the presence of m6A in miR-200b-3p. In order to take into consideration the influence of these three parameters on the level of adenosine methylation of miR-200c-3p, we have calculated what we called the α FM^{score}. For each GBM samples, +1 was affected when the expression of α KG, FTO and METTL3 is predicted to increase the N6-adenosine methylation *i.e.* when the α KG and FTO expressions are lower or equal to the median value of our cohort and when METTL3 expression is higher than the median value of our cohort. -1 was affected when the expression of α KG, FTO and METTL3 is predicted to decrease the N6-adenosine demethylation *i.e.* when the α KG and FTO expressions are higher than the median value of our cohort and when METTL3 expression is lower or equal to the median value of our cohort. For example, a GBM harboring a high level of α KG and FTO and a low level of METTL3 has a α FM^{score} equal to +1, while another GBM harboring a low level of α KG and FTO and a low level of METTL3 has a α FM^{score} equal to +3. Thus, we noted that the α FM^{score} and the percentage of presence of m6A in miR-200b-3p were significantly correlated in our collection of 32 GBM ($p=0.0006$) (**Figure 1L**).

Taken together, our data support the idea that METTL3, FTO and α KG are involved in the regulation of the N6-adenosine methylation of miR-200b-3p.

The N6-adenosine methylation of miR-200b-3p limits its translational repressor function towards anti-apoptotic players and confers poor prognosis in GBM patients

XIAP^{mRNA} being identified as a target of miR-200b-3p (according to the miRTarBase website), we next investigated whether there is a link between miR-200b-3p^{exp}, miR-200b-3p^{%m6A} and the XIAP expression in our collection of 32 GBM samples.

Our study did not correlate miR-200b-3p^{exp} and the XIAP expression when all GBM samples were considered ($p=0.8803$) (**Figure 2A**).

We then extended our study by dividing our samples in 3 groups by taking into consideration the adenosine methylation percentage of miR-200b-3p (**Figure 2B**). Group#1 included samples with miR-200b-3p^{%m6A} >10%. Group#2 included samples with a percentage miR-200b-3p^{%m6A}<10% and miR-200b-3p^{exp} inferior to the median (miR-200b-3p^{exp-low}). Group#3 included samples with miR-200b-3p^{%m6A}<10 and an expression level of miR-200b-3p superior to the median (miR-200b-3p^{exp-high}).

For all samples having miR-200b-3p^{%m6A}<10 (group#2 and #3), we noted that XIAP expression is inversely correlated with miR-200b-3p^{exp} (**Figures 2B and S5**). This data is consistent with the dogma saying that miRNA is a post-transcriptional repressor.

Surprisingly, we noted that the average of XIAP expression of group#1's samples is higher than the ones of the two other groups (**Figure 2B**). These results suggest that miR-200b-3p regulates XIAP expression when its sequence does not contain m6A (or a level inferior to 10%) and that the m6A presence in miR-200b-3p could abrogate the post-transcriptional repressor function of this miRNA.

To investigate this hypothesis, U251 cells were treated with an unspecific oligonucleotide (negative control), miR-200b-3p^{mimetic} or m6A-modified miR-200b-3p^{mimetic}. As expected, we did not observe any change in XIAP expression when cells were treated with unspecific oligonucleotide, while XIAP expression strongly decreased when cells were treated with miR-200b-3p^{mimetic} (**Figure 2C**). Interestingly, we noted that this decrease is less efficient when cells were treated with the same quantity of m6A-modified miR-200b-3p^{mimetic} (**Figure 2C**). Thus, it appears that the presence of m6A in miR-200b-3p abrogates the post-transcriptional repressor function of this miRNA toward XIAP^{mRNA}.

We next performed Cross-Linking Immunoprecipitation and qPCR (CLIP-qPCR) analyses to determine whether the adenosine-methylation of miR-200b-3p influences the endogenous formation of 3'UTR-mRNA-XIAP/miR-200b-3p duplex. In our assays, immunoprecipitation is performed *via* an antibody directed against GW182 and TNRC6B (i.e. two proteins of the RISC complex having a central role in miRNA-mediated silencing), and qPCRs were performed to detect the enrichment/presence of miRNA and 3'UTR^{mRNA} on the GW182- and TNRC6B-mediated co-immunoprecipitation products. CLIP-qPCRs were performed from samples with knock-down of METTL3 in order to estimate the impact of the loss of adenosine-methylation on the GW182- and TNRC6B-mediated co-immunoprecipitation of miRNAs and mRNAs. The miR-150-5p/3'UTR-mRNA-EP300 duplex was considered as a

control. The choice of this control was dictated by the fact that miR-150-5p is not adenosine-methylated and the fact that miR-150-5p targets 3'UTR-mRNA-EP300.

We first noted that miR-150-5p and 3'UTR-mRNA-EP300 were present in GW182- and TNRC6B- mediated co-immunoprecipitation products, and this independently of the METTL3 knock-down (**Figure 2D and Figure 2E**). Secondly, we noted that the METTL3 knock-down increased the presence of miR-200b-3p and 3'UTR-XIAP in the GW182- and TNRC6B-immunoprecipitates (**Figure 2D and Figure 2E**). Thus, these last results indicate that the METTL3-mediated adenosine-methylation status of miR-200b-3p influences the endogenous formation of 3'UTR-mRNA-XIAP/miR-200b-3p duplex.

By affecting the expression of XIAP, an apoptotic player, our data suggest that the expression level and the N6-adenosine methylation level of miR-200b-3p could affect the intrinsic apoptosis level of tumors. To investigate this hypothesis, we analyzed the Caspase/DEVDase activity as a marker of the intrinsic apoptosis level of tumors. Our work indicates that tumors harboring the miRNA-200b-3p^{exp-low} signature or the miR-200b-3p^{%m6A>10%} signature have a lower intrinsic apoptosis level (**Figure 2F**).

Finally, we observed that patients whose tumors harbored the miRNA-200b-3p^{exp-low} signature or the miR-200b-3p^{%m6A>10%} signature have a lower survival outcome than the other GBM patients (**Figure 2G**).

m6A-miR-200b-3p appears as a promising tool in anti-GBM therapy

Based on the fact that the miR-200b-3p affects the intrinsic apoptosis level, we extended our study by investigating whether miR-200b-3p and m6A-miR-200b-3p could be used as a therapeutic tool. For this purpose, the miR-200b-3p- and m6A-miR-200b-3p-induced cell death was measured from a panel of cells representing human brain cells (astrocytes (HAST40), neurons (RN33b) and astrocytoma (U87)). We included in this panel U87^{IDH1mut} cells since IDH1 mutation is observed in GBM. Besides, we observed that the presence of IDH1 mutation decreased α KG and increased the adenosine methylation of miR-200b-3p in a context of the FTO and METTL3 expression level being unchanged (**Figure 3A**). Meclofemalic acid was also used as a FTO inhibitor ²¹. Because peripheral blood is the place where exposure to chemicals occurs, PBMC (peripheral blood mononuclear cells) were also included in our study. Firstly, our data indicated that miRNA-200b-3p induced cell death in

all cells with the exception of neuron (RN33b cell line) (**Figure 3B**). Secondly, we observed that m6A-miR-200b-3p induced cell death in U87 cells, but not in U87^{IDH1mut}, U87^{Meclofemalic}, PMBC, neurons and astrocytes (**Figure 3B**). In other terms, these data suggest that the ability of m6A-miR-200b-3p to induce cell death occurs in cancer cells and not in non-cancerous cells like PMBC, neurons and astrocytes. Based on our knowledge, the absence of massive m6A-miR-200b-3p-induced cell death in U87^{IDH1mut} could be associated to the fact that these cells have a lower quantity of α KG, i.e. a lower quantity of the enzyme co-factor (FTO) catalyzing the adenosine demethylation of miR-200b-3p. Besides, the fact that the meclofemalic acid treatment abrogated the m6A-miR-200b-3p-induced cell death in U87 cells confirmed the involvement of FTO in this process (**Figure 3B**).

We have then investigated the putative anti-GBM effect of m6A-miR-200b-3p in an *in vivo* model of GBM. For this purpose, U87-induced GBMs were generated by xenograft in mice. When the volume of the U87-induced GBMs was close to 100mm³, three mice were randomly untreated, treated with temozolomide (TMZ) and/or with m6A-miR-200b-3p (**Figure 3C**). The option to use TMZ is due to the fact that this alkylating agent is the chemotherapeutic agent included in the current standard care protocol in GBM treatment²².

By comparing the effect of the TMZ treatment with the effect of the m6A-miR-200b-3p treatment, we could clearly see that the m6A-miR-200b-3p treatment has similar efficiency than the TMZ-25mg/kg treatment (**Figure 3D**). We also noted that the m6A-miR-200b-3p+TMZ-25mg/kg treatment has the same efficiency than the TMZ-50mg/kg treatment (**Figure 3D**).

miR-200b-3p could also be used as a therapeutic tool in other cancer types

The above data are focused on the XIAP regulation by miR-200b-3p, but it is well known that one miRNA has multiple targets. Consequently, we next investigated whether the adenosine methylation of miR-200b-3p could abrogate its translational repressor function towards other putative protein targets than XIAP. Among the putative protein targets of miR-200b-3p (according to the miRTarBase website²³), we focused our study on two other apoptotic players (Bcl-2 (B-cell lymphoma 2, Uniprot#P10415) and Caspase-2 (cysteine-dependent aspartate-directed proteases 2, Uniprot#P42575)), two epigenetic players (EZH2 (Enhancer of zeste homolog 2, Uniprot#Q15910) and DNMT1 (DNA (cytosine-5)-methyltransferase 1,

Uniprot#P26358)) and a negative immune checkpoint PD-L1 (Programmed cell death 1 ligand 1, Uniprot#Q2NZQ7). Our data indicated that the presence of m6A in miRNA-200b-3p also abrogated the translational repressor function of miR-200b-3p toward Bcl-2 and PD-L1 (**Figure 4A**).

Finally, we investigated whether the ability of m6A-miR-200b-3p to induce cell death was specific of U87 cells. For this purpose, cancerous cell lines representative of several cancers were transfected with m6A-miR-200b-3p (U251 and T98G for glioblastoma, A549 and H1975 for lung, MCF7 and T47D for breast, OE21 for esophagus, OV90 and SKOV3 for ovaries). Four non-cancerous cell lines were also included in our study. Four hours after cells transfection, we noted that all cells were transfected with similar quantity of m6A-miR-200b-3p since the range of increase of miR-200b-3p expression was homogeneous (10-13 fold induction) (**Figure 4B**). Then, we noted that cell death occurred in cells having the ability to adenosine-demethylate miR-200b-3b *i.e.* in U251, A549, T47D and SKOV3 (**Figure 4B**). The absence of cell death in other cell lines and particularly in non-cancerous cell lines was explained by the inability of these cells to adenosine-methylate miR-200b-3b (m6A enrichment transfected/control being equal to 1) (**Figure 4B**).

Taken together, all these last results are consistent with the fact that m6A-miR-200b-3p appears as a promising tool in anti-GBM therapy.

DISCUSSION

Recent investigations concerning the description of the molecular mechanisms of bases modification of miRNAs have provided meaningful progresses in the understanding of regulation of the miRNAs biogenesis and functionality. Thus, after the studies of Alarcón *et al.* (2015), Berulava *et al.* (2015) and Konno *et al.* (2019), our study reports the presence of m6A in miRNAs *via* the realization of RNA immunoprecipitation with an anti-m6A-antibody followed by RT-qPCR^{5,6,7}. Despite these posterior studies, our investigation harbors several innovative points.

First, our work indicates that the adenosine methylation of miR-200b-3p abrogates its translational repressor function towards its putative targets such as XIAP, Bcl-2 and PD-L1. The works published by Alarcón *et al.* (2015) and Berulava *et al.* (2015) report the existence of 2 different consensus sequences for the m6A methylation in pri-miRNAs (UGAC) and in mature miRNAs (ADRA)^{5,6}. Interestingly, we noted that the miRNA-200b-3p sequence

contains a sequence matching one of the consensus (**Table S2**). We also noted that the miR-200b-3p sequence contains a sequence matching the consensus sequence binding by METTL3/WTAP defined by Ping *et al.* (2014)¹² (**Table S2**). From a certain perspective, this last point can also constitute an argument supporting the role of METTL3 in the adenosine methylation of miRNAs.

The work of Berulava *et al.* (2015) indicate that FTO plays a crucial role in the demethylation of miRNAs⁶. Our data complete this by indicating that the presence of α KG also acts as a non-negligible player in the demethylation of miRNAs.

In addition to these 2 initial reports, our study shows that the presence of m6A acts as an inhibitor of the post-transcriptional repressor function of miRNAs. Mechanistically, our data indicate that the presence of m6A limits the formation of miRNA/mRNA duplex. Our study is also distinguished from the first two studies by its clinical translational study effort using a cohort of cancer patients. Indeed, our study is the first to mention that the level of N6-adenosine methylation of a miRNA (in association with the expression level of this miRNA) acts as a biomarker characterizing GBM patients with a poor survival. Our study is also distinct to the one recently published by Konno *et al.* (2019) since Konno and colleagues considerate the adenosine methylation of miR as a tool to distinguish early pancreatic cancer patients from healthy controls with an extremely high sensitivity and specificity; while in our article the adenosine methylation of miR-200b-3p is associated with a prognosis value of response for GBM patients⁷ and could have a therapeutic function.

The work of Berulava *et al.* (2015) and the one of Yuan *et al.* (2014) introduce a debate about the impact of the adenosine methylation of miRNA on their stability^{6,24}. Our data focusing on miR-200b-3p seems to indicate that the adenosine methylation of this miRNA does not impact on its expression. Indeed, the modulation of its adenosine methylation level *via* siRNA directed against FTO and METTL3 or *via* chemical components does not affect its expression. However, this finding being obtained on one miRNA, it is not possible to generalize a rule about the impact of the adenosine methylation on the miRNA stability.

By observing that the adenosine methylated miR-200b-3p was not recruited to the RISC complex, our data reinforces the idea that the adenosine methylation of miRNA appears as molecular mechanism governing the miRNA functionality *via* the regulation of the duplex formation between miRNA and mRNA. More generally, our data support the idea that

nucleotide modification occurring in miRNA or in 3'UTR-mRNA alters the formation of miR/3'UTR-mRNA duplex, such as reported by Lockhart *et al.* (2019) ²⁵.

By reporting that m6A methylation of miRNAs could act as a biomarker characterizing GBM patients with a poor survival, our data open the idea that the molecular actor writing this epitranscriptomic signature (METTL3 according to our data) could be used as a target for the development of epidrugs. Indeed, this point of view is already discussed since METTL3 promotes oncogene translation ²⁶.

During the last decade, miRNA mimics and molecules targeting miRNAs (anti-miRs) have shown promising results in preclinical development ^{27,28}. Four arguments strongly support the idea that the adenosine-methylated form of miR-200b-3p could be used as a promising therapeutic tool. First, m6A-miR-200b-3p is apoptogenic by itself *via* the repression of XIAP, an anti-apoptotic protein. Secondly, our data indicate that m6A-miR-200b-3p promotes cell death in cancerous cells such as U87 (but also in other cancer cell lines) and not in non-cancerous cells such as neurons, PBMC, astrocytes and hepatocytes. Thirdly, our *in vivo* data indicate that m6A-miR-200b-3p has an anti-tumor growth effect in an *in vivo* model of GBM. Fourthly, our *in vivo* data also indicate that the m6A-miR-200b-3p/TMZ combination permits to limit the dose of TMZ since the m6A-miR-200b-3p/TMZ-25mg/kg combination has the same anti-tumor growth effect than the use of the TMZ-50mg/kg treatment. Thus, all these arguments define the adenosine-methylated form of miR-200b-3p as the prodrug form of this miRNA. More interestingly, our data indicate that its conversion under an active form occurs in cancer cells but not in non-cancerous cells. This observation is highly promising since it can be translated such as the fact that only cancerous cells have the "tools" (FTO and α KG) to activate the prodrug form of miR-200b-3p. Thus, the adenosine-methylated form of miRNAs could be considered such as a manner to limit the off-targets effect of miRNA therapy associated with the relative lack of addressing of miRNA-based therapy against the cancer cells ²⁹. Our data also introduce the idea that the presence of IDH1 mutations could be considered such as a biomarker excluding the use of adenosine-methylated form of miRNAs since cells presenting IDH1 mutations have a low level of α KG. Concretely, the first reading of this idea might exclude the use of m6A-miR-200b-3p treatment in less than 10% of primary GBM and in 6-10% of de novo AML, as example ^{30,31}. However, this point is available when the m6A-miR-200b-3p treatment is envisioned as single treatment since its

combination with BAY1436032 (a pan-mutant IDH1 inhibitor ³²) restored its ability to promote cell death (**Figure S6**).

In conclusion, our study opens a new area in the understanding of epigenetic modifications concerning miRNA and in the development of innovative epidrugs. Indeed, since several years chemical modifications of RNAs (*i.e.* epitranscriptomic) are defined such as central players in the control of messenger and ncRNA activity ³³. Our data reinforce this idea by showing that the adenosine methylation of miRNAs abrogates their post-transcriptional repressive function. By initiating the idea that adenosine-methylated miRNA could be used as a prodrug, our work provides the base for the development of a new pathway of anti-cancer therapeutic strategies targeting miRNA. Thus, in the future years, the understanding of the mechanisms involved in the epigenetic regulation of miRNA could improve patient stratification and the development of successful miRNA-based therapeutic strategies.

MATERIALS and METHODS.

miRNA extraction

miRNA extractions were performed using the NucleoSpin[®] miRNA kit (Macherey Nagel, France) according to the manufacturer's instructions.

miRNA and siRNA transfection

Briefly, 6×10^5 cells were seeded in each well of 4-well plates. Transfection was performed using HiPerFect Transfection Reagents (Qiagen, France) and 10 ng miR (Qiagen, France) or 10nm of Silencer[®] siRNA (Thermo Fisher, France), according to the manufacturer's recommendations. For siRNA controls, transfection control (HiPerfect Transfection Reagent only) and a negative control (Silencer[®] Negative control#1 siRNA) had been used. For miR controls, transfection control (HiPerfect Transfection Reagent only) and an oligo (miScript Inhibitor Negative Control; Qiagen, France) had been used.

Acellular METTL3 methylation assay

METTL3-including complexes were immunoprecipitated from cellular lysate obtained after sonication and the use of CHAPS buffer (40 mM HEPES, pH 7.4, 120 mM NaCl, 1% CHAPS, 1 mM EDTA, supplemented with protease and phosphatase inhibitors). Immunoprecipitations *were performed using* Catch and Release v2.0 Reversible Immunoprecipitation System (Merck, France) and anti-METTL3 (Abcam, France). IgG (Abcam, France) was used as control. Elutions from IP were performed using the non-denaturing Elution Buffer according to the manufacturer's instructions. Then 30µL of elution were used in METTL3 enzymatic assay. METTL3 enzymatic assay was conducted in reaction buffer (20 mM Tris pH 7.5, 1 mM DTT, 0.01% Triton X-100, 40U/100ml buffer RNaseOUT). The reaction mixture contained unmethylated mimic miR-200b-3p with biotin tag and SAM. Enzymatic assay reactions were incubated overnight at room temperature on shaker. After streptavidin isolation, the presence of N6-adenosine methylation was determined by dot blot. Dots were then incubated with anti-m6A and anti-adenosine (as loading control) antibodies overnight. For signal detection secondary HRP antibodies were used and signal was detected on ChemiDoc MP (Bio-Rad, France).

RNA-immunoprecipitation for miRNA

For immunoprecipitation of RNA, two rounds using 5 µg of anti-m6A antibody (Abcam, France) and 5 µg of small RNA were performed. The reaction was carried out using Dynabeads Protein G Immunoprecipitation kit with some modifications (ThermoFisher Scientific, France) such as described by Berulava et al. (2015) ⁶. As a control, immunoprecipitation was performed using IgG (Abcam, France) instead of anti-m6A antibody. miRs obtained from m6A immunoprecipitation were reverse transcribed using miRScript II RT kit (Qiagen, France) and analyzed using the miScript miRNA PCR Array Human Cancer Pathway kit (Qiagen, France) according to the manufacturers' instructions. Fold enrichment was next calculated using Ct value obtained from RT-qPCR performed with input miR, IP-IgG and IP-m6A and the $2^{-\Delta\Delta Ct}$ formula.

Cross-linking immunoprecipitation (CLIP)

CLIP were performed using RiboCluster Profiler RIP-Assay (CliniScience, France) from 10 millions per sample of UV crosslinked cells (150 mJ/cm² of UVA (365 nm) according to the manufacturer's instructions. IP were performed in presence of 15µg of anti-GW182

(#RN033P, CliniScience, France) and anti-TNRC6B (#9913, Merck-Millipore, France) for overnight at 4°C.

Quantitative PCR of miRNA

For miRNA expression analysis and detection from product of RIP performed with anti-m6A antibody, RNA was reverse transcribed using miRScript II RT kit and analyzed by qPCR with the miScript SYBR Green PCR Kit using the specific hsa-miR miScript Primer Assays (Qiagen, France) according to the manufacturers' instructions.

ELISA

Proteins extracts were obtained by using RIPA Lysis and Extraction Buffer (Thermo Scientific, France) in accordance with the manufacturer's instructions. XIAP (Human) Cell-Based ELISA Kit (Abnova, Taiwan), Alpha Ketoglutarate (alpha KG) Assay Kit (ab83431) (Abcam, France) Human FTO ELISA Kit (68ELH-FTO) (Tebu-Bio, France) Methyltransferase like 3 (METTL3), ELISA Kit (MBS9326769) (My BioSource, USA), CST - PathScan® Total Ezh2 Sandwich ELISA Kit (Ozyme, France), EpiQuik Dnmt1 Assay Kit (EpiQuik Dnmt1 Assay Kit, Euromedex/EpiGentek, France), Human Bcl-2 ELISA Kit (Abcam, France), Caspase-2 ELISA Kit (Tebu-Bio, France) and PathScan® Total PD-L1 Sandwich ELISA Kit (Ozyme, France) were performed according to the manufacturer's instructions.

Tumor xenografts in nude mice

Cells were harvested by trypsinization, washed and resuspended in saline buffer. Cell suspensions were injected s.c. into the flank of 7-8-week-old mice (Janvier, France) in 100 µl of sterile PBS. Tumor volume based on caliper measurements was calculated using the modified ellipsoidal formula ($Tumor\ volume = 1/2(length \times width^2)$).

The experimental procedures with animals were in accordance with the guidelines of Institutional Animal Care and the French National Committee of Ethics. In addition, all experiments were conducted according to the Regulations for Animal Experimentation at the "Plateforme Animalerie" in the "Institut de Recherche en Santé de l'Université de Nantes (IRS-UN)" and approved by the French National Committee of Ethics.

Cell lines.

U87, U87^{IDH1mut}, RN33b and A549 cells were obtained from the American Type Culture Collection (ATCC, Molsheim, France). HASTR040/astrocytes were obtained from Clonexpress (Gaithersburg, USA). OE21 cells were obtained from Sigma (France). HEP10 cells were obtained from ThermoFisher (France). MCF7 and T47D cells were provided by the Dr P. Juin's lab. SKOV3 cells were provided by the Dr E. Scottet's lab. OV90 cells were provided by the Dr R. Spisek's lab.

Statistical analysis.

All experiments were done at least in triplicates. Significance of the differences in means were calculated using Student-t-test while correlations were determined using Pearson-test. Survival curves were plotted according to Kaplan-Meier method and compared log rank test.

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Author contributions

PFC designed and coordinated the project.

JB, AS, AN, GCB and PFC performed all experiments.

FMV, AS, BO, DH and PFC interpreted and discussed the data.

PFC wrote the first version of the manuscript and all authors reviewed and approved it.

Conflict of interest statement

Authors declare that they have no financial relationship with the organization that

sponsored the research.

Ethics approval and consent to participate

The experimental procedures using animals were in accordance with the guidelines of Institutional Animal Care and the French National Committee of Ethics. In addition, all experiments were conducted according to the Regulations for Animal Experimentation at the “Plate-forme Animalerie” of “Institut de Recherche en Santé de l'Université de Nantes (IRS-UN) and approved by the French National Committee of Ethics (Agreement number: B44278).

Patient material as well as records (diagnosis, KPS, age, sex, date of death) was used with confidentiality according to French laws and recommendations of the French National Committee of Ethic. In addition, patient material and experiments using this material are conducted according to the regulations of “the Réseau des tumorothèques du Cancéropôle Grand-Ouest” and more particularly with the regulations of “Réseau Gliome”. All patients provided informed consent in accordance with the Helsinki Declaration.

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Figures legends

Figure 1: The m6A methyltransferase METTL3, the m6A demethylase FTO and alpha-ketoglutarate regulate the N6-adenosine methylation of miR-200b-3p

A. Each bar represents the relative expression level of miR-200b-3p (miR-200b-3p^{exp}) in 32 samples of GBM. After miR extraction from tumors, RT-qPCR were performed to evaluate the relative expression level of miRNA-200b-3p by using non tumor brain samples as reference and SNORD6.1 as housekeeping miR.

B. Correlation between the relative expression level of miR-200b-3p and the percentage of N6-adenosine methylation in miR-200b-3p (miR200b-3p^{%m6A}). This percentage was calculated *via* the realization of RNA immunoprecipitation performed with an anti-m6A antibody followed by qPCR analysis (miRIP^{m6A}-qPCR) (as previously described by Berulava *et al.* 2015). Each square symbolizes a GBM patient.

- C.** Absence of correlation between the relative expression level of FTO and the percentage of N6-adenosine methylation in miR-200b-3b. Human FTO ELISA Kit (Tebu-Bio, France) was used to estimate the FTO expression. Each square symbolizes a GBM patient.
- D.** Absence of correlation between the relative presence of alpha-ketoglutarate (α KG) and the percentage of N6-adenosine methylation in miR-200b-3p. Alpha-ketoglutarate Assay Kit (Abcam, France) was used to estimate the relative presence of α KG. Each square symbolizes a GBM patient.
- E.** High percentage of N6-adenosine methylation in miR-200b-3p is observed in GBM harboring a low level of FTO and α KG. Each square symbolizes a GBM patient. Red squares symbolize the average \pm standard deviation of the two considered subgroups.
- F.** Impact of the down-regulation of FTO on the adenosine methylation percentage of miR-200b-3p^{%m6A} calculated through the realization of miRIP^{m6A}-qPCR as previously described.
- G.** Impact of the alpha-Ketoglutarate (α KG) treatment on the adenosine methylation percentage of miR-200b-3p (miRNA-200b-3p^{%m6A}).
- H.** Impact of the Meclofenamic acid (MA) treatment on miRNA-200b-3p^{%m6A} in U87 cells. After MA treatment (15 μ M/24h, Santa Cruz, France), the miRNA-200b-3p^{%m6A} was calculated through the realization of miRIP^{m6A}-qPCR as previously described.
- I.** Correlation between the relative expression level of METTL3 and the percentage of N6-adenosine methylation in miR-200b-3p. Each square symbolizes a GBM patient.
- J.** Dot blot illustrating the presence of adenosine methylation in mimetic miR-200b-3p in presence of METTL3 immunoprecipitation product. Adenosine detection is used as control. S1/S2/S3 are three independent experiments. Ctrl: synthetic miR-200b-3p adenosine unmethylated, IP-IgG: produce of IP performed with anti-IgG. (Absence of adenosine signal indicates that miRNA was not unspecifically immunoprecipitated). IP-METTL3: produce of IP performed with anti-IgG. Produces of IP (immunoprecipitation) were obtained in non-denaturing condition in order to conserve enzymatic activity according to the manufacturer's indications.
- K.** The METTL3 knock-down (by siRNA approach) decreases the percentage of adenosine methylation of miR-200b-3p.
- L.** α FM^{score} reflects the METTL3, FTO and α KG expression levels in the 32 GBM samples. Each circle symbolizes a GBM patient. More α FM^{score} is higher more the percentage of adenosine methylation of miR-200b-3p is higher.

Figure 2: The N6-adenosine methylation of miR-200b-3p limits its translational repressor function toward anti-apoptotic players and confers poor prognosis in GBM patients.

A. Absence of correlation between the relative expression level of XIAP and miRNA-200b-3b (miR200b-3p^{exp}) in all 32 patients included in our study. Human XIAP ELISA Kit (Abcam, France) was used to estimate the relative expression level of XIAP. Each square symbolizes a GBM patient.

B. Samples were stratified according to the miR-200b-3p^{exp} and miR-200b-3p^{m6A} parameters in order to distinguish the 3 indicated groups. Each box represents a sample/patient. For each group, the average of XIAP expression was analyzed with Human XIAP ELISA Kit (Abcam, France) was calculated and represented on the graph.

C. Impact of the *in vitro* N6-adenosine methylation of miRNA-200b-3p^{mimetic} on the expression of the XIAP (Human XIAP ELISA Kit, Abcam, France).

D. Cross-Linking Immunoprecipitation and qPCR (CLIP-qPCR) investigate the 3'UTR/XIAP and 3'UTR/EP300 (internal control) enrichments on GW182, TNRC6B and IgG (negative control). Experiments were performed using the RiboCluster Profiler kit (CliniScience, France) according to the manufacturer's instructions.

E. Cross-Linking Immunoprecipitation and qPCR (CLIP-qPCR) investigate the miR-150-5p (internal control) and miR-200b-3p enrichments on GW182, TNRC6B and IgG (negative control). Experiments were performed using the RiboCluster Profiler kit (CliniScience, France) according to manufacturer's instructions.

F. For each samples, DEVDase activity was estimated such as previously described³⁴. Each open square symbolizes a sample. Blue square symbolizes the average of samples having miR-200b-3p^{m6A}>10% or miRNA-200b-3p^{exp-low}. Grey square symbolizes the average of samples having miR-200b-3p^{m6A}<10% or miRNA-200b-3p^{exp-high}.

G. Kaplan-Meier representation of survival curves for GBM patients those tumors are characterized by a miR-200b-3p^{m6A}>10% or a miRNA-200b-3p^{exp-low} (in blue) and by a miR-200b-3p^{m6A}<10% and a miRNA-200b-3p^{exp-high} (in grey).

Figure 3: The N6-adenosine methylation of miR-200b-3p selectively induces apoptosis in cancer cells and has an anti-tumor growth effect.

A. IDH mutation in U87 cells induces a decrease of α KG rate and an increase of miR-200b-3p^{%m6A} without FTO or METTL3 expression modification.

B. miR-200b-3p promotes cell death by itself in cancerous and non-cancerous cells (excepted neuron RN33b), while miR-200b-3b induced apoptosis by itself in U87 cells, only. The LDH-Cytotoxicity Assay Kit (Abcam, France) is used to estimate the cell death 24h after the m6A-miR-200b-3b incubation.

C. Representation of our *in vivo* model of U87-induced GBM. Mice were xenografted with U87 cells. When tumor volume was equivalent to 100mm³, mice were treated with TMZ and/or miR-200b-3p five days per week. Tumor volumes were measured after 3 weeks of treatment.

D. Impact of the adenosine-methylated form of miR-200b-3p on the tumor growth in mice model.

Figure 4: miR-200b-3p could also be used as a therapeutic tool in other cancer types

A. Protein expression of different targets of mir-200b-3p has been studied by ELISA in U87 cells treated with unspecific oligo or miR-200b-3p adenosine methylated or not. miR-200b-3p decreases Bcl-2 and PD-L1 expression only when not methylated and has no effect on other proteins studied.

B. In cell lines transfected with m6A-miR-200b-3p, cell death is induced in several cancer cell line types, when cell lines are able to demethylate this miR.

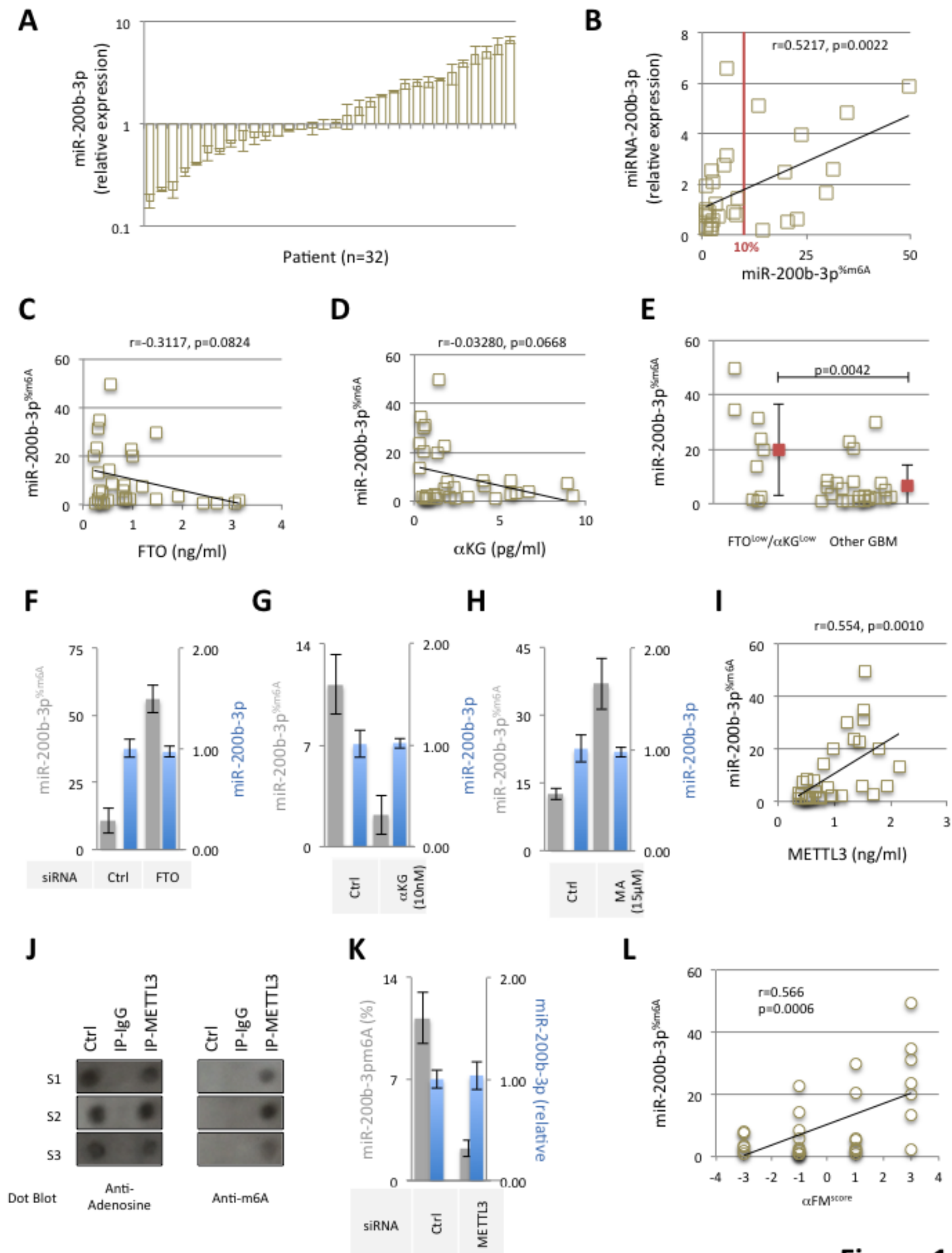
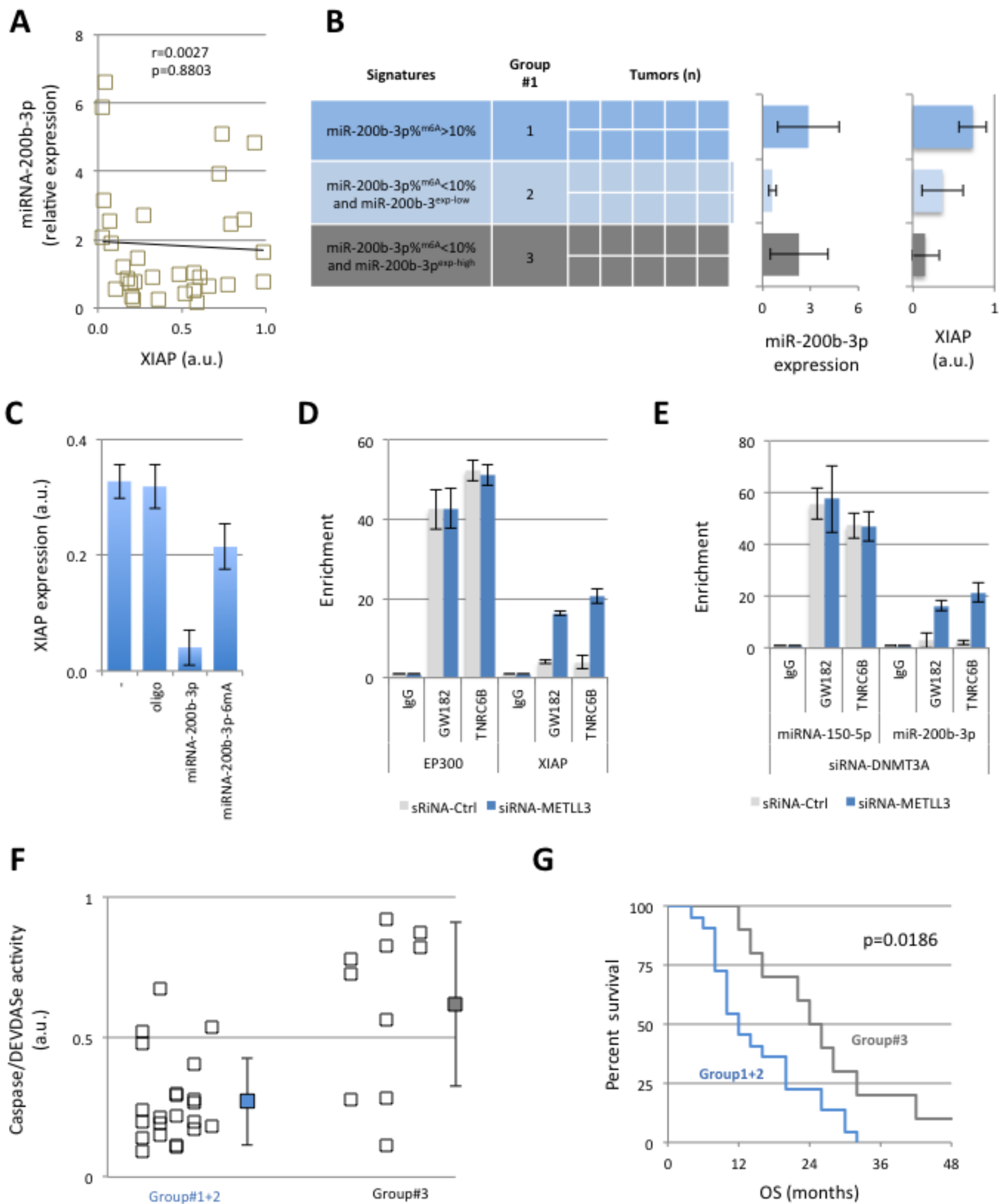
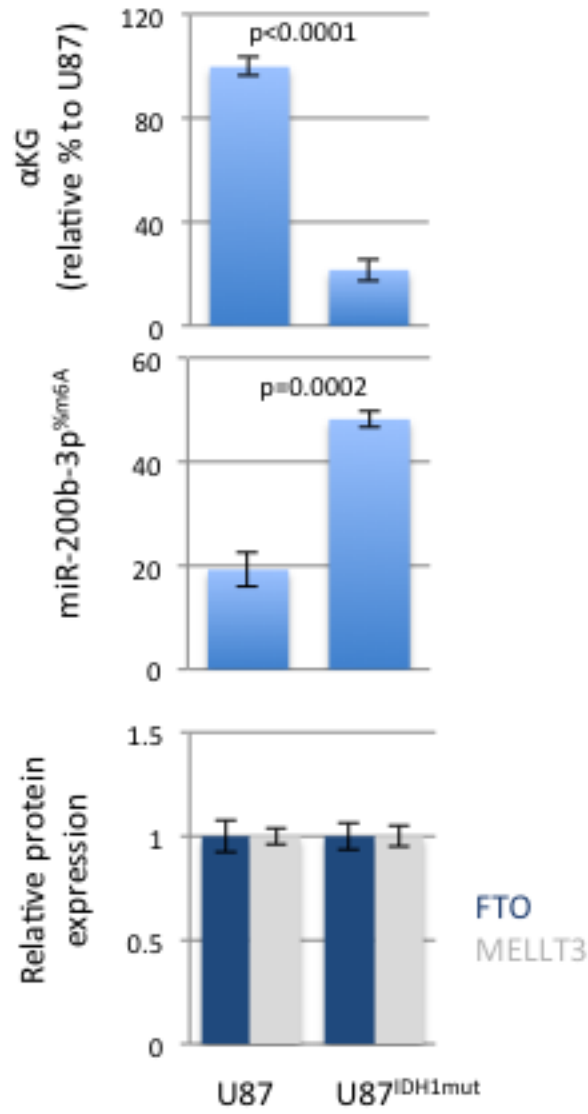
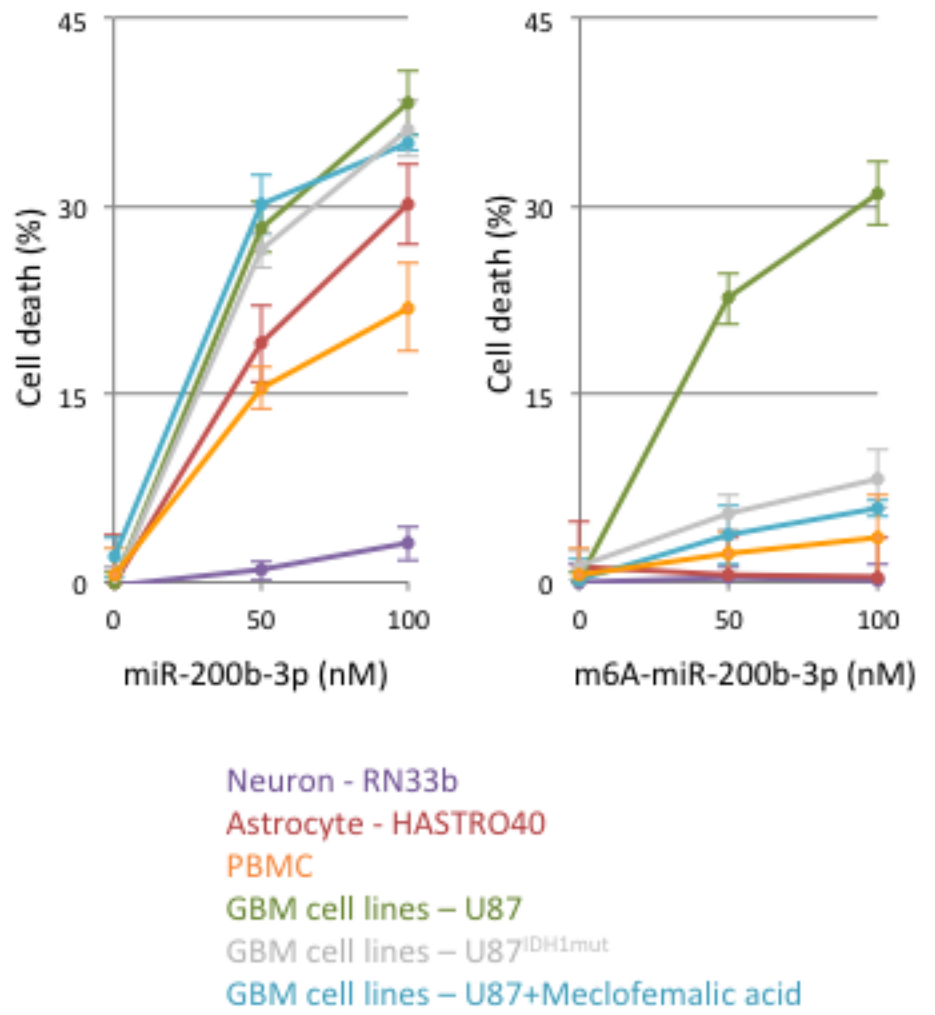
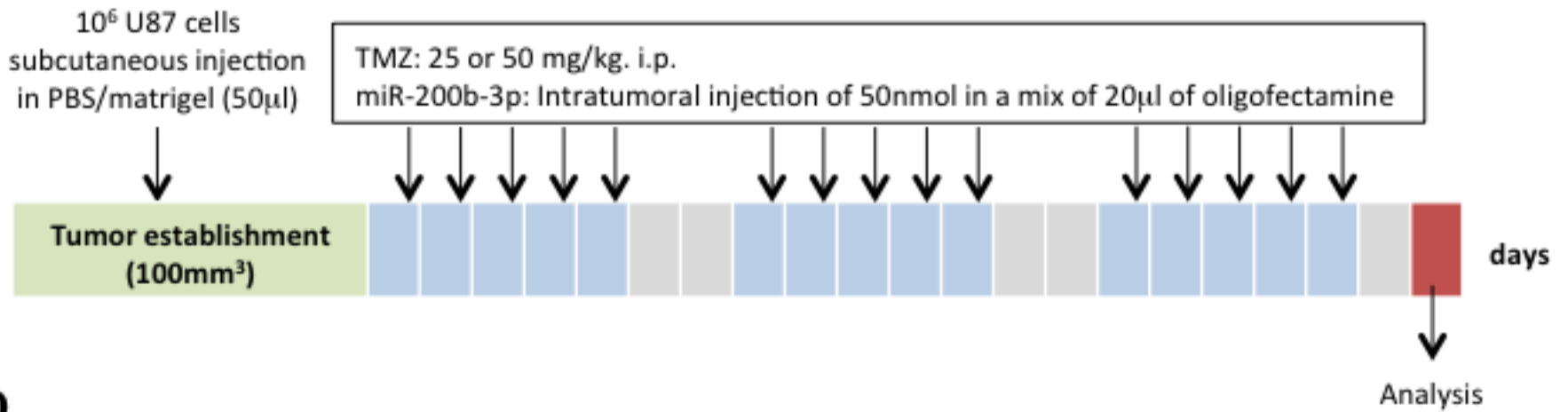
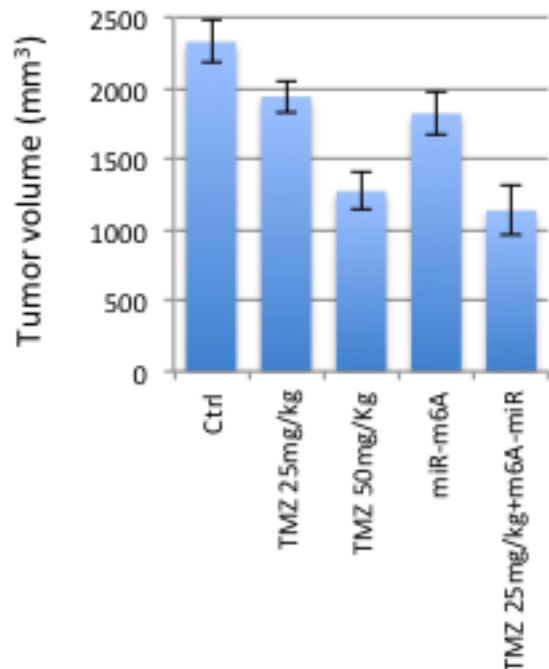


Figure 1



A**B****C****D****Figure 3**

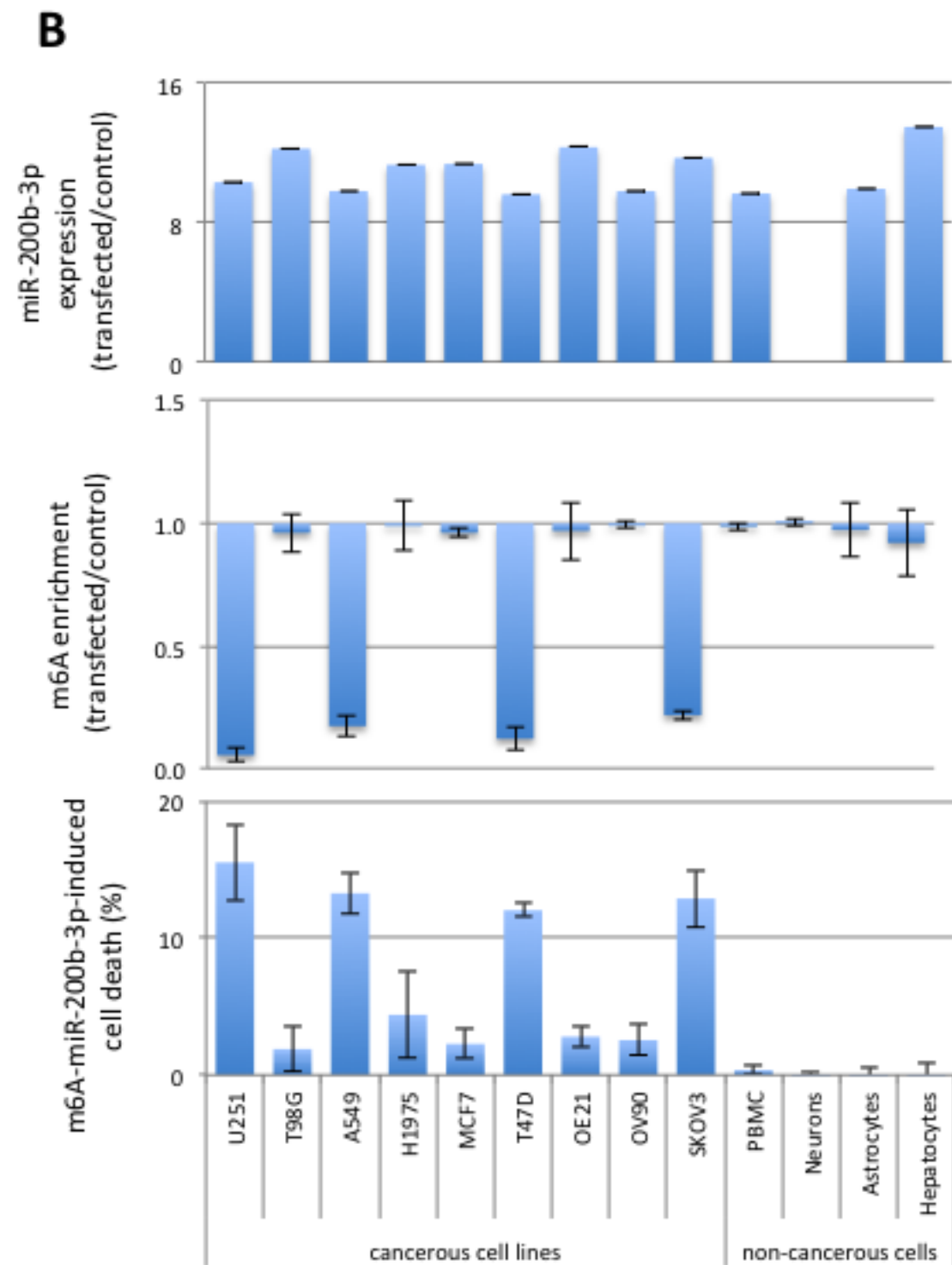
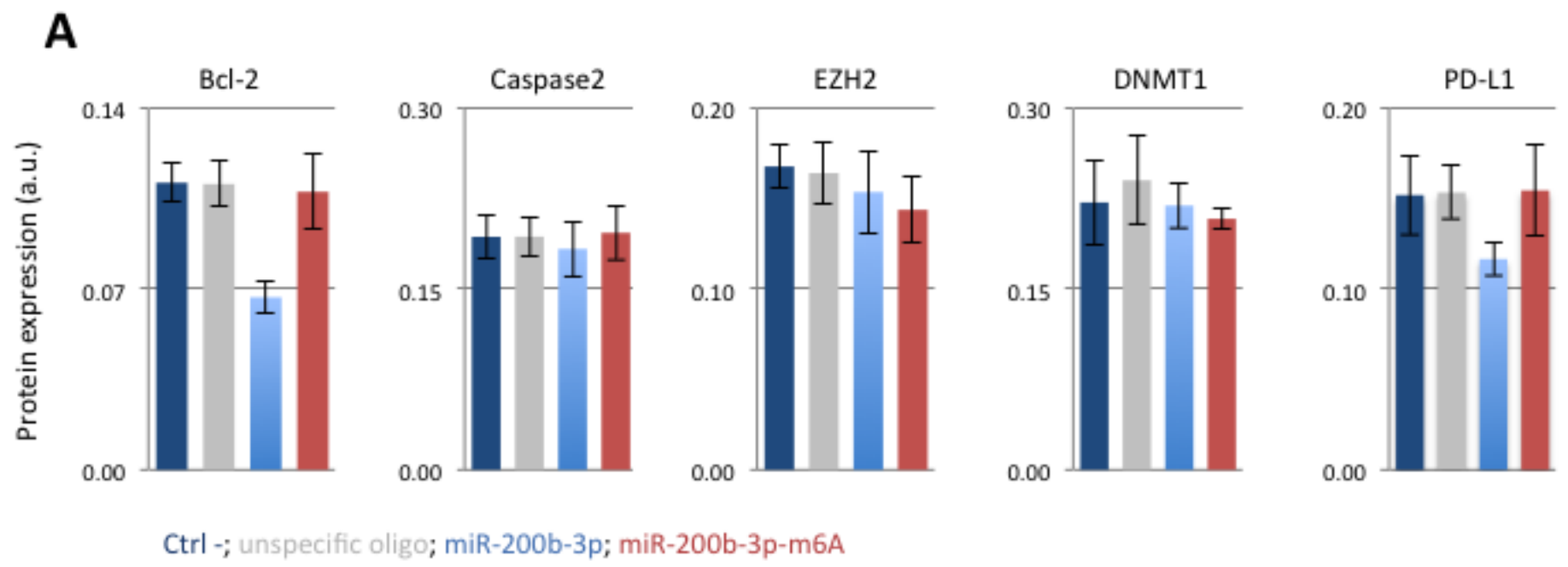


Figure 4

Graphical abstract

