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Targeting miR-21 inhibits *in vitro* and *in vivo* multiple myeloma cell growth

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Abstract

Purpose—Deregulated expression of microRNAs (miRNAs) plays a role in the pathogenesis and progression of multiple myeloma (MM). Among upregulated miRNAs, miR-21 has oncogenic potential and therefore represents an attractive target for the treatment of MM.

Experimental design—Here, we investigated the *in vitro* and *in vivo* anti-MM activity of miR-21 inhibitors.

Results—Either transient enforced expression or lentivirus-based constitutive expression of miR-21 inhibitors triggered significant growth inhibition of primary patient MM cells or IL-6-dependent/independent MM cell lines and overcame the protective activity of human bone marrow stromal cells. Conversely, transfection of miR-21 mimics significantly increased proliferation of MM cells, demonstrating its tumor promoting potential in MM. Importantly, upregulation of miR-21 canonical validated targets (PTEN, Rho-B and BTG2), together with functional impairment of both AKT and ERK signaling, were achieved by transfection of miR-21 inhibitors into MM cells. *In vivo* delivery of miR-21 inhibitors in SCID mice bearing human MM xenografts expressing miR-21 induced significant anti-tumor activity. Upregulation of PTEN and

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downregulation of p-AKT were observed in retrieved xenografts following treatment with miR-21 inhibitors.

Conclusions—Our findings show the first evidence that *in vivo* antagonism of miR-21 exerts anti-MM activity, providing the rationale for clinical development of miR-21 inhibitors in this still incurable disease.

Keywords

multiple myeloma; plasma cell leukemia; miR-21; miR-21 inhibitor; microRNA

Introduction

Multiple myeloma (MM) is a genetically complex hematologic malignancy characterized by abnormal infiltration of clonal plasma cells in the bone marrow (BM) (1, 2). Despite novel insights into the pathobiology of the disease and the availability of new research platforms and therapeutics (3–6), innovative treatment strategies are urgently needed. A major area of investigation is the human BM microenvironment (hBMM), which plays an essential role promoting growth, survival and drug resistance in MM (7). Moreover, within the hBMM tumor cells progressively accumulate genetic aberrations leading to relapsed and refractory disease (8). Currently, there is clear evidence that changes in gene copy number, chromosomal translocations, mutations, as well as transcriptional and epigenetic events during the evolutionary process of MM (9, 10) result in profound deregulation of the microRNA (miRNA) network (11, 12), which in turn leads to aberrant translation of messenger RNAs (mRNAs) and cell signaling.

miRNAs are small, non-coding RNAs of 19–25 nucleotides, which regulate gene expression by inducing degradation or translation inhibition of target mRNAs primarily through base pairing to partially or fully complementary sites in the 3'UTR (13). Aberrant expression of miRNAs occurs widely in human cancers, including both solid tumors and hematological malignancies (11, 12, 14). Deregulated miRNAs may act as oncogenes (Onco-miRNAs) or tumor-suppressors (TS-miRNAs) (14–16), with the former generally upregulated and the latter downregulated in cancer cells. Since miRNAs regulate several mRNAs relevant in cancer promotion, targeting of deregulated miRNAs in cancer cells is emerging as a novel promising therapeutic approach in a variety of malignancies (17, 18), including MM (19–25). Among those miRNAs involved in tumorigenesis, miR-21 plays a key role in tumor progression and is significantly upregulated in several human cancers (26). It has been recently shown that the Epstein–Barr virus (EBV)-encoded EBNA2, which is needed for the transforming capacity of B cells *in vitro*significantly increase miR-21 expression (27). Interestingly, a genetically engineered mouse model showed that constitutive tissue-specific overexpression of miR-21 resulted in a pre-B cell lymphoma (28). Conversely, inhibition of miR-21 induces antiproliferative and apoptotic effects as well as enhances sensitivity to anti-tumor agents including gemcitabine, docetaxel, temozolomide and 5-fluorouracil (29–31). These findings strongly support the notion that miR-21 represents a potential therapeutic target in human cancer. In the last few years, there is increasing evidence for the role of miR-21 in pathogenesis of plasma cell dyscrasias. Upregulation of miR-21 has been found in both MGUS and MM (32); in the latter, miR-21 is regulated by IL-6 through Stat3-

pathway activation (33), and a Stat3/miR-21 positive feedback loop has been demonstrated (34). Moreover, miR-21 induces resistance to MM cell apoptosis triggered by dexamethasone, doxorubicin or bortezomib (35). Importantly, adhesion of MM cells to human bone marrow stromal cells (hBMSCs) triggers upregulation of miR-21 expression, suggesting its role in BM-mediated growth, survival and drug resistance (35). This MM cell growth and survival promoting activity of miR-21 suggests that it represents an attractive novel therapeutic target.

In this report, we characterized the anti-MM activity and the molecular events triggered by miR-21 inhibition in patient MM cells and MM cell lines, which were maintained even when tumor cells were adherent to patient BMSCs *in vitro*. We then demonstrated the *in vivo* cytotoxicity and mechanisms of action of miR-21 inhibitors in a murine xenograft model of human MM, providing the framework for its clinical development.

Materials and Methods

Reagents and cell culture

MM cell lines were cultured in RPMI-1640 (Gibco, Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (Lonza Group Ltd., Switzerland) and 1% penicillin/streptomycin (Gibco, Life Technologies, Carlsbad, CA). The IL-6 dependent MM cell line INA-6 (kindly provided from Dr Renate Burger, University of Erlangen-Nuernberg, Erlangen, Germany) was cultured in the presence of rhIL-6 (R&D Systems, Minneapolis, MN), as previously reported (36). Following informed consent approved by our University Hospital Ethical Commitee, primary patient MM cells (ppMM cells) were isolated from BM aspirates by Ficoll-Hypaque density gradient sedimentation followed by antibody mediated positive selection using anti-CD138 magnetic activated cell separation microbeads (Miltenyi Biotech, Gladbach, Germany). Purity of immunoselected cells were assessed by flow cytometric analysis using a phicoeritrin conjugated CD38 mAb (CD38-PE; Imgenex, San Diego, CA) by standard procedures (37, 38). Human BM stromal cells (hBMSCs) were obtained by long-term culture of BM mononuclear cells (39). For co-culture, 1×10^5 ppMM cells were seeded on 2×10^4 hBMSCs for 24 to 48 hours in 96-well plates. RNA samples of normal healthy bone marrow-derived plasma cells (nPCs) were purchased (AllCells, CA, US).

Overexpression and inhibition of miR-21 in MM cells

Pre-miR-21 miRNA precursor molecules and miR-21 inhibitors were purchased from Ambion (Applied Biosystems, CA, US) and were used to enforce or to antagonize mir-21 expression, respectively, at a final concentration of 100nM. Pre-miR precursor negative control and anti-miR miRNA inhibitor negative control were obtained from Ambion (Applied Biosystem, CA, US). $1x10^6$ cells were transfected using Neon® Transfection System (Invitrogen, CA, US), (1 pulse at 1050 V, 30 ms), and the transfection efficiency evaluated by flow cytometric analysis relative to a FAM dye labeled anti-miR negative control reached 85–90%. The same conditions were applied for transfection of MM cells with 10 micrograms of the p3x FLAG-PTEN (kindly provided by Prof. Giuseppe Viglietto, *Magna Graecia* University, Catanzaro, Italy) or with the same amount of the empty p3x

FLAG-CMV-7.1 vector. When co-transfected, we used 100nM of synthetic miR-21 or miR-NC together with 10 micrograms of p3x FLAG--PTEN or the same amount of empty p3x FLAG-CMV-7.1 vector.

Cell proliferation assays

Cell growth was evaluated by Trypan blue exclusion cell count and BrdU proliferation assay. Electroporated cells were incubated for 4 hours in 6 well plates; after harvesting, they were plated in 24 well plates for Trypan blue exclusion cell count and in 96 well plates for BrdU proliferation assay. Cells were counted at 24 hours intervals. BrdU uptake was measured every 24 hours by the DELFIA cell proliferation assay, and luminescence was detected using a Victor 4 plate reader (Perkin Elmer. Waltham, Massachusetts). Each sample was run at least in triplicate.

Survival assay

Cell survival was evaluated by MTT assay in 96-well plates. In brief, transfected cells were seeded at a density of 1×10^4 cells per well in 100 ul of culture medium. Every 24 hours, 10 ul of 5 mg/ml MTT (Dimethyl thiazolyl diphenyl tetrazolium, Sigma) reagent were added to each well, and cells were further incubated for 4 h at 37°C. Then medium was removed, and 100 ul of DMSO (dimethyl sulfoxide) were added to each well to dissolve the formazan. The optical density (OD) was evaluated at wave length of 560 nm. Wells without cells (DMSO alone) were used as blank, and experiments were repeated at least three times. Data represent the mean ± SD of 3 independent experiments.

Colony formation assay

Clonogenity was evaluated by a colony formation assay in methylcellulose-based medium (Methocult H4100, StemCell Technologies), following manufacturer's instructions. 2×10^2 cells/mL electroporated cells were seeded in 24 well plates and incubated for three weeks; colony formation was then evaluated by counting colonies of >100 cells. The experiments were repeated at least three times. Data represent the mean ± SD of 3 independent experiments.

Quantification of IL-6 production

The concentration of IL-6 in culture medium was measured by enzyme-linked immunosorbent assay (ELISA, Quantikine Rat IL-6; R&D Systems). hBMSCs were electroporated; following incubation periods of 24 – 48 hours, aliquots of culture medium were collected and subjected to a specific ELISA for IL-6.

Reverse transcription and quantitative real-time PCR

Total RNA containing miRNAs and mRNAs was extracted from cells with Trizol Reagent (Invitrogen, CA, US), according to the manufacturer's instructions. All RNA extractions were carried out in a sterile laminar flow hood using RNase/DNase-free laboratory ware. The integrity of total RNA was verified by nanodrop (Celbio Nanodrop Spectrophotometer nd-1000). The single-tube TaqMan miRNA assay (Applied Biosystems, CA, US) was used to detect and quantify mature miR-21, using ViiA7 RT reader (Applied Biosystems, CA,

US); the protocol was performed for 40 cycles at 95°C for 3 min, 95°C for 15 s, and 60°C for 30 s. miR-21 expression was normalized on RNU44, and then expressed as fold change ($2^{\Delta\Delta Ct}$). For mRNA dosage studies, oligo-dT-primed cDNA was obtained through the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and then used as a template to quantify PTEN (Hs01026371_m1), Rho-B (Hs1085292_m1), and BTG2 (Hs1105077_m1) levels by TaqMan assay (Applied Biosystems, CA, US); normalization was performed with GAPDH (Hs03929097_g1). Comparative real-time polymerase chain-reaction (RT-PCR) was performed in triplicate, including no-template controls. Relative expression was calculated using the comparative cross threshold (Ct) method.

Protein extraction and western blotting analysis

Total proteins were extracted with a lysis buffer (Tris-HCl 15 mM pH7.5, NaCl 120 mM, KCl 25 mM, Tryton x-100 0.5 %) and addition of Halt Protease Inhibitor Single-Use Cocktail 1X (Thermo SCIENTIFIC). After lysis in ice for 30 min, the solution was disrupted by gentle pipetting followed by 3x 10 sonication cycles; the lysate was centrifuged at 12,000 rpm for 20 min, and the supernatant was collected. For western blotting analysis, 60 μg of lysate were separated by electrophoresis on Mini Protean TGX precast gels (4–20%) and electro-transferred onto a nitrocellulose membrane (Invitrogen, Carlsbad, CA, USA). The membranes were blocked for 1 hour in 5% milk at room temperature, and then incubated overnight at 4°C in milk 5% with the following antibodies: PTEN (A2B1) (Santa Cruz); Phospho-Akt (Ser473) Rabbit mAb (Cell Signaling); AKT (pan) (11E7) Rabbit mAb (Cell Signaling); Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Rabbit mAb (Cell Signaling); p44/42 MAPK (Erk1/2) Rabbit mAb (Cell Signaling); γ Tubulin Antibody (C-20) goat polyclonal; GAPDH-HRP rabbit polyclonal IgG (Santa Cruz). Membranes were washed 3 times in PBST, and then incubated with a secondary antibody conjugated with horseradish peroxidase in 0.5% milk for 2 hours at room temperature. After 3 washes with PBSTween, chemiluminescence was detected using Pierce ECL Western Blotting Substrate (cat. 32109, Pierce, USA). Intensity of the bands was analyzed using Quantity One analyzing system (Bio-Rad, USA).

Virus Generation and Infection of MM cells

MM cells stably expressing miR-21 inhibitor were transduced by the lentiviral vector miRZip-21 anti-miR-21 construct (System Biosciences, CA, US). The supernatant was collected 48h post-transfection and was centrifuged (3,000xg for 15 min at 4°C) to remove cell debris; it was then passed through a 0.45-μm filter and used for two rounds of transduction of U-266 and MM.1S cells ($1x10^6$) in the presence of 8 μg/ml polybrene (Sigma-Aldrich, US). MM cells underwent three rounds of infection (8 hours each round), and transduced cells were selected in medium containing 1 μg/ml puromycin.

Animals and *in vivo* model of human MM

Male CB-17 severe combined immunodeficient (SCID) mice (6– to 8-weeks old; Harlan Laboratories, Inc., Indianapolis) were housed and monitored in our Animal Research Facility. All experimental procedures and protocols had been approved by the Institutional Ethical Committee (Magna Graecia University) and conducted according to protocols approved by the National Directorate of Veterinary Services (Italy). In accordance with

institutional guidelines, mice were sacrificed when tumors reached 2 cm in diameter or in the event of paralysis or major compromise, to prevent unnecessary suffering. Animal experimental procedures have been performed as in previous reports (40–42). Briefly, mice were sc inoculated with $1x10^6$ OPM-2 cells, and treatment started when palpable tumors became detectable, approximately 3 weeks following injection of MM cells. Each dose contained 20 μg of NLE-formulated (MaxSuppressor In Vivo RNA-LANCEr II) synthetic oligo which equals 1 mg/kg per mouse, as previously described (21). Treatments were performed intratumorally (i.t.) every two days for a total of 8 injections.

Statistical Analysis

Each experiment was performed at least 3 times and all values are reported as means ±SD. Comparisons between groups were made with student's t-test, while statistical significance of differences among multiple groups was determined by GraphPad software (www.graphpad.com). Graphs were obtained using SigmaPlot version 12.0. p-value of less than 0.05 was accepted as statistically significant.

Results

1. miR-21 expression in MM cell lines and primary patient MM cells

By real-time PCR, we evaluated the miR-21 expression in INA-6, MM.1S, NCI-H929, RPMI-8226, OPM-2, U-266 and KMS-26 MM cell lines as compared to nPCs. Among these cell lines, we found variable miR-21 expression: KMS-26, U-266 and OPM-2 showed the highest, whereas other cells expressed very low levels of miR-21 (Fig. 1A). Notably, we found > 3 fold increase in miR-21 expression when INA-6 cells, which are IL-6-dependent, were cultured adherent to hBMSCs (Fig. 1B). Consistent with data achieved in cell lines, miR-21 levels were upregulated in ppMM cells as compared to nPCs; moreover, ppMM cells further increased miR-21 expression (> 4.18 fold) when cells were cultured adherent to hBMSCs (Fig. 1C), demonstrating that miR-21 in MM is indeed upregulated by the BM *milieu*.

2. Transfected miR-21 inhibitors exert anti-MM effects *in vitro*

To study the anti-MM activity of miR-21 inhibition, we transfected tumor cells with miR-21 inhibitors. By trypan blue exclusion cell count and BrdU proliferation assay, we found significantly decreased cell growth of MM cell lines highly expressing miR-21 levels (KMS-26, U-266 and OPM-2) (Fig. 2A-B). In these cells, we also observed reduction of cell survival by MTT assay (Fig. 2C). Conversely, miR-21 inhibitors did not affect cell proliferation or survival of MM cell lines with low miR-21 expression (NCI-H929, MM.1S, RPMI-8226) (Fig. S2).

3. Lentiviral transduced miR-21 inhibitors affect MM cell proliferation and clonogenicity

We next evaluated the effects of constitutive inhibition of miR-21. U-266 cells (expressing high miR-21) or MM.1S cells (expressing low miR-21) were transduced with a lentiviral vector carrying a miR-21 inhibitory sequence or with a lentiviral empty vector. By BrdU proliferation assay, we found significantly decreased cell growth of U-266 cells transduced with miR-21 inhibitors as compared to controls (Fig. 3B). In contrast, no significant effects

on cell proliferation were observed in MM.1S cells transduced with miR-21 inhibitors (Fig. 3A). Constitutive expression of miR-21 inhibitors in U-266 cells also significantly inhibited colony formation in methylcellulose cultures (75% reduction in colonies, Fig. 3B), whereas clonogenicity of MM.1S cells was not affected (Fig. 3A). Taken together, these results show that miR-21 inhibition obtained either by transfection or transduction of miR-21 inhibitors exerts anti-proliferative activity in MM cells. The growth-inhibitory activity strongly relies on high basal miR-21 expression, since MM cells with low miR-21 were not affected.

4. miR-21 inhibition modulates the expression and activity of several signaling molecules

miR-21 is known as a growth promoting and anti-apoptotic factor in several human cancers through the targeting of multiple tumor suppressor genes (26). Among validated targets of miR-21, PTEN, Rho-B and BTG2 are the most studied and involved in cell cycle progression and/or apoptosis regulation (43–45). We therefore evaluated the effects of miR-21 inhibition on the expression of these targets and found that these genes were upregulated at mRNA level after transfection of miR-21 inhibitors, as compared to control cells transfected with scrambled sequences (miR-NC inhibitors) (Fig. 3C). Moreover, by western blotting analysis, we found a marked upregulation of PTEN protein expression 24 – 48 hours after cell transfection with miR-21 inhibitors (Fig. 3D). Notably, we found that both p-AKT and p-ERK were reduced by miR-21 inhibitors, while total AKT and ERK were unaffected (Fig. 3E).

5. Enforced expression of miR-21 mimics enhances proliferation of MM cells

To establish the oncogenic role of miR-21 in our model, we evaluated the effects of transiently enforced expression of synthetic miR-21 mimics in tumor cells. Specifically, we examined whether miR-21 mimics exerted a growth promoting activity in MM.1S (low miR-21) or in U-266 (high miR-21) cells. We found that miR-21 mimics enhanced growth of MM.1S (Fig. 4B) cells, whereas no effects were observed in U-266 cells (Fig. 4A). We then evaluated whether miR-21 overexpression downregulated canonical targets. As shown in Figure 4C, qRT-PCR analyses demonstrated a decrease in PTEN, BTG2 and Rho-B mRNA expression (78%, 62%, and 42%, respectively). Moreover, western blotting analysis showed that levels of PTEN protein were reduced in MM.1S cells overexpressing miR-21 compared to controls (Fig. 4D). To investigate if PTEN downregulation mediates the growth promoting activity of miR-21, we transfected MM.1S cells with an expression vector encoding PTEN gene lacking regulatory 3’ UTR sequence. We demonstrated that transfected cells indeed overexpressed PTEN gene which was not downregulated by miR-21 (Fig. 4E). Importantly, the PTEN rescue completely abrogated the miR-21 growth promoting activity (Fig. 4B). We conclude that the proliferative effect of miR-21 is dependent on PTEN suppression in MM cells.

6. miR-21 inhibitors antagonize the hBMSCs protective role on MM cells

It is well known that the human BM *milieu* strongly support survival and proliferation of MM cells. Since miR-21 expression in MM cells was significantly enhanced by adherence of cells to hBMSCs (Fig. 1B-C), we next evaluated whether miR-21 inhibition could overcome the supportive effects of the human BM *milieu*. To this aim, we evaluated the

anti-tumor activity of miR-21 inhibition in a context closely resembling the intramedullary stage of MM. Specifically, we cultured IL-6-dependent MM cell line INA-6 adherent to hBMSCs and enforced the expression of miR-21 inhibitors in in one or both cell types. As shown in Figure 6A, miR-21 inhibition affected viability of MM cells to a similar extent as does hBMSCs deprivation. miR-21 inhibition was also observed in INA-6 cells cultured in an IL-6-enriched culture medium (Fig. 5A). We wondered whether this effect was due to miR-21 inhibition directly in INA-6 cells, in hBMSCs, or in both. Therefore, we investigated the effects of miR-21 inhibition in INA-6 cells co-cultured with non transfected hBMSCs and found that the anti-tumor effect was similar to that obtained when miR-21 inhibition was induced in both cell types (Fig. 5B). In contrast, no effects were observed when miR-21 was inhibited only in hBMSCs adherent to INA-6 cells (Fig. 5B). Furthermore, viability and IL-6 secretion in culture medium were not affected by miR-21 inhibition in hBMSCs (data not shown). Finally, ppMM cells were cultured adherent to hBMSCs and then exposed to miR-21 inhibitors, which were able to overcome the supportive effect of human BM *milieu* (Fig. 5C). On these findings, we conclude that miR-21 inhibitors abrogates the supporting activity of hBMSC on MM cell lines and ppMM cells.

7. *In vivo* delivery of miR-21 inhibitors exert anti-MM activity against human MM xenografts

Finally, we studied the *in vivo* anti-tumor potential of miR-21 inhibitor oligonucleotides in NOD/SCID mice bearing human MM xenografts. Based on *in vitro* findings, we induced OPM-2 xenografts into a cohort of 12 mice. When tumors became palpable, mice were randomized into 2 groups and treated intratumorally with miR-21 inhibitors or scrambled inhibitors (miR-NC inhibitors). As shown in Figure 6A, repeated injection of miR-21 inhibitors (1mg/Kg; 8 injections, 2 days apart), significantly reduced growth of MM xenografts. Importantly, we next evaluated if the anti-proliferative activity of miR-21 inhibitors was related to modulation of miR-21 canonical targets. Consistent with our *in vitro* experiments, we found upregulation of PTEN both at mRNA and protein levels in retrieved tumors from animals treated with miR-21 inhibitors (Fig. 6B-C). Moreover, inhibition of miR-21 significantly reduced the phosphorylation of AKT, a down-stream target of PTEN and key mediator of tumor cell survival (Fig. 6C), suggesting that the *in vivo* anti-MM activity of miR-21 inhibitors is related to PTEN upregulation and AKT activation impairment within tumors.

Discussion

In this report, we demonstrate that antagonism of miR-21 by oligonucleotide inhibitors exerts anti-tumor activity *in vitro* and *in vivo* against human MM xenografts in SCID/NOD mice. To our knowledge, this is the first evidence of a successful *in vivo* treatment with miR-21 inhibitors in a murine xenograft model of human MM, which has important potential clinical applications. We show that efficacy of strategies based on miR-21 inhibition is dependent upon miR-21 expression levels in MM cells. Indeed, in MM cells expressing high miR-21 levels (KMS-26, U-226 and OPM-2), miR-21 inhibitors reduce cell proliferation, survival and clonogenicity. In contrast, no anti-MM effects were observed in cells with low endogenous miR-21 expression. These data suggest that miR-21 expression is

a potential biomarker predictive of therapeutic response to miR-21 inhibitors to be validated in future clinical trials.

The oncogenic role exerted by miR-21 in MM pathogenesis is predicted upon its upregulated levels even at early stages of disease. This notion is further supported by the role of miR-21 in IL-6/Stat3 signaling, a central pathway for MM cell growth and drug resistance (33, 46, 47). In this report, we demonstrate increased proliferation in MM cells transfected with miR-21 mimics, further supporting this view. Consistent with prior reports (35), we found that miR-21 expression in MM cells is upregulated by adherence to hBMSCs. Importantly, we demonstrate that the anti-tumor properties of miR-21 inhibitors were not attenuated either by exogenous IL-6 or by adherence of ppMM cells or MM cell lines to hBMSCs. These findings strongly suggest the clinical potential of targeting miR-21. Specifically, the human BM *milieu* induces resistance to conventional therapies including melphalan, doxorubicin and dexamethasone (7); in contrast, upregulation of miR-21 promoted by the BM suggests that targeting this miRNA may be even more active against MM cells in this *milieu*.

We investigated the molecular basis of miR-21 inhibitor anti-MM activity. It is well known that tumor suppressor genes, including PTEN, BTG2 and Rho-B, are validated targets of miR-21 (26). Here we demonstrated that miR-21 inhibition triggered upregulation of these genes in MM cell lines, whereas miR-21 mimics had the opposite effect. Modulation of PTEN expression and activity may be of particular clinical relevance in MM, since PTEN mutations are rare events in this disease (43) and PTEN downregulates AKT and ERK activity (48), thereby decreasing MM cell proliferation, survival, and drug resistance (49). Importantly, upregulation of miR-21, p-AKT and p-ERK in MM cells mediates hBMM-induced tumor cell growth and survival (7), and a p-AKT/miR-21 positive feedback loop has been already suggested (50). It is therefore of note that miR-21 inhibitors upregulate PTEN and decrease AKT and ERK phosphorylation in MM cells in our study. By transfection of a PTEN expression vector not containing 3’ miRNA target sequence which could be not downregulated by miR-21, we demonstrated that PTEN suppression was indeed essential for miR-21-induced proliferative activity. Moreover, while miR-21 inhibitors produced a prolonged growth inhibitory activity with pERK impairment at 48h, pAKT was strongly suppressed only at 24 h and almost recovered at 48h. We interpretate these findings as a rebound activation of pAKT which does not translate in growth rescue.

In conclusion, our results both further highlight the oncogenic role of miR-21 in MM and provide the framework for clinical development of miR-21 inhibitors to improve patient outcome in MM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Statement of translational relevance

Oncogenic miRNAs are an emerging target for the treatment of human cancer. We investigated the therapeutic potential of miR-21 inhibitors in human multiple myeloma (MM). The translational relevance of our study relies in the strong anti-MM activity of miR-21 inhibitors, which is not abrogated by exogenous IL-6 or cell adherence to human bone marrow stroma, taking into account the dependency of MM from the bone marrow *milieu*. Moreover, we provide evidence of a successful *in vivo* treatment with miR-21 inhibitors in a murine xenograft model of human MM, offering a framework for clinical development of miR-21 inhibitors in MM. Notably, this therapeutic activity is dependent upon miR-21 expression, which may represent a predictive biomarker to miR-21 inhibitors in this still incurable disease.

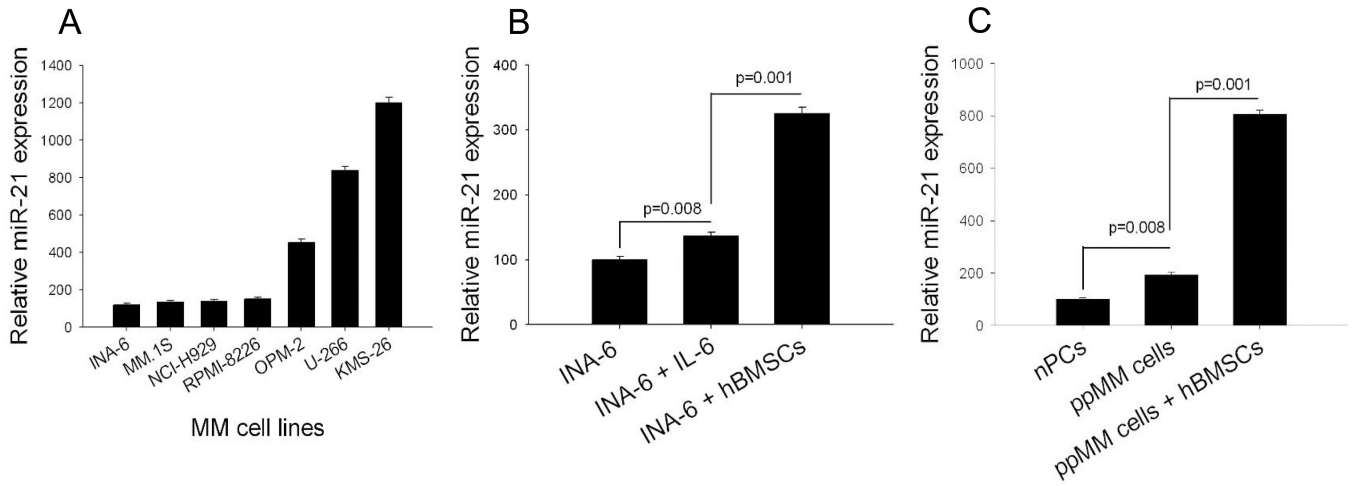

Figure 1. MiR-21 expression in MM patient cells and cell lines

(**A**) Quantitative RT-PCR analysis of miR-21 expression using total RNA from 7 MM cell lines and bone marrow-derived plasma cells (nPCs) from healthy donors. Raw Ct values were normalized to RNU44 housekeeping snoRNA and expressed as ΔΔCt values relative to miR-21 levels in nPCs. Values represent mean ±SE of three different experiments. (**B**) Quantitative RT-PCR of miR-21 expression in INA-6 cells cultured in the presence or absence of IL-6 (2.5 ng/mL) or co-cultured with hBMSCs, and then immunopurified by immunomagnetic sorting with anti-CD138 beads. Raw Ct values were normalized to RNU44 housekeeping snoRNA and expressed as ΔΔCt values calculated using the comparative cross threshold method. miR-21 expression levels in INA-6 cells cultured in the absence of IL-6 were set as an internal reference. Values represent mean ±SE of three different experiments. (**C**) Quantitative RT-PCR of miR-21 expression in nPCs (n=3) and ppMM (n=3) cells before and 6 hours after seeding on hBMSCs, and then immunopurified by immunomagnetic sorting with anti-CD138 beads. Raw Ct values were normalized to RNU44 housekeeping snoRNA and expressed as ΔΔCt values calculated using the comparative cross threshold method. MiR-21 expression levels in nPCs were used as an internal reference. Data are the average of three independent experiments performed in triplicate. P values were obtained using two-tailed *t* test.

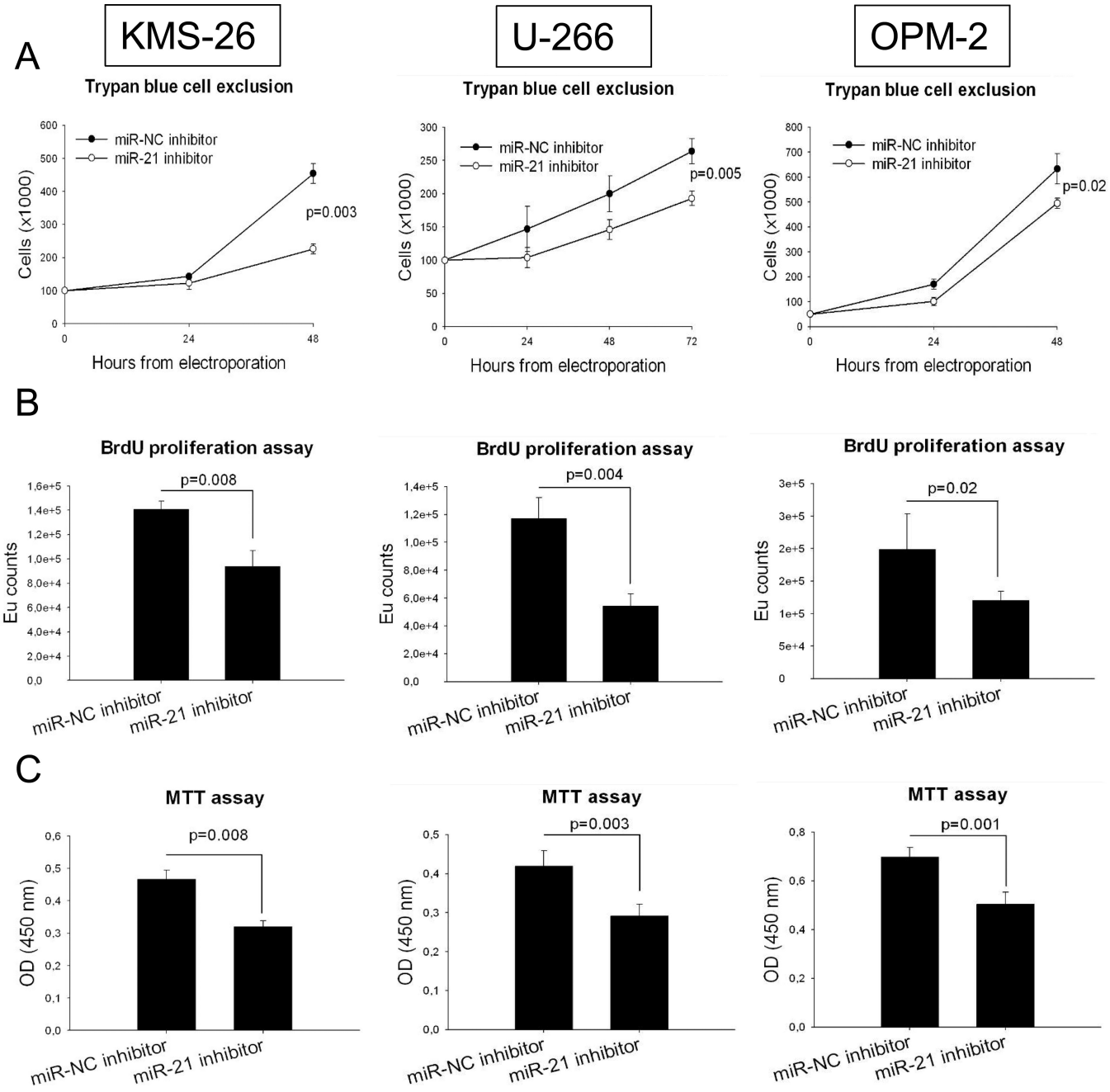

Figure 2. Effects of ectopic expression of miR-21 inhibitors in MM cell lines

(**A**) Trypan blue exclusion growth curves were generated from KMS-26, U-266 and OPM-2 cells transfected with miR-21 inhibitors or scrambled controls (miR-NC inhibitors). (**B**) BrdU proliferation assay was performed in KMS-26, U-266 and OPM-2 cells 48h after transfection with miR-21 inhibitors or scrambled controls. (**C**) MTT survival assay was performed in KMS-26, U-266 and OPM-2 cells 48h after transfection with miR-21 inhibitors or scrambled controls. Averaged values ±SD of three independent experiments are plotted. P values were obtained using two-tailed *t* test.

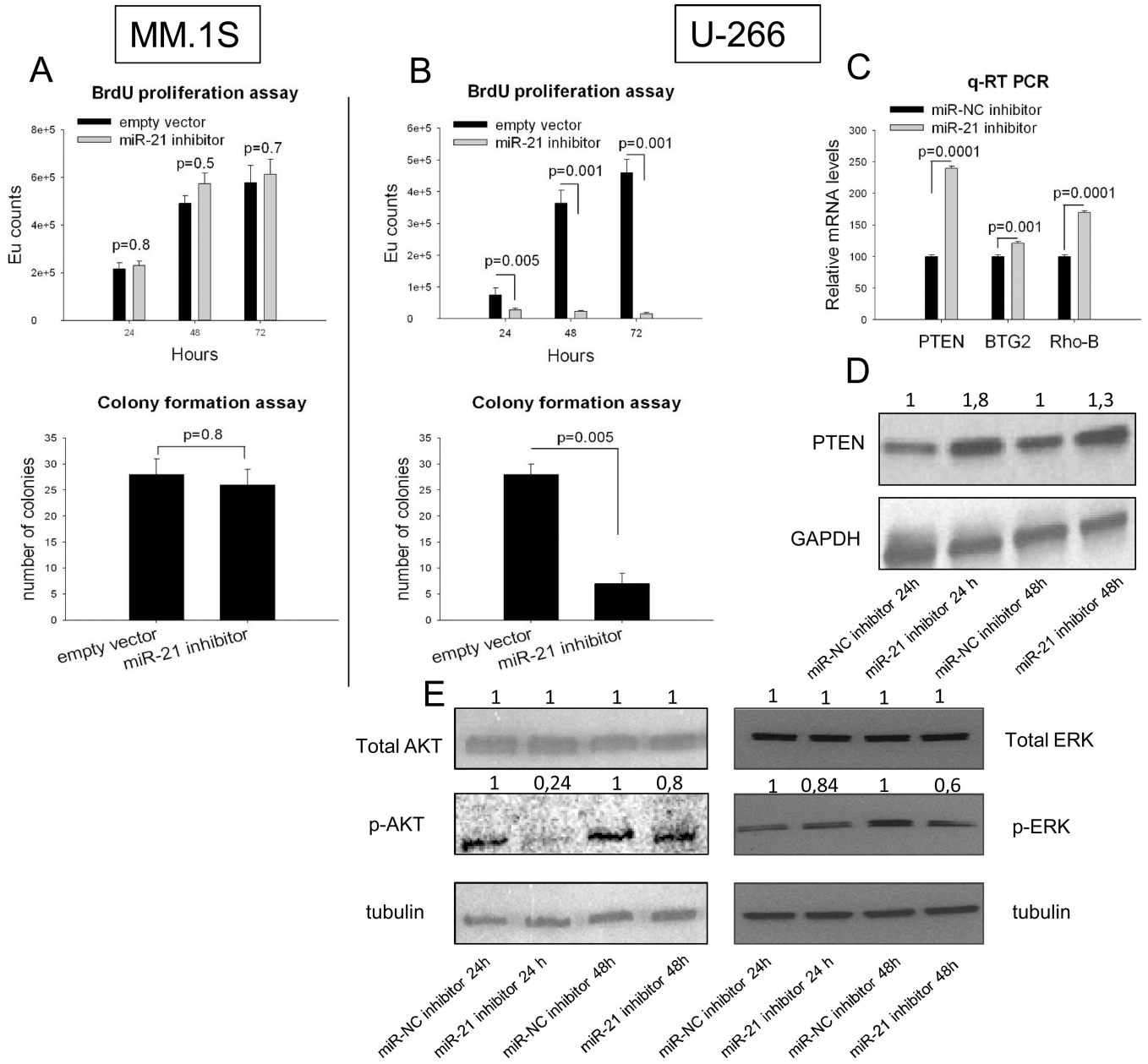

Figure 3. Activity of miR-21 inhibitors in MM cell lines

BrdU proliferation assay and colony formation assay of (**A**) MM.1S and (**B**) U-266 cells transduced with a lentivirus carrying either the empty vector or miR-21 inhibitory sequences. Averaged values ±SD of three independent experiments are plotted. P values were obtained using two-tailed *t* test. (**C**) qRT-PCR of PTEN, BTG2 and Rho-B expression was performed in U-266 cells 48 hours after transfection with either miR-21 inhibitors or scrambled controls (miR-NC inhibitors). The results are shown as average mRNA expression after normalization with GAPDH and ΔΔCt calculations. Data represent the average ±SD of 3 independent experiments. (**D**) Western Blot of PTEN 24 – 48 hours after transfection of U-266 cells with miR-21 inhibitors or scrambled controls. (**E**) Levels of total AKT, total ERK, p-AKT and p-ERK 24 – 48 hours after transfection of U-266 cells with

either miR-21 inhibitors or scrambled controls. Relative protein level values are derived from densitometric scan.

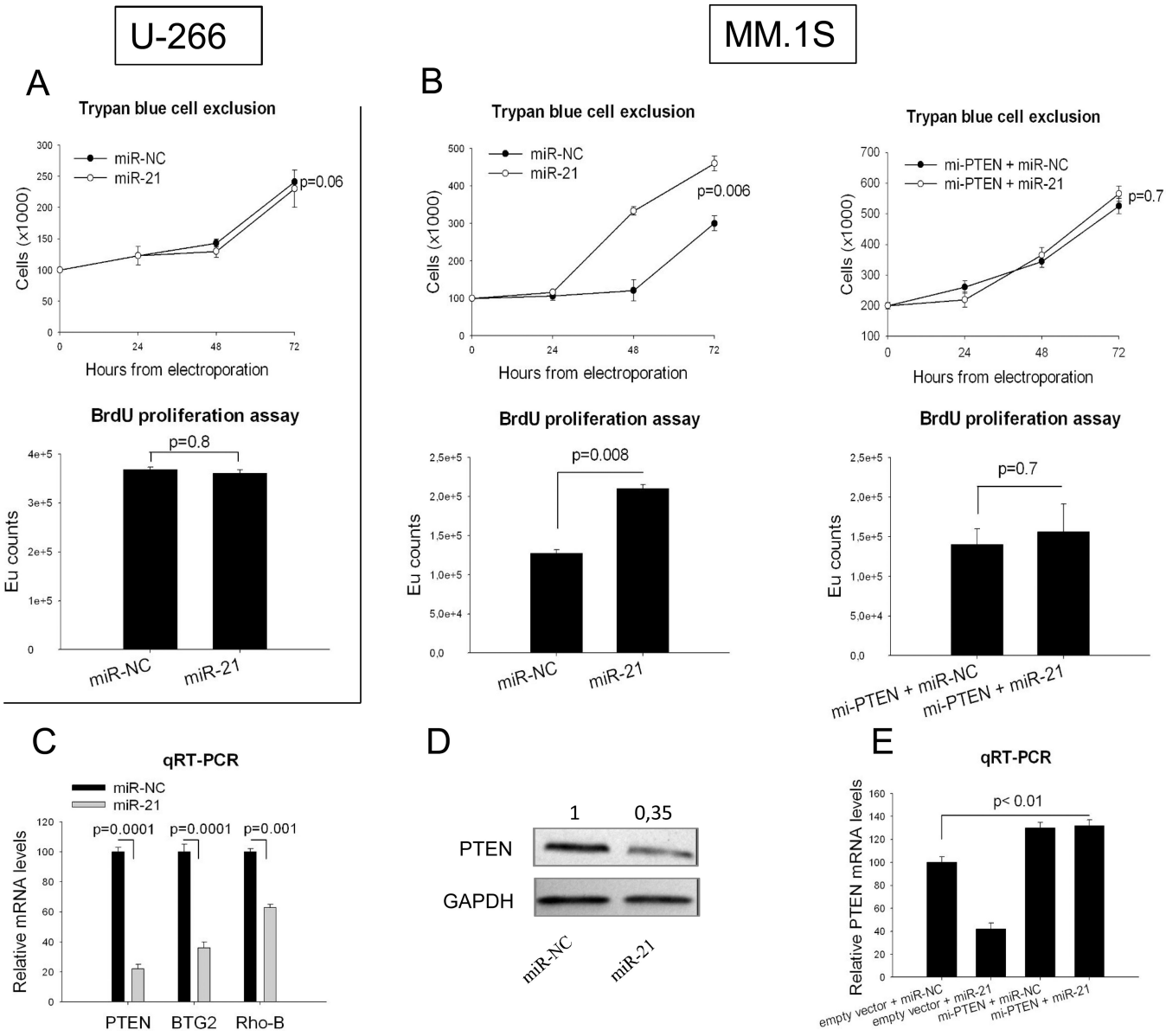

Figure 4. Effects of transient enforced expression of miR-21 mimics in MM cell lines

Growth curves and Brdu uptake (48 h time point) of (**A**) U-266 and (**B**) MM.1S cells transfected with either miR-21 mimics or scrambled controls (miR-NC). Enforced expression of miR-21 mimics was also triggered in MM.1S cells overexpressing a miRNA insensitive PTEN construct (mi-PTEN). (**C**) qRT-PCR of PTEN, BTG2 and Rho-B expression levels was done 48 hours after transfection of MM.1S cells with either miR-21 mimics or scrambled controls. The results shown are average mRNA expression after normalization with GAPDH and ΔΔCt calculations. (**D**) Western Blot of PTEN 48 hours after transfection of MM.1S cells with either miR-21 mimics or scrambled controls. Relative protein level values are derived from densitometric scan. (**E**) qRT-PCR of PTEN expression levels in MM.1S cells co-transfected with either mi-PTEN or an empty vector and miR-21 mimics or scrambled controls (48h time point). The results shown are average mRNA

expression after normalization with GAPDH and ΔΔCt calculations. Data represent the average ±SD of 3 independent experiments.

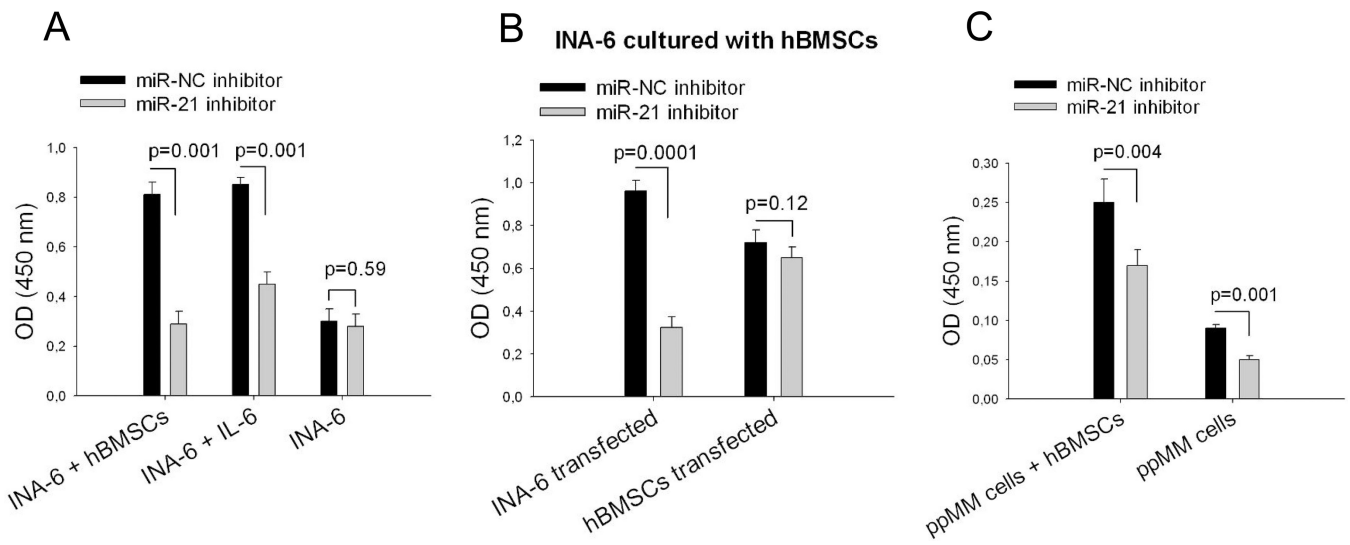

Figure 5. miR-21 inhibition antagonizes pro-survival effect of BM *milieu*

(**A**) MTT assay of INA-6 cells cultured adherent to hBMSCs, in IL-6-enriched culture medium, or in IL-6-free culture medium. The assay was performed 48 hours after transfection of co-cultured cells with miR-21 inhibitors or scrambled controls (miR-NC inhibitors). (**B**) MTT assay of INA-6 cells cultured with hBMSCs was performed 48 hours after either INA-6 cells or hBMSCs were independently transfected with miR-21 inhibitors or scrambled controls. (**C**) MTT assay performed in ppMM cells cultured in the presence or absence of hBMSCs. The assay was performed 48 hours after transfection with miR-21 inhibitors or scrambled controls. Averaged values ±SD of three independent experiments are plotted including. P values were obtained using two-tailed *t* test.

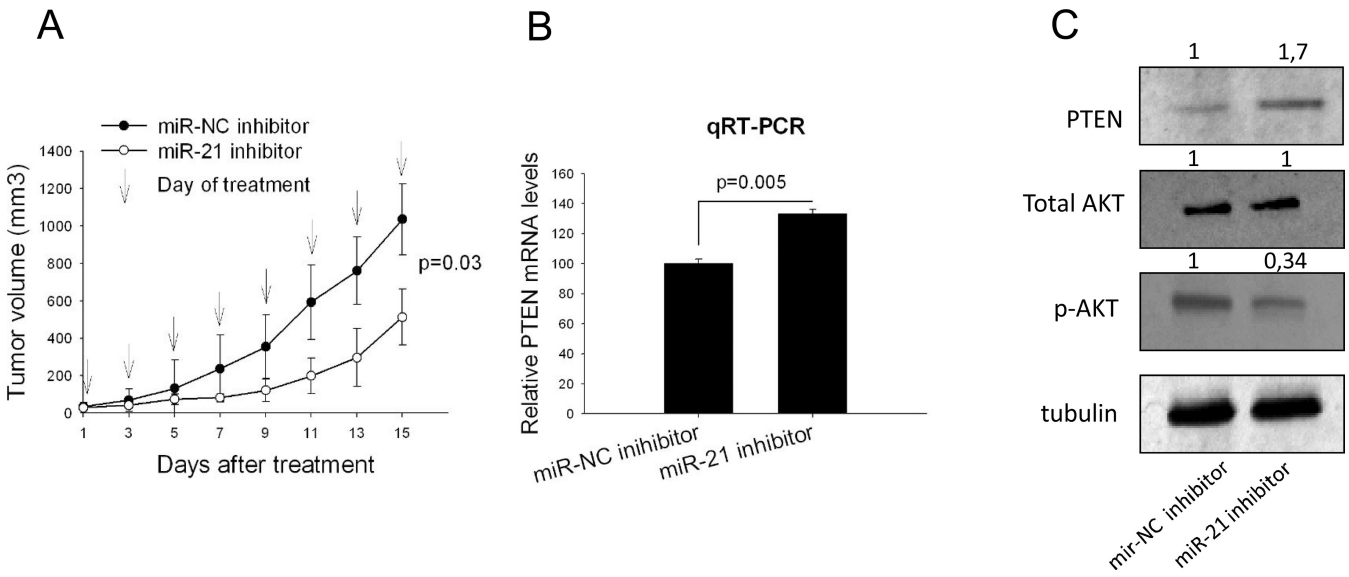

Figure 6. MiR-21 inhibition antagonizes MM tumor growth in vivo

(**A**) *In vivo* tumor growth of OPM-2 xenografts intratumorally-treated with miR-21 inhibitors or scrambled controls (miR-NC inhibitors). Palpable subcutaneous tumor xenografts were treated every 2 days with 20 μg of miR-21 inhibitors for a total of 8 injections (indicated by arrows). A separate control group of tumor-bearing animals was injected with scrambled controls. Tumors were measured with an electronic caliper every 2 days, and averaged tumor volume of each group ±SD are shown. P values were obtained using two-tailed *t* test. (**B**) Quantitative RT-PCR of PTEN expression in lysates from retrieved OPM2 xenografts. The results shown are average mRNA expression levels after normalization with GAPDH and ΔΔCt calculations. Data represent the average ±SD of 3 independent experiments. (**C**) Western Blot of PTEN, total AKT and p-AKT levels in lysates from a representative retrieved OPM-2 xenograft. Relative protein level values are derived from densitometric scan.