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Untersuchungen zum Einfluss intronischer miRNAs auf Expression und Funktion ihrer Host-Gene an den Beispielen miR-641/AKT2 und miR-744/MAP2K4 in malignen Gliomen

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Meinen Eltern und Katrin

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Eidesstattliche Versicherung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Thema “Untersuchungen zum Einfluss intronischer miRNAs auf Expression und Funktion ihrer Host-Gene an den Beispielen miR-641/AKT2 und miR-744/MAP2K4 in malignen Gliomen” selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe. Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

München, d. 05.02.2019

Max Hübner

Inhaltsverzeichnis

I. Abkürzungsverzeichnis

miRNA	Mikro-RNA
mRNA	messenger RNA
AGO	Argonaute
DICER	Dicer1, ribonuclease III
TRBP	RISC loading complex RNA binding subunit
RISC	RNA-induced Silencing Complex
AKT2	AKT serine/threonine kinase 2
MAP2K4	Mitogen-Activated Protein Kinase Kinase 4
EGF	Epidermal Growth Factor
TGFB1	Transforming Grwoth Factor, Beta 1
MAPK	Mitogen-Activated Protein Kinase
PIK3R3	phosphoinositide-3-kinase regulatory subunit 3
MAPKAP1	mitogen-activated protein kinase associated protein 1
NFAT5	Nuclear Factor of Activated T cells
SMAD	Small Mothers Against Decapentaplegic
DVL2	Disheveled2

II. Publikationsliste

1. **Hübner M**, Hinske CL, Effinger D, Wu T, Thon N, Kreth F-W, et al. Intronic miR-744 Inhibits Glioblastoma Migration by Functionally Antagonizing Its Host Gene MAP2K4. Cancers . 2018;10. doi:10.3390/cancers10110400

2. Hinske LC, Heyn J, **Hübner M**, Rink J, Hirschberger S, Kreth S. Intronic miRNA-641 controls its host Gene's pathway PI3K/AKT and this relationship is dysfunctional in glioblastoma multiforme. Biochem Biophys Res Commun. 2017;489: 477–483.

1. Einleitung:

Das humane Genom besteht zu ca. 98% aus Sequenzen, die nicht für Proteine kodieren [1–3]. Man nahm zunächst an, dass diese Sequenzen durch Mutationen und virale Infektionen in die DNA integriert wurden und evolutionär bedingte Abfallprodukte ohne relevante biologische Funktion darstellen. Wenige Jahre später wurden nicht-kodierende DNA-Abschnitte identifiziert, die die Genexpression induzieren [4–6] oder inhibieren [7–9] können. Obwohl diese Abschnitte für kein Protein kodieren, besitzen sie für Gene charakteristische regulative Elemente, wie Promotoren, Enhancer oder Silencer und können daher auch transkribiert werden. Mit der Entdeckung des für die larvale Entwicklung essentiellen *lin-4*-Gens in *C. Elegans* konnten Lee et al. erstmals ein nicht proteinkodierendes Gen identifizieren, dessen Transkriptionsprodukt eine Gesamtlänge von ca. 20 Nukleotiden besitzt und die Expression des Gens *lin-14* direkt inhibieren kann. Dies geschieht über komplementäre Basenpaarung des *lin-4*-Transkripts mit dem 3`-Ende des *lin-14*-Transkripts [10]. Diese Erkenntnis war bahnbrechend, und 2001 zeigten die Arbeiten von Lagos-Quintana et al., dass viele dieser nichtkodierenden RNA-Sequenzen -fortan als “mikro-RNAs” (miRNAs) bezeichnet- hochkonserviert vorliegen und in einer Vielzahl von Organismen in verschiedenen Geweben differenziell exprimiert werden [11]. In den folgenden Jahren erschienen zunehmend mehr Arbeiten, die den Stellenwert von miRNAs in unterschiedlichen Krankheitsbildern, wie Autoimmunerkrankungen oder Tumoren beschrieben: von ca. 400 PubMed-Einträgen zum Thema “miRNA” in 2005 stieg die Anzahl auf über 11,000 Einträge im Jahr 2017. Nach heutigem Stand sind mehr als 2000 mit rechnerbasierten Algorithmen identifizierte, teilweise auch experimentell

validierte miRNA-Sequenzen des Menschen bekannt [12,13]. Das Konzept von miRNAs als essentiellen Regulatoren der Genexpression ist mittlerweile fest etabliert: man geht davon aus, dass die Expression von ca. 60% aller Gene durch miRNAs beeinflusst wird [14].

MiRNAs inhibieren die Genexpression ihrer Zielgene durch direkte Interaktion mit der 3`untranslatierten Region (3`UTR). Zunächst wird ein RNA-Proteinkomplex aus miRNA und den Proteinen AGO, Dicer und TRBP gebildet. Dieser RNA-Induced Silencing Complex (RISC) interagiert mit dem 3`UTR der Ziel-mRNA, wobei die miRNA die exakte Lokalisation des RISC bestimmt. Durch Basenpaarung entsteht ein doppelsträngiges miRNA-RNA Hybridmolekül, das durch die intrinsische Endonukleaseaktivität des RISC degradiert wird. Es steht nun weniger mRNA für die Proteintranslation zur Verfügung [15,16] (Abb. 1).

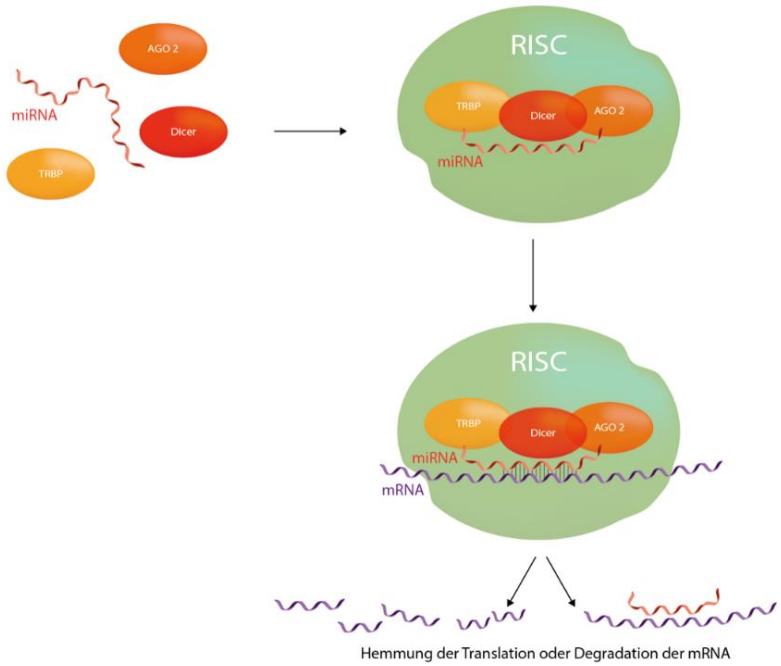

Abb. 1: Vereinigung der Proteine AGO2, DICER, TRBP sowie einer miRNA zum RNA-Induced Silencing Complex (RISC). Die miRNA fungiert hierbei als Leitstruktur für die korrekte Platzierung des RISC am 3`UTR der Ziel-mRNA. Diese wird nach Anlagerung des RISC durch die Ribonukleaseaktivität des Proteins AGO2 degradiert.

Alternativ wird die Genexpression durch translationale Repression beeinflusst. Hierbei lagert sich der RISC ebenfalls an die mRNA an und interferiert mit der Proteintranslation, ohne jedoch die mRNA zu degradieren [17,18].

Die von einer miRNA regulierten Gene stehen oftmals in funktionellem Bezug. Dies generiert ein komplexes regulatorisches Netzwerk, in dem die miRNA die Signaltransduktion durch paralleles Targeting mehrerer funktionell verwandter Gene entscheidend beeinflussen kann [19–21]. Schon für eine physiologische Embryonalentwicklung sind miRNAs unverzichtbar: mehrere Studien zeigen, dass bestimmte miRNAs, abhängig vom Stand der Embryonalentwicklung, differenziell exprimiert werden und wichtige Prozesse, wie die Entwicklung von Gehirn [22] oder Herz [23,24] maßgeblich mitbestimmen. Eine komplette Inhibition der miRNA-Synthese führt in embryonalen Stammzellen zu verstärkter Apoptose und frühzeitiger Seneszenz [25,26]. In adulten Zellen sind miRNAs ebenfalls an der Regulation nahezu sämtlicher Signalkaskaden beteiligt [27,28]. Es liegt daher nahe, dass die Dysregulation von einer oder mehreren miRNAs an der Pathogenese bestimmter Erkrankungen maßgeblich sein kann.

MiRNAs sind im humanen Genom auf ganz unterschiedliche Arten codiert: ähnlich proteinkodierenden Genen können miRNAs als eigenständiges Gen mit eigenem Promotor vorliegen und autonom transkribiert werden. In ca. 50% der Fälle sind miRNAs innerhalb eines anderen Gens, dem sogenannten Host-Gen, lokalisiert [29]. In den meisten Fällen besitzen diese intragenischen miRNAs keine eigenen regulatorischen Elemente und werden zusammen mit ihren Host-Genen zunächst als pre-mRNA transkribiert [29]. Während des anschließenden Spleißvorgangs wird die

meist intronisch liegende miRNA-Sequenz durch Spleißen aus der mRNA entfernt und

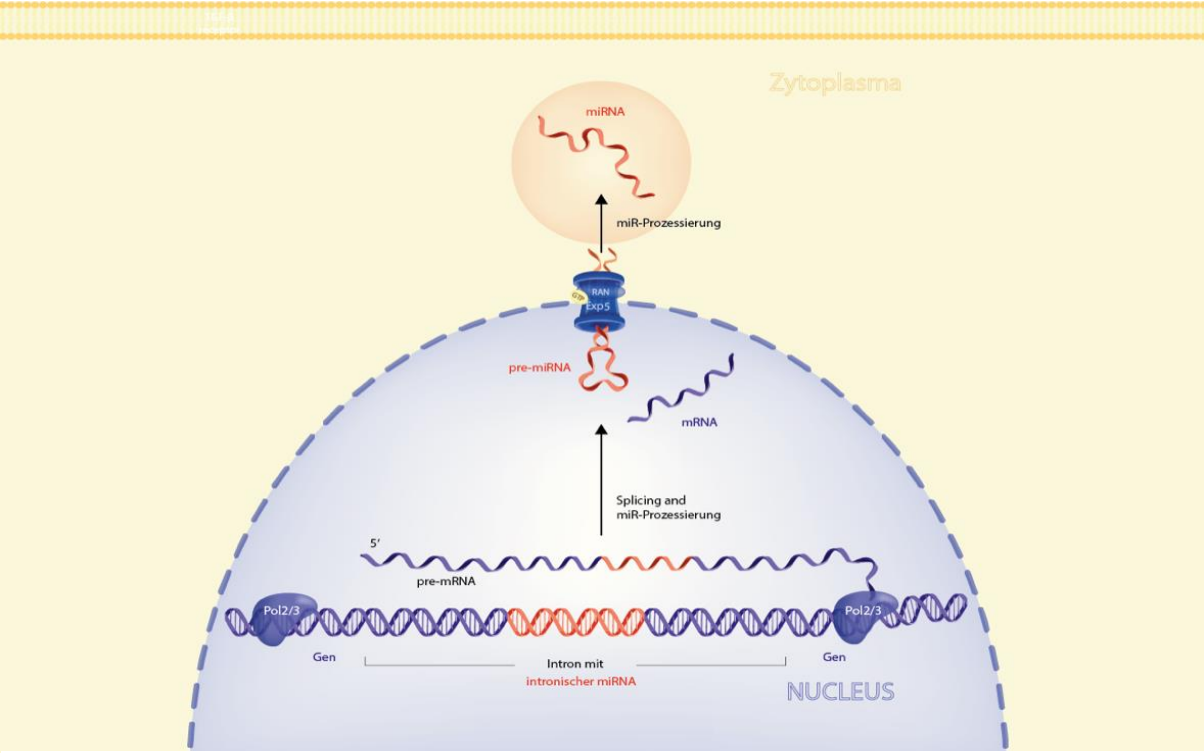

Abb. 2: Prozessierung einer intronischen pre-miRNA zu einer reifen miRNA: Nach Transkription der pre-mRNA wird die pre-miRNA durch Spleißen von der mRNA getrennt und Exportin5-abhängig aus dem Nukleus in das Zytoplasma transportiert. Hier wird die pre-miRNA enzymabhängig zu einer reifen miRNA prozessiert.

zu einer reifen miR prozessiert (Abb.2). Bioinformatische Analysen legen nahe, dass diese transkriptionellen Kopplungen im Genom eine funktionelle Relevanz besitzen und dass das jeweilige Host-Gen durch die intragenisch kodierte miRNA über unterschiedliche Rückkopplungs- (Feedback-) mechanismen beeinflusst wird [20]. Diese Feedback-Mechanismen könnten entweder durch direkte Interaktion mit dem Host-Gen (direktes Feedback bzw. Feedback erster Ordnung, Abb. 3), oder über Inhibition von Genen, die Genexpression bzw. Aktivierungsstatus des Host-Gens regulieren (indirektes Feedback bzw. Feedback zweiter Ordnung, Abb. 4) vermittelt werden.

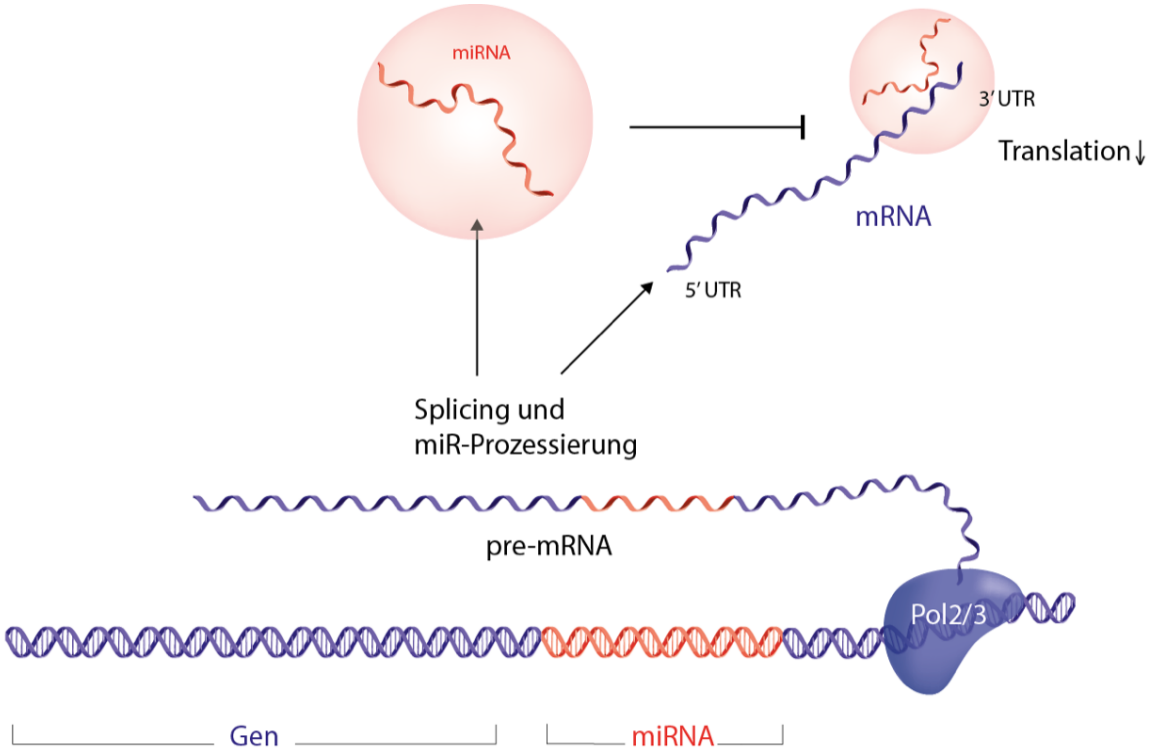

Abb. 3: Direktes Feedback einer intronischen miR (rot): Nach der Transkription entsteht aus der pre-mRNA eine reife mRNA (blau) sowie eine pre-miR. Diese wird weiter zu einer reifen miRNA prozessiert und inhibiert durch direkte Interaktion mit dem 3`UTR ihres Host-Gens dessen Expression.

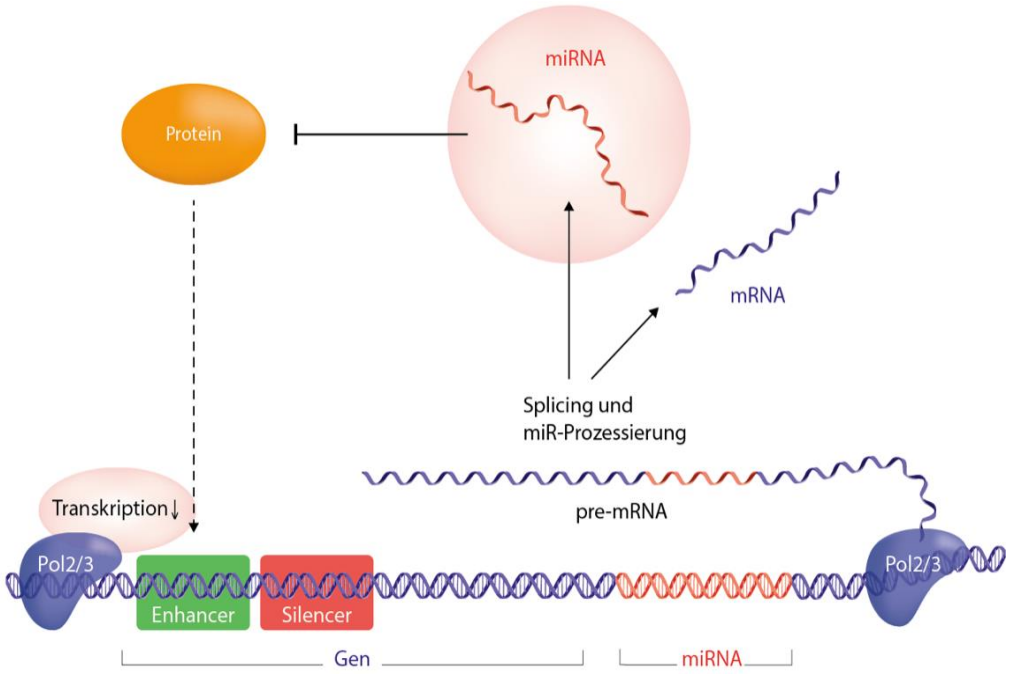

Abb 4:

Indirektes Feedback einer intronischen miR (rot): Nach Transkription und Spleißen inhibiert die reife miRNA die Expression von Genen, die Transkription oder Aktivität des Host-Gens beeinflussen.

Diese Rückkopplungsmechanismen scheinen nicht nur über ein einziges Target vermittelt, sondern durch Interaktion der miRNA mit funktionell ähnlichen Targets potenziert zu werden. Dies generiert ein komplexes funktionelles Netzwerk mit miRNA und Host-Gen als zentralen Bestandteilen.

Aktuell existieren nur wenige Arbeiten, die diese auf *in-silico*-Analysen gegründeten Vermutungen für einzelne ausgewählte miRNAs experimentell validieren und Host-Gen-augmentierende (positives Feedback) [30] bzw. -antagonisierende Effekte (negatives Feedback) [31–33] der miRNA nachweisen konnten.

Insbesondere die Expression von Genen, die Zellproliferation, -differenzierung oder -migration steuern und daher potentielle Onkogene (sogenannte Protoonkogene) darstellen, muss jedoch eng reguliert sein. Intragenisch kodierte miRNAs könnten hierbei einen zusätzlichen physiologischen Kontrollmechanismus zur Feinsteuerung der Gentranskription darstellen und einer inadäquaten Gentranskription oder -aktivität von Proto-Onkogenen entgegenwirken. Alterationen der miRNA Expressionsprofile können bei einer Vielzahl von Tumorerkrankungen nachgewiesen werden [34–36]. Hiervon sind ebenfalls intronische bzw. intragenische miRNAs betroffen [37]. Bislang existieren jedoch nur wenige Studien, die einen funktionellen Zusammenhang zwischen intragenischen miRNAs und protoonkogenen Host-Genen während der Tumorentstehung herstellen und charakterisieren konnten.

2. Fragestellung

In der vorliegenden Arbeit sollte anhand von zwei bekannten Proto-Onkogenen, AKT2 (AKT Serine/Threonine Kinase 2) [38,39] und MAP2K4 (Mitogen-Activated Protein

Kinase Kinase 4) [40–44], sowie ihren intronischen miRNAs untersucht werden, ob eine funktionelle Beziehung im Sinne eines Feedback-Loops zwischen Host-Gen und intronischer miRNA besteht. Dies ist von besonderem Interesse, da AKT2 die Zellproliferation induziert und durch Wachstumsfaktoren wie EGF (Epidermal Growth Factor) oder TGFB1 (Transforming Growth Factor, Beta1) über Rezeptortyrosinkinasen aktiviert werden kann [45]. Eine verstärkte Expression bzw. Überaktivierung von AKT2 ist in den unterschiedlichsten Tumorarten beschrieben [46] und führt nicht nur zu verstärkter Proliferation und Migration [47], sondern auch zur Inhibition der Apoptose [48,49].

Ähnlich wie AKT2 reguliert MAP2K4 durch die Beteiligung im MAPK-Signalweg ebenfalls tumorigene Prozesse wie Proliferation und Metastasierung, aber auch zelluläre Anpassungsvorgänge, die in Tumorzellen beispielsweise als Reaktion auf Hypoxie [50,51] oder Chemotherapie stattfinden [52,53].

Insbesondere sollen die folgenden Fragen adressiert werden:

- Besteht ein funktioneller Zusammenhang zwischen intronischen miRNAs und Host-Gen und liegt dieser in malignen Zellen eventuell entkoppelt vor?
- Können durch Deregulation intragenischer miRNAs physiologisch vorhandene Kontrollmechanismen zur Genexpression und -aktivität des Host-Gens deaktiviert bzw. inhibiert werden?
- Wird durch Modulation der miRNA-Expression Phänotyp und Malignität der Zellen beeinflusst?

3. Zusammenfassung

Gene, die essenzielle zelluläre Funktionen, wie Apoptose oder Proliferation regulieren, müssen einer engen transkriptionellen Kontrolle unterliegen. MiRNAs, die in Introns dieser Gene gelegen sind, könnten hier wichtige regulative Funktionen erfüllen, indem sie –direkt oder indirekt- die Expression ihrer sogenannten „Host-Gene“ kontrollieren. Die vorliegende Arbeit befasste sich mit der Frage, ob intronisch lokalisierte miRNAs einen regulativen Einfluss auf ihre Host-Gene ausüben können und ob diese Kontrollmechanismen in Tumoren verändert oder sogar inaktiviert sind. Dies wurde an zwei bekannten tumorigenen Kinasen, AKT2 und MAP2K4, und den jeweils intronisch gelegenen miRNA-641 und miRNA-744 mittels real-time-PCR, RNA-Interferenz-Experimenten, Proteinanalysen, Immunhistochemie, Reportergenassays, etablierten Langzeit-Zellkulturen, primären Zelllinien aus Glioblastomgewebe und Glioblastom-Biopsaten untersucht. Es konnte gezeigt werden, dass beide Kinasen durch ihre intronischen miRNAs im Sinne einer indirekten negativen Rückkopplung inhibiert werden: miR-641 hemmt über die Targets PIK3R3, MAPKAP1 und NFAT5 die Phosphorylierung und damit die Aktivität ihres tumorigenen Host-Gens AKT2; miRNA-744 inhibiert durch Targeting von TGFB1 die Aktivität des SMAD- und MAPK-Signalwegs. Weiterhin konnte gezeigt werden, dass beide miRNAs in Glioblastomen - im Vergleich zu normalem Hirngewebe- stark reprimiert sind. Für die zugehörigen miRNA-Zielgene PIK3R3, NFAT5 und TGFB1 wurde im Tumorgewebe eine verstärkte Expression gefunden.

Die vorliegende Promotionsarbeit konnte damit an zwei Beispielen ein wichtiges Prinzip expressioneller Kontrolle nachweisen: intronische miRNAs können über

komplexe funktionelle Netzwerke ihre Host-Gene regulieren. Die Störung dieser negativen Feedback-mechanismen kann die intrazelluläre Signalgebung maßgeblich beeinflussen und die Tumorprogression begünstigen. Diese Ergebnisse können zur Entwicklung miRNA-basierter zukünftiger Therapiestrategien beitragen.

4. Summary

Genes that regulate essential cellular functions, such as apoptosis or proliferation, must be subject to tight transcriptional control. MiRNAs located in introns of these genes could fulfill important regulatory functions by directly or indirectly controlling the expression of their host genes.

The present work dealt with the question of whether intronically localized miRNAs can exert a regulatory influence on their host genes and whether these control mechanisms are impaired or even inactivated in tumors. This was investigated in two known tumorigenic kinases, AKT2 and MAP2K4, and the intronically located miRNA-641 and miRNA-744 by real-time PCR, RNA interference experiments, protein analysis, immunohistochemistry, reporter gene assays, established long-term cell cultures, primary cell lines from glioblastoma tissue and glioblastoma biopsies. It could be shown that both kinases -indeed- are inhibited by their intronic miRNAs via indirect negative feedback: miR-641 inhibits phosphorylation via the targets PIK3R3, MAPKAP1 and NFAT5 and thus the activity of its tumor host gene AKT2; miRNA-744 inhibits the activity of the SMAD and MAPK signaling pathway by targeting TGFB1. We further found that both miRNAs are strongly repressed in glioblastomas as compared to normal brain tissue. In contrast, the miRNA target genes PIK3R3, NFAT5 and TGFB1 were induced in tumor tissue.

The present doctoral thesis was thus able to demonstrate an important principle of expressional control: intronic miRNAs can regulate their host genes via complex functional networks. The disruption of these negative feedback mechanisms can significantly influence intracellular signalling promote tumour progression. These results may contribute to the development of miRNA-based future therapeutic strategies.

5. Veröffentlichung I:

cancers

MDPI

Article

Intronic miR-744 Inhibits Glioblastoma Migration by Functionally Antagonizing Its Host Gene MAP2K4

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Abstract: Background: The second intron of Mitogen-Activated Protein Kinase Kinase 4 (MAP2K4), an important hub in the pro-invasive MAPK pathway, harbors miR-744. There is accumulating evidence that intronic micro-RNAs (miRNAs) are capable of either supporting or restraining functional pathways of their host genes, thereby creating intricate regulative networks. We thus hypothesized that miR-744 regulates glioma migration by interacting with its host's pathways. Methods: Patients' tumor specimens were obtained stereotactically. MiR-744 was overexpressed in U87, T98G, and primary glioblastoma (GBM) cell lines. Cell mobility was studied using migration and Boyden chamber assays. Protein and mRNA expression was quantified by SDS-PAGE and qRT-PCR. Interactions of miR-744 and 3'UTRs were analyzed by luciferase reporter assays, and SMAD2/3, p38, and beta-Catenin activities by TOP/FOPflash reporter gene assays. Results: As compared to a normal brain, miR-744 levels were dramatically decreased in GBM samples and in primary GBM cell lines. Astrocytoma WHO grade II/III exhibited intermediate expression levels. Re-expression of miR-744 in U87, T98G, and primary GBM cell lines induced focal growth and impaired cell mobility. Luciferase activity of 3'UTR reporter constructs revealed the pro-invasive factors TGFB1 and DVL2 as direct targets of miR-744. Re-expression of miR-744 reduced levels of TGFB1, DVL2, and the host MAP2K4, and mitigated activity of TGFB1 and DVL2 downstream targets SMAD2/3 and beta-Catenin. TGFB1 knock-down repressed MAP2K4 expression. Conclusion: MiR-744 acts as an intrinsic brake on its host. It impedes MAP2K4 functional pathways through simultaneously targeting SMAD-, beta-Catenin, and MAPK signaling networks, thereby strongly mitigating pro-migratory effects of MAP2K4. MiR-744 is strongly repressed in glioma, and its re-expression might attenuate tumor invasiveness.

Keywords: glioblastoma; migration; microRNAs; MAP2K4

1. Introduction

Micro-RNAs (miRNAs) are short RNA molecules with an established role as important epigenetic regulators of the transcriptome via recognition of base-complementary signals in the 3' untranslated regions of target mRNAs [1,2]. Interestingly, the majority of human miRNA genes are located within non-coding regions of protein-coding genes [3,4]. It also appears that this colocalization leads to

coregulation in several instances, either through cotranscription and therefore coexpression, or via shared cis-regulatory elements [5]. Increasing evidence has begun to unravel the shades of a mechanism bearing an important role in the regulation of central cellular pathways, rather than just being a biological pendent of an information compression algorithm. However, we are only on the verge of understanding its impact on health and disease [6,7]. The Mitogen-Activated Protein Kinase (MAPK) pathway is one of such central signal transduction pathways, regulating a plethora of essential cellular functions, including proliferation, differentiation, and modulation of gene expression. One of its members, Mitogen-Activated Protein Kinase Kinase 4 (MAP2K4), is a well-known tumorigenic kinase with an established role in metastasis, invasion, and cancer progression [8–10]. *MAP2K4* in turn hosts the intronic microRNA *hsa-miR-744*, located in the second intron of its host. Even though MAP2K4 has long been recognized as a potent proto-oncogene, little is known about its intronic miRNA. Most importantly, the relationship between MAP2K4 and miR-744 is as yet completely uncharacterized. Due to the central and established role of MAP2K4 in tumor biology [11,12], we hypothesized miR-744 to be guilty by association, possibly augmenting or antagonizing MAP2K4s tumor-promoting effects.

In the following manuscript, we provide new and unprecedented evidence of a significant role of miR-744 in the regulation of its host gene MAP2K4 in human glioma. This is accomplished via controlling both expression and functional aspects of its host gene. We show how miR-744 inhibits cell migration and invasion, a key characteristic of glioblastoma (GBM), via targeting three essential cellular pathways. We finally validate our hypothesis in cell line experiments as well as patients´ tissue samples. We believe that our findings shed some more light on the as yet blurry contours of the mechanisms underlying tumor development through dysregulation of cellular signaling, and portray miR-744 as a central molecule in the formation of GBM.

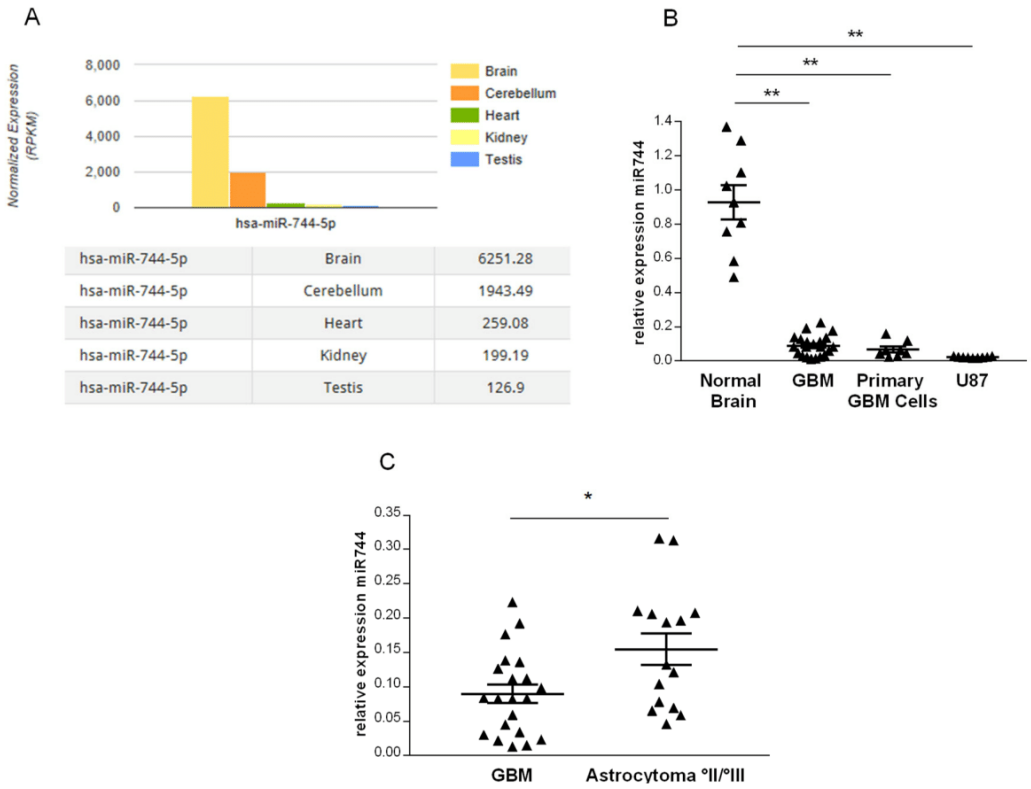
2. Results

2.1. miR-744 Is Strongly Repressed in Human Glioma

To identify human tissues in which miR-744 may fulfill important regulatory functions, we used the intragenic microRNA database miRIAD (http://bmi.ana.med.uni-muenchen.de/miriad/) [4], and screened for tissues with high expression levels of this miRNA. As depicted in Figure 1A, among five different tissues deposited in miRIAD (heart, testis, kidney, cerebellum, and brain), human brain exhibited the highest expression levels of miR-744, which suggests its implication in the maintenance of homeostatic conditions in the central nervous system. To investigate our initial hypothesis, we next quantified miR-744 in stereotactically obtained GBM specimens and primary cell lines obtained from open GBM resections by qRT-PCR, and found a dramatic reduction of miR-744 as compared to normal brain tissue (Figure 1B; GBM samples: reduction by 90.3% $\pm$ 14.7%, primary GBM cell lines: reduction by 92.7% $\pm$ 7.3%; n = 9 for normal brain tissue, n = 21 for GBM samples, n = 8 for primary GBM cell lines; $p < 0.01$). Also, we could detect reduced expression of miR-744 in U87 cells, a human GBM cell line (Figure 1B; reduction by 97.7% $\pm$ 6%, n = 9, $p < 0.001$).

Collectively, this data shows that miR-744 is highly expressed in human brain tissue, whereas it is almost entirely repressed in GBM.

To assess the expression of miR-744 in human glioma of different grades, we quantified miR-744 in 15 stereotactically obtained WHO II/III tumors by qRT-PCR. As depicted in Figure 1C, we found miR-744 also to be repressed; however, expression levels were significantly higher as compared to GBM (48% $\pm$ 20%; WHO II/III: n = 15, GBM: n = 21, $p = 0.034$). It thus appears that miR-744 expression is inversely correlated with tumor grade and may contribute to increased tumor aggressiveness.



hsa-miR-744-5p	Brain	6251.28
hsa-miR-744-5p	Cerebellum	1943.49
hsa-miR-744-5p	Heart	259.08
hsa-miR-744-5p	Kidney	199.19
hsa-miR-744-5p	Testis	126.9

Figure 1. miR-744 is strongly repressed in glioma. MiR-744 expression was quantified by qRT-PCR. U47 served as the endogenous reference. (**A**) Expression levels of miR-744 in five different tissues. (**B**) Expression of miR-744 in normal brain tissue (NB) (n = 9), glioblastoma (GBM) (n = 21), primary GBM cell lines (n = 9), and U87 cells (n = 9), $p < 0.001$. (**C**) Expression of miR-744 in WHO grade °II/°III glioma (n = 15) compared to GBM (n = 21), $p = 0.034$. ** $p < 0.001$; * $p < 0.05$.

2.2. Overexpression of miR-744 Reduces Migration of GBM Cells

It is a frequently occurring phenomenon that tumors down-regulate, or even hamper, the expression of genes that are not useful for malignant transformation. Our next aim was to understand the reasons for miR-744 downregulation in human GBM, and thus we investigated the biological functions of miR-744 in glioma cells. To this end, we transiently re-expressed miR-744 in U87, T98G, and primary GBM cell lines by transfection of the respective premiR and assessed the resulting phenotype. Surprisingly, we could not detect any alterations of apoptosis or proliferation after transfection of miR-744 (data not shown). 2D wound closure assays however, revealed a strong impact of miR-744 on cellular migration, which was markedly attenuated in miR-744 transfected cells (Figure 2A). To study the long-term effects of miR-744 on cellular migration, we constructed a miR-744 delivering expression vector, and stably transfected U87 GBM cells (Figure 2D, left panel). As shown in Figure 2B, overexpression of miR-744 leads to a decrease in cellular spreading and induces focal growth, pointing towards an alteration of cellular mobility. Importantly, 2D migration and Boyden Chamber assays revealed a less invasive phenotype (Figure 2C,D, right panel; reduction of 46% ± 5.8%, n = 4, $p = 0.029$). Taken together, this data shows that miR-744 inhibits migration of GBM cells.

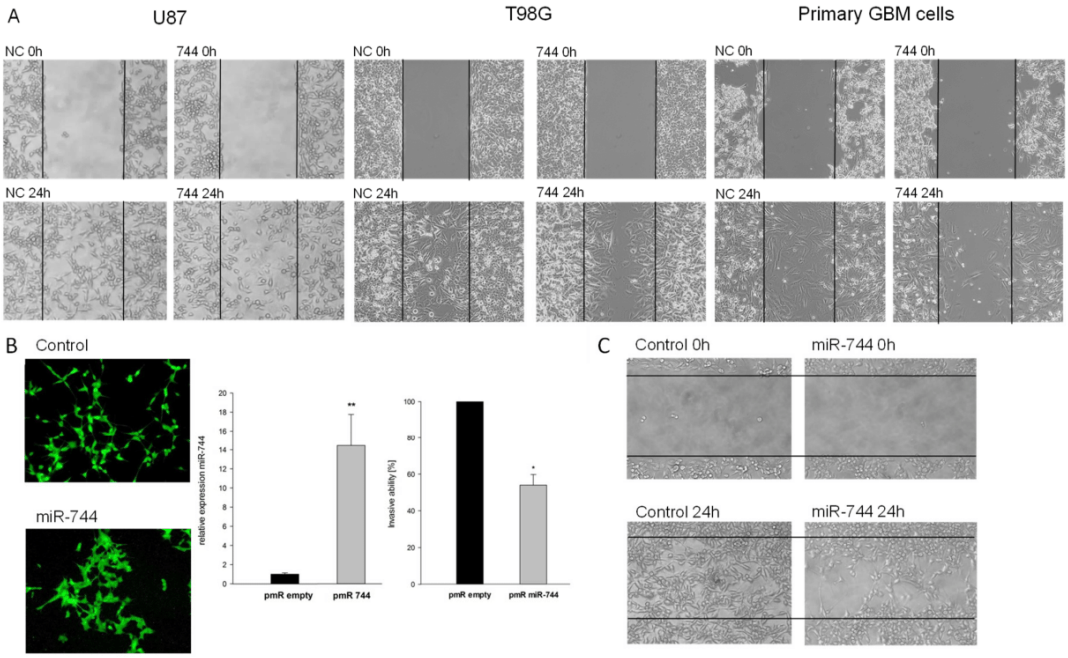


Figure 2. Overexpression of miR-744 induces focal cell growth and inhibits invasion and migration. (**A**) 2D migration assays of transiently miR-744 transfected cells (left panel: U87; middle panel: T98G; right panel: primary GBM cell lines) at start and after 24 h. Lines mark the initially cell-free area. A typical example of 3 experiments is shown. (**B**) Fluorescence microscopy of control and stably miR-744 overexpressing cells. (**C**) 2D migration assays of stably transfected cells; depicted are start state and after 24 h of incubation. Lines mark the cell-free area. A typical example of 3 similar experiments is shown. (**D**) Left panel: MiR-744 levels after stable transfection (induction 14.4-fold ± 6.0, n = 5, $p < 0.001$). Right panel: Invasion after stable transfection of U87 cells, measured with Collagen-coated Boyden Chamber invasion assays (n = 4, $p = 0.029$). ** $p < 0.001$; * $p < 0.05$.

2.3. TGFB1 and DVL2 are Direct Targets of miR-744, Regulate Migration in GBM Cells, and are Induced in Tissue of Human Malignant Glioma

To identify direct targets of miR-744 possibly accounting for the detected phenotypic alterations, we next combined in silico target prediction and pathway analysis to extract mRNAs (a) containing miR-744 binding sites in their 3′ untranslated region (3′UTR), and (b) being involved in the regulation of cellular mobility. This prompted us to investigate Transforming Growth Factor Beta 1 (TGFB1) and Dishevelled2 (DVL2) in detail, as these were the most promising predicted targets of miR-744 with respect to a supposed role in GBM migration [13,14]. To test this assumption, we quantified mRNA and protein expression levels of TGFB1 and DVL2 in U87 cells stably overexpressing miR-744, and indeed detected a marked decrease of TGFB1 and DVL2 mRNA (reduction of 38% ± 5.6% and 33.6% ± 4.9%, respectively, n = 5, $p < 0.05$), and protein expression (reduction of 35.6% ± 8.3% and 36.8% ± 5.3%; Figure 3A, left and middle panels). To provide experimental proof that both genes are bona fide targets of miR-744 in GBM, we performed luciferase reporter gene assays on vector constructs containing the full-length 3′-UTR of either TGFB1 or DVL2. As shown in Figure 3A (right panel), co-transfection of miR-744 mimic and reporter constructs diminished luciferase activity by 37% and 47%, respectively, compared to miR scrambled control (n = 5, $p < 0.05$), thereby proving that both genes are direct targets of miR-744. Transient knock-down of both TGFB1 and DVL2 by specific siRNAs (knock-down efficiency: Supplementary Figure S1) reduced the migratory capabilities of GBM cells in 2D migration assays (Figure 3B), thereby closing the anticipated functional loop; miR-744 impairs migration of human glioma cells by direct targeting of TGFB1 and DVL2.

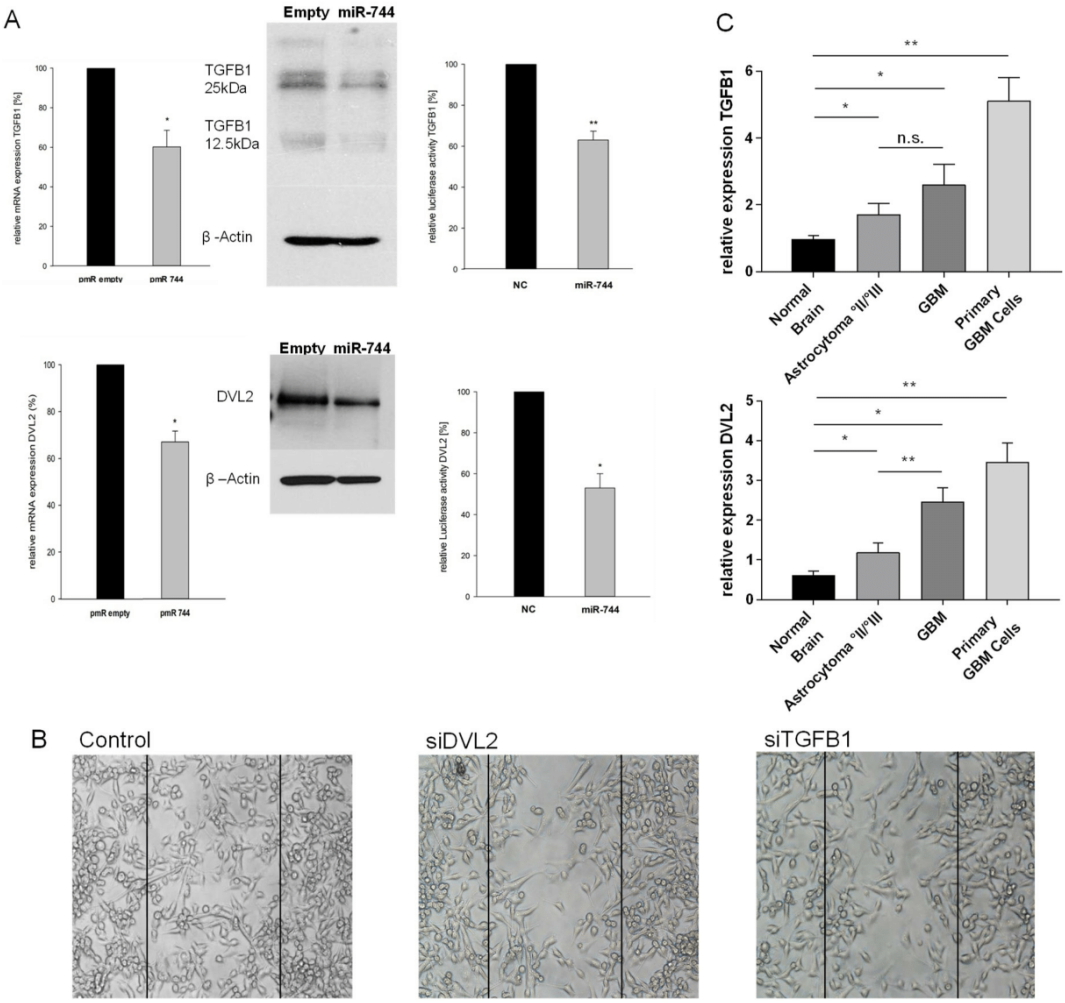


Figure 3. Transforming Growth Factor Beta 1 (TGFB1) and Dishevelled2 (DVL2) are direct targets of miR-744, regulate migration, and are induced in GBM and in Astrocytoma °II/°III. (**A**) Left and middle panels: mRNA and protein expression of TGFB1 and DVL2 in U87 cells stably transfected with miR-744 or empty vector control, respectively (n = 5, $p < 0.05$). Right panels: TGFB1 and DVL2 3′UTR Luciferase Reporter Gene activity after co-transfection of the respective reporter vectors with miR-744 or with scrambled control, respectively (DVL2: −47% ± 3,2%, TGFB1: −37% ± 2%, n = 5, $p < 0.05$). All Luciferase and mRNA experiments were performed in triplicates. (**B**) 2D migration assay after transient knock-down of DVL2 and TGFB1 in U87 cells. One representative example of 3 experiments is shown. (**C**) Expression of DVL2 and TGFB1 mRNA in Astrocytoma °II/°III biopsies (n = 10, $p < 0.05$), GBM biopsies (DVL: n = 17, $p = 0.001$; TGFB1: n = 39, $p = 0.015$), and primary GBM cell lines (n = 8, $p < 0.001$), as compared to normal brain tissue (n = 9). ** $p < 0.001$; * $p < 0.05$. n.s. = not significant.

In GBM and Astrocytoma °II/°III specimens, as well as in primary GBM cells, we found significantly increased expression levels of DVL2 and TGFB1. These results are in line with the detected down-regulation of miR-744 (Figure 3C; DVL2: GBM, induction 3.35-fold ± 0.14, $p = 0.002$; primary GBM cells, induction 5.4-fold ± 0.46, $p < 0.001$; normal brain tissue (NB), n = 9; GBM, n = 17; primary GBM cells, n = 8. TGFB1: GBM, induction 2.19-fold ± 0.09, $p = 0.015$; primary GBM cells, induction 4.33-fold ± 0.69, $p < 0.001$; NB, n = 9; GBM, n = 39; primary GBM cells, n = 8). Notably, GBM exhibited higher levels of TGFB1 and DVL2 as compared to Astrocytoma °II/°III (Figure 3; TGFB1:

induction 1.5-fold ± 0.36, p = n.s.; DVL2: induction 2.1-fold ± 0.3, $p < 0.001$; n = 10 for Astrocytoma °II/°III).

Hence, this data indicates that miR-744 puts the brake on the expression of DVL2 and TGFB1, which both play an important role as promoters of migration in GBM.

2.4. Via Repression of DVL2 and TGFB1, miR-744 Regulates Beta-Catenin and SMAD-Signaling Pathways

We next set out to gain insight into the molecular pathways underlying the observed phenotypic alterations. TGFB1 is assumed to enhance cellular mobility through SMAD-dependent induction of pro-invasive factors such as Matrix Metalloproteinases (MMPs) [15,16], while DVL2 represents a central inducer of beta-Catenin signaling [17]. Both pathways induce epithelial-to-mesenchymal transition (EMT), and enhance tumor cell migration [14,17]. Consequently, we next assessed the activation status of SMAD2/3 and beta-Catenin upon overexpression of miR-744, as these transcription factors represent important downstream effector molecules of TGFB1 and DVL2 [16,18], respectively. As depicted in Figure 4A, miR-744 overexpression resulted in a marked reduction of the transcriptionally active forms of SMAD2/3 and beta-Catenin (SMAD2/3: reduction of 52% ± 15.8%; active beta-Catenin: reduction of 38.6% ± 5.7%). In addition, immunohistochemistry showed a significantly weaker staining for transcriptional active beta-Catenin upon overexpression of miR-744 as compared to controls (Figure 4B). In line with these results, subsequent analysis of beta-Catenin-mediated transcriptional activity by Lef/Tcf luciferase reporter gene assay revealed a significantly reduced activity in cells stably overexpressing miR-744 (Figure 4C; −46.1% ± 10.7%, n = 5, $p < 0.01$).

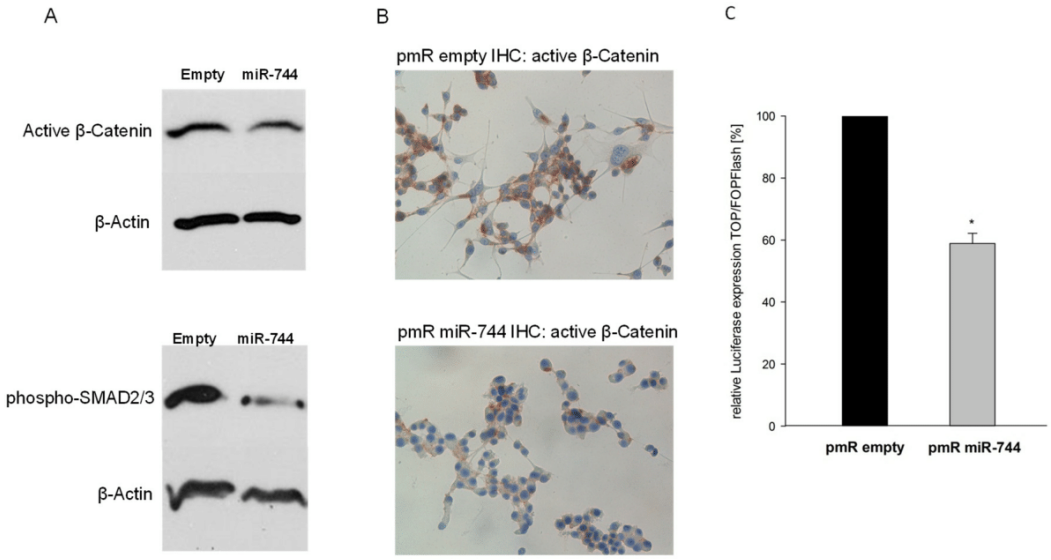


Figure 4. Stable overexpression of miR-744 represses activity of TGFB1 and DVL2 downstream effector molecules SMAD- and β-Catenin. Experiments were conducted after stable overexpression of miR-744 as compared to empty vector controls. (**A**) SDS-PAGE of active β-Catenin and phosphorylated SMAD 2/3. (**B**) Immunohistochemistry staining of active β-Catenin. (**C**) β-Catenin-dependent transcriptional activity of Lef/Tcf, measured with TOP/FOPFlash Luciferase Reporter Gene Assay (n = 4, $p = 0.007$). * $p < 0.05$.

Taken together, our results show that miR-744 via DVL2 and TGFB1 ameliorates invasive properties of GBM cells by down-regulation of beta-Catenin and SMAD-signaling.

2.5. miR-744 Reduces It's Host Gene MAP2K4 Through TGFB1-Mediated Negative Feedback

So far, our results have suggested that miR-744 acts as a counterpart of its host gene MAP2K4, an enhancer of cancer progression [8,10]. It thus appeared likely that a negative feedback relationship between miR and host might exist. To test this assumption, we assessed MAP2K4 expression in U87 cells stably overexpressing miR-744, and indeed found reduced mRNA and protein levels (Figure 5A; mRNA: −66.2% ± 7.9%; protein: −56.2% ± 9.6%; n = 5, $p < 0.05$). Concordantly, miR-744 overexpression attenuated activity of MAP2K4s downstream effector p38. As the 3'UTR of MAP2K4 does not contain any putative binding sites of miR-744, the host obviously is not directly targeted. We thus hypothesized that miR-744 indirectly represses its host by targeting an activator of the host gene. As TGFB1 is known to activate p38 MAPK, we speculated that TGFB1 might also fulfill a function as an enhancer of MAP2K4 signaling. Gene-specific knock-down of TGFB1 (Figure 5C, right panel) significantly decreased MAP2K4 mRNA and protein levels, thereby strongly supporting this assumption (Figure 5B; −47.7% ± 3.8%, n = 4, $p = 0.004$). Not unexpectedly, intronic miR-744 was also significantly affected by TGFB1 knock-down (Figure 5C, left panel; −38.1% ± 9.5%, n = 4, $p = 0.015$).

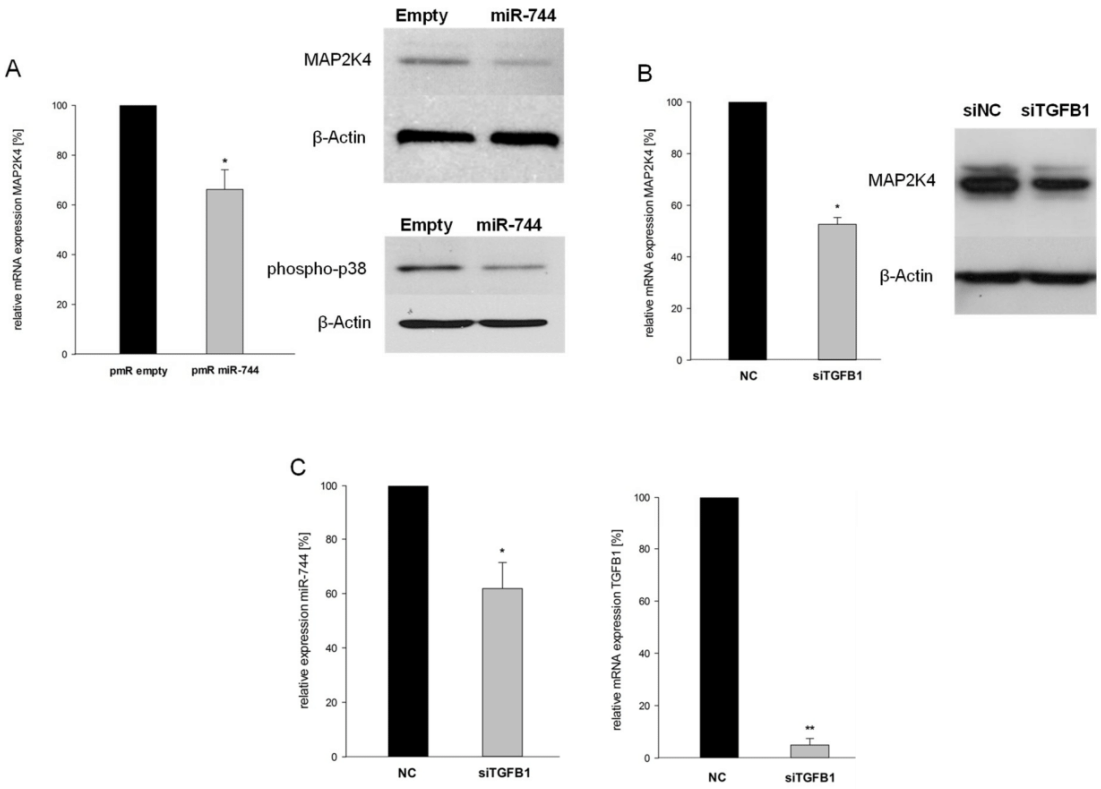


Figure 5. miR-744 reduces its host gene Mitogen-Activated Protein Kinase Kinase 4 (MAP2K4) through TGFB1-mediated negative feedback. (**A**) mRNA expression of MAP2K4 (left panel), and protein expression of MAP2K4 and its downstream target phospho-p38 MAPK (right panel), after stable overexpression of miR-744 as compared to empty vector controls (n = 5, $p = 0.003$). (**B**) mRNA (left panel) and protein expression (right panel) of MAP2K4 after knock-down of TGFB1 (n = 5, $p = 0.004$). (**C**) Expression of miR-744 (left panel) and TGFB1 (right panel) after knock-down of TGFB1 (miR-744: n = 5, $p = 0.015$; TGFB1: n = 5, $p < 0.001$). ** $p < 0.001$; * $p < 0.05$.

These results sketch a regulatory loop: TGFB1 simultaneously enhances transcription of MAP2K4 and miR-744. The latter directly targets TGFB1, which as a negative feedback results in concurrent repression of MAP2K4 and miR-744 (Figure 6). These results highlight the close regulatory relationship between intronically located miRs and their host genes.

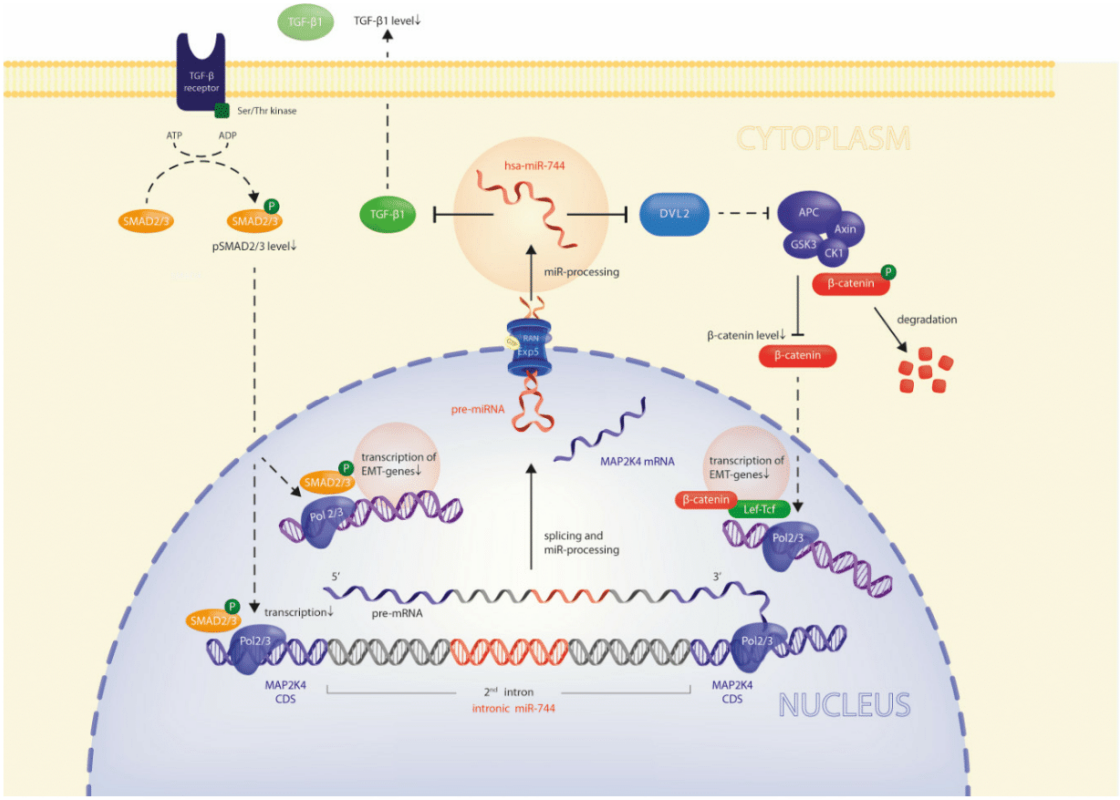


Figure 6. Model of miR-744 effects on it's host gene´s expression and on tumor cell migration. Direct repression of DVL2 and TGFB1 by miR-744 inhibits SMAD- and beta-Catenin-dependent transcriptional activity, respectively, thus reducing expression of EMT- and pro-invasive genes. Moreover, miR-744 constitutes a second-order negative feedback loop on its host gene MAP2K4 through direct repression of TGFB1 levels, concomitantly repressing host gene and miR-744 expression.

3. Discussion

In recent years, evidence has accumulated that colocalization of intronic miRNA and the host gene is not a random choice by nature, but rather fulfills important functional tasks within the host genes' pathways. Several studies have experimentally proven in different contexts that intronic miRNAs are capable of either supporting or restraining functional pathways of their host genes, thereby creating intricate regulative networks [19,20]. As about half of human miRNAs reside in introns of protein-coding genes, these examples may signify a more general biological principle. A very recent study however, has suggested low prevalence of functional association between host and intronic miRNAs in general, but has assumed a key role of this type of regulation in cellular signaling pathways requiring tight control [21]. To date, experimental evidence supporting this view is scarce. As an example of potential high interest, we investigated miR-744, located in the second intron of the tumorigenic kinase MAP2K4, and its impact on it's host gene's functional networks.

Expression patterns of miRNAs are highly heterogeneous among cell types, and pronounced expression levels of a certain miRNA within a specific tissue is likely to reflect functional relevance. After analysis of different tissues, we found brain tissue to be the most suitable for further analyses, due to its high miR-744 expression. With respect to the role of MAP2K4 as a driver of malignancy, we assessed miR-744 expression in human brain tissue, in Astrocytoma WHO grade II/III, and in the most malignant brain tumor (i.e., GBM). Interestingly, we detected a gradual tumor-grade-dependent loss of expression, with almost completely lost miR-744 expression levels in GBM. These findings made us assume that miR-744 is a gatekeeper of oncogenic signaling during the malignization process. Indeed, stable re-expression of miR-744 in GBM cells resulted in a significantly more benign phenotype,

with tumor cells growing focally, and migrating less. To uncover the mechanisms underlying these phenotype changes, we stably overexpressed miR-744 in GBM cells, and evaluated potential miR-744 target genes fulfilling the criteria of (a) being involved in the regulation of cell motility and (b) harboring potential miR-744 binding sites in their 3'UTRs. We identified and experimentally validated DVL2 and TGFB1 to be directly regulated by miR-744 in GBM cells. We were further able to show that specific knock-down of these target genes impaired cellular migration similar to miR-744 overexpression. In line with these results, we found that down-regulation of miR-744 in human GBM is accompanied by a marked increase in DVL2 and TGFB1 expression levels, indicating clinical relevance of these functional networks.

DVL2 and TGFB1 have repeatedly been shown to act as inductors of epithelial-to-mesenchymal transition (EMT) [13,22,23], one of the hallmarks of cancer progression. During EMT, induction of Matrix-Metalloproteinases initiates breakdown of the extracellular matrix and reduces cellular adhesion, which results in enhanced migratory capacity and invasiveness [24,25]. Particularly in GBM, TGFB1 is one of the most powerful cytokines secreted by the tumor itself. It has repeatedly been shown that GBM-derived TGFB1 induces EMT via activation of transcription factors of the SMAD family, thereby increasing migration of GBM cells into the surrounding brain tissue. It thus represents a hallmark of GBM progression [26–28]. In addition, DVL2, as a key enhancer of beta-Catenin transcriptional activity, represents another orchestrator of EMT [29–31]. In GBM, the DVL2-beta-catenin signaling axis has been found to be markedly activated, which significantly contributes to the extremely invasive nature of these tumors [13,32,33]. To this end, we tested whether inhibition of DVL2 and TGFB1 by overexpression of miR-744 exerted the supposed impact on the respective downstream effector molecules, beta-Catenin and SMAD. Based on immunohistochemistry, protein analyses, and reporter gene assays, our results provided evidence that miR-744 hampers GBM cell migration via concurrent reduction of SMAD- and beta-Catenin signaling. Thus, downregulation of miR-744 during gliomagenesis may be an effective tumor intrinsic mechanism to support tumor progression by simultaneously affecting different oncogenic signaling pathways.

This negative impact of miR-744 on tumor cell migration strongly contrasts with the functions of its host gene, MAP2K4, which has been shown to profoundly enhance cancer cell migration and metastasis [8,10]. It can thus be concluded that that miR-744 acts as functional antagonist of its host, thereby keeping a molecular balance. As stable overexpression of miR-744 resulted in significantly reduced MAP2K4 levels, we assumed that miR-744 may not only control its host functionally, but also on the level of gene expression through negative feedback. The concept of intronic miRs regulating their host genes by direct or indirect feedback loops has been experimentally proven in several different contexts [6,34]. Effects are achieved either directly by targeting of the host's 3'UTR (first order negative feedback), or indirectly by targeting an interposed gene that subsequently affects the host gene's expression (second order negative feedback). As the 3'UTR of MAP2K4 does not contain any predicted miR-744 binding sites, we focused on the identification of a second order negative feedback. In this regard, our finding that stable overexpression of miR-744 attenuated the activity of MAP2K4's downstream effector p38 prompted us to come back to TGFB1 as an important activator of p38 MAPKs [35,36]. Indeed, we could show that gene-specific knock-down of TGFB1 significantly decreased MAP2K4 expression. Concomitantly, miR-744 levels were decreased, supporting the notion of a negative feedback loop acting as an "intrinsic transcriptional brake" to prevent inadequate transcription of MAP2K4. Inhibition of TGFB1 by miR-744 thus fulfills a dual role in mediating effects of this intronic miRNA on its host MAP2K4: (i) Functional antagonization via blocking SMAD-signaling, thereby reducing glioma migration and invasion; and (ii) control of the tumorigenic host's expression levels.

It is a limitation of the current study that the molecular mechanisms enabling this fundamental switch remain elusive. However, due to the assumable multi-layered nature of these processes, this question may be addressed in further research projects.

4. Materials and Methods

4.1. Bioinformatics

Analysis of potential miR-mRNA interactions was performed using the public databases TargetScan, PITA, miRIAD, and picTAR. Potential direct interactions were considered probable when 2 or more algorithms returned a positive target prediction. In-silico analysis of miR expression levels was conducted using the intragenic microRNA database miRIAD (http://bmi.ana.med.uni-muenchen.de/miriad/). Involvement of target genes in tumor-associated pathways was evaluated using the KEGG database (www.genome.jp/kegg/pathway.html).

4.2. Human Tissue Samples

Tissue samples (n = 21, GBM; n = 15, WHO II/III Astocytoma; n = 8, primary GBM cell lines; and n = 9, normal brain) were obtained and processed as described previously [7]. Written informed consent was given by all patients, and the study protocol was approved by the Institutional Review Board of the Ludwig-Maximilians University of Munich, Germany (approval number: 216/14). For this study, only specimens from patients diagnosed with primary GBM, LOH 1p/19q negative, and IDH wild type were used.

4.3. RNA Extraction and cDNA Synthesis

RNA was extracted using the RNAqueous or miRvana Isolation kit (Ambion, Waltham, MA, USA), followed by DNAse treatment (Turbo DNAse, Ambion) according to the manufacturer's instructions. RNA amount and quality was assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Equal amounts of RNA were transcribed using Oligo-dT Primers, Random Hexamers (Qiagen, Venlo, Netherlands), dNTPs, RNAse OUT, and Superscript® III Reverse Transcriptase (Invitrogen, Waltham, MA, USA), following the manufacturer's instructions.

4.4. Quantitative RT-PCR

Quantitative analysis of mRNA levels was performed on a LightCycler 480 (Roche Diagnostics, Penzberg, Germany) using 10 ng of cDNA/well. Succinate Dehydrogenase Subunit A (SDHA) and TATA Box Binding Protein (TBP) were used as reference genes. Quantitative real-time PCR (qRT-PCR) was conducted using the primers (Metabion, Martinsried, Germany) and UPL Probes (Roche Diagnostics) provided in Supplementary Table S1. All assays were designed intron spanning. qRT-PCR conditions comprised initial denaturation for 10 Minutes (95 °C), and 50 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 1 s. Quantification cycle (C_q) values were calculated employing the "second derivative maximum" method computed by the LightCycler® software.

4.5. Quantification of miRNA Expression

Mir-744 expression was studied using TaqMan miRNA assays (Applied Biosystems, Waltham, MA, USA) according to manufacturer's instructions. MiR-744 and reference gene expression was measured in technical duplicates. U47 served as an endogenous reference. All patient samples were calibrated using a sample of normal brain tissue.

4.6. SDS-PAGE

Cells were lysed in cell lysis buffer containing protease and phosphatase inhibitors (Cell Signaling Technologies, Danvers, MA, USA). Protein concentrations were assessed through BCA assays (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Forty micrograms of the protein extracts were electrophoresed on 10% SDS-PAGE gels and electroblotted on PVDF-membranes. Nonspecific binding was blocked with 5% Bovine Serum Albumin (BSA) in TBS-Tween-20 (TBST) (Sigma, St.Louis, MO, USA). Antibodies for MAP2K4 (Cat. No. 9152),

TGFB1 (Clone 56E4, Cat. No. 3709), DVL2 (Clone 30D2, Cat. No. 3224), and β-Actin (Clone 13E5, Cat. No. 4970) (all Cell Signaling Technologies) were diluted in TBST with 1% BSA. β-Actin served as the loading control. Immunoreactivity was assessed using horseradish peroxidase-labeled goat anti-mouse or goat anti-rabbit antibodies (Cell Signaling Technologies).

4.7. Cell Culture

U87 and T98G GBM cells were purchased from the American Type Cell Culture Collection (ATCC). Cells were maintained in DMEM (Gibco) with 10% FCS (Biochrom AG), 2% L-Glutamine, 1% Penicillin/Streptomycin, 1% MEM NEAA (Invitrogen), and 1% Sodium Pyruvate (PAA). HEK-293 cells (ATCC) were maintained in DMEM with 10% FCS, 2% L-Glutamine, 1% Penicillin/Streptomycin, and 1% MEM NEAA. Primary GBM cell lines were obtained from patients undergoing open GBM resection, according to the study protocol mentioned above. Tumor tissue was dissociated using the Brain Tumor Dissociation Kit P (Miltenyi, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. Primary GBM cells were cultivated in MACS Neuro Medium supplemented with Neuro Brew-21 without Vitamin A (Miltenyi).

4.8. Cloning of Reporter Constructs

The 3'UTRs of TGFB1 and DVL2 were amplified using genomic DNA and primer with XhoI and NotI, or PmEI and XhoI, restriction sites. Primer sequences are supplied in Supplementary Table S2. PCR products were cloned into the psiCHECK2 vector (Promega, Mannheim, Germany). Correct sequences were verified by Sanger sequencing (Eurofins Operon, Ebersberg, Germany). Plasmids were purified using the Qiaprep Spin Plasmid Miniprep Kit (Qiagen) and the Pure Yield Plasmid Midiprep System (Promega). DNA concentrations were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).

4.9. Cloning of miRNA Expression Vector

The pre-miR sequence was amplified using genomic DNA and specific primers (sequences supplied in Supplementary Table S2). The resulting amplicon was cloned into the pmRZs-Green1 vector (Promega).

4.10. Transfections

Transfections were conducted using the NEON electroporation device (Life Technologies, Waltham, MA, USA). Transient transfection with microRNA precursors (premiR, Thermo Fisher Scientific) or siRNA (Dharmacon, Lafayette, CO, USA) was carried out at final concentrations of 50 nM (premiR) or 100 nM (siRNA), and 250,000 cells per well. Cells were incubated for 36 h at 37 °C and 5% CO_2 in an antibiotics-free medium. Stable transfection was performed using 1 million cells and 10 μg plasmid/well. After incubation for 12 h in antibiotics-free medium, cells were seeded in DMEM containing 750 μg/mL Geneticin (Life Technologies). Stable transfection was analyzed by flow cytometry (Attune, Life Technologies). Monoclonal cell lines were obtained by single-cell picking. Overexpression of miR-744 was assessed through TaqMan assays. Co-transfection of luciferase reporter plasmids and premiR™ was carried out using 100,000 HEK-293 cells and 1 μg of Psi-CHECK™2 plasmid. All transfection experiments were performed in triplicate.

4.11. Reporter Gene Assays

After 36 h of incubation, co-transfected cells were harvested, washed twice, and resuspended in 20μl medium. Luminescence was measured with the MicroLumat Plus (Berthold Technologies, Bad Wildbad, Germany) using the Dual-Glo Luciferase Assay system (Promega), according to manufacturer's instructions. All experiments were performed in triplicate.

4.12. Migration Assays

70,000 cells/well were seeded in 2-well culture inserts (Ibidi, Martinsried, Germany) and incubated for 24 h at 37 °C with 5% CO_2 in a humidified incubator. Inserts were removed, cells were washed with cell culture media, and pictures were obtained using an inverted microscope (Carl Zeiss, Jena, Germany). Cells were incubated for 24 h in a cell culture incubator. Pictures were obtained and cells were fixed with methanol.Invasive properties were studied with the Cytoselect Assay, Collagen I, Colorimetric Format (Cell Biolabs, San Diego, CA, USA). 100.000 Cells were resuspended in 250 µL FCS-free media and seeded into the upper compartment of the boyden chamber. DMEM containing 10% FCS was added to the lower compartment. After incubation for 24 h in a cell culture incubator, non-invasive cells were removed with cotton swabs, invasive cells were stained, lysed, and the optical density was determined. Experiments were performed in duplicate.

4.13. Immunohistochemistry

100,000 cells were seeded on glass slides (Falcon) and incubated for 2 days. Cells were washed with PBS, fixed with ice-cold acetone, and washed with TBST. Endogenous peroxidase was blocked (1% H_2O_2, 10 minutes), cells were washed twice with TBST, and incubated in 5% normal goat serum for 1h. Antibodies were diluted in Antibody Diluent (Cell Signaling Technologies) at a concentration of 1:800 and incubated at 4 °C overnight. Cells were washed twice with TBST and Signal Stain Boost IHC Detection Reagent was added (Cell Signaling Technologies). After washing with TBST, Signal Stain DAB substrate was added for 45 s. Thereafter, cells were washed with TBST. Counterstaining was performed with Haematoxylin (Sigma Aldrich).

4.14. Statistics

All data is presented as the mean ± SEM. p-values were calculated using student's t-tests. Statistical analyses were performed using SigmaPlot 12.0 (Systat Software). p-values below 0.05 were considered statistically significant (* $p < 0.05$; ** $p < 0.001$).

5. Conclusions

Taken together, we here uncovered a new regulatory circuit consisting of the tumorigenic host gene MAP2K4 and its intronically located miR-744; miR-744 acts as an intrinsic brake on its host by counterbalancing both its expression and function. In human glioma, this circuit is disrupted, leading to an invasion-promoting constellation where miR-744 is almost completely repressed while its host is induced.

Our data underscores the necessity to gain further profound insights into the networks of intronic miRNAs and their hosts, which is particularly important with respect to potential clinical approaches. Counteracting the shortfall of miR-744 in GBM pharmacologically, for example, might be an innovative clinical direction to pursue.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6694/10/11/400/s1, Table S1: Primer sequences for qRT-PCR; Table S2: Primer sequences for molecular cloning; Figure S1: Knock-down efficiency after transient transfection of U87 GBM cells with DVL2 or TGFB1 siRNA, as analyzed by qRT-PCR.

Author Contributions: M.H. designed and performed experiments, collected, analyzed, and interpreted data, and wrote the manuscript; C.L.H. designed and performed research, analyzed and interpreted data, and wrote the manuscript; D.E. performed experiments and interpreted data; T.W. planned and performed research; N.T. contributed patient samples and helped to revise the manuscript. F.-W.K. contributed patient samples and interpreted data, and participated in writing the manuscript; S.K. designed the study, analyzed and interpreted data, wrote the manuscript, and supervised the study.

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Conflicts of Interest: The authors declare no conflict of interest.

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Intronic miRNA-641 controls its host Gene's pathway PI3K/AKT and this relationship is dysfunctional in glioblastoma multiforme

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ABSTRACT

MicroRNAs have established their role as important regulators of the epigenome. A considerable number of human miRNA genes are found in intronic regions of protein-coding host genes, in many cases adopting their regulatory circuitry. However, emerging evidence foreshadows an unprecedented importance for this relationship: Intronic miRNAs may protect the cell from overactivation of the respective host pathway, a setting that may trigger tumor development. AKT2 is a well-known proto-oncogene central to the PI3K/AKT pathway. This pathway is known to promote tumor growth and survival, especially in glioblastoma. Its intronic miRNA, hsa-miR-641, is scarcely investigated, however. We hypothesized that miR-641 regulates its host AKT2 and that this regulation may become dysfunctional in glioblastoma.

We found that indeed miR-641 expression differs significantly between GBM tissue and normal brain samples, and that transfection of glioma cells with miR-641 antagonizes the PI3K/AKT pathway. Combining clinical samples, cell cultures, and biomolecular methods, we could show that miR-641 doesn't affect AKT2's expression levels, but down-regulates kinases that are necessary for AKT2-activation, thereby affecting its functional state. We also identified NFAT5 as a miR-641 regulated central factor to trigger the expression of these kinases and subsequently activate AKT2.

In summary, our study is the first that draws a connecting line between the proto-oncogene AKT2 and its intronic miRNA miR-641 with implication for glioblastoma development.

1. Introduction

MiRNAs are small, non-coding RNA molecules that post-transcriptionally regulate target mRNAs [1,2]. These targets are recognized via base-pair complementarity to the 3′-UTR of the mRNA, leading to mRNA decay [3,4] or translational repression [5]. Most human miRNA genes are located within intronic regions of protein-coding host genes [6], presumably with the evolutionary advantage of adopting their hosts' transcriptional regulatory circuitry [7]. Accumulating evidence supports a functional implication of intronic miRNAs on their host genes via both direct and indirect regulation [8–11]. A prominent case has recently been described: The well-known proto-oncogene Erbb2 is functionally antagonized by its intronic miRNA miR-4728, which has immediate prognostic impact [11].

Another gene well-known for its importance in tumor development is AKT Serine/Threonine Kinase 2 (AKT2) [12,13]. Its proto-oncogenicity has been attributed to increased cell survival through reduced apoptosis and increased chemoresistance [12,14–16]. Elevated phospho-AKT levels in turn are a typical trait of glioblastoma multiforme (GBM), a highly malignant neoplasia. Despite intense efforts to find AKT-targeting therapies, treatment options are still very limited [17]. AKT2 however harbors intronic miR-641 immediately in its first intronic region. While there is a plethora of literature investigating AKT2, miR-641 is yet largely

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uncharacterized. One study reported that its methylation, among other miRNAs, and subsequent silencing might be permissive of cervical cancer development, hence claiming an overall tumor-suppressive function [18].

In the current manuscript we therefore investigated, if hsa-miR-641 plays a role in GBM via regulation of its host gene AKT2. We hypothesized that a) miR-641 is tumor-suppressive in GBM and b) it negatively regulates the PI3K/AKT pathway, promoting apoptosis.

2. Materials and methods

2.1. Human tissue samples

GBM tissue samples were obtained and processed as described previously [19]. Written informed consent was given by all patients, and the study protocol was reviewed and approved by the Institutional Review Board of the Ludwig Maximilians University Munich, Germany (approval number: 216/14).

2.2. RNA isolation

Tissue preparation and isolation of total RNA was done using either the RNAqueous® Micro Kit or the mirVana miRNA Isolation Kit with subsequent DNase treatment (Turbo DNase, Ambion) as described previously [20]. Purity and quantity of the isolated RNA were measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific).

2.3. Quantitative RT-PCR

cDNA was synthesized from equal amounts of total RNA (1 μg) using Superscript III reverse transcriptase (Invitrogen) and oligo (dT) and random hexamer primers following the supplier's instructions. Quantitative analyses of mRNA levels were performed on a Light Cycler 480 (Roche Diagnostics) using either UPL probes and specific primers or specific single assays (Table S1, Roche Diagnostics, Penzberg). Data were normalized to the reference genes SDHA and TBP [21].

2.3.1. Quantification of miRNA expression

Expression of miR-641 was quantified using TaqMan miRNA assays (Applied Biosystems) following the manufacturer's protocol; U47 served as endogenous control.

2.4. Western blot analysis and phospho-Akt (Ser474p-Akt) ELISA

35 μg of total protein extracts were electrophoresed in 8% SDS–PAGE and electroblotted onto PVDF membranes. Non-specific binding sites on the membrane were blocked using 5% Bovine Serum Albumin (BSA) in TBS-Tween. Phospho-Akt Rabbit mAb (Cell Signaling Technology Inc., Danvers, MA) were diluted in PBST supplemented with 1% BSA. β-actin (Cell Signaling Technology Inc.) served as a loading control. Immunoreactive bands were visualized using horseradish peroxidase-labeled goat anti-mouse or goat anti-rabbit antibodies (Cell Signaling Technology Inc.).

The Phospho-ELISA (PathScan® Phospho-Akt2 Sandwich ELISA kit, Cell Signaling Technology, Inc.) was used according to the manufacturer's instructions.

2.5. Cell cultures conditions

HEK 293 cells were maintained under standard conditions as previously described [22].

2.6. Flow cytometric analysis of cell apoptosis

After transfection of U87 cells with pre-miR-641 or NC, cells were incubated for 48 h. Thereafter, apoptosis was induced by incubating the transfected U87 cells in serum-free media and a controlled anaerobic atmosphere (anaerobic chamber) for 24 h [23].

Apoptosis was analyzed using Annexin-V-Fluorescein and Propidium Iodide (PI) according to manufacturer's protocol (Annexin-V-FLUOS Staining Kit, Roche Diagnostics GmbH, Mannheim, Germany). Cells were thereafter analyzed immediately by flow cytometry using an Attune Acoustic Focusing Cytometer (Life Technologies, Carlsbad, USA).

2.7. Cloning of vector constructs

The psiCHECK-2 Vector (PromegaMadisonWIUSA) was used for generation of 3'-untranslated region (3′-UTR) reporter constructs as described before [24]. All plasmids were verified by sequence analysis (MWG Biotech, Ebersberg, Germany). Cloning primers are provided in Table S1.

2.8. Cell transfections and luciferase assay

Cell transfections were performed by electroporation using the Neon™ transfection system (Invitrogen, Life Technologies, Darmstadt, Germany) as previously described [24]

2.9. Statistical and bioinformatics analysis

All statistical analyses were performed using SigmaStat 12.0 (Systat Software, Chicago, USA). Every statistical analysis was started with testing for normal distribution using the Shapiro Wilk Test. Further analyses were performed with Student's t-test for all data with normal distribution and the nonparametric Mann-Whitney Rank Sum Test otherwise. Values are expressed as mean ± standard deviation (SD). p-values < 0.05 were considered as statistically significant. The public resource miRIAD was used to assess miR-641 target interaction predictions as well as protein-protein interactions [6].

3. Results

3.1. MiR-641 increases apoptosis in vitro and its expression is reduced in GBM

In human malignant glioma, increased AKT2 expression inhibits apoptosis and promotes malignancy [14]. To test our hypothesis that miR-641 may act as a negative feedback regulator of its host genes' AKT2 pathways, we transfected glioma cells with either miR-641 or negative control (NC), and induced apoptosis by incubating the transfected cells in serum-free media under controlled anaerobic atmosphere. As shown in Fig. 1A, apoptosis significantly increased after miR-641 transfection (1.48 ± 0.2; $n = 6$; $p < 0.01$).

When we compared the expression of miR-641 in primary human glioblastoma tissue specimen to normal brain tissue, we found significantly decreased miR-641 levels in glioblastoma (0.39 ± 0.2; $n = 12$; $p < 0.01$; Fig. 1B). These findings sketch a scenario, in which miR-641 may attenuate ATK2's antiapoptotic traits and that this interaction seems to be disrupted in human GBM.

3.2. MiR-641 regulates its host gene AKT2 by influencing its phosphorylation state

To test whether miR-641 may directly target its host gene AKT2,

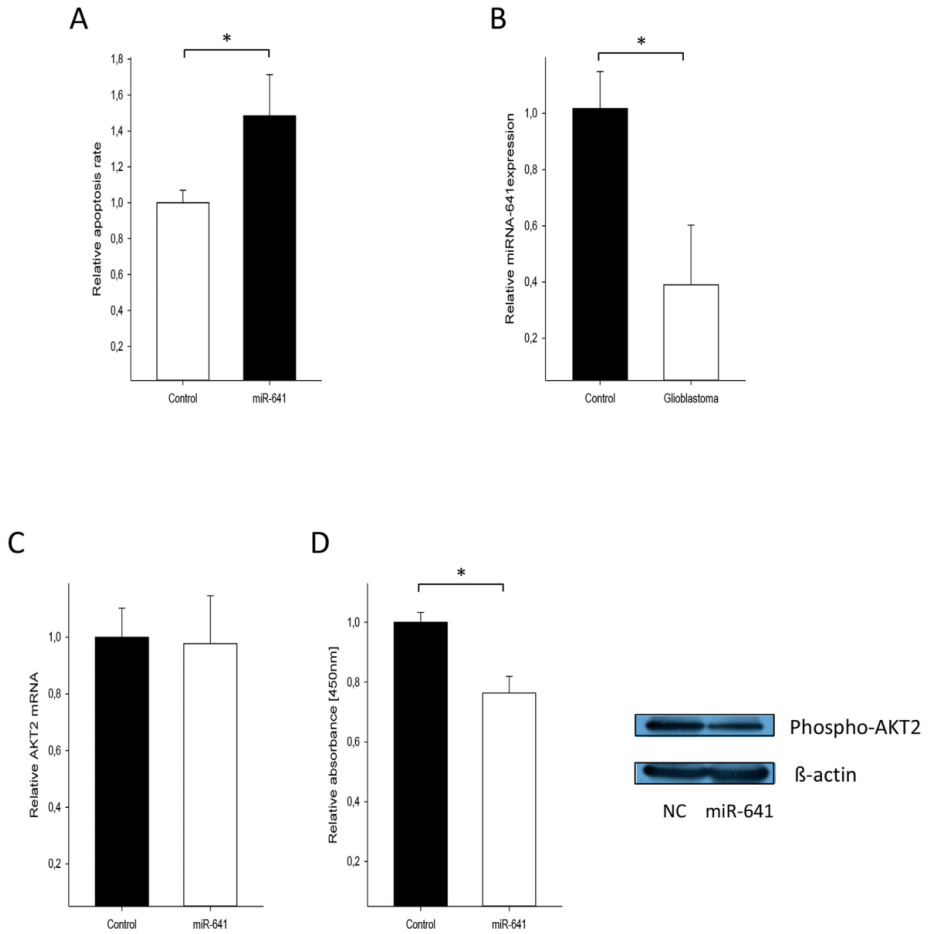

Fig. 1. MiR-641 increases apoptosis *in vitro* and its expression is reduced in GBM. (**A**) U87 cells were transfected with miR-641 and scrambled control (Control) and apoptosis rate was determined. (**B**) Expression of miR-641 was determined via rt-PCR in tissue samples of Glioblastoma and normal brain tissue (Control) for n = 12 samples. (**C**) Relative AKT2 mRNA expression levels determined via rt-PCR after transfection of GBM cells with miR-641. (**D**) AKT2-Phosphorylation changes after transfection of miR-641 were quantified using ELISA (left panel) and phospho Western Blots (right panel; a representative blot is shown). If applicable, all data are given as means ± SD; *p < 0.01, n = 6.

we transfected human U87 cells with either miR-641 mimics or NC and assessed AKT2 mRNA levels. As shown in Fig. 1C, AKT2 mRNA expression did not significantly change after transient transfection with miR-641. These findings indicate that miR-641 does neither directly target AKT2, nor indirectly regulates its expression.

We next hypothesized that miR-641 may affect the function of AKT2 by modulation of its phosphorylation. We thus transfected the human GBM cell line T98G with miR-641 or NC and analyzed phosphorylation of AKT2 using ELISA and Western Blot. Transient transfection of T98G with miR-641 resulted in a significant decrease of AKT2 phosphorylation (ELISA: 0.76 ± 0.05; n = 4; p < 0.01, Fig. 1D, left panel, Western Blot, right panel). These results provide evidence that the phosphorylation state and thus the activity of AKT2 is regulated by its intronic miR-641.

3.3. *PIK3R3 and MAPKAP1 are direct targets of miR-641*

We next set out to identify the miR-641 target genes that would explain these effects. It is well known that PIK3R3, mTOR, RICTOR, PDK2 and MAPKAP1 are involved in the phosphorylation of AKT2 [25–28]. We thus analyzed the impact of miR-641 on the expression of these genes. As shown in Fig. 2A, transient transfection of U87 cells with miR-641 resulted in a significant decrease of PIK3R3 (0.49 ± 0.1; n = 6; p < 0.01), PDK2 (0.72 ± 0.2; n = 6; p < 0.01) and MAPKAP1 mRNA (0.73 ± 0.2; n = 6; p < 0.01) expression, while mTOR and RICTOR were not influenced (not shown). Since these results suggest that PIK3R3, PDK2, and MAPKAP1 could be direct targets of miR-641, we searched for predicted binding sites of miR-641, which we found in the 3'-UTR of PIK3R3 and MAPKAP1, but not in PDK2. We then analyzed a potential direct targeting of PIK3R3 and MAPKAP1 by luciferase reporter assay. After transient transfection of HEK-293 cells with miR-641, luciferase activity of both PIK3R3 (0.72 ± 0.1, n = 6; p < 0.01, Fig. 2B) and MAPKAP1 (0.85 ± 0.1; n = 6; p < 0.01, Fig. 2B) was significantly reduced, which indicates that both genes are direct targets of miR-641.

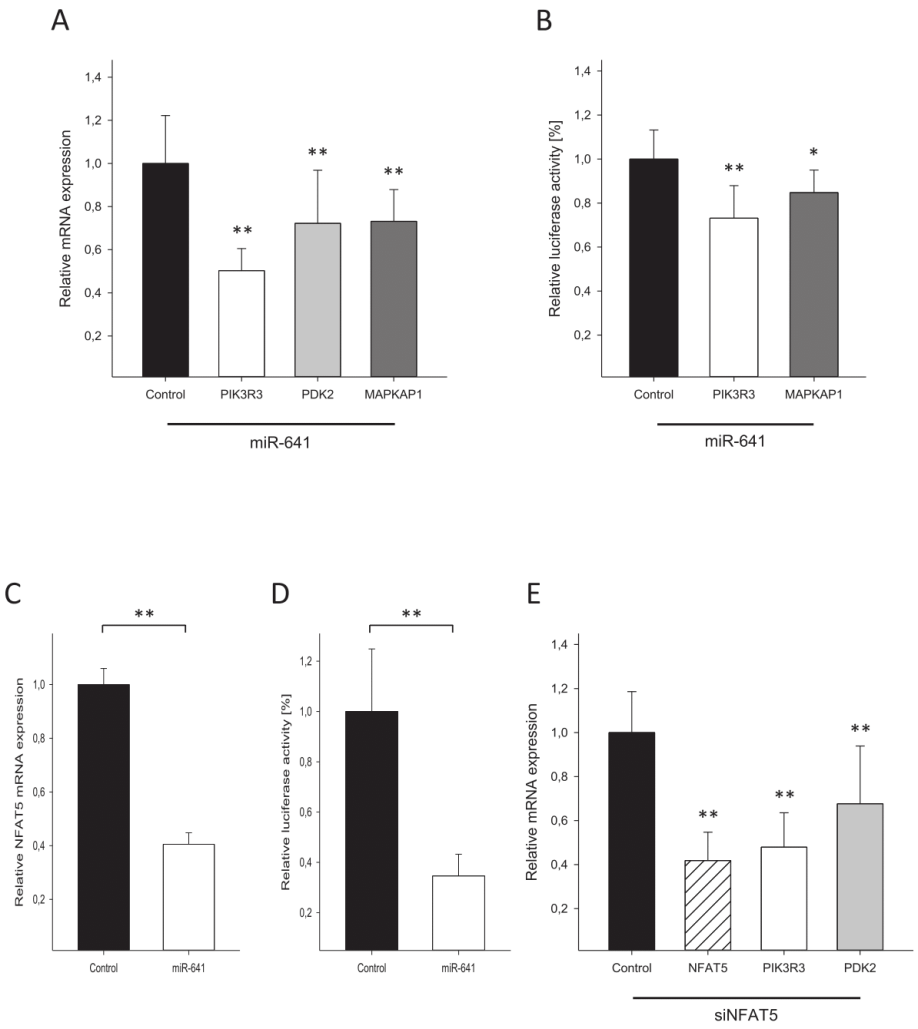

Fig. 2. MiR-641 regulates mRNA expression of genes involved in the phosphorylation of AKT2. (**A**) Relative mRNA expression of PIK3R3, PDK2, MAPKAP1 after transfection of miR-641 or control. (**B**) Relative Luciferase activity of vector constructs containing the 3′-UTR of PIK3R3 and MAPKAP1 after transfection of miR-641 or control. (**C**) Relative NFAT5 mRNA expression after transfection of miR-641. (**D**) Relative Luciferase activity of a vector construct containing the 3′-UTR of NFAT5 after transfection of miR-641. (**E**) Relative mRNA expression of NFAT5, PIK3R3, and PDK2 after transfection of siNFAT5. Data are given as means ± SD; n = 6, $^{*}p < 0.05$, $^{**}p < 0.01$.

3.4. MiR-641 regulates PDK2 and PIK3R3 via targeting NFAT5

Since we were devoid of evidence that miR-641 directly regulates PDK2, the influence of miR-641 on its expression levels may be caused indirectly via the PIK/AKT pathway's interactome. Recent research provided evidence that the PIK/AKT pathway and nuclear factors of activated T-cells (NFATs) are closely interrelated [29–31]. We found predicted high-probability binding sites for miR-641 within the 3'-UTR of the NFAT5 transcript.

To investigate this potential interaction, we transfected human U87 cells with either miR-641 mimics or NC, and determined the expression of NFAT5 mRNA. As shown in Fig. 2C, NFAT5 mRNA expression significantly decreased after transient transfection with miR-641 compared to NC (0.41 ± 0.1; n = 6; $p < 0.01$). Moreover, luciferase activity of a NFAT5 3'-UTR-containing vector was significantly reduced after transfection of HEK-293 cells with miR-641 (0.32 ± 0.1; n = 6; $p < 0.01$, Fig. 2D), thereby providing evidence of a direct targeting.

As expected, knock-down of NFAT5 by specific siRNA (0.42 ± 0.1; n = 6; $p < 0.01$, Fig. 2E) resulted in a significant decrease of PDK2 mRNA expression (0.67 ± 0.2; n = 6; $p < 0.01$, Fig. 2E). The expression of PIK3R3 was also affected (0.48 ± 0.2; n = 6; $p < 0.01$, Fig. 2E), which indicates that it is both directly and indirectly regulated by miR-641.

3.5. SiNFAT5 and siPIK3R3 affect the phosphorylation of AKT2

We next aimed at validating the supposed effects of the NFAT5-PIK3R3-axis on the phosphorylation state of AKT2. To this end, we transfected T98G cells with either siNFAT5 or siPIK3R3, and analyzed AKT2 phosphorylation using ELISA and Western Blot.

As shown in Fig. 3, AKT2 phosphorylation was significantly decreased after transient transfection of T98G with siNFAT5 (ELISA: 0.70 ± 0.04; n = 4; $p < 0.05$, Fig. 3A, left panel) or siPIK3R3 (ELISA: 0.71 ± 0.03; n = 4; $p < 0.05$). These results were confirmed by Western Blot (Fig. 3A, right panel).

Taken together, these experiments show that NFAT5 is a central hub mediating miR-641 effects on AKT2 phosphorylation.

3.6. Differential expression of PIK3R3 and NFAT5 in human glioblastoma

Finally, to further corroborate the relevance of the regulatory circuitry consisting of intronic miR-641, and its targets influencing the phosphorylation status of its host gene, AKT2, we determined the mRNA expression of PIK3R3 and NFAT5 in primary human glioblastoma tissue specimen compared to normal brain tissue. As

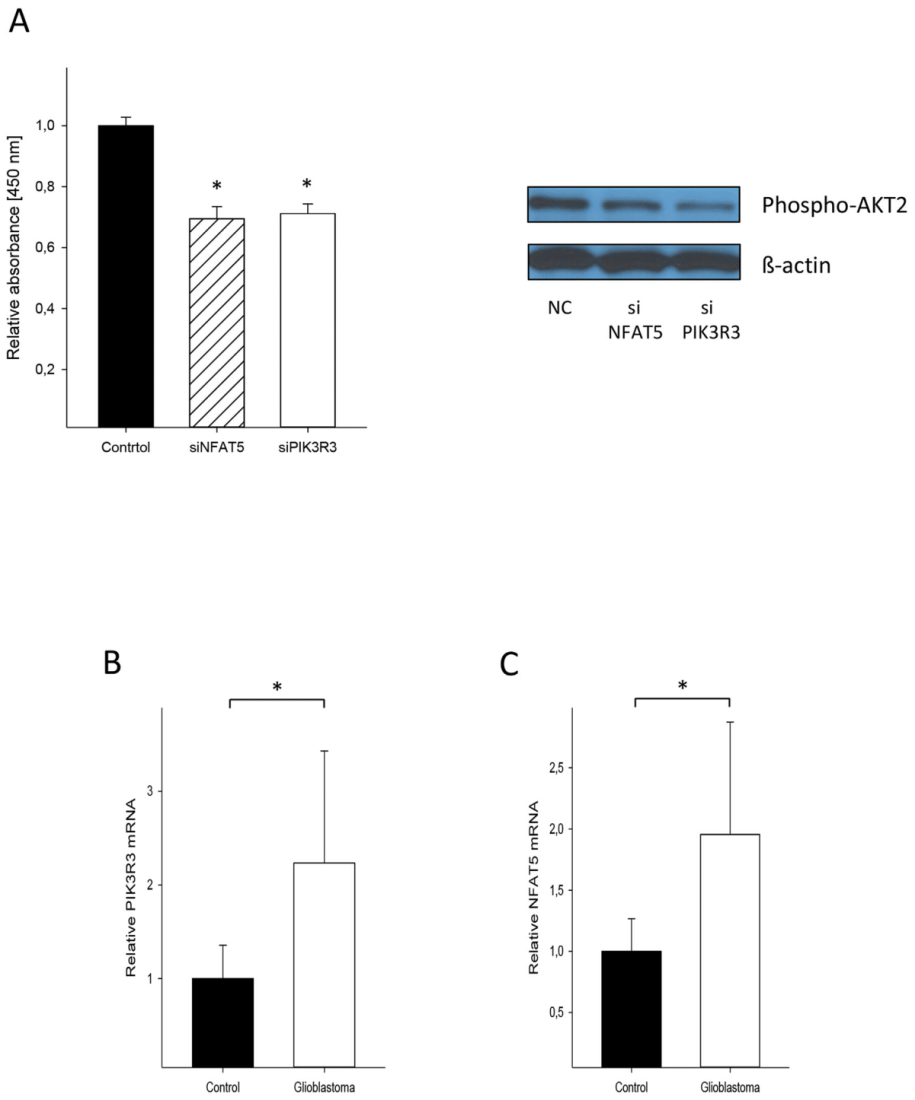

Fig. 3. NFAT5 and PIK3R3 affect the phosphorylation of AKT2 *in vitro* and their expression is increased in GBM. (**A**) AKT2 phosphorylation after knock-down of NFAT5 and PIK3R3 by specific siRNAs (n = 4) (ELISA: left panel; representative Western Blot: right panel). (**B**) Expression of PIK3R3 and (**C**) NFAT5 mRNA (n = 12) in tissue samples of GBM and normal brain. Data are given as means ± SD; *$p < 0.01$.

shown in Fig. 3, expression of PIK3R3 and NFAT5 mRNA was significantly higher in human glioblastoma specimen compared to normal brain tissue (PIK3R3: 2.23 ± 1.1; n = 12; $p < 0.01$; Fig. 3B, NFAT5: 1.96 ± 0.9; n = 12; $p < 0.05$, Fig. 3C).

These results let us to propose the following model (illustrated in Fig. 4): miR-641 acts as a physiologic inhibitor of its host gene AKT2 by targeting several genes (directly or indirectly via NFAT5) that determine the phosphorylation state of AKT2. In human GBM, this negative feedback loop is disrupted. While AKT2 expression is increased, miR-641 is significantly suppressed. This suppression results in an increased expression of PIK3R3 and NFAT5 mRNA, and, second line, of PDK2, thereby enhancing AKT2 phosphorylation and thus further supporting malignancy.

4. Discussion

Ever since their description, miRNAs have been identified as influential regulators of the transcriptome [2,4]. A significant fraction of human miRNA genes reside within intronic regions of protein-coding host genes [6]. While individual transcription is still possible, expression of these miRNAs is influenced by the regulatory circuitry of their host genes in many cases [7,32]. It is therefore not surprising that increasing evidence suggests also a functional coupling between intronic miRNAs and their host genes. Examples of first-order negative feedback loops, in which intronic miRNAs directly target their host genes' transcripts have been published for ZFR and CTDSP2 [8,9]. However, the probably more interesting setting is a higher-order feedback, in which a miRNA interferes with its host gene's network [33]. The scenario gains even more weight, when the host gene is central to cell signaling pathways linked to cancer pathogenesis. Just recently, intronic miR-4728-3p has been described as a negative regulator of its host gene ERBB2, also showing that reduced miRNA expression correlated with poor prognosis in breast cancer [11].

Another prominent proto-oncogene harboring an intronic miRNA is found in the PI3K/AKT signaling pathway. Central to this pathway, as the name reveals, are the phosphoinositide kinases (PIK) that activate downstream serine-threonine-kinases (AKT1-3) via phosphorylation. It is crucial for cell growth and survival and inhibits apoptosis [12,14–16]. It is also known to be proto-oncogenic and activated in many human tumors. Especially glioblastoma is known to yield an overactivation of the PI3K/AKT with a high amount of phosphorylated, activated AKT [12,13]. One of the central members, AKT2, contains the intronic miRNA miR-641 that is yet largely uncharacterized and the functional relationship to its host gene is so far unknown.

Due to its central position, we hypothesized that miR-641 exerts control over its host gene's pathway, and that this control is distorted in GBM. Indeed, we observed significantly reduced levels of miR-641 in GBM compared to normal brain tissue, accompanied by increased levels of AKT2, which conforms to our hypothesis. In the next step we sought to investigate, whether miR-641 directly targets AKT2, hence controlling its host gene's expression levels. However, AKT2's 3′-UTR doesn't contain predicted binding sites for miR-641 and transfection of GBM cells with miR-641 did not alter AKT2 mRNA levels. These findings exclude a regulatory feedback loop mediated by reduction of AKT2 expression. Despite constant AKT2 expression levels after miR-641 overexpression, we observed a 50% increase in apoptotic cells, congruent with a functional antagonism of the PI3K/AKT pathway. Therefore, we investigated, if AKT2 activation would be altered by miR-641. AKT2 is activated through phosphorylation of the Ser473 residue in the "hydrophobic motif" of the carboxy-terminal region [34]. We indeed observed a significant reduction in phospho-AKT2 in reaction to miR-641 overexpression, suggesting a higher-order negative feedback loop similar to the one described for ERBB2 and its intronic miRNA [11].

To explore this observation, we investigated a set of five kinases known to phosphorylate and activate AKT2: PIK3R3, mTOR, RICTOR, PDK2, and MAPKAP1 [25–28]. MiR-641 overexpression lead to decreased levels of PIK3R3, PDK2, and MAPKAP1, while mTOR and

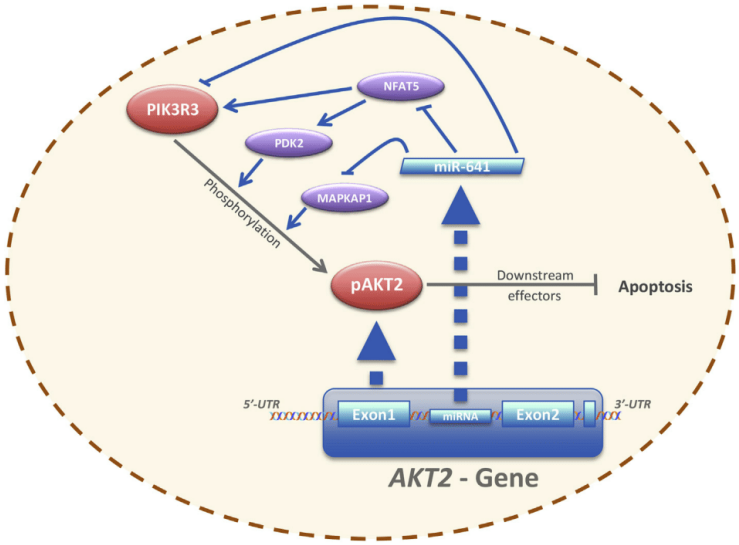

Fig. 4. Model of intronic negative feedback regulation. MiR-641 regulates the activation of its host gene AKT2 via (i) directly via targeting of the activating kinases PIK3R3 and MAPKAP1, and (ii) indirectly, via targeting of NFAT5, which controls the expression of the activating kinases PIK3R3 and PDK2. Consequently, miR-641 antagonizes the apoptosis-block induced by AKT2-overactivation.

RICTOR expression remained constant. Especially PIK3R3 levels dropped dramatically. After searching for potential miRNA binding sites, we identified MAPKAP1 and PIK3R3 as direct targets of miR-641, even though the effect was rather modest. However, PDK2 was not directly targeted by miR-641. Therefore we set out to search among predicted miR-641 targets for transcription factors known or predicted to interact with PDK2 and PIK3R3. The most likely candidate we found was NFAT5. NFAT family members are key factors in the regulation of cellular functions [29]. Their dysregulation can lead to malignant growth through interaction with the PI3K/AKT pathway [29–31]. Overexpression of NFAT5 promotes survival of astrocytes but also inhibits apoptosis in these cells [29,35]. After validation of the interaction between miR-641 and NFAT5, we could show that specific silencing of NFAT5 via siRNA lead to reduction of PIK3R3 and PDK2, and consequently to reduced AKT2 phosphorylation. In summary, our results show that miR-641 regulates its host gene's activation state via direct and indirect regulation of PIK3R3, as well as direct targeting of MAPKAP1 and indirect targeting of PDK2.

To the best of our knowledge, this is the first study to elucidate the relationship between the well-known proto-oncogene AKT2 and its intronic miRNA miR-641. Furthermore, we were able to demonstrate a potential clinical significance for glioblastoma, a highly malignant tumor that still lacks sufficient treatment options.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2017.05.175

Transparency document

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