Expression inactivation of SMARCA4 by microRNAs in lung tumors

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Abstract

SMARCA4 is the catalytic subunit of the SWI/SNF chromatin-remodeling complex, which alters the interactions between DNA and histones and modifies the availability of the DNA for transcription. The latest deep sequencing of tumor genomes has reinforced the important and ubiquitous tumor suppressor role of the SWI/SNF complex in cancer. However, although SWI/SNF complex plays a key role in gene expression, the regulation of this complex itself is poorly understood. Significantly, an understanding of the regulation of SMARCA4 expression has gained in importance due to recent proposals incorporating it in therapeutic strategies that use synthetic lethal interactions between SMARCA4-MAX and SMARCA4-SMARCA2. In this report, we found that the loss of expression of SMARCA4 observed in some primary lung tumors, whose mechanism was largely unknown, can be explained, at least partially by the activity of microRNAs (miRNAs). We reveal that SMARCA4 expression is regulated by miR-101, miR-199 and especially miR-155 through their binding to two alternative 3’UTRs. Importantly, our experiments suggest that the oncogenic properties of miR-155 in lung cancer can be largely explained by its role inhibiting SMARCA4. This new discovered functional relationship could explain the poor prognosis displayed by patients that independently have high miR-155 and low SMARCA4 expression levels. In addition, these results could lead to application of incipient miRNA technology to the aforementioned synthetic lethal therapeutic strategies.
Introduction

The SWI/SNF complex is a chromatin-remodeling complex that uses the energy of adenosine triphosphate (ATP) hydrolysis to modify the interactions between DNA and histones. In this way it modifies the availability of the DNA information to the transcriptional machinery, resulting in altered gene expression (1,2). Increasing amounts of evidence demonstrates that some of the components of the SWI/SNF complex are tumor suppressors and are involved in human cancer development. Importantly, functional alterations of SWI/SNF complex factors are ubiquitous in tumor types, underlining the importance of this complex in carcinogenesis (3,4). The human SWI/SNF is a complex composed of a catalytic subunit that has chromatin-remodeling activity itself and a group of 9–15 variable subunits that modulate its activity. SMARCA4 (also known as BRG1) is a catalytic ATPase/helicase subunit of the SWI/SNF complex. SMARCA4 has been widely studied for its association with cancer (5). In a previous report, we determined the importance of the inactivation of SMARCA4 in lung cancer cell lines. Remarkably, we found that 35% of non-small cell lung cancer (NSCLC) cell lines bear homozygous SMARCA4 inactivating mutations (6). These results reveal SMARCA4 to be one of the genes most frequently mutated in NSCLC cancer cell lines, highlighting the tumor suppressor activity of SWI/SNF. We also described the first somatic mutation of SMARCA4 in primary tumors (7). However, although SMARCA4 mutations were found frequently in lung cancer cell lines, we were unable to find this same frequency of mutations in primary tumors (7). Further studies, using more powerful sequencing techniques such as ultra-deep pyro-sequencing (8), displayed similar results with a mutation frequency of 5–10%. However, a tissue microarray of 245 NSCLC tumors detected the loss of expression of SMARCA4 in the 48% of the tumors, in contrast with normal tissue that express SMARCA4 in all cases (9). Intriguingly, the loss of the expression of SMARCA4 in primary tumors is similar to that observed in cell lines. Thus, as there exists an unknown mechanism that contributes to the functional inactivation of SMARCA4.

During carcinogenesis, cells develop different mechanisms to inactivate gene expression of tumor suppressor genes. Promoter hypermethylation is one of the most studied mechanisms of epigenetic silencing in tumor development. We previously studied whether SMARCA4 expression was inactivated by this mechanism by studying the promoter hypermethylation status of 52 lung cancer primary tumors and 10 lung cancer cell lines studied using methylation-specific PCR (MSP) and bisulfite sequencing. We concluded that promoter hypermethylation was not a mechanism of SMARCA4 expression inactivation (7).

MicroRNAs (miRNAs) are a class of small RNA molecules that negatively regulate gene expression at the post-transcriptional level by binding to complementary sequences in 3′ untranslated regions (UTRs), ushering in a renewed appreciation of the regulatory capabilities of non-coding RNA (ncRNA). Today, miRNAs are increasingly seen as important regulators of gene expression, and they have been shown to play an important role in human pathology, including cancer (10–12). Importantly, aberrant levels of miRNAs often result in loss of differentiation, a hallmark of cancer. Not surprisingly, therefore, dysfunctions of the miRNA pathway affect many cellular processes that are routinely altered in cancer, such as differentiation, proliferation, apoptosis, metastasis and senescence (10,11).

We developed the first animal model exhibiting oncogenic addiction to a miRNA (13), underscoring the key role of miRNAs in tumor development in vivo. Interestingly, recent studies have observed that miRNAs are able to very tightly control expression of SWI/SNF complex protein members (14,15). Therefore, it is plausible to believe that miRNAs could contribute to the loss of expression of SMARCA4 during lung development. The aim of this work is to determine whether SMARCA4 expression is lost in lung cancer primary tumors because of repression by miRNAs.

Results

SMARCA4 expression is regulated by miRNAs

Experimentally determining the sequence of the 3′ UTR of SMARCA4

Most studies predicting binding sites for miRNAs use non-empirically determined 3′UTRs. Since it was recently established that many genes may have alternative UTRs (16), this is not a trivial matter since the predicted 3′UTR or the one annotated in unsupervised databases is often not complete. To investigate the regulation of SMARCA4 expression by miRNAs, we experimentally determined the sequence of the 3′UTR of SMARCA4. For this purpose, we optimized a protocol of Rapid Amplification of 3′cDNA Ends (3′RACE), to amplify the possible 3′UTRs of SMARCA4 using as template, RNA of normal lung tissue. This experiment revealed two main 3′UTR fragments that we named ‘short’ and ‘long’ (Fig. 1A). The bands were extracted from the gel and sequenced, and found to correspond to two alternative SMARCA4 3′UTRs of a size of 242 and 466 nucleotides (nt), respectively, from stop codon to the polyA tail.

To study the relative usage and prevalence of these SMARCA4 3′UTRs in lung cancer, we studied a panel of five primary tumors and found that they each displayed the same pattern of two 3′UTRs (Supplementary Material, Fig. S1). We also studied the 3′UTR patterns in a panel of nine NSCLC cell lines (Fig. 1D). Calu3, H460 and H441 cells, previously sequenced and found to have no SMARCA4 mutations (6), displayed full-length SMARCA4 protein expression (Fig. 1E) and exhibited the two 3′UTRs of SMARCA4 found in primary tumors (Fig. 1D and Supplementary Material, Fig. S1B). The H2126 cell line that has a tumor-specific amino-acid substitution (W764R) and displays full-length SMARCA4 protein (Fig. 1E) also expresses both 3′UTRs. Interestingly, the cell lines H23, A427, H522, A549 and H1299 that have SMARCA4 mutations that are predicted to cause truncated proteins expressed none of the native 3′UTRs described above, and sequencing of the alternative products revealed only nonspecific sequences (Fig. 1E).

miR-21 does not affect expression of SMARCA4 in lung tumor cells

We started to test the possibility of the inactivation of SMARCA4 by miRNAs by testing those miRNAs already known to regulate SMARCA4 from the published literature. To date, there is only one report on this matter. That study identified SMARCA4 as an miR-21 target in prostate and lymphoma tumors (17). We tested whether SMARCA4 expression was affected by miR-21 in our different lung cell lines and found no involvement of miR-21 in regulation of SMARCA4 expression (Supplementary Material, Figs S2 and 3 and Fig. 3A). Consequently, we decided to determine which miRNAs are able to regulate SMARCA4 expression in lung tumors.

Identifying predicted miRNAs’ binding sites in the SMARCA4 3′UTR

We experimentally determined the 3′UTR length of SMARCA4 to properly assess its putative miRNA binding sites. We used four different bioinformatic tools (DIANA-mT, miRanda, mirWalk and Targetscan) to predict miRNA-binding sites. We selected the four top-ranked miRNAs commonly found in the different
prediction algorithms (miR-7, miR-101, miR-155 and miR-199) to use for functional analysis. Significantly, the predicted miRNA-binding sites are located in conserved regions among mammalian genomes (Fig. 1B and C). Interestingly, although miR-21 was described as a SMARCA4 regulator (17), none of the 472 putative miRNA-binding sites predicted by the combination of DIANA-mT, miRanda, miRWalk and Targetscan algorithms detect a binding site for miR-21.

**Functional validation of the miRNAs**

To validate the selected miRNAs predicted to regulate SMARCA4 expression, we performed a variety of strategies. Firstly, we developed a functional miRNA-binding test using Luciferase-SMARCA4-3’ UTR constructs. For this purpose, we cloned the two different SMARCA4 3’UTRs that we found previously (long and short) in pGL4.75 plasmid behind the Renilla Luciferase reporter gene. To normalize the transfection, we utilized pGL3-control that expresses Firefly Luciferase. We used as model A549, a NSCLC cell line, because it has no SMARCA4 expression (Fig. 1E) due to a frameshifting homozygous mutation that we described previously (6). In this genetic background, with no wild-type SMARCA4 mRNA to confound these assays (Supplementary Material, Fig. S3C), we tested for functional binding of the following miRNAs: miR-7, miR-101, miR-155, miR-199 and included let-7, miR-21 as controls, by transfecting miRNA mimics with the luciferase plasmids. Although miR-21 was not predicted by any of the four algorithms, we tested this miRNA because it was previously described to regulate SMARCA4 expression in some prostate and lymphoma tumors (17). We used let-7 as a negative control, because this miRNA was not predicted to bind to these 3’UTRs. We also used a scrambled miRNA mimic as an additional negative control. From these assays, we clearly observed that miR-21, let-7 and miR-7 (Supplementary Material, Fig. S2) did not affect the expression of the luc SMARCA4-3’UTR constructs. miR-101 and miR-199 showed an effect on the long 3’UTR of SMARCA4, but interestingly these miRNAs did not affect the short SMARCA4 3’UTR (Fig. 2). Finally, as was predicted by the algorithms, miR-155 caused a change in reporter gene expression when either the short or long SMARCA4 3’UTRs were used. Importantly, miR-155 was the most efficient miRNA in inhibition of luc SMARCA4-3’UTR expression. These results suggest that miR-101, miR-199 and miR-155 have the potential to regulate SMARCA4 expression through binding to its 3’UTR.
Once we determined that miR-101, miR-155, miR-199, but not miR-7, miR-21, let-7 or the miRNA scramble was able to regulate the SMARCA4 3’UTR, we extended these observations by examining the endogenous expression of SMARCA4. For this purpose, we cannot use the A549 cell line, because, as we have indicated above, SMARCA4 expression is inactivated by mutation (Fig.1E). It was difficult to find an NSCLC lung cancer cell line with wild-type expression of SMARCA4, since many of them bear inactivating mutations of SMARCA4. We previously sequenced the full SMARCA4 mRNA coding sequence from H441 and H460 cell lines and found no mutations (6). Western blot also showed that these cell lines displayed full length of SMARCA4 protein (Fig.1E). Thus, we transfected miR-101, miR-155, miR-199 whose binding sites are not present in the short 3’UTR don’t change significantly the luciferase expression.

Figure 2. (A) Luciferase assays performed in the A549 lung cancer cell line to functionally test miRNA-binding sites of predicted miRNAs on SMARCA4 3’UTR-long: miR-101, miR-155 and miR-199 inhibit luciferase expression significantly. (B) Luciferase assays performed in the A549 lung cancer cell line to functionally test miRNA-binding sites of predicted miRNAs on SMARCA4 3’UTR-short: miR-155 whose binding site is present in the 3’UTR significantly inhibits luciferase expression. miR-101 and miR-199 whose binding sites are not present in the short 3’UTR don’t change significantly the luciferase expression.

miR-155 is necessary and sufficient to repress the SMARCA4 3’UTR

As miR-155 was the only microRNA able to regulate both long and short SMARCA4 3’UTRs very effectively, we studied their functional relationships further. To test the necessity of miR-155 to regulate the 3’UTR of SMARCA4, we performed the opposite experiment. In cells that overexpress miR-155, we tested whether the introduction of anti-miR-155 results in an increase of SMARCA4 expression. For this objective, we first performed screening to find a cell line that overexpresses miR-155 to use as a model. Five NSCLC lung cancer cell lines were tested for miR-155 expression using TaqMan qPCR. The H1299 cell line was chosen as model because of its high level of miR-155 expression and lack of SMARCA4 expression (Supplementary Material, Fig. S3B and C). When we transfected H1299 cells with luciferase-SMARCA4 3’UTR constructs and anti-miR-155, we observed a significant de-repression of the luciferase activity compared with the use of scramble as control (Fig. 3B). These results are in agreement with the hypothesis that SMARCA4 is negatively regulated by miR-155 expression.

To test the hypothesis that miR-155 acted through the predicted miR-155 complementary elements in the SMARCA4 3’UTR, we altered the putative binding site of miR-155. For this purpose, we substituted the miR-155 binding site (AGCAUUA) with a let-7 binding site (CTACCTC) using a PCR-based mutagenesis protocol (18). Consequently, with this substitution, we observed significant down-regulation in luciferase only with the transfection of let-7 (P = 0.002), but not with transfection with scramble or miR-155 (Fig. 3C right). In the luciferase construct with the native 3’UTR, we observed significant repression
miR-155’s role in cell proliferation in lung tumors can be explained through its role-regulating SMARCA4 expression

miR-155 is up-regulated in lung cancer (19) and has also prognostic value, being able to predict recurrence and survival in lung cancer (20,21). We studied the significance of SMARCA4 inhibition by miR-155 in the tumoral phenotype. For this purpose, we used the H441, a NSCLC that express SMARCA4 (Fig. 1E) and has no mutation in its sequence (6). H441 cell line was transfected with mature miR-155 or a control scramble miRNA in presence of a plasmid that codified SMARCA4 but without its 3’UTR (i.e. without miRNA complementary sites and therefore not regulated by miR-155) or a control vector. We measured cell viability after 48 h using a MTT assay (Fig. 4A). The cells that were transfected with miR-155 and control plasmid proliferated significantly 60% faster (P-value = 0.0083) than the ones transfected with the scramble miRNA and the control plasmid demonstrating the oncogenic role of miR-155. However, this phenotype of enhanced proliferation mediated by miR-155 was largely rescued (in a 82.5%) with the transfection of SMARCA4 without 3’UTR (and therefore insensitive to miR-155), suggesting that the oncogenic activities of miR-155 in this cell line was related largely to its role inhibiting SMARCA4.

Correlation of miR-155 and SMARCA4 expression in lung primary tumors

Since we had compelling data about the functional relationship between miR-155 and SMARCA4 in cell lines, we tested whether this relationship extended to primary tumors. In an initial approach, we used expression microarray data of 69 NSCLC tumors that we published previously (22) using a cDNA expression microarray by competitive hybridization (CNIO-Oncohip) and whose data are uploaded on the NCBI’s Gene Expression Omnibus database (accession number GSE8569). Importantly, the expression data of the transcript MIR155HG (that contains the precursor RNA of miR-155) and SMARCA4 are negatively correlated
($r = -0.53$), and this correlation is very significant ($P = 0.00000924$) (Fig. 4B left). Next, we studied the correlation using external expression data obtained from 44 NSCLC in the data set GSE19188. Despite the fact that a different expression platform (Affymetrix Human Genome U133 Plus 2.0 Array) was used, the expression data of the transcript MIR155HG and SMARCA4 were also similarly negatively correlated ($r = -0.54$) and very significant ($P = 0.00017229$) (Fig. 4A middle). However, to study this correlation more accurately, we analyzed the expression of SMARCA4 and mature miR-155 in an independent set of tumors. For this purpose, we extracted the RNA of 13 samples of NSCLC and performed a specific qPCR to detect the mature miR-155 and SMARCA4 expression levels (Fig. 4B). Significantly, the negative correlation of miR-155 and SMARCA4 was remarkably high, with $r = -0.93$, with a $P$-value of 0.00000181. Importantly, it is known that lung primary tumors (19) overexpress miR-155, and therefore, these results validate our hypothesis that high miR-155 levels can contribute to inactivate SMARCA4 expression and aid tumor progression.

**Discussion**

The latest deep-sequencing efforts on tumor genomes have underlined the important and ubiquitous role of the SWI/SNF complex and cancer (3,4). However, although SWI/SNF complex plays a key role in gene expression, the regulation of SWI/SNF complex itself is poorly understood. In this report, we show that expression SMARCA4, encoding the SWI/SNF catalytic subunit, is regulated by miRNAs through two alternative 3'UTRs (Fig. 1). Importantly, our data demonstrate that the loss of expression of SMARCA4 observed in primary lung tumors (9,23), whose mechanism was unknown to date, could be explained by the activity of miRNAs, in particular miR-155.

The identification of miRNAs and their roles adds another layer to the complexity of control of gene regulation. Many studies suggest that dysregulation of miRNAs are an important step in development of several cancers, including lung cancer. miR-155 is located in the chromosomal region 21q21.3 within a conserved region of the non-coding MiR-155 host gene (MIR155HG) formerly...
called B-cell integration cluster (BIC). Overexpression of miR-155 has been observed in several tumor types including lung tumors (19). miR-155 is a well-known oncogenic miRNA; among its targets, mostly studies in hematological malignancies are the proteins SHIP1 and C/EBPbeta (24-26). In this work, we determined for first time that miR-155 is able to regulate both 3’UTRs of SMARCA4 and down-regulate their expression very effectively, both in Luciferase-SMARCA4-3’UTR constructs assays and on the native SMARCA4 protein. Importantly, we demonstrated that the oncogenic activity of miR-155 in H441 NSCLC cell line can be explained largely because of its role inhibiting SMARCA4 expression (Fig. 4A). Significantly, this work functionally connects for first time two important genes in tumor development: miR-155 and SMARCA4. Interestingly, SMARCA4 expression is lost in lung primary tumors, and importantly, this loss of expression has prognostic value (8,23). Conversely, miR-155 is up-regulated in lung cancer (19) and has also prognostic value, being able to predict recurrence and survival in lung cancer (20,21). We have demonstrated that miR-155 levels are very significantly negatively correlated with SMARCA4 expression (r = −0.93, P = 0.0000018) in lung primary tumors. Since SMARCA4 is a target of miR-155, it may explain, at least partially, the prognostic value of SMARCA4.

Significantly, the regulation of SMARCA4 expression has progressively gained importance due to a recently proposed therapeutic strategy (27,28) whereby synthetic lethality with SMARCA4 could be a viable cancer option. Importantly, the results of this work show that SMARCA4 expression can be modified using incipient miRNA technology (Supplementary Material, Fig. S4). Restoration of SMARCA4 expression is known to promote differentiation and to suppress tumor progression in cancer cell lines (29,30) and may have a therapeutic value in primary tumors. We have demonstrated that the inhibition of miR-155 using anti-miR-155 could restore levels of SMARCA4 expression and therefore could have therapeutic utility (Supplementary Material, Fig. S4I).

Conversely, inhibition of SMARCA4 expression has demonstrated therapeutic utility under some circumstances. Very recently, several papers have underscored the importance of SMARCA4 expression and proposed several therapeutic strategies in lung cancer based on SMARCA4 inhibition due to synthetic lethal interactions (27). In this way, the loss of SMARCA4 in lung cancer cells in which MAX is inactive resulted in cell death that suggests a synthetic lethal interaction (31). Therefore the use of miR-155 to inhibit SMARCA4 expression during these conditions may have therapeutic value (Supplementary Material, Fig. S4I).

The chromatin-remodeling complex SWI/SNF includes one SMARCA2 or SMARCA4 molecule as its catalytic subunit. Recently, several reports have identified SMARCA2 (also called BRM) as a critical synthetic lethal target in SMARCA4-deficient cancer (32,33). In this way inhibiting SMARCA2 expression suppressed the growth of SMARCA4-deficient cancer cells relative to SMARCA4-proficient cancer cells, inducing senescence. Interestingly, it was reported that miR-199a-5p and -3p target SMARCA2 (15) inhibiting its expression. Thus, we could speculate that the use of these miRNAs may have a therapeutic value in SMARCA4-deficient tumors (Supplementary Material, Fig. S4III). Notably, SMARCA2 attenuation due to post transcription suppression occurs often in tumor cells, in which this event contributes to their oncogenic potential (15,34). Using the same concept of synthetic lethality, we could target SMARCA4 using miR-155 in SMARCA2-deficient cells as a therapeutic strategy (Supplementary Material, Fig. S4IV).

In conclusion, the results obtained in this work could open the door to the development of new therapeutic strategies described above (Supplementary Material, Fig. S4) using miRNA-based technology.

Materials and Methods

Determining 3’UTRs of SMARCA4

We utilized the 3’ RACE (35) protocol to detect the different 3’UTRs of SMARCA4. Briefly, 1 μg total RNA was used to generate cDNA (iScript, Bio-Rad) according to the manufacturer’s instructions using the primer GACCTGAGTCGACATCG and SMARCA4-specific primer GACGGCTCAGAAGTGGCA. The PCR product from PCR was resolved in agarose gel and sequenced. For a loading control for RT-PCR at non-saturating conditions, we used the gADPH primers TCTCTTGTCCCAGGTCGCCAG (forward) and AGCCCCAGCTTCTCCA (reverse).

Cloning the long and short SMARCA4 3’UTRs

We amplified the short and long SMARCA4 3’UTRs by PCR using a high fidelity Taq polymerase (Phusion DNA Polymerases, Thermo Fisher). For the short 3’UTR, we used the primers AAAATCTTA GACCCCCGACATTCCAAGTCGACCC (forward) and TTTTCT TAGACTGCCAACATCCTCTTCTCCTCTCTTTTA (reverse). For the long 3’UTR, we used the primers AAAAACTGAGCCCCGACATTCCA AGTCTCGACCC (forward) and TTTTCTCAAGGCTGTGGCTTT TGTGGTGGTTTA (reverse). Once amplified, the PCR products were purified and digested with Xbal. Next, we digested pGL4.75 (Promega) with Xbal, dephosphorylated the ends with rapid alkaline phosphatase. Subsequently, the digested amplicons were introduced into the plasmid using T4-ligase. The final plasmid inserts were sequenced to ensure the correct sequence.

Cell lines

Calu3, H522, H23, H1299, H2126, A427, A549, H441, H460, H1299, H1975 (lung cancer cell lines) and HeLa were acquired from the American Type Culture Collection (ATCC, Rockville, MD) and maintained using the recommended media.

Transfection and luciferase assays

Cells were plated in 24-well plates to get a 70% confluence at the time of transfection. Each well was transfected with 600 ng expression vector containing full-length 3’UTR of SMARCA4 (pGL4.75 3’UTR SMARCA4) and 200 ng pGL3-control luciferase reporter vector and 30 nM miRNA mimics using lipofectamine 2000 according to the manufacturer’s instructions. The miRNA mimics used: hsa-miR-21 (PM10206), hsa-miR-101 (PM11414), hsa-miR-199 (PM10893), hsa-miR-7 (PM10047), hsa-let-7 g (PM11758), hsa-miR-155 (PM12601), miRNA negative control (miR-scr), hsa-anti-miR-155 (AM12601) and anti-miR negative control (AM17010) were purchased from Ambion. The cells were harvested 24 h after transfection and assayed using the Dual-Glo Luciferase Reporter Assay Kit (Promega) in a Glomax Luminometer (Promega).

3’UTR mutagenesis assays

The mutagenesis was performed adapting the site-directed mutagenesis by Overlap Extension protocol described in (18). The PCR reactions were performed with Phusion Hot Start II High-Fidelity DNA polymerase (Thermo Scientific) and the primers
Total RNA was isolated from 13 NSCLC lung tumor samples using the TRIzol reagent (Invitrogen). Ten nanograms of total RNA were retro-transcribed using the TaqMan miRNA reverse transcription kit (ABI) using specific primers for hsa-miR-155 (ref. 2623, Applied Biosystems) and the normalization control RNU6B (ref. 1093, Applied Biosystems). miRNA-specific qPCR was performed using Master Mix TaqMan Gene Expression (Applied Biosystems) and the specific TaqMan probes for miR-155 and RNU6B following the manufacturer’s instructions. Quantification was determined using the Standard curve method. To quantify SMARCA4 expression levels, cDNA was synthesized using the iScript Select cDNA Synthesis kit (BIO-RAD) and random primers following the manufacturer’s instructions. 3.5 ng of cDNA was mixed within the iTaq Universal SYBR Green Supermix (BIO-RAD) and 1 µM of both forward (5’-TACCCTCAGGAACACTTCTCC-3’) and reverse (5’-ACCCGATCTCCATTTCCCGTT-3’) primers. The reaction was normalized with the TATA-binding protein (TBP) using the primers forward (5’-TACCCTCAGGAACACTTCTCC-3’) and reverse (5’-GACCAAGGTTTACGAATGTCC-3’) primers. Quantification was determined using the Standard curve method. The specificity of the reaction was verified on SYBR Green qPCR by melt curve analysis. Quantitative PCR reactions were performed using an ABI Prism 7500 System (Applied Biosystems), and data processing was performed using ABI SDS v2.04 software (Applied Biosystems).

**Protein extraction and western blot**

Cells were recollected in RIPA buffer. Lysates were centrifuged at 14 000 rpm at 4°C for 10 min. The supernatants were used for western blot assay, and the protein concentration was measured by BCA Protein Assay (Thermo Scientific, USA). The amount of protein loaded per lane was 50 µg. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions was performed in a Mini Protean II electrophoresis system (Bio-Rad, Richmond, USA). The samples were mixed with a sample buffer that contained 62.5 mM Tris–HCl at pH 6.8, 20 g l−1 SDS, 100 ml−1 glycerol, 25 g l−1 β-mercaptoethanol and 0.045 mmol l−1 bromophenol blue and then heated for 5 min at 100°C. Polyacrylamide gels were prepared using 5% SDS-PAGE under denaturing conditions. After electrophoresis, the gel was stained with 0.045 mmol l−1 Tris–HCl, 100 mM NaCl, 2.5 mM KCl, pH 7.6, 1 ml l−1 Tween 20 and 30 g l−1 denatured fat-free milk at pH 7.6. Membranes were incubated with antibodies against SMARCA4 (1:200, Santa Cruz Bio-technology, Inc., sc-10768) and β-actin (1:20 000, Sigma Aldrich; A5441) during 2 h at RT. After three washes with PBS-T, membranes were incubated with a HRP-conjugated antibody anti-rabbit IgG or anti-mouse IgG (1:10 000, DAKO) for 1 h at RT. Blots were developed using the ECL Plus Western blot detection system (GE Healthcare). The specific signals were measured in a chemiluminescence detector (Image Quant Las 4000).

The levels of SMARCA4 expression were shown as arbitrary intensity units of each band compared with arbitrary intensity units of actin. The values represent SMARCA4/β-actin ratio ± SD of at least three separate experiments. The specific signals were quantified using the Multi Gauge programme (Fuji Film Europe).

**Cell proliferation by MTT**

Cell proliferation was assessed using the MTT (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. H441 cells were counted, and 1 × 10⁴ cells were placed into single wells in a 96-well plate. Cells were transfected the next day with plasmid (pCDNA4 or pCDNA4-SMARCA4) and miRNAs (miR-155 or scramble) in triplicate using lipofectamine 2000. After 48 h, MTT was added to all the wells at a final concentration of 0.5 mg ml⁻¹. Following 2-h incubation, the formazan generated was dissolved with 100 µl dimethyl sulfoxide per well. Optical density was measured on a GLOMAX plate reader (GLOMAX® Multidetection System) at 560 nm. Absorbance was proportional to the number of viable cells.

**Statistical analysis**

Data are shown as the median ± the standard deviation (SD). The statistical significance and Pearson correlation coefficients were determined by Student’s t-test using SPSS software.

**Supplementary Material**

Supplementary Material is available at HMG online.

Conflict of Interest statement. None declared.

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