Research article

MicroRNA-1911-3p targets mEAK-7 to suppress mTOR signaling in human lung cancer cells

Daniela Baccelli Mendonça a,b, Joe Truong Nguyen a,b, Fatima Haidar a,b, Alexandra Lucienne Fox a,b, Connor Ray a,b, Halimah Amatullah a,b, Fei Liu a, Jin Koo Kim c, Paul H. Krebsbach c,*

a Department of Biologic and Materials Sciences, University of Michigan, Ann Arbor, MI, 48105, USA
b Biointerfaces Institute, University of Michigan, Ann Arbor, MI, 48105, USA
c Section of Periodontics, University of California, Los Angeles, School of Dentistry, Los Angeles, CA, 90095, USA

ARTICLE INFO

Keywords:
MicroRNA-1911-3p
mEAK-7
mTOR signaling
Lung cancer
Cell proliferation
Cell migration
Cell biology
Gene expression
Gene regulation
Cancer research
Oncology

ABSTRACT

Regulation of mTOR signaling depends on an intricate interplay of post-translational protein modification. Recently, mEAK-7 (mTOR associated protein, eak-7 homolog) was identified as a positive activator of mTOR signaling via an alternative mTOR complex. However, the upstream regulation of mEAK-7 in human cells is not known. Because microRNAs are capable of modulating protein translation of RNA in eukaryotes, we conducted a bioinformatic search for relevant mEAK-7 targeting microRNAs using the Exiqon miRSearch V3.0 algorithm. Based on the score obtained through miRSearch V3.0, the top predicted miRNA (miR-1911-3p) was studied. miR-1911-3p mimics decreased protein levels of both mEAK-7 and mTORC1 downstream effectors p-S6 and p-4E-BP1 in non-small cell lung carcinoma (NSCLC) cell lines H1975 and H1299. miR-1911-3p levels and mEAK7 mRNA/mTOR signaling levels were negatively correlated between normal lung and NSCLC cells. miR-1911-3p directly interacted with mEAK7 mRNA at the 3'UTR to negatively regulate mEAK-7 and significantly decreased mTOR localization to the lysosome. Furthermore, miR-1911-3p significantly decreased cell proliferation and migration in both H1975 and H1299 cells. Thus, miR-1911-3p functions as a suppressor of mTOR signaling through the regulation of mEAK7 mRNA translation in human cancer cells.

1. Introduction

Mammalian EAK-7, or MTOR associated protein, eak-7 homolog (mEAK-7), is a positive regulator of mTOR signaling that functions through the S6K2/4E-BP1 axis in human cells [1]. mEAK-7 is expressed predominantly in metastatic human cancer and forms a novel mTOR complex involving DNA-PK to promote S6K2 signaling and suppress 4E-BP1 [2]. It is well known that aberrant mTOR signaling results in diverse diseases such as diabetes type II, neurological disorders, and cancer [3], and thus a more complete understanding of the interactions of mTOR and mEAK-7 may be important to preventing or treating these human conditions.

mTOR is a member of the phosphatidylinositol-3 kinase-related kinase (PIKK) family [4]. PIKKs typically have redundant functions and include ataxia-telangiectasia mutated (ATM), ataxia- and Rad3-related (ATR), and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) [5]. PIKKs share redundancies to effectively regulate cell metabolism, DNA repair pathways, and genome surveillance. mTOR signaling is defined by two multi-protein complexes. mTOR complex 1 (mTORC1) targets S6K1/4E-BP1 and mTOR complex 2 (mTORC2) targets Akt [3]. Both complexes function in the lysosome, an essential cellular compartment for mTOR signaling [6, 7]. mEAK-7 is anchored at the lysosomal membrane and forms an alternative mTOR complex with DNA-PK to regulate the lysosome [1, 2]. We have posited that this novel complex is a third member of known mTOR complexes, mTORC3. Other research groups have identified an astrocyte-specific mTOR complex [8] and a rapamycin insensitive mTOR complex in human cancer cells [9]. These findings demonstrate the existence of multiple mTOR complexes and suggest the possibility that additional cell-type or disease-specific mTOR complexes may exist.

MicroRNAs (miRNAs) are short, non-coding RNA molecules that mediate gene silencing by guiding an intricate series of protein

* Corresponding author.
E-mail address: pkrebsbach@dentistry.ucla.edu (P.H. Krebsbach).

https://doi.org/10.1016/j.heliyon.2020.e05734
Received 2 June 2020; Received in revised form 3 November 2020; Accepted 11 December 2020
2405-8440/© 2020 The Author(s). Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
complexes to target specific motifs in the 3’ untranslated region (UTR) of messenger RNAs (mRNAs) [10]. The first miRNA, named lin-4, was reported in *Caenorhabditis elegans*, and was shown to be required for post-transcriptional repression of lin14, a protein that regulates the timing of several developmental processes in worms [11]. It was later discovered that this class of mRNA regulation was essential for eukaryotic development and was discovered in many different animal phyla [12]. Collectively across eukaryotes, these conserved RNA molecules are known as miRNAs [13]. Recently, it has been appreciated that mTOR signaling may also be modulated by post-transcriptional regulation.

**Table 1. Exiqon miRSearch for potential mEAK-7 (KIAA1609) related miRNAs.**

| # | microRNA Accession number |
|---|--------------------------|
| 1 | has-miR-1911-3p MIMAT0007886 |
| 2 | has-miR-1233-5p MIMAT0022943 |
| 3 | has-miR-1294 MIMAT0005884 |
| 4 | has-miR-149-3p MIMAT0004609 |
| 5 | has-miR-202-3p MIMAT002811 |
| 6 | has-miR-3192-5p MIMAT0015076 |
| 7 | has-miR-3649 MIMAT0018069 |
| 8 | has-miR-4314 MIMAT0016868 |
| 9 | has-miR-4529-3p MIMAT0019068 |
| 10 | has-miR-4648 MIMAT0019710 |

Figure 1. miR-1911-3p is an effector for mEAK-7 and mTOR signaling in human lung cancer cells. H1975 (A) and H1299 (B) cells were transfected with miR-control or miR-1911-3p mimic for 48 h to evaluate mEAK-7 and mTOR signaling. GAPDH was used as a loading control. See full images in Supplementary Figure S1. Expression of miR-1911-3p (C) and MEAK7 (D) in H1299, H1975, and BEAS-2B cells was quantified by qPCR. microRNA and mRNA levels were normalized to RNU6-1 and GAPDH, respectively. Data are expressed as means ± SD of 3 independent experiments. n.s., not significant. (E) Expression of mEAK-7 and mTOR signaling in H1299, H1975, and BEAS-2B cells was analyzed by Western blotting. β-actin was used as a loading control. See full images in Supplementary Figure S2. All experiments were repeated at least three times.
through miRNA targeting mTOR and upstream regulators of mTOR [14]. Within diverse cancer types, miRNA expression profiles are substantially different compared to their normal tissue counterparts, and miR-21 overexpression directly activates aberrant mTOR signaling in many solid cancers [14, 15].

In this study, we hypothesized that miRNAs regulate MEAK-7 to modify mTOR signaling in cancer cells. We investigated the extent to which miR-1911-3p regulates mTOR signaling through MEAK-7.

2. Materials and methods

2.1. Cell culture

Human non-small lung cancer cell lines H1975 (ATCC CRL-5908) and H1299 (ATCC CRL-5803) were obtained from American Type Tissue Collection (ATCC) and maintained in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific (TFS), cat# 11995-073) containing 10% fetal bovine serum (FBS) (TFS, cat#10437-036). A human bronchial epithelial cell line BEAS-2B (ATCC CRL-9609) was obtained from ATCC and maintained in Bronchial Epithelial Cell Growth Basal Medium (Lonza, CC-3171) containing growth factors and supplements from BEGM BulletKit (Lonza, CC-4175).

2.2. miRNA or plasmid transfection

miRNA negative control (TFS, cat# 4464059), miR-1911-3p mimic (TFS, cat# 4464067), and miR-1911-3p inhibitor (TFS, cat# 4464085) were obtained from TFS, pmirGLO Dual-Luciferase miRNA Target Expression Vector was purchased from Promega (cat# E1330). On the day prior to transfection, 50,000 cells were seeded into 60-mm tissue culture plates. Then, cells were transiently transfected with 100 nM of miRNAs for 48 h, using Lipofectamine RNAiMAX Transfection Reagent (TFS, cat# 13778-150). For plasmid transfection, we used FUGENE 6 Transfection reagent (Promega, cat# E2691). For miRNA and DNA co-transfection experiments, both transfection reagents were added.

2.3. Cell proliferation and migration

Cells were seeded at a density of 500,000 into 60-mm tissue culture plates and grown for 24 h. Cells were transfected with 100nM of miR-control or miR-1911-3p mimic. For cell proliferation, 48 h after transfection, 200,000 cells were seeded into 100-mm tissue culture plates and counted after days 3 and 5 using the LUNA Automated Cell Counter (Logos Biosystems (LB), cat# L10001) along with LUNA Cell Counting Slides (LB, cat# L12003) and AO-PI dye (LB: cat# F23001). For cell migration, 48 h after transfection, cells were seeded at 50,000 cells per well into CIM 16-well plates (ACEA Biosciences, cat# 0565817001). Cell migration was captured in real time for 4 h using the xCelligence System, RTCA DP instrument (ACEA Biosciences, cat# 00380601050) and processed by RTCA Software 2.0.

2.4. Microarray and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using miRNeasy Mini kit (Qiagen, cat# 217004), according to manufacturer's instructions. Microarray analysis was performed by the Advanced Genomics Core (University of Michigan). The quality of each RNA sample was analyzed using a 2100 Bioanalyzer (Agilent Technologies). Biotinylated cDNA were prepared from 400 ng of total RNA using the GeneChip Whole Transcript PLUS Reagent Kit (Applied Biosystems) according to the Affymetrix Plus Whole Transcript probe protocol. For data processing, the Robust Multi-Array Average (RMA) method was used to fit log2 expression values to the data using the oligo bioconductor package in R version 3.3.0. The bioinformatics analysis of microarray data was performed by iPathwayGuide software of Advaita Corporation (www.Advaitabio.com). For qRT-PCR, 1 µg of RNA was reverse transcribed using SuperScript VILO cDNA synthesis kit (TFS, cat#
11754050), and cDNA was used for qRT-PCR using mEAK-7 (TLD1) primer (TFS, cat# 4331182) in an Applied Biosystems 7900HT Real Time PCR System (TFS). mRNA levels were normalized to ACTB or GAPDH as a housekeeping gene. For microRNA analysis, microRNA was isolated using a Mirpremier microRNA isolation kit (Sigma, cat# SNC50-1KT), according to manufacturer's instructions. MicroRNAs (100 ng) were first polyadenylated and then reverse transcribed using MystiCq microRNA cDNA Synthesis Mix kit (Sigma, cat# MIRRT). cDNA was used for qRT-PCR using miR-1911-3p primer (GeneCopoeia, cat# HRNQP0266) and MystiCq microRNA SYBR Green qPCR ReadyMix (Sigma, cat# MIRRM00) in a CFX96 Real Time PCR System (Bio-Rad). microRNA levels were normalized to RNU6-1 (MystiCq microRNA qPCR control primer, Sigma, cat# MIRCP00001).

2.5. Western blot

Cells were harvested and lysed with cold NP-40 lysis buffer (50 mM tris, 150 mM NaCl, and 1.0% NP-40 at pH 8.0). 50 μg of protein lysate was separated in Novex WedgeWell 4-20% gradient tris-glycine gels (TFS, cat# XP04205BOX), and transferred to PVDF membranes. Membranes were blocked with 5% non-fat dry milk in 1x TBST, incubated with primary antibodies overnight at 4 °C, and then with HRP-conjugated secondary antibodies for 1 h at room temperature. Blots were developed using SuperSignal West Pico (TFS, cat# 34078) or Femto (TFS, cat#34095) Chemiluminescent Substrate Solution. The primary antibodies used were: mouse monoclonal antibody against mEAK-7 (KIAA1609), which was obtained from Origene Technologies (clone OTI12B1, formerly 12B1, cat# TA501037). All antibodies from Cell Signaling Technology were: GAPDH (cat# 2118S), β-actin (4970), (Ser235/236) p-S6 (2211), (Ser240/244) p-S6 (2215), S6 ribosomal protein (2217), (Thr37/46) p–4E-BP1 (9459), (Ser65) p–4E-BP1 (9451), (Thr70) p–4E-BP1 (13396), and 4E-BP1 (9452). Antibodies p-S6, S6, and 4E-BP1 were used at 1:3000 dilution and the remainder at 1:1000 dilution in 5% bovine serum albumin (BSA) in TBST buffer with 0.04% sodium azide. Secondary antibodies for immunoblot analysis: 1:4000 dilution for an α-mouse IgG horseradish peroxidase (HRP) conjugate (Promega, cat# W4021) and 1:7500 dilution for an α-rabbit IgG HRP conjugate (Promega, cat# W4011).

Table 3. The most impacted pathways obtained from the microarray analysis.

| Ranking | Pathway name       | # genes (Deregulated|All) | p-value |
|---------|--------------------|-------------------|------|---------|
| 1       | Influenza A        | 26/77             | 5.43E-06 |
| 2       | Hepatitis C        | 23/71             | 6.68E-06 |
| 3       | Cell cycle*        | 24/69             | 7.34E-06 |
| 4       | MAPK signaling     | 35/138            | 1.05E-05 |
| 5       | IL-17 signaling    | 18/47             | 2.17E-05 |
| 6       | MicroRNA in cancer | 30/142            | 2.73E-05 |
| 7       | Epstein-Barr virus infection | 27/94 | 5.60E-05 |
| 8       | DNA replication    | 11/26             | 7.27E-05 |
| 9       | Ferroptosis        | 8/16              | 1.76E-04 |
| 10      | Metabolic          | 99603             | 1.87E-04 |
| 11      | mTOR signaling*    | 1968              | 1.98E-04 |
| 12      | PI3K-Akt signaling*| 34/147            | 3.90E-04 |
| 13      | Lysosome*          | 14/51             | 0.002  |

* mTOR signaling related pathway.

Table 4. The deregulated mTOR signaling genes obtained from the microarray analysis.

| Ranking | Gene symbol | LogFC | p-value |
|---------|-------------|------|---------|
| 1       | SGK1        | 1.128| 3.45E-05 |
| 2       | DDT4        | -1.321| 1.41E-04 |
| 3       | WNT7B       | -1.288| 1.58E-04 |
| 4       | MIOS*       | -1.013| 1.64E-04 |
| 5       | PRKCG       | 1.038| 1.70E-04 |
| 6       | SLC3A2*     | -0.9| 2.03E-04 |
| 7       | P6K3R3      | 0.708| 2.67E-04 |
| 8       | FGDS        | 0.885| 3.15E-04 |
| 9       | ATP6V1C2*   | 0.885| 3.91E-04 |
| 10      | SLC7A5*     | -0.657| 5.84E-04 |
| 11      | LRBP5       | 0.649| 7.80E-04 |
| 12      | RRAGA*      | -0.765| 8.91E-04 |
| 13      | RPTOR       | 0.702| 0.001  |
| 14      | WNT9A       | 0.8| 0.002  |
| 15      | RPS6KA1     | -0.671| 0.003  |
| 16      | IRS1        | 0.698| 0.004  |
| 17      | TNF         | -0.589| 0.005  |
| 18      | LAMTOR3*    | -0.692| 0.014  |
| 19      | FNIP2*      | 0.594| 0.026  |

* The genes related to lysosomal regulation of mTOR by amino acids.
2.6. Cloning of 3’-UTR of MEAK7 and miRNA analysis via luciferase assay

Oligonucleotides containing the 3’-UTR of MEAK7 mRNA with the potential miR-1911-3p binding site, were cloned into Dual-Luciferase miRNA Target Expression Vector pmirGLO (Promega). The resulting ligated product (pmirGLO-3’-UTR-MEAK7) was transformed into DH5α competent cells (TFS, cat# EC0112). Plasmid DNA was confirmed by DNA sequencing (University of Michigan DNA Sequencing Core). Cell were co-transfected with 100 nM of miRNA-control or miRNA-1911-3p mimic in combination with 0.5μg of pmirGLO or pmirGLO-3’-UTR-MEAK7. After 48 h, cells were lysed in passive lysis buffer. Dual Luciferase Assay (Promega, cat# E1910) was performed according to the manufacturer's instructions. Luciferase activities were normalized with basal Renilla luciferase activities. The empty pmirGLO vector (pmirGLO Control) was set to 100% for luciferase activity.

2.7. Cell immunofluorescence analysis

Cells transfected with control miRNA or 1911-3p miRNA were seeded (62,500 cells/cm²) into a two-well Nunc Lab-Tek II Chamber Slide System (TFS, catalog #12-565-5) for 24 h. Then, cells were fixed with Z-Fix solution (Anatech LTD, catalog #170) for 10 min at room temperature, washed three times in PBS, and incubated with the following: unmasking solution (PBS, 2N HCl, and 0.5% Triton X-100) for 10 min, permeabilization solution (PBS and 0.02% Triton X-100) for 10 min, and 5% BSA for 1 h. Cells were incubated overnight at 4 °C with primary antibodies. Next, slides were washed with PBS and incubated in secondary antibodies for 1 h at room temperature. A Nikon Ti2 Eclipse confocal microscope (×60 oil magnification) was used to capture images. Images were captured with 1/32 frames/s, 1024 × 1024 image capture, 1.2 airy units, 0x line averaging, appropriate voltage, and power settings optimized per antibody. No modification was done, except image sizing reduction for figure preparation. Identical threshold settings captured images across three to five individual fields (10–15 cells) per condition, with the data representing at least three independent experiments. For quantitative analyses, confocal images in six random fields were used for counting the number of cells showing mTOR/LAMP2 colocalization. Primary antibodies for immunofluorescence were as follows: LAMP2 (1:1500, SCB, catalog #sc-18822) and mTOR (1:1000, CST, catalog #2983S). Both antibodies were used with a
working volume of 1.5 ml in 5% BSA in PBS. Secondary antibodies for immunofluorescence were as follows: Anti-mouse IgG (H + L), F (ab’)2 Fragment (Alexa Fluor® 488 Conjugate) (CST, catalog #4408) and anti-rabbit IgG (H + L), F (ab’)2 Fragment (Alexa Fluor® 594 Conjugate) (CST, catalog #8889). Both antibodies were used at a concentration of 1:1500 with a working volume of 1.5ml in 5% BSA in PBS. DAPI stain was used for DNA.

2.8. Statistical analysis

For statistical analysis, data are presented as mean ± S.E or S.D. Significance of the difference between two measurements was determined by unpaired Student’s t-test. Paired Student’s t-test was used for cell proliferation and migration. All experiments were repeated at least three times in all cell lines.

3. Results

3.1. miR-1911-3p decreases mEAK-7 protein levels to suppress mTOR signaling

Several cancer cell lines synthesize mEAK-7 protein [1]. Interestingly, while mEAK-7 protein is predominantly detected in human cancer cell lines, it is not consistently expressed in all cancers. To investigate the extent to which miRNAs modify mEAK-7 expression, the Exiqon database was assessed with the Exiqon miRSearch V3.0 algorithm (www.exiqon.com/miRSearch), and miR-1911-3p was identified as a top predicted target for mEAK-7 (Table 1). Non-small cell lung carcinoma (NSCLC) cells from ATCC, H1975 and H1299 were used for this study. To test the effect of this potential miRNA on mEAK-7 expression and mTOR signaling, H1975 and H1299 cells were treated with 100 nM mimics of...
this miRNA or miRNA controls for 48 h in DMEM medium containing 10% serum. miR-1911-3p mimics resulted in a significant decrease in mEAK-7 protein levels, thereby suppressing mTOR signaling through (Ser235/236) p-S6, (Ser 240/244) p-S6, (Thr 37/46) p-4E-BP1, (Ser 65) p-4E-BP1, and (Thr70) p-4E-BP1 in H1975 (Figure 1A) and H1299 cells (Figure 1B). Thus, we have identified a novel miRNA that targets MEAK7 to suppress mTOR signaling.

To investigate the basal expression levels of miR-1911-3p and MEAK7 in lung cancer and normal lung cells, miR-1911-3p and MEAK7 expression was examined by quantitative RT-PCR in H1299, H1975, and human bronchial epithelial cell BEAS-2B. miR-1911-3p expression levels were low in both H1299 and H1975 cells, but higher in BEAS-2B cells (BEAS-2B:H1299, fold change: 1.88; BEAS-2B:H1975, fold change: 2.33) (Figure 1C). In contrast, MEAK7 mRNA levels were higher in both H1299 and H1975 cells compared to BEAS-2B cells (H1299:BEAS-2B, fold change: 2.37; H1975:BEAS-2B, fold change: 2.85) (Figure 1D). Furthermore, the protein levels of mEAK-7 and mTOR signaling were significantly higher in both H1299 and H1975 cells compared to BEAS-2B cells (Figure 1E). Thus, these data showed a negative correlation between miR-1911-3p levels and MEAK7/mEAK-7/mTOR signaling levels in normal lung and NSCLC cells.

3.2. miR-1911-3p targets MEAK7 mRNA at 3'-UTR

MicroRNAs are a class of small, non-coding RNAs that have important regulatory roles in biological processes such as cell metabolism, proliferation, differentiation, migration, and apoptosis [16, 17]. miRNAs most often interact with the 3'-UTR of the target mRNA and either block translation into protein, or promote RNA degradation [16, 17]. miRNAs have also been associated with the onset and progression of human pathological conditions, including several types of cancer, exhibiting either oncogenic or suppressor functions, depending on the genes they regulate and the cellular context. A linkage between miRNAs and NSCLC progression has been described and specific miRNAs promote tumor metastasis [18, 19, 20, 21]. Interestingly, a single miRNA can regulate more than one target gene and this is particularly important in cancer because it is a heterogenic disease and typically cannot be treated by targeting a single gene [22]. Cancer related miRNAs and their target genes have important clinical applications as well [22].

To test whether miR-1911-3p regulates MEAK7 mRNA and other target genes in NSCLC cells, H1975 cells were transfected with miR-control or miR-1911-3p mimic for 48 h. Total RNA was isolated and microarray analysis was performed. A volcano plot of the array data demonstrated that 1,370 genes were significantly and differentially expressed in miR-1911-3p transfected cells compared to controls (Figure 2A) with the data represented in terms of their measured expression change (x-axis) and the significance of the change (y-axis). Microarray data demonstrated that MEAK7 levels were down-regulated by more than 2-fold (Figure 2B). For other target genes, microarray analysis revealed the top deregulated genes by miR-1911-3p (Table 2). The most positively regulated gene was EGR1. EGR1 functions as a transcriptional regulator [23]. EGR1 induces the tumor cell apoptosis via upregulating tumor suppressors, NAG-1 and PTEN directly. Studies suggested that EGR1 functions as a tumor suppressor gene [23]. The most
negatively regulated gene was PEG10. PEG10 is highly expressed in a variety of cancers containing lung cancer compared to normal tissues and promotes cancer proliferation and metastasis [24]. These data indicated that miR-1911-3p regulates both a tumor suppressor gene (EGR1) and oncogene (PEG10) to inhibit tumorigenesis. In addition, microarray analysis determined the most impacted pathways by miR-1911-3p (Table 3). miR-1911-3p significantly regulated the genes involving in mTOR signaling related pathways (cell cycle, PI3K-Akt signaling, and lysosome) and mTOR signaling pathway. In the deregulated mTOR signaling genes, seven of nineteen genes (MIOS, SLC3A2, ATP6V1C2, SLC7A5, RRAGA, LAMTOR3, and FNIP2) were genes related to lysosomal regulation of mTOR by amino acids (Table 4), suggesting that miR-1911-3p may target other mTOR signaling related genes as well as MEAK7 directly or indirectly. Interestingly, microarray data suggested that miR-1911-3p mimic selectively increased lysosome-related gene expression (Figure 3A) and selectively decreased cell cycle-related gene expression (Figure 3B) compared to other signaling-related gene expression (Figure 3, C-E).

With evidence that miR-1911-3p was capable of regulating MEAK7 mRNA levels, we sought to confirm the extent to which miR-1911-3p directly targeted the 3’-untranslated region (3’-UTR) of MEAK7 mRNA. The target relationship between MEAK7 mRNA and miR-1911-3p was investigated using a Dual Luciferase Assay. The 3’-UTR sequences of MEAK7 mRNA were cloned into a pmirGLO dual-luciferase miRNA target expression vector and the empty pmirGLO vector (pmirGLO Control) was set to 100% for luciferase activity. There was no change in luciferase activity when H1975 or H1299 cells were co-transfected with pmirGLO-3’-UTR MEAK7 and miRNA controls (Figure 4, A and B). However, when H1975 or H1299 cells were co-transfected with pmirGLO-3’-UTR MEAK7 and miR-1911-3p mimic, luciferase activity was decreased by about 50% (Figure 4, A and B). In the presence of miR-1911-3p inhibitor and pmirGLO-3’-UTR mEAK7, luciferase activity was not significantly increased (Figure 4, C and D) because both H1975 and H1299 cells have low levels of miR-1911-3p (Figure 1C). These results demonstrate that miR-1911-3p negatively regulated MEAK7 mRNA by binding to its 3’-UTR (Figure 4E).

3.3. miR-1911-3p impairs co-localization of mTOR to the lysosome

We have previously found that mEAK-7 knockdown impaired mTOR localization to the lysosome [1], demonstrating that mEAK-7 is important for mTOR localization. Because miR-1911-3p negatively regulated...
MEAK7 mRNA and protein levels, we hypothesized that miR-1911-3p may also influence mTOR localization to the lysosome. H1299 cells were transfected with miR-control or miR-1911-3p mimic for 72 h. miR-1911-3p significantly decreased mTOR/LAMP2 (lysosomal marker) colocalization (Control:miR-1911-3p, fold change: 4.75) (Figure 5, A and B), confirming that miR-1911-3p is also important for mTOR localization to the lysosome through MEAK-7.

3.4. miR-1911-3p suppresses cell proliferation and migration in NSCLC cells

mTOR signaling is well known for its ability to regulate cell proliferation [25] and cell migration [26]. Likewise, MEAK-7 has been shown to be an essential effector of cell proliferation and migration in human cancer cells [1]. Because miR-1911-3p negatively regulated MEAK-7 expression and subsequent mTOR signaling, lung cancer cell proliferation and migration were analyzed in response to miR-1911-3p. H1975 and H1299 cells were transfected with miR-control or miR-1911-3p mimic for 48 h. miR-1911-3p significantly decreased cell proliferation (Figure 6, A and B) and migration (Figure 6, C and D) in both H1975 and H1299 cells. Thus, miR-1911-3p suppressed mTOR signaling through down-regulation of MEAK-7, resulting in reduced cell proliferation and migration (Figure 7).

4. Discussion

Recent studies suggest multiple functions of miRNAs in NSCLC. For example, miR-99b acts as a tumor suppressor in NSCLC by directly targeting fibroblast growth factor receptor 3 [27]. Another study reported that miR-320a-3p regulates cell metastasis and invasion as a tumor suppressor through PI3K/Akt inactivation in NSCLC [28], and bioinformatic and functional analyses have been used to identify potential miRNAs and their regulatory mechanism in NSCLC [29] showing that some target genes of downregulated miRNAs are associated with PI3K-Akt signaling pathway, among other pathways. VEGFA and MYC were regulated by downregulated upregulated miRNAs. In this study, we observed that miR-1911-3p caused a down-regulation of VEGF and MYC, which were involved with PI3K-Akt and mTOR signaling pathway (Figure 3, C and D). Besides PI3K-Akt and mTOR regulation, miRNAs influence Transforming Growth Factor-β (TGf-β) signaling at multiple levels and the dysregulation of TGf-β1 signaling is often linked to several diseases, including cancer [30]. We found that miR-1911-3p caused an up-regulation of TGf-β1 in NSCLC (Figure 3B).

Because mTOR is a validated therapeutic target for cancer, miRNAs inhibiting mTOR signaling may provide a novel approach to facilitate an integrated anti-cancer therapy [22]. For example, miR-125b-5p is a potential therapeutic target of cancers with hyperactivated mTORC1 [31]. We recently identified a novel positive regulator of mTOR signaling, which we named MEAK-7 [1]. Here, we demonstrated that miR-1911-3p directly targets the 3’-UTR of MEAK7 mRNA and negatively regulates MEAK7 mRNA and MEAK-7 expression. Therefore, miRNA 1911-3p is able to significantly reduce MEAK-7-mediated mTORC1 signaling and suppress cell proliferation and migration in NSCLC cells, indicating a role for this miRNA as a tumor suppressor gene. To our knowledge, this is the first report linking miR-1911-3p to NSCLC and more importantly, linking miR-1911-3p to suppressing mTOR signaling through MEAK-7. Thus it will be important to determine if manipulation of miR-1911-3p expression could have a potential therapeutic application in lung cancer.

Declarations

Author contribution statement

Paul H. Krebsbach: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Daniela Baccelli Mendonça: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Joe Truong Nguyen: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Fatima Haidar, Alexandra Lucienne Fox, Connor Ray, Halimah Amatullah, Jin Koo Kim: Performed the experiments; Analyzed and interpreted the data.

Fei Liu: Analyzed and interpreted the data.

Funding statement

This work was supported by the National Institute of Dental and Craniofacial Research (1F30DE026048-01, R01-DE016530, and T32-DE007057); the Stuart & Barbara Padnos Research Award from the Comprehensive Cancer Center at the University of Michigan.

Data availability statement

Data included in supplemental material.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2020.e05734.

Acknowledgements

Figure 7 was created with BioRender.

References

[1] J.T. Nguyen, C. Ray, A.L. Fox, D.B. Mendonca, J.K. Kim, P.H. Krebsbach, Mammalian EAK-7 activates alternative mTOR signaling to regulate cell proliferation and migration, Sci. Adv. 4 (2018) eaao6838.
[2] J.T. Nguyen, F.S. Haidar, A.L. Fox, C. Ray, D.B. Mendonca, J.K. Kim, P.H. Krebsbach, MEAK-7 forms an alternative mTOR complex with DNA-PKcs in human cancer, Sciencce 17 (2019) 190–207.
[3] P.A. Saxton, D.M. Sabatini, mTOR signaling in growth, metabolism, and disease, Cell 168 (2017) 960–976.
[4] J. Kunz, R. Henriqués, U. Schneider, M. Deuter-Reinhard, N.R. Movva, M.N. Hall, Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression, Cell 73 (1993) 585–596.
[5] A.N. Blackford, S.P. Jackson, ATM, ATR, and DNA-PK: the trinity at the heart of the DNA damage response, Mol. Cell. 66 (2017) 801–817.
[6] E. Arias, H. Koga, A. Díaz, E. Mocholi, B. Patel, A.M. Cuervo, Lysosomal miR122/ PHEPP1/Akt regulate chaperone-mediated Autophagy, Mol. Cell. 59 (2015) 270–284.
[7] C. Betz, M.N. Hall, Where is mTOR and what is it doing there? J. Cell Biol. 203 (2013) 563–574.
[8] L.J. Smithson, D.H. Gutmann, Proteomic analysis reveals GTI1 as a novel mTOR complex component critical for mediating astrocyte survival, Genes Dev. 30 (2016) 1383–1388.
[9] F.C. Harwood, R.I.K. Geltink, B.P. O’Rahilly, M. Cardone, L. Janke, D. Finkielstein, I. Einit, L. Paul, P.J. Houghton, G.C. Grosveld, ETV7 is an essential component of a rapamycin-insensitive mTOR complex in cancer, Sci. Adv. 4 (2018) 1–18.
[10] L.F.R. Gebert, I.J. MacRae, Regulation of microRNA function in animals, Nat. Rev. Mol. Cell Biol. 20 (2019) 21–37.
[11] Y. Hong, R.C. Lee, V. Ambros, Structure and function analysis of LIN-14, a temporal silencing of let-7 heterochronic regulatory RNA, Nature 408 (2000) 86.
[12] A.E. Pasquinelli, B.J. Reinhart, F. Slack, M.Q. Martindale, M.I. Kurodak, B. Maller, J. Finnerty, J.C. Corbo, M. Levine, P. Leahy, E. Davidson, G. Ruvkun, Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA, Nature 408 (2000) 86–89.
[13] N.C. Lau, L.P. Lim, E.G. Weinstein, D.P. Bartel, An Abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans, Science 294 (2001) 858–862.
[14] Y. Zhang, B. Huang, H.Y. Wang, A. Chang, X.F.S. Zheng, Emerging role of MicroRNAs in mTOR signaling, Cell. Mol. Life Sci. 74 (2017) 2613–2625.
S. Volinia, G.A. Calin, C.-G. Liu, S. Ambis, A. Cimmino, F. Petrocca, R. Visone, M. Iorio, C. Boldrini, M. Ferracin, R.L. Punteit, N. Yamaihara, G. Lanza, A. Scarpa, A. Vecchione, M. Negrini, C.C. Harris, C.M. Croce, A microRNA expression signature of human solid tumors defines cancer gene targets, Proc. Natl. Acad. Sci. Unit. States Am. 103 (2006) 2257–2261.

L. He, G.J. Hannon, MicroRNAs: small RNAs with a big role in gene regulation, Nat. Rev. Genet. 5 (2004) 522–531.

M.R. Fabian, N. Sonenberg, W. Filipowicz, Regulation of mRNA translation and stability by microRNAs, Annu. Rev. Biochem. 79 (2010) 351–379.

D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, Cell 116 (2004) 281–297.

Q. Li, Y. Han, C. Wang, S. Shan, Y. Wang, J. Zhang, T. Ren, MicroRNA-125b promotes tumor metastasis through targeting tumor protein 53-induced nuclear protein 1 in patients with non-small-cell lung cancer, Canc. Cell Int. 15 (2015) 84.

D. Li, Y. Wei, D. Wang, H. Gao, K. Liu, MicroRNA-26b suppresses the metastasis of non-small cell lung cancer by targeting MIEN1 via NF-kappaB/MMP-9/VEGF pathways, Biochem. Biophys. Res. Commun. 472 (2016) 465–470.

X. Wu, T. Liu, O. Fang, W. Dong, F. Zhang, L. Leach, X. Hu, Z. Luo, MicroRNA-708-5p acts as a therapeutic agent against metastatic lung cancer, Oncotarget 7 (2016) 2417–2432.

A.G. Badler, D. Brown, J. Stoudemire, P. Lammers, Developing therapeutic microRNAs for cancer, Gene Ther. 18 (2011) 1121–1126.

T.T. Li, M.R. Liu, D.S. Pei, Friend or foe, the role of EGR-1 in cancer, Med. Oncol. 37 (2019) 7.

T. Xie, S. Pan, H. Zheng, Z. Luo, K.M. Tembo, M. Jamal, Z. Yu, Y. Yu, J. Xia, Q. Yin, M. Wang, W. Yuan, Q. Zhang, J. Xiong, PEG10 as an oncogene: expression regulatory mechanisms and role in tumor progression, Canc. Cell Int. 18 (2018) 112.

R.J. Dowling, I. Topisirovic, T. Alain, M. Bidinosti, B.D. Fonseca, E. Petroulakis, X. Wang, O. Larsson, A. Selvaraj, Y. Liu, S.C. Kuzma, G. Thomas, N. Sonenberg, mTORC1-mediated cell proliferation, but not cell growth, controlled by the 4E-BPs, Science 328 (2010) 1172–1176.

A.C. Hsieh, Y. Liu, M.P. Eddin, N.T. Ingolia, M.R. Janes, A. Sher, E.Y. Shi, C.R. Stumpf, G. Chiswensen, M.J. Bonham, S. Wang, P. Ren, M. Martin, K. Jessen, M.E. Feldman, J.S. Weissman, K.M. Shokat, G. Rommel, B. Ruggero, The translational landscape of mTOR signalling steers cancer initiation and metastasis, Nature 485 (2012) 55–61.

J. Kang, S.Y. Lee, S.Y. Lee, Y.J. Kim, J.Y. Park, S.J. Kwon, M.J. Na, E.J. Lee, H.S. Jeon, J.W. Son, microRNA-99b acts as a tumor suppressor in non-small cell lung cancer by directly targeting fibroblast growth factor receptor 3, Exp. Ther. Med. 3 (2012) 149–153.

W. Zhao, Q. Sun, Z. Yu, S. Mao, Y. Jin, J. Li, Z. Jiang, Y. Zhang, M. Chen, P. Chen, D. Chen, H. Xu, S. Ding, Z. Yu, MI-R-320a-3p/ELF3 axis regulates cell metastasis and invasion in non-small cell lung cancer via PEI/K Akt pathway, Gene 670 (2018) 31–37.

F. Song, Z. Xuan, X. Yang, X. Ye, Z. Pan, Q. Fang, Identification of key microRNAs and hub genes in non-small-cell lung cancer using integrative bioinformatics and functional analyses, J. Cell. Biochem. 121 (2020) 2690–2703.

E. Abedini Bakhshmand, B.M. Soltani, Regulatory effect of hsa-miR-5590-3P on TGFbeta signaling through targeting of TGFbeta-R1, TGFbeta-R2, SMAD3 and SMAD4 transcripts, Biol. Chem. 400 (2019) 677–685.

C. Zhang, X. Wan, S. Tang, K. Li, Y. Wang, Y. Liu, Q. Sha, X. Zha, Y. Liu, miR-125b-5p/STAT3 pathway regulated by mTORC1 plays a critical role in promoting cell proliferation and tumor growth, J. Canc. 11 (2020) 919–931.