Regulation of human PAX6 expression by miR-7

M Needhamsen  
*Edith Cowan University*

Robert B. White  
*Edith Cowan University, r.white@ecu.edu.au*

Keith M. Giles

Sarah A. Dunlop

Meghan G. Thomas  
*Edith Cowan University, m.thomas@ecu.edu.au*

10.4137/EBO.S13739

Regulation of Human PAX6 Expression by miR-7

Maria Needhamsen1,2, Robert B. White1,2,4, Keith M. Giles3, Sarah A. Dunlop2 and Meghan G. Thomas1,2

1Parkinson’s Centre (ParkC), School of Medical Sciences, Edith Cowan University, Joondalup, Western Australia, Australia. 2Experimental and Regenerative Neurosciences (EaRN), School of Animal Biology, University of Western Australia, Crawley, Western Australia, Australia. 3Harry Perkins Institute of Medical Research, Nedlands, Western Australia, Australia. 4School of Anatomy, Physiology and Human Biology, University of Western Australia, Crawley, Western Australia, Australia.

ABSTRACT: The paired box gene 6 (PAX6) is a powerful mediator of eye and brain organogenesis whose spatiotemporal expression is exquisitely controlled by multiple mechanisms, including post-transcriptional regulation by microRNAs (miRNAs). In the present study, we use bioinformatic predictions to identify three candidate microRNA-7 (miR-7) target sites in the human PAX6 3′ untranslated region (3′-UTR) and demonstrate that two of them are functionally active in a human cell line. Furthermore, transient transfection of cells with synthetic miR-7 inhibits PAX6 protein expression but does not alter levels of PAX6 mRNA, suggesting that miR-7 induces translational repression of PAX6. Finally, a comparison of PAX6 3′-UTRs across species reveals that one of the functional miR-7 target sites is conserved, whereas the second functional target site is found only in primates. Thus, the interaction between PAX6 and miR-7 appears to be highly conserved; however, the precise number of sites through which this interaction occurs may have expanded throughout evolution.

KEYWORDS: miR-7, PAX6, miRNA, 3′-UTR


ACADEMIC EDITOR: Jike Cui, Associate Editor

TYPE: Original Research

FUNDING: MGT was funded by Edith Cowan University’s Office of Research and Innovation, the Faculty of Computing, Health and Science, and the Vario Health Institution. RBW was funded by a Raine Medical Research Foundation priming grant and an Early Career grant from the Cancer Council of Western Australia. SAD was funded by an NHMRC Principal Research’s fellowship (1002347). MN received a postgraduate research scholarship from Edith Cowan University, and KG was the recipient of a Royal Perth Hospital Medical Research Foundation’s fellowship.

COMPETING INTERESTS: Authors disclose no potential conflicts of interest.

COPYRIGHT: © the authors, publisher and licensee Libertas Academica Limited. This is an open-access article distributed under the terms of the Creative Commons CC-BY-NC 3.0 License.

CORRESPONDENCE: m.thomas@ecu.edu.au

Introduction

MicroRNA (miRNA) regulation of protein expression adds a subtle layer of complexity that fine-tunes on top of numerous modes of protein production and destruction. miRNAs typically bind to specific sites within the 3′ untranslated regions (3′-UTRs) of their target mRNAs, to impart post-transcriptional silencing, either through translational repression and/or mRNA decay.1,2 In animals, miRNAs typically exhibit partial complementarity base pairing to their mRNA targets, with the “seed region” of six to eight nucleotides toward the 5′ end of the miRNA being important for target specificity.3 miRNA target efficacy is predicted to increase with the number of Watson-Crick matches to the seed region and is also dependent on base composition at nucleotide position 1.2,4 For example, a perfect match to nucleotides 2-8, referred to as a “7Mer-m8” target site, has a higher hierarchy rating compared to a “7Mer-A1” target site, which only confers Watson-Crick base pairing to nucleotides 2-7.3 Noticeably, the latter has an A at nucleotide position 1, which again rates it higher than a “6Mer” with the same 2-7 seed match, but a different number 1 nucleotide (C, G, or T).4 However, other factors such as AU-rich neighbor nucleotides and accessibility may also influence site-efficacy.5,6 Hence, miRNAs target specific genes for post-transcriptional regulation, fine-tuning protein expression levels during critical processes such as cell proliferation, differentiation, and maturation.7

The functional roles of microRNA-7 (miR-7) have been extensively studied, as it is highly conserved, being detected in 81 species (October 2013: http://www.mirbase.org),8-10 and controls multiple cell signaling networks, including
epidermal growth factor receptor,\textsuperscript{11} insulin-like growth factor,\textsuperscript{12} Hedgehog,\textsuperscript{13} and the mammalian target of rapamycin (mTOR) signaling pathways.\textsuperscript{14} miR-7 is also a critical regulator of multiple regulatory genes, including paired box gene 6 (\textit{PAX6}) in mice.\textsuperscript{8,9} \textit{PAX6} encodes a neurogenic transcription factor, which is highly conserved, both structurally and functionally, and is critical for neural tube polarization, brain regionalization, and eye formation.\textsuperscript{15–17} \textit{PAX6} is widely co-expressed with miR-7 in several tissues including the forebrain, pancreas, and retina where it is involved in regulating cellular differentiation.\textsuperscript{8–10}

Given the functional importance of the miR-7/\textit{PAX6} regulatory apparatus in controlling cell fate in mouse, we sought to evaluate whether this mechanism was conserved in humans. Herein we use bioinformatics to predict three miR-7 target sites in the human \textit{PAX6} 3′-UTR and reporter gene assays to confirm the functional capacity of two of these predicted target sites. Further, we compare the human \textit{PAX6} 3′-UTR region across species and identify a conserved miR-7 3′-UTR target site present in many species, as well as a second functional miR-7 target site in the human \textit{PAX6} 3′-UTR that is specific to primates.

Materials and Methods

Prediction of miR-7 target sites. 3′-UTRs were identified using the “Spidey” freeware, which is part of the NCBI toolkit (http://www.ncbi.nlm.nih.gov/spidey/). Target sites for hsa-miR-7 within human \textit{PAX6} 3′-UTR (GenBank accession number: NM_001127612) were predicted using TargetScan software (release 6.2)\textsuperscript{4} with the following search criteria: species: human, human \textit{Entrez gene} symbol: \textit{PAX6}, and \textit{miRNA} name: hsa-miR-7. Target sites for miR-7 in 3′-UTRs of other species (GenBank accession numbers given in Supplementary Table 1) were predicted using a “Find in This Sequence” function available at GenBank to search for the miR-7 seed region (5′-GUCUUC-3′). Notably, only species that encode miR-7 (mirbase.org) are included in the analysis. Nucleotide position numbers for 3′-UTRs are listed in Supplementary Table 1.

Luciferase reporter constructs. Luciferase reporter plasmids were generated by ligating annealed DNA oligonucleotides containing each of the three predicted \textit{PAX6} 3′-UTR hsa-miR-7 7mer targets sites (NM_001127612.1; m\#1: nt. 2389–2441, m\#2: nt. 3613–3664, and A1\#3: nt. 4170–4216) to the pMIR-REPORT luciferase plasmid (Ambion) via HindIII and SpeI restriction sites. Mutated (Mut) miR-7 seed target sites with three nucleotide substitutions were generated following the same procedure (for sequences refer to Supplementary Table 2). A perfect hsa-miR-7 target site (forward: 5′-CAACCAATAAT\textsuperscript{-CCTACTTCTTCCA-3′} and reverse: 5′-TGGAAGAC-TAGTTATTTGGTG-3′) inserted in the pGL3-control (Promega) firefly luciferase reporter vector was used as a control as previously described.\textsuperscript{11,18} Sequences of all plasmids were confirmed by Sanger DNA sequencing.

Cellculture. HeLa and HEK293 cells were obtained from the American Type Culture Collection (ATCC) and maintained at 37 °C and 5% CO\textsubscript{2} in Dulbecco’s modified Eagle’s medium (DMEM)/F-12-GlutaMAX (Life Technologies) supplemented with 5% fetal bovine serum (Serana) and 100 units/mL of Penicillin/Streptomycin (Life Technologies).

Luciferase assays. HEK293 cells in 24-well plates were co-transfected with 1) firefly luciferase reporter plasmid DNA (100 ng) containing either \textit{PAX6} 3′-UTR WT or Mut miR-7 target sites (7mer-m\#1, 7mer-m\#2, and 7mer-A1\#3), 2) a control pRL-CMV \textit{Renilla} luciferase reporter plasmid (5 ng), and 3) precursor miRNAs (30 nM) purchased from Ambion, corresponding to human miR-7 (ID: PM10047) or a \textit{miRNA} negative control (miR-NC) (ID: AM17110) using Lipofectamine 2000 (Invitrogen). Cells were harvested 24 hours post-transfection and assayed using a Dual-Luciferase Reporter Assay System (Promega) and an EnSpire Multimode Plate Reader (PerkinElmer). For data analysis, firefly luciferase activity was firstly normalized to \textit{Renilla} (transfection control) and then hsa-miR-7 was normalized to miR-NC transfected cells.

Transfection. HeLa cells were seeded 24 hours before transfection with miR-7 or miR-NC \textit{miRNA} precursor molecules (30 nM) using RNAi/MAX transfection reagent (Invitrogen). Cells were harvested at 24 hours (for RNA) or 72 hours (for protein) post-transfection.

Western blotting. HeLa cells were lysed by sonication (4 × 5 seconds) in ice-cold lysis buffer (0.1% SDS, 10 mM Tris–HCl pH 7.5) containing 1× complete inhibitor cocktail tablet (Roche) and protein concentrations determined by Bradford assay (Bio-Rad). Samples (70 μg) were electrophoretically separated on a 12.5% polyacrylamide gel, transferred to a Hybond-P membrane (Amersham Biosciences), and probed with anti-PAX6 (1:1,000; Abcam Clone AD2.38) or anti-β-actin (1:10,000; Sigma-Aldrich Clone AC-15) mouse monoclonal antibodies before detection with the commercially available Qlot 625 system (Molecular Probes) and UV light using Universal Hood II (Bio-Rad) for visualization of a representative image. A colored ladder was used to determine protein size (Marker Precision Plus Protein Kaleidoscope standard; Bio-Rad).

RT-qPCR. Total RNA was extracted (SV Total RNA Isolation System; Promega), quality assessed, and quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific) with threshold cutoff for DNA contamination (260/280) at 2.0. cDNA was synthesized (SuperScript III; Invitrogen) using 250 ng RNA and random hexamers according to the manufacturer’s instructions. qPCR assays were performed using a Rotor-Gene Q (Qiagen) with SYBR-Green chemistry (GoTaq qPCR Master Mix; Promega) and previously published gene-specific primers: \textit{PAX6}\textsuperscript{19–21} forward: 5′-TCTTTGCTTGGAAATCCG-3′; and reverse: 5′-CTGCCGTATTTGATCG-3′; glyceraldehyde 3-phosphate dehydrogenase (\textit{GAPDH})\textsuperscript{22–25}
forward: 5′-GAAGGTGAAGGTCTCGAGTC-3′ and reverse: 5′-GAAGATGTGATGGAGATTTG-3′. Primerspecificity was tested using gel electrophoresis (to verify single band and amplicon size), DNA sequencing, and melting profile. A no-reverse transcription control was included in each run for detection of potential PCR contamination. Normalized PAX6 expression relative to GAPDH was calculated using the 2−ΔΔCt method.26

Statistical analysis. Data are presented as mean of three independent experiments (n = 3) + standard deviation (SD). Significance (P < 0.05) was assessed using a paired t-test (RT-qPCR) and ANOVA Tukey’s post-hoc analysis (luciferase assay) in R (version 3.0.2).27

Results and Discussion

The human PAX6 3′-UTR contains two functional miR-7 target sites. There are seven verified human PAX6 transcripts in the NCBI database, which result from alternative splicing and alternative initiation of transcription. However, it is noteworthy that they all share identical 3′-UTRs accounting for approximately 75% of the total mRNA sequence, which allows for the suggestion that PAX6 expression is possibly regulated by miRNAs. Using TargetScan software,2 we identified three miR-7 target sites: one of which (m8#1) has previously been shown to regulate PAX6 expression in mouse2 and two additional miR-7 target sites (m8#2 and A1#3) were predicted when poorly conserved target sites were considered (Fig. 1A). Two of the three predicted miR-7 target sites within the PAX6 3′-UTR contained 7mer-m8 seed matches (m8#1 and m8#2), whereas the third target site contained a lower hierarchy 7mer-A1 seed match (A1#3) (Fig. 1B). Noticeably, the A1#3 target site has a mismatch at position 8 in the miR-7 sequence (Fig. 1B), which has been shown to be essential for functional miR-7-target binding.28

To examine the functional significance of the three predicted human miR-7 target sites within the PAX6 3′-UTR, we generated firefly luciferase reporter constructs containing each of these individual sites, as well as Mut constructs of each. We then co-transfected HEK293 cells with the firefly and Renilla (transfection control) luciferase constructs and either hsa-miR-7 or miR-NC (a negative control) precursor miRNAs to assess target specificity. To confirm the reliability of our assay, we first measured luciferase activity of a construct containing a perfect miR-7 target site (5′-CAACAAAAT-CACTAGCTTCCA-3′), which resulted in a 99% reduction in luciferase activity (Renilla normalized) compared to cells transfected with miR-NC precursors (Fig. 1C). Assessment of cells transfected with PAX6 3′-UTR target site firefly luciferase reporter plasmids revealed that two of the three miR-7 target sites (m8#1 and m8#2) were functionally active, each yielding a significant reduction in luciferase activity of approximately 45% with miR-7 relative to their Mut target

Figure 1. The human PAX6 3′-UTR contains two specific, functional miR-7 target sites. (A) Prediction of miRNA-7 target sites within the human PAX6 3′-UTR. Black line (top) illustrates the length of the PAX6 3′-UTR (k = kilobase), and asterisks illustrate predicted miR-7 target sites (m8#1, m8#2, and A1#3). (B) Alignment of predicted human PAX6 miR-7 target sites (top) to miR-7 (bottom). The seed target region (GUCUCC) is in bold, and the seed matches, target sites, and positions are given. (C) Luciferase assays using firefly reporter constructs with predicted (WT: wild type and Mut) miR-7 7mer target sites (m8#1, m8#2, and A1#3) and a perfect miR-7 target site (PT) as a positive control. The firefly luciferase reporters were co-transfected into HEK293 cells with a Renilla luciferase reporter as a transfection control and either miR-7 or miR-NC precursors. Relative luciferase expression (firefly normalized to Renilla) values of hsa-miR-7 were normalized to miR-NC transfected controls, and is given as mean + SD. The relative luciferase activity (firefly normalized to Renilla) of the positive control was calculated as miR-7 relative to miR-NC and is given as mean + SD, *P < 0.05.
site reporters (Fig. 1C). Both of these active sites contained 7mer-m8-predicted miR-7 target sites, whereas the third site (7mer-A1#3) did not show any regulation by miR-7. Our study therefore confirms previous findings, showing that Watson-Crick base pairing to nucleotide position 8 is essential for functional miR-7-target binding.²⁸

Taken together, these data indicate that the PAX6 3'UTR contains two 7 mer-m8 miR-7 seed sites. A third predicted 7 mer-A1 miR-7 target site that did not comply with Watson-Crick pairing at nucleotide 8 failed to show functional activity in luciferase reporter assays.

PAX6 protein expression is regulated by miR-7 in human cells. To assess whether miR-7 regulates PAX6 protein expression in human cells, we transiently transfected HeLa, a human cervical cancer cell line, with miR-7 precursor molecules and measured PAX6 levels by Western blotting. Compared with a negative miRNA precursor molecule (miR-NC), miR-7 reduced expression of PAX6 in HeLa cells (Fig. 2A). To determine whether miR-7 reduced PAX6 protein levels through interfering with the translational pathway or mediating mRNA decay, we assessed endogenous PAX6 mRNA levels by RT-qPCR; however, we saw no significant difference in PAX6 mRNA expression between HeLa cells transfected with hsa-miR-7 or miR-NC miRNA precursor molecules (Fig. 2B).

Taken together, these data indicate that miR-7 regulates PAX6 protein levels in human cells, but has no significant effect on mRNA levels, which suggests that miR-7 interferes with the translational pathway rather than mediating PAX6 mRNA decay.

Diversification of PAX6 3' UTR miR-7 target sites across species. Recent experiments in mice have demonstrated that miR-7 directly represses the expression of PAX6 through a single miR-7 target site in the mouse PAX6 3' UTR.⁸⁹ Given our identification of a second functional miR-7 target site (7mer-m8) in human PAX6 3'UTR compared to mouse, we examined the evolutionary conservation of PAX6 3'UTRs and their miR-7 target sites across species, and found that the length of PAX6 3'UTRs varied considerably and that this length seemed to be associated with the number of predicted miR-7 target sites (Fig. 3A). For example, in primates, human (Homo sapiens) and rhesus monkey (Macaca mulatta), PAX6 has relatively long 3'UTRs (accounting for more than 70% of the total mRNA sequences), and we demonstrated the existence of two functional 7mer-m8 miR-7 target sites within these regions. In comparison, the 3'UTRs of pig (Sus scrofa), rodents (Mus musculus and Rattus norvegicus), frog (Xenopus tropicalis) and fish (Danio rerio) account for between 30 and 39% of total PAX6 mRNA length and contain only one predicted miR-7 target site (Fig. 3A). In other species such as chicken (Gallus gallus) and fruit fly (Drosophila melanogaster), where the 3' UTR accounts for less than 20% of the total PAX6 mRNA sequence, no miR-7 target sites were predicted (Fig. 3A). Notably, in zebrafish, there are two duplicated PAX6 gene variants, PAX6a and PAX6b, with distinct 3'UTR lengths, accounting for 34%-39% of the total mRNA lengths, respectively (Fig. 3A). PAX6a contains a single predicted miR-7 target site, whereas no site was predicted in the shorter 3'UTR of the PAX6b variant, suggesting that perhaps only PAX6a is post-transcriptionally regulated by miR-7. Also, the fruit fly has multiple PAX6 gene loci, from which six different PAX6 3'UTRs are transcribed; none have a predicted miR-7 target site in the 3'UTR despite one transcript having a 3'UTR that accounts for more than 45% of the total mRNA sequence (Fig. 3A), suggesting that miR-7 might not regulate PAX6 expression levels in fruit flies. These results are in agreement with previous work that suggested the expansion of 3'UTR sequences increases with organism complexity throughout evolution.²⁹

In summary, given our finding that miR-7 can regulate PAX6 expression via two miR-7 target sites in a human cell line, future experiments are needed to examine the physiological importance of this regulatory system, in particular, examining whether there is a combinatorial effect between the two functional human miR-7 target sites (m8#1 and m8#2).

Figure 2. miR-7 reduces PAX6 protein expression in human cells without inducing PAX6 mRNA decay. (A) Western blot analysis of HeLa cells transfected with hsa-miR-7 (lane 1) or control non-coding miR-NC miRNA (lane 3) using antibodies against PAX6 (top) and β-actin (bottom). A protein marker (lane 2) confirmed the correct size of PAX6 (48 kDa) and β-actin (42 kDa) proteins. (B) RT-qPCR analysis of PAX6 mRNA isolated from HeLa cells transfected with miRNA precursors corresponding to hsa-miR-7 (miR-7) and a negative control (miR-NC). PAX6 message levels were normalized relative to GAPDH and are presented as mean ± SD.

Needhamsen et al

A

B

miR-7
miR-NC

PAX6

miR-7

miR-NC

miR-NC

PAX6

β-Actin

Normalized expression relative to GAPDH

miR-7

miR-NC

0

0.2

0.4

0.6

0.8

1.0

1.2

1.4

Figure 2. miR-7 reduces PAX6 protein expression in human cells without inducing PAX6 mRNA decay. (A) Western blot analysis of HeLa cells transfected with hsa-miR-7 (lane 1) or control non-coding miR-NC miRNA (lane 3) using antibodies against PAX6 (top) and β-actin (bottom). A protein marker (lane 2) confirmed the correct size of PAX6 (48 kDa) and β-actin (42 kDa) proteins. (B) RT-qPCR analysis of PAX6 mRNA isolated from HeLa cells transfected with miRNA precursors corresponding to hsa-miR-7 (miR-7) and a negative control (miR-NC). PAX6 message levels were normalized relative to GAPDH and are presented as mean ± SD.
or if a dysfunctional miR-7/PAX6 regulatory system promotes tumorigenesis, as both factors are highly associated with cancer.\textsuperscript{30–34}

**Conclusions**

In conclusion, we provide the first demonstration that miR-7 regulates the expression of PAX6 protein in human cells, and show that this regulation occurs via two target sites within the \textit{PAX6} 3′-UTR: the first site is highly conserved across species, whereas the second functional miR-7 site is conserved only in primates. This finding leads to the intriguing possibility that the expanded 3′-UTR found in human \textit{PAX6} may allow for a tighter control of \textit{PAX6} expression by miR-7 than that already documented in mouse.

**Author Contributions**

MN, RBW, KMG, SAD, and MGT conceived and designed the experiments. MN, RBW, KMG, and MGT analyzed the
REFERENCES

Supplementary Data

Table 1. miR-7 regulation of PAX6.

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>VARIANTS</th>
<th>ACCESSION NUMBERS</th>
<th>3’UTR (START-STOP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homo Sapiens</td>
<td>N/A</td>
<td>NM_001127612.1</td>
<td>1739–6883</td>
</tr>
<tr>
<td>Macaca mulatta</td>
<td>N/A</td>
<td>NM_001266257.1</td>
<td>1711–5908</td>
</tr>
<tr>
<td>Sus scrofa</td>
<td>N/A</td>
<td>NM_001244172.1</td>
<td>1728–2643</td>
</tr>
<tr>
<td>Mus musculus</td>
<td>N/A</td>
<td>NM_001244198.1</td>
<td>1597–2619</td>
</tr>
<tr>
<td>Rattus norvegicus</td>
<td>N/A</td>
<td>NM_013001.2</td>
<td>1430–2191</td>
</tr>
<tr>
<td>Gallus gallus</td>
<td>N/A</td>
<td>NM_205066.1</td>
<td>1641–1961</td>
</tr>
<tr>
<td>Xenopus tropicalis</td>
<td>N/A</td>
<td>NM_001006762.1</td>
<td>1651–2361</td>
</tr>
<tr>
<td>Danio rerio</td>
<td>PAX6a</td>
<td>NM_131304.1</td>
<td>1845–2809</td>
</tr>
<tr>
<td></td>
<td>PAX6b</td>
<td>NM_131641.1</td>
<td>1575–1733</td>
</tr>
<tr>
<td>Drosophila Melanogaster</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eyeless (Ey)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Variant a</td>
<td>NM_079899.4</td>
<td>2160–2287</td>
</tr>
<tr>
<td></td>
<td>Variant c</td>
<td>NM_00127215.1</td>
<td>2109–3870</td>
</tr>
<tr>
<td></td>
<td>Eyegone (Eyg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Variant a</td>
<td>NM_001014582.1</td>
<td>2350–2586</td>
</tr>
<tr>
<td></td>
<td>Variant b</td>
<td>NM_001274833.1</td>
<td>2030–2303</td>
</tr>
</tbody>
</table>

Notes: List of PAX6 GenBank accession numbers, N/A = “not applicable.” Nucleotide position numbers for the 5’end (start) and 3’end (stop) of the 3’-UTRs are listed.

Table 2. Oligonucleotides used for generating firefly luciferase constructs.

<table>
<thead>
<tr>
<th>TARGET SITES</th>
<th>OLIGONUCLEOTIDES (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>m8#1</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>SE  AGCTTTCTGAGGATTTTCTAGGAGAAGACAAATACTTACATTGTCATATAAAACAAATT</td>
</tr>
<tr>
<td></td>
<td>AS  CTAAGTTTTAGTTATGAAAAATTGGATATTGGCTTTTCCATTAGAAATCTCAGAA</td>
</tr>
<tr>
<td></td>
<td>Mut SE  AGCTTTCTGAGGATTTTCTAGGAGGCAAATACTTACATTGTCATATAAAACAAATT</td>
</tr>
<tr>
<td></td>
<td>AS  CTAAGTTTTAGTTATGAAATGAAATTTGGCTTTTCCATTAGAAATCTCAGAA</td>
</tr>
<tr>
<td>m8#2</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>SE  AGCTAGTGAAGTTTGCAAGGAAGACTTACTGTATTTGACTTATATGTGGCACATA</td>
</tr>
<tr>
<td></td>
<td>AS  CTAAGCTGTGCAACAGATATAAGTGCAATACAGATAAGTGTGCAACAGATACACT</td>
</tr>
<tr>
<td></td>
<td>Mut SE  AGCTAGTGAAGTTTGCAAGGAAGGCTTTACTGTATTTGACTTATATGTGGCACATA</td>
</tr>
<tr>
<td></td>
<td>AS  CTAAGCTGTGCAACAGATATAAGTGCAATACAGATAAGGCTTTACTGTATTTGCAACAGATACACT</td>
</tr>
<tr>
<td>A1#3</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>SE  AGCTAAAAATATCTACCTCTGGAAGATGTGTCAAAAACATTCCTGCAGATACACCA</td>
</tr>
<tr>
<td></td>
<td>AS  CTAAGCTGAGGATCTGGAGGAGTTTTGACACCATCTTCCAGGATGGAGATTTTG</td>
</tr>
<tr>
<td></td>
<td>Mut SE  AGCTAAAAATATCTACCTCTTGGAAGGTTGGTGTCAAAAACATTCCTGCAGATACACCA</td>
</tr>
<tr>
<td></td>
<td>AS  CTAAGCTGAGGATCTGGAGGAGTTTTGACACCATCTTCCAGGATGGAGATTTTG</td>
</tr>
</tbody>
</table>

Notes: List of sense (SE) and antisense (AS) oligonucleotides used for generating firefly luciferase constructs containing WT and Mut miR-7 7mer target sites (m8#1, m8#2, and A1#3). Italic, bold, and underlined nucleotides represent restriction recognition-, miR-7 target- and Mut sites, respectively.