

RESEARCH ARTICLE

In Vivo Imaging of miR-221 Biogenesis in Papillary Thyroid Carcinoma

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Abstract

Purpose: To investigate the overexpression of miR-221 in papillary thyroid carcinoma (PTC), we developed a Gaussia luciferase (Gluc) system regulated by miR-221.

Procedures: Quantities of primary or mature miR-221 in normal thyroid cells (HT-ori3) and in PTC (NPA, TPC-1) were measured by quantitative real-time polymerase chain reaction. Cytomegalovirus (CMV)/Gluc-3xPT_miR221, which included three perfect complementary target sequences repeats of miR221 in the 3'-untranslated region of Gluc, was transfected into cells with pre-miR-221 or anti-miR-221 and Gluc activities were then compared *in vitro* and *in vivo*.

Results: Primary or mature miR-221 were overexpressed in PTC as compared with HT-ori3. In cells transfected with the Gaussia luciferase reporter system (CMV/Gluc-3xPT_miR221), Gluc activities were regulated according to miR-221 levels *in vitro* and *in vivo*.

Conclusions: These results suggest that the devised CMV/Gluc-3xPT_miR221 system may be a useful tool for monitoring quantities of endogenous miR-221 in cells or living organisms.

Key words: Gaussia luciferase, MicroRNA, Papillary thyroid carcinoma, Bioluminescence image

Introduction

MicroRNAs (miRNAs) represent a class of endogenous, small, noncoding but functional RNAs of 19–23 nt. The miRNAs *lin-4* and *let-7* acted as regulators of developmental timing in *Caenorhabditis elegans* and were first discovered in 1993 and 2000, respectively [1–4]. Since then, several hundred miRNAs have been discovered in vertebrates and invertebrates. Primary miRNAs (pri-miRNAs) are transcribed by RNA polymerase II and processed in the nucleus by the RNase III enzyme Droscha into hairpin-shaped precursor miRNAs (pre-miRNA) of 70–100 nt. These pre-miRNAs are then transported to the cytoplasm by exportin-5 and further processed into mature miRNAs of 19–23 nt by

cytoplasmic RNase III endonuclease Dicer complex and RNA-induced silencing complex [3, 5, 6]. Mature miRNAs perfectly, or near perfectly, bind to complementary sequences in the 3'-untranslated regions (UTRs) of target mRNAs and cause either translational repression or mRNA degradation, depending on their degrees of complementarity or homology with target genes [7, 8].

Several reports have demonstrated that miRNAs have diverse functions, e.g., they participate in the regulation of cellular differentiation, proliferation, apoptosis, and development. Examples of such miRNAs include miR-1 and miR-133, which regulate muscle differentiation; miR-181, which regulates hematopoietic stem cell differentiation to B-lineage cells; miR-375, which is involved in mammalian pancreatic islet-cell development and in the regulation of insulin secretion; miR-132, miR-124, and miR-9, which regulate the neuronal system; and miR-14, a cell death suppressor [3, 9–11].

Furthermore, deficiencies or excesses of miRNA functions in tissues have been correlated with a number of clinically important diseases. In particular, miRNAs function as tumor suppressors or oncogenes by participating in the aberrant expression, deletion, amplification, or mutation of genes in human carcinogenesis [10, 12–15]. Of these many miRNAs, miR-146b, miR-221, and miR-222 are upregulated in papillary thyroid carcinoma (PTC), the most common malignant thyroid cancer. In fact, PTC represents ~80% of all thyroid malignancies [2, 16–20].

Generally, methods like Northern blotting, reverse transcriptase polymerase chain reaction (RT-PCR), and microarray analysis, which are commonly used to quantify miRNAs *in vitro* and *in vivo*, are time consuming, laborious, nonreproducible, and invasive. On the other hand, optical imaging techniques using bioluminescent reporter genes offers a noninvasive means of performing real-time analyses at the molecular level in living animals [21–23].

Bioluminescence imaging involves the detection of light emitted from cells or tissues by an enzymatic reaction between luciferase and its substrate. Moreover, background luminescence levels are minimal in most animals because no light is emitted in the absence of an enzymatic reaction. Firefly luciferase (Fluc), Renilla luciferase (Rluc), and Gaussia luciferase (Gluc) have been commonly used as bioluminescence reporter proteins in living small animals. Fluc reacts with beetle D-luciferin (benzothiazole) and emits light about a 562 nm [24, 25], whereas Rluc and Gluc react with coelenterazin and emit at about 480 nm. Of these, Gluc has the advantage that it emits light at an intensity 1,000-fold greater than native Rluc or Fluc. Furthermore, Gluc is the smallest luciferase known and is stable at elevated temperatures [24, 26]. In this study, we quantified endogenous primary and mature miR-221 levels in papillary thyroid carcinoma using real-time PCR and developed a Gaussia luciferase reporter system for the *in vitro* quantification and *in vivo* qualitative imaging of PTC specifically expressed miR-221.

Materials and Methods

Cell Lines and Culture Conditions

The human papillary thyroid cancer cell lines, TPC-1 and NPA, were kindly provided by Dr. Park (Department of Internal Medicine, Seoul National University College of Medicine) and the normal human thyroid cell line, HT-ori3, was kindly provided by Dr. Kim (Radiation Health Research Institute). TPC-1 cells were maintained in Dulbecco's minimum essential medium supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin,

and 10% fetal bovine serum (FBS). HT-ori3 and NPA cells were maintained in RPMI 1640 supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 10% FBS. All cells were incubated at 37°C in a 5% CO₂ humidified chamber.

Relative Quantifications of Primary miR-221 and Mature miR-221

Total RNA was isolated from HT-ori3, NPA, and TPC-1 cells using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. Total RNA was then reverse transcribed in a final volume of 20 µl containing 1 µl of oligo (dT), 4 µl of 5_{first} strand buffer, 2 µl of 0.1 mol/l DTT, 1 µl of 10 mmol/l deoxynucleotide triphosphate mix, and Moloney murine leukemia virus reverse transcriptase (Invitrogen, USA). Real-time PCR analyses of pri-miR-146b, miR-221, or miR-222 were then performed in a total volume of 25 µl containing 12.5 µl of SYBR Premix Ex TaqTM(2×; Takara, Japan), 100 ng of cDNA, 10 pmol of forward primer, and 10 pmol of reverse primer using an iCycler (Bio-Rad, USA) at 95°C for 3 min and 40 cycles of 95°C for 15 s and 56°C for 30 s. β-actin was used as an internal control. The primers used are shown in Table 1.

Small RNA was isolated from cultured cells, i.e., HT-ori3, NPA, or TPC-1, using *mir*-VanaTM miRNA isolation kits (Ambion, USA), and then subjected to cDNA synthesis and qRT-PCR of mature miR-221 using the *mir*-VanaTM qRT-PCR primer Set and the *mir*-VanaTM qRT-PCR miRNA kit (both from Ambion, USA). PCRs were performed in triplicate using an iCycler (Bio-Rad, USA) and SYBR Premix Ex TaqTM(2×; Takara, Japan) at 95°C for 3 min and 40 cycles of 95°C for 15 s and 60°C for 30 s. The relative amounts of each mature miRNA were normalized versus the U6 snRNA primer set (Ambion, USA) using the equation $2^{-\Delta\Delta CT}$, where $\Delta CT = (C_{TmiRNA} - C_{TU6RNA})$, $\Delta\Delta CT = (\Delta CT - \Delta C_{TmiRNA \text{ of HT-ori3}})$.

Constructs and Transfection

We constructed cytomegalovirus (CMV)/Gluc-3xPT_miR221, which included three perfect complementary target sequence repeats (3xPT) of mature miR-221 after the Gluc stop codon, by annealing and subcloning the oligonucleotides shown in Table 2. Annealing was performed using a mixture of 200 pmol of oligonucleotides and 48 µl of annealing buffer containing 1× TE buffer and 50 mM NaCl, using the following conditions: 95°C for 4 min, 70°C for 10 s, followed by slow cooling in a 70°C water bath for 90 min. The annealed oligonucleotide was subcloned into the XhoI and XbaI sites of CMV/Gluc vector (Targetingsystems, USA).

The constructed plasmids were transfected into HT-ori3, NPA, and TPC-1 cells. Briefly, cells were plated into 24-well plates the day before transfection at a seeding density of 1×10^5 cells/well.

Table 1. The primers used for RT-PCR or real-time PCR

GenBank	Gene symbol	Forward primer	Reverse primer	Temperature (°C)
MI0003129	pri-miR-146b	5'-CCACCTGGCACTGAGAAGTCAA-3'	5'-GAGCCCCCTGACGTCTTTGAGCATAA-3'	56
MI0000298	pri-miR-221	5'-GCTACATTGCTCTGCTGGGTTTCA-3'	5'-AGCTTTCTTGCGGTCCTTTCTCTG-3'	56
MI0000299	pri-miR-222	5'-TCTGGCTACTGGGTCTCTGATG-3'	5'-TCCTCCCCCTTGTAGTATTGAA-3'	56

Table 2. The oligonucleotides cloned in 3'UTR of Gluc

ID	Oligonucleotide sequence
3xPT_miR221 Forward	5'- <i>tcgaga</i> atctagt GAAACCCAGCAGACAATGTAGCT tagta GAAACCCAGCAGACAATGTAGCT tagta GAAACCCAGCAGACAATGTAGCT t-3'
3xPT_miR221 Reverse	5'- <i>ctaga</i> AGCTACATTGTCTGCTGGGTTT Cacta AGCTACATTGTCTGCTGGGTTT Cacta AGCTACATTGTCTGCTGGGTTT Cactagattc-3'

The italicized sequences indicate the enzyme sites, XhoI or XbaI
The bold sequences indicate the binding sites of mature miR-221

Transfection was performed using Lipofectamine Plus reagent (Invitrogen, USA), 0.3 µg of DNA, 4 µl of Plus reagent, and 1 µl of Lipofectamine per well. Forty nanomolars of pre-miR-221 (Ambion, USA) or 80 nM of anti-miR-221 (Ambion, USA) were cotransfected with 0.3 µg of CMV/Gluc-3xPT_miR221; CMV/Gluc was used as a control. All transfections were carried out in quadruplicate.

Gaussia Luciferase Assays

Thirty hours after transfection, cells were washed with phosphate buffered saline (PBS) and treated with 100 µl of lysis solution. Supernatants were plated in microplates and Gaussia luciferase activities were measured using a Wallac1420 VICTOR3V (PerkinElmer Life and Analytical Sciences, Shelton, CT, USA) using a luciferase assay kit (TargetingSystems, USA). All results are displayed as means ± standard deviation (SD; $n=4$).

In Vivo Visualization of Gaussia Luciferase Expression in Cells

All experimental animals were housed under specific pathogen-free conditions and handled in accord with the guidelines issued by the Seoul National University Hospital-Institutional Animal Care and Use Committee.

NPA cells were plated into 10-cm dishes the day before transfection at a seeding density of 2×10^6 cells/dish. Forty nanomolars of pre-miR-221 (Ambion, USA) or 80 nM of anti-miR-221 (Ambion, USA) were then cotransfected with 2 µg of CMV/Gluc-3xPT_miR221 using Lipofectamine Plus reagent (Invitrogen, USA). Cells transfected with CMV/Gluc were used as controls. After 24 h of transfection, cells were counted, and 2×10^6 NPA cells were suspended in 100 µl of PBS and implanted subcutaneously into nude mice, as follows: cells transfected with CMV/Gluc were implanted into right shoulders, cells transfected with CMV/Gluc-3xPT_miR221 into left thighs, and cells cotransfected with CMV/Gluc-3xPT_miR221 and pre-miR-221 or CMV/Gluc-3xPT_miR221 and anti-miR-221 were implanted into right thighs.

To investigate Gluc activity regulation by miR-221 in mice over 6 days, cells prepared using the methods described above were implanted into nude mice, as follows: cells transfected with CMV/Gluc were implanted into left thighs, cells cotransfected with CMV/Gluc-3xPT_miR221 into right thighs, cells transfected with CMV/Gluc-3xPT_miR221 into left thighs, and cells cotransfected with CMV/Gluc-3xPT_miR221 plus pre-miR-221 or with CMV/Gluc-3xPT_miR221 plus anti-miR-221 were implanted into right thighs.

The IVIS100 imaging system (Xenogen, USA), which includes an optical charge coupled device (CCD) camera mounted on a light-tight specimen chamber, was used for the data acquisition and analysis. Briefly, coelenterazine (Biotium, Inc., USA) was diluted to 5 µg/50 µl in PBS before use, and this was subcutaneously injected into the sites of previous NPA cell injections. A mouse was placed in a specimen chamber with a mounted CCD camera and light emitted by luciferase in mice was then measured. Gray scale photographic images and bioluminescent color images were superimposed using LIVINGIMAGE V. 2.12 software (Xenogen, USA) and IGOR image analysis software. Bioluminescence signals are expressed as photons per cubic centimeter per second per steradian ($P/cm^2/s/sr$).

Results

MiR-221 was Highly Expressed in Papillary Thyroid Carcinoma

To identify a good microRNA candidate for imaging gene regulation in carcinoma, we examined the expressions of miR-146b, miR-221, and miR-222, which have all been reported to be highly expressed in PTC, by RT-PCR and real-time PCR in HT-ori3 cells (a normal thyroid cell line) and in NPA and TPC-1 cells (papillary thyroid cancer cell lines). RT-PCR detected primary miR-221 (pri-miR-221) overexpression in PTC cells, but the other miRNAs were not distinguishable (Fig. 1a). To quantify the expressions of pri-miRNAs in NT or PTC cells, real-time PCR was performed. Levels of each primary miRNA were normalized versus β -actin. pri-miR-146b expressions in NPA and TPC-1 cells were 2.7 and 2.2 times lower, respectively, than in HT-ori3 cells. pri-miR-221 expressions in NPA and TPC-1 cells were 2.24 and 1.5 times higher, respectively, than in HT-ori3 cells. pri-miR-222 expression in NPA cells was 1.3 times higher than that in HT-ori3 cells, and in TPC-1 cells, it was a half that in HT-ori3 cells (Fig. 1b).

Based on the qRT-PCR results of primary miR-146b, miR-221, and miR-222 and the observed overexpression of miR-221 in both PTC cell lines (NPA and TPC-1), it was selected for further analysis. MiR-221 is intergenic microRNA located on chromosome Xp11.3, in which it clusters with miR-222. Moreover, the sequence of mature miR-221 is evolutionarily well conserved from worm to man [16, 17]. We quantified the endogenous levels of mature miR-221 in HT-ori3, NPA, and TPC-1 cells by real-time PCR (normal-

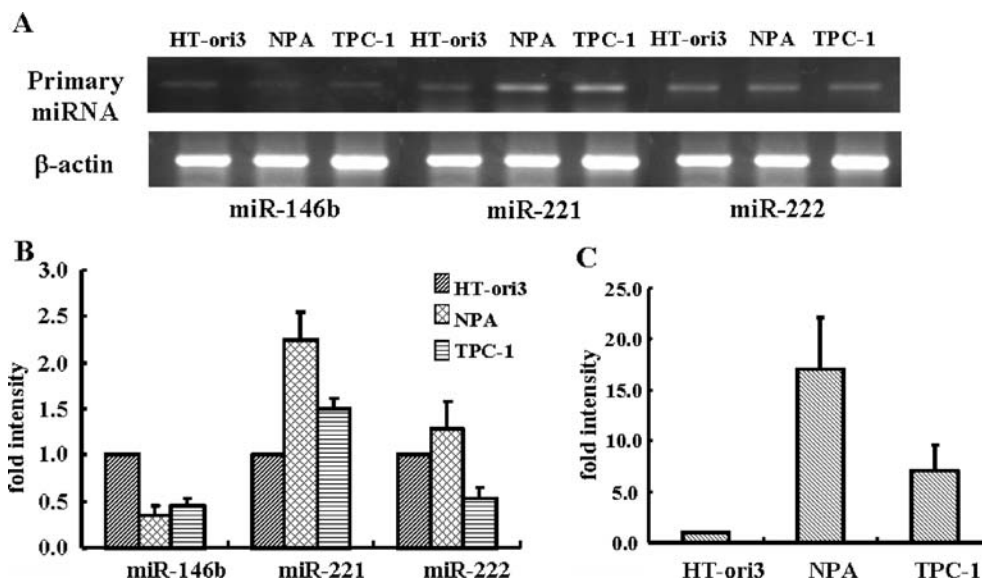


Fig. 1. Quantitative comparisons of primary and mature miRNAs. **a** RT-PCR of primary miRNA-146b, miRNA-221, and miRNA-222 in HT-ori3, NPA, or TPC-1 cells. RT-PCR was performed using total RNAs and primers for the different primary miRNAs. β -actin was used as a loading control. **b** Real-time PCR of primary miRNA-146b, miRNA-221, and miRNA-222. The relative amounts of primary miRNAs were normalized versus β -actin using the equation $2^{-\Delta\Delta C_T}$, where $\Delta C_T = (C_{TmiRNA} - C_{T\beta-actin})$ and $\Delta\Delta C_T = (\Delta C_T - \Delta C_{TmiRNA \text{ of HT-ori3}})$. The amount of pri-miRNAs in HT-ori3 was set at 1. The relative amounts of pri-miRNAs in NPA and TPC-1 are shown. The data displayed are means \pm SD (fold intensity). **c** Quantifications of mature miRNA-221 in HT-ori3, NPA, and TPC-1 cells. Real-time PCR was performed using small RNAs from each cell line and mature miR-221 primers. Amounts of mature miR-221 were normalized versus U6 snRNA using the equation $2^{-\Delta\Delta C_T}$, where $\Delta C_T = (C_{TmiRNA} - C_{TU6RNA})$ and $\Delta\Delta C_T = (\Delta C_T - \Delta C_{TmiRNA \text{ of HT-ori3}})$. The relative amounts of mature miR-221 in NPA and TPC-1 cells are shown. Data are displayed (fold intensity differences) and are expressed as means \pm SD.

ized versus U6) and found that its levels in NPA and TPC-1 cells were 17 and seven times greater, respectively, than its levels in HT-ori3 cells (Fig. 1c).

MiR-221—Directed the Repression of Gaussia Luciferase Reporter Gene Bearing Complementary Sequences of Mature miR-221

Gene expression analyses of miR-221 by PCR are meaningful, but do not readily allow the *in vitro* or *in vivo* noninvasive imaging of miR-221 function. To monitor the endogenous expression levels of mature miR-221 in PTC, we constructed a CMV/Gluc-3xPT_miR221, which included CMV promoter and three perfect complementary target sequence repeats of mature miR-221 in the 3'UTR of Gluc. When miR-221 was present in cells, Gluc activities, after transfecting CMV/Gluc-3xPT_miR221, were repressed by binding between mature miR-221 and the 3xPT in the 3'UTR of Gluc, which resulted in mRNA destabilization. Thus, repressed Gluc activities indirectly represented levels of mature miR-221 in cells.

Initially, CMV/Gluc-3xPT_miR221 was examined to determine whether it was repressed by gradually increasing amounts of exogenously derived pre-miR-221 in HeLa cells (a human cervical carcinoma cell line) and in HT1080 cells (a human fibrosarcoma cell line), which both express low endogenous levels of mature miR-221 (Fig. 2a). When CMV/Gluc-3xPT_miR221 was cotransfected with 0, 10, 20,

40, or 80 nM of cognate pre-miRNA into HeLa or HT1080 cells, Gluc activities from CMV/Gluc-3xPT_miR221 were found to be dose dependently repressed.

To further establish a link between Gluc repression and the presence of endogenous miR-221 in PTC, HT-ori3, NPA, and TPC-1 cells were transfected with CMV/Gluc-3xPT_miR221 vector. Transfection with only CMV/Gluc-3xPT_miR221 induced a significant reduction in Gluc activity in HT-ori3, NPA, and TPC-1 cells versus transfection with CMV/Gluc. In addition, Gluc expression from CMV/Gluc-3xPT_miR221 was more repressed in TPC-1 and NPA cells than in HT-ori3 cells. To confirm that Gluc repression by CMV/Gluc-3xPT_miR221 was due to miR-221, CMV/Gluc-3xPT_miR221 was cotransfected with either exogenous pre-miR-221 or anti-miR-221. Cotransfection of CMV/Gluc-3xPT_miR221 with 40 nM of exogenous pre-miR-221 showed additional repression of Gluc in all three cell lines. Conversely, the Gluc activities of CMV/Gluc-3xPT_miR221 in HT-ori3, NPA, and TPC-1 cells were dramatically retrieved after cotransfection with 80 nM of anti-miR-221 (Fig. 2b).

In Vivo Visualization of Gaussia Luciferase Expression Controlled by miR-221

To detect the miR-221-mediated repression of Gluc activity resulting from CMV/Gluc-3xPT_miR221 in PTC *in vivo*, we imaged nude mice at 5 h after subcutaneously implanting $2 \times$

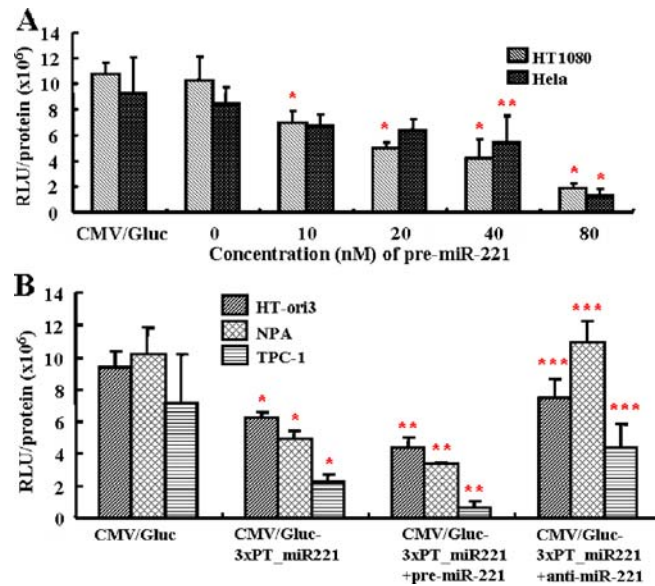


Fig. 2. *In vitro* Gaussia luciferase activities regulated by miR-221. **a** Gaussia luciferase activity was repressed dose dependently by exogenous miR-221 in HeLa and HT1080 cells. CMV/Gluc-3xPT_miR221 was cotransfected with four different doses of pre-miR-221 (0, 10, 20, 40, or 80 nM) into HeLa or HT1080 cells. * $P < 0.01$; ** $P < 0.05$ (versus CMV/Gluc). **b** Gaussia luciferase activities regulated by endogenous or exogenous miR-221, or anti-miR-221 in HT-ori3, NPA, or TPC-1 cells. CMV/Gluc-3xPT_miR221 was cotransfected with 40 nM of pre-miR-221 or 80 nM of anti-miR-221 into cells. * $P < 0.02$ (CMV/Gluc versus CMV/Gluc-3xPT_miR221); ** $P < 0.02$ (CMV/Gluc versus CMV/Gluc-3xPT_miR221 + pre-miR-221); *** $P < 0.05$ (CMV/Gluc-3xPT_miR221 versus CMV/Gluc-3xPT_miR221 + anti-miR-221). Gluc assays were conducted after transfecting for 30 h. Data are displayed as means \pm SD (RLU/mg protein; $n=4$). *RLU* relative light units.

10^6 of NPA cells transfected with CMV/Gluc, CMV/Gluc-3xPT_miR221, and CMV/Gluc-3xPT_miR221 with pre-miR-221, or CMV/Gluc-3xPT_miR221 with anti-miR-221 (Fig. 3). Gluc activities from CMV/Gluc-3xPT_miR221 were significantly lower than that from the positive control (CMV/Gluc), because the endogenous level of mature miR-221 was overexpressed in PTC. Furthermore, cotransfection with CMV/Gluc-3xPT_miR221 and pre-miR-221 further reduced Gluc expression due to the presence of exogenously derived miR-221 (Fig. 3a). However, anti-miR-221 transfected into NPA cells inhibited the binding of endogenous mature miR-221 to CMV/Gluc-3xPT_miR221 and reduced this repression of Gluc activity (Fig. 3b). Normalized fold expressions determined by region of interest (ROI) analysis showed that the Gluc activities of CMV/Gluc-3xPT_miR221 were 0.39 times and 0.4 times lower, respectively, than that of CMV/Gluc due to endogenously presented miR-221, and 0.25 times lower by exogenous pre-miR-221 and 1.73 times higher by the addition of anti-miR-221 from each right thigh compared with that of CMV/Gluc from right shoulder (Fig. 3c,d).

To investigate *in vivo* the real-time imaging of miR-221 production and specificity related to PTC, Gluc activities in mice were monitored for 6 days and subjected to ROI analysis (Fig. 4). CMV/Gluc-3xPT_miR221 transfected cells showed higher Gluc repression over time than CMV/Gluc transfected cells (positive control; Fig. 4a). To investigate the specificity of the miR-221-regulated repression of CMV/

Gluc-3xPT_miR221, mice were implanted with NPA cells transfected with CMV/Gluc-3xPT_miR221 and CMV/Gluc-3xPT_miR221 plus pre-miR-221 (Fig. 4b), or CMV/Gluc-3xPT_miR221 and CMV/Gluc-3xPT_miR221 plus anti-miR-221 (Fig. 4c). The Gluc activities in mice transfected with CMV/Gluc-3xPT_miR221, but without the cotransfection of exogenously derived pre-miR-221 or anti-miR-221 into NPA cells, were normally repressed. However, Gluc expression repression in mice was augmented by pre-miR-221, and its activity was almost undetectable after 3 days. On the other hand, Gluc expression was increased by anti-miR-221, and this was much more resistant to the repressive effect of endogenous miR-221 over the 6-day observation period. Fold ratios of ROI normalized versus CMV/Gluc-3xPT_miR221 showed that Gluc repression by endogenous miR-221 or exogenous pre-miR-221 and the recovery of Gluc expression by anti-miR-221 were maintained for 6 days even though Gluc repression by endogenous miR-221 was unstable at 2 days (Fig. 4d).

Discussion

The expressions and functions of microRNAs have been correlated with aspects of cellular metabolism, such as the regulation of cellular differentiation, proliferation, apoptosis, and development, and with clinically important diseases, such as cancer, cardiovascular diseases, neurological diseases, viral diseases, and metabolic disorders [3, 9–11].

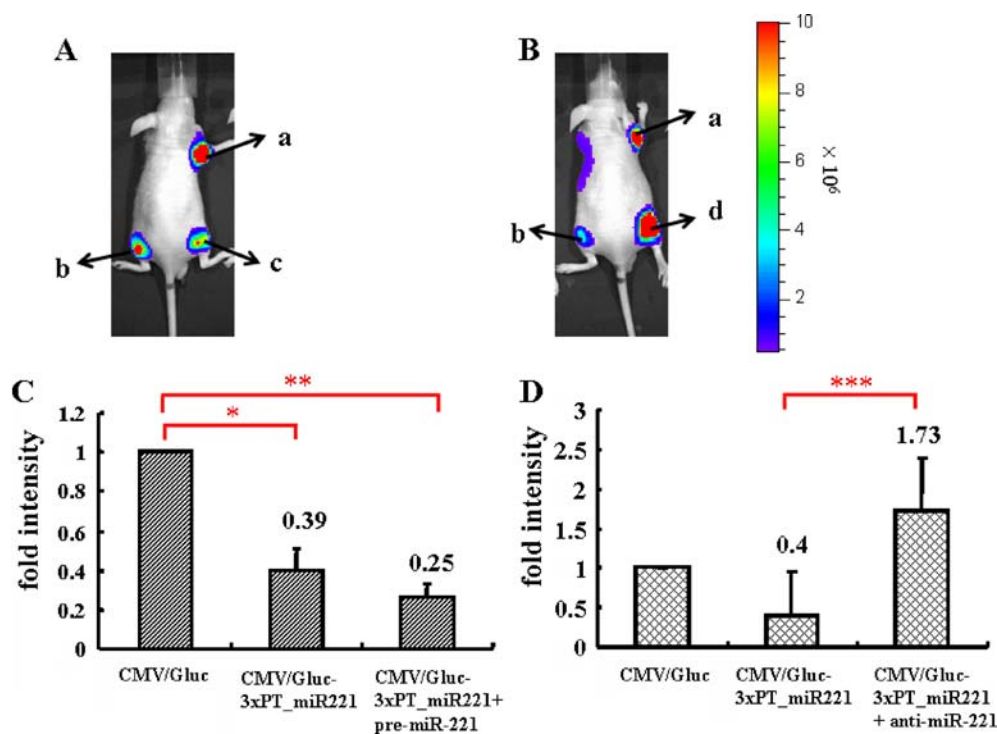


Fig. 3. *In vivo* visualization and ROI analysis of Gluc activities controlled by miR-221 in nude mice bearing NPA cells. Images were obtained at 5 h after implanting NPA cells subcutaneously into nude mice. **a, c** Repression of Gluc activities by endogenous or exogenous miR-221. **b, d** Recovery of Gluc activities by anti-miR-221. **a** Cells transfected with CMV/Gluc; **b** cells transfected with CMV/Gluc-3xPT_miR221; **c** cells cotransfected with CMV/Gluc-3xPT_miR221 and 40 nM of pre-miR-221; **d** cells cotransfected with CMV/Gluc-3xPT_miR221 and 80 nM of anti-miR-221 ($n=3$ mice/group). **c, d** The ROIs of sites were measured and normalized versus those of the CMV/Gluc control (**a**). * $P=0.012$; ** $P=0.014$; *** $P=0.027$. Data are displayed as means \pm SD (fold intensity).

MicroRNAs are up- or downregulated in many human cancers and may function as oncogenes or tumor suppressor genes during carcinogenesis [12–15]. Of the many miRNAs known, miR-221 has been recently reported to be highly expressed in PTC [16–20]. In the present study, we found a significant correlation between miR-221 and PTC by using qRT-PCR and our luciferase reporter system to follow gene expression. In terms of mature miR-221 expression, NPA cells (a PTC cell line) expressed 17-fold higher levels than in HT-ori3 (a NT cell line). This numerical value is considerably higher than the 2.24-fold difference found for primary miR-221. To image the gene expressions of primary and mature miR-221, a 1.8-kb upstream region of miR-221 (containing a well-conserved GC-rich region by comparative genome mapping of the University of California Santa Cruz database) was cloned into the promoterless Fluc vector, pGL3_basic. Unfortunately, promoter analysis of five different fragments of the 1.8-kb upstream region did not show any carcinoma-directing promoter elements in the NPA or TPC-1 cell lines, which suggested that the enhancer elements are located outside this 1.8 kb region (data not shown). This discrepancy in the expressions of endogenous primary and mature miR-221 in NPA cells might be due to an unknown process that converts primary to mature miR-

221 in cancer cells [15]. Moreover, this could be associated with the cytoplasmic storage of miRNAs in P-bodies or some other mechanism required for the rapid turnover of primary miRNAs to mature miRNAs.

The CMV/Gluc-3xPT_miR221 luciferase reporter system we devised to monitor miR-221 in PTC showed great specificity for the miR-221-regulated imaging of exogenous pre-miR-221 or anti-miR-221. Gluc expression *in vitro* was downregulated by more than twofold by binding between endogenous miR-221 and 3xPT, and the addition of exogenous pre-miR-221 in cancer cells dose dependently augmented the repression of Gluc activity, whereas the addition of anti-miR-221 into cancer cells showed the retrieval of repressed Gluc activity. *In vivo* bioluminescence images of Gluc activity produced the same result, i.e., endogenous or exogenous miR-221 downregulated Gluc activity, and this repression was retrieved by anti-miR-221. *In vivo* Gluc expression in cells transiently cotransfected with CMV/Gluc-3xPT_miR221 and pre-miR-221 or anti-miR-221 almost disappeared after 1 week. Thus, for the long-term noninvasive imaging of miRNA-related gene expression, cell lines stably expressing these vectors should be devised to overcome this limitation, which would enable dynamic changes in miRNA-directed Gluc

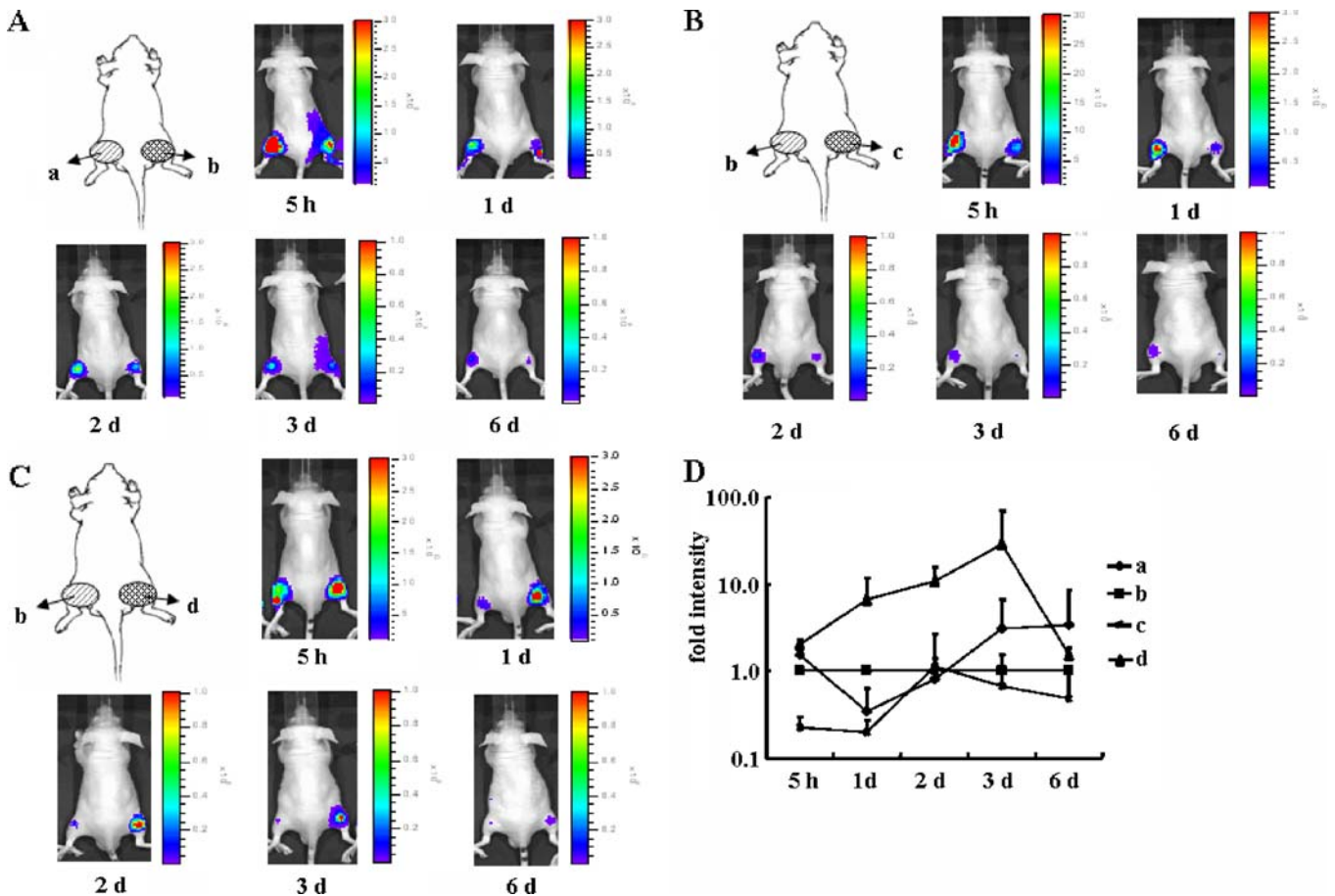


Fig. 4. Real-time monitoring of Gluc activities controlled by miR-221 in nude mice bearing NPA cells for 6 days. Images were obtained at 5 h and at 1, 2, 3, and 6 days after implanting NPA cells subcutaneously into nude mice. **a** Repression of Gluc activity by endogenous miR-221. **b** Comparisons of the Gluc repressing activities of endogenous and exogenous miR-221. **c** Recovery of Gluc activity by anti-miR-221. **a** Cells transfected with CMV/Gluc; **b** cells transfected with CMV/Gluc-3xPT_miR221; **c** cells cotransfected with CMV/Gluc-3xPT_miR221 and 40 nM of pre-miR-221; **d** cells cotransfected with CMV/Gluc-3xPT_miR221 and 80 nM of anti-miR-221 ($n=3$ mice/group). **d** Comparisons were made using ROIs of bioluminescence images at the five-time points. All data were normalized versus the ROIs of cells transfected with CMV/Gluc-3xPT_miR221. Data are displayed as means \pm SD (fold intensity).

activity to be observed long term. The present study demonstrates the capability of the luciferase system for *in vitro* quantitative assessment and for the *in vivo* qualitative visualization of miR-221 expression in PTC. Furthermore, *in vitro* and *in vivo* imaging analyses based on the described luciferase system provide an understanding of the biogenesis of miRNAs and of their functions in carcinoma and in cellular metabolism, without having to sacrifice animals.

The bioluminescence imaging method used in the present study is highly sensitive at detecting low levels of gene expression due to the absence of background, whereas bioluminescence imaging using reporter genes like firefly luciferase, Renilla luciferase, and Gaussia luciferase is limited in terms of its clinical applications due to the immunogenicity of nonhuman imaging reporter enzymes. To overcome this limitation, human-derived reporter genes based on dopamine receptor type 2, somatostatin receptor,

sodium iodide symporter, norepinephrine transporter, human mitochondrial thymidine kinase type 2, transferrin receptor, or ferritin could be devised for the nuclear or MR imaging of miRNA biogenesis *in vivo*.

Conclusion

MiR-221 was found to be overexpressed in PTC, and the devised Gluc reporter system, which was developed to quantify mature miR-221 in PTC, was found to be useful for monitoring the expressions and functions of miRNAs noninvasively. In addition, we believe that this system could be used to investigate the regulation of miRNAs in various tissues during carcinogenesis in living organisms.

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References

- Lee RC, Feinbaum RL, Ambros V (1993) The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell* 75:843–854
- Lu J, Getz G, Miska EA et al (2005) MicroRNA expression profiles classify human cancers. *Nature* 435:834–838
- Kloosterman WP, Plasterk RH (2006) The diverse functions of microRNAs in animal development and disease. *Dev Cell* 11:441–450
- Reinhart BJ, Slack FJ, Basson M et al (2000) The 21-nucleotide *let-7* RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* 403:901–906
- Bushati N, Cohen SM (2007) microRNA functions. *Annu Rev Cell Dev Biol* 23:175–205
- Kim VN (2005) MicroRNA biogenesis: coordinated cropping and dicing. *Nat Rev Mol Cell Biol* 6:376–385
- Zeng Y, Yi R, Cullen BR (2003) MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms. *Proc Natl Acad Sci U S A* 100:9779–9784
- Valencia-Sanchez MA, Liu J, Hannon GJ, Parker R (2006) Control of translation and mRNA degradation by miRNAs and siRNAs. *Genes Dev* 20:515–524
- Obermosterer G, Leuschner PJ, Alenius M, Martinez J (2006) Post-transcriptional regulation of microRNA expression. *Rna* 12:1161–1167
- Weiler J, Hunziker J, Hall J (2006) Anti-miRNA oligonucleotides (AMOs): ammunition to target miRNAs implicated in human disease. *Gene Ther* 13:496–502
- Brennecke J, Hipfner DR, Stark A, Russell RB, Cohen SM (2003) *bantam* encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene *hid* in *Drosophila*. *Cell* 113:25–36
- Osada H, Takahashi T (2007) MicroRNAs in biological processes and carcinogenesis. *Carcinogenesis* 28:2–12
- Tsuchiya S, Okuno Y, Tsujimoto G (2006) MicroRNA: biogenetic and functional mechanisms and involvements in cell differentiation and cancer. *J Pharmacol Sci* 101:267–270
- Zhang B, Pan X, Cobb GP, Anderson TA (2007) microRNAs as oncogenes and tumor suppressors. *Dev Biol* 302:1–12
- Esquela-Kerscher A, Slack FJ (2006) Oncomirs—microRNAs with a role in cancer. *Nat Rev Cancer* 6:259–269
- Pallante P, Visone R, Ferracin M et al (2006) MicroRNA deregulation in human thyroid papillary carcinomas. *Endocr Relat Cancer* 13:497–508
- Felli N, Fontana L, Pelosi E et al (2005) MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor down-modulation. *Proc Natl Acad Sci USA* 102:18081–18086
- He H, Jazdzewski K, Li W et al (2005) The role of microRNA genes in papillary thyroid carcinoma. *Proc Natl Acad Sci U S A* 102:19075–19080
- Nikiforova MN, Tseng GC, Steward D, Diorio D, Nikiforov YE (2008) MicroRNA expression profiling of thyroid tumors: biological significance and diagnostic utility. *J Clin Endocrinol Metab* 93(5):1600–1608
- Gillies JK, Lorimer IA (2007) Regulation of p27Kip1 by miRNA 221/222 in glioblastoma. *Cell Cycle* 6:2005–2009
- Dobrovinn M, Serganova I, Mayer-Kuckuk P, Ponomarev V, Blasberg RG (2004) Multimodality *in vivo* molecular-genetic imaging. *Bioconjug Chem* 15:1376–1388
- Lee JY, Kim S, Hwang DW et al (2008) Development of a dual-luciferase reporter system for *in vivo* visualization of MicroRNA biogenesis and posttranscriptional regulation. *J Nucl Med* 49(2):285–294
- Ottobriani L, Ciana P, Biserni A, Lucignani G, Maggi A (2006) Molecular imaging: a new way to study molecular processes *in vivo*. *Mol Cell Endocrinol* 246:69–75
- Gould SJ, Subramani S (1988) Firefly luciferase as a tool in molecular and cell biology. *Anal Biochem* 175:5–13
- Brasier AR, Tate JE, Habener JF (1989) Optimized use of the firefly luciferase assay as a reporter gene in mammalian cell lines. *Biotechniques* 7:1116–1122
- Tannous BA, Kim DE, Fernandez JL, Weissleder R, Breakefield XO (2005) Codon-optimized *Gaussia* luciferase cDNA for mammalian gene expression in culture and *in vivo*. *Mol Ther* 11:435–443