Utilization of microRNAs with decreased expression levels in response to X-ray irradiation for fine-tuning radiation-controlled gene regulation

AKIHIRO MORII[1*], RYOHEI OGAWA[2*], AKIHIKO WATANABE[1], ZENG-GUO CUI[3], ICHIRO TAKASAKI[4], NOBUTAKA DOI[2], TAKASHI KONDO[2] and HIDEKI FUSE[1]

Departments of [1]Urology, [2]Radiological Sciences and [3]Public Health, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama; [4]Division of Molecular Genetics Research, Life Science Research Center, University of Toyama, Toyama, Japan

Received January 8, 2013; Accepted March 12, 2013

DOI: 10.3892/ijmm.2013.1360

Abstract. We previously developed a promoter that was responsive to radiation by randomly combining *cis*-elements of transcription factors that are activated in response to radiation in prostate cancer cells. The promoter enhanced the expression of the luciferase gene linked downstream by more than 10-fold 12 h after X-ray irradiation at 10 Gy. However, without radiation, it still significantly drove its expression. To suppress expression while retaining its enhancement in response to radiation, we focused our attention on microRNAs (miRNAs). miRNAs are a group of non-coding RNAs approximately 22 nucleotides long that control gene expression by binding to a target sequence residing on the 3'-untranslated region (3'UTR) of a target gene. We identified 8 miRNAs that were downregulated in response to X-ray irradiation, and inserted artificial target sequences composed of randomly combined complementary sequences into 3 representative miRNAs into the 3'UTR of the luciferase gene. The target sequences suppressed the expression, and released the expression, after X-ray irradiation, as expected. When we combined an artificial target sequence with the radiation-responsive promoter, it resulted in a clear-cut gene regulation of expression that was greater than that induced by the promoter alone.

Introduction

Although radiation therapy has been applied for prostate cancer, it has been pointed out that improvement of treatment strategies is necessary to manage the local recurrence of prostate cancer after radiotherapy. In addition, the side effects of radiation therapy are always a concern. Gene therapy is being considered as one of the new procedures for prostate cancer treatment. Prostate cancer is a particularly suitable malignancy to study, due to its relatively accessible location and the specificity of its gene products (1). However, there are still problems that need to be solved before gene therapy can become a general therapeutic procedure for prostate cancer.

It has been pointed out that combining gene therapy with radiation may lead to a novel, more efficient therapy with fewer side effects as the strategies can compensate for each other's defects (2). Thus, we developed promoters that are activated in response to radiation, wherein radiation stimulation may control the expression of therapeutic genes. We demonstrated that it was possible to efficiently obtain radiation-responsive promoters by linking the TATA box signal to randomly combined DNA fragments containing *cis*-elements of transcription factors activated by radiation. However, the resulting promoters tended to be cell type-specific (3). Therefore, we constructed a radiation-responsive promoter for prostate cancer cells with *cis*-elements of transcription factors activated in prostate cancer cells by radiation. In addition, these were successfully improved by randomly introducing point mutations. The promoter that was designated clone 880-8 enhanced the expression of the luciferase gene linked downstream more than 10-fold 12 h following 10 Gy X-ray irradiation *in vitro* in prostate cancer cells. In the case of malignant LNCaP cells implanted into a mouse, the promoter also significantly enhanced the luciferase gene expression after X-ray irradiation, although to a lesser degree. However, in an *in vitro* simulation study of suicide gene therapy, when a prodrug was applied at high concentrations, significant cell death was observed even without X-ray irradiation (4).

microRNAs (miRNAs) are low-molecular-weight RNAs (19-24 nucleotides in length) that do not code for polypeptides and control gene expression mainly by interfering with the translation process after binding target sequences complimentary to the miRNA seed sequence present in messenger RNAs. Over 1,000 miRNAs have been identi-

Correspondence to: Dr Ryohei Ogawa, Department of Radiological Sciences, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama 930-0194, Japan
E-mail: ogawa@med.u-toyama.ac.jp

*Contributed equally

Key words: microRNA, promoter, gene expression, radiation

fied in human cells and are reported to be involved in many important biological processes, such as development, differentiation, cell proliferation and cell death in a tissue type-specific manner (5).

It was recently reported that expression of many miRNAs is altered in response to stimuli. For instance, many cancer cells have altered miRNA expression profiles in response to radiation (6-8). In addition, the expression of the let-7 family, known as antimirs, increases after stimulation with radiation (9). These results suggest that changes in miRNA expression may be involved in the response of cells to radiation (10). Furthermore, it has been reported that other stimuli, including anticancer drugs (11), ultrasound (10), hyperthermia (12) and hypoxia (13) also induce changes in miRNA expression levels, suggesting that miRNAs may be involved in the adaptive response of cells to various types of stimuli.

Several reports have indicated that changes in miRNA expression may be useful for controlling gene expression for tissue-specific or cancer-specific gene therapy. Research has shown that inserting copies of a target sequence of an miRNA that is not expressed in the target tissues into the 3'-untranslated region (3'UTR) of a gene of interest resulted in gene expression in a target tissue-specific manner (15-17).

In this study, we analyzed the miRNA expression changes in a prostate cancer cell line after radiation stimulation. We focused our attention on the miRNAs that significantly decreased in response to radiation, and introduced copies of their complementary target sequences into the 3'-UTR of our gene of interest to see if it could lead to radiation-mediated gene expression control. In addition, we combined target sequences with a radiation-responsive clone (880-8) promoter, so that it could lead to fine-tuned gene expression control, thus suppressing gene expression in the absence of radiation, while increasing such expression after radiation stimulation.

Materials and methods

Cells and bacteria. LNCaP cells from human prostate carcinoma were used throughout this study. The cell line was purchased from Health Science Research Resources Bank (Tokyo, Japan). Cells were grown and maintained in RPMI-1640 medium supplemented with 10% fetal calf serum and appropriate antibiotics at 37˚C in a 5% CO_2 atmosphere. The other cell line used in this study was AmphoPack293 (Takara Bio, Inc., Ohtsu, Japan), which is a genetically modified cell line used for the generation of recombinant retrovirus particles. It was derived from HEK293 cells, and the envelope gene and gag gene of the moloney murine leukemia virus were inserted into the genome to provide the viral proteins for packaging. A retrovirus genome-like RNA containing the Ψ sequence transcribed from a vector introduced into the cells is packaged in a virus-like particle and buds into the culture medium. The particles carry amphotropic envelope protein on the surface so that they can recognize the ram 1 receptor. Therefore, they infect a broad range of mammalian cells.

The DH5α strain of *Escherichia coli* (Nippon Gene; Toyama, Japan) was used for the DNA manipulation experiments. *E. coli* cells were grown in LB medium at 37˚C. All medium components were purchased from BD Diagnostics (Sparks, MD, USA). DNA manipulation experiments with *E. coli* were performed according to the methods described by Sambrook and Russell (18).

RNA extraction and miRNA microarray analysis. Total RNA was extracted from control cells as well as cells exposed to radiation 6 h post-exposure using the miRNeasy Extraction kit. Samples were treated with DNase I (RNase-free DNase kit), (both from Qiagen Inc., Valencia, CA, USA) for 15 min at room temperature during the procedure as directed in the instructions to remove residual genomic DNA.

miRNA expression was analyzed using a GeneChip system with the miRNA array ver. 1.0 or 2.0 (Affymetrix Inc., Santa Clara, CA, USA). Samples for array hybridization were prepared using the HRT FlashTag RNA Labeling kit for Affymetrix GeneChip miRNA arrays (Genisphere Inc., Hatfield, PA, USA) following a procedure described in the product manual. Briefly, 1 μg of total RNA was labeled with biotin through ligation with 3DNA dendrimer. The labeled RNA was hybridized to the GeneChip array at 48˚C for 16 h. The arrays were washed, stained with streptavidin-phycoerythrin and scanned using a probe array scanner. The scanned chip data were converted to digitized information using the miRNA QC Tool application (ver. 1.0.33.0 or 1.1.1.0).

Quantitative real-time PCR. To evaluate the miRNA expression, quantitative real-time PCR was performed. Total RNA was collected from LNCaP cells using the miRNeasy Mini kit (Qiagen Inc.) and treatment with DNase I according to the manufacturer's instructions, as described above. cDNAs were synthesized using the extracted RNA as templates, using a miScript Reverse Transcription kit (Qiagen Inc.) according to the manufacturer's instructions. The miRNA expression analysis was performed using a Mx3000P QPCR System (Agilent Technologies Inc., Santa Clara, CA, USA) with the synthesized cDNA. Quantitative PCR measurement by real-time monitoring of SYBR-Green integration into the synthesized DNA was performed during the PCR process: incubation at 95˚C for 15 min, and then 40 thermal cycles for reactions at 94˚C for 15 sec, 55˚C for 30 sec and 70˚C for 30 sec, followed by reactions at 55˚C for 30 sec and 95˚C for 30 sec with a miScript SYBR-Green PCR Kit (Qiagen Inc.). After the PCR process, the dissociation temperature of the synthesized DNA fragments was also determined by monitoring the release of SYBR-Green from the denatured DNA to confirm the integrity of the synthesized DNA fragment. The primers used for the PCR reaction were selected from a miRNA primer library provided in the miScript Primer assay (Qiagen Inc.). We used a specific primer to detect human U1A small nuclear RNA expression as an internal control. Relative standard curves representing several 10-fold dilutions of cDNA from a representative sample were used for the linear regression analysis for other samples.

Vector construction. The initial assessment of the introduction of target sequences into the 3'UTR of the luciferase gene was performed using vectors constructed based on pmirGLO (Promega Corp., Madison, WI, USA), in which the 3'UTR of the firefly luciferase gene carries a multi-cloning site and is driven by the mouse PGK promoter, and the *Renilla* luciferase gene was aligned in tandem with the firefly luciferase gene

and is driven by the SV40 promoter. We inserted DNA fragments containing target sequences into the multi-cloning site to see whether the target sequences affected the luciferase expression after radiation.

We constructed two types of target sequences. One was composed of randomly combined complimentary sequences of the 3 miRNAs that were chosen due to the decreases in their expression in response to radiation, and the other was composed of 2 or 4 copies of a single complimentary sequence of 1 of the 3 miRNAs. To construct target DNA fragments composed of randomly combined complimentary sequences, we synthesized DNA fragments with a sticky end sequence that was not palindromic (5'-agtg-3') so that the complimentary sequences would have to be unidirectionally aligned. The synthesized target sequences for miR-92-1-5p were as follows: 5'-aggttgggatcggttgcaatgctcact-3' and 5'-agcattgcaaccgatcccaacctagtg-3; for miR-19b, 5'-tgtgcaaatccatgcaaaactgacact-3' and 5'-tcagttttgcatggatttgcacaagtg-3'; miR-503, 5'-tagcagcgggaacagttctgcagcact-3' and 5'-ctgcagaactgttcccgctgctaagtg-3'. These fragment pairs were annealed in annealing buffer (100 mM NaCl, 50 mM Tris-Cl, pH 7.5) by incubating them at 95˚C for 5 min and then gradually decreasing the temperature to 4˚C. The annealed pairs were treated with polynucleotide kinase at 37˚C for 60 min, and then equimolar amounts were mixed. We also synthesized a pair of DNA fragments containing a *Nhe*I recognition site (5'-gtacgctagcagtg-3' and 5'-gctagcgtaccact-3') and a one containing *Sal*I and *Eco*RI recognition sites (5'-gaattcgtcgacagtg-3' and 5'-gtcgacgaattccact-3') and similarly treated them for annealing and kinase activity. They were also mixed at equimolar concentrations.

The target mixture and the restriction site mixture were further mixed at ratios of 20:1, 10:1 and 5:1 and then separately ligated. Elongated DNA fragments were digested with *Nhe*I and *Sal*I, and purified using spin columns. They were then inserted into the *Nhe*I and *Sal*I sites of pmirGLO. We judged that an insert carrying the *Eco*RI site was cloned in the right direction. We examined 9 plasmid clones designated as pmirGLO5-1, 5-2, 5-3, 5-4, 10-1, 10-2, 20-1, 20-2, 20-3, according to the mixing ratios.

We then constructed target fragments containing 2 or 4 copies of the complimentary sequences of the 3 miRNAs. For miR-19b, 5'-ctagctcagttttgcatggatttgcacacagctcagttttgcatggatttgcacaaagcttg-3' and 5'-tcgacaagctttgtgcaaatccatgcaaaactgagctgtgtgcaaatccatgcaaaactgag-3' were annealed and treated with kinase, and then were inserted into the *Nhe*I and *Sal*I sites of pmirGLO to construct pmirGLO-19b-2. Furthermore, 5'-tcgactcagttttgcatggatttgcacacagctcagttttgcatggatttgcacagaattcgc-3' and 5'-ggccgcgaattctgtgcaaatccatgcaaaactgagctgtgtgcaaatccatgcaaaactgag-3' were annealed and treated with kinase, and inserted into the *Sal*I and *Not*I sites of pmirGLO-19b-2 to construct pmirGLO-19b-4. As for miR-503, 5'-ctagcctgcagaactgttcccgctgctacagcctgcagaactgttcccgctgctaaagcttg-3' and 5'-tcgacaagctttagcagcgggaacagttctgcaggctgtagcagcgggaacagttctgcagg-3' were annealed and treated with kinase, and then were inserted into the *Nhe*I and *Sal*I sites of pmirGLO to construct pmirGLO-503-2. Furthermore, 5'-tcgacctgcagaactgttcccgctgctacagcctgcagaactgttcccgctgctagaattcgc-3' and 5'-ggccgcgaattctagcagcgggaacagttctgcaggctgtagcagcgggaacagttctgcagg-3' were annealed and treated with kinase, and inserted into the *Sal*I and *Not*I sites of pmirGLO-503-2 to construct pmirGLO-503-4. For miR-92a-1-5p, 5'-ctagcagcattgcaaccgatcccaacctcagcagcattgcaaccgatcccaacctaagcttg-3' and 5'-tcgacaagcttaggttgggatcggttgcaatgctgctgaggttgggatcggttgcaatgctg-3' were annealed and treated with kinase, and inserted into the *Nhe*I and *Sal*I sites of pmirGLO to construct pmirGLO-92a-1-5p-2. In addition, 5'-tcgacagcattgcaaccgatcccaacctcagcagcattgcaaccgatcccaacctgaattcgc-3' and 5'-ggccgcgaattcaggttgggatcggttgcaatgctgctgaggttgggatcggttgcaatgctg-3' were annealed and treated with kinase, and inserted into the *Sal*I and *Not*I sites of pmirGLO-92a-1-5p-2 to construct pmirGLO-92a-1-5p-4.

Transient transfection and X-ray irradiation. The constructed plasmid vectors were transfected into cells using the Effecten reagent (Qiagen Inc.) according to the manufacturer's instructions. One and a half million cells were washed once with pre-warmed RPMI-1640, and resuspended with 1.5 ml of the medium. An Effecten complex containing 1.0 of a plasmid vector, and in some cases 10 ng phRL-TK (Promega Corporation), was added to each dish. The cells were harvested and resuspended with 5 ml of pre-warmed RPMI-1640 medium after incubation at 37˚C for more than 4 h, and then were re-incubated at 37˚C overnight. A 35-mm cell culture dish was seeded with $3.0x10^5$ of the incubated cells in 2 ml of pre-warmed RPMI-1640 and then the cells were subjected to X-ray irradiation. Each cell culture dish was placed on the turning table of an X-ray generator (MBR-1520-3; Hitachi Medical Technology Corp., Tokyo, Japan) and irradiated with 5 to 15 Gy X-ray at 5 Gy/min.

Luciferase assay. At various times after X-ray irradiation, cells were washed once with PBS, and 300 μl of passive lysis buffer from the Dual Luciferase Assay kit (Promega Corp.) was added to lyse the cells. Cells were incubated at room temperature for 15 min. A volume of 10 μl of cell lysate supernatant was mixed with 50 μl of Luciferase Assay Reagent II from the kit to measure the luminescence generated by the firefly luciferase. Immediately following this, 50 μl of Stop & Glow reagent from the kit was added to the mixture to measure the luminescence generated by the *Renilla* luciferase expressed from phRL-TK or pmrGLO. The amount of luciferase gene expression was determined as relative luminescence units (RLU), where the value of luminescence from the firefly luciferase was divided by that of the *Renilla* luciferase expressed in the same lysate. The increase or decrease in the luciferase expression was expressed as the fold-activity, where the RLU value of a sample of treated cells was divided by that of an identically prepared sample without treatment.

In the case of a combination of stable transfection to express firefly luciferase and transient transfection to express *Renilla* luciferase, when X-ray irradiation was applied to the cells, the cell proliferation would be modified, possibly affecting the ratio of firefly and *Renilla* luciferase activities, regardless of gene expression efficiency. We thus employed a single luciferase assay for such cases, using the protein concentration of the cell lysate as a reference for standardization. At various times after X-ray irradiation, cells were washed once in PBS, and 300 μl of passive lysis buffer from the Dual Luciferase Assay kit was added to lyse the cells.

The cells were incubated at room temperature for 15 min. A volume of 10 μl of cell lysate supernatant was mixed with 50 μl of Luciferase Assay Reagent II included in the kit to measure the luminescence generated by the firefly luciferase. The protein concentration was determined by the Bradford method with 5-20 μl of the cell lysate using a BioRad protein assay kit (Bio-Rad, Hercules, CA, USA).

Recombinant retrovirus vector construction and stable transfection. After the initial screening of pmirGLO derivative vectors with miRNA target insertion, we excised 2 miRNA target sequences out of agarose gels that had been subjected to electrophoresis of pmirGLO-10-1 and pmirGLO-503-4 after digestion with *Nhe*I and *Eco*RI. Each of these fragments was inserted into the *Xba*I and *Eco*RI sites of pRet-880-8-luc to generate pRet-880-8-luc-10-1 and pRet-880-8-luc-503-4.

One million AmphoPack293 cells (Takara Bio, Inc.) were seeded onto a 60-mm collagen-coated cell culture dish, and the following day, they were transfected using a CalPhos™ Mammalian Transfection kit (Takara Bio, Inc.) with 5 mg of each of the constructed retrovirus-generating vectors. The virus-containing conditioned medium was collected 48 h after transfection and passed through a 0.45-mm filter to remove debris. Polybrene (Sigma-Aldrich Inc., St. Louis, MO, USA) was added to the filtered medium at a final concentration of 7.0 μg/ml. This prepared solution was used as a virus source to infect $1x10^6$ LNCaP cells. The infected cells were concentrated by treatment with 0.5 μg/ml puromycin, to which infected cells were resistant, in order to establish a stably transfected cell line.

Statistical analysis. All values are expressed as the means ± standard deviations. Differences were assessed with the Student's unpaired t-test. For comparisons of more than two groups, a one-way analysis of variance (ANOVA) was used. Statistical significance was established at a value of $P<0.05$.

Results

Changes in the miRNA expression profile following X-ray irradiation. First, we investigated the miRNA profile in the LNCaP cells using the miRNA array ver. 1.0 of the GeneChip system developed by Affymetrix Inc. Total RNA was extracted from LNCaP cells 4 h after X-ray irradiation at 5 or 10 Gy. The results showed that the expression levels of many of the miRNAs were altered following X-ray irradiation, suggesting that they may be involved in the adaptive responses of cells to radiation, consistent with reports showing that miRNAs are involved in the cellular response to radiation (19).

We focused on the miRNAs downregulated after X-ray irradiation, since we intended to determine whether such miRNAs can be applied to increase X-ray irradiation-mediated gene expression. We identified 15 miRNAs that were downregulated in the cells after exposure to 5 Gy (Table I) or 10 Gy (Table II) irradiation. In addition, we obtained another list of downregulated miRNAs by using the miRNA array ver. 2.0 with the total RNA extracted from LNCaP cells after 10 Gy X-ray irradiation (Table III).

Table I. Radiation-induced changes in expression of miRNAs in LNCaP cells 4 h after X-ray irradiation at 5 Gy.

miRNA[a]	Change ratio	Relative expression value (without radiation)
has-miR-126	0.80	27.60
hsa-miR-20b	0.80	106.11
hsa-miR-203	0.80	50.80
hsa-let-7g	0.82	73.09
hsa-miR-30b	0.83	57.43
hsa-miR-30a	0.85	37.12
hsa-miR-19b	0.85	295.98
hsa-miR-29a	0.85	44.77
hsa-miR-25	0.85	202.84
hsa-miR-183	0.86	59.72
hsa-miR-20a	0.86	706.08
hsa-miR-1826	0.87	2295.58
has-miR-1207-5p	0.87	91.50
has-miR-105	0.87	31.77
hsa-miR-181a	0.88	40.73

[a]The listed miRNAs demonstrated significant changes in their relative expression values before and/or after radiation.

We chose miR-181a, -1207-5p, -20b, -19b, -3156, -3175, -92a-1*, -503 and -224 for the subsequent quantitative real-time PCR analyses for confirmation of the change in expression and to assess the time course of the expression decrease. We chose these miRNA since they exhibited larger downregulation ratios after irradiation according to the microarray results, and exhibited higher expression in cells without radiation exposure. We also had to consider the availability of primers for the real-time PCR analysis. Regarding Tables I and II, we gave a higher priority to Table II, but chose miRNAs listed in both of the Tables. According to the results (Fig. 1), the data concerning miR-181a obtained with the GeneChip and real-time PCR were conflicting. Although the expression levels of miR-3156 and -3175 decreased initially for a few hours, they increased rapidly by 12 h after X-ray irradiation. Regarding miR-20b and -1207, their decreases in expression reached a trough level 6 h after X-ray irradiation. The expression levels of miR-92a-1* and miR-19b reached their greatest decrease 12 h after X-ray irradiation, while miR-224 and miR-503 continued to decrease for up to 24 h after X-ray irradiation. Out of these miRNAs, we chose miR-503, miR-92a-1* and miR-19b to evaluate the potential of using their changes in expression for gene expression control due to their large ratios of decrease.

Effects of target sequences composed of complimentary sequences on the luciferase expression levels following X-ray irradiation. We constructed plasmid vectors containing the firefly luciferase gene whose 3'-UTR carried a variety of target sequences composed of complimentary sequences of the chosen miRNA(s). As described in Materials and methods, we constructed two types of target sequences. One was

Table II. Radiation-induced changes in expression of miRNAs in LNCaP cells 4 h after X-ray irradiation at 10 Gy.

miRNA[a]	Change ratio	Relative expression value (without radiation)
has-miR-181a	0.59	40.73
has-miR-1207-5p	0.60	91.50
hsa-miR-193a-5p	0.72	77.39
hsa-mi-638	0.73	138.32
hsa-miR-502-3p	0.75	67.38
hsa-miR-138-1*	0.77	25.87
hsa-miR-181b	0.78	44.83
hsa-miR-500	0.78	57.41
hsa-miR-20b	0.78	106.11
hsa-miR-19b	0.79	295.98
hsa-miR-767-5p	0.80	44.19
hsa-miR-149	0.80	50.79
has-miR-30a	0.81	37.12
has-miR-193b*	0.81	23.68
hsa-miR-720	0.81	131.87

[a]The listed miRNAs demonstrated significant changes in their relative expression values before and/or after radiation.

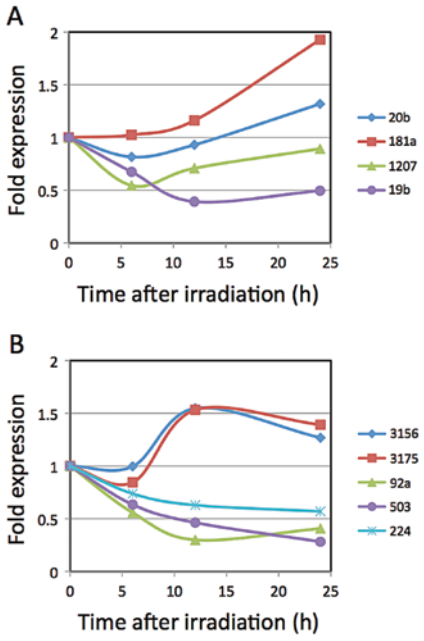

Figure 1. Kinetics of miRNA expression after X-ray irradiation. LNCaP cells were irradiated with 10 Gy X-rays. Total RNAs were extracted at 0, 6, 12 and 24 h after irradiation, and the cDNAs were synthesized for the real-time PCR analyses. (A) Expression levels of miR-20b, -181a, -1207-5p and -19b, which were chosen according to the results of the GeneChip miRNA array ver. 1.0, were analyzed by real-time PCR. (B) Expression levels of miR-3156, -3175, -92a-1*, -503 and -224, which were chosen according to the results of the GeneChip miRNA array ver. 2.0, were analyzed by real-time PCR.

composed of randomly combined complimentary sequences of the chosen miRNAs and the other comprised multi-copies

Table III. Radiation-induced changes in expression of miRNAs in LNCaP cells 6 h after X-ray irradiation at 10 Gy.

miRNA[a]	Change ratio	Relative expression value (without radiation)
has-miR-3156	0.39	23.71
hsa-miR-3175	0.50	77.52
hsa-miR-139-3p	0.59	20.19
hsa-lmiR-483-5p	0.64	21.66
hsa-miR-198	0.66	21.39
hsa-miR-92a-1*	0.67	81.86
hsa-miR-30c-2*	0.68	28.61
hsa-miR-503	0.68	95.41
hsa-miR-224	0.69	48.22

[a]The listed miRNAs demonstrated a significant change in their relative expression values before and/or after radiation.

of tandemly-aligned complimentary sequence of the miRNA. We designated vectors containing the former target sequences as pmirGLO-miRT#5-1, 5-2, 5-3, 5-4, 10-1, 10-2, 20-1, 20-2 and 20-3, and designated the latter as pmirGLO-miRT#19b-2, 19b-4, 92a*-2, 92a*-4, 503-2 and 503-4. Each was transfected into LNCaP cells and irradiated with 10 Gy X-rays. The cell lysates were subjected to a dual luciferase assay 9 h after X-ray irradiation (Fig. 2).

We observed suppressed expression of the luciferase gene in all of the plasmids containing target sequences. In addition, when transfected cells were irradiated, the enhancement ratios of luciferase expression increased compared to those of cells transfected with pmirGLO. In the case of vectors containing complementary sequences of a single miRNA, as expected, vectors containing 4 copies of the complementary sequences showed higher enhancement ratios for miR-92a* and miR-503, although this was opposite in the case of the vectors containing complementary sequences for miR-19b. These results suggest that the mRNA conformation may be involved in the expression, presumably as a result of sequestering the target sequences. Among the targets composed of a single miRNA complementary sequence, pmirGLO-miRT#503-4 showed the highest enhancement, which was 1.9-fold that without the target sequences. In addition, among the randomly combined complementary sequences, pmirGLO-miRT#10-1 was the highest, and showed 1.5-fold enhancement compared to that of pmirGLO without a target sequence.

Nucleotide sequence analysis revealed that the miRT#10-1 target sequence was 218 nucleotides long, and was composed of 8 complementary sequences, and as shown in Fig. 3, 3 were complementary sequences for miR-19b and miR-503, and the rest were composed of 2 copies of miR-92a-1* complementary sequences.

Control of the luciferase gene expression using the combination of a radiation-responsive promoter and miRNA target sequence. We then combined the clone 880-8 promoter (a previously constructed radiation-responsive promoter) and the target sequences contained in pmirGLO-miRT#503-4

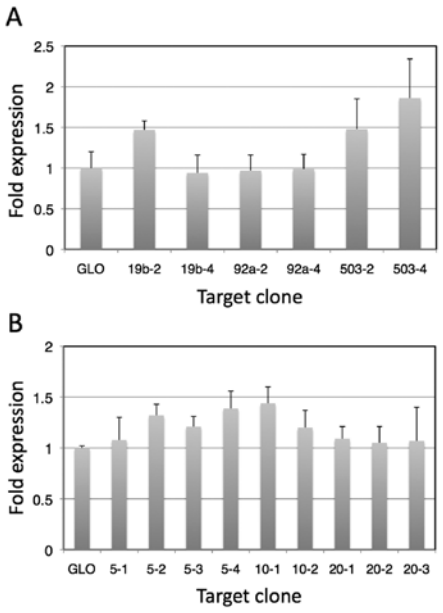

Figure 2. Effects of target constructs on the luciferase expression in response to X-rays. LNCaP cells transiently transfected with a derivative of pmirGLO carrying a constructed target sequence in the 3'UTR of the firefly luciferase gene were irradiated with 10 Gy X-rays, and a luciferase assay was conducted 9 h later to determine whether the target affects the expression of the luciferase gene. (A) The pmirGLO derivatives used were those carrying a target composed of 2 or 4 tandemly aligned copies of the complementary sequence of a single miRNA. (B) The pmirGLO derivatives used were those carrying a target composed of randomly combined complementary sequences of 3 miRNAs.

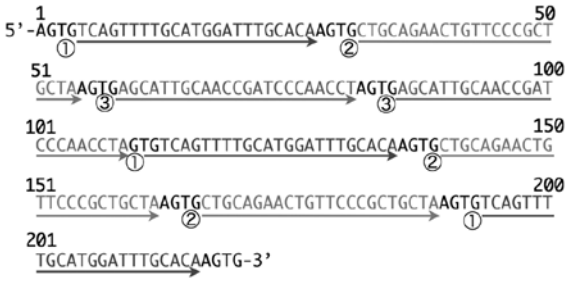

Figure 3. Nucleotide sequences of the target sequence in pmirGLO-miRT#10-1. The incorporated complementary sequences are underlined and numbered 1, 2 and 3 to indicate miR-19b, miR-503 and miR-92a-1*, respectively. The directions of the arrows represent the directions of the complementary sequences that were uniformly aligned due to their sticky end sequence that was not palindromic.

and pmirGLO-miRT#10-1. The target sequences were PCR-amplified and cloned downstream of the luciferase gene of pRet-880-8-luc, which was a vector used to construct a recombinant retrovirus expressing the luciferase gene under control of the clone 880-8 promoter. The promoter is efficiently responsive to X-ray irradiation, but its basal activity is a problem since it expresses genes linked downstream even in the absence of radiation, and it killed cells at a high concentration of prodrug in an *in vitro* suicide gene therapy simulation study (4). Thus, by combining these features, we wished to determine whether the target sequences could suppress the basal activity while retaining the peak activity after X-ray

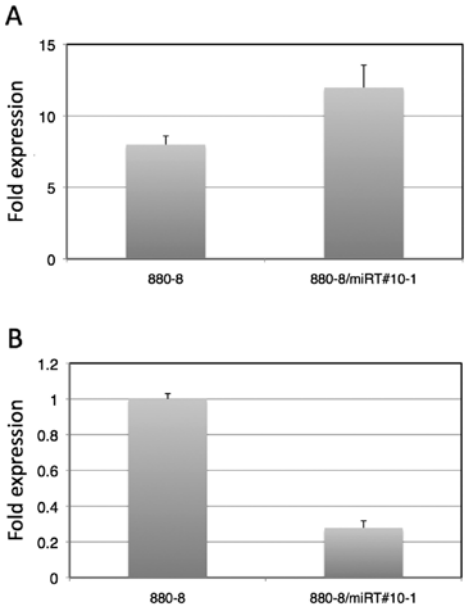

Figure 4. Coordinated gene expression control by the clone 880-8 promoter and miRT#10-1, an miRNA target sequence. (A) The suppression of the basal expression (expression without radiation enhancement) of the luciferase gene driven by the clone 880-8 promoter was mediated by miRT#10-1. (B) Radiation stimulation inhibited the miRT#10-1-mediated suppression due to the decrease in miRNA expression, resulting in a greater enhancement of the expression of the luciferase gene.

irradiation. We designated a vector constructed by introducing the target sequence of pmirGLO-miRT#503-4 into pRet-880-8-luc as pRet-880-8-luc-miRT#503-4, and another vector with the target sequence of pmirGLO-miRT#10-1 as pRet-880-8-luc-miRT#10-1. Recombinant retroviruses were constructed and infected into LNCaP cells to generate stably transfected cells. We then obtained stably transfected LNCaP cells designated LNCaP-880-8-luc-miRT#10-1 using pRet-880-8-luc-miRT#10-1. However, we could not obtain stably transfected cells with pRet-880-8-luc-miRT#503-4, although the reason remains unknown. It is possible that the target sequence may contain some type of signal sequence affecting the stability of the recombinant virus.

As shown in Fig. 4A, 9 h after the LNCaP-880-8-luc-miRT#10-1 cells were irradiated with 10 Gy X-rays, the luciferase activity was enhanced 12-fold to that of the level of the LNCaP-880-8-luc-miRT#10-1 cells without X-ray irradiation. This was an ~1.5-fold enhancement compared to that of the LNCaP-880-8-luc without a target sequence. Fig. 4B shows a comparison of the luciferase activities of LNCaP-880-8-luc and LNCaP-880-8-luc-miRT#10-1 without X-ray irradiation, indicating that there was a 70% suppression of luciferase expression by the target sequence contained in LNCaP-880-8-luc-miRT#10-1. As expected, the target sequence of miRNAs was able to be used for fine-tuning the gene expression control induced by another mechanism.

Discussion

In the present study, we initially investigated change in the miRNA profiles in LNCaP cells after radiation. We confirmed that there were several miRNAs that exhibited

changes in their expression levels in the prostate cancer cell line after X-ray irradiation as determined by the microarray and quantitative real-time PCR analyses. In addition, reports have suggested that expression levels of miRNAs are altered in response to stimuli including ultrasound (10), anticancer agents (11), hyperthermia (12) and hypoxia (13). As Simone *et al* (20) demonstrated that the oxidative stress induced after radiation alters the expression levels of miRNAs, the changes in miRNA expression after radiation that were observed in our study may have been caused by oxidative stress. It was also suggested that such miRNA expression changes may be involved in the responses of cells to stimulation, including apoptosis (3). In our case, several miRNAs may have played a role in the response of the cells to radiation.

We employed two different versions of GeneChip® microarrays for the miRNA profile analyses. The lists of miRNAs shown in Tables I and III were substantially different from each other. The reason for the differences remains unknown, but may have been derived from inter-experimental differences in techniques or the differences between the two versions of the arrays (e.g. sensitivity, sample numbers). We consider that it may have been due to inter-experimental differences, since most of the miRNAs (8 out of 9) listed as being decreased after radiation were confirmed to be decreased after X-ray irradiation by real-time PCR.

We focused on the miRNAs that exhibited decreased expression levels after radiation to determine whether their target sequences could be applied for gene expression regulation. Few reports exist concerning the use of miRNAs to regulate gene expression. For instance, Xie *et al* (17) found that the introduction of a target sequence from a liver-specific miRNA into the 3'UTR of a gene in an expression vector for the central nervous system resulted in suppression of the expression in the liver after systemic administration. In the present study, we attempted to use radiation-responsive miRNAs rather than tissue-specific miRNAs. We introduced the target sequences of miRNAs whose expression was decreased following radiation into a gene of interest so that the gene could be suppressed under normal conditions, but retain a higher level of expression following radiation exposure. We demonstrated that this was possible. However, the degree of miRNA expression changes induced by radiation was limited. Thus, we applied the system for fine-tuning another gene regulation system.

The system was combined with a radiation-responsive promoter that was previously developed by us. The promoter was sensitively responsive to radiation *in vitro*, and *in vivo*, and its transcriptional activity was activated after radiation. However, the promoter was active to some extent even without radiation. Thus, in a suicide gene therapy simulation study *in vitro*, cells were killed in the presence of a high concentration of prodrug even without radiation. We inserted two target sequences that enhanced gene expression after radiation into the 3'UTR of the luciferase gene under control of the radiation-responsive promoter. The transfected cells successfully showed gene regulation that was more strict than that by LNCaP-880-8-luc with the promoter alone, with approximately one-third of the basal activity (activity without radiation) and a 1.5-fold increase in the enhancement ratio after radiation.

We did not obtain stably transfected cells with the luciferase gene containing a target sequence of miRT#503-4, which exhibited the best results in the transient transfection experiment among the various constructed target sequences, since all of the cells were killed during the process of concentrating the transfected cells by cultivating them in medium containing puromycin. Although the reason for the cell death remains unknown, we considered that the sequence may have affected the stability of the recombinant retrovirus. We are still trying to obtain stably transfected LNCaP cells with the luciferase gene carrying the target sequence under the control of the clone 880-8 promoter.

Our present findings showed that this method utilizing changes in the expression levels of miRNAs in response to stimulation may be used for fine-tuning a gene regulation system, even though it may be difficult to apply alone for the purpose of obtaining sufficient gene regulation. The stimulation-responsive promoter developed using our method was associated with some basal activity. Thus, this method utilizing miRNA expression changes should be useful for promoters developed according to our method. At present, we are aiming to devise a more effective application of miRNA expression change for developing new gene regulation systems.

Acknowledgements

This study was supported, in part, by Grants-in-Aid for Scientific Research (C) (21500403) and for Young Scientists (B) (20377253 and 25861412) from the Japan Society for the Promotion of Science, and, in part, by a Collaborative Research Project of the Wakasa Wan Energy Research Center. The authors thank Dr Loreto B. Feril Jr for his critical reading of the manuscript.

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