Biotechnology Reports 4 (2014) 5–13

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Effect of small interfering RNAs on matrix metalloproteinase 1 expression

Gen-Hung Chen [a], Chun-Hua Huang [a], Huang-Yao Luo [a], Shann-Tzong Jiang [b,c,*]

[a] Department of Cosmetic Science, Providence University, 200, Chung-Chi Rd., Taichung 43301, Taiwan
[b] Department of Food & Nutrition, Providence University, Taiwan
[c] Department of Food Science, National Taiwan Ocean University, Keelung 202, Taiwan

ARTICLE INFO

Article history:
Received 1 May 2014
Received in revised form 11 July 2014
Accepted 15 July 2014
Available online 20 July 2014

Keywords:
Matrix metalloproteinase 1
Small interfering RNAs
MMP1 gene expression

ABSTRACT

Three small double strand siRNAs (506-MMP1, 859-MMP1 and 891-MMP1), each contains 25–26 nucleotides, with high specific to human MMP1 were designed according to mRNA sequence of human MMP1 (NCBI, NM_002421). To monitor the MMP1 gene expression, the total RNAs of human skin fibroblast (Detroit 551, BCRC 60118) were extracted. One human matrix metalloproteinase 1 (MMP1) partial sequence cDNA, included all the three siRNA target sequences, amplified specifically via RT-PCR and PCR reactions, and three synthesized siRNA target DNAs were cloned individually into pAcGFP1-N3 with green fluorescent protein (GFP). These reporter plasmids were then transfected individually into malignant melanoma (MeWo, BCRC 60540) and the GFP was detected after 48 h. Fluorescence results indicated that the 859 siRNA revealed highest inhibitory ability (almost 90%), and was, accordingly, transfected into MeWo cells. According to the real-time quantitative PCR and western blot, the exhibition ability to silence MMP1 gene expression was 85–89%.

1. Introduction

Matrix metalloproteinase 1 (MMP1), the member of MMP family, is a kind of zinc and calcium-dependent endopeptidase and collagenase that are able to degrade essentially all extracelluar matrix (ECM) components, such as basement membranes, collagen, and fibronectin [23,16,24]. The human MMPs family, which consists of at least 26 proteases, can be divided into several subgroups according to their structure and substrate specificity [22,28]. These subfamilies include collagenases, gelatinases, stromelysins, matrilysins, and membrane-type MMPs (MT-MMPs), among others. MMPs play an important role in both physiological and pathological conditions, including tissue regeneration, wound repair, reproduction, arthritis, atherosclerosis, and autoimmune blistering disorders of the skin [3]. MMPs have also been implicated in carcinogenesis because of their ability to degrade ECM, which is a key event in cancer progression [7]. Growing evidence has shown that MMPs can facilitate tumor growth, invasion, and metastasis in various cancers [7]. The ECM is composed of collagen and elastin, and is very important for creating the cellular environments during morphogenesis, tissue repair and remodeling [28,16]. Degradation of ECM in skin tissue would cause skin wrinkle [8].

The human MMPs family, which consists of at least 26 proteases, can be divided into several subgroups according to their structure and substrate specificity [22,28]. These subfamilies include collagenases, gelatinases, stromelysins, matrilysins, and membrane-type MMPs (MT-MMPs). MMPs play an important role in both physiological and pathological conditions, including tissue regeneration, wound repair, reproduction, arthritis, atherosclerosis, and autoimmune blistering disorders of the skin [3]. It also plays an important role on the malignant tumor metastasis. The proliferation of host tumor cell is usually accompanied by simultaneous cancer cells migration that enable them to reach the target tissue [18]. During malignant tumor proliferation, the neoplastic cells firstly attach to the underlying basement membrane. After being degraded by proteases produced by malignant cells, tumor cells pass through the basement membrane, spread into adjacent connective tissue. They proliferate to form a metastasis in target tissue and induce angiogenesis [2].

MMP1 (collagenase-1), located on chromosome 11q22, is an important member of the MMP family that specifically degrades a

* Corresponding author at: Department of Food & Nutrition, 200, Section 7, Taiwan BLVD, Salu, Taichung 43301, Taiwan. Tel.: +886 4 26328001x15375; fax: +886 426530027.
E-mail address: stjiang@pu.edu.tw (S.-T. Jiang).

http://dx.doi.org/10.1016/j.btre.2014.07.003

major component of the ECM, type I collagen, as well as other fibrillar collagens of types II, III, V, and IX [13,32]. The MMP1 gene is expressed in various kinds of normal cells, often at low levels under physiological conditions. However, MMP1 gene expression increases dramatically in a large number of malignancies, including head and neck cancer [25]. It is worth to mention that MMP1 is very important to metastasis and invasion of tumor cells because of its capability of degrading fibrillar collagen type I and III [26], which consequently breaks the ECM molecules.

RNA interference (RNAi) is a phenomenon of sequence-specific post-transcriptional gene silencing (PTGS) that is a conserved biological response. It was first discovered by plant biologists in 1980, but its molecular mechanism kept unknown until the late 1990. Andrew Fire and Ceaig Mell studied the nematode, *Caenorhabditis elegans*, and found that it is a specific silencing of genes, highly homologous in sequence to the delivered double-stranded RNA (dsRNA) [6]. This kind of process was considered to be a defense mechanism against viral pathogens or uncontrolled transposon mobilization [19,31]. The mechanism of RNAi essentially involves the effectors, short (21–28 nucleotides) dsRNA, and then degrades the target mRNA. RNAi is mediated by siRNAs that are processed from long dsRNAs of exogenous or endogenous origin by a cytoplasmic ribonuclease-III type called dicer [21]. The resulting siRNAs are about 21–23 nucleotides (nt) long and then incorporated into nuclease complex, a RNA-inducing silencing complex (RISC). The antisense strand of siRNA serves as a template for RISC to recognize, then targets and cleaves the mRNA containing a sequence identical to that of the siRNA, which can consequently then rapidly degrade mRNA [29]. In this study, 3 small double strand siRNAs (506-MMP1, 859-MMP1 and 891-MMP1), each contains 25–26 nucleotides, with 30–50% of GC content and high specific to human MMP1 were designed and synthesized according to mRNA sequence of human MMP1 (NCBI, NM_002421) and factors affecting RNA interfering efficiency from previous studies [1,27,12,14,20]. To directly evaluate the silencing efficiency of three siRNAs in living cells, four target genes (a long one contained all three siRNA target DNAs and three individual target DNAs) – green fluorescent fusion protein (GFP) report plasmids were created and examined (Fig. 1). Finally, the quantitative PCR and western blot analyses were also carried out to evaluate the mRNA and protein expression quantity, respectively, of endogenous MMP1 in MeWo fibroblast cells.

2. Materials and methods

2.1. Cell line and cell culture

Human embryonic skin, Detroit 551 (BCRC 60118) and malignant melanoma of human skin, MeWo (BCRC 60540) were purchased from Bioresource Collection and Research Center (BCRC). The Detroit 551 cells were grown in a culture medium A [Minimum Essential Medium Alpha Medium powder (α-MEM, Gibco BRL) supplemented with 10% fetal bovine serum (FBS), 1.5 g/L sodium bicarbonate (Sigma)], while the MeWo cells were grown in the same culture medium A with 1.0 mM sodium pyruvate. All cells were maintained in a humidified 37 °C incubator with 5% CO_2.

2.2. RNA isolation and cDNA synthesis

Detroit 551 cells were incubated in 75 mL flask in cultured medium A for 3–4 days. Total RNA were isolated from Detroit 551 cells by using UltraspecII RNA™ isolation kit (Biotecx, Houston, TX) according to the manufacturer's instructions. The first strand

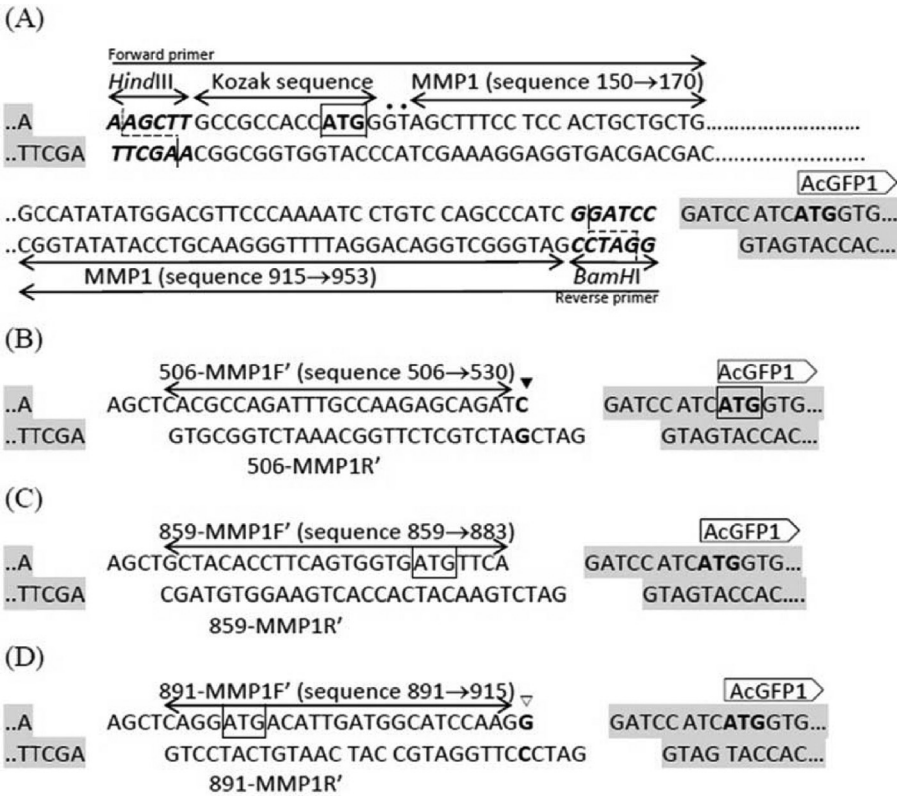

Fig. 1. Construction of MMP1-AcGFP1-N3 reporter plasmids. The sequence number was according to mRNA sequence of human MMP1 (NCBI, NM_002421). Bordered ATP indicated the translation initiation codons. Shading indicated the sticky ends of multiple cloning sites of pAcGFP1-N3 vector, which was cut by *Hind*III and *BamH*I restriction enzymes.

of cDNA was obtained using reverse transcription-PCR. The forward (5′-ATGCACAGCTTTCCTCCACTGCT) and reverse (5′-TCAATTTTTCCTGCAGTTGAACCAGCTAT) primers, used for PCR amplification of human MMP1 cDNA (1410 bp), were designed according to sequences 144–166 and 1525–1553 of NCBI: NM_002421, respectively. Amplification was performed using proofreading polymerase (Gibco BRL products, Cat. No. 10,480-010) by PCR reactions, including preincubation at 95 °C for 5 min, followed by 25 cycles of 30 s at 95 °C, 40 s at 50 °C, 2 min at 72 °C and a final extension at 72 °C for 10 min, in a DNA thermal cycler (PerkinElmer, GeneAmp PCR system 2700). PCR products were identified using electrophoresis on 1.5% agarose gels and carried out by ethidium bromide (EtBr). The PCR product of human MMP1 was first cloned into pGEM-T Easy vector (Promega) and then transformed into *Escherichia coli* Top 10. After blue/white selection and midi preparation, the DNA sequence between T/A cloning sites of human MMP1 cDNA- pGEM-T Easy vector was sequenced by Minshin Biotech Co., Ltd. (Taipei, Taiwan).

2.3. Construction of target MMP1 gene reporter plasmid

Three small double strand DNAs, being with 25–26 nucleotides each, high specific to human MMP1 and 30–50% of GC content, were predicted to be a nice target for RNA interference according to the mRNA sequence of human MMP1 (NCBI, NM_002421) and factors affecting RNA interfering efficiency from previous studies [1,27]. To evaluate the interference efficacy of potential siRNA sequences, which were predicted to be able to block MMP1 gene expresses, one green fluorescent protein (GFP) coding plasmid, pAcGFP1-N3 vector, was used as a reporter system. A MMP1 partial cDNA, including all the three potent siRNA target sequences, was constructed to the reporter vector. As shown in Fig. 1, the MMP1 cDNA (831 bp), containing sequence 150–953 of MMP1 cDNA (NM_002421), one Kozak sequence [15,4] and two restriction sites (*Hind*III and *BamH*I), incorporated to facilitate subcloning of the PCR product, were amplified by PCR. The human MMP1 cDNA-pGEM-T Easy vector was used as template; and the following two oligonucleotides, AAGCTTGCCGCCACCTGGGTAGCTTTCCTCCACTGCTGCTG and GGATCCGATGGGCTGGACAGGATTTTGGGAACGTCCATATATGGC, were used as forward and reverse primers, respectively. After PCR reaction, the product was first purified and cloned into pGEM-T Easy vector (Promega), and then transformed into *E. coli* Top 10. After blue/white selection and sequence analysis, the target DNA was subcloned into pAcGFP1-N3 vector (Clontech Laboratories, Inc.), downstream the immediate early promoter of CMV ($P_{CMV\ IE}$) and before the green fluorescent protein AcGFP1 coding sequences, using *Hind*III and *Bam*HI cutting sites.

According to our preliminary experiments (data not shown), the intensity of the fluorescence, expressed from MMP1 partial cDNA-pAcGFP1-N3 plasmid (Fig. 1A), was not perfect enough for the following assay, if the length of insert target gene was too long. Therefore, the construction of MMP1 target gene reporter plasmid was divided into three parts: 506-MMP1, 859-MMP1, and 891-MMP1. As shown in Fig. 1, the 3′-ends of forward and reverse oligonucleotides were complementary (underlined) for each other, they annealed to each other after cooling down from 95 °C to 50 °C. After annealing of each pairs of oligonucleotides, two 5′-sticky ends at each annealed double strand oligonucleotide were created and, following, they were ligated into the *Hind*III and *BamH*I restriction sites of pAcGFP1-N3 vector. Primers used in this study were as following:

- 506-MMP1 forward: AGCTCACGCCAGATTTGCCAAGAGCAGATC
- 506-MMP1 reverse: GATCGATCTGCTCTTGGCAAATCTGGCGTG
- 859-MMP1 forward: AGCTGCTACACCTTCAGTGGTGATGTTCA
- 859-MMP1 reverse: GATCTGAACATCACCACTGAAGGTGTAGC
- 891-MMP1 forward: AGCTCAGGATGACATTGATGGCATCCAAGG
- 891-MMP1 reverse: GATCCCTTGGATGCCATCAATGTCATCCTG

2.4. SiRNAs design and chemical synthesis

According to mRNA sequence of human MMP1 (NCBI number: NM_002421) and the general approach in designing siRNAs for silencing, 26 segments with 30–50% of GC content and 19–25 nt of double-stranded siRNAs are preferred. Accordingly, 3 sequences considering to have high efficiency of silencing were synthesized. Following were the target sequences of siRNA, relative to the sequence of human MMP1 (NM_002421) in NCBI web.

- Target sequence 506–530 (506 siRNA): AUCUGCUCUUGGCAAAUCUGGCGUG
- Target sequence 859–883 (859 siRNA): UGAACAUCACCACUGAAGGUGUAGC
- Target sequence 891–915 (891 siRNA): CUUGGAUGCCAUCAAUGUCAUCCUG

2.5. Transfection of reporter plasmid and siRNA

The living colors pAcGFP1-N3 vector (Clontech Laboratories, Inc.) was chosen as report system, which encoding the green fluorescent protein (GFP) under the CMV promoter. To evaluate the efficiency of siRNAs silencing, 1×10^6 MeWo cells were first inoculated into each well of 24-well plate and cultured in culture medium for 24 h. Following 1 μg of reporter plasmid 506-MMP1, 859-MMP1 or 891-MMP1 were transfected individually into the cultured MeWo cells using Xfect™ Transfection Reagent (Clontech Laboratories, Inc.) and cultured continuously for the next 24 h, and then the designed target siRNA (506 siRNA, 859 siRNA and 891 siRNA) or negative control siRNA (neg-siRNA) were transfected correspondingly using Xfect™ siRNA Transfection Reagent (Clontech Laboratories, Inc.). After cultivation of the following 24 h, the GFP expression was analyzed using Olympus CKX41 fluorescent microscope and ELISA reader (BioTek synergy HT). Cells with GFP expression indicated it was successful in construction of target gene reporter plasmid. Cells with an apparent absence of green fluorescence indicated gene silencing. Cell viability assay was performed right after the fluorescent analysis. The protocol of transfection of reporter plasmid was according to the manufacturer's instruction (Clontech).

2.6. MeWo cells transfected with synthesized siRNA to knockdown endogenous MMP1 gene

The experiment of knockdown endogenous MMP1 gene was performed in MeWo cells. MeWo cell is human melanoma cell and the morphology is fibroblast, therefore, it can express the MMP1 protein. Exogenous delivery of siRNA duplexes to mammalian cells was carried out with the Xfect™ siRNA Transfection Reagent (Clontech Laboratories, Inc.) in a 24 well plate, which was developed for the delivery of siRNA. Absence of transfection reagents, siRNA duplexes were not taken up by cells. The protocol was according to the manufacturer's instruction (Clontech).

2.7. Western blot analysis

After transfection with 859 siRNA and further 24 h incubation, cells were lysed in a mammalian cell lysis buffer (Clontech Laboratories, Inc.). Western blot analysis was then performed using conventional protocols. In brief, protein concentration was

determined with Bradford assay (Bio-Rad) with bovine serum albumin as a standard (Sigma). Equal amounts of total protein were then separated on 12% polyacrylamide gels by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membrane. Antibodies and dilutions used in this study included anti-MMP1 (1:1000 dilution, Millipore, Billerica, MA, USA) and anti-GAPDH (1:2000 dilution, Millipore, Billerica, MA, USA). After being washed extensively, the membranes were incubated with goat anti-rabbit IgG peroxidase conjugate antibody (1:10000 dilution) for 1 h at room temperature and developed with chemiluminescent HRP substrate (Millipore, Billerica, MA, USA). Membranes probed for hMMP1 were re-probed for GAPDH to normalize for loading and/or quantification errors and to allow comparisons of target protein expression or inhabitation to be made. Band density was measured by photoimage (Fusion-SL2-3500WL, Vilber Lourmat, France, www.vilber.com).

2.8. Cell viability assay

To detect the potential toxicity to the cell during the experiments, the cell viability was determined in 24 well plates. After specified periods of cell incubation (48 h post-transfection), 0.5 mL of MTT solution (1.5 mg/mL) was added to each well and incubated at 37 °C for 4 h. After removal of media, 0.5 mL of DMSO was added and the absorbance at 540 nm was measured. The viabilities were normalized to the absorbance of non-treated cells.

2.9. Quantitative real-time PCR

The expression of MMP1 mRNA was analyzed by real time-PCR assay. After transfection for 24 h, total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, California, USA) with the RNA quality (UV reader, 260/280 ratio > 1.7) and triplicate assessment by using microplate (BioTeck, USA). First-strand cDNA was synthesized using 1 μg of total RNA by reverse-transcription using iScript™ cDNA synthesis kit (Bio-rad, California) as instructed by the manufacturer. For real-time PCR analysis of MMP1 and GAPDH gene expression was carried out using iQ™ SYBR® Green Supermix (Bio-rad, California), the primers used were:

- MMP1 forward: AGTCAAGTTTGTGGCTTATGGA
- MMP1 reverse: TTGTCACTGAAGCTGCTCTC
- GAPDH forward: GTCGGAGTCAACGGATTT
- GAPDH reverse: CAACAATATCCACTTTACCAGAG

Briefly, the reaction mixture containing 2 μL cDNA, 1 μL forward primer (0.5 μM), 1 μL reverse primer (0.5 μM), 10 μL iQ SYBR Green Supermix, and 6 μL RNase-free water was prepared. The real-time PCR program was set as follows: initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, and then 61 °C for 30 s. Finally, the melting curve program was performed at the end of each reaction. The relative levels of mRNA expression was assessed by the comparative Ct method (DDCT method), which normalize the mRNA level of negative control to that of reference gene GAPDH.

3. Results and discussion

3.1. Human MMP1 cDNA synthesis and construction of MMP1 target gene reporter plasmid

To construct MMP1 target reporter plasmid, as shown in Fig. 1, the MMP1 cDNA (sequence 150–953) fused with Kozak sequence [15,4] at 5′-end to initiate translation process and incorporated 2 restriction sites to facilitate subcloning reaction was first amplified

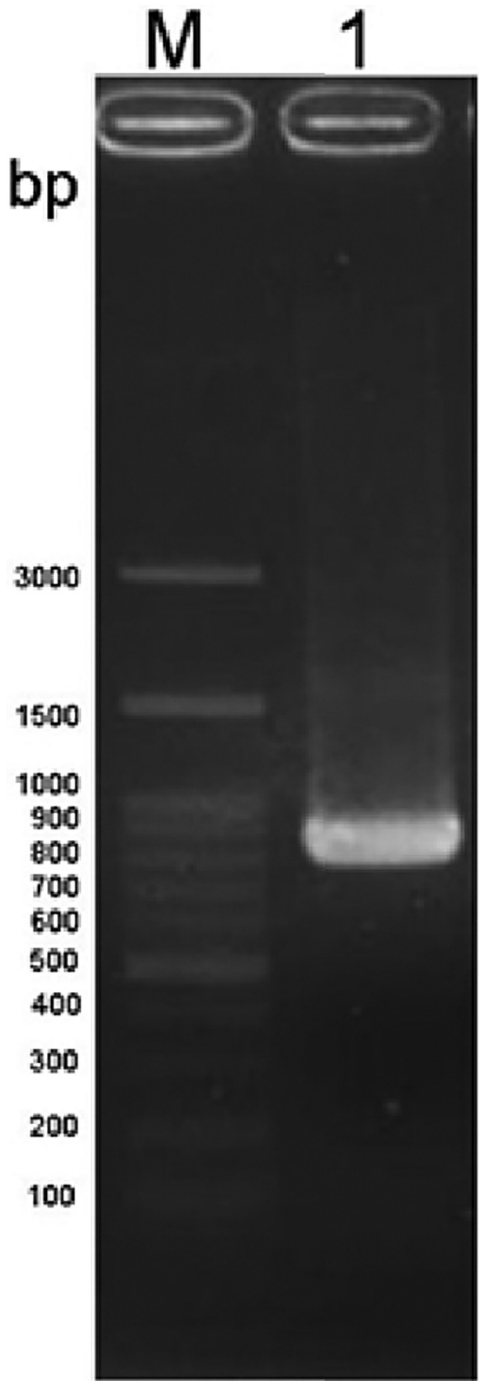

Fig. 2. Agarose gel analysis of PCR product of human fibroblast cell MMP1 cDNA. Lane M: 100 bp DNA marker; lane 1: PCR product (MMP1 831 bp), EtBr staining was carried out after electrophoresis in a 1.5% agarose gel.

(831 bp fragment) by PCR (Fig. 2) and subcloned into pAcGFP1-N3 vector, using *Hind*III and *Bam*HI cutting sites, downstream the immediate early promoter of CMV ($P_{CMV\ IE}$) and followed in frame by the green fluorescent protein AcGFP1 coding sequences. Although the partial MMP1-AcGFP1 fusion DNA could be transcribed under control of CMV promoter, and translated by Kozak sequence, the fluorescent intensity was not satisfied (data not shown). It might be because the molecular of N-terminal fused MMP1 partial protein was too large, which consequently affected the green fluorescent protein folding or its function. To overcome this issue, three potent siRNA target DNAs, 506-MMP1, 859-MMP1 and 891-MMP1 as shown in Fig. 1B–D, were constructed

individually to pAcGFP1-N3 plasmid. Since the length of target gene was about 25–26 bp, forward and reward oligonucleotides were annealed by cooling down from 95 °C to 50 °C in PCR machine to form a double strand and ligated to pAcGFP1-N3 vector, which was precut by *Hind*III and *Bam*HI. As shown in Fig. 1B, the 506-MMP1 (sequence 506–530) had no translation initiation codon "ATG" and its last 2 codes were "AT". One cytidylic acid "C" was extended at the 3′-end of 506-MMP1F′ oligonucleotide, as indicated by "q", to avoid translation initiation codon "ATG" been created after ligated with *BamH*I, since the created "ATG" would be used as translation initiation codon, and frame shift mutation would happen in the following codons of AcGFP1. As shown in Fig. 1C and D, the "ATG" codon was existed in 506-MMP1 (sequence 506–530) and 859-MMP1 (sequence 859–883) DNA sequences, as indicated by border, and would be used as translation initiation codon for expression of the N-MMP1 oligopeptide- AcGFP1 fusion fluorescence proteins. As shown in Fig. 1D, one guanylic acid "G" extended at the 3′-end of 891-MMP1F′ oligonucleotide, as indicated by "s", was designed to avoid frame shift mutation in the following codons of AcGFP1.

3.2. Optimal transfection concentration of reporter plasmid and suitable time for fluorescence assay

To determine the optimal transfection concentration of reporter plasmid and suitable time for fluorescence assay, the MeWo cells were treated with 25 μL Xfect™ Transfection Reagent. The fluorescent expression of cells at 24, 48 and 72 h post transfection of 506-MMP1-pAcGFP1-N3 increased with the duration of incubation time (Fig. 3). Nevertheless, it increased with the increase of vector concentration (0.5, 0.75, 1.0, and 1.5 μg) (Fig. 4). According to the data obtained, the highest fluorescent intension was observed at 72 h incubation time, while the optimal concentration for the reporter vector concentration was 1.0 μg. Although the 72 h was determined to be the optimal time, the 48 h also had considerable fluorescent intensity for the following interfering experiments. To avoid contamination during cell cultivation and for the consideration of cell life-time, 48 h incubation time and 1.0 μg of the reporter vector concentration was chosen for the following experiments.

3.3. Estimation of MMP1 gene silencing efficacy by siRNAs using reporter system

MMP1 partial cDNA-pAcGFP1-N3, 506-MMP1-pAcGFP1-N3 (506 plasmid), 859-MMP1- pAcGFP1-N3 (859 plasmid), and 891-MMP1- pAcGFP1-N3 (891 plasmid) plasmids were used to evaluate the gene silencing efficacy according to intensity of green fluorescence expressed from these reporter systems. The 506, 859, and 891 plasmids, target 506 siRNA, 859 siRNA, 891 siRNA, and a negative control siRNA (neg siRNA) (Invitrogen) with GC content of 48% (similar to that of target siRNA between 45% and 55%) were transfected separately into MeWo cells. Since Xfect™ Transfection Reagent would cause cells toxicity and affect fluorescent expression, cell viability was examined by MTT reagent right after the treatment of fluorescent assay to exclude deviation. The

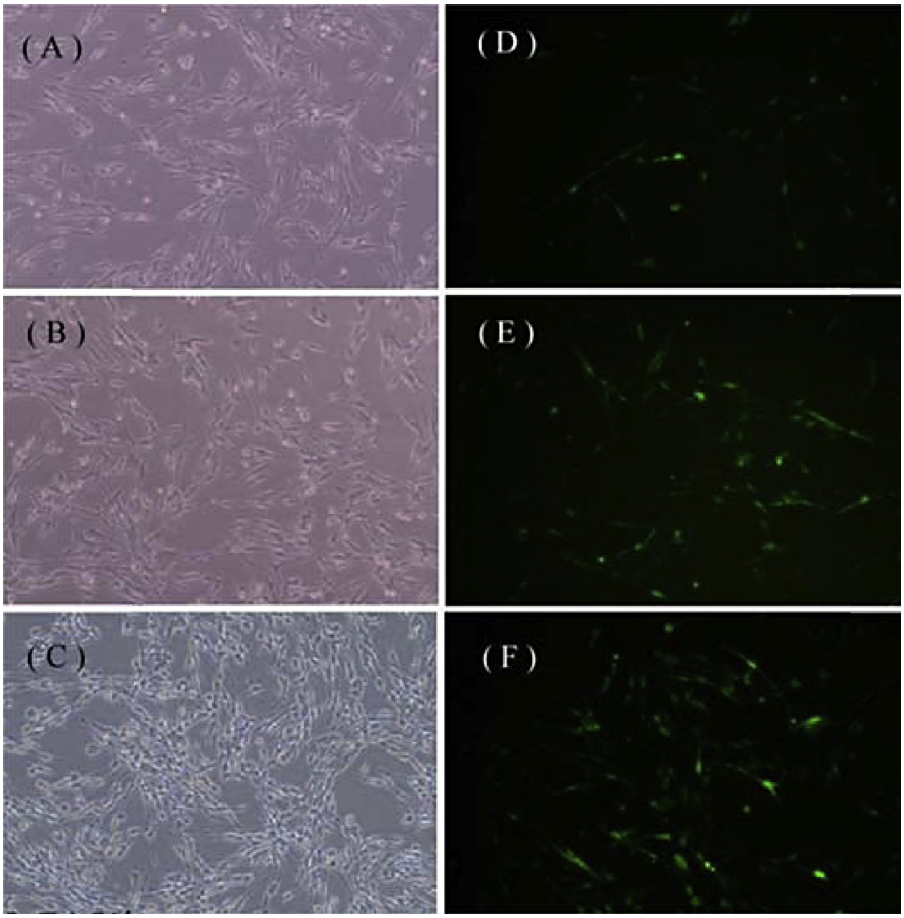

Fig. 3. The image of the fluorescent intensity and cell number of MeWo cell transfected with 0.5 μg of 506-MMP1-pAcGFP1-N3 reporter plasmid by Xfect reagent and cultured for 24, 48 and 72 h. A–C are the light micrographs taken at 24, 48 and 72 h after transfection, respectively. D–F are the fluorescence images corresponding to A–C. All the micrographs were taken using Olympus CKX41 fluorescent microscope.

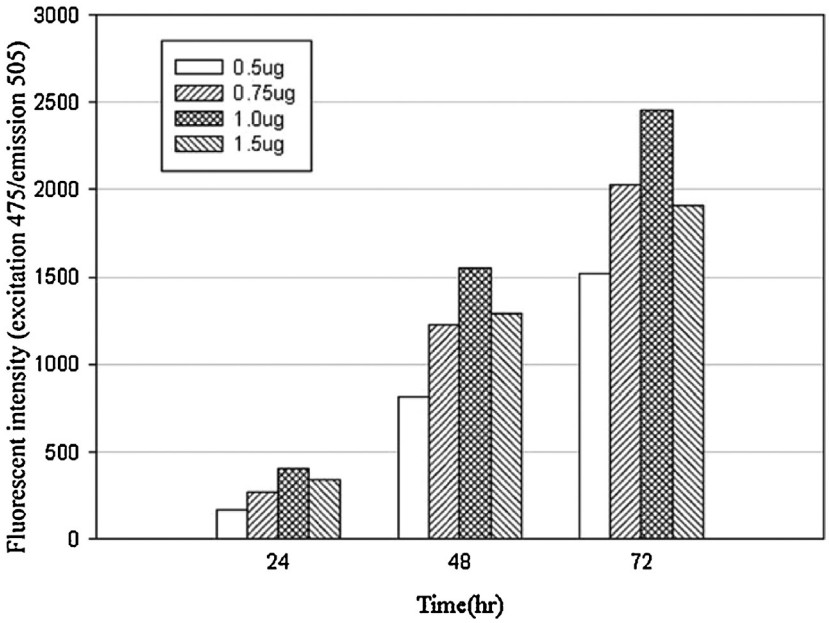

Fig. 4. The optimal transfection concentration of 506-MMP1- pAcGFP1-N3 reporter plasmid and suitable time for fluorescence assay. The fluorescent intensity of the MeWo cells transfected with different concentration (0.5, 0.75, 1.0 and 1.5 μg) of 506-MMP1-pAcGFP1-N3 reporter plasmid by Xfect reagent and cultured for 24, 48 and 72 h was quantified using ELISA reader (BioTek synergy HT). The wavelengths of excitation and emission were 475 and 505 nm, respectively.

fluorescent expression of each cell was obtained from the fluorescent expression divided by cell viability, and the fluorescent expression of control group (no siRNA) was used as background to ensure non-specific complementation or other genes inhibition.

Furthermore, to emphasize that the designed target siRNAs caused the influence effect, the MeWo cells transfected with different concentrations of neg siRNA were assayed. According to the fluorescent photos and the statistical data of the results, no

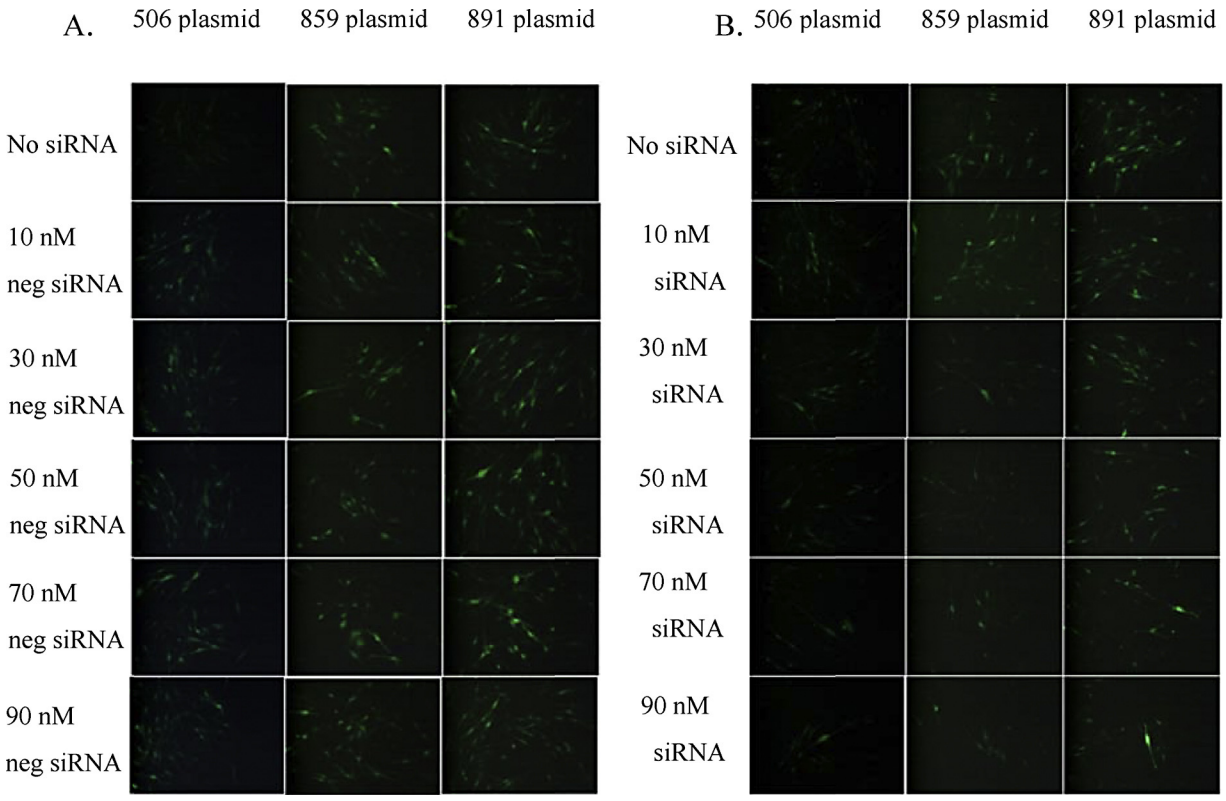

Fig. 5. Fluorescent micrographs of MeWo cells, transfected with target gene reporter plasmid (506 plasmid, 859 plasmid or 891 plasmid) and target siRNA (506 siRNA, 859 siRNA, 891 siRNA) or neg siRNA. These fluorescent micrographs were taken at 48 h post-transfection. (A) MeWo cells were first transfected with 1 μg of individual target gene reporter plasmid, and incubation for 24 h following transfected with different concentration (0, 10, 30, 50, 70, 90 nM) of neg siRNA. After further incubation of 24 h, the MeWo cells were analyzed for GFP expression. (B) All the transfection and cultivation conditions were the same as (A), but replaced the neg siRNA as individual designed target siRNA. All the micrographs were taken using Olympus CKX41 fluorescent microscope.

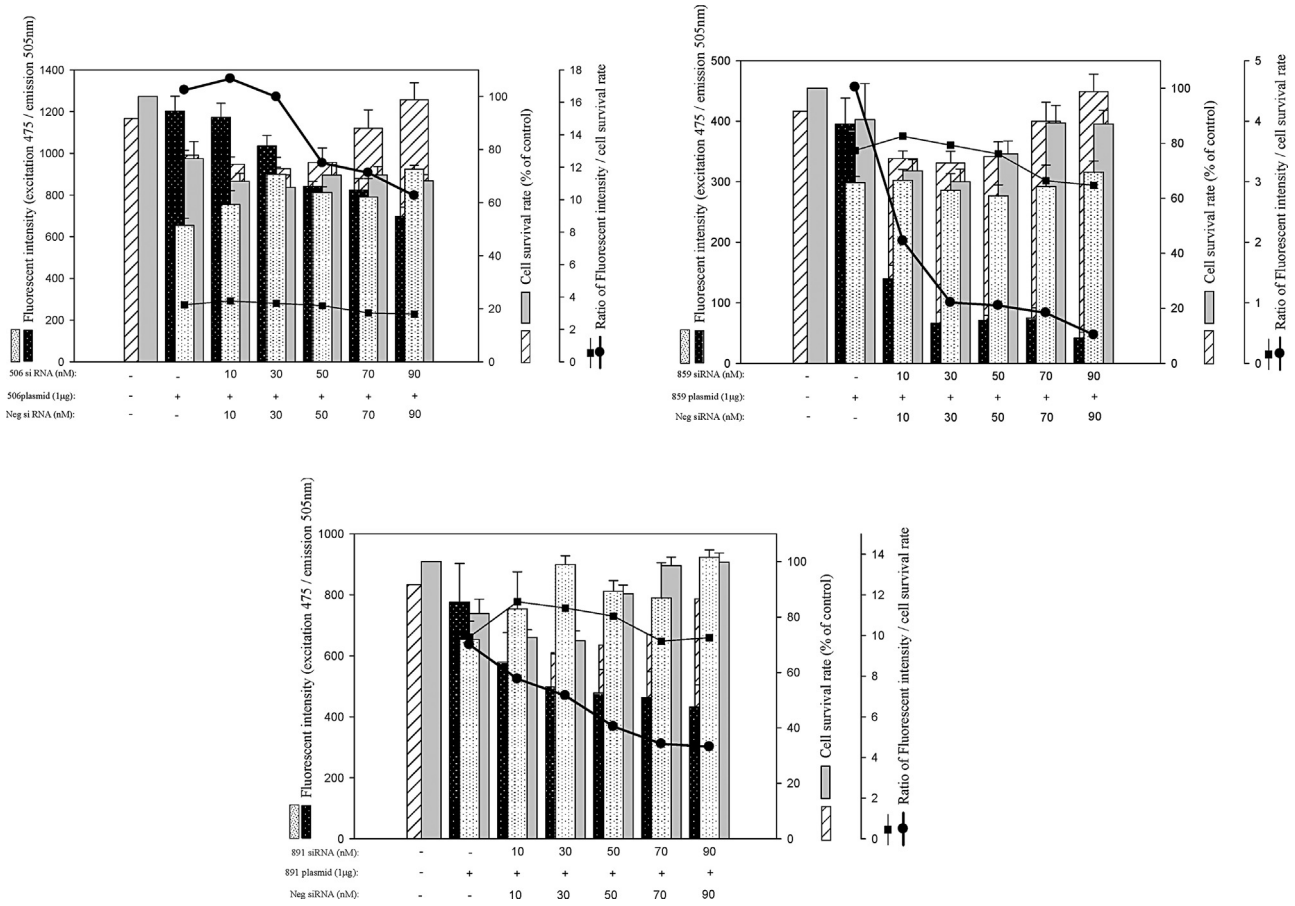

Fig. 6. The statistical analysis of the interfering efficiency of target designed siRNA using the GFP reporter system. The MeWo cells transfected successively (with an interval of 24 h) with target gene reporter plasmid (506 plasmid (A), 859 plasmid (B) or 891 plasmid (C)) and neg siRNA or target siRNA (506 siRNA (A), 859 siRNA (B) or 891 siRNA (C)) were used as reporter system. All the transfection and cultivation conditions were the same as Fig. 5. MeWo cells transfected with 1 μg target gene reporter plasmid and different concentration (0, 10, 30, 50, 70, 90 nM) of neg siRNA or target designed siRNA. After further incubation for 24 h, cells were analyzed for the fluorescent intensity using ELISA reader (BioTek synergy HT) and cell viability by MTT assay.

significant changes in fluorescent intensity and cell survival rate of MeWo cells transfected with different concentrations of neg siRNA was obtained (Fig. 5A and Fig. 6). However, when treated with the designed target siRNA, the fluorescent expression decreased with the increase of siRNA concentration and the influence efficiency was more significant (Fig. 5B and Fig. 6), suggesting it was dose-dependent. These phenomena suggested that these three target siRNAs were able to specifically and distinctly silence the endogenous MMP1 gene expression. According to the data obtained, the inhibition of 506 siRNA was 39.2%, while those of 859 siRNA and 891 siRNA were 89.4% and 54.1%, respectively (Fig. 6). The best interference effect was observed on 859 siRNA, up to 89.4% of inhibition rate.

3.4. Quantitative PCR analysis of the interference efficacy of various concentrations siRNAs on endogenous MMP1 gene expression

Ishii et al. [10] reported that MeWo fibroblast cell (BCRC 60540), a kinds of human melanoma cells, can express spontaneously the endogenous MMP1 protein, and the expression quantity could be enhanced by exposure to nitric oxide (NO) or *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) in a dose-dependent manner. Therefore, the MeWo cells were employed as target cells in quantitative PCR and western blot analysis to investigate whether the evaluation of effective designed siRNAs by the GFP reporter systems was able to interfere with the expression of MMP1 mRNA or protein. According to the results of preliminary experiments, Figs. 7 and 8, the expression quantity and the siRNA interfering efficiency of endogenous mRNA or protein of MMP1 in MeWo cells without induction by NO or SNAP was visible in the blanks (without siRNA treatment). Accordingly, the MeWo cells used in this study were not treated with NO or SNAP to avoid influence by other factors.

To confirm the interference efficacy of siRNAs against endogenous MMP1 gene expression in MeWo cells, the quantitative PCR (real-time PCR) was prepared. The MeWo cells were transfected with various concentrations (10, 30, 50, 70 or 90 nM) of 506 siRNA, 859 siRNA and 891 siRNA, separately. The total RNA was extracted and cDNA were synthesized as described in the methodology. The remained MMP1 mRNA was analyzed by real-time quantitative polymerase chain reaction (RT-PCR) and agarose gel electrophoresis analysis.

According to the results of preliminary experiments, when the concentration of any one of the three designed siRNAs was higher than 100 pmol, the interference efficacy was not consistent and had high standard deviation between repetitive experiments (data not shown). This might be attributed to short half-life and instability of siRNAs. The phenomenon was similar to [11], they had suggested that when the concentration of siRNA was higher than 100 nM, it could cause off-target effect, resulting in the error judgments of the experimental results, so in this study, all the concentration of siRNA used in different tests was ranged from 10 to 90 nM.

As shown in Fig. 7, the endogenous MMP1 mRNA could be interfered with 506 siRNA and 859 siRNA, but the interference

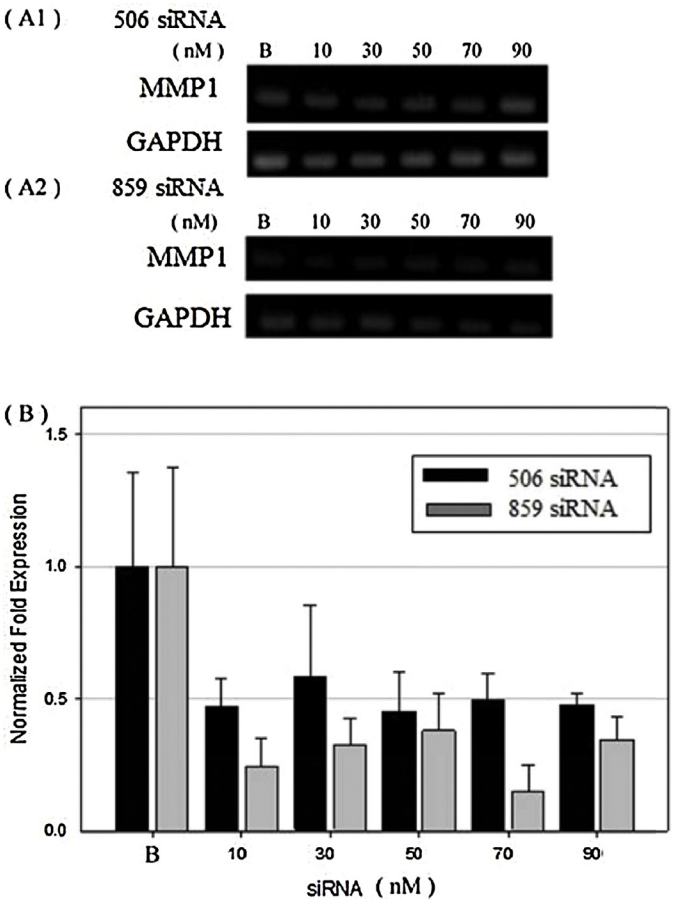

Fig. 7. Analysis of endogenous MMP1 gene transcription by quantitative-PCR. MeWo cells were treated with different concentration (0, 10, 30, 50, 70, 90 nM) of target designed siRNA (506 siRNA and 859 siRNA) and incubated for 24 h. After incubation, the total RNA was extracted using TRIzol Reagent. The cDNA was synthesized using 1 μg of total RNA by reverse-transcription using iScript™ cDNA synthesis kit. MMP1 and GAPDH gene expression was carried out using Quantitative-PCR/iQ™ SYBR® Green Supermix (Bio-rad, California). (A) was the 1.5% agarose gel electrophoresis assay of the PCR products of different siRNA treatments. (B) was the quantified results of the expression quantity of relative expression of the MMP1 mRNA normalized against GAPDH mRNA and compared to blank (without treatment of siRNA).

efficacy of various concentrations of siRNAs on endogenous MMP1 gene expression were not dose-dependent. The inhibition rates of 506 siRNA and 859 siRNA against endogenous MMP1 gene expression were 55% and 85%, respectively (Fig. 7). Regarding to the 891 siRNA, its interference efficacy was not constant, dose-dependent with large standard deviation among many repetitive experiments (data not shown). It was, therefore, not included in Fig. 7. This might be attributed to instability of the 891 siRNAs, non-specific complementation or other unknown siRNA interfering progress. According to the above results of quantitative PCR, the 859 siRNA had better interference efficiency on endogenous MMP1 gene expression in MeWo cells, and consequently been proceeded the following western blot analysis.

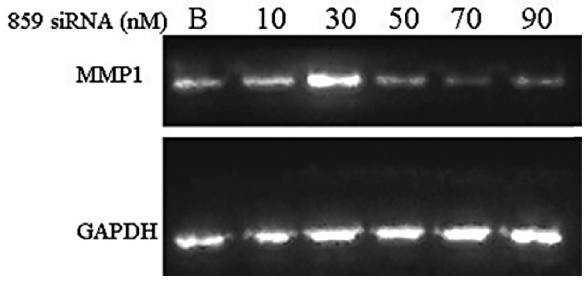

Fig. 8. Analysis of endogenous MMP1 protein expression by western blot. MeWo cells were treated with different concentration (0, 10, 30, 50, 70, 90 nM) of the 859 siRNA and incubated for 24 h. After incubation, cells were lysed in a mammalian cell lysis buffer. Western blot analysis was then performed using conventional protocols.

3.5. Western blot analysis of protein silencing

The inhibition rates of 859 siRNA against endogenous MMP1 gene expression in MeWo cells transfected with various concentrations of 859 siRNA (10, 30, 50, 70 or 90 nM) were determined and found that 90 nM of 859 siRNA had highest inhibition rate, 89.4%, on endogenous MMP1 protein expression (Fig. 8).

Whereas many study had focused and proved on factors affecting siRNA interfering efficiency [1,27,12,14,20], and many software for design and prediction of siRNA [5,9,17,30] have been developed. In this study, the MMP1-pAcGFP1-N3 reporter/MeWo cells reporter system had been created and the interference efficacy of three novel designed siRNAs against MMP1 had been evaluated. According to the results of MMP1-pAcGFP1-N3/MeWo cells reporter system, all the three target siRNAs were able to silence the target MMP1- GFP fused gene expression and the inhibition rate of 506 siRNA, 859 siRNA and 891 siRNA were 39.2%, 89.4% and 54.1%, respectively. The 859 siRNA exhibited the highest gene silencing activity in 859-MMP1- pAcGFP1-N3 reporter system. Further confirmation of the interference efficacy of the 859 siRNA against endogenous MMP1 gene expression was performed in MeWo cells using quantitative PCR (Fig. 7) and western blot (Fig. 8) analyses. It exhibited 85 (quantitative PCR) and 89% (western blot) inhibition rates of endogenous MMP1 gene and protein expression, respectively. These results were in accordance with the assay by MMP1-pAcGFP1-N3/MeWo cells reporter system, suggesting that the data evaluated by the reporter system were reliable, although it is regrettable that the long MMP1 partial cDNA-AcGFP1-N3 reporter plasmid (Fig. 1A), contained all three siRNA target DNAs, is not suitable.

These data not only provide the basic data for siRNA technology, but also obtain the small interfering RNAs (siRNA or shRNA) with inhibition of matrix metalloproteinase 1 (MMP1) gene expression. In the future, the 859 siRNA may be applied as anti-wrinkle reagent in cosmetic industry and anti-tumor metastasis reagents in medical applications, and it is actually on-going in our laboratory, currently.

References

[1] L. Aagaard, J.J. Rossi, RNAi therapeutics: principles, prospects and challenges, Adv. Drug Deliv. Rev. 59 (2–3) (2007) 75–86.
[2] R. Ala-aho, V.M. Kahari, Collagenases in cancer, Biochimie 87 (3–4) (2005) 273–286.
[3] C. Amălinei, I. Căruntu, S. Giuşcă, R. Bălan, Matrix metalloproteinases involvement in pathologic conditions, Rom. J. Morphol. Embryol. 51 (2) (2010) 215–228.
[4] M. De Angioletti, G. Lacerra, V. Sabato, C. Carestia, β+45 G→C: a novel silent β-thalassaemia mutation, the first in the Kozak sequence, Br. J. Haematol. 124 (2) (2004) 224–231.
[5] P. Dudek, D. Picard, TROD: T7 RNAi oligo designer, Nucleic Acids Res. 32 (Suppl. 2) (2004) W121–W123.
[6] A. Fire, S. Xu, M.K. Montgomery, S.A. Kostas, S.E. Driver, C.C. Mello, Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*, Nature 391 (6669) (1998) 806–811.
[7] J. Freije, M. Balbín, A. Pendás, L. Sánchez, X. Puente, C. López-Otín, Matrix metalloproteinases and tumor progression, Adv. Exp. Med. Biol. 532 (2003) 91–107.
[8] A. Gupta, C.D. Kaur, M. Jangdey, S. Saraf, Matrix metalloproteinase enzymes and their naturally derived inhibitors: novel targets in photocarcinoma therapy, Age. Res. Rev. 13 (0) (2014) 65–74.

[9] A. Henschel, F. Buchholz, B. Habermann, DEQOR: a web-based tool for the design and quality control of siRNAs, Nucleic Acids Res. 32 (Suppl. 2) (2004) W113–W120.
[10] Y. Ishii, T. Ogura, M. Tatemichi, H. Fujisawa, F. Otsuka, H. Esumi, Induction of matrix metalloproteinase gene transcription by nitric oxide and mechanisms of MMP-1 gene induction in human melanoma cell lines, Int. J. Cancer 103 (2) (2003) 161–168.
[11] A.L. Jackson, P.S. Linsley, Noise amidst the silence: off-target effects of siRNAs? Trends Genet. 20 (11) (2004) 521–524.
[12] B. Jagla, N. Aulner, P. Kelly, D. Song, A. Volchuk, A. Zatorski, D. Shum, T. Mayer, D. De Angelis, O. Ouerfelli, U. Rutishauser, J. Rothman, Sequence characteristics of functional siRNAs, RNA 11 (6) (2005) 864–872.
[13] E. Kerkelä, U. Saarialho-Kere, Matrix metalloproteinases in tumor progression: focus on basal and squamous cell skin cancer, Exp. Dermatol. 12 (2) (2003) 109–125.
[14] A. Khvorova, A. Reynolds, S.D. Jayasena, Functional siRNAs and miRNAs exhibit strand bias, Cell 115 (2) (2003) 209–216.
[15] M. Kozak, An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs, Nucleic Acids Res. 15 (20) (1987) 8125–8148.
[16] H. Nagase, J.F. Woessner Jr., Matrix metalloproteinases, J. Biol. Chem. 274 (31) (1999) 21491–21494.
[17] Y. Naito, T. Yamada, K. Ui-Tei, S. Morishita, K. Saigo, siDirect: highly effective, target-specific siRNA design software for mammalian RNA interference, Nucleic Acids Res. 32 (Suppl. 2) (2004) W124–W129.
[18] S.K. Patra, S. Bettuzzi, Epigenetic DNA-methylation regulation of genes coding for lipid raft-associated components: a role for raft proteins in cell transformation and cancer progression (review), Oncol. Rep. 17 (6) (2007) 1279–1290.
[19] B.J. Reinhart, F.J. Slack, M. Basson, A.E. Pasquinelli, J.C. Bettinger, A.E. Rougvie, H.R. Horvitz, G. Ruvkun, The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans, Nature 403 (6772) (2000) 901–906.
[20] A. Reynolds, D. Leake, Q. Boese, S. Scaringe, W. Marshall, A. Khvorova, Rational siRNA design for RNA interference, Nat. Biotech. 22 (3) (2004) 326–330.
[21] P. Sandy, A. Ventura, T. Jacks, Mammalian RNAi: a practical guide, Biotechniques 39 (2) (2005) 215–224.
[22] M.D. Sternlicht, Z. Werb, How matrix metalloproteinases regulate cell behavior, Annu. Rev. Cell Dev. Biol. 17 (1) (2001) 463–516.
[23] W.G. Stetler-Stevenson, L.A. Liotta, D.E. Kleiner, Extracellular matrix 6: role of matrix metalloproteinases in tumor invasion and metastasis, FASEB J. 7 (15) (1993) 1434–1441.
[24] W.G. Stetler-Stevenson, A.E. Yu, Proteases in invasion: matrix metalloproteinases, Semin. Cancer Biol. 11 (2) (2001) 143–153.
[25] A. Stokes, J. Joutsa, R. Ala-aho, M. Pitchers, C.J. Pennington, C. Martin, D.J. Premachandra, Y. Okada, J. Peltonen, R. Grénman, H.A. James, D.R. Edwards, V.-M. Kähäri, Expression profiles and clinical correlations of degradome components in the tumor microenvironment of head and neck squamous cell carcinoma, Clin. Cancer Res. 16 (7) (2010) 2022–2035.
[26] Y. Suzuki, I. Someki, E. Adachi, S. Irie, S. Hattori, Interaction of collagen molecules from the aspect of fibril formation: acid-soluble, alkali-treated, and MMP1-digested fragments of type I collagen, J. Biochem. 126 (1) (1999) 54–67.
[27] Y. Takahashi, M. Nishikawa, Y. Takakura, Nonviral vector-mediated RNA interference: its gene silencing characteristics and important factors to achieve RNAi-based gene therapy, Adv. Drug Deliv. Rev. 61 (9) (2009) 760–766.
[28] R. Visse, H. Nagase, Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry, Circ. Res. 92 (8) (2003) 827–839.
[29] K. Yavari, M. Taghikhani, M.G. Maragheh, S.A. Mesbah-Namin, M.H. Babaei, Knockdown of IGF-IR by RNAi inhibits SW480 colon cancer cells growth in vitro, Arch. Med. Res. 40 (4) (2009) 235–240.
[30] B. Yuan, R. Latek, M. Hossbach, T. Tuschl, F. Lewitter, siRNA selection server: an automated siRNA oligonucleotide prediction server, Nucleic Acids Res. 32 (Suppl. 2) (2004) W130–W134.
[31] S. Zhang, B. Zhao, H. Jiang, B. Wang, B. Ma, Cationic lipids and polymers mediated vectors for delivery of siRNA, J. Control. Release 123 (1) (2007) 1–10.
[32] B.L. Ziober, M.A. Turner, J.M. Palefsky, M.J. Banda, R.H. Kramer, Type I collagen degradation by invasive oral squamous cell carcinoma, Oral Oncol. 36 (4) (2000) 365–372.